Bioremediation Journal

ISSN: 1088-9868 (Print) 1547-6529 (Online) Journal homepage: http://www.tandfonline.com/loi/bbrm20

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To cite this article: Muna Ibrahim, Essam A. Makky, Nina Suhaity Azmi & Jamil Ismail (2018) Impact of incubation period on biodegradation of petroleum hydrocarbons from refinery wastewater in Kuantan, Malaysia by indigenous bacteria, Bioremediation Journal, 22:1-2, 52-61, DOI: 10.1080/10889868.2018.1476453

To link to this article: https://doi.org/10.1080/10889868.2018.1476453

BIOREMEDIATION JOURNAL, 2018
VOL. 22, NO. 1-2, 52–61
https://doi.org/10.1080/10889868.2018.1476453

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Impact of incubation period on biodegradation of petroleum hydrocarbons from refinery wastewater in Kuantan, Malaysia by indigenous bacteria

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ABSTRACT

This work studied the biodegradation of petroleum hydrocarbons (PHCs) extracted from refinery wastewater to produce industrially important by-products at different incubation periods. Two out of 13 bacterial isolates, KRD2 and KRA4 were isolated. Dichloromethane was used to extract the PHC, and gas chromatography-mass spectrometry (GC-MS) analysis revealed that the refinery wastewater PHC was successfully biodegraded using the selected bacterial isolates within 15 days of incubation. Both KRD2 and KRA4 isolates degraded all 13 initially extracted PHC compounds within 5 days, except C13BD and C9BD, which produced 6 and 4 compounds as secondary metabolites with peak area percentages of 1.58, 1.38, 0.85, 29.94, 7.59, and 11.16% and 3.55, 2.88, 52.31, and 6.14%, respectively. These metabolites have been reported in industrial and medical applications. After 10 days, only 6 and 8 compounds were degraded by both isolates, respectively, and C11PAD compound was produced, as well as C5PAD, C7PAD, and C13PAD. After 15 days, it was clear that all the initial PHC compounds have been completely degraded by both isolates. Metabolites C5PAD, C6PAD, C8PAD, and C13PAD were produced by KRD2, and metabolites C5PAD, C6PAD, C8PAD, and C9PAD were produced by KRA4 at different peak areas. The alignment revealed that the KRA4 isolate was included in the genus *Chryseobacterium gambrini*, while KRD2 isolate was successfully identified as *Mycobacterium confluentis* using the Biolog microbial identification system. The incubation period evidently affected biodegradation process by indigenous degraders. These effective bacteria were shown to be of great potential for further application in biodegradation technology of PHC contaminated refinery wastewater to produce industrially important by-products.

KEYWORDS

Biodegradation; *Chryseobacterium gambrini*; GC-MS; metabolites; *Mycobacterium confluentis*; petroleum refinery wastewater

Introduction

There are four classes of petroleum hydrocarbons (PHCs), which are aromatics, saturates, asphaltenes (fatty acids, phenols, ketones, porphyrins, and esters), and resins (amides, quinolines, carbazoles, pyridines, and sulfoxides) (Colwell, Walker, and Cooney 1977). Petroleum biodegradation depends on environmental conditions and microbial community in addition to the amount and type of hydrocarbon content of petroleum. The availability of microorganisms that can degrade oil pollutants is the major problem that limits their biodegradation. When PHC compounds bind to soil components, their removal or degradation is usually difficult (Barathi and Vasudevan 2001). The susceptibility of hydrocarbons to microbial attacks differs, and generally is ranked in the order: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Perry 1984; Ulrici 2000). Some hydrocarbons may not even be degraded at all, such as high molecular weight polycyclic aromatic hydrocarbons (PAHs) (Das and Chandran 2010). PHCs are regarded as priority pollutants which are highly resistant to degradation as a result of their low reactivity (Varjani 2017). When petroleum products are accidentally released into the environment, they are of particular concern because their components are carcinogenic and neurotoxic. In addition to this, they are toxigenic and mutagenic. Each year, over 3 million tons of oil is accidentally discharged into the sea, out of which oil pumping, transport and refining operations account for about 20% while non-tanker shipping and natural seepage account for 25%. Illegal oil activities account for more than half (55%) of the annual oil spillage (Golyshin et al. 2003).

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Based on the approach presented by PETRONAS Chemicals Fertilizer Kedah Sendirian Berhad (Sdn Bhd), a wholly owned subsidiary of Petronas Chemicals Group (PCG); they established their pilot Reed Bed wastewater treatment facility at their existing plant in Kedah, Malaysia. The pilot reed bed currently complements the wastewater treatment system already in existence. The reed bed wastewater treatment system, which was developed on a two-acre piece of land utilizes microorganisms and wetland vegetation to minimize wastewater pollutants concentration. These microorganisms break down and transform dangerous contaminants into less toxic or non-toxic forms. Moreover, the reed plants contribute significantly to the creation of an aerobic environment needed for the survival of the microorganisms (PCG 2014). The wide environmental distribution, resistance to biodegradation, the potential for bioaccumulation, and harmful effects of the bacteria represent significant concerns. Further economic consequences such as soil and water quality deterioration have been mitigated by the implementation of potential treatments (Kadri et al. 2017). Various organisms such as bacteria (Ferreira et al. 2015; Hamamura, Ward, and Inskeep 2013), fungi (Young et al. 2015; Li et al. 2005), and algae can degrade hydrocarbon pollutants (Varjani and Upasani 2013). The process of bioremediation is an innovative technique which involves the degradation or reduction of dangerous organic pollutants to less toxic compounds such as CO_2, CH_4, H_2O, and biomass by microorganisms, without posing any effect on the environment (Varjani and Srivastava 2015). One of the major mechanisms of bioremediation is biodegradation, which involves the use of oleophilic microbes to remove hydrocarbon pollutants from the environment (Macaulay and Rees 2014; Varjani and Upasani 2016; Varjani and Upasani 2013). Industries that engage mainly in the refining of crude oil and production of fuels, petrochemical intermediates, and lubricants generate reasonable amounts of petroleum refinery effluents (PRE) as wastes (Harry 1995).

