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Improved PAHs removal performance by a defined bacterial consortium of indigenous *Pseudomonas* and actinobacteria from Patagonia, Argentina

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ABSTRACT

Defined mixed cultures consisting of *Pseudomonas* and actinobacteria strains showed interesting polycyclic aromatic hydrocarbons (PAHs) removal values, improving greatly removal efficiency of strains in pure culture. These bacterial mixed cultures were synthesized with bacteria isolated from contaminated sites, previously evaluated regarding of naphthalene, phenanthrene and pyrene removal when operating at flask scale. The results had shown that monocultures of *Pseudomonas monteilii* P26 and *Pseudomonas* sp. N3 could degrade efficiently low molecular weight (LMW) PAHs but they did not show interesting high molecular weight (HMW) PAHs removal capabilities. On the other hand, the actinobacteria *Rhodococcus* sp. P18, *Gordonia* sp. H19 and *Rhodococcus* sp. F27 were able to degrade relatively efficiently HMW PAHs but they did not remove LMW PAHs from culture medium. The combination of four of these five strains (called C15 mixed culture) removed 100% of naphthalene and phenanthrene, and showed the highest pyrene biodegradation activity with removal values close to 42%, almost 6-times higher than those values recorded with the strains in pure culture. In addition, this defined mixed culture showed also bioemulsifying activities in presence of the tested hydrocarbons, which notably helps to solubilize them during the biodegradation process. We could confirm that highest pyrene degradation rate was observed when bioemulsifying activity was maximum in the mixed culture. Our results reveal that defined mixed culture C15 presents synergistic activity for effective removal of a mix of PAHs, improving the biodegradation process in a valuable approach.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of chemical compounds consisting of two or more fused aromatic rings, which are produced by natural and anthropogenic sources (Soclo et al., 2000). The persistence of PAHs in the environment is largely due to their low aqueous solubility, which also results in its association with particulate and sedimentary material and low bioavailability (Johnsen et al., 2005). Microbial treatment of PAHs-contaminated environments is one of the most important, economic and efficient processes that industries might apply instead of physicochemical treatments (Haritash and Kaushik, 2009). Therefore, bioremediation can be a sustainable alternative to PAHs complete degradation or transformation into non-hazardous forms and many researchers have been focusing on this particular approach.

PAHs are usually present in the environment as a mixture of several aromatic compounds, where each PAH has the capacity to influence others affecting their bioavailability and increasing the difficulty for microbial degradation (Marsili, 2000). In a real event of oil contamination, usually low molecular weight (LMW) PAHs as naphthalene and phenanthrene are easily degradable, while high molecular weight (HMW) PAHs are really difficult to degrade (Commendatore et al., 2000).

Abbreviations: PAH, polycyclic aromatic hydrocarbon; LMW, low molecular weight; HMW, high molecular weight.

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One of the most important biological strategies used by PAHs-degrading microorganisms to enhance hydrocarbon bioavailability is the release of molecules with emulsifying activity to culture medium (Ron and Rosenberg, 2002). The addition of synthetic emulsifiers was used to improve hydrocarbon biodegradation (Grimberg, 1996; Willumsen et al., 2001), but bioemulsifiers were confirmed to be more selective, biodegradable and eco-friendly (Poremba et al., 1991).

A large number of bacteria capable of degrading a wide range of PAHs have been isolated from contaminated sites (Mrozik, 2003; Janbandhu and Fulekar, 2011; Zhong et al., 2011) belonging to different genera such as *Burkholderia*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* among others (Moscoso et al., 2012). However, not many genera have been reported as both LMW and HMW polycyclic hydrocarbons degraders (Song et al., 2011). Some recent studies have employed the individual degradation capabilities of different bacterial genera to define mixed cultures with cooperative interactions, in order to improve the efficiency of degradation on a mixture of PAHs used as substrate (Mrozik, 2003; Chávez et al., 2004; Janbandhu and Fulekar, 2011; Zhong et al., 2011; Mikesková et al., 2012).

