Fluorene Degrading Bacteria from Indonesian Marine Environment

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Abstract. Fluorene is one of the Polycyclic Aromatic Hydrocarbons (PAHs) that has toxic, mutagenic, and gives a persistent effect in the environment. One of the alternative ways that can be done to overcome environmental problems due to contamination of fluorene is biodegradation using marine bacteria. There are 25 marine bacterial isolates from Laboratory of Biocatalyst and Fermentation (LBF), Research Center for Biotechnology LIPI Cibinong, Bogor which have capability to degrade fluorene. Selection of these 25 marine bacterial isolates showed that LBF-1-0144 isolate was the good relatively in degrading fluorene. This isolate was able to grow in 1500 ppm fluorene containing medium with an initial cell OD₆₀₀nm of 2.5 and growth percentage of 58.652%. Molecular identification results based on the 16S rDNA gene of the LBF-1-0144 showed that this isolate had similarity of 96% with Bacillus subtilis strain 168.

1. Introduction
Fluorene is the largest PAH compounds of fossil fuels and their derivatives. These compounds can generally be found in the atmosphere, clean water, riverside and marine sediments. Fluorene have high levels of toxicity for fish and water algae [1] and also has mutagenic properties and its carcinogenic [2], therefore, it is important to carry out the effort processing a polluted environment fluorene. Marine bacteria have a role in all biogeochemical cycles and regulation of the balance of the marine environment and utilized in bioremediation of polluted environments. Marine bacteria have a high diversity, but only little information is known. About 90-99% of marine microorganisms cannot be cultured. This is due to physiology, growth, environmental conditions such as unknown physical and chemical factors [3].

Monna et al., [4] reported Staphilococcus auriculans DBF63 able to degrade fluorene on petroleum contaminated soil. Casellas et al., [1] also reported that Arthrobacter sp. strain F101 capable to degrade 92.6% of fluorene. According to Hatmanti and Darmayati [5], Bordetella sp. F2 isolated from the waters of Jakarta Bay is able to use fluorene as a carbon source and has very good growth in the Arabian Light Crude Oil (ALCO). Eriksson et al., [6] reported that Pseudomonas fluorescens that had been isolated from polluted soil could use fluorene and some other PAH’s compounds as carbon sources for growth.
This research aims to obtain marine bacterial isolates that can degrade fluorene based on their growth in media containing fluorene and molecular identification based on the 16S rDNA gene of marine bacterial isolates with the highest ability of life on media containing fluorene.

2. Materials and methods

2.1. Microorganisms, chemicals and media
Isolates used in this study were the collection in Laboratory of Biocatalyst and Fermentation, Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong Bogor, West Java, Indonesia i.e. LBF-1-0010, LBF-1-0013, LBF-1-0022, LBF-1-0027, LBF-1-0030, LBF-1-0060, LBF-1-0062, LBF-1-0102, LBF-1-0103, LBF-1-0114, LBF-1-0118, LBF-1-0127, LBF-1-0128, LBF-1-0129, LBF-1-0130, LBF-1-0131, LBF-1-0133, LBF-1-0134, LBF-1-0135, LBF-1-0136, LBF-1-0137, LBF-1-0138, LBF-1-0142, LBF-1-0143 and LBF-1-0144. The chemicals and media were purchased from TCL Tokyo Kasei (fluorene), BD Difco (marine agar), Himedia (marine broth), PE, PET Japan (artificial sea water).

2.2. Methods

2.2.1. Screening of marine degrading fluorene bacteria. The screening tests of marine bacteria degrading fluorene were conducted by sublimation method [7]. Isolates capable to degrade fluorene confirmed by formation of clear zone or colour change and/or both. The positive isolates resulted on sublimation test were confirmed by growth test on ASW medium (contains of NaCl 22.1g; MgCl$_2$.6H$_2$O 9.9g; Na$_2$SO$_4$.3.9g; KCl 0.61g; NaHCO$_3$. 0.19g; KBr 96mg; Na$_2$B$_4$O$_7$.10H$_2$O 78mg; SrCl$_2$. 13mg; NaF 3mg; LiCl 1mg; KI 81µg; CoCl$_2$.4H$_2$O 0.6µg; AlCl$_3$.6H$_2$O 8µg; FeCl$_3$.6H$_2$O 5µg; Na$_2$WO$_4$.2H$_2$O 2µg; and (NH$_4$6Mo$_7$O$_24$.4H$_2$O 18µg in 1L solution) to know cell amount along incubation time. The initial OD$_{600nm}$ cell was 0.5 and 50 ppm fluorene inoculated on liquid ASW medium with total volume 3 mL incubated on 30°C, 150rpm along 7 days incubation. The sample culture were taken on day 0, 3 and 7 incubation time to be calculate the cell number using spectrophotometer UV-Vis on 600nm. The best growth of isolate used for the next method.

