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POLYAROMATIC HYDROCARBONS UTILIZATION BY A *Pseudomonas* STRAIN

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Abstract

Polyaromatic hydrocarbons (PAHs) are major environmental pollutants, which are well known for their toxic effects on the organisms. However, different *Pseudomonas* strains are able to use these toxic compounds as growth substrate. *Pseudomonas aeruginosa* strain IBB_{Po16} was able to grow on nutrient-rich and minimal media in the presence of several toxic PAHs, such as naphthalene, methylnaphthalene, phenanthrene, fluorene. *P. aeruginosa* IBB_{Po16} cells exhibited a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs, as compared with growth on minimal medium. Naphthalene was less toxic for *P. aeruginosa* IBB_{Po16} cells, compared with methylnaphthalene, phenanthrene, and fluorene. *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs produced specific extracellular secondary metabolites (i.e., surfactants, pigments). *P. aeruginosa* IBB_{Po16} cells grown in the presence of naphthalene possess *ndoM* (naphthalene dioxygenase) and *rhlAB* (rhamnosyltransferase 1) genes, whereas *C23DO* (catechol 2,3-dioxygenase) and *pahDO* (PAH dioxygenase) genes were not detected in this Gram-negative bacterium.

Key words: *Pseudomonas, PAHs, secondary metabolites*.

INTRODUCTION

Petroleum and petroleum products are important energy resources used in industry and in our daily life. Consequently, petroleum hydrocarbons are major pollutants of the environment (Al-Wasify and Hamed, 2014). Presence of polyaromatic hydrocarbons (PAHs) in soil and water is a major environmental problem, because most of them are toxic, mutagenic and/or carcinogenic for humans and other animals (Bugg et al., 2000; Al-Wasify and Hamed, 2014). PAHs are formed during pyrolysis of petroleum and petroleum products and they are components of petrogenic materials (e.g., crude oil, refined oil products, coal, tar, etc.) (Pies et al., 2008). Extensive research was carried out on the fate of these contaminants in the environment and in bioremediation systems (Foght and Westlake, 1988; Bugg et al., 2000; Al-Wasify and Hamed, 2014). Bacteria are considered as one of the dominant hydrocarbon-degrading organism found in the environment (Al-Wasify and Hamed, 2014).

PAHs which are compounds with two and more fused aromatic rings are rich sources of carbon and energy. Consequently, these compounds are used as growth substrate by some bacteria (Bugg et al., 2000; Norman et al., 2004; Zhang et al., 2011; Al-Wasify and Hamed, 2014).

Different *Pseudomonas aeruginosa* strains were isolated from sites polluted with petroleum and petroleum products and these bacteria were able to produce extracellular secondary metabolites (i.e., surfactants, pigments) which enhance their competetiveness and survival.

It is well known that pyocyanin pigment has antimicrobial activity against a variety of microorganisms (Norman et al., 2004).

Furthermore, some *P. aeruginosa* strains produced rhamnolipid biosurfactant (mixtures of mono- and di-rhamnolipids) which enhance the biodegradation of numerous toxic hydrocarbons (Cameotra and Singh, 2009; Abdel-Mawgoud et al., 2009).

The aim of this study was to investigate the capability of *P. aeruginosa* IBB_{Po16} cells to grow on nutrient-rich and minimal media in the presence of several toxic PAHs, such as naphthalene, methylnaphthalene, phenanthrene and fluorene. The production of extracellular secondary metabolites (i.e., surfacetants, pigments) by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs was also evaluated in this study.

MATERIALS AND METHODS

Polyaromatic hydrocarbons (PAHs) growth substrate experiments. *P. aeruginosa* strain IBB_{Po16} was inoculated into nutrient-rich LB medium (Heipieper et al., 1992). Flask was incubated 24 h at 30°C on a rotary shaker (200 rpm).

Overnight bacterial cultures were spotted (20 µl) on nutrient-rich (Heipieper et al., 1992) and minimal (Stancu and Grifoll, 2011) agar media. PAHs (i.e., naphthalene, methylnaphthalene, phenanthrene, fluorene) were supplied in the vapor phase. Controls were prepared in the same way but without addition of PAHs. Petri plates were sealed and incubated for 24-48 h at 30°C.