Recent works concerning pollutants biodegradation by mixed cultures have been reviewed. Senthilvelan et al. (2014) tested *Pseudomonas* and *Staphylococcus* strains as pure and mixed culture for degradation of phenol, showing mixed culture as a better strategy in all assayed conditions. Also, Jin et al. (2012) reported that mixed cultivation of three bacterial strains *Arthrobacter* sp. NB1, *Serratia* sp. NB2 and *Stenotrophomonas* sp. NB3 could enhance the degradation of nitrobenzene compared with pure cultivation. Similarly, a bacterial consortium (TJ-2) composed by three *Pseudomonas* strains, showed an aromatic amine mineralization higher than pure strains in decolorization of azo dyes (Barsing et al., 2011). In PAHs biodegradation, Trzesicka-Mlynarz and Ward (1995) studied a consortium of *Pseudomonas putida*, *Flavobacterium* sp. and *Pseudomonas aeruginosa* which showed higher removal of less water soluble PAHs than strains in pure culture. Janbandhu and Fulekar (2011) tested a microbial consortium with interesting phenanthrene and other PAHs degradation capabilities. At the same time, other researchers reported the use of mixed culture as an alternative strategy for PAHs remediation (Arun and Eyini, 2011; González et al., 2011; Simarro et al., 2011).

In the present study, sixteen combinations of bacterial strains belonging to *Pseudomonas*, *Rhodococcus* and *Gordonia* genera were evaluated according to LMW and HMW PAHs removal capabilities in liquid media. The aim of this work was to improve the degradation process of a mix of PAHs by enhancing individual biodegradation performances after the formulation of a defined mixed culture, in which synergistic activity were demonstrated.

Materials and methods

Chemicals, culture media and strains

Naphthalene, phenanthrene and pyrene (>99% purity) used in degradation experiments were purchased from Sigma–Aldrich Co. (St. Louis, MO, US). All chemicals used in this study were analytical grade and acquired from standard manufacturers. PAHs stock solutions were 25 mM in acetone.

Pseudomonas strains were previously isolated from oil polluted marine sediments according to their capability to degrade naphthalene and phenanthrene in pure culture (Isaac et al., 2013).

Gram-positive actinobacteria were enriched and isolated from Patagonia contaminated soil. Enrichment cultures were performed by suspending sediment samples (10 g) in 10 mL of minimal medium (MM) which contains in g L^{-1}: $(NH_4)_2SO_4$, 0.5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; $FeSO_4$, 0.01; pH 7.0 ± 0.2 and supplemented with a mixture of PAHs (naphthalene, phenanthrene and pyrene, 0.2 mM each). The enrichment cultures were incubated 15 days at 30 °C and 150 rpm in an orbital shaker. PAHs were added in order to supply either, hydrocarbon loss or consumption, and incubated for additional 15 days at similar conditions. Subsequently 1 mL of sample was withdrawn and serially diluted in sterile physiologic solution. Dilutions were plated onto Starch Casein (SC) agar medium g L^{-1}: starch, 10; casein powder, 1; agar, 15; sea water 50% and pH 7.2 ± 0.2 and MM media supplemented with nalidixic acid and cycloheximide (both 10 μg mL^{-1}) in order to inhibit growth of Gram-negative bacteria and fungi respectively (Ravel et al., 1998). Plates were incubated at 30 °C for 7 days and colonies were purified by streaking them onto JPP agar medium (Riva Mercadal et al., 2010) without antibiotic.

Phylogenetic identification of isolates

Sequence analysis of the 16S rRNA gene of the isolates P26 and N12 allowed to determine their relationship with the genus *Pseudomonas*. P26 strain was closely related to *Pseudomonas monteilii* CIP 104883T (Genebank ID NR024910) (99.6%), while N12 strain was closely related to *Pseudomonas xanthomarina* KMM 1447T (AB176954) (99.3%) (Isaac et al., 2013).

