

## SCREENING OF MARINE BACTERIA CAPABLE OF DEGRADING VARIOUS POLYAROMATIC HYDROCARBONS

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### Abstract

This research aims to screen and to identify marine bacteria able to degrade Polyaromatic Hydrocarbons (PAHs). 7 bacterial isolates were screened for the ability to degrade 6 PAHs (phenanthrene, naphthalene, dibenzothiophene, fluorene, phenothiazine, and pyrene). This screening step was conducted by sublimation method, in which the color change or clear zone appearance was used as an qualitative indicator to show the capability of the selected isolate to degrade PAHs. Two bacterial isolates, designated as LBF-1-0102 and LBF1-0103, showed capability in degradating the PAHs tested. In particular, the isolate LBF-1-102 was potential in degrading phenanthrene, naphthalene, dibenzothiophene, fluorene, phenothiazine, and pyrene. The isolate LBF-1-0103 showed the ability in degrading 2 (two) PAHs, i.e. dibenzothiophene and fluorene. Based on partial 16S rDNA analysis, LBF-1-0102 and LBF-1-0103 were identified as *Pseudomonas balerica* and *Brachybacterium* sp., respectively.

**Keywords:** biodegradation, marine bacteria, PAH

### 1. Introduction

Crude oil is a complex mixture of hydrocarbons containing more than 17.000 compounds (Marshall & Rodger, 2004). Therefore, oil spill contamination in the environment can release some organic pollutants, such as alkanes, BTEX (Benzene, toluene, ethyl benzene, xylene), and Polycyclic Aromatic Hydrocarbon (PAH). PAHs are aromatic compounds consisting of two to eight conjugated ring systems. They can have a range of substituents such as alkyl, nitro, and amino groups in their structure (Pampanin & Sydnes, 2013).

Polycyclic aromatic hydrocarbons are unique pollutants in both aquatic and terrestrial ecosystems which are related to some pyrogenic and petrogenic resources. PAH compounds are hydrophobic, toxic, mutagenic, and environmentally persistent. More than 16 kinds of the compounds are also carcinogenic to marine organisms which may be transferred to human through seafood consumption (Meador et al., 1995).

Bioremediation is an alternative technology that use microorganisms for solving environmental problems. In addition, bioremediation is believed to

be non-invasive and relatively cost-effective technology. Bioremediation utilizes the metabolic versatility of microorganisms to degrade hazardous pollutants. One of the bioremediation goal is to transform organic pollutants into harmless metabolites or mineralize the pollutant into carbon dioxide and water (Alexander, 1999). Biodegradation is a very broad field and involves uses of a wide range of microorganism to break chemical bonds. Several reports on isolation and characterization of PAH degrading bacteria or detection of gene associated with PAH degradation have been released (Cheung & Kinkle, 2001; Ahn et al., 1999; Kim et al., 2003; Lisdiyanti et al., 2011; Thontowi & Yopi, 2013).

In recent years, several genera of marine bacteria have been reported to catabolize PAHs for carbon and energy, which include *Marinobacter* (Gauthier et al., 1992), *Cycloclasticus* (Dyksterhouse et al., 1995), *Neptunomonas* (Hedlund et al., 1999), and *Pseudoalteromonas* (Hedlund et al., 2006). In addition, several other genera capable of PAH degradation, including *Sphingomonas*, *Pseudomonas*, *Burkholderia*, *Mycobacterium*, have been isolated

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from marine or estuarine sites (Berardesco et al., 1998). For example, PAH-degrading bacterium *Alteromonas alvinellae* Bt05 was isolated from Jakarta Bay, Indonesia (Thontowi et al., 2013). In our previous works, we have isolated oil-degrading marine bacteria as pure stock cultures by medium enrichment of oil substrate methods (Yopi et al., 2006). However many of them have not been characterized and identified yet. This study aims to screen and to identify marine oil-degrading bacterial isolates exhibiting PAH degradation ability.