Mycobacteria are a group of eubacteria that belong to a larger group of Gram-positive nocardioform *Actinobacteria*, which include *Corynebacterium, Nocardia, Rhodococcus, Gordonia*, and *Dietzia* (Stackebrandt et al. 1997). The organisms in the genus *Mycobacteria* are aerobic, acid-fast, rod-shaped bacteria that primarily contain mycolic acid in their cell wall. They are common

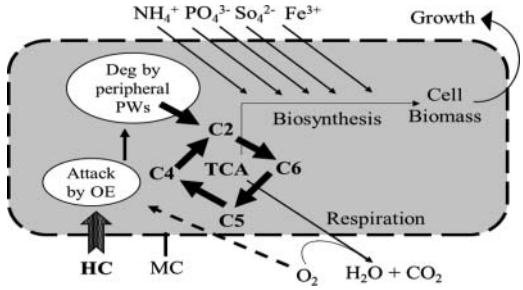

Figure 1. The main steps of hydrocarbon degradation assimilated by microbial cell under aerobic conditions. HC: Hydrocarbon; MC: Microbial Cell; TCA: Tricarboxylic acid Cycle; OE: Oxygenase Enzyme; Deg: Degradation; PWs: Pathways; C2: 2 carbon atoms compound (Acetyl-COA); C6: 6 carbon atoms compound (e.g., Citrate); C5: 5 carbon atoms compound (e.g., α-Keto-glutarate); C4: 4 carbon atoms compound (e.g., Oxaloacetate). (Source: This study).

saprophytes that are widely distributed in the environment (Primm, Lucero, and Falkinham 2004). The species of genus *Chryseobacterium* are typically pigmented from yellow to orange, rod-shaped, Gram-negative, non-spore forming, non-motile, and aerobic (Chaudhary and Kim 2017). Bacterial species of genera *Achromobacter, Acinetobacter, Azoarcus, Brevibacterium, Cellulomonas, Corynebacterium, Flavobacterium, Marinobacter, Mycobacterium, Micrococcus, Nocardia, Ochrobactrum, Pseudomonas, Stenotrophomaonas*, and *Vibrio* spp are reportedly hydrocarbon degraders (Varjani et al. 2015; Varjani and Upasani 2016). The proposed steps in hydrocarbon degradation by microbial cells are illustrated in (Figure 1). The purpose of this study is to isolate, screen, and identify bacterial isolates from the Kuantan River (KR) hydrocarbon-contaminated water for the biodegradation of PHCs extracted from refinery wastewater into compounds with high medical and industrial importance.

Materials and methods

Sample collection and preparation

Refinery wastewater (RW) samples were collected from BASF Petronas Chemicals Sdn Bhd, Kuantan, Pahang, 26100, Malaysia within 4 months (January 2016 to April 2016). Water samples (2 L) were collected in glass bottles and covered with screw caps. The collected water samples were immediately sent to the laboratory at Faculty of Industrial Sciences & Technology (FIST), Universiti Malaysia Pahang

(UMP) for analysis and liquid-liquid extraction (LLE) as described by (Eaton et al. 1998; Ibrahim et al. 2017) using Fioroni filter paper (90 mm diameter) under vacuum pump and then stored at 4°C for further use within 1 week.

Medium preparation and extraction

An enrichment medium (Bushnell Haas Broth) was prepared as follows: (gl^{-1}): $MgSO_4$, 0.2; $CaCl_2$, 0.02; NH_4NO_3, 1.0; KH_2PO_4, 1.0; K_2HPO_4, 1.0; and $FeCl_3$, 0.05 in demineralized water (Prabhakaran et al. 2014). Nutrient agar (NA) (Hardy Diagnostics, USA) and broth (NB) media (Merck, Germany) were used to culture the bacterial isolates from PHC contaminated water. All media were autoclaved for 15 min at 121°C. A bacterial suspension (25 ml) using (0.5 McFarland standard) was prepared in 50 ml Falcon tube, centrifuged and incubated at 37°C for 24 h under static conditions. The bacterial suspension (5 ml) was inoculated in 50 ml enrichment medium as described previously at pH 7.2 and in 0.75 ml of extracted petroleum hydrocarbon (sterilized using syringe filter 0.4 μm pore size) incubated at 37°C for 5, 10, and 15 days. At the end of the incubation period, the medium was extracted three times with a separating funnel (Pyrex 6402-1L Brand 6402) using dichloromethane at a ratio of 1:8 v/v and evaporated down to at least 3 ml using a rotary evaporator. The absorbance (Abs) was then recorded at 245 nm using a UV-VIS spectrometer (GENESYS 10S). The degradation percentage was calculated using Equation (1).

$$\text{PHC degradation}(\%) = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100 \quad (1)$$

The organic phase was carefully poured into a beaker and 10 g of anhydrous sodium sulphate (Merck) (annealed at 400°C for 4 h at a heating rate of 3°C min^{-1}) was added. The extract was concentrated to a volume of 2 ml using the rotary evaporator (Rotavapor ® R-300 System) at speed 3 and 40°C heat, after which it was analyzed with a gas chromatograph. All experiments were performed in triplicate.

Bacterial isolate identification

The bacterial isolates selected (KRD2 and KRA4) were inoculated into NB medium at 37°C for 48 h, subjected to Gram staining properties and identified using a Biolog microbial identification system and molecular technique. Each well in the biological microplates was inoculated with 150 ml of bacterial suspension isolate and incubated for 24 h at 25°C. The plates were evaluated on a microplate auto reader (Bio-tek EL311) at 590 nm, and the outputs were compared in Microlog software (Biolog release 3.5). The test panel contains a standardized set of micro methods comprising 94 biochemical tests for the profiling and identification of a wide range of Gram-negative and Gram-positive bacteria. The bacterial isolates were molecularly identified using 16S rRNA gene amplification. The genomic DNA of the selected isolates was extracted using a G-spimTM Genomic DNA extraction kit (iNtRON, Korea) following the instructions of the manufacturer. Each genomic DNA served as a template for 16S rRNA gene amplification. The Polymerase chain reaction (PCR) reaction was done with the aid of universal primers 518F 5`CCAGCAGCCGCGGTAATACG3` and 800R 5`TACCAGGGTATCTAATCC3`. The PCR mixtures (50 μl) were freshly prepared in a sterile PCR tube according to a method described by Makky et al. (2016). The PCR products were run on a 1.2% agarose gel electrophoresis to determine the size of the PCR products. These were then sent to 1st BASE Molecular Biology Services after DNA sequencing had been performed. The 16S rRNA sequence was aligned and compared to the sequences deposited in the Gen Bank database at the National Center for Biotechnology Information (NCBI) using a blast analysis tool. Next, phylogenetic tree construction was allocated.

