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Research Article

Enrichment and Identification of Phenanthrene-Degrading Bacteria Isolated from the Oil-Stained Engine Sediment in the Mangrove Swamps of Thailand

Aiya Chantarasiri*
Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok, Rayong Campus, Rayong, Thailand

* Corresponding author. E-mail: aiya.c@sciee.kmutnb.ac.th DOI: 10.14416/j.asep.2020.04.003
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Abstract
Polycyclic aromatic hydrocarbons (PAHs) are persistent pollutants that contaminate various environments. The biodegradation method conducted by bacteria is a promising alternative approach to remediate PAHs. Mangrove bacteria play a role in biogeochemical cycles and are able to degrade PAHs. The aims of this study included the enrichment, isolation, and characterization of phenanthrene (or tricyclic PAH)-degrading bacteria from some oil-stained engine sediments in a mangrove swamp in Rayong Province, Thailand. Ten isolated phenanthrene-degrading bacteria with a high ability for biodegradation were phenotypically and genetically identified. They are considered proteobacteria in the genera of *Achromobacter*, *Comamonas*, and *Pseudomonas*. The phenanthrene biodegradation results revealed that *Pseudomonas* sp. strain MP6-0207 could degrade phenanthrene 52.35 ± 0.28% of 100 mg/L of phenanthrene supplemented in saline Bushnell Haas Medium within 7 days. Its cells could adhere to the hydrophobic structure of phenanthrene with a BATH value of 45.17 ± 0.29% and produced biosurfactant to solubilize phenanthrene with E_{24} value of 32.33 ± 2.52%. The knowledge gained from this study offers a novel report on the isolation of phenanthrene-degrading bacteria from the mangrove swamp in Rayong Province, Thailand and could be used for future PAHs bioremediation in the contaminated sites.

Keywords: Biodegradation, Mangrove, Phenanthrene-degrading bacteria, Polycyclic aromatic hydrocarbon

1 Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are major components of fossil fuels and can be produced by incomplete thermal decomposition of organic matter such as coal, diesel, gasoline and plant biomass [1], [2]. PAHs are widely distributed in water and soil ecosystems from anthropogenic activities such as domestic sewage, oil material extractions and industrial wastes involved in medicine, paint, plastic, and insecticide manufacturing [3]. PAHs represent a significant public health risk to living organisms because of their carcinogenic, mutagenic and toxigenic properties [4]. Therefore, they are considered Persistent Organic Pollution (POP) [5]. There are more than 100 various PAHs and 16 PAHs have been included on the list of priority pollutants formulated by the U.S. Environment Protection Agency (USEPA) [6]. Phenanthrene, a low-molecular-weight tricyclic PAH comprised of fossil fuels, is one of the 16 toxic PAHs on the list of priority pollutants [7]. It does not pose a risk to human health. However, it has shown to be toxic to fish and algae in aquatic and marine environments [8]. Since its toxicity and recalcitrant degradation, the remediation studies of this tricyclic PAH have been significantly developed and investigated. PAHs-remediation difficulties are mainly caused by their chemical structure, including complexity, hydrophobicity and

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low water solubility [9]. Bioremediation relies on the microbial degradation of toxic compounds and is an alternative, effective remediation approach to detoxifying phenanthrene from contaminated environments [1]. PAH-bioremediation is considered environmentally clean technology because it shows partial or complete degradation at a lower cost with greater safety compared to physicochemical remediation [4], [10]. It is the major route for PAHs removal from contaminated sites and offers *in situ* biodegradation of PAHs [3]. Many microorganisms have been defined as PAH-bioremediation microbes and have been isolated from various environments such as the ocean, soil and mangroves [11], [12]. To improve the knowledge of PAH bioremediation by microorganisms and their removal efficiencies, more study has been carried out.

In Thailand, PAHs with 3 to 7 benzene rings contaminating concentration in coastal and riverine sediments were low to moderate compared to globally [13]. However, the contamination mainly increases from fossil fuel combustion and motor vehicle exhaust [7]. The Rayong River Estuary, the research area in this study, is a major urban area with a dense population (approximately 4,000 persons per square kilometer). The area is located in Rayong Municipality in the Eastern region of Thailand. It is an important ecological area covered with mangrove swamps on the east coast of the country. It plays an important role in the petrochemical industry zone named the Eastern Seaboard Industrial Estate, which is near the estuary. Therefore, this estuary area has become chronically contaminated with hazardous waste and pollutants, including phenanthrene.