## 2. Material and Methods

### 2.1. Bacterial Isolates, Media, and Culture Condition

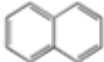
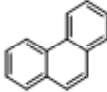
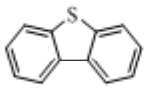
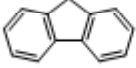
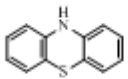
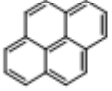
Isolates used in this study are the selected oil-degrading marine bacteria obtained from Laboratory of Biocatalyst and Fermentation (LBF) Collection, Research Center for Biotechnology, Indonesian Institutes of Sciences. The bacteria were previously isolated from Cilacap sea water and Marina Beach, Jakarta, Indonesia. Marine Agar (MA) and Marine Broth (MB) were used to culture the isolates. The microorganisms were incubated on a rotary shaker, temperature 30 °C, 200 rpm for 24 h. The pH of all

media were adjusted to 7.5. Artificial Sea Water Agar (ASWA) was used to inoculate the isolates in pre-degradation test by sublimation methods (Alley and Brown, 2000). Whereas, the growth assay of bacteria in liquid media was conducted in ASW containing 50 ppm of each PAH compound.

### 2.2. Screening of Isolates

To screen the marine bacterial isolates able to degrade PAH compounds, we carried out the preliminary degradation assay by sublimation test. Sublimation test was conducted for PAH compounds due to their dissolution properties as described by Alley and Brown (2000). The bacteria were incubated for 7 to 14 days. Sublimation test was carried out on solid ASW medium and 6 PAHs compounds (naphthalene, phenanthrene, dibenzothiophene, fluorene, phenothiazine, and pyrene). ASW medium contained NaCl 22.1 g, MgCl<sub>2</sub>·6H<sub>2</sub>O 9.9 g, CaCl<sub>2</sub>·2H<sub>2</sub>O 1.5 g, Na<sub>2</sub>SO<sub>4</sub> 3.9 g, KCl 0.61 g, NaHCO<sub>3</sub> 0.19 g, KBr 96 mg, Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O 78 mg, SrCl<sub>2</sub> 13 mg, NaF 3 mg, LiCl 1 mg, KI 81 µg, MnCl<sub>2</sub>·4H<sub>2</sub>O 0.6 µg, CoCl<sub>2</sub>·6H<sub>2</sub>O 2 µg, AlCl<sub>3</sub>·6H<sub>2</sub>O 8 µg, FeCl<sub>3</sub>·6H<sub>2</sub>O 5 µg, Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O 2 µg, and (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>21</sub>·4H<sub>2</sub>O 18 µg per liter. The condition of sublimation test for each

Table 1. Sublimation Test Condition

PAH compounds		Melting Temperature (°C)	Time (min.)
Naphthalene		80	0,5
Phenanthrene		97-100	5
Dibenzothiophene		95-105	510
Fluorene		115	3-5
Phenothiazine		130-140	30-60
Pyrene		145-148	30-60

compound was shown in Table 1. The bacteria that grew and gave clear zone and/or color change on solid media indicated capability in PAH degradation.

### 2.3. Growth Test

The growth test of the potential isolates in ASW medium containing PAH aims to confirm the positive candidates of bacteria that able to degrade PAH as described by Juhasz et al. (1997) with modification. The growth test was performed using ASW medium which supplemented with 50 ppm of PAH compounds in triplo replication. The isolates were precultured in MB medium and incubated in a rotary shaker at 30 °C, 200 rpm for 24 h. The inoculation was performed in test tubes containing 5 mL of both medium and each tube was inoculated with 0,2 OD (optical density) at 600 nm of bacterial inoculum for pre culture. The medium was supplemented with 50 ppm of PAH compounds. The tubes were incubated at 30 °C for a period of 7-14 days. In this study, we analyzed bacterial growth and/or color change as an indicator of biodegradation daily. As a control, we used culture medium without PAH content with inoculum of isolate and culture medium containing PAH without bacterial inoculum.