Gas chromatography-mass spectrometry (GC-MS) analysis

The samples were analyzed using an Agilent technology 7890A gas chromatograph (GC) with a 7693 autosampler. The GC was equipped with a DB-5MS capillary column of (30 m × 250 μm film thickness × 0.25 mm, Agilent), coupled to an Agilent MSD 5975C mass spectrometer (MS), and operating with helium carrier gas. The injector was operated in splitless

mode. The sample was introduced into the GC at 1 μl aliquot by the auto-sampler. The oven temperatures for the GC injector and detector were kept at 250°C and 325°C, respectively. The column temperature was initially set at 70°C and held for 2 min before being increased by 5°C min^{-1} to 280°C and held for 10 min. GC-MS was used for the separation and identification of petroleum hydrocarbons according to a modified version of Shao et al. (2015) method.

Results and discussion

Samples analysis and bacterial isolation

A total of 13 bacterial isolates were isolated from hydrocarbon-contaminated water in the Kuantan River (KR). All were screened and only two bacterial isolates (KRD2 and KRA4) were successfully able to degrade the PHC extracted from refinery wastewater samples with 27.27% and 22.22% degradation and PHC concentrations of 166.15 and 177.69 μl ml^{-1}, respectively. GC-MS analysis of dichloromethane extracts from RW and KR samples was recorded and showed the presence of 13 compounds, 8 of which belonged to PHC. The pH of the samples recorded before and after bacterial degradation were 11.5 and 7.9, respectively. The presence or absence of these compounds was analyzed again after bacterial degradation at incubation periods of 5, 10, and 15 days by the KRD2 and KRA4 isolates. Our findings agree with (Romero et al. 1998) who isolated *Pseudomonas aeruginosa* strain from a stream heavily polluted by a petroleum refinery exhibited active growth at high phenanthrene dosages, with complete removal of the pollutant after a period of 30 days.

PHC degradation by selected bacterial isolates

The PHCs extracted from refinery wastewater were subjected to biodegradation by indigenous bacteria at different incubation periods and compared in the present study. After 5 days of incubation, all compounds disappeared, indicating biodegradation action, except C_{13}BD compound (Table 1), which could not be degraded by KRD2. Incubation for 5 days with this isolate resulted in the production of six compounds (C_1PAD, C_2PAD, C_4PAD, C_5PAD, C_7PAD, and C_{10}PAD) (Table 2) as secondary metabolites with peak area percentages of 1.58, 1.38, 0.85, 29.94, 7.59, and 11.16% with the same isolate and incubation time. Guanidine, N-methyl-N′-nitro-N-nitroso (C_2PAD) was identified in this study as a degradation product. This compound was used in a wide range of nano-synthesis and dendrimer formation applications from materials to biology (Tahir, Badshah, and Hussain 2015). In addition, guanidines are now identified as compounds with varied and numerous applications (Tahir, Badshah, and Hussain 2015). After 10 days of incubation, seven compounds, namely C_1BD, C_2BD, C_4BD, C_5BD, C_6BD, C_7BD, and C_{13}BD (Table 1) could not be degraded or assimilated by the selected bacterial isolates. Only C_{11}PAD compound (Table 2) was produced at PA% of 2.29, and this compound has wide industrial applications (Van et al. 2016). After 15 days of incubation, there was a change in the biodegradation conditions, as all the initial compounds were observed to have been successfully biodegraded due to the microbial action. Moreover, four compounds (C_5PAD, C_6PAD, C_8PAD, and C_{13}PAD) (Table 2) were produced and known for medical and industrial applications (Varjani 2017). Phthalate (C_5PAD) was also identified in the present study after 5, 10, and 15 days at PA% of 29.94, 4.96, and 57.44, respectively. The initial PHC compounds percentages before bacterial degradation were represented in (Table 1) and all compounds produced by KRD_2 isolate were represented in (Table 3). The results of the present study on the effect of incubation period on the biodegradation of PHC were comparable to the results of other studies reported earlier. Similarly, *Mycobacterium* was previously isolated and it metabolized 61.5% of the hydrocarbon after 5 days, as reported by Rehmann et al. (1998) who isolated the *Mycobacterium* spp. strain KR2 from PAH-contaminated soil at a gasworks plant. This strain metabolized up to 60% of the added pyrene (0.5 mg ml^{-1}) as a sole energy and carbon source within 8 days of incubation at 20°C. According to (Varjani 2017), the degradation products were identified as 4-5-phenanthrene dicarboxylic acid, Cis-4,5-pyrene dihydrodiol, 2-carboxybenzaldehyde,

1-hydroxy-2-naphthoic acid, protocatechuic acid, and phthalic acid. Due to the favorable properties and low cost of Di-(2-ethyl-hexyl-phthalate (DEHP), it is widely employed in the production of polyvinyl chloride (PVC) for use as a plasticizer. This family of chemicals is found in many products, including building materials, clothing, household furnishings, pharmaceuticals, nutritional supplements, cosmetics, medical devices,

Table 1. Initial screening of by-products produced using GC-MS analysis from extracted PHCs biodegradation at different incubation periods by KRD2 and KRA4 isolates.

					PA (%) at different incubation periods (days)					
					KRD2			KRA4		
No.	Compound code	[3]MF	[2]PA%	[4]Cd%	5	10	15	5	10	15
1	[1]C_1BD	1,6-heptadiyne-1,7-diylbis (trimethyl Silane) [$C_{13}H_{24}Si_2$]	2.61	12.70	—	5.25	—	—	—	—
2	C_2BD	l-Methionine, n-heptafluorobutyryl-, isohexyl ester [$C_{15}H_{22}F_7NO_3S$]	4.70	22.88	—	4.65	—	—	—	—
3	C_3BD	2-methyl-[$C_{11}O_4N_5H_{15}$], Adenosine	4.58	22.29	—	—	—	—	—	—
4	C_4BD	4-[(trimethylsilyl)amino]-, trimethylsilyl ester, Benzoic acid [$C_{13}H_{23}NO_2Si_2$]	5.98	29.11	—	7.87	—	—	7.31	—
5	C_5BD	3-[2-Hydroxy-3-(3-hydroxy-phenylamino)-propyl]-1,2,3,4,5,6-hexahydro-1,5-methano-pyrido[1,2-a] [1,5] diazocin-8-one [$C_{20}H_{25}N_3O_3$]	7.56	36.80	—	15.73	—	—	7.55	—
6	C_6BD	N-(2,3-dimethylphenyl)-,oxiranylmethyl ester, Carbamic acid [$C_{12}H_{15}NO_3$]	9.19	44.74	—	13.29	—	—	9.07	—
7	C_7BD	2-[4-Acetamidophenylsulfonyl]-1,4-naphthoquinone [$C_{18}H_{13}NO_5S$]	10.84	52.77	—	15.52	—	—	—	—
8	C_8BD	1,3,5-Triazine-2,4-diamine, N,N′-diethyl [$C_7H_{13}N_5$]	6.13	29.84	—	—	—	—	—	—
9	C_9BD	Methyl 2-methyl-2-(methoxy-3-hydroxypropoxy) amino-propanoate [$C_9H_{19}NO_5$]	10.25	49.90	—	—	—	4.93	—	—
10	C_{10}BD	N-Benzyl-N-ethyl-p-isopropylbenzamide [$C_{19}H_{23}NO$]	7.13	34.71	—	—	—	—	9.44	—
11	C_{11}BD	3-Octene, (Z)- [C_8H_{16}]	7.08	34.46	—	—	—	—	—	—
12	C_{12}BD	2,2′-(2,2′-Oxybis(ethane-2,1-diyl) bis (oxy)) bis(ethane-2,1-diyl) bis(3,5,5-trimethylhexanoate) [$C_{26}H_{50}O_7$]	20.54	100	—	—	—	—	—	—
13	C_{13}BD	trans-2,3-Methylenedioxy-β-methyl-β-nitrostyrene [$C_{10}H_9NO_4$]	3.41	16.60	3.45	3.63	—	—	2.89	—