Mangrove swamps are a unique coastal wetland ecosystem that can be found along tropical and subtropical coastlines [14]. They provide many ecological services such as the degradation of contaminants, nutrient cycling, pollution trapping, reduction of coastal erosion, and the storage of surface runoff [12], [15]. The mangrove sediments are considered a natural pollution trap for such PAHs as well as a biodiversity hotspot for PAH-degrading microorganisms. Therefore, they are noticeable resources for isolating PAH-degraders, including phenanthrene degrading bacteria [16]. Microorganisms play a role in biogeochemical cycles. Most phenanthrene-degrading bacteria isolated from mangrove sediments belong to the genera *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Gordonia*, *Marinobacter*, *Microbacterium*, *Mycobacterium*, *Novosphingobium*, *Ochrobactrum*, *Paracoccus*, *Pseudomonas*, *Ralstonia*, *Rhodococcus*, *Staphylococcus*, *Stenotrophomonas*, *Terrabacter* and *Vibrio* [12], [17]–[21]. However, information concerning the effective bacteria for phenanthrene bioremediation isolated from the local mangrove ecosystems is still needed. Knowledge of removal efficiencies by phenanthrene-degrading bacteria is still required as well.

For this study, phenanthrene degrading-bacteria were enriched and isolated from mangrove swamp sediments in the Rayong River Estuary of Eastern Thailand. They were biochemically and genetically identified and analyzed for their biodegradable ability. The purpose was to determine the potential of the bacteria for possible use in sustainable bioremediation and related applications.

2 Materials and Methods

2.1 *Sediment samples*

Sediment samples were randomly collected from Phra Chedi Klang Nam mangrove swamps in Rayong River Estuary, Rayong Municipality, Thailand (12° 39′N, 101° 14′E). The sampling site covers an area of 75,000 m^2. The dominant plants in the sampling site included *Avicennia* sp. and *Rhizophora* sp. Samples were collected during the winter season in September 2016. Samples were taken from the surface sediment at different locations in the swamps because the pollutants in the surface sediments are higher than in the bottom sediments [22]. The samples were aseptically kept at 4°C and taken for bacterial isolation within 24 h of collection.

2.2 *Bacterial enrichment and primary screening of phenanthrene-degrading bacteria*

Phenanthrene-degrading bacteria were enriched in Bushnell Haas Medium, pH 6.50 (HiMedia, India) supplemented with 6.90 mg/L of NaCl (the average pH values and salinity of sampling sites). The purpose of appropriate NaCl supplementation in the medium was suggested by Wongwongsee *et al*. [21]. Ten grams of sediment samples were suspended in 90 mL of enriched medium and incubated at 30 ± 1°C (the

average temperature of sampling sites) for 24 h on the shaking incubator at 150 rpm. Subsequently, 10 mL of the suspended medium was inoculated in 90 mL of saline Bushnell Haas Medium supplemented with 100 mg/L of phenanthrene (Alfa Aesar, UK) and incubated at 30 ± 1°C for 7 days on the shaking incubator. Phenanthrene was supplemented as external environmental stress. The cultured medium was then serially inoculated to fresh saline-Bushnell Haas Medium supplemented with phenanthrene 5 more times. The total time for the screening experiment was 42 days.

The active bacterial communities for degradation of phenanthrene were primarily selected using the measurement of residual phenanthrene in culture medium after 7 days of the last inoculation (on experimental day 42). The extraction of residual phenanthrene in culture medium using *n*-hexane (Sigma-Aldrich, USA) and the quantification of residual phenanthrene by spectrophotometric analysis at 293 nm (Abs_{293}) was conducted from previously described [23], [24]. The negative control was the saline-phenanthrene Bushnell Haas Medium inoculated with *Escherichia coli* strain TISTR074 (Thailand Institute of Scientific and Technological Research, Thailand).

Percentage of phenanthrene degradation was calculated using the following formula: Phenanthrene degradation (%) = {1– (Abs_{293} on the final day/Abs_{293} on the initial day)} × 100

2.3 *Bacterial isolation and secondary screening of phenanthrene-degrading bacteria*

The most active bacterial community was isolated by spread plating on saline Bushnell Haas Medium Agar, pH 6.50 sprayed with 100 mg/L of phenanthrene solution and incubation at 30 ± 1°C for 7 days. Morphologically dissimilar bacterial colonies were selected and streak plated for purification onto the Tryptone Soya Agar (HiMedia, India), pH 6.50, supplemented with 6.90 mg/L of NaCl. Phenanthrene degrading performance of each bacterial isolate was confirmed by culture in saline Bushnell Haas Medium supplemented with 100 mg/L of phenanthrene. The extraction and quantification of residual phenanthrene in the culture medium were assayed using the methods described above.