### 2.4. Identification of 16S rDNA Partial Gene

Gene sequence coding for 16S rRNA was partially amplified by PCR using universal primers, forward primer (9F: 5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer (1510R: 5'G GC TACC TTGT TACGA-3') (Nilsson & Strom, 2002). The PCR operating condition was performed as described by Lu et al. (2003) with minor modifications as follows: 95 °C, 2 min ( 1 cycle); 95 °C, 30 sec; 65 °C, 1min; 72 °C, 2 min (10 cycles); 95 °C, 30 sec; 55 °C, 1 min; 72 °C, 2 min (30 cycles); 72 °C, 2 min ( 1 cycle). The PCR product was separated using 1% agarose gel electrophoresis followed with Ethidium Bromide staining (Sigma, St. Louis, USA).

### 2.5. DNA Sequencing and Sequence Analysis

The PCR products were then sent to First Base Company for sequencing. The sequencing products were then analyzed using Bioedit software. To identify the isolated bacteria, the 16S rRNA consensus sequence was then compared with 16S rRNA gene sequences from the public GeneBank, EMBL, and DDBJ data bases using the advanced gapped n-BLAST program, version 2.1. The program was run via internet through the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/blast>). Multiple alignment and

phylogenic tree were visualized by the NJ plot program (Thompson et al., 1994) and Mega 3.1 ABI *sequencer software* (Kumar et al., 2004).

## 3. Results and Discussion

### 3.1. Screening of Isolates and Growth Test

Hydrocarbon-degrading bacteria in saline environment have attracted attentions for decades (Gutierrez et al., 2013; Wang et al., 2014). 7 isolates was screened of oil degrading bacteria isolated from Cilacap and Marina Beach, Indonesia. The screening was performed on six PAHs compounds (naphthalene, phenanthrene, fluorene, dibenzothiophene, phenothiazine, and pyrene). Positive PAH degradation in sublimation test was indicated with clear zone formation and/or color change of medium around the isolate. Two isolates (LBF-1-0102 and LBF-1-0103) showed positive in degrading PAH compounds. LBF-1-1-0103 isolate positively degrading 2 (two) PAH compounds, i.e. dibenzothiophene and fluorene, while LBF-1-0102 isolate was potential in degrading of phenanthrene, naphthalene, dibenzothiophene, fluorene, phenothiazine, and pyrene (Figure 1).

Screening of the 7 isolates assayed showed that LBF-1-0102 and LBF-1-0103 are positively capable to degrade PAH compounds. As showed in Table 2, LBF-1-0102 isolate showed better capability in degrading PAH than LBF-1-0103 isolate. This isolate able to degrade both of low molecular weight (LMW) PAH compounds such as; naphthalene, phenanthrene, dibenzothiophene, fluorene, and also high molecular weight (HMW) PAH such as phenothiazine and pyrene. While LBF-1-0103 isolate only able to degrade 2 LMW PAH compounds, i.e.: dibenzothiophene and fluorene. The growth test confirmed the positive result of the sublimation test (Figure 2).

This result indicated that both LBF-1-0102 and LBF-1-0103 isolates use PAH compounds as sole carbon and energy source. In this study, we found that LBF-1-0102 produced a bigger clear zone than LBF-1-0103. LBF-1-0103 isolate showed a positive result in the sublimation and growth test for dibenzothiophene and fluorene, however LBF-1-0102 isolate showed a better color change in the test. LBF-1-0102 started to change the color of ASW medium containing dibenzothiophene and fluorene in day 0. In addition, the color changes were looked clearer and more concentrated in day 5 and day 11 (data not shown). While isolate LBF-1-0103 started to change the color of dibenzothiophene and fluorene-containing medium in day 3. If is assumed that isolate LBF-1-