Note. All values represented in the table indicate the presence or absence (-) of the initial compound at the peak area within different incubation periods; [1]CBD: Compound before degradation; [2]PA%: Peak area percentage; [3]MF: Molecular formula; [4]Cd%: Compound percentage.

children's toys, dentures, glow sticks, food packaging, automobiles, modeling clay, lubricants, waxes, insecticides, and cleaning materials (Heudorf, Mersch-Sundermann, and Angerer 2007). Fumaric acid (C_8PAD) was produced in the present study and known as an effective treatment of psoriasis has also been reported by Van et al. (2016). This compound was found upon degradation after 15 days by KRD_2 isolate at PA of 1.60%. Some bacteria such as *Mycobacterium* sp. (Kelley, Freeman, and Cerniglia 1990) can oxidize PAHs through a cytochrome P450 monooxygenase enzyme mediated system to form trans-dihydrodiols. These results indicate that may be our isolates in the present study can also produce the same type of enzyme to enhance the PHCs degradation.

PAHs can be degraded by several bacteria such as *Rhodococcus* sp., *Mycobacterium sp*., *Pseudomonas alcaligenes*, and *Sphingomonas* sp. The majority of these bacteria can grow on low molecular weight PAHs, such as fluorene, naphthalene, and phenanthrene. However, within the last few years, several organisms (especially *Mycobacterium*) have been grown on four-ring PAHs. These bacteria can adhere to hydrophobic PAHs owing to their hydrophobic surfaces, leading to their bulk transfer inside the cells (Seo, Keum, and Li 2009). Similarly, *Mycobacterium* was reported by Walter et al. (1991) and Trzesicka-Mlynarz (1995), they found that Benzo(α)pyrene (Bap) was degraded by different bacteria, including *Rhodococcus* sp., *Mycobacterium*, and a mixed culture of *Pseudomonas* and *Flavobacterium* after 30 days of incubation.

PHCs biodegradation by KRA_4 isolate was recorded after different incubation periods in this study. Evidently, all 13 initial compounds were successfully degraded after 5 days, except the C_9BD compound (Table 1). Some compounds were produced and identified using GC-MS analysis (C_1PAD, C_2PAD, C_3PAD, and C_4PAD) as shown in (Table 2). By increasing the incubation period to 10 days, the GC-MS results indicated that only 8 compounds out of 13 were successfully degraded and completely disappeared (Table 1). At the same time, three compounds were produced and identified by GC-MS (i.e., C_5PAD, C_7PAD, and C_{13}PAD), as shown in (Table 2). The long incubation period up to 15 days

Table 2. GC-MS results of industrially important by-products produced after PHCs refinery wastewater biodegradation by KRD2 and KRA4 isolates at different incubation periods.

			[1]PA (%) at different incubation periods (days)						
			KRD2			KRA4			
No.	Compound code	[2]MF	5	10	15	5	10	15	Compound application
1	[3]C_1PAD	1-nitro-3-(trifluoromethyl)-Benzene [$C_7H_4F_3NO_2$]	1.58	—	—	3.55	—	—	Industrial solvents (Chaudhary and Kim 2017).
2	C_2PAD	N-methyl-N′-nitro-N-nitroso, Guanidine [$C_2H_5N_5O_3$]	1.38	—	—	2.88	—	—	Multiple biological uses of chemistry (Harry 1995).
3	C_3PAD	Hexadecanoic acid, methyl ester [$C_{17}H_{34}O_2$]	—	—	—	52.31	—	—	Medical- industrial uses Rehmann et al. 1998).
4	C_4PAD	Dimethylcarbamothioic acid, *O*-isopropyl ester [$C_6H_{13}NOS$]	0.85	—	—	6.14	—	—	—
5	C_5PAD	Bis(2-ethylhexyl) phthalate [$C_{24}H_{38}O_4$]	29.94	4.96	57.44	—	8.55	60.87	Industrial uses (Chaudhary and Kim 2017).
6	C_6PAD	3-buten-2-one, 4-(1-aziridinyl)-4-(dimethylamino) [$C_8H_{14}N_2O$]	—	—	2.10	—	—	4.75	—
7	C_7PAD	1,1,1,5,5,5-hexamethyl-3,bis [(trimethylsilyl)oxy]-Trisiloxane [$C_{12}H_{36}O_4Si_5$]	7.59	—	—	—	6.02	—	Industrial uses (Chaudhary and Kim 2017).
8	C_8PAD	monoamide, N-(2methoxyphenyl)-, isohexyl ester Fumaric acid [$C_{17}H_{23}NO_4$]	—	—	1.60	—	—	3.06	Food-medicine, psoriasis-Medical Rehmann et al. 1998).
9	C_9PAD	4-Fluorohistamine [$C_5H_8FN_3$]	—	—	—	—	—	4.25	—
10	C_{10}PAD	3-Trimethylsilyloxystearic acid, trimethylsilyl ester [$C_{24}H_{52}O_3Si_2$]	11.16	—	—	—	—	—	Steel, petrochemical coal, Fertilizer (Harry 1995).
11	C_{11}PAD	9-Octadecenamide, (Z)-, Oleic acid amide [$C_{18}H_{35}NO$]	—	2.29	—	—	—	—	Industry uses, Consumer uses (Kadri et al. 2017).
12	C_{12}PAD	3-Amino-2-phenazinol ditms [$C_{18}H_{25}N_3OSi_2$]	—	—	—	—	5.17	—	—
13	C13PAD	2,5-bis(1,1-dimethylethyl)-Phenol [$C_{14}H_{22}O$]	—	—	6.66	—	—	13.91	Petrochemical industries (Kadri et al. 2017).