2.4 *Biochemical and genetical identification of the bacterial isolates*

All phenanthrene-degrading bacteria were identified by standard morphological investigation, biochemical test, and 16S rRNA gene sequence analysis. Biochemical characterization was determined using a KB013 identification kit (HiMedia, India). For genetic identification, genomic DNA of each bacterial isolates was extracted with the heat treatment [25]. Polymerase Chain Reaction (PCR) amplification of 16S rRNA genes was performed using the OnePCR™ reaction mixture (Bio-Helix, Taiwan). Primers used for the amplification included the universal forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and the universal reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Sigma-Aldrich, Singapore). PCR was performed in a Mastercycler® Nexus (Eppendorf, Germany) and amplification conditions were carried out according to the previous method of Ferbiyanto *et al*. [26]. Approximately 1,500-bp PCR products were analyzed using a 1.5% (w/v) OmniPur® agarose gel (Calbiochem, Germany), visualized by Novel Juice (Bio-Helix, Taiwan) and sequenced by the nucleotide sequencing service of Macrogen Inc. (Seoul, Korea). Sequence similarity analysis of the resulting 16S rRNA genes was performed at the National Center for Biotechnology Information (NCBI) database using the BLASTn program. The phylogenetic tree was generated and visualized by the SeaView program version 4.6.4 [27] and FigTree program version 1.4.3 (Institute of Evolutionary Biology, University of Edinburgh, UK). The phylogenetic tree was generated by the neighbor-joining (NJ) method with 100,000 bootstrap replications. Ten 16S rRNA gene sequences of isolated phenanthrene-degrading bacteria from this study were deposited in the GenBank database under the accession numbers MH174318 to MH174326 and MH174342.

2.5 *Determination of emulsification activity (E_{24})*

The analysis of E_{24} was modified from previously described [3]. The phenanthrene-degrading bacteria were cultured in Nutrient Broth (HiMedia, India), pH 6.50, for 18 h at 30 ± 1°C and centrifuged at 4,000 ×g for 15 min to obtain the cell-free culture solution. The aliphatic petroleum hydrocarbon *n*-hexadecane

(Alfa Aesar, UK) was added to the same volume of the cell-free culture solution, vortexed for 2 min and left to stand for 24 h at room temperature. The E_{24} was determined as the percentage of the height of the emulsion layer divided by the overall height of the mixture. The negative control was the uninoculated nutrient broth mixed with *n*-hexadecane solution, while the positive control was *Pseudomonas putida* strain TISTR1522 (Thailand Institute of Scientific and Technological Research, Thailand).

2.6 *Determination of bacterial adhesion to hydrocarbon (BATH)*

BATH assay or classical microbial adhesion to hydrocarbon (classical MATH) assay was previously described by Zoueki *et al*. [28]. The phenanthrene-degrading bacteria were cultured in nutrient broth, pH 7.00, for 18 h at 30 ± 1°C and centrifuged at 4,000 ×g for 15 min to obtain the bacterial cells. The cells were resuspended in sterile 0.85% (w/v) NaCl solution and centrifuged to remove traces of the culture medium. The bacterial cells were resuspended in sterile 0.85% (w/v) NaCl solution to an absorbance of 1.0 at 600 nm (Abs_0). Then 5 mL of the bacterial suspension was vortexed with 0.3 mL of *n*-hexadecane for 2 min and left to stand for 15 min at room temperature. The bacterial suspension layer was carefully pipetted to avoid taking in the *n*-hexadecane layer and measured for the final absorbance at 600 nm (Abs_f). BATH index was calculated using the following formula: BATH index (%) = $\{1– (Abs_f/Abs_0)\} \times 100$. The negative control was the sterile NaCl solution mixed with *n*-hexadecane solution, while the positive control was *P. putida* strain TISTR1522.

2.7 *Preliminary PAH degradation assessments of bacteria*

The phenanthrene-degrading bacteria were cultured in saline Bushnell Haas Medium, pH 6.50, supplemented with various PAH compounds to preliminarily determine PAH biodegradation performance besides the biodegradation of phenanthrene. The bacteria were cultured in saline Bushnell Haas Medium supplemented with 100 mg/L of anthracene (three fused benzene rings) and pyrene (four fused benzene rings) (Alfa Aesar, UK). All cultures were incubated at 30 ± 1°C for 7 days on the shaking incubator. PAH degradation was indirectly assessed by measurement of the bacterial growth with optical density at 600 nm (OD_{600}) after incubation time.

2.8 *Statistical analysis*

The statistical analysis in this study was analyzed by One-Way analysis of variance (One-Way ANOVA) followed by Tukey's test with a 95% confidence interval using a free statistical software, R version 3.6.1 (The R Foundation, Austria).

3 Results and Discussion

3.1 *Sediment samples*

The collection of samples was specifically focused on the oil-stained engine or oil film floating sediments in mangrove swamps of the Rayong River Estuary. Engine oil is basically composed of many aliphatic, aromatic, heterocyclic and polycyclic hydrocarbons. Ten interesting samples were found and collected from the sampling site. These samples were likely impacted by chronic hydrocarbon contamination from local fishing boats and nearby petrochemical industries. The soil texture of all samples was sandy clay with dark brown color. The average temperature, pH value and salinity of sediment samples were 30 ± 1°C, 6.50 ± 0.01, and 6.90 ± 0.05 mg/L, respectively.

3.2 *Isolation and screening of phenanthrene-degrading bacteria*

The saline-phenanthrene Bushnell Haas Medium appeared turbid and yellow-colored compared to the control medium. It was defined as active media thriving in the communities of phenanthrene-degrading bacteria. The turbidity of culture media was related to the number of bacterial cells. The yellow-colored culture media resulted from the byproducts of the phenanthrene degrading pathway by bacteria, such as catechol and related phenolic compounds. The culture media and negative control after incubation for 7 days of the last subculture (on experimental day 42) are shown in Figure 1.

