BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBON (PAH), PHENANTHRENE BY MARINE BACTERIUM *THALASSOSPIRA* SP. C.260

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ABSTRACT

Phenanthrene is a polycyclic aromatic hydrocarbon (PAH) compound that is known to be reported toxic to marine flora and fauna. Remediation of this environmental pollutant using chemical and physical methods causes environmental issues. Bioremediation using marine has been applied to degrade such various PAH compounds. Screening of marine microorganism in degrading this recalcitrant is very importance for bioremediation application in Indonesian waters. The purpose of this study was to screen and isolate bacterial with potential application in biodegradation of phenanthrene and other harmful PAH in marine environments. Several potential bacteria strains were isolated from oil contaminated sea water in Cilacap area. Sequence analysis using 16S rRNA gene marine bacterium strain C.260 showed 96% sequence homology to sequence of *Thalassospira* sp. In biodegradation of phenanthrene, within 28 days experiments, this bacterium degraded 50% and 99.75% of phenanthrene in medium with and without enrichment with NPK fertilizer respectively. Using sublimation method, this bacterium also degradeds phenothiazine, fluoranthene, and dibenzothiophene.

Keywords: Phenanthrene, biodegradation, and *Thalassospira* sp.

INTRODUCTION

Phenanthrene is polycyclic aromatic hydrocarbon (PAH) that has three benzene-aromatically chain. Stereochemicaly, phenanthrene is a simplest PAH that previously was applied in modeling of PAH enzymology studies. This compound exposed in contaminated soil (Kim *et al.*, 2005; Supaka *et al.*, 2001; Zao *et al.*, 2009) *as* well as estuarine water. Although it didn't show the mutagenic and carcinogenic effect, this compound was reported toxic to diatom, gastropod, shell, crustacean, and fish (Kiyohara *et al.*, 1994). The Registry of Toxic Effect of Chemical Substances (RTECS) data showed LD50 of this compound in mouse intravenous at 59 mg/kg., and LC50 in the *Artemia salina* was at 677 ìg/l. Although municipal and industrial wastes contribute for PAH contamination in the marine environment, oil spill is the most important source of this recalcitrant. Crude oil and processed oil contained 0.2–7% of PAH compounds (Neff, 1985) Indonesia has high occurrence of oil spill accident in the marine environment that there are six accidents in Kepulauan Seribu in 2004 (jatam.org, 2004). Study on bioremediation of PAH's contamination in the Indonesian sea area must be done urgently.

Phenanthrene could be degraded by soil bacteria through naphtalene pathway. The compound is converted to the oxides form of 1-hydroxy-2-naphtoic acid and subsequently oxides to 1, 2-dihydronaphtalene. This compound was later degraded to form salicylic acid through the naphtalene pathway and enter to the Creb's cycle for mineralization (Kiyohara *et al.*, 1994). Recent study of degradation phenanthrene showed that soil bacteria, *Pseudomonas stutzeri* ZP2, degraded more than 90% of phenanthrene with addition of tween 80 addition could degrade more than 90% at any concentration, ranging from 250–1000 ppm in 6 days (Zhao *et al.*, 2008).

The availabilities of Indonesian microorganisms in degrading polyaromatic hydrocarbon in

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marine environment are very poorly known. In this work, we investigated the potential of Indonesian marine bacteria in degrading phenanthrene by adding NPK fertilizer to stimulate the bacteria growth. NPK fertilizer is a local cost plants fertilizer and could be obtained easily in Indonesia.

MATERIALS AND METHODS

Sea waters with oil contamination were collected from Cilacap coastal waters. The samples were enriched using ONR-7 media and polycyclic aromatic hydrocarbons, (phenanthrene, fluoranthene, and phenothiazine at concentration 1000 ppm) and Cepu crude oil. The composition of ONR-7 in 1 L media was showed by Table 1. Media for isolation and preservation were 10% Marine Agar (Difco) (Table 2), 15 agar in 1L distilled, water and 200 ppm 200il Arabian Crude Oil. 100% SWP medium contained 0.1g NH4NO3, 0.02g K2HPO4, and 0.002 Fe(III) cytrate were used for growing bacteria in biodegradation analysis rate.

"Grow more" USA NPK fertilizer with ratio nitrogen:phospor:kalium (20:20:20) was used for stimulating bacterial growth. Polycyclic aromatics hydrocarbons used in this research were phenanthrene, dibenzothiophene, fluoranthene, phenothiazine, and fluorene purchased from Wako pure chemical.

