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Selection and Identification of Polyaromatic Hydrocarbon Degrading Bacteria

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Abstract: There are many industries which produces large amount of poly-aromatic hydrocarbons (PAHs). Bioremediation of PAHs contaminated environment needs to be done because of the carcinogenic and mutagenic properties of PAHs. Five marine bacterial isolates from Indonesian territory which had high potential for PAHs (phenanthrene and pyrene) degradation were analyzed. Five strains, named M2292, M128, C318, C19 and C15, have been identified by 16S rDNA sequence analysis and were identified as *Ochrobactrum oryzae* (99% homology with NR_042417.1), *Bacillus subtilis* (99 % homology with HQ851067.1), *Bacillus subtilis* (99 % homology with JN587510.1), *Bacillus subtilis* (100 % homology with JN587510.1) and *Bacillus pumilus* (99% homology with JN315777.1), respectively. The initial dioxygenase genes of the five PAH-degrading bacteria were investigated and revealed that all the isolates possessed the *nidA* and *nahAc* gene encoding the initial dioxygenase required for pyrene and phenanthrene degradation.

Key words: PAHs degrading bacteria · Dioxygenase · Phenanthrene · and Pyrene

INTRODUCTION

Economic growth in developing countries have many effects, such as increasing oil exploitation as one energy resource and also increasing growth of other industries, such as paper, pesticide and petrochemical industries. This kind of industries produce large amount of poly-aromatics hydrocarbons (PAHs) [1]. PAHs are toxic environmental pollutants that are known or suspected carcinogens or mutagens. Pyrene represents one of most abundant high-molecular weight PAHs and biodegradation of HMW-PAHs compound with four or more rings, such as pyrene, are more difficult than low molecular weight PAHs (LMW-PAHs) [2].

Study of complete and integrated biochemical pathways of pyrene degradation shows phenanthrene to be the most intermediate product of degradation [3, 4]. Unlike HMW-PAHs, phenanthrene does not pose a risk to human health, since phenantrene exhibit no genotoxic or carcinogenic effect, but it has toxic effect to fish and algae [1].

The biodegradation ability and dioxygenase gene of PAH-degrading bacteria were investigated and most of the potential isolates belonged to the genera of *Pseudomonas*, *Sphingomonas*, *Mycobacterium*, *Rhodococcus*, *Burkholderia*, *Arthrobacter* [5, 6]. The ability of *Bacillus* genera to degrade PAHs was also reported. *Bacillus pumilus* and *Bacillus subtilis* are able to grow and utilize efficiently PAHs such as naphthalene, phenanthrene, fluoranthene and pyrene [7]. Previous researchers also reported capability of *Bacillus cereus* to degrade PAHs pyrene [8].

To investigate dioxygenase gene involving in PAHs degradation, another previous researchers was screened and the presence of three bacterial ring-hydroxylating dioxygenase genes were confirmed. The *ndoB*, *phnAc* and *nidA* gene was used for naphthalene, phenanthrene and pyrene degradation respectively [9]. The other researchers have suggested the usage of *nidA* and *nahAc* gene that are prevalent in the PAH degrading bacteria. The *nidA* and *nahAc* were useful for determining the present of PAH dioxygenase gene [10].

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In Indonesia, there are many industrial areas produces large amount of PAHs which expose to the environment and affects human health and aquatic ecosystems and become a severe hazard. Therefore, there is an urgent requirement to remove PAHs contamination from the environment. One of the most effective and efficient way to remove this contamination is bioremediation by using selected microorganism locally isolated from Indonesian territorial that have high capability to degrade PAHs.

The present study aims is done for isolation and genetic identification of indigenous PAHs degrading bacteria and further is done for searching of the responsible dioxygenase genes for PAH degradation in those bacterial isolates.

MATERIALS AND METHODS

Isolation of Microorganisms: Microorganism used in this study, M2292, M128, C318, C19 and C15, were isolated from marine area of Indonesia. The strains were collected to Research Center for Biotechnology, Indonesian Institute of Science.