All active media were primarily determined by the percentage of phenanthrene degradation by

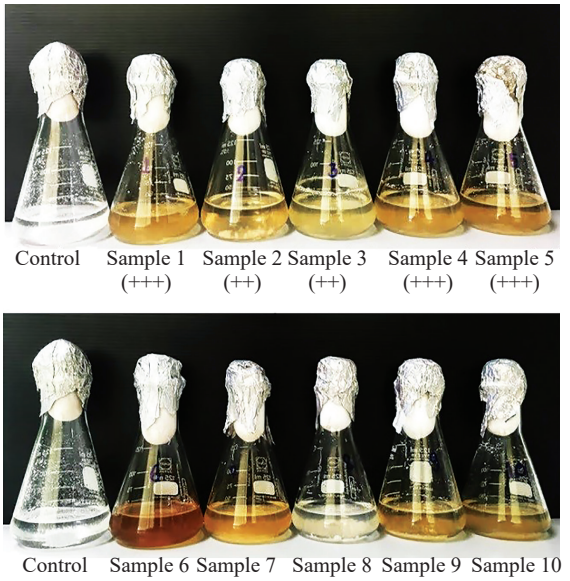

Figure 1: The color of saline-phenanthrene Bushnell Haas Medium on the last subculture day (on experimental day 42). "+" represents the yellow-colored intensity, which assumes the quantity of byproducts from phenanthrene degrading pathway by the bacterial community. Control was the culture medium inoculated with *E. coli* strain TISTR074.

spectrophotometric analysis. The result is shown in Figure 2. The biodegradation performance of bacterial communities ranged from 12.90 ± 0.09 to 90.29 ± 1.16% of the concentration of phenanthrene by 100 mg/L, whereas the control was 3.61 ± 0.26%. The sediment sample No.6 exhibited the maximum of degradation by 90.29 ± 1.16% directly according to the deep yellow-colored intensity of culture media. Therefore, the bacterial community of the sediment sample No.6 was believed to aid in the remediation of phenanthrene. This bacterial community was isolated, named and screened for phenanthrene degradation performance.

Ten bacterial isolates were isolated from the sediment sample No.6 by spread plating on saline Bushnell Haas Medium Agar and sprayed with phenanthrene solution. They were named bacterium MP6-0101 to bacterium MP6-0208. The formation of clear zones by biodegradation performance could not be investigated within 7 experimental days, but could be investigated

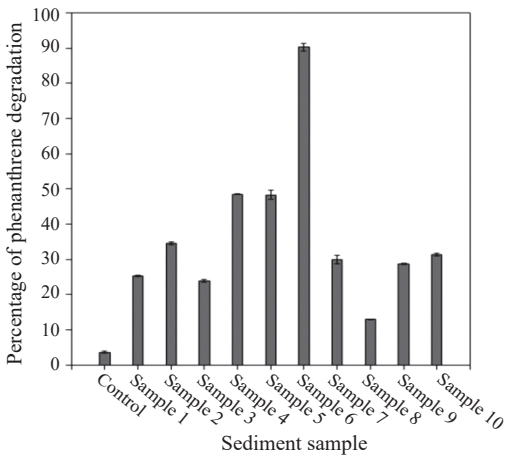

Figure 2: Percentage of phenanthrene degradation by active bacterial communities. Experiments were conducted in triplicate.

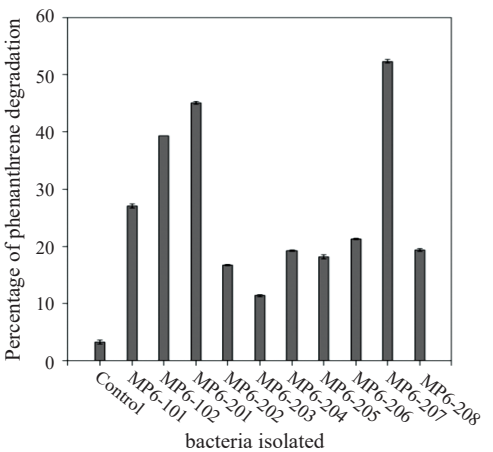

Figure 3: Percentage of phenanthrene degradation by active bacteria isolated from the sediment sample No.6. They were experimented in triplicate.

after 21 days of incubation. Wongwongsee *et al*. [21] that reported the PAH-degrading bacteria isolated from mangrove sediments in Thailand showed the resulting clear zones on agar plates sprayed with phenanthrene after 15 to 22 days of incubation. All isolated bacteria were secondarily determined by the percentage of phenanthrene degradation by spectrophotometric analysis. The results are shown in Figure 3. The degradation performance of isolated bacteria ranged from 11.41 ± 0.17 to 52.35 ± 0.28%

of the concentration of phenanthrene by 100 mg/L, whereas the control was 3.24 ± 0.37%. Interestingly, bacterium MP6-0207 alone could degrade 52.35 ± 0.28% of the phenanthrene within 7 days.