Other strains were identified in this study. DNA extraction and PCR amplification of 16S rRNA gene were performed according to Weisburg et al. (1991). Due to 16S rDNA analysis limitations, the selected isolates were also subjected to partial sequencing of protein-encoding genes. The *gyrB* gene (Fukushima et al., 2002) in Gram negative bacteria was amplified by PCR according to Kazunori et al. (2003), while the protocol descripted by Shen et al. (2006) was used in Gram positive actinobacteria. The *cpn60* gene (Goh et al., 1997, 2000; Brousseau et al., 2001) was also amplified by PCR, according to Hill et al. (2006).

Sequencing was conducted directly on PCR products using Macrogen sequencing service (Macrogen Inc., Korea). Sequences analysis for strains identification was performed according to Tindall et al. (2010). The isolates were identified according to the databanks of 16S rRNA sequences of the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012) and Ribosomal Database Project (https://rdp.cme.msu.edu/) (Cole et al., 2013). The sequences were analyzed with Chromas software (Technelysium, Tewantin, Australia).

The identity and similarity to the nearest neighbor of *gyrB* and *cpn60* genes sequences were obtained by using the BLAST (Basic Local Alignment Search Tool) algorithm (Altschul et al., 1990) at the National Center for Biotechnology Information (NCBI).

The nucleotide sequences identified in this study were deposited in the EMBL nucleotide sequence database (GenBank/EMBL/DDBJ) under the accession numbers LN680634, LN680635, LN680636, LN680637.

Screening for PAH degrading bacteria

For degradation assays, bottles containing 5 mL of JPP medium supplemented with 0.2 mM of naphthalene, phenanthrene, or pyrene were inoculated with 5% v v^{-1} of cell suspension. Bottles were incubated in the dark, at 30 °C on a rotary shaker (180 rpm) for 48 h or 21 days, for LMW and HMW respectively. Before bacterial inoculation, PAH solution was added and acetone was permitted to evaporate at room temperature. All experiments were conducted in triplicate and a control sterile flask was prepared for each treatment in order to evaluate abiotic loss. Hydrocarbon removal was quantified considering PAH loss occurred in the control flask.

After incubation, residual PAHs were extracted by adding 10 mL of acetone and filtered using a 0.22 μm – nylon membrane (Microclar, Argentina). The samples were stored at −20 °C until analysis. PAHs analysis was carried out by RP-HPLC using a Waters e2695 HPLC equipment coupled to a PDA detector (Waters 2998, Waters Corporation, MA, USA) operating at fixed wavelength ($\lambda = 276$ nm). Filtered solutions were injected into C18 μBondapak HPLC column (4.6 × 250 mm, 50 Å pore size, 5 μm particle size). A methanol/water (9:1 v v^{-1}) solution was the mobile phase and a flow rate of 1 mL min^{-1} were established during 25 min (Manohar et al., 2001). PAHs quantification was calculated by applying the external standard method.

Bioemulsifier activity

The ability to form stable emulsions after mechanical mixing between organic and aqueous phases was used to detect bioemulsifier production by bacteria (Cooper and Goldenberg, 1987). Bioemulsifier substances released by bacteria were determined by testing the formation of emulsions of kerosene into an aqueous phase. Cell-free supernatant of each strain after growing on naphthalene, phenanthrene or pyrene was mixed (1:1 v v^{-1}) with kerosene in glass tubes. The mixture was vigorously mixed by vortex for 2 min and left to settle. The emulsification index (EI) was calculated as the ratio between the height of the emulsified layer (mm) and the total height of the liquid column (mm). Finally, the emulsion was stable if the EI was greater than 50% after being left to settle for 24 h (Bosch et al., 1988). Tween 20 was used as positive control and distilled water and JPP medium were used as negative and medium controls.