Overnight bacterial cultures were inoculated (100 µl) into nutrient-rich and minimal liquid media. Then, the tested PAHs (i.e., naphthalene, methylnaphthalene, phenanthrene, fluorene) were supplied in a concentration of 100 mg l^{-1} (dissolved in dichloromethane) to the cell suspensions. Controls were prepared in the same way but without addition of PAHs. Flasks were sealed and incubated for 24-216 h at 30°C on a rotary shaker (200 rpm). The growth of the cells in the presence of PAHs was determined by measuring optical density at 660 nm (OD_{660}) using a SPECORD 200 UV-visible spectrophotometer (Analytik Jena, Jena, Germany). The cell growth in the presence of PAHs was investigated also by spot assay. The bacterial cultures (20 µl) were spotted on nutrient-rich agar. Petri plates were incubated for 24 h at 30°C.

Extracellular secondary metabolites. CTAB methylene blue agar (Siegmund and Wagner, 1991), King A and King B agar (King et al., 1954) were used to detect rhamnolipid surfactants, pyocyanin and pyoverdin (fluorescein) pigments, respectively. The bacterial cultures (20 µl) were spotted on these selective media. Petri plates were incubated for 24-48 h at 30°C. The colonies which produced rhamnolipid surfactants were surrounded by dark blue halos on CTAB agar. The colonies which produced pyocyanin pigment were blue-green on King A and the colonies which produced pyoverdin were yellow-green fluorescent on King B agar. Emulsification activity (E_{24}) assay was used to quantify the surfactants in the cell-free culture broths (Abdel-Mawgoud et al., 2009).

Polymerase chain reaction (PCR). Genomic DNA was extracted from the cell pellets with Pure Link genomic kit (Invitrogen). For PCR amplification, 1 µl of DNA extract was added to a final volume of 25 µl reaction mixture, containing: 5 µl 5×GoTaq flexi buffer, 5 µl $MgCl_2$, 0.5 µl dNTP mix, 0.5 µl specific primers (23CAT-f/23CAT-r, Mesarch et al., 2000; ISPGRLE1B/ISPGRRI1B, Meyer et al., 1999; ndoM-f/ndoM-r, Márquez-Rocha et al., 2005; rhlA-f/rhlB-r, Medina et al., 2003), and 0,125 µl GoTaq G2 hot start polymerase (Promega). PCR was performed with a Mastercycler pro S (Eppendorf, Hamburg, Germany). The PCR program consisted in initial denaturation for 10 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 43°C, 50°C or 58°C for 30 sec, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. After separation on 1.5% (w/v) TBE agarose gel (Sambrook et al., 1989) and staining with fast blast DNA stain (Bio-Rad) the PCR products were analyzed.

Reagents used during this study were procured from Merck (Darmstadt, Germany), Sigma-Aldrich (Saint-Quentin-Fallavier, France), Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA, USA) or Bio-Rad Laboratories (Hercules, CA, USA). The PCR primers were purchased from Integrated DNA Technologies (Coralville, IA, USA) and Invitrogen (Carlsbad, CA, USA).

RESULTS AND DISCUSSIONS

The strain used in this study was *P. aeruginosa* IBB_{Po16} (GenBank accession number KT315654). This *P. aeruginosa* strain which produced some extracellular secondary metabolites (i.e., rhamnolipid surfactants, pyocyanin and pyoverdin pigments) was formerly isolated by us from Poeni oily sludge (Stancu, 2017).

Polyaromatic hydrocarbons (PAHs) growth substrate experiments. *P. aeruginosa* IBB_{Po16} was able to grow on nutrient-rich and minimal agar media when several PAHs, such as naphthalene, methylnaphthalene, phenanthrene, and fluorene were supplied in vapor phase (Figure 1a, 1b). *P. aeruginosa* IBB_{Po16} cells

showed a higher growth when they were inoculated on nutrient-rich agar medium in the presence of PAHs (100%), as compared with growth on minimal agar (10%). *P. aeruginosa* IBB_{Po16} cells inoculated on nutrient-rich agar medium in the presence of PAHs produced the pyoverdin (yellow-green fluorescent) pigment (Figure 1a) visible under UV light (366 nm), while on minimal agar medium the pigment production was not observed. Was not surprising to observe such changes, because the bacteria which are exposed to toxic hydrocarbons under nutrient limited condition, generally exhibit different physiological changes (Sikkema et al., 1995; Bugg et al., 2000; Norman et al., 2004).