Phenanthrene concentration in study was determined using Gas Chromatography Shimadzu type GC 17 A (Company, Country). PCR PC-818 Astec, Electrophoreses Mupid-exu Advance, centrifuge, and transluminator for 16S ribosomal RNA were used for gene analysis

Table 1. Composition of 1L ONR-7 media

Ingredients	Grams
NaCl	22.79g
Na2SO4	3.98g
TAPSO {3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid}	1.3g
KCl	0.72g
NH4Cl	0.27g
Na2HPO4.7H2O	89 mg
NaBr	83 mg
NaHCO3	31g
H3BO3	27g
NaF	2.6g
CaCl2.2H2O	1.46g
MgCl2.6H2O	11.18g
SrCl2.6H2O	24mg
FeCl2.4H2O	2mg

Screening and Isolation of the Potential Bacteria

The potential PAH degrading bacteria were isolated from Cilacap sea water contaminated area. Approximately 4 ml sea water sample was enriched in ONR-7 media with 3 kinds of PAHs, arabian crude oil, and Cepu crude oil (each PAH/crude oil in each tube containing 4 mL ONR-7 medium). Cultures were incubated at room temperature in rotary shaker incubator. After 2 weeks of incubation, an aliquot of each culture solution spread on agar media and laid on phenanthrene, fluoranthane, and phenothiazine vapor using sublimation method (Alley & Brown, 2000). Those PAH powder were put on the glass plate and stained on the hotplate at 90–95°C ,5 minute for phenanthrene, 105°C in 5 minute for fluoranthene, and 135°C in 30 minute for phenotiazin. Each of agar plate was laid to catch those PAH vapor with ice cooler for sublimation. The growth of *Thalassospira* sp. C.260 was determined using Spectronic 21D Milton Roy. The DNA concentration was determined using Spectrophotometer UV-visible-1700 Shimadzu.

After one week incubation, the potential bacteria appeared by clear zone around the colony. Bacteria colony was isolated and purified for further experiment. The purified colonies were screened for the PAH (phenanthrene, fluoranthene, dibenzothiphene, and phenothiazine)

Table 2. The composition of 10% Marine Agar (Difco)in 1L media

Ingredients	Grams
peptone	0.5g
yeast extract	0.1g
Ferric citrate	0.01g
NaCl	1.945g
MgSO4	0.324g
CaCl2	0.18g
KCl	0.055g
KBr	0.008g
NaHCO3	0.016g
SrCl2	3.4mg
H3BO3	2.2mg
NaSiO2	0.4mg
NaF	0.24mg
NH4NO3	0.16mg
Na2PO4	0.8mg

bio-degradation using sublimation methode. Preservation of purified potential bacteria was done using glycerol stock method with 1/10 Marine Agar medium contained Arabian crude oil.

The Characterization of Potential Bacterium

The potential strain CECC-Ft1.14 was characterized using analysis of 16S ribosomal RNA gene. DNA extraction was done using instagene matrix reagent with the following method. Spectrophotometer UV-visible-1700 Shimadzu for determining DNA concentration. Approximately 1 loop of bacterial colony was put on 250 il sterile MiliQ water and centrifuged at 12000 rpm. Pellet was dissolved using 50 il instagene reagents, followed by incubating this solution at 56°C during 45 minute. Solution was mixed at high speed vortex for 10 minutes, and incubated at 100°C for 8 minutes to heat sock the DNA. DNA template was collected by centrifugation of the solution at 12,000 rpm for 5 minutes at 4°C. DNA was kept at -20°C before further experiment. PCR reaction was carried out with PCR mixtures containing 2 il DNA template, 25 il Gotag, 0.5 il primer 9F, 0.5 il primer 1510R, and 22 il miliQ water. The 16S rRNA gene was amplified using forward primer 9F (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer 1510R (GGCTAGGTTGTTACGACTT-5') (reference). The PCR cycles was set as 95°C, 2 minutes (1 cycle); 95°C, 30 second; 65°C, 1 second; 72°C, 2 minutes (10 cycle); 95°C, 30 second; 55°C, 1 minute; 72°C, 2 minutes (30 cycle); and 72°C, 2 minutes (1 cycle). PCR product was detected using gel electrophoreses at 100 mA with 1% agarose in phosphat buffer. Etylene bromide solution was used for visualling DNA band. The 16S rRNA gene was purified and sent for sequencinged. Sofware bioedit was used for editing the raw data sequence comparison using Blast NCBI database. Phenanthrene concentration in this study was determined using Gas Chromatography Shimadzu type GC17-A.