Antagonism test

Antagonistic effects between selected strains was tested on solid JPP medium supplemented with 0.3 mM of a mix of naphthalene, phenanthrene and pyrene, according to the technique described by Bell et al. (1980). A strain was considered to be antagonistic if cross-inhibition of growth against the others was observed. Thus, antagonism among the selected strains was assessed by considering all possible combinations.

Hydrocarbon removal performance by mixed cultures

Different mixed cultures were synthesized by combination from two up to five strains. Mixed cultures were formulated by combining naphthalene and phenanthrene degrading bacteria with pyrene degrading strains, in all cases. Biodegradation of a mix of PAHs was evaluated in 125-mL Erlenmeyer flasks containing 30 mL of JPP medium. Naphthalene, phenanthrene and pyrene were added at 0.1 mM each. Flasks were capped with cellulose stoppers and inoculated (5% v v^{-1}) with actively growing cells. Pure microorganisms were inoculated at a final concentration of 0.05 g L^{-1} for actinobacteria and 0.025 g L^{-1} for *Pseudomonas* strains. In the screening assay, all sixteen defined mixed cultures were composed by the same proportion (v v^{-1}) of each individual strain. Cultures were incubated in darkness for seven days in orbital shaker at 30 °C and 180 rpm. Residual hydrocarbon was evaluated by RP-HPLC as previously described. The samples were prepared by triplicate including a sterile negative control.

Mixed culture versus pure culture removal performance

PAHs removal performance was evaluated according to the efficiency (E %), calculated as follows:

$$E\ (\%) = \frac{\left(H_i - H_f\right)}{H_i} \cdot 100\%$$

H_i is the hydrocarbon concentration (μM) at time t_i, H_f is the residual hydrocarbon concentration (μM) at time t_f, t_i is the initial time of the experiment ($t = 0$ h), t_f is the total incubation time (h). The loss of hydrocarbon by abiotic factors was considered in all cases.

To compare PAHs degrading performance of defined mixed cultures versus pure cultures, relative removal activity (observed/expected E %) was determined according to a modification of the data analysis technique described by Fuentes et al. (2011). The observed E % values of each mixed culture were compared with the expected removal activity, calculated as the average of the E % of each individual strain forming the complex biological system.

Statistical analysis

Experiments were performed by triplicate and the values correspond to mean values with standard deviation lower than 15%. All the results were analyzed using MINITAB 17 (PA, USA). One-way analysis of variance (ANOVA) was used to test the significant differences between responses. When significant differences were found, Turkey post-test was used to separate the responses into groups. Tests were considered significantly different a $P < 0.05$.

Biodegradation kinetics and bioemulsifier production

As a first step, the effect of inoculum in the PAH removal efficiency of the selected mixed culture was evaluated. Two different strategies were applied for combining bacteria strains for the formulation of the selected mixed culture. Strategy A consisted in put together the same amount of biomass (UFC) of each pure culture. Because *Pseudomonas* growth rate, and also removal of LMW PAHs, is faster than actinobacteria, strategy B consisted of duplicate the biomass of those pyrene degrading actinobacteria while *Pseudomonas* inoculum was halved.

Kinetics of PAHs degradation was followed with the selected mixed culture, in 125-mL Erlenmeyer flask containing 30 mL of JPP medium. Naphthalene, phenanthrene and pyrene were added at 0.2 mM each. Flasks were capped with cellulose stoppers and inoculated (5% v v^{-1}) with actively growing cells. Cultures were incubated in darkness for 14 days in orbital shaker at 30 °C and 180 rpm. Samples were withdrawn by sacrificing cultures at different times to monitor PAHs removed and bioemulsifier production.

Results

Selection of naphthalene, phenanthrene and pyrene degrading bacteria

In order to formulate a defined mixed culture able to efficiently degrade a mix of LMW and HMW PAHs, bacteria isolated from contaminated sites were evaluated regarding the removal of naphthalene, phenanthrene and pyrene from the culture medium. *P. monteilii* P26 and *P. xanthomarina* N12 were previously isolated, identified and reported as PAHs degrading bacteria, removing 100% of naphthalene and 65 and 43% of phenanthrene respectively (Isaac et al., 2013).