Culture Media: Growth of M2292, M128, C318, C19 and C15 strains on pyrene and phenantrene were determined in solid medium with composition (L^{-1}) 20 g nutrient agar, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 1 ml MgSO4 1 M and 2.5 ml micro-element that contained (L^{-1}) 53 mg $MnCl_4 H_2O$, 31 mg H_3BO_3, 36 mg $CoCl_2$ $6H_2O$, 10 mg $CuCl_2$ $2H_2O$, 30 mg Na_2MoO_4 $2H_2O$ and 50 mg $ZnCl_2$. The pH of this medium was adjusted to 7.0 and the solution was sterilized in high pressure sterilizer for 20 min at 121°C. The ethanol solution of 20 mg/L pyrene and phenanthrene was added to sterilize mineral salt solution and the solution could be used after the ethanol had been completely volatilized.

Selection of Bacteria: Five isolates, M2292, M128, C318, C15 and C19, were spread on agar plate contained 20 mg/l pyrene and phenanthrene. The growing strains were selected as the candidate of pyrene and phenanthrene degrading bacteria.

Identification of Bacteria: In order to identify M2292, M128, C318, C19 and C15 strains, 16S rDNA gene sequence analysis was carried out. Genomic DNA extraction was done by using Miobio-laboratories kit. The 16S rDNA gene fragment was amplified by PCR using the set of primer: 16S-F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 16S-R (5'-CGGCTACCTTGTTACGACTTC-3'). The PCR conditions (35 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C) were performed with PCR system 9700 from Applied Bio-system. The PCR product were then purified and linked to the pMD20 vector. The DNA sequence of the positive clone with 16S rDNA gene fragment was compared with the available database (Genbank) using the BLAST program at the National Center for Biotechnology Information (NCBI) website.

Table 1: PCR Primer for the detection of initial PAH-degrading dioxygenese gene [10]

Primer	Sequence
nidA - first PCR	Forward primer:
	Nid-for. TCCRMTGCCCDTACCACGG
	Reverse primer:
	Nid-rev1 GAASGAYARRTTSGGGAACA
nidA - nested PCR	Forward primer:
	Nid-for. TCCRMTGCCCDTACCACGG
	Reverse primer:
	Nid-rev2 GCGSCKRKCTTCCAGTTCG
*nahA*c - first PCR	Forward primer:
	Nah-for TGCMVNTAYCAYGGYTGG
	Reverse primer:
	Nah-rev 1 CCCGGTARWANCCDCKRTA
nahAc- nested PCR	Forward primer:
	Nah-for TGCMVNTAYCAYGGYTGG
	Reverse primer:
	Nah-rev 2 CRGGTGYCTTCCAGTTG

Detection of PAH-Degrading Dioxygenase: Bacterial DNA was extracted by the bacterial DNA extraction (Miobio-laboratories kit). The presence of the initial dioxygenase gene was detected base on PCR amplification. The primer for detection of *nahAc* and *nidA* in M2292, M128, C318, C19 and C15 were listed in Table 1.

Components for the PCR were 5 µL buffer, 4 µL dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µL DNA template, 0.25 µL Ex Taq DNA polymerase and sterile distilled water to a final volume 50 µL. The PCR cycle condition were 94°C for 3 min, then 40 cycles (first PCR) or 30 cycles (nested PCR) of 94°C 45 s, 55°C 45 s and 72°C 45 s and final extension at 72°C for 5 min.

PCR amplification of *nidA* and *nahAc* gene was performed using genomic DNA of M2292, M128, C318, C19 and C15 strains as template. The first round PCR products was used as template for second PCR. The first and second round PCR products were analysed by electrophoresis in 2 % agarose gel.

RESULTS

Selection of Bacteria: Strains which have potential for pyrene and phenanthrene degradation are listed in Table 2 and 3.

The M2292 strain found to be grown fastest on solid medium containing phenanthrene and the colonies were visible within 24 hours (Figure 1) followed by M128, C318, C19 on solid medium containing both pyrene and phenanthrene within 48 hours.