Cultivation for Determining Phenanthrene Biodegradation Rate

The selected strain was precultured into 4 ml of 100% Marine Broth contained Arabian crude oil and incubated at 30° C for 2 days. Approximately 1.5 ml of the precultured solution was poured on the 150 ml liquid artificial sea water (swp) media contained A: 150 ppm phenanthrene, B: 500 ppm phenanthrene and "Grow More" (NPK) fertilizer, and C: control medium, NPK and without inoculating the potential bacteria. Each treatment was carried out in triplicate for data validation. Cultures were incubated at 30°C for 1 month in rotary shaker incubator. Growth was checked and monitored weekly by determining the liquid absorbance at ë 660 nm using spectrophotometer. Determination of phenanthrene biodegradation rate was done by harvesting approximately 5 ml culture solution at 0 and 28 days cultivation.

Quantitative Analysis of Phenanthrene Degradation Rate

The harvesting culture at 0 and 28 days cultivation was extracted using dichloromethane. Organic phase that containing phenanthrene was evaporated and dried using nitrogen gas. Approximately 1 ml of dichloromethane was added to the dried extract for quantitative analysis. The quantitative analysis of phenanthrene biodegradation was done using Gas Chromatography. The Gas Chromatography condition was set as followed: temperature injection was 240°C, detector FID (with temperature 300°C), column TR-50MS (containing 50% phenyl polysilphenylene-siloxane) with 0.25micrometer im film thickness, 30 m length, 0.32 mm ID. Column temperature is set as follows: 0, 60, 120 with temperature increasing rate 20°C/min, hold time 15 min. Column flow was 6,01 ml/min, total flow 83 ml/min. Gas carrier was nitrogen with pressure 146 kPa.

RESULTS

Screening and Isolation of the Potential Bacteria

One of the potential marine bacterium, C.260 strain isolated from Cilacap contaminated sea water showed potential to degrade phenanthrene, was selected in this study. This bacterium was isolated using "Cepu crude oil" enrichment method and isolated on SWP agar media that was vaporized with fluoranthene (After enriched with cepu crude oil during one week, the solution was spread on agar plate and vaporized using fluorathene).This bacterium has clear white bone colony that could degrade phenanthrene, as well as phenotiazine, flouranthene, and dibenzothiophene (Figure 1).

Figure 1. The colony profile of C.260 in 20% marine agar medium containing Arabian Crude Oil for preservation

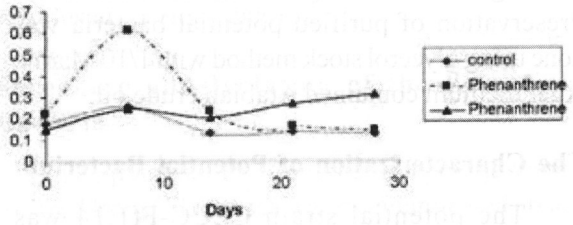
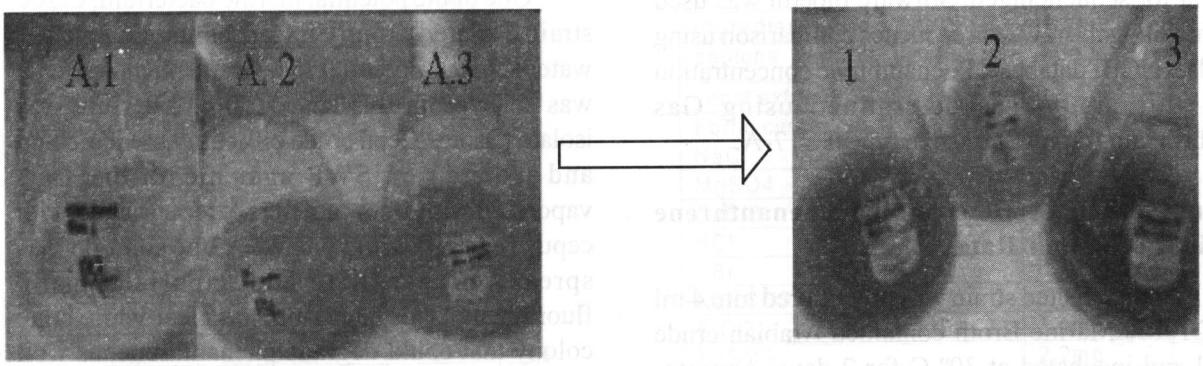


Figure 2. The Growth of C.260 in phenanthrene substrate.