Sequence analysis of 16S rRNA gene of the isolate N3 allowed us to confirm its relationship with *Pseudomonas* genus. This strain was closely related to *Pseudomonas kunmingensis* HL22-2T (JQ246444) (99.2%). N3 strain was able to remove 100% of naphthalene and 79% of phenanthrene after 48 h of incubation in JPP medium (Fig. 1A).

Actinobacteria strains were also recovered from chronically hydrocarbon-contaminated soil in Patagonia, Argentina. Macroscopic and microscopic observations, chemotaxonomic analysis, and 16S rDNA sequencing confirmed the placement of these strains within Actinobacteria class. According to 16S rRNA sequence analysis, actinobacteria isolates P7, P18, H19, F27, HT1A, HT2B and HT3N showed closest relationship with *Arthrobacter*, *Rhodococcus* and *Gordonia* genera. P7, HT1A and HT2B strains were closest related to *Arthrobacter gandavensis* R5812^T (AJ316140) (100%), *Arthrobacter oryzae* KV-651T (AB279889) (99.6%) and *Arthrobacter oxydans* DSM 20119^T (X83408) (100%) respectively. On the other hand, P18 16S rRNA sequence showed 100% of identity to *Rhodococcus wratislaviensis* NCIMB13082^T (Z37138), while both F27 and HT3N were related to *Rhodococcus ruber* DSM43338^T (X80625) (100%). Finally, H19 strain was related to *Gordonia alkanivorans* NBRC16433^T (99.6%). These actinobacteria strains were not able to remove naphthalene and phenanthrene efficiently, but some of them were able to remove up to 19% of pyrene in 21 days. P18, H19 and F27 actinobacteria strains showed the higher pyrene remotion from culture medium. These strains were capable to grow on pyrene only if other carbon and energy source was available (data not shown). However, although this hydrocarbon was not essential for growth, the ability to remove it appears to be intact (Fig. 1B).