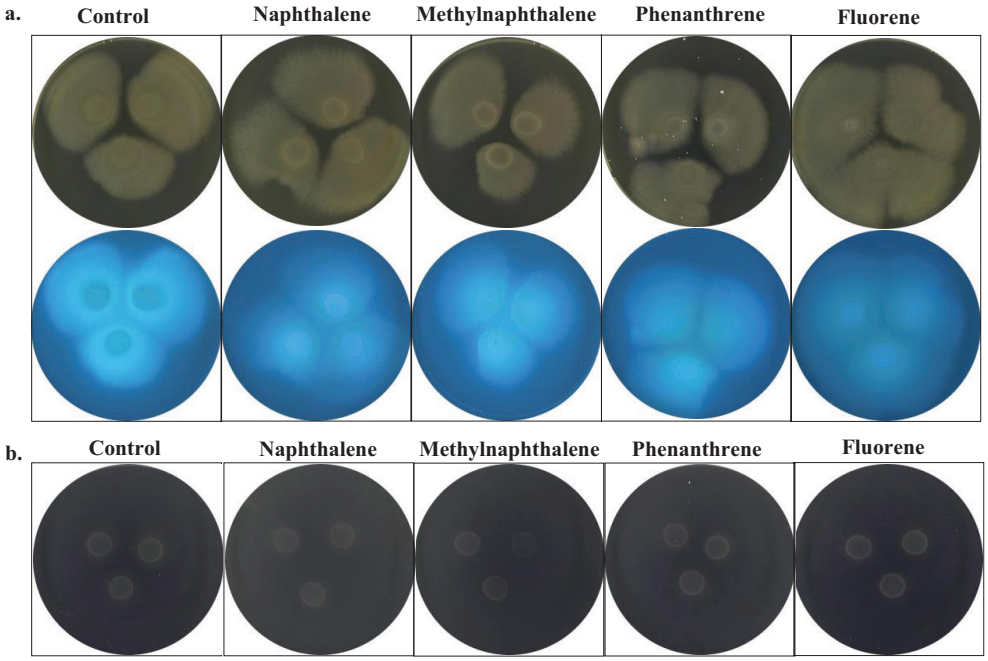

Figure 1. *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (in vapor phase). a. Nutrient-rich agar medium; plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side). b. Minimal agar medium; plates were observed under visible white light (500 nm).

P. aeruginosa IBB_{Po16} cells were also able to grow on nutrient-rich and minimal media in the presence of 100 mg l^{-1} PAHs (naphthalene, methylnaphthalene, phenanthrene, fluorene) (Table 1). As observed in previously assay, *P. aeruginosa* IBB_{Po16} cells showed a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs (OD increased by 0.21-1.55 times), as compared with growth on minimal medium (OD increased by 0.13-0.63 times). According with literature (Bugg et al., 2000), PAHs have high octanol-water partition coefficients (log P_{OW}) and partition readily into organic phases. The ordering of phospholipids in bacterial membranes creates a hydrophobic region in the interior of the membrane that can act as a reservoir for accumulation of hydrophobic compounds, including of toxic PAHs (Bugg et al., 2000). As a result of accumulation of hydrophobic compounds in lipid membranes, loss of membrane integrity and alterations in enzyme activities have been described (Sikkema et al., 1995). It is well known that naphthalene (with log P_{OW} = 3.31), methylnaphthalene (log P_{OW} = 3.81), phenanthrene and fluorene (log P_{OW} = 4.49) are low molecular weight PAHs that contain two or three benzene rings. However, they belongs to the same group, naphthalene was less toxic for *P. aeruginosa* IBB_{Po16} (OD increased by 0.14-1.55 times), compared with methylnaphthalene, phenanthrene, and fluorene (OD increased by 0.13-1.24 times).

