Biosurfactant-producing and anthracene-degrading bacteria from oil contaminated soil

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Abstract. Anthracene, together with other polycyclic aromatic hydrocarbons (PAHs), is a persistent and toxic soil contaminant because of its low water solubility and high hydrophobicity. The effectiveness of PAH degradation could be enhanced by using biosurfactant. In this study, we isolated bacteria that were able to degrade anthracene and also produce biosurfactant. Samples were taken from oil contaminated soil at Jakarta Bay. Isolation was done in a Minimal Salt Medium (MSM) supplemented with 1% crude oil. Screening methods for biosurfactant production include Blood Hemolysis, Drop Collapse Assay and Oil Displacement Assay. Three isolates which have biosurfactant production and anthracene degradation capability was identified based on 16SrRNA gene. These isolates were identified as Ochrobactrum intermedium AMA9, Ochrobactrum tritici CHA60, and Pseudomonas stutzeri CRA7. Based on GC-MS analysis, Ochrobactrum intermedium AMA9 showed high ability to degrade 84.79% anthracene in liquid MSM medium supplemented by 100 ppm anthracene after seven days incubation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are dangerous contaminants in diverse environments because it has carcinogenic and mutagenic effects [1]. Release of PAHs into the environment can lead to the contamination of the natural resources. Their complex molecular structure and low solubility in water, limit the application of conventional remedial techniques [2].

The biodegradation of PAHs can be considered on one hand to be a part of the normal processes of the carbon cycle, and on the other hand as the removal of man-made pollutants from the environment. The use of microorganisms for bioremediation of PAH-contaminated environments seems to be an attractive technology for restoration of polluted sites [3]. A bioremediation method is considered as an economical and safe approach for the environment [4].

However, PHAs are generally recalcitrant and resistant to microbial attack due to their low solubility and bioavailability. Therefore, these compounds are highly persistent in the environment and are bioaccumulated in organisms [5]. The application of biosurfactants in the remediation of organic compounds, such as hydrocarbons, aims at increasing their bioavailability (biosurfactant-enhanced bioremediation) or mobilizing and removing the contaminants by pseudosolubilisation and emulsification in a washing treatment.

Biosurfactants are a surface active compounds produced by a variety of microorganisms. They are generally composed of a hydrophilic part, consisting of amino acid or peptide anions or cations, mono- or polysaccharides, and a hydrophobic part consisting of saturated, unsaturated or fatty acids [6].
Utilization of biosurfactant-producing and hydrocarbon-degrading microbes could enhance the effectiveness of bioremediation as biosurfactant plays a key role by making hydrocarbons available for degradation. Isolating the bacteria with necessary performance for degradation of organic pollutants such as anthracene in soil and water ecosystems, can be a solution for improving the microbial population in areas contaminated by hydrocarbons. In this study, we investigated the bacterial isolates associated with biosurfactant production and anthracene degradation potentiality. Biosurfactant production by PAHs degrading bacteria may enhance the bioavailability of PAHs in the environment.

2. Methods

2.1. Enrichment culture and isolation of bacteria

Samples were collected from oil contaminated soil in oil contaminated soil at fishing boat harbor, Cilincing area, North Jakarta, Indonesia. A five grams of soil samples were diluted in 250 mL flask with 100 mL of mineral salt medium (MSM) enriched with 0.1% crude oil. This medium containing (g/L): 3.0 g of KH₂PO₄, 6.0 g of Na₂HPO₄, 1.0g of NH₄Cl, 0.5 g of NaCl, 1.0 mL of 1M MgSO₄, and 2.5 mL of a trace element solution (g/L): 23 mg of MnCl₂·2H₂O, 36 mg of CoCl₂·6H₂O, 30 mg of MnCl₂·H₂O, 31 mg of H₃BO₃, 10 mg of CuCl₂·2H₂O, 30 mg of Na₂MoO₄·2H₂O, 20 mg of NiCl₂·6H₂O and 50 mg ZnCl₂ with pH 7.0. Incubation was done on a rotary shaker (100 rpm) at room temperature. After four days of incubation, the samples were inoculated into MSM plate agar with 0.1% crude oil by spread plate method. The isolates were selected based on morphology of colony and were stored as pure culture. Screening for biosurfactants production was done using following screening methods.