Bacterial cells may be grown and remain viable in a liquid medium better than a solid medium (agar plates). Liquid media are utilized for the growth of large numbers of bacteria. The culture on the agar plates hardly grew since the medium and bacteria easily dehydrated. Therefore, the active bacterial isolates from the formation of the clear zones on saline Bushnell Haas Medium Agar could be investigated after 21 days of incubation but could only be investigated within seven days of incubation in liquid saline Bushnell Haas Medium.

Phenanthrene degradation by the bacterial community of sediment sample No.6 at 90.29 ± 1.16% provided more efficiency than each active bacteria isolate in the range of 11.41 ± 0.17 to 52.35 ± 0.28%. It was believed that the active bacteria dwelling in sediment sample No.6 synergistically degraded phenanthrene. In a natural environment, bacteria have evolved strategies to utilize PAHs. The observed pattern of the PAHs utilization is not only as a result of bioavailability, but also of the metabolic interactions and cometabolic effects. Cometabolism is an important strategy of the PAHs degradation of many bacteria [6]. Cui *et al*. [5] reported that the biodegradation of pyrene and fluoranthene by the bacterial consortium increased from 63% to 76% and 65% to 83% compared with that of the pure culture.

3.3 *Identification of the phenanthrene-degrading bacteria*

The identification of phenenthrene-degrading bacteria isolated from the sediment sample No.6 was first determined by phenotypic identifications involving standard morphological investigation and classical biochemical tests. All bacterial isolates comprised aerobic bacteria with light cream-colored and transparent colonies. Colony morphology was circular-shaped for the entire margin and convex elevation. Bacterial cells were rod-shaped, Gram-negative and motile. Most of the bacteria were malonate, citrate and arginine utilization-positive. Bacteria were classified into three groups based on the resulting biochemical profiles: bacterial group 1 was positive with malonate utilization, citrate utilization, nitrate reduction, catalase activity, and arginine utilization, bacterial group 2 was positive with malonate utilization, citrate utilization, catalase activity and arginine utilization and bacterial group 3 was positive with malonate utilization, citrate utilization, nitrate reduction, and arginine utilization. The morphology of colonies and biochemical characterizations are shown in Tables 1 and 2.

Table 1: Colony morphology of phenanthrene-degrading bacteria

Bacterial Isolate	Shape	Margin	Elevation
MP6-0101	Circular	Entire	Convex
MP6-0102	Circular	Entire	Convex
MP6-0201	Circular	Entire	Convex
MP6-0202	Circular	Entire	Convex
MP6-0203	Circular	Entire	Convex
MP6-0204	Circular	Entire	Convex
MP6-0205	Circular	Entire	Convex
MP6-0206	Circular	Entire	Convex
MP6-0207	Circular	Entire	Convex
MP6-0208	Circular	Entire	Convex

Table 2: Biochemical profile of phenanthrene-degrading bacteria

Bio chemical Test*	Bacterial Isolate No. MP6-									
	0101	0102	0201	0202	0203	0204	0205	0206	0207	0208
1	+	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	-	-	-	-	-
5	+	-	+	+	+	-	+	+	+	+
6	+	+	+	+	+	+	+	+	+	-
7	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-

*Biochemical test. 1: Malonate utilization, 2: Voges Proskauer's test, 3: Citrate utilization, 4: β-galactosidase test, 5: Nitrate reduction, 6: Catalase activity, 7: Arginine utilization, 8: Sucrose utilization, 9: Mannitol utilization, 10: Glucose utilization, 11: Arabinose utilization, 12: Trehalose utilization. "+" represents a positive reaction and "-" represents a negative reaction.

The phenotypic identifications were not sufficient to identify bacterial genera. The bacteria were genetically identified by amplification and analysis of the 16S rRNA gene. The genotypic results of isolated bacteria belong to three genera: *Achromobacter*, *Comamonas*, and *Pseudomonas* as shown in Table 3. Seven bacteria from bacterial group 1 exhibited the homology to *Pseudomonas* sp. with 93 to 100% identity including MP6-0101, MP6-0201, MP6-0202, MP6-0203, MP6-0205, MP6-0206, and MP6-0207. Bacteria MP6-0102 and MP6-0204 from bacterial group 2 were identified as *Achromobacter* sp. with 92% identity. Bacteria MP6-0208 from bacterial group 3 was *Comamonas* sp. with 99% identity. The phylogenetic tree of isolated bacteria is shown in Figure 4. All 16S rRNA gene sequences from this study have been deposited in the GenBank database under the accession numbers MH174318 to MH174326 and MH174342, as mentioned above in the Materials and Methods section.