2.2.2. Optimization of fluorene concentration. Isolate with capability for fluorene degrade were screened base on inoculation in various fluorene concentration, i.e. 0 ppm, 50 ppm, 100 ppm, 250 ppm, 500 ppm, 750 ppm and 1000 ppm. The initial cell were OD$_{600nm}$ 0.5 on total volume 3 mL. Inoculums were incubated in 30°C, 150rpm along 7 days incubation. Each culture was sampled every day along incubation to analyze cell number per-day on 600nm using spectrophotometer UV-Vis. Fluorene concentration that showed best growths of marine bacteria were used for next step.

2.2.3. Optimization of marine bacteria cell number. All isolates were inoculated on liquid ASW medium with optimum concentration of fluorene and variation of isolate cell number of marine bacteria i.e. OD$_{600nm}$ 1, 2.5, 5, 10, 15 and 20. The isolates were incubated on temperature 30°C and 150 rpm of agitation within 7 days incubation. Each culture was sampled every day to analyze cell number per-day using spectrophotometer UV-Vis on wave length 600nm.

2.2.4. Molecular identification of marine marine degrading bacteria. Identification of bacteria were conducted by analysis of partial 16S rDNA genes. The genes were amplified from genomic DNA using the universal primer set 27F(5′GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTGTTACGACTT-3′). The thermal cycling parameters were a min, hot start at 95°C, 2 min (1 cycle), followed by 95°C, 2 min (1 cycle); 95°C, 30 sec; 65°C, 1 min; 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 by cyber green staining. The sequences were determine directly using conserved bacterial 16S rDNA sequencing primers by First Base. The sequencing product was analyzed using Basic Local Alignment Search Tool (BLAST) program. The
program was run via internet through the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Result and discussion

3.1. Screening marine bacteria degrading fluorene

Twenty five marine bacteria isolates were sublimed on ASW-agar medium and incubated for seven days on 30ºC. The positive isolates from sublimation test were confirmed by the clear zone and/or colour change formation on the medium (Figure 1). The sublimation was carried out due to insolubility characteristic of fluorene [7]. According to Kumar et al., [8] the positive isolates capable of degrading fluorene will form a clear zone or colour change from colourless to yellow and/or both. The positive isolates resulted were 17 isolates (Table 1 and Figure 1).

The growth test of marine bacteria in 50 ppm of fluorene showed that they have optimum growth in 3rd day of incubation. The result of growth test was shown in the Figure 2. Isolate LBF-1-0144 has relative good growth in 50 ppm fluorene medium among others, it is accordance with sublimation test. This isolate began to form the clear zone on sublimation test on the 3 hour after sublimation was done (Figure 3), so it was used for the next test.

![Table 1](image)

| No | Isolate     | Result | Clear zone | Colour change |
|----|-------------|--------|------------|---------------|
| 1  | LBF-1-0010  | ✓      |            |               |
| 2  | LBF-1-0013  | ✓      |            |               |
| 3  | LBF-1-0022  | ✓      | ✓          |               |
| 4  | LBF-1-0027  | ✓      |            |               |
| 5  | LBF-1-0030  | ✓      |            |               |
| 6  | LBF-1-0060  | ✓      |            |               |
| 7  | LBF-1-0062  | ✓      |            |               |
| 8  | LBF-1-0102  | ✓      |            |               |
| 9  | LBF-1-0114  | ✓      |            |               |
| 10 | LBF-1-0127  | ✓      |            |               |
| 11 | LBF-1-0130  | ✓      |            |               |
| 12 | LBF-1-0133  | ✓      |            |               |
| 13 | LBF-1-0134  | ✓      |            |               |
| 14 | LBF-1-0135  | ✓      |            |               |
| 15 | LBF-1-0137  | ✓      | ✓          |               |
| 16 | LBF-1-0143  | ✓      |            |               |
| 17 | LBF-1-0144  | ✓      |            |               |