2.2. Screening methods for biosurfactant production

2.2.1. Hemolytic activity. The first screening test for identification and isolation of biosurfactant-producing bacteria was the hemolysis test. Each bacterial isolate was streaked on blood agar plates and incubated at 30 °C for 2 days. Hemolytic activity as indicator of biosurfactant production was showed by define clear zone around the colonies [7].

2.2.2. Drop collapse assay. Screening of biosurfactant production through drop collapse assay relies on the destabilization of a liquid drop on the hydrocarbon surface by cell-free extract containing biosurfactant. This method based on Bodour and Miller-Maier by modification [8]. Strains were inoculated in 5 ml of nutrient broth and incubated for 48 hours at 37°C. After incubation, cultures were centrifuge for 20 minutes at 20,000 rpm to remove the cells. The drop collapse test was conducted by using 96-well micro plate. Wells was coated with 2µl of used motor oil and were equilibrated for 1 hour to ensure uniform oil coating. The supernatant of each strain was transferred into the centre of oil on the well. Biosurfactant production was considered positive when the drop diameter was at least 0.5 mm larger than those produced by distilled water as negative controls [9,10].

2.2.3. Oil spreading assay. Twenty mililiter of distilled water was added to 15 cm petri dish followed by addition of 20 µL of crude oil to the surface of the water. A cell free culture broth (10 µL) was then added to the oil surface. If biosurfactant is present in the cell free culture broth, the oil will be displaced with an oil free clearing zone [11]. Distilled water was used as a negative control in which no oil displacement or clear zone was produced and Tween 20 was used as the positive control.

2.3. DNA isolation and identification of bacteria by analysis of 16S rRNA

Molecular identification of bacteria was done by 16S rRNA gene sequence analysis. Bacterial isolates were grown in NB medium for 48 hours. The DNA isolation process was carried out according to the PureLink® Genomic DNA kit (Invitrogen) protocol using 640 µL bacterial culture. The 16S rRNA gene fragment was amplified by PCR using the sets of primer 63f (59-CAGGCC TAA CAC ATG CAA GTC-39) and reverse primer 1387r (59-GGG CGG WGT GTA CAA GGC-39) described by Marchesi et al.
[12]. Amplification was performed in thermocycler with 30 cycles consisting of 94 °C for 15 s, 53 °C for 30 s, and 72 °C for 60 s. The PCR products were analyzed by electrophoresis in 1% agarose gel. All sequences obtained were compared to available databases using the Basic Local Alignment Search Tool (BLAST). The nucleotide sequence was aligned using Bioedit version 7.2.5. The phylogenetic analyses were conducted using Geneious Basic version 5.6.5 software.

2.4. Bacterial growth and anthracene degradation
The concentration of anthracene used is 50 and 100 mg/L. The stock solutions of anthracene were made in acetone. Selected bacterial cultures with a cell concentration of 10^4 cfu/mL were inoculated as much as 5% into a 250 mL flask containing 100 mL MSM supplemented with anthracene as sole carbon source. All flasks were incubated in a rotary shaker at a speed of 100 rpm at room temperature for 7 days. Bacterial growth was observed by taking 100 µL of bacterial culture every 24 hours, then inoculated into a medium Plate Count Agar using the Pour Plate Method. Medium without inoculum was used as negative control. The concentration of anthracene residual in the supernatant was measured using GC-MS (Shimadzu QP2010).