Note. All values represented in the table indicate the presence or absence (-) of the compound at the peak area within different incubation periods; [1]PA%: Peak area percentage; [2]MF: Molecular formula; [3]CPAD: Compound produced after degradation.

showed that all 13 compounds were completely degraded and disappeared and about four compounds were produced and identified (C_5PAD, C_6PAD, C_8PAD, and C_9PAD) at different PA% (Table 2). Palmitic acid (C_3PAD) was produced by KRA4 after 5 days at PA of 52.31%. Albuquerque et al. (2005) described the specific use of hexadecanoic ester derivatives for the effective control of the Groundnut earwig pest and the difference in the specificity of the synthesized ester derivatives in attracting both sexes also indicates their probable

Table 3. The industrially important by-products percentage produced after PHCs refinery wastewater biodegradation by KRD2 and KRA4 isolates at different incubation periods.

		Compounds produced (%) at different incubation periods (day)					
		KRD2			KRA4		
No.	Compound produced code	5	10	15	5	10	15
1	C_1PAD	5.27	0.0	0.0	6.78	0.0	0.0
2	C_2PAD	4.60	0.0	0.0	5.50	0.0	0.0
3	C_3PAD	0.0	0.0	0.0	100	0.0	0.0
4	C_4PAD	2.88	0.0	0.0	11.73	0.0	0.0
5	C_5PAD	100	100	100	0.0	100	100
6	C_6PAD	0.0	0.0	3.65	0.0	0.0	7.80
7	C_7PAD	25.35	0.0	0.0	0.0	70.40	0.0
8	C_8PAD	0.0	0.0	2.78	0.0	0.0	5.02
9	C_9PAD	0.0	0.0	0.0	0.0	0.0	6.98
10	C_{10}PAD	37.27	0.0	0.0	0.0	0.0	0.0
11	C_{11}PAD	0.0	46.16	0.0	0.0	0.0	0.0
12	C_{12}PAD	0.0	0.0	0.0	0.0	60.46	0.0
13	C_{13}PAD	0.0	0.0	11.59	0.0	0.0	22.85

resemblance to the pheromone components of *Caryedon serratus*. Moreover, Palmitic acid has a wide range of industrial applications, including soap and cosmetics production as well as food additives (Simakova et al. 2009). Therefore, several species of microorganisms are pooled to form a microbial consortium with enhanced enzymatic capacities that will increase the rate of PAH degradation (Gupta, Kumar, and Pal 2016). In the present study, other compounds with such applications are fumaric acid, monoamide and N-(2methoxyphenyl)-, isohexyl ester, which were produced by KRA_4 isolate after 15 days at PA of 3.06%, while Phenol, 2,5-bis(1,1-dimethylethyl)- was produced at PA of 13.91%. The initial PHC compounds percentages before bacterial degradation were represented in (Table 1) and all percentages of the compounds produced by KRA_4 isolate were shown in (Table 3). According to (Hasanoğlu 2013; Naeem and Ouyang 2013), phenol compounds are currently the commonest organic pollutants in wastewater due to the steady increase in industrialization and these compounds are extensively used in various industries, such as the petrochemical and chemical-allied industries.

Several recent studies have examined the mechanisms on how the bacteria degrade the petroleum hydrocarbons. For example, *Mycobacterium* is able to degrade pyrene and phenanthrene by K-region dihydrodiol intermediates (Rehmann et al. 1998; Dean-Ross and Cerniglia 1996; Moody et al. 2001; Vila et al. 2001; Cerniglia 1992). The bacterial degradation mechanisms could be interpreted as PHCs tend to be sorbed on the water sediments, desorption of PHCs from these sediments may lead to a higher concentration of PHCs near the water sediment interface. Also, the bacteria could be attached to the interface, this will increase contact chances between bacteria and PAHs. Moreover, the vinyl groups may be easily attacked by a cytochrome P450 and dioxygenase enzymes by *Mycobacterium* system (Heitkamp et al. 1988b; Pothuluri and Cerniglia 1994). According to the above discussion, we can conclude that the long incubation period may affect the biodegradation rate, due to the PHCs increased sediment content in the water system as well as other metabolites.

Identification of bacterial isolates

Two selected bacterial isolates were identified using Gram reaction and the results showed that bacterial isolate KRD2 was Gram-positive while KRA4 was Gram-negative. The genomic materials of the samples were

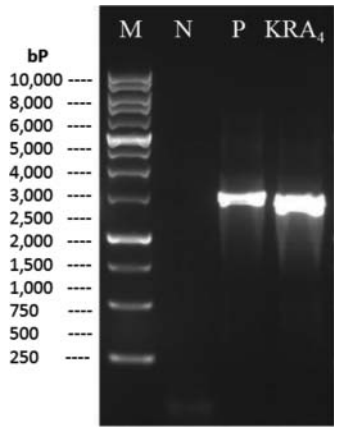

Figure 2. PCR-DGGE gel with 16S rDNA fragments derived from KRA4 isolate and *E. coli* in pure culture, as follows: Lane M: Marker; Lane N: PCR no template control (water to replace DNA template; Lane P: Positive control (DNA extracted from *E. coli* is used as template).

extracted using a G-spin TM DNA extraction kit. The DNA of the extracts was detected by gel electrophoresis and the two bacterial samples were amplified using 16S rRNA. The PCR reaction outcome was analyzed using gel electrophoresis (Figure 2). Based on denaturing gradient gel electrophoresis (DGGE), the KRA4 lane showed a thick band, potentially due to the high 16S rRNA gene concentration of approximately 1500 bp compared to the DNA marker in lane M. The 16S rRNA partial sequencing of KRA4 isolate was shown in (Figure 2). Based on these observations, a phylogeny tree was constructed using the neighbor joining method (Figure 3), and the nucleotide sequence of 16S rDNA from KRA4 isolate was 1459 bp as presented in (Figure 4).