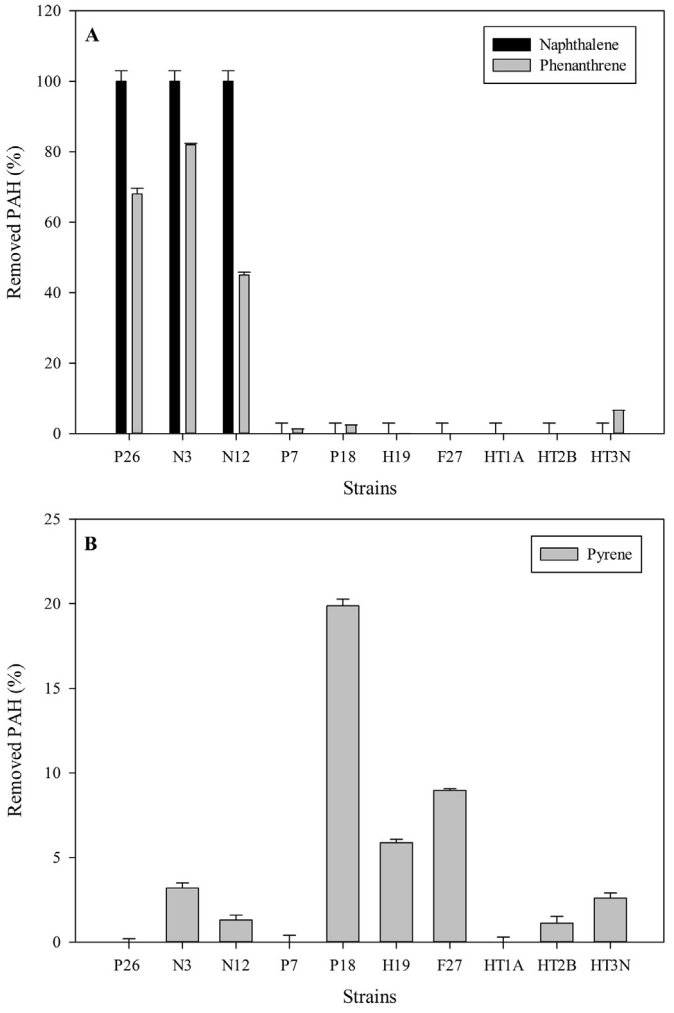

Fig. 1. PAHs removal by strains in pure culture. Naphthalene and phenanthrene remotion (A) was determined after 48 h of incubation. Pyrene remotion (B) was determined after 21 days of incubation. Strains: *P. monteilii* P26, *Pseudomonas* sp. N3, *P. xanthomarina* N12, *Arthrobacter* sp. P7, *Rhodococcus* sp. P18, *Gordonia* sp. H19, *Rhodococcus* sp. F27, *Arthrobacter* sp. HT1A, *Arthrobacter* sp. HT2B and *Rhodococcus* sp. HT3N. Hydrocarbon abiotic loss was considered in all cases. Values are the average of triplicate samples.

In addition to *P. monteilii* P26, the strains N3, P18, H19 and F27 were selected based on the interesting obtained results of LMW and HMW PAHs degradation. For these selected strains, an additional phylogenetic analysis based on protein-encoding genes was done.

Sequence analysis of both *gyrB* (1200 bp) and *cpn60* (620 bp) genes in N3 strain showed 99 and 98% identity with *Pseudomonas stutzeri* IMVB-177 (AB039417) and *P. stutzeri* (Y13828), respectively.

In actinobacteria H19 and F27, *gyrB* gene sequences allowed us to confirm the results obtained with 16S rDNA sequence analysis. Whereas H19 was closest related to *G. alkanivorans* DSM44369 (Genbank ID AB438179) (100%), the strain F27 was related to *R. ruber* IFO15591 (Genbank ID AB014174) (99%). The *gyrB* gene could not be amplified in P18 strain in assayed conditions. Sequence analysis of *cpn60* gene confirmed the assignation of all three actinobacteria strains to *Nocardiaceae* family (Lu and Zhang, 2012). P18, H19 and F27 strains were related to *R. ruber* (EF685255) (99%), *Gordonia amarae* DSM43392 (Genbank ID JQ613502) (97%) and *Rhodococcus rhodochrous* 372 (JQ356851) (94%).

Bioemulsifier activity and antagonism test

Bioemulsifier production was determined as emulsification index (EI) in all assayed strains. *P. monteilii* P26 and *Pseudomonas* sp. N3 supernatants produced stable and compact emulsions at 1 h and retained 78 and 85% of the initial height of emulsion after 24 h, respectively (Table 1). The emulsifying activity and the removal capabilities demonstrated by both microorganisms make these *Pseudomonas* strains promising candidates to form the defined mixed culture. *Arthrobacter* sp. P7 produced low emulsifying activity at 1 h, which was retained for 24 h. However, because this strain did not remove significantly PAHs at the previous assays, it was not chosen to compose the defined mixed culture.

Since surface active bacterial substances can often inhibit the growth of other microorganisms (Sartoros et al., 2005), antagonism among the strains was evaluated using a simplistic *in vitro* approach. No cross-inhibition was observed among the strains, confirming that it is possible to mix any of them in a defined mixed culture.

PAHs removal by mixed cultures

Five strains were selected to formulate a mixed culture for LMW and HMW PAHs biodegradation. *P. monteilii* P26 and *Pseudomonas*

Table 1
Bioemulsifier production by individual strains. Emulsification index at 1 (EI-1 h) and 24 h (EI-24 h) and emulsion stability (ES) were determined in all strains. Values are presented as average of triplicate samples. Tween 20 was used as positive control. Water and JPP medium were also evaluated to avoid false results.