Identification Bacteria: Genome extraction from Isolate M2292, M128, C318, C15 and C19 were done by using Mio Bio Laboratories Kit (give the company's name). The extracted genome from this isolate have molecular weight for about 19329 bp (Figure 2A). PCR amplification of 16S rDNA gene was performed using genomic DNA of strains M2292, M128, C318, C15 and C19 as template. The PCR products, about 1489 bp, were separated by electrophoresis in a 1% agarose gel. The length of the DNA fragment was in accordance with primer designing region size as shown in Figure 2B.

To verify ligation process of 16S rDNA from strains M2292, M128, C318, C19 and C15 to pMD20 vector had been done correctly, blue-white selection and PCR screening method has been carried out, Figure 3.

The bands at about 1489 bp represent positive clone of *E. coli DH5α* was inserted with 16S rDNA from M2292, M128, C318, C19 and C15 strains.

To identify whether the correct gene inserted in the plasmid, cutting plasmid was done by using *AvaI* and *HindIII* restriction enzymes (Figure 4).

After cutting by using *AvaI* and *hindIII* restriction enzymes, it was found the correct inserted DNA at about 1489 bp and 2800 bp bands. The 1489 bp bands represent 16S rDNA from M2292, M128, C318, C19 and C15 strains and the 2800 bp bands represent the DNA of pmD20 vector. The Polymerase chain reaction (PCR) was performed for sequencing the 16S rDNA of M2292, M128, C318, C19 and C15 strains. The sequence was submitted to the GenBank data base to search for similarity with the sequences of other bacteria. Table 4 shown 16S rDNA sequence analysis results of those strains compared with other bacteria.

The M2292 strain has high potential ability for phenanthrene degradation, has 99 % similarity to *Ochrobactrum oryzae* with accession no. NR042417.1, but *Ochrobactrum oryzae* NR042417.1 capability for phenanthrene degradation has not reported yet.

Table 2: Capacity of strains to grow in solid medium containing 20 mg/l pyrene

	Growth in solid media contains pyrene		
Strains	24 h	48 h	72 h
M2292	-	-	+
M128	-	+	++
C318	-	+	++
C19	-	+	++
C15	-	-	+

Table 3: Capacity of strains to grow in solid medium containing 20 mg/l phenanthrene

	Growth in solid media contains phenanthrene		
Strains	24 h	48 h	72 h
M2292	+	++	+++
M128	-	+	++
C318	-	+	++
C19	-	+	++
C15	-	-	+

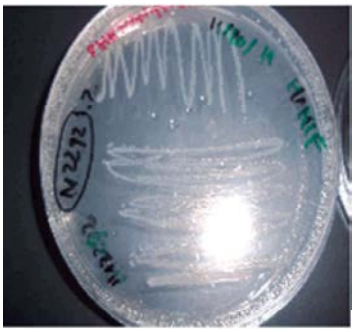

Fig. 1: Strain M2292 grew on solid medium containing phenanthrene

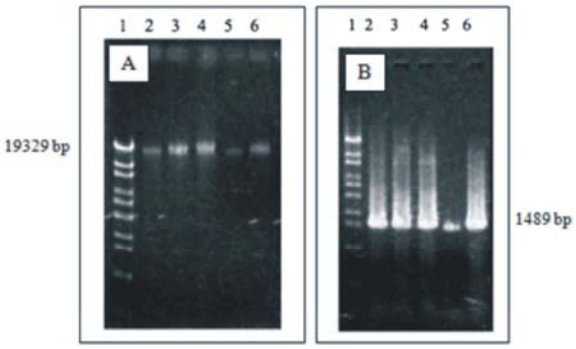

Fig. 2: [A].Genome of potential strains (±19329 bp); [B]. 16S rDNA potential isolate (±1489 bp). (1. standard, 2. C19, 3. M128, 4. C318, 5. M2292, 6. C15).