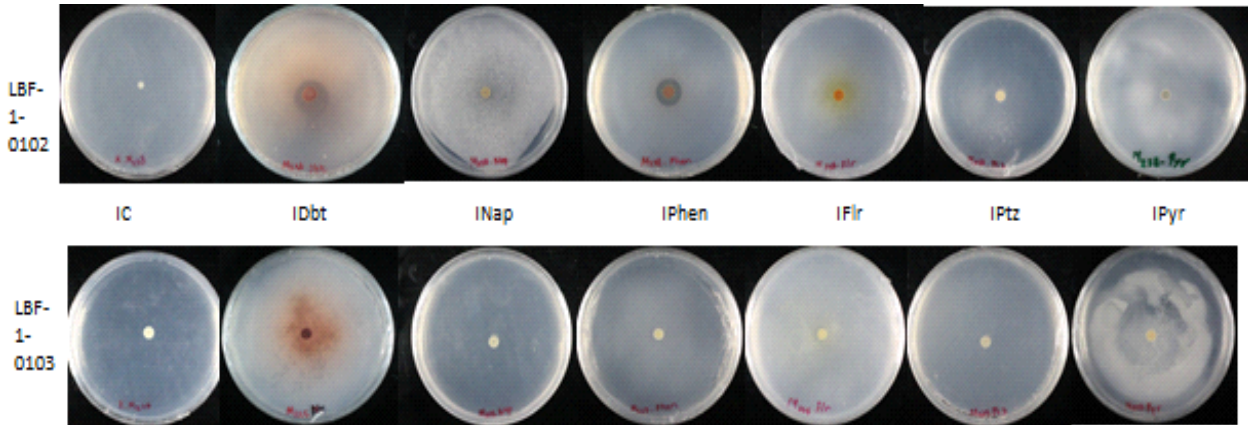


Figure 1. Appearance of Clear Zone and/or Color Change in Biodegradation of PAH Compounds (from left to the right: Control of isolate (IC), isolate on dibenzothiophene (IDbt), naphthalene (INap), phenanthrene (IPhen), fluorene (IFlr), phenothiazine (IPTz), and pyrene (IPyr) by Isolates LBF-1-0102 and LBF-1-0103. The picture was taken on day 7 of the incubation.

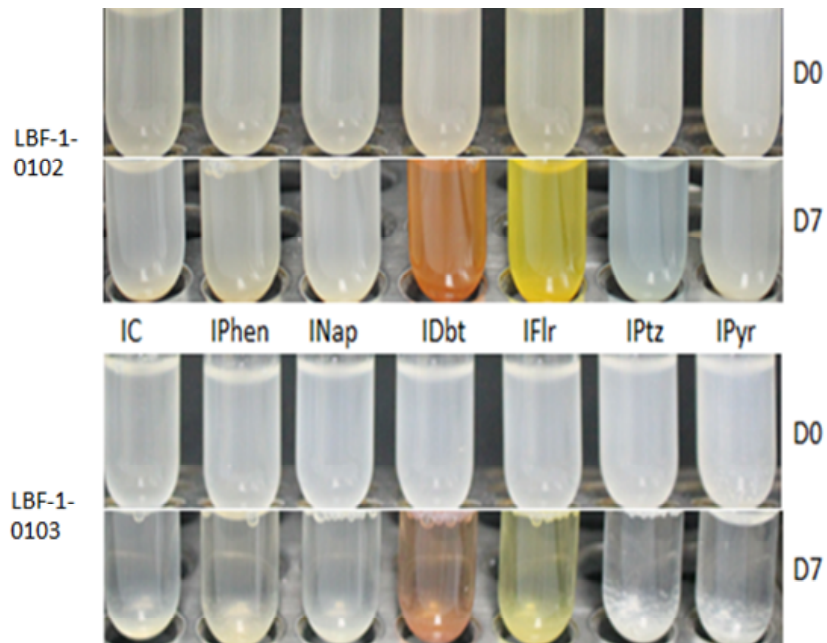


Figure 2. The Growth Test of LBF-1-0102 and LBF-1-0103 Isolates in a medium containing PAH. D0: Day 0; D7: Day 7 of incubation; IC= isolate control; IPhen=isolate culture+phenanthrene; INap=isolate culture+ naphthalene; IDbt= isolate culture+dibenzothiophene; IFlr= isolate culture+fluorene; IPTz= isolate culture+phenothiazine; IPyr=isolate culture+pyrene.

0102 degraded PAH in higher rate than isolate LBF-1-0103. However, in order to confirm this result, it is important to perform further study by quantitatively test the biodegradation using gas chromatography.