3. Result and discussion
Biodegradation of hydrophobic pollutant such as polycyclic aromatic hydrocarbon is limited due to its poor availability, which is a potential problem of the bioremediation process in contaminated areas. Some microorganisms improve bioavailability of biodegradable organic matter by production of biosurfactants. They help to disperse the hydrocarbons, increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby stimulating the growth of bacteria and the rate of bioremediation [13].

The blood hemolysis, drops collapse assay, and oil spreading assay were chosen as screening methods to detect biosurfactant production due to their simplicity, low cost, quick implementation and use of relatively common equipment. In this present study, three bacterial isolates which were obtained from oil contaminated soil in fishing boat harbor, Cilincing area, North Jakarta, have ability to produce biosurfactant based on screening result. These bacteria could also grow in MSM agar plate supplemented with PAH anthracene as a sole carbon source (Table 1). It is assumed that biosurfactant produced by bacteria facilitated the availability of anthracene so that they can be used to grow.

**Table 1.** Growth of bacterial isolates on MSM agar plate with anthracene.

| No | Isolates | Gram type | Growth in Anthracene Medium | Type of growth* |
|----|----------|-----------|----------------------------|----------------|
| 1  | AMA9     | negative  | +                          | fast           |
| 2  | CHA60    | negative  | +                          | fast           |
| 3  | CRA7     | negative  | +                          | moderate       |

*: positive growth
*) Fast: < 7 days, moderate: 7-14 days, slow: > 14 days

Identification of isolates was done by 16S rRNA analysis using forward primer 63f (59-CAGGCC TAA CAC ATG CAA GTC-39) and reverse primer 1387r (59-GGG CGG WGT GTA CAA GGC-39). The expected size of the fragment sequenced from the 16S rRNA gene was 1300 bp (Figure 1). The identity of the selected biosurfactant-producing and anthracene degrading bacteria based on 16S rDNA gene sequences are shown in Table 2.
Table 2. Identification result of biosurfactant-producing and anthracene degrading bacteria based on analysis of 16SrRNA gene.

| No | Isolates | Species                  | Closest Gene Bank Homolog | Similarity (%) |
|----|----------|--------------------------|---------------------------|----------------|
| 1  | AMA9     | *Ochrobactrum intermedium* | NR_113812.1               | 99%            |
| 2  | CRA7     | *Pseudomonas stutzeri*    | NR_074829.1               | 99%            |
| 3  | CHA60    | *Ochrobactrum tritici*    | NR_114148.1               | 99%            |

Based on the type of growth and performance in screening of biosurfactant production, *Ochrobactrum intermedium* AMA9 was selected for further analysis. *Ochrobactrum* is a Gram-negative, strictly aerobic, catalase and oxidase positive and usually single-cell with rod shape [14]. Several species of this genus are known to be able to degrade polycyclic aromatic hydrocarbon anthracene, phenanthrene and fluorene [15].

The growth of *O. intermedium* AMA 9 bacteria on MSM medium with anthracene 50 and 100 mg/L as a single carbon source was observed in seven days and it showed a similar pattern. The number of bacterial cells (log cfu/mL) increased up to day five and then the growth was begun to decline. The highest cell counts at both anthracene concentrations were reached on day 4 (Figure 2).

Figure 1. Agarose gel electrophoresis of 16S rRNA products (1300 bp): a. AMA9, b. CRA7, c. CHA 60.

Figure 2. Growth of *Ochrobactrum intermedium* AMA9 in liquid MSM supplemented with anthracene.
The ability to grow on anthracene medium was followed by a high capability to degrade that compound. After 5 days of incubation, this strain was able to degrade anthracene to 81.72% at a concentration of 50 mg/L and 88.73% at a concentration of 100 mg/L. Degradation on day 7 was 88.73% at anthracene concentrations of 50 mg/L and 84.79% at concentrations of 100 mg/L (Figure 3). This result was higher than that achieved by *Brachybacterium paraconglomeratum* BMIT637C that was 67.11% on day 8 [16]. Differences in ability may be due to differences in enzyme activity that play a role in the degradation process. Bacteria from the genus Ochrobactrum have a potential as hydrocarbon degrader besides the genera Ralstonia, Burkholderia and Corynebacterium [17]. Furthermore, the *O. intermedium* bacteria in this study have been known to produce biosurfactants which can increase hydrocarbon degradation.