From the alignment, isolate KRA4 was obtained to include in the genus *Chryseobacterium gambrini*. However, isolate KRD2 could not be identified using the molecular technique but with the Biolog microbial identification system, it was successfully designated as *Mycobacterium confluentis*. According to (Guo et al. 2017), they reported that the ryegrass root (soil-holding root system) enhanced the functional bacterial diversity in the early stages (0–10 days). However, the effect of ryegrass root may have stimulated the expression of GP and GN PAH-RHDa genes after 40 days and indicated that ryegrass increased the degradation of PAHs by promoting bacterial diversity, increasing the abundance of total bacteria PAHs degraders, and stimulating RHDa gene expression (Guo et al. 2017). Other studies report that bacteria are the most predominant microorganisms among others in either *in situ* or *ex situ* biodegradation processes, indicating that bacteria are the main agents

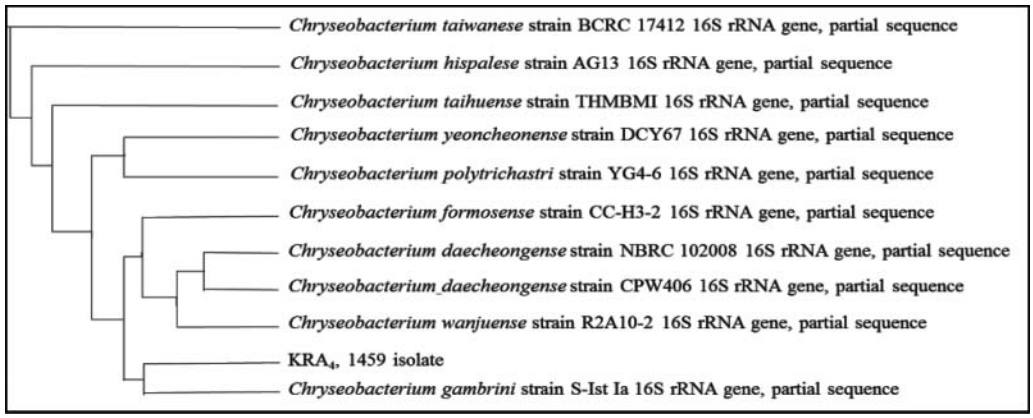

Figure 3. Phylogenic tree based on partial 16S rRNA sequences, showing the relationship between isolate KRA4 and other species belonging to the genus *Chryseobacterium*. The tree was constructed using the Clustal-x and neighbor joining method.

GGCTCAGGATGAACGCTAGCGGGAGGCCTAACACATGCAAGCCGAGCGGTAGAGATTC	60
TTCGGAATCTTGAGAGCGGCGCACGGGTGCGGAACACGTGTGCAACCTGCCTTTATCTG	120
GGGGATAGCCTTTCGAAAGGGAGATTAATACCCCATAATATACTGAGTGGCATCACTTA	180
TTATTGAAAACTCCGGTGGATAGAGATGGGCACGCGCAAGATTAGATAGGTTGGTGAG	240
GTAACGGCTCACCAAGTCAATGATCTTTAGGGGGCCTGAGAGGGTGATCCCCCACACTG	300
GTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATG	360
GGTGAGAGCCTGATCCAGCCATCCCGCGTGAAGGACGACGCCCTATGGGTGTAAACTC	420
CTTCTTTTGTACAGGGATAAACCTATTTACGTGTAAATAGCTGAAGGTACTGTACCAAT	480
AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATC	540
CGGATTTATTGGGTTTAAAGGGTCCGTAGGCGGATCCGTAAGTCAGTGGTGAAATCTCA	600
TAGCTTAACTATGAAACTGCCATTGATACTGCGGGTCTTGAGTAAGGTAGAAGTAGCTG	660
GAATAAGTAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGAACACCAATTGCG	720
AAGGCAGGTTACTATGTCTTAACTGACGCTGATGGACGAAAGCGTGGGGAGCGAACAG	780
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGCTAACTCGTTTTTGGTTTTTCGGA	840
ATCAGAGACTAAGCGTTTGTGATAAGTTAGCCACCTGGGGAGTACGAACGCAAGTTTGT	900
TTCTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGTTTAATTCGATG	960
ATACGCGAGGAACACTTACCAAGACTTAAGGGAATTGACAGATTTAGAAATAGATCCT	1020
CCTTCGGGCAATTTTCAAGGTGCTGCATGTGTCGTCAGCTCGTGCCGTGAGGTGTTAGG	1080
TTAAGTCCTGCAACGAGCGCAACCCCTGTCACTAGTTGCCATCATTCAGTTGGGGACTC	1140
TAGTGAGACTGCCTACGCAAGTAGAGAGGAAGGTGGGGATGACGTCAAATCATCACGG	1200
CCCTTACGTCTTGGGCCACACACGTAATACAATGGCCGGTACAGAGGGCAGCTACACA	1260
GCGATGTGATGCAAATCTCGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGAC	1320
TCTATGAAGCTGGAATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGTTCC	1380
CGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGTCTGGGGTACCTGAAGTCGGTG	1440
ACCGTAACAGGAGCTGCCTAGGGTAAAACAGGTAACTAGGGCTAAGTCGTAACAAG	1459

Figure 4. The nucleotide sequence of 16S rDNA from KRA4, 1459 bp isolate.

responsible for PAH degradation (Velayutham et al. 2012). Two efficient PAH-degrading strains isolated have been identified as *Pseudomonas stutzeri* and *Bacillus subtilis* (Chaudhary and Kim 2017). The use of native bacteria with PAH-utilization capabilities in a contaminated environment has proven to be an eco-friendly approach (Velayutham et al. 2012). Members of genus *Chryseobacterium* have been isolated from a wide variety of habitats, including plants, soil, tree roots, clinical specimens, animal bodies, fresh water, wastewater, compost, sludge, sediments, and dairy products (Chaudhary and Kim 2017).

Conclusions

This study demonstrates that PHCs were successfully degraded by KRD2 and KRA4 bacterial isolates at different incubation periods with a degradation rate up to 92.31% of the initial PHC compounds within 5 days, and decreased to 46.15% and 61.54% within 10 days, respectively. Meanwhile, after 15 days, a complete degradation, then (100%) was achieved. Most of the microbial degradation products reported were shown to have wide industrial and medical applications in food and pharmaceutical industries. The two isolates were successfully identified as *Mycobacterium confluentis* and *Chryseobacterium gambrini*, respectively. Conclusively, this study strongly recommends the potential microbial degradation of PHCs in refinery wastewater to produce industrially important by-products and to reduce environmental pollution.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The author would like to thank Faculty of Industrial Sciences & Technology (FIST), Universiti Malaysia Pahang (UMP) for technical assistance during this research. Moreover, the financial support from PGRS160393 is highly acknowledged and appreciated.