### 3.2. Molecular Identification and Phylogenic Analysis

Isolate LBF-1-0102 and LBF-1-0103 were identified based on their 16s rDNA sequence analysis. Approximately, 1,114 bp of LBF-1-102 and LBF-1-0103

gene was sequenced (Figure 3). Isolate LBF-1-0102 was related to *Pseudomonas balearica* BerOc6 (AM421029) with 97% identity, whereas isolate LBF-1-0103 was related to *Brachybacterium* sp. with 98% identity (Ohnishi et al. 2011). The phylogenic tree for LBF-1-1-0102 and LBF-1-0103 are shown in Figure 4.

LBF-1-0102 isolate was identified as *P. balearica* (Figure 4). *P. balearica* LBF-1-0103 was isolated from Indonesian marine sea water. This strain was reported

Table 2. Screening of PAHs biodegrading bacteria

Isolates Code	Sublimation Test						Growth Test					
	Nap	Phen	Dbz	Flr	Ptz	Pyr	Naph	Phen	Dbz	Flr	Ptz	Pyr
LBF-1-00135	-	-	-	-	-	-	-	-	-	-	-	-
LBF-1-0102	+++	+++	+++	+++	+	++	+++	+++	+++	+++	++	++
LBF-1-0103	-	-	++	++	-	-	-	-	++	++	-	-
LBF-1-0105	-	-	-	-	-	-	-	-	-	-	-	-
LBF-1-0116	-	-	-	-	-	-	-	-	-	-	-	-
LBF-1-0119	-	-	-	-	-	-	-	-	-	-	-	-
LBF-1-0120	-	-	-	-	-	-	-	-	-	-	-	-

Note: Nap: Naphthalene; Phen: Phenanthrene; Dbz: Dibenzothiophene; Flr: Fluorene; Ptz: Phenothiazine; Pyr: Pyrene

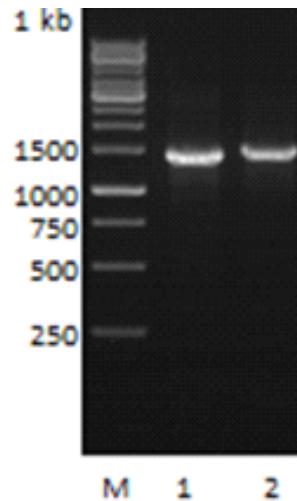


Figure 3. 16s rDNA gene PCR amplification for LBF-1-0102 and LBF-1-0103. 1= LBF-1-0102 and 2=LBF-1-0103.

as naphthalene degrader (Rosselló-Mora et al., 1994; Bannasar et al., 1996). Another paper reported the ability of the strain in methylmercury and tributyltin removal (Lee et al., 2012). LBF-1-0103 isolate was identified as *Brachybacterium* sp. Recently, members in the genus *Brachybacterium* have been reported to be involved in the degradation of hydrocarbons such as alkane (Wang et al., 2010) and naphthalene (Velmurugan & Arunachalam 2009). In addition to that, the genome sequence analysis of this hydrocarbon degradation-isolate has also been done (Wang et al., 2014).

For the next step, analysis of the remaining PAHs after biodegradation process will be carried out by Gas Chromatography (GC). This assay is important to

determine the characterization of these two PAH biodegrading isolates.

#### 4. Conclusion

Based on this study, it can be concluded that isolates LBF-1-0102 and LBF-1-0103 are potential to be used as degrader of PAH compounds. Isolate LBF-1-0102 has capability to degrade naphthalene, phenanthrene, dibenzothiophene, fluorene, phenothiazine, and pyrene, meanwhile isolate LBF-1-0103 is potential to degrade dibenzothiophene and fluorene. From visual monitoring, it is also assume that isolate LBF-1-0102 has better rate to degrade dibenzothiophene and fluorene than isolate LBF-1-0103.