Table 3: Identity percentage of 16S rRNA gene sequences for the isolated phenanthrene-degrading bacteria

Bacterial Isolate	Closely-related Bacteria	GenBank Accession No.	Identity (%)
MP6-0101	*Pseudomonas guariconensis* strain Pg-CW13	MK880382.1	99.92
MP6-0102	*Achromobacter* sp. strain Fo40	MF155647.1	91.51
MP6-0201	*Pseudomonas plecoglossicida* strain RL105	MN234078.1	97.04
MP6-0202	*Pseudomonas taiwanensis* strain SR4	KY514158.1	100.00
MP6-0203	*Pseudomonas taiwanensis* strain SR4	KY514158.1	99.69
MP6-0204	*Achromobacter xylosoxidans* strain TOC68	KC010531.1	92.48
MP6-0205	*Pseudomonas plecoglossicida* strain pgE 16S	MK519202.1	96.83
MP6-0206	*Pseudomonas* sp. strain MSSRFPD38	KY849354.1	95.04
MP6-0207	*Pseudomonas* sp. strain ST14	MK934484.1	92.98
MP6-0208	*Comamonas aquatica* strain KUGK120	MF354014.1	99.01

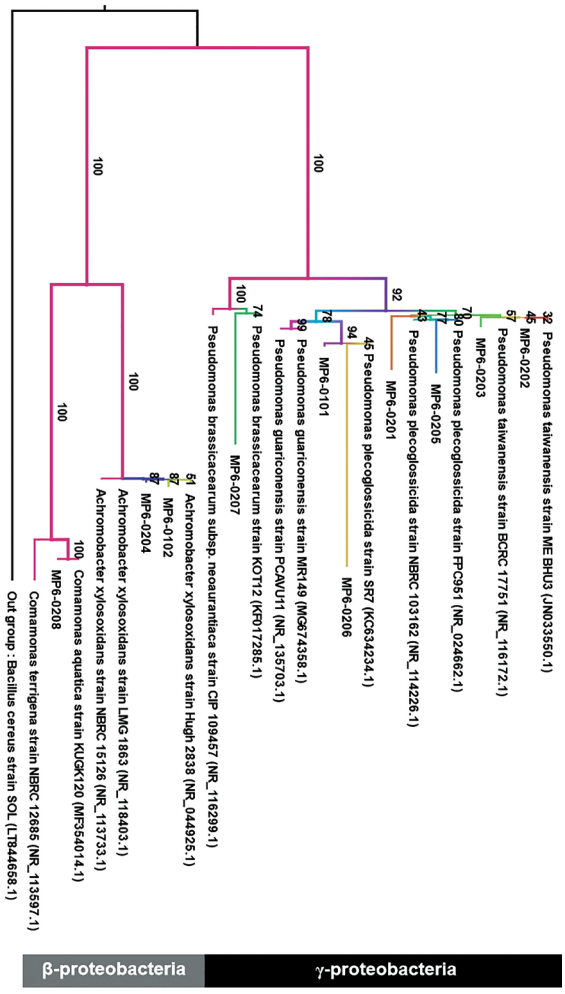

Figure 4: Phylogenetic tree of 16S rRNA of the phemanthrene-degrading bacteria. The neighbor-joining (NJ) method with 100,000 bootstrap replications was used in infer tree topology. The phylogenetic tree was generated and visualized by SeaView program version 4.6.4 and FigTree program version 1.4.3.

Achromobacter is a genus of bacteria in the class of β-proteobacteria. Their cells are rod-shaped, Gram-negative and motile. They are aerobe and found in soil, freshwater, and marine water [29]. It conforms to the characteristics and habitat of *Achromobacter* sp. strain MP6-0102 and *Achromobacter* sp. strain MP-0204 isolated in this study. Several reports have shown *Achromobacter* sp. could biodegrade several PAHs.

For example, *A. xylosoxidans* strain N6 isolated from the contaminated soils of industrial zone in Qatar could degrade anthracene, naphthalene and phenanthrene [30], and *Achromobacter* sp. strain LH-1 achieved 94% degradation with 100 mg/L phenanthrene [31]. *Achromobacter* sp. strain SSK4 isolated from mangrove sediments in Thailand achieved 58.7% degradation with 100 mg/L phenanthrene within 14 days [21]. *A. xylosoxidans* strain AY189752 has been reported degrade 73.2% of 200 mg/mL phenanthrene within 21 days [32].

Comamonas is a genus of bacteria in the class of β-proteobacteria. Their cells are aerobe, rod-shaped, Gram-negative and motile [33]. They are isolated from mud, soil, and water [34]. It complies with the characteristics and habitat of *C. aquatica* strain MP6-0208 isolated from mangrove sediments in this study. *Comamonas* sp. has been reported to be a PAHs degrader. *C. testosteroni* strain GZ39 isolated from river sediment has the ability to degrade anthracene, naphthalene and phenanthrene [35].