Table 1. Growth of *P. aeruginosa* IBB_{Po16} in the presence of PAHs (100 mg l^{-1})

Variant	Cell growth									
	Nutrient-rich medium					Minimal medium				
	24 h	48 h	72 h	144 h	216 h	24 h	48 h	72 h	144 h	216 h
Control	1.12	1.34	1.80	2.22	2.18	0.19	0.20	0.21	0.81	0.39
Naphthalene	0.83	0.99	1.01	1.55	1.35	0.14	0.15	0.17	0.63	0.32
Methylnaphthalene	0.24	0.45	0.66	1.22	0.58	0.17	0.19	0.22	0.51	0.32
Phenanthrene	0.22	0.44	0.71	1.24	0.65	0.13	0.14	0.14	0.45	0.30
Fluorene	0.21	0.41	0.75	1.13	0.67	0.15	0.16	0.18	0.48	0.29

Cell growth, the OD at 660 nm was measured, and the values from the table represent the ratio between OD at 24, 48, 72, 144 or 216 h and OD at 0 h.

P. aeruginosa IBB_{Po16} cells inoculated on nutrient-rich and minimal media produced the pyoverdin pigment visible under UV light (366 nm). As could be observed in Figure 2, the colony fluorescence decreases when *P. aeruginosa* IBB_{Po16} cells were grown for 144 h in the presence of the PAHs (especially on minimal medium).

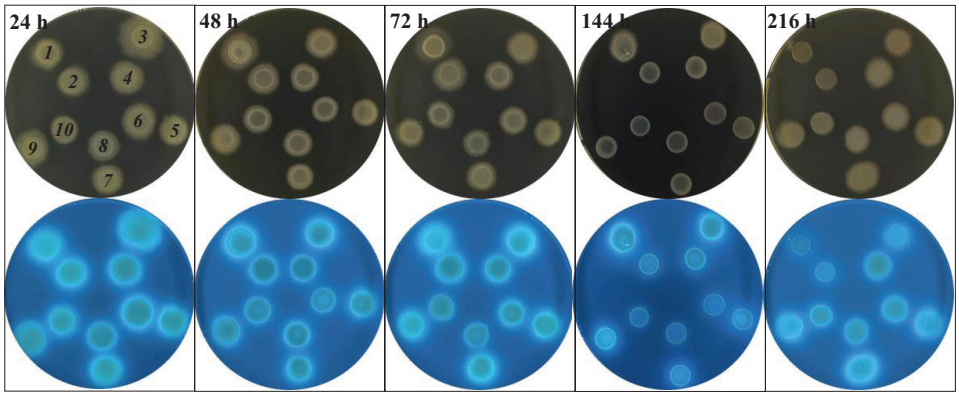

Figure 2. *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (100 mg l^{-1}). Nutrient-rich medium (***1***, ***3***, ***5***, ***7***, ***9***); minimal medium (***2***, ***4***, ***6***, ***8***, ***10***); control (***1***, ***2***), naphthalene (***3***, ***4***), methylnaphthalene (***5***, ***6***), phenanthrene (***7***, ***8***), fluorene (***9***, ***10***); plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side).

Extracellular secondary metabolites. The production of extracellular secondary metabolites (i.e., surfactants, pigments) by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs (naphthalene, methylnaphthalene, phenanthrene, fluorene) was further investigated (Figure 3).

A dark blue halo was observed (under visible white light) on CTAB methylene blue agar for *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs, as well as for the control cells. As expected, *P. aeruginosa* IBB_{Po16} produced rhamnolipid surfactants, and their emulsification activity was very good (data not shown). Like other PAHs with two or three benzene rings, naphthalene, methylnaphthalene, phenanthrene, and fluorene are extremely resistant to nucleophilic attack and they are also recalcitrant to biodegradation due to very less solubility in water (Das et al., 2007). However, the biosurfactants produced by some bacteria, including by different *Pseudomonas* strains, increase the solubility of several PAHs, facilitating their assimilation and utilization by bacteria (Das et al., 2007).

P. aeruginosa IBB_{Po16} cells grown in the presence of PAHs produced pyoverdin (yellow-green fluorescent) pigment on both King A and King B agar media (Figure 3). We observed that the pyoverdin pigment production increased when *P. aeruginosa* IBB_{Po16} cells were grown on nutrient-rich and minimal media in the presence of the PAHs, as compared with respective controls.