Previous researcher [11] reported at GenBank database that the *B. subtilis* strain K21 16S ribosomal RNA gene with acession no. JN587510.1 has antimicrobial

Table 4: Five PAH-degrading bacterial strains isolated from Indonesian territorial

Bacterial strains	Bacterial name (% Similarity)	GenBank accession No.
M2292	*Ochrobactrum oryzae* (99%)	NR_042417.1
M128	*Bacillus subtilis* (99%)	HQ851067.1
C318	*Bacillus subtilis* (99%)	JN587510.1
C19	*Bacillus subtilis* (100%)	JN587510.1
C15	*Bacillus pimilus* (99%)	JN315777.1

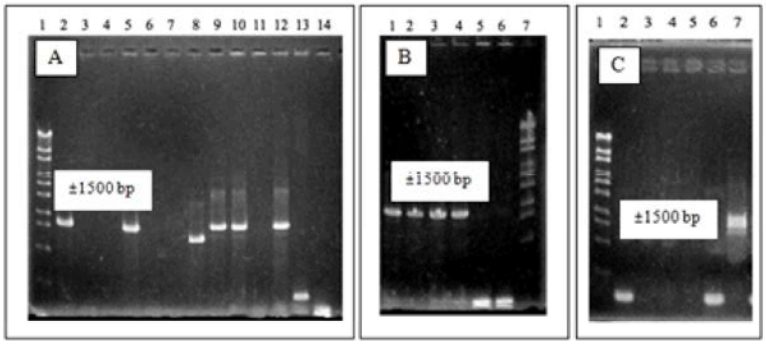

Fig. 3: Selection positive clone with 16S rDNA gene fragment using PCR screening. [A]. (1. Std. λ, 2. M2292-1, 3. M2292-2, 4. M2292-3, 5. M2292-4, 6. M2292-5, 7. M128-1, 8. M128-2, 9. M128-3, 10. M128-4, 11. C318-1, 12. C318-2, 13. C318 no 3, 14. M128-4); [B]. (1. C15-1, 2. C15-2, 3. C15-3, 4. C15-4, 5. C19-1 6. C19-2, 7. Std. λ); [C]. (1. Std. λ, 2. C19-1, 3. C19-2, 4. C19-3, 5. C19-4, 6. C19-5, 7. C19-6)

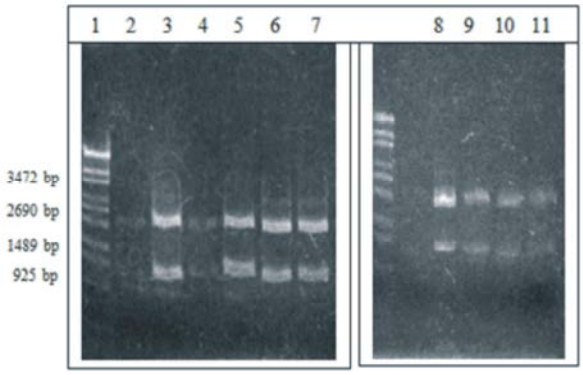

Fig. 4: Cutting Plasmid using *Ava I* and *Hind III* (1.Std. λ, 2.M2292-1, 3.M2292-2, 4. M128-1, 5.M128-2, 6.C318-1, 7.C318-2, 8.C19-1, 9.C19-2, 10.C15-1, 11.C15-2)

properties, but *B. subtilis* C318 and *B. subtilis* C19 wich has 99 % and 100 % similarity with JN587510.1 respectively show the different activity. Our result reveals that the *B. subtilis* M128 and C19 has capability for PAHs degradation.

The M128 strain also have capability for PAHs degradation, it has 99 % similarity to thermophilic bacteria *B. subtilis* strain NBY44 16S ribosomal RNA gene with accession no. HQ851067.1 which reported at GenBank database [12]. But this information has not reported that their strain has capability for PAHs degradation.

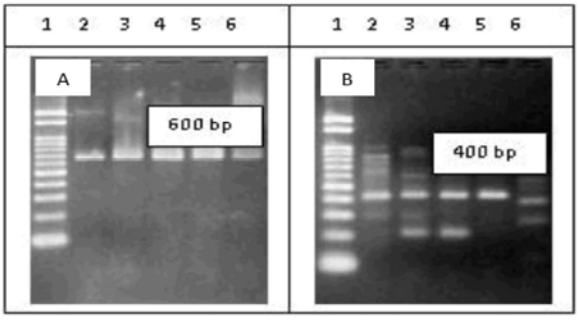

Fig. 5: Nested PCR product,1.Standard, 2.M2292, 3. M128, 4.C318, 5.C19, 6.C15. [A] *nidA* gene (600 bp); [B] *nahAc* gene (400 bp).