**Figure 3.** Degradation of anthracene in liquid MSM by *Ochrobactrum intermedium* AMA9.

The initial reactions in the degradation of anthracene are catalyzed by multicomponent dioxygenases that incorporate both atoms of molecular oxygen into the PAH nucleus to produce cis-dihydrodiols. *Pseudomonas* spp. and *Sphingomonas yanoikuyae* B1 initially oxidize anthracene in the 1,2 position to form (1R,2S)-cis-1,2-dihydroxy-1,2-dihydroanthracene, which is subsequently converted to 1,2 dihydroxyanthracene. The compound is then converted into 2-hydroxy-3-naphthoic acid, salicylate, and catechol which is degraded into simple aliphatic compounds through the same pathway as naphthalene degradation [18].

### 4. Conclusions
Three strains of bacteria isolated from fishing boat harbor have been determined to produce biosurfactants and also have the ability to degrade anthracene hydrocarbon with *Ochrobactrum intermedium* AMA9 strain showed the highest activity in degrading anthracene. Their ability makes them potential candidates for bioremediation application.

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### References
[1] Kanaly R A and Harayama S 2000 Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria *J Bacteriol.* **182** (8) 2059-2067

[2] Hesham A E, Alami S A, Khan S, Mahmoud M E and Mahmoud H M 2009 Isolation and molecular genetic characterization of yeast strain able to degrade petroleum polycyclic...
aromatic hydrocarbons Afr. J. Biotechnol. 8 (10) 2218-2223

[3] Mrozik A, Piotrowska-Seget Z and Labuzek S 2003 Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons Polish J Environ Studies 12 15-25

[4] Pawar A N, Ugale S S, More M G, Kokani N F and Khandelwal S R 2013 Biological degradation of naphthalene: a new era J Bioremed & Biodeg. 4 (7) 1-5

[5] Simarro R, González N, Bautista L F and Molina M C 2013 High molecular weight PAH biodegradation by a wood degrading consortium at low temperatures FEMS Microbiol. Ecol. 83 438–449

[6] Banat I M, Franzetti A, Gandolfi I, Bestetti G, Martinotti M G, Fracchia L, Smyth T J and Marchant R 2010 Microbial biosurfactants production, applications and future potential Appl Microbiol Biotechnol. 87 427–444

[7] Carrillo P G, Mardaraz C, Pitta-Alvarez S I and Giulietti A M 1996 Isolation and selection of surfactant-producing bacteria World J Microbiol Biotechnol. 12 (1) 82–4

[8] Alnor D, Frimodt-Meller N, Espersten F and Frederiksen W 1993 Infections with the unusual human pathogens Agrobacterium species and Ochrobactrum anthropi Clin Infect Dis. 18 (6) 914-920

[9] Ron E and Rosenberg E 2001 Natural roles of biosurfactants Environ Microbiol. 3 (4) 229-236

[10] Youssef N H, Duncan K E, Nagle D P, Savage K N, Knapp R M and McInerney M J 2004 Comparison of methods to detect surfactant production by diverse microorganisms J Microbiol Methods 56 (3) 339–47

[11] Marchesi J R, Sato T, Weightman A J, Martin T A, Fry J C, Hiom S J and Wade W G 1998 Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA Appl Environ Microbiol. 64 (2) 795-799

[12] Mooney J D, James P F, Daniel R D and Cerniglia C E 2001 Degradation of phenanthrene and anthracene by cell suspensions of Mycobacterium sp. strain PYR-1 Appl Environ Microbiol. 64 (4) 1476