Cultivation for Determining Phenanthrene Biodegradation Rate

Phenanthrene biodegradation rate was determined using very minimum nutrient (only artificial sea water/swp) with phenanthrene as a sole energy and carbon source. This medium was designed for conditioning bacterium to produce oxygenase as the primary enzyme in the phenanthrene or PAH metabolism. The growth of bacterium C.260 in phenanthrene as a substrate was detected using visible spectrophotometer method to determine absorbance at 660 nm (Figure 2). Growth of C.260 in phenanthrene as a substrate was added with and without "Grow More" Fertilizer. The square line (-. o.-) indicated growth of C.260 in phenanthrene substrate with NPK fertilizer enrichment while triangle line (-²%-) presented bacterial growth in phenanthrene substrate without fertilizer. Compare with culture without NPK fertilizer, culture with NPK enrichment showed highest in biomass productivity. The visualization of phenanthrene biodegradation in the fermentation flask is presented in Figure 3.

The color of C.260 liquid fermentation changed after 28 days cultivation. Figure 3 (A) showed fermentation profile at 0 day cultivation and Figure 3 (B) is after 28 days cultivation. The white clear solution in flask 3. A1 contained SWP media without enriched with NPK fertilizer. Figure 3. A 2 was control media with NPK fertilizer without inoculums and Figure 3 A 3 was inoculums C.260 with NPK fertilizer. Color of flask 3. A 1 changed to the red- brown after 28 days cultivation (Figure 3. B 1) while, inoculum with NPK (A 3) changed to the green color (Figure B.3). No changes were observed in control media (Figure 3.B2).



A. fermentation at 0 day., 1:C.260 without NPK, 2 control.3:C.260 with NPK

B. fermentation at 28 day., 1:C.260 without NPK, 2 control.3::C.260 with NPK

Figure 3. Fermentation profile of C.260 at 0 day cultivation (A) and after 28 days (B).

Quantitative Analysis of Phenanthrene Degradation rate

Quantitative analysis using gas chromatography is shown in Figure 4. Amount of phenanthrene remaining in the fermentation solution at 0 day cultivation and after 28 days cultivation was presented by gas-chromatogram. Peak of phenanthrene appeared at 24.3 minutes in gas chromatogram. Figure 4 described the profile of gas chromatogram of remaining phenanthrene in 2 experiments. Experiment I was cultivating C.260 in medium SWP without NPK, while experiment II was adding NPK in SWP medium. In experiment I (Figure 4.A) concentration of phenanthrene at 0 day was 149 p.p.m (intensity ±3000000) and the remaining phenanthrene is at 75 p.p.m after 28 days cultivation (intensity ±1500000). Phenanthrene degradation efficiency in experiment I was approximately 50% after 1 month cultivation.

Experiment II (Figure 4.B) showed that in the beginning (0 day cultivation), the phenanthrene concentration was 514 p.p.m, and after 28 days the remaining phenanthrene was 1 p.p.m, Close to 100% of phenanthrene could be degraded in culture with NPK enrichment.

The Characterization of Potential Bacterium

Polymerase chain of reaction product of 16S ribosomal RNA gene was showed in Figure 5. The size of 16S rRNA gene of CECC-Ft1.14 was 1,500 bp. This PCR product was purified and used for sequencing. Using Blast database, 16S ribosomal RNA gene sequence of isolated bacteria was 96% (1312/1364 base) similar with *Thalassospira lucentensis* strain QMT2. The comparing data of sequences was shown in Table 3.

Table 3 showed that symbol of dash (I) explains the similarity of sequences. Analyzed data showed that this potential bacterium was identified as *Thalassospira* sp.