Funding

Financial support (PGRS160393).

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References

Albuquerque, M. L., I. Guedes, P. Alcantara Jr., S. G. Moreira, N. N. M. Barbosa, D. S. Correa, and S. C. Zilio. 2005. Characterization of Buriti (*Mauritia flexuosa* L.) oil by absorption and emission spectroscopies. *Journal of the Brazilian Chemical Society* 16 (6A):1113–17. doi:10.1590/S0103-50532005000700004.

Barathi, S., and N. Vasudevan. 2001. Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environment International* 26 (5):413–16. doi:10.1016/S0160-4120(01)00021-6.

Cerniglia, C. E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3:351–68. doi:10.1007/BF00129093.

Chaudhary, D. K., and J. Kim. 2017. *Chryseobacterium nepalense* sp. nov., isolated from oil-contaminated soil. *International Journal of Systematic and Evolutionary Microbiology* 67 (3):646–52. doi:10.1099/ijsem.0.001680.

Colwell, R. R., J. D. Walker, and J. J. Cooney. 1977. Ecological aspects of microbial degradation of petroleum in the marine environment. *CRC Critical Reviews in Microbiology* 5 (4):423–45. doi:10.3109/10408417709102813.

Das, N., and P. Chandran. 2010. Microbial degradation of petroleum hydrocarbon contaminants: An overview. *Biotechnology Research International* 2011:13.

Dean-Ross, D., and C. E. Cerniglia. 1996. Degradation of pyrene by *Mycobacterium flavescens*. *Applied Microbiology and Biotechnology* 46:307–12. doi:10.1007/s002530050822.

Eaton, A. D., L. S. Clesceri, A. E. Greenberg, M. A. H. Franson. 1998. Standard methods for the examination of water and wastewater. American Public Health Association (APHA), American Water Works Association, Water Environment Federation.

Makky, E. A., M. Z. Shaiful Zahid, M. Al-Matar, and M. M. Yusoff. 2016. Preliminary phylogenetic analysis of thermophilic bacteria producing protease isolated from Kuantan Malaysia. *Journal of Biotechnology Science Research (JBSR)* 3 (5):166–74.

Ferreira, L., E. Rosales, M. Sanromán, and M. Pazos. 2015. Preliminary testing and design of permeable bioreactive barrier for phenanthrene degradation by *Pseudomonas stutzeri* CECT 930 immobilized in hydrogel matrices. *Journal of Chemical Technology and Biotechnology* 90 (3):500–06. doi:10.1002/jctb.4338.

Golyshin, P. N., V. A. M. Dos Santos, O. Kaiser, M. Ferrer, Y. S. Sabirova, H. Lünsdorf, and A. Pühler. 2003. Genome sequence completed of *Alcanivorax borkumensis*, a hydrocarbon-degrading bacterium that plays a global role in oil removal from marine systems. *Journal of Biotechnology* 106 (2):215–20. doi:10.1016/j.jbiotec.2003.07.013.

Guo, M., Z. Gong, R. Miao, J. Rookes, D. Cahill, and J. Zhuang. 2017. Microbial mechanisms controlling the rhizosphere effect of ryegrass on degradation of polycyclic aromatic hydrocarbons in an aged-contaminated agricultural soil. *Soil Biology and Biochemistry* 113:130–42. doi:10.1016/j.soilbio.2017.06.006.

Gupta, G., V. Kumar, and A. K. Pal. 2016. Biodegradation of polycyclic aromatic hydrocarbons by microbial consortium: A distinctive approach for decontamination of soil. *Soil and Sediment Contamination: An International Journal* 25 (6):597–623. doi:10.1080/15320383.2016.1190311.

Hamamura, N., D. M. Ward, and W. P. Inskeep. 2013. Effects of petroleum mixture types on soil bacterial population dynamics associated with the biodegradation of hydrocarbons in soil environments. *FEMS Microbiology Ecology* 85 (1):168–78. doi:10.1111/1574-6941.12108.

Harry, M. 1995. *Industrial pollution handbook*. New York: McGraw Hill. Inc.

Hasanoğlu, A. 2013. Removal of phenol from wastewaters using membrane contactors: Comparative experimental analysis of emulsion pertraction. *Desalination* 309:171–80. doi:10.1016/j.desal.2012.10.004.

Heitkamp, M. A., J. P. Freeman, D. W. Miller, and C. E. Cerniglia. 1988b. Pyrene degradation by a Mycobacterium sp.: Identification of ring oxidation and ring fission products. *Applied and Environmental Microbiology* 54:2556–65.

Heudorf, U., V. Mersch-Sundermann, and J. Angerer. 2007. Phthalates: Toxicology and exposure. *International Journal of Hygiene and Environmental Health* 210 (5):623–34. doi:10.1016/j.ijheh.2007.07.011.

Kadri, T., T. Rouissi, S. K. Brar, M. Cledon, S. Sarma, and M. Verma. 2017. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by fungal enzymes: A review. *Journal of Environmental Sciences (JES)* 51:52–74 doi:10.1016/j.jes.2016.08.023.

Kelley, I., J. P. Freeman, and C. E. Cerniglia. 1990. Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp. *Biodegradation* 1 (4):283–90. doi:10.1007/BF00119765.

Li, P., H. Li, F. Stagnitti, X. Wang, H. Zhang, Z. Gong, and C. Austin. 2005. Biodegradation of pyrene and phenanthrene in soil using immobilized fungi *Fusarium* sp. *Bulletin of Environmental Contamination & Toxicology* 75 (3):443–50. doi:10.1007/s00128-005-0773-1.

Macaulay, B. M., and D. Rees. 2014. Bioremediation of oil spills: A review of challenges for research advancement. *Annals of Environmental Science* 8:9–37.

Moody, J. D., J. P. Freeman, D. R. Doerge, and C. E. Cerniglia. 2001. Degradation of phenanthrene and anthracene by cell

suspensions of *Mycobacterium* sp. strain PYR-1. *Applied and Environmental Microbiology* 67:1476–83. doi:10.1128/AEM.67.4.1476-1483.2001.

Ibrahim, M., E. A.. Makky, S. Zareen, T. F. Abdelhadi, N. S. Azmi, and J. Ismail. 2017. Bioremediation of PAHS from industrial refinery effluents to highly valuable transformed compounds. Paper presented at the FGIC 1st Conference on Governance & Integrity, 2017 "Innovation & Sustainability Through Governance," Yayasan Pahang, Kuantan, Malaysia, April 3 – 4 2017. 323–36. ISBN 978-967-2054-37-5

Naeem, K., and F. Ouyang. 2013. Influence of supports on photocatalytic degradation of phenol and 4-chlorophenol in aqueous suspensions of titanium dioxide. *Journal of Environmental Sciences* 25 (2):399–404. doi:10.1016/S1001-0742(12)60055-2.