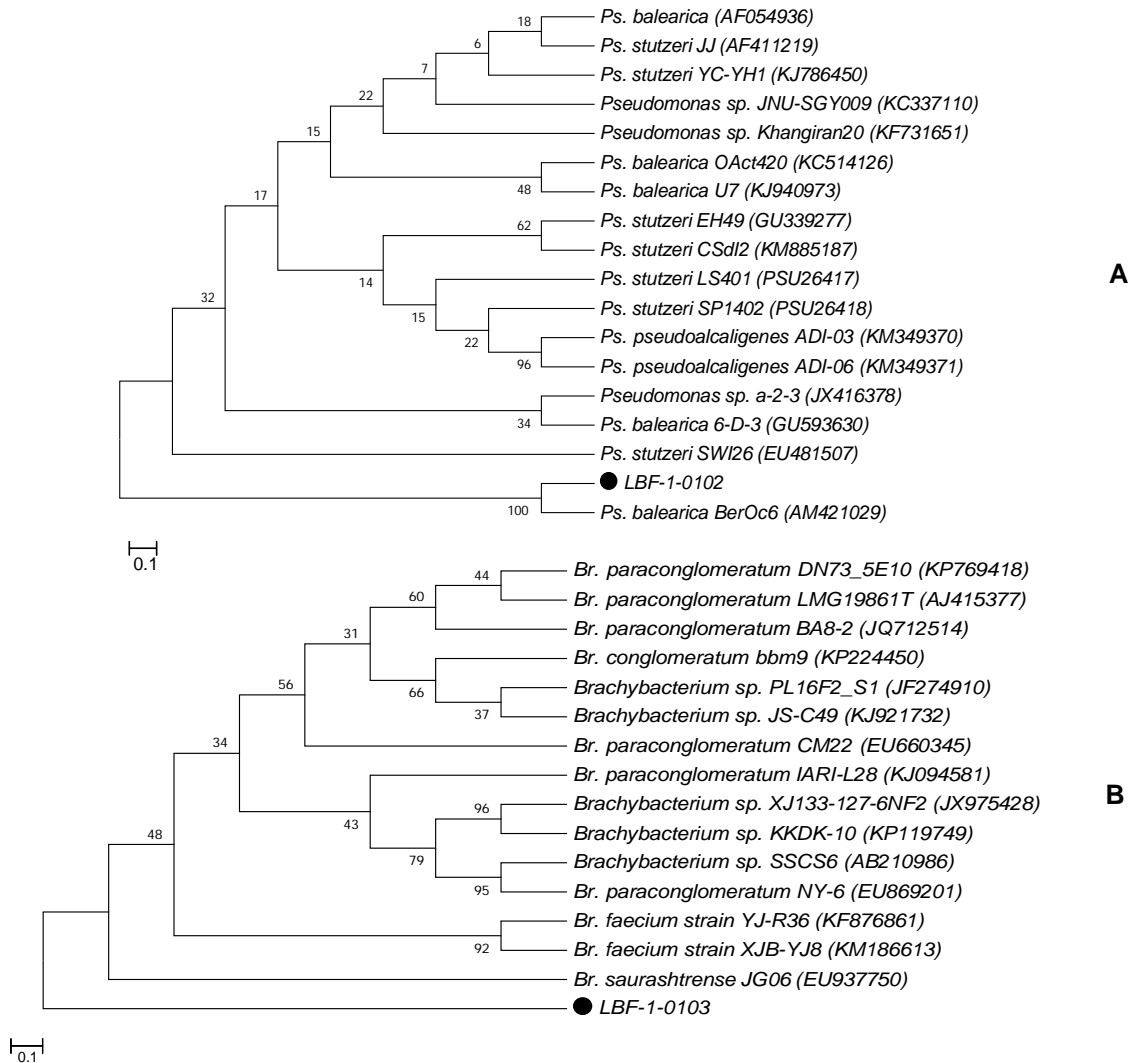


Figure 4. Phylogenetic tree of LBF-1-0102 isolate (A) and LBF-1-0103 isolate. The phylogenetic neighbors obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 3.1 software. Bar, 2% and 5% estimated sequence divergence.

### Acknowledgments

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