3.2. Optimization of fluorene concentration

Optimization of fluorene concentration for isolate LBF-1-0144 were done with the variation of fluorene concentration of 0, 50, 100, 250, 500, 750, 1000, 1500 and 2000 ppm. The isolate has the best growth on the 3rd day of incubation. From Figure 4, it is known that this isolate has 78.13% growth on the 1500 ppm fluorene medium on 30ºC and 150 rpm agitation speed. It is indicate that isolate LBF-1-0144 able to utilize fluorene as carbon source in the medium containing 1500 ppm of fluorene, because the medium has been used is the minimum medium that only contain fluorene as carbon source. According to Mrozik et al., [9], the mechanism of metabolism fluorene by *Pseudomonas* sp. strain F274 produced six major metabolites. Five of them were identified as 9-fluorenol, 9-fluorenone, 1,1-dihydroxy-1-hydro-9-fluorenone, 4-hydroxy-3,4-benzocoumarin and phthalic acid (Figure 5). Meanwhile, on the medium containing 2000 ppm of fluorene this isolate has the low percentage of growth that is 4.492%. The PAHs that are not used as a carbon source by the marine bacteria will accumulated and further become toxic source for it is bacteria [10].
Figure 1. Several visualizations of sublimation test (A) clear zone (B) colour change (C) clear zone and colour change (D) control of fluorene (E) control of isolate.

Figure 2. Growth of the marine bacteria degrading fluorene graphic.
Day 0 (after 3 hours of sublimation test)  
Day 3  
Day 7

**Figure 3.** Visualization of isolate LBF-1-0144 on sublimation test.

**Figure 4.** Optimization of fluorene concentration on isolate LBF-1-0144.

### 3.3. Optimization of marine bacteria initial cell number

Optimization of marine bacteria initial cell number was done with variation of the initial cell number of OD$_{600nm}$ 0, 2.5, 5, 10, 15 and 20. It shown that initial cell number OD$_{600nm}$ 1 dan 2.5 have the best value on 3rd day of incubation, on the other hand the initial cell number of OD$_{600nm}$ 5, 10, and 15 have increased on the 7th day of incubation, and marine bacteria with initial cell number of OD$_{600nm}$ 20 was not growth along incubation day (Figure 6). The percentage of growth in initial cell number OD$_{600nm}$ 2.5 shown the 7th day was lower than the 3rd day, it is indicated in the 7th day of incubation periods the medium was used up by the bacteria, it is inducing some of bacteria may dyed in the 7th day due to substrate reduction.

Isolate LBF-1-0144 with the initial cell number of OD$_{600nm}$ 2.5 growth well on the 1500 ppm fluorene medium with percentage growth of 58.652% on the 3rd day of incubation. In the other hand, the initial cell number of OD$_{600nm}$ 5, 10, 15 and 25 did not have the increasing growth as shown on Figure 6. This is due to the lack of the nutrient in the media for cell metabolism because of the high number of initial cell. According to Atlas [11] more bacteria cell number will cause accelerated of nutrient deficiency. A small concentration of nutrients can be a barrier to microorganisms in carrying out metabolism [12].
Figure 5. Mechanism of metabolism fluorene by Pseudomonas sp. strain F274 [9]

Figure 6. Optimization initial cell number of marine bacteria LBF-1-0144.
3.4. Molecular identification of marine bacteria degrading fluorene

Molecular identification of LBF-1-0144 with PCR 16S rDNA method show that the amplified DNA was purified (Figure 7). Isolate LBF-1-0144 was related to Bacillus subtilis strain 168 with 96% similarity on BLAST program (Table 2). According to Earl et al., [13] Bacillus subtilis founded in various environmental conditions both on land and in the waters. Bacillus sp. is the dominant genus that is able to degrade PAHs in Indonesian marine waters [14] and they can degrade 40% pyren and 50% benzo[a]pyren along 4 day incubation on 30°C [15].

![Figure 7](image.jpg)

**Figure 7.** Amplification of 16S rDNA gene on agarose gel 1% (M) marker 1 kb Ladder Takara (1) LBF-0144 gene and (K) control negative.

| Closest strain           | Accession No   | Max Score | E value | Similarity |
|--------------------------|----------------|-----------|---------|------------|
| Bacillus subtilis strain 168 | NR_102783.1 | 2043      | 0.0     | 96%        |
| Bacillus subtilis strain BCRC 10255 | NR_116017.1 | 2037      | 0.0     | 95%        |
| Bacillus mojavensis strain NBRC 15718 | NR_112725.1 | 2026      | 0.0     | 95%        |
| Bacillus axarguensis strain LMG 22476 | NR_115929.1 | 2026      | 0.0     | 95%        |
| Bacillus mojavensis strain IFO15718 | NR_024693.1 | 2026      | 0.0     | 95%        |

4. Conclusions

Isolate of marine bacteria LBF-1-0144 collection Laboratory of Biocatalyst and Fermentation, Research Center of Biotechnology LIPI has good relativity to growth on the medium containing 1500 ppm fluorene with initial cell number of OD\(_{600}\)nm 2.5 with percentage of growth 58.652% on the 3rd day of incubation. This isolate has 96% similarity with Bacillus subtilis strain 168.

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6. References

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