Pseudomonas is a genus of bacteria in the class of γ-proteobacteria. Their cells are rod-shaped, Gram-negative and motile. They are aerobe and colonize in a wide range of habitats [36]. The PAHs-degrading *Pseudomonas* was isolated and characterized from various environments, not only the PAHs-contaminated area, but also mangrove sediments. It conforms to experimental data of *Pseudomonas* strains isolated in this study (e.g., *P. guariconensis* strain MP6-0101, *Pseudomonas* sp. strain MP6-0201, *P. taiwanensis* strain MP6-0202, *P. taiwanensis* strain MP6-0203, *Pseudomonas* sp. strain MP6-0205, *Pseudomonas* sp. strain MP6-0206 and *Pseudomonas* sp. strain MP6-0207). *Pseudomonas* sp. have been intensively studied and are undoubtedly one of the best PAHs degraders. They are able to degrade PAHs like anthracene, naphthalene, and phenanthrene by well-detailed catabolic pathways of synergistic dioxygenase enzymes [8]. *Pseudomonas* isolated from soil has recently been reported to be a phenanthrene degrader at 86% of the PAHs removal rate within 72 incubation days [37].

High throughput nucleotide sequencing techniques have been used to analyze the microbial communities in environments and showed that proteobacteria were dominantly represented in estuarine and mangrove sediments [38], [39]. Recently, the autochthonous microbial community in hydrocarbon-contaminated mangrove sediments in Thailand has been explored by the genetic throughput technique. It was revealed that the dominant PAHs-degrading bacteria dwelling in contaminated sediments were α-proteobacteria, γ-proteobacteria (e.g., *Pseudomonas* sp. and *Acinetobacter* sp.) and δ-proteobacteria [12].

3.4 *Emulsification activity (E_{24}) and bacterial adhesion to hydrocarbon (BATH) of ten bacterial isolates*

The biodegradation of PAHs can be facilitated by microbial biosurfactants. It serves as a solubilizer of PAHs, which has a strong hydrophobic structure molecule. When the solubility of PAHs was increased, the degradation rate was concurrently promoted [40]. Emulsification activity (E_{24}) is a reliable method for determining the effectiveness of biosurfactants [41]. Bacterial adhesion to hydrocarbon assay (BATH) is a rapid method for determining bacterial cell surface hydrophobicity [42]. It is used to determine the affinity of bacterial cells that adhere to hydrophobic PAHs. The E_{24} and BATH assays were examined for all bacteria isolated from the sediment sample No.6. The results are shown in Table 4.

Pseudomonas sp. has good emulsification and cell surface hydrophobicity, especially *Pseudomonas* sp. strain MP6-0207 and *Pseudomonas* sp. strain MP6-0201. *Pseudomonas* sp. strain MP6-0207 has the highest value of E_{24} and BATH by $32.33 \pm 2.52\%$ ($p < 0.001$) and $45.17 \pm 0.29\%$ ($p < 0.001$), respectively. These results were consistently related to their good phenanthrene degradation performance. According to a previous report, eighteen PAHs-degrading bacteria were isolated from the Persian Gulf. Their E_{24} values ranged from 10.27% to 88.60%, while BATH values ranged from 5.71% to 73.58%, which were directly related to the percentage of PAH degradation [3]. It could be supposed that *Pseudomonas* sp. strain MP6-0207 and *Pseudomonas* sp. strain MP6-0201 capably adhered to phenanthrene molecules and produced biosurfactants to dissolve them. *Pseudomonas* sp. is one of the well-known biosurfactant-producing bacteria isolated from various environments [43]. For example, *P. aeruginosa* strain S5 isolated from cooking wastewater was determined to be an efficient glycolipid biosurfactant-producing bacteria and PAHs-degrading bacteria with E_{24} value of 48.9% [41].

Table 4: Emulsification activity (E_{24}) and bacterial adhesion activity (BATH) of isolated PAHs-degrading bacteria. They were experimented in triplicate

Isolated Bacteria	E_{24} (%)	BATH (%)
P. guariconensis strain MP6-0101	18.50 ± 0.87^{bc}	15.43 ± 0.60^{e}
Achromobacter sp. strain MP6-0102	19.83 ± 0.29^{bc}	22.50 ± 0.50^{f}
Pseudomonas sp. strain MP6-0201	22.67 ± 2.08^{c}	29.77 ± 0.75^{g}
P. taiwanensis strain MP6-0202	14.67 ± 3.51^{ab}	12.07 ± 0.12^{d}
P. taiwanensis strain MP6-0203	10.00 ± 1.00^{a}	5.87 ± 0.15^{b}
Achromobacter sp. strain MP6-0204	11.33 ± 0.58^{a}	12.17 ± 0.76^{d}
Pseudomonas sp. strain MP6-0205	10.67 ± 0.58^{a}	10.17 ± 0.29^{c}
Pseudomonas sp. strain MP6-0206	13.00 ± 2.65^{a}	12.60 ± 0.53^{d}
Pseudomonas sp. strain MP6-0207	32.33 ± 2.52^{d}	45.17 ± 0.29^{h}
C. aquatica strain MP6-0208	12.00 ± 1.73^{a}	4.27 ± 0.64^{a}
Control*	21.50 ± 1.32^{c}	21.43 ± 0.75^{f}