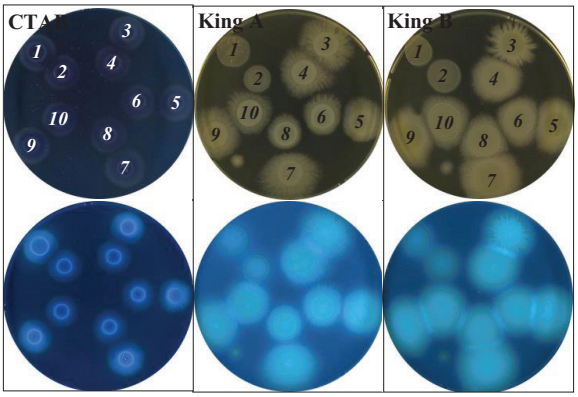

Figure 3. Production of extracellular secondary metabolites by *P. aeruginosa* IBB_{Po16} grown in the presence of PAHs (100 mg l^{-1}). Nutrient-rich medium (***1***, ***3***, ***5***, ***7***, ***9***); minimal medium (***2***, ***4***, ***6***, ***8***, ***10***); control (***1***, ***2***), naphthalene (***3***, ***4***), methylnaphthalene (***5***, ***6***), phenanthrene (***7***, ***8***), fluorene (***9***, ***10***); plates were observed under visible white light (500 nm, upper side) and UV light (366 nm, down side).

The production of pyocyanin (light blue-green) pigment by *P. aeruginosa* IBB_{Po16} cells grown in the presence of PAHs was acquired, when the plate with King A agar medium, was incubated for 24 h at 37°C.

Polymerase chain reaction (PCR). Genomic DNA extracted from *P. aeruginosa* IBB_{Po16} cells grown on nutrient-rich medium in the presence of naphthalene was used as template for PCR amplification of *C23DO* (catechol 2,3-dioxygenase), *pahDO* (PAH dioxygenase), and *ndoM* (naphthalene dioxygenase) catabolic genes, and *rhlAB* (rhamnosyltransferase 1) genes (Figure 4). According with literature, the *C23DO*, *pahDO* and *ndoM* genes are responsible for aromatic hydrocarbon metabolism (Mesarch et al., 2000; Meyer et al., 1999; Márquez-Rocha et al., 2005), and *rhlAB* genes are involved in synthesis of rhamnolipid surfactants (Medina et al., 2003).

The annealing temperatures were 43°C, 50°C and 58°C when primers for these genes were used for PCR amplification. In the DNA extracted from *P. aeruginosa* IBB_{Po16} cells grown in the presence of naphthalene only *ndoM* (642 bp) and *rhlAB* (216 bp) genes were detected, whereas *C23DO* (238 bp) and *pahDO* (900 bp) catabolic genes were not detected in this bacterium.

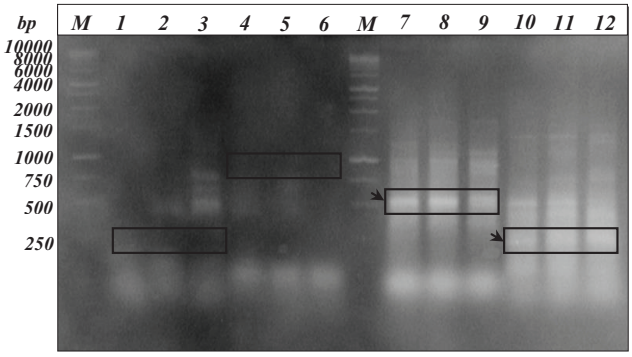

Figure 4. Detection of *C23DO, pahDO, ndoM*, and *rhlAB* genes in DNA extracted from *P. aeruginosa* IBB_{Po16} grown on nutrient-rich medium in the presence of naphthalene (100 mg l^{-1}). 1 kb DNA ladder, Promega (***M***); *C23DO* (expected fragment size 238 bp) gene (***1-3***); *pahDO* (900 bp) gene (***4-6***); *ndoM* (642 bp) gene (***7-9***); *rhlAB* (216 bp) gene (***10-12***); primers annealing of 43°C (***1***, ***4***, ***7***, ***10***), 50°C (***2***, ***5***, ***8***, ***11***), 58°C (***3***, ***6***, ***9***, ***12***).