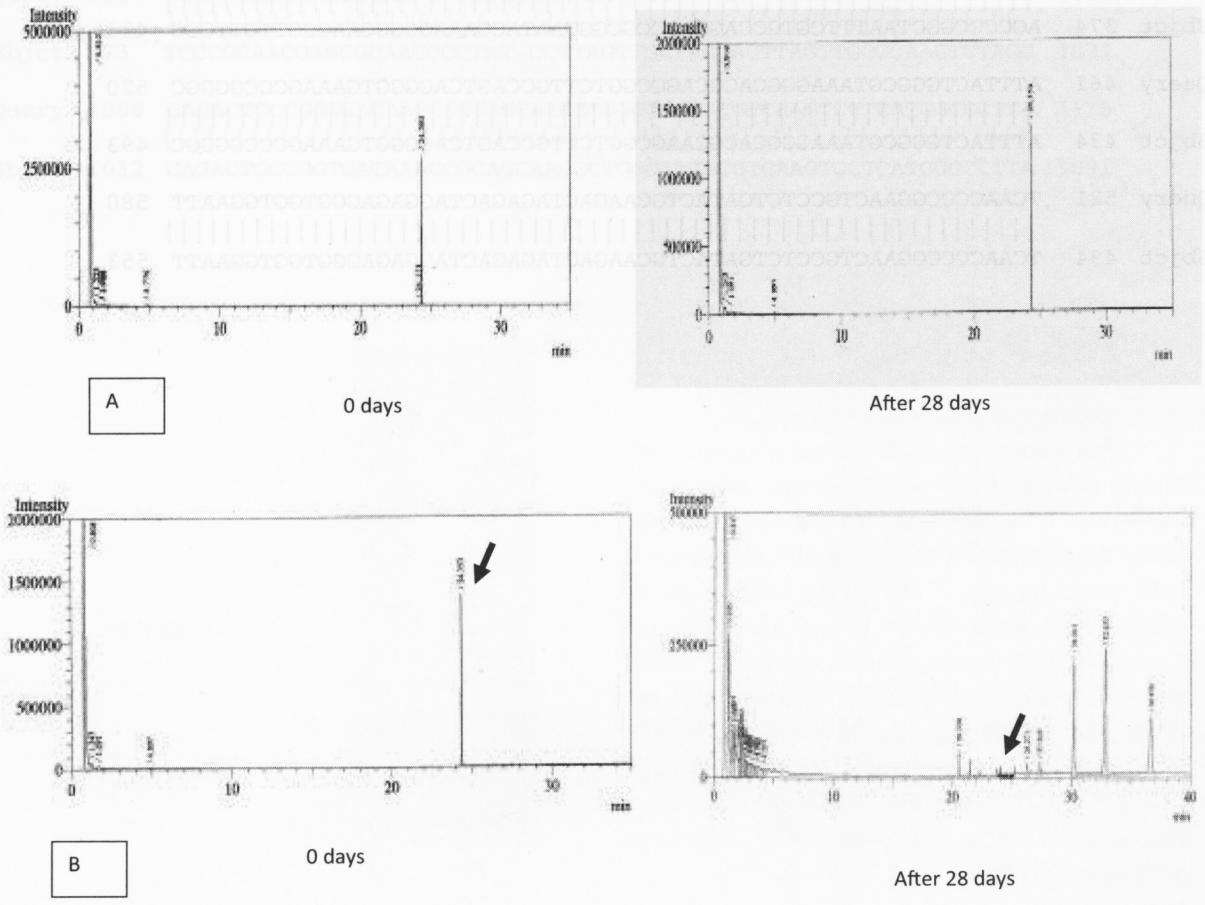


Figure 4. Gas chromatogram of phenanthrene remaining in C.260 fermentation without NPK fertilizer (A) and with NPK (B).

Table 3. showed that symbol of dash (I) explain the similarity of sequences. Analyzed data showed that this potential bacterium was identified as *Thalassospira* sp.

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Query  41   GAGTGGCGCACGGGTGAGTAACGCGTGGGGACCTACCTCTTAGTGGGGGATAACGGTTGG  100
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The previous research by Xu *et al.* (2004) also showed that the presence of fertilizer osmocote (containing N & P mineral) was able to accelerate the biodegradation of aliphatics and PAHs in oil contaminated sediment under natural field condition in an intertidal shore environment. Based on their experiment, they reported that the PAHs with ring number from two-to six in osmocote treated were respectively 2.71 ; 2.48; 3.2; 2.14; and 2.20 fold which was higher than control without osmocote.

The Characterization of Potential Bacterium

The homology of 16S ribosomal RNA sequences of marine bacterium C.260 was 96% comparing to the *Thalassospira lucentensis*. Approximately, 96 similarity of sequences indicated marine bacterium C.260 was the same genus and different species with *Thalassospira lucentensis*. The further analysis is recommended to know that this bacterium is a novel species.

Finally, this research showed that by enriching NPK to the medium cultivation of *Thalassospira sp.,* the efficiency of phenanthrene biodegradation by this bacteria increases from 50% (without NPK) to 99.75 % (with NPK) during 28 day cultivation.

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