**P. putida* strain TISTR1522. The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the isolated bacteria.

In this study, *Achromobacter* sp. strain MP6-0102 showed quite good emulsification and cell surface hydrophobicity, whereas *Achromobacter* sp. strain MP6-0204 and *C. aquatica* strain MP6-0208 were moderate. *Achromobacter* sp. and *Comamonas* sp. also hypothetically produce biosurfactant to dissolve phenanthrene molecules. A previous report showed that *A. xylosoxidans* strain GSMSR13B could produce biosurfactant and was considered hydrocarbon-degrading bacteria with E_{24} value of 28.7% [44]. In the same way, *C. terrigena* strain AS47 isolated from soil and water contaminated with palm oil in Thailand could produce biosurfactant with E_{24} value of 29.5% when grown in MSM medium supplemented with crude palm oil [45].

Importantly, *Achromobacter* sp., *Comamonas* sp. and *Pseudomonas* sp. are Gram-negative bacteria. Their outer cell membranes can tolerate and uptake PAHs better than Gram-positive bacteria [46]. Therefore, Gram-negative bacteria could be considered promising bacteria for remediating PAHs.

3.5 *Preliminary PAH degradation assessments of bacteria*

All bacteria isolated from the sediment sample No.6 were grown and cultured in saline Bushnell Haas Medium supplemented with 100 mg/L of anthracene and pyrene. The bacterial growth levels were measured by optical density at 600 nm (OD_{600}) at the end of incubation time and considered as indirect PAH degradation performance. Growth levels of isolated PAHs-degrading bacteria are shown in Table 5.

Table 5: Growth (OD_{600}) of isolated PAHs-degrading bacteria cultured in saline Bushnell Haas Medium supplemented with 100 mg/L of anthracene (Anth.) and pyrene (Pyr.). They were experimented in triplicate

Isolated Bacteria	Anth.	Pyr.
P. guariconensis strain MP6-0101	0.419 ± 0.007^{cd}	0.283 ± 0.008^{c}
Achromobacter sp. strain MP6-0102	0.332 ± 0.021^{a}	0.251 ± 0.031^{b}
Pseudomonas sp. strain MP6-0201	0.336 ± 0.004^{ab}	0.337 ± 0.000^{d}
P. taiwanensis strain MP6-0202	0.409 ± 0.002^{c}	0.325 ± 0.005^{d}
P. taiwanensis strain MP6-0203	0.407 ± 0.001^{c}	0.352 ± 0.002^{d}
Achromobacter sp. strain MP6-0204	0.381 ± 0.003^{bc}	0.234 ± 0.002^{ab}
Pseudomonas sp. strain MP6-0205	0.391 ± 0.014^{bc}	0.341 ± 0.004^{d}
Pseudomonas sp. strain MP6-0206	0.461 ± 0.009^{d}	0.217 ± 0.007^{a}
Pseudomonas sp. strain MP6-0207	0.458 ± 0.004^{d}	0.212 ± 0.001^{a}
C. aquatica strain MP6-0208	0.389 ± 0.002^{bc}	0.326 ± 0.005^{d}

The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the isolated bacteria.

All bacteria showed sufficient growth in the culture medium. *Pseudomonas* sp. strain MP6-0206 and *Pseudomonas* sp. strain MP6-0207 exhibited the highest growth in the medium supplemented with anthracene ($p < 0.01$), whereas *Pseudomonas* sp. strain MP6-0201, *P. taiwanensis* strain MP6-0202, *P. taiwanensis* strain MP6-0203, *Pseudomonas* sp. strain MP6-0205 and *C. aquatica* strain MP6-0208

($p < 0.001$) exhibited the highest growth in the medium supplemented with pyrene. It was preliminarily supposed that all bacteria could catabolize anthracene and pyrene as the sole carbon source for subsistence by their PAHs-degrading enzymes. Most effective PAHs-degrading bacteria often have the ability for biodegradation, not only for phenanthrene, but also other low molecular weight PAHs. *P. putida* strain AFS-3 has been reported to biodegrade and use naphthalene, phenanthrene and chrysene supplemented in culture medium as the sole carbon source, resulting in the turbidity of the medium [47]. However, residual anthracene and pyrene after bacterial biodegradation processes should be further confirmed by high-throughput technologies such as gas chromatography.

4 Conclusions

The isolation and identification of phenanthrene-degrading bacteria obtained from oil-stained engine sediments in a mangrove swamp, Rayong Province, Thailand were reported. *Pseudomonas* sp. strain MP6-0207 was considered to be effective phenanthrene biodegrading bacteria in this study with 52.35 ± 0.28% of 100 mg/L of phenanthrene within 7 days. The phenanthrene degradation process in the culture medium was directly related to bacterial cell surface hydrophobicity and the effectiveness of bacterial biosurfactant. *Pseudomonas* sp. strain MP6-0207 could preliminarily degrade anthracene and pyrene. These bacteria can be helpful in the bioremediation of phenanthrene and other low molecular weight PAHs contaminated sites.

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