Samples	EI-1	EI-24	ES
Water	–	–	–
JPP	–	–	–
Tween 20	83.77	66.82	79.72
P26	58.21	45.67	78.45
N12	–	–	–
N3	63.33	53.89	85.09
P7	13.01	12.82	98.53
P18	–	–	–
F27	–	–	–
H19	–	–	–
HT1A	–	–	–
HT2B	–	–	–
HT3N	–	–	–

–: bioemulsifier activity was not detected.

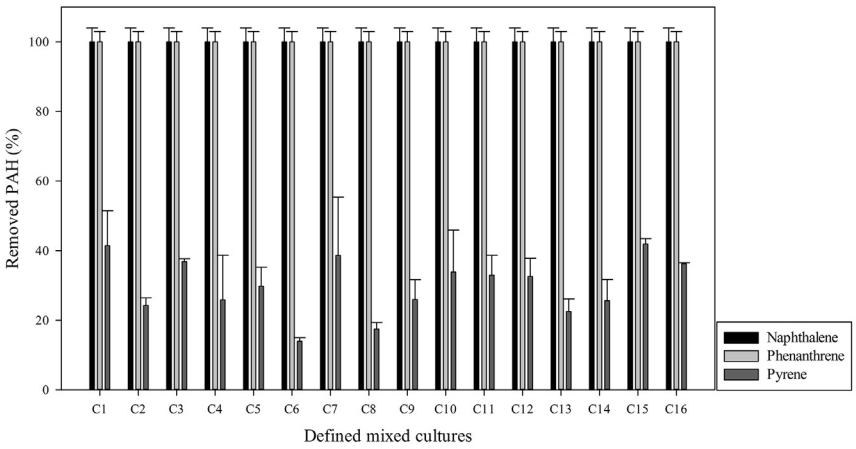

Fig. 2. PAHs removal by mixed cultures. Mixed cultures C1–C16 represent different combinations of five pure strains: *P. monteilii* P26, *Pseudomonas* sp. N3, *Rhodococcus* sp. P18, *Gordonia* sp. H19 and *Rhodococcus* sp. F27. Residual naphthalene, phenanthrene and pyrene were evaluated after seven days. Values are the average of triplicate samples.

sp. N3 showed interesting LMW PAHs degradation but they have difficulties to degrade HMW PAHs. On the other hand, the actinobacteria *Rhodococcus* sp. P18, *Gordonia* sp. H19 and *Rhodococcus* sp. F27 were able to degrade relatively efficiently HMW PAHs, while they could not remove low molecular weight PAHs from culture medium. In addition, *P. monteilii* P26 and *Pseudomonas* sp. N3 showed bioemulsifying activities in presence of the tested hydrocarbons, increasing PAHs bioavailability during the degradation process. According to PAHs degrading capabilities, emulsifying activity and negative antagonism, the strains *Rhodococcus* sp. P18, *Gordonia* sp. H19, *Rhodococcus* sp. F27, *P. monteilii* P26 and *Pseudomonas* sp. N3 were selected to formulate defined mixed cultures for PAHs removal.

Sixteen different combinations (C1–C16) of two to five of these strains were studied in a culture medium with a mix of PAHs and their removal capability was determined (Fig. 2). PAHs *E* % was calculated and the results were compared with the expected activity, calculated as the average of the removal activity of each pure culture (Table 2). All mixed cultures assayed showed maximum *E* % values for naphthalene degradation, in accordance with expected results. Also, all consortia evaluated reached 100% efficiency in phenanthrene degradation, increasing in most cases the expected value calculated from the individual performance. Pyrene removal values by mixed cultures of two strains were higher than those obtained with the corresponding pure cultures. Combinations of *P. monteilii* P26/*Rhodococcus* sp. P18 (C1) showed pyrene removal value of 41%, whereas *P. monteilii* P26 showed no pyrene removal capability when was grown in pure culture. Interesting C3 (*P. monteilii* P26/*Gordonia* sp. H19) removed 25% of pyrene, improving the expected performance more than 4 times. Maximum removal of the mix of hydrocarbons by three strains mixture was obtained in a defined mixed culture of *P. monteilii* P26/*Rhodococcus* sp. P18/*Rhodococcus* sp. F27 (C7) capable to degrade near 39% of pyrene from culture medium.