Petronas Chemicals Group (PCG) 2014. 17th Annual general meeting, Kuala Lumpur Convention Centre, Jalan Ampang, 50088 Kuala Lumpur, Malaysia.

Perry, J. J. 1984. *Microbial metabolism of cyclic alkanes in petroleum microbiology*, R. M. Atlas, Ed., 61–98, New York, NY, USA: Macmillan.

Pothuluri, J. V., and C. E. Cerniglia. 1994. Microbial metabolism of polycyclic aromatic hydrocarbons. In *Biological degradation and bioremediation of toxic chemicals*, ed. G. R. Chaudhry, 92–124. Dordrecht: Kluwer.

Prabhakaran, P., A. Sureshbabu, S. Rajakumar, and P. Ayyasamy. 2014. Bioremediation of crude oil in synthetic mineral salts medium enriched with aerobic bacterial consortium. *International Journal of Innovative Research in Science Engineering and Technology* 3:9236–42.

Primm, T. P., C. A. Lucero, and J. O. Falkinham. 2004. Health impacts of environmental mycobacteria. *Clinical Microbiology Reviews* 17 (1):98–106. doi:10.1128/CMR.17.1.98-106.2004.

Rehmann, K., H. P. Noll, C. E. Steinberg, and A. A. Kettrup. 1998. Pyrene degradation by *Mycobacterium* sp. strain KR2. *Chemosphere* 36 (14):2977–92. doi:10.1016/S0045-6535(97)10240-5.

Romero, M., M. Cazau, S. Giorgieri, and A. Arambarri. 1998. Phenanthrene degradation by microorganisms isolated from a contaminated stream. *Environmental Pollution* 101 (3):355–59. doi:10.1016/S0269-7491(98)00056-6.

Seo, J. S., Y. S. Keum, and Q. X. Li. 2009. Bacterial degradation of aromatic compounds. *International Journal of Environmental Research and Public Health* 6 (1):278–309. doi:10.3390/ijerph6010278.

Shao, Y., Y. Wang, X. Wu, X. Xu, S. Kong, L. Tong, and B. Li. 2015. Biodegradation of PAHs by Acinetobacter isolated from karst groundwater in a coal-mining area. *Environmental Earth Sciences* 73 (11):7479–88. doi:10.1007/s12665-014-3920-3.

Simakova, I., O. Simakova, P. Mäki-Arvela, A. Simakov, M. Estrada, and D. Y. Murzin. 2009. Deoxygenation of palmitic and stearic acid over supported Pd catalysts: Effect of metal dispersion. *Applied Catalysis A: General* 355 (1):100–108. doi:10.1016/j.apcata.2008.12.001.

Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey. 1997. Proposal for a new hierarchic classification system, *Actinobacteria classis* nov. *International Journal of Systematic and Evolutionary Microbiology* 47 (2):479–91.

Tahir, S., A. Badshah, and R. A. Hussain. 2015. Guanidines from 'toxic substances' to compounds with multiple biological applications–Detailed outlook on synthetic procedures employed for the synthesis of guanidines. *Bioorganic Chemistry* 59:39–79. doi:10.1016/j.bioorg.2015.01.006.

Trzesicka-Mlynarz, D., and O. Ward. 1995. Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures, obtained from PAH-contaminated soil. *Canadian Journal of Microbiology* 41 (6):470–76. doi:10.1139/m95-063.

Ulrici, W. 2000. Contaminated soil areas, different countries and contaminants, monitoring of contaminants. *Biotechnology Set, Second Edition* 11:5–41.

van Geel, M., P. van de Kerkhof, A. Oostveen, E. de Jong, and M. Seyger. 2016. Fumaric acid esters in recalcitrant pediatric psoriasis: A prospective, daily clinical practice case series. *Journal of Dermatological Treatment* 27 (3):214–20. doi:10.3109/09546634.2015.1088131.

Varjani, S. J., and V. N. Upasani. 2013. Comparative studies on bacterial consortia for hydrocarbon degradation. *International Journal of Innovative Research in Science, Engineering and Technology* 2 (10) 5377–83.

Varjani, S. J., and V. N. Upasani. 2016. Biodegradation of petroleum hydrocarbons by oleophilic strain of Pseudomonas aeruginosa NCIM 5514. *Bioresource Technology* 222:195–201. doi:10.1016/j.biortech.2016.10.006.

Varjani, S. J., D. P. Rana, A. K. Jain, S. Bateja, and V. N. Upasani. 2015. Synergistic *ex-situ* biodegradation of crude oil by halotolerant bacterial consortium of indigenous strains isolated from on shore sites of Gujarat. *India International Biodeterioration & Biodegradation* 103:116–24. doi:10.1016/j.ibiod.2015.03.030.

Varjani, S. J., and V. K. Srivastava. 2015. Green technology and sustainable development of environment. *Renewable Resources Journal* 3 (1):244–49.

Varjani, S. J. 2017. Microbial degradation of petroleum hydrocarbons. *Bioresource Technology* 223:277–86. doi:10.1016/j.biortech.2016.10.037.

Velayutham, T., V. Arutchelvan, S. Nagarajan, and V. Muralikrishnan. 2012. Isolation and identification of polycyclic aromatic ydrocarbon–degrading bacteria from crude oil exploration bore well sludge. *Bioremediation Journal* 16 (3):141–46. doi:10.1080/10889868.2012.687413.

Vila, J., Z. López, J. Sabaté, C. Minguillón, A. M. Solanas, and M. Grifoll. 2001. Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Applied and Environmental Microbiology* 67:5497–505. doi:10.1128/AEM.67.12.5497-5505.2001.

Walter, U., M. Beyer, J. Klein, and H. J. Rehm. 1991. Degradation of pyrene by *Rhodococcus* sp. UW1. *Applied Microbiology and Biotechnology* 34 (5):671–76. doi:10.1007/BF00167921.

Young, D., J. Rice, R. Martin, E. Lindquist, A. Lipzen, I. Grigoriev, and D. Hibbett. 2015. Degradation of bunker C fuel oil by white-rot fungi in sawdust cultures suggests potential applications in bioremediation. *PLOS One* 10 (6):e0130381. doi:10.1371/journal.pone.0130381.