CONCLUSIONS

P. aeruginosa strain IBB_{Po16} was able to grow on nutrient-rich and minimal media in the presence of tested PAHs (i.e., naphthalene, methylnaphthalene, phenanthrene, fluorene). *P. aeruginosa* IBB_{Po16} cells exhibited a higher growth when they were inoculated on nutrient-rich medium in the presence of PAHs, as compared with growth on minimal medium.

Naphthalene was less toxic for *P. aeruginosa* IBB_{Po16} cells, compared with methylnaphthalene, phenanthrene, and fluorene.

P. aeruginosa IBB_{Po16} cells grown in the presence of naphthalene possess *ndoM* and *rhlAB* genes, which are involved in naphthalene degradation and rhamnolipid surfactants synthesis, respectively.

P. aeruginosa IBB_{Po16} cells grown in the presence of PAHs produced some extracellular secondary metabolites, such as rhamnolipid surfactants, pyocyanin and pyoverdin pigments, which are well recognized for their multiple applications (e.g., bioremediation of petroleum polluted sites).

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REFERENCES

Abdel-Mawgoud A.M., Aboulwafa M.M., Hassouna N.A.-H., 2009. Characterization of rhamnolipid produced by *Pseudomonas aeruginosa* isolate Bs20. Applied Biochemistry and Biotechnology, 157:329-345.

Al-Wasify R.S., Hamed S.R., 2014. Bacterial biodegradation of crude oil using local isolates. International Journal of Bacteriology, doi:10.1155/2014/863272.

Bugg T., Foght J.M., Pickard M.A., Gray M.R., 2000. Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Applied and Environmental Microbiology, 66:5387-5392.

Cameotra S.S., Singh P., 2009. Synthesis of rhamnolipid biosurfactant and mode of hexadecane uptake by *Pseudomonas* species. Microbial Cell Factories, 8, doi:10.1186/1475-2859-8-16.

Das P., Mukherjee S., Sen R., 2007. Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin. Chemosphere, 72:1229-1234.

Foght J.M., Westlake D.W.S., 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. Canadian Journal of Microbiology, 34:1135-1141.

Heipieper H.J., Diefenbac, R., Keweloh H., 1992. Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. Applied and Environmental Microbiology, 58:1847-1852.

King E.O., Ward M. K., Raney D. E., 1954. Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine, 44:301-307.

Márquez-Rocha F.J., Olmos-Soto J., Rosano-Hernández M.C., Muriel-Garcia M., 2005. Determination of the hydrocarbon-degrading metabolic capabilities of tropical bacterial isolates. International Biodeterioration and Biodegradation, 55:17-23.

Medina G., Juárez K., Valderrama B., Soberón-Chávez G., 2003. Mechanism of *Pseudomonas aeruginosa* RhlR transcriptional regulation of the rhlAB promoter. Journal of Bacteriology, 185:5976-5983.

Mesarch M.B., Nakatsu C.H., Nies L., 2000. Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR. Applied and Environmental Microbiology, 66:678-683.

Meyer S., Moser R., Neef A., Stahl U., Kampfer P., 1999. Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology, 145:1731-1741.

Norman R.S., Moeller P., McDonald T.J., Morris P.J., 2004. Effect of pyocyanin on a crude-oil-degrading microbial community. Applied and Environmental Microbiology, 70:4004-4011.

Pies C., Ternes T.A., Hofmann T., 2008. Identifying sources of polycyclic aromatic hydrocarbons (PAHs) in soils: distinguishing point and non-point sources using an extended PAH spectrum and *n*-alkanes. Journal of Soils and Sediments, 8:312-322.

Sambrook J., Fritsch E.F., Maniatis T., 1989. Molecular Cloning, A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Siegmund I., Wagner F., 1991. New method for detecting rhamnolipids excreted by *Pseudomonas* species during growth on mineral agar. Biotechnology Techniques, 5:265-268.

Sikkema J., de Bont J.A.M., Poolman B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews, 59:201-222.

Stancu M.M., Grifoll M., 2011. Multidrug resistance in hydrocarbon-tolerant Gram-positive and Gram-negative bacteria. The Journal of General and Applied Microbiology, 57:1-18.

Zhang Z., Hou Z., Yang C., Ma C., Tao F., Xu P., 2011. Degradation of *n*-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresource Technology, 102:4111.4116.