Strain C15 has potential for PAHs degradation, this strain has 99 % similarity to *B. pumilus* strain AUCAB16 with acession no. JN315777.1, but *B. pumilus* AUCAB16 capability for PAHs degradation has also not reported yet.

Detection of PAH-Degrading Dioxygenase: Product of PCR amplification of the dioxygenase genes *nidA* and *nahAc* were detected in all isolate M2292, M128, C318, C19 and C15, as shown in Figure 5.

The high ability to remove PAHs from all isolate was carried out by *dioxygenase* produced by all five bacteria strains in aerobic condition, Table 5 shows that dioxygenase gene is belonged to all five strains.

Table 5: Detection of dioxygenase gene in PAH-degrading bacteria

		Dioxygenase gene	
Strain	Bacterial name	*nidA*	*nahAc*
M2292	*Ochrobactrum oryzae*	+	+
M128	*Bacillus subtilis*	+	+
C318	*Bacillus subtilis*	+	+
C19	*Bacillus subtilis*	+	+
C15	*Bacillus pumilus*	+	+

+ sign indicates the PCR product was detected and

– sign means PCR product was not detected.

DISCUSSION

From the result of this study, it has been found that all five isolates have capability to degrade PAHs. *Bacillus subtilis* M128, C318 and C19 could grow fast in medium contains pyrene and phenanthrene. Furthermore, from above result they contain both *nidA* and *nahAc* dioxygenase gene which was responsible in PAHs degrading activity. The ability of *Bacillus subtilis* to degrade PAHs was also reported by other researchers which was reported *Bacillus subtilis* BMT4i started degrading Benzo[a]Pyrene (BaP) after 24 hours and continued up to 28 days achieving maximum degradation of approximately 84.7 % [13]. Another researchers also found the ability of *B. subtilis* to transform pyrene and benzo[a]pyrene [14].

Strain M2292 which was also contain both of dioxygenese gene, identified as *Ochrobactrum oryzae*, grows faster in medium contains phenanthrene. Another research result also reported the ability of *Ochrobactrum sp.* BAP5 to utilize polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, pyrene and fluoranthene as the sole carbon and energy source [15]. Strain *Ochrobactrum* sp BAP5 could grow in mineral salt medium with 50 mg/L of Benzo[a]Pyrene (BaP) and degrade about 20% BaP after 30 days of incubation.

Strain C15 was identified as *Bacillus pumilus* and this strain has capability to degrade pyrene and phenanthrene. Toledo also found that *B. pumilus* and *B. subtilis* can tolerate and remove different PAHs naphthalene, phenanthrene, fluoranthene and pyrene as sole carbon source [7].

As additional information, ability of *Sphingomonas* and *Mycobacterium* strains to degrade PAHs by analyzing the initial *dioxygenase gene* using *nahAc*, *phnAc*, *nidA* and *pdoB* primer was performed [6]. *Mycobacterium* has higher degradation ability than the *Sphingomonas* strains. Here it is because *Mycobacterium* strains possessed the *dioxygenase nidA* gene, while *nahAc* and *phaAc* were not detected in the *Sphingomonas*. Furthermore, similar to *Mycobacterium* strain, all five isolates, M2292, M128, C318, C19 and C15 which were capable utilize PAHs as sole carbon source, have both of dioxygenase gene *nidA* and *nahAc.*

CONCLUSION

All of five isolates that were identified as *Ochrobactrum oryzae* M2292, *Bacillus subtilis* M128, *Bacillus subtilis* C318, *Bacillus subtilis* C19 and *Bacillus pumilus* C15 have capability to degrade PAHs pyrene and phenanthrene because those strains possessed the dioxyrenese *nidA* and *nahAc* gene which are responsible for initial attack of PAHs degradation.

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