Mixed culture of *P. monteilii* P26, *Pseudomonas* sp. N3, *Gordonia* sp. H19, and *Rhodococcus* sp. F27 (C15) showed the highest pyrene biodegradation activity with removal values close to 42%, almost 6-times higher than removal values obtained with these strains in pure culture, whose removal did not exceed 9% (Table 2). According to hydrocarbon removal efficiency and relative activity observed, C15 consortium was selected for further biodegradation tests.

Four strains defined mixed culture (C15) biodegradation assays

The application of the two different strategies of inoculation of pure strains for the formulation of mixed culture, allowed to determine that no difference on growth and PAHs removal was

Table 2
PAHs removal by defined mixed cultures. Expected values were calculated as an average of removal capabilities of strains in pure culture. Means that do not share a letter are significantly different.

Mixed culture	Strains	Nah E%		Relative activity	Phe E%		Relative activity	Pyr E%		Relative activity
		Expected	Observed	Obs/exp	Expected	Observed	Obs/exp	Expected	Observed	Obs/exp
C1	P26|P18	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	19.87 ± 2.02	41.43 ± 10.03	2.08 ^{ab}
C2	P26|F27	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	8.97 ± 1.36	24.27 ± 2.17	2.70 ^{abcdefgh}
C3	P26|H19	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	5.87 ± 0.79	25.26 ± 1.40	4.29 ^{abcdefg}
C4	N3|P18	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	19.88 ± 2.02	25.87 ± 12.81	1.30 ^{abcdefg}
C5	N3|F27	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	8.97 ± 1.362	29.79 ± 4.45	3.32 ^{abcdef}
C6	N3|H19	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	5.87 ± 0.79	13.93 ± 1.02	2.37 ^{fghi}
C7	P26|P18|F27	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	14.42 ± 1.69	38.64 ± 16.71	2.68 ^{abc}
C8	P26|P18|H19	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	12.87 ± 1.41	17.46 ± 1.90	1.36 ^{efghi}
C9	P26|F27|H19	100 ± 0.0	100 ± 0.2	1.00	68.00 ± 4.00	100 ± 0.25	1.47	7.42 ± 1.08	25.95 ± 5.71	3.49 ^{abcdefg}
C10	N3|P18|F27	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	14.42 ± 1.69	33.84 ± 12.04	2.35 ^{abcde}
C11	N3|P18|H19	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	12.87 ± 1.41	32.94 ± 4.36	2.55 ^{abcde}
C12	N3|F27|H19	100 ± 0.0	100 ± 0.2	1.00	100 ± 6.00	100 ± 0.25	1.00	7.42 ± 1.08	32.59 ± 5.23	4.39 ^{abcde}
C13	P26|N3|P18|F27	100 ± 0.0	100 ± 0.2	1.00	84.00 ± 2.00	100 ± 0.25	1.19	14.42 ± 1.69	22.52 ± 3.59	1.56 ^{cdefghi}
C14	P26|N3|P18|H19	100 ± 0.0	100 ± 0.2	1.00	84.00 ± 2.00	100 ± 0.25	1.19	12.87 ± 1.41	25.61 ± 6.09	1.98 ^{abcdefg}
C15	P26|N3|F27|H19	100 ± 0.0	100 ± 0.2	1.00	84.00 ± 2.00	100 ± 0.25	1.19	7.42 ± 1.08	41.91 ± 1.54	5.64 ^{a}
C16	P26|N3|P18|F27|H19	100 ± 0.0	100 ± 0.2	1.00	84.00 ± 2.00	100 ± 0.25	1.19	11.58 ± 1.39	36.26 ± 0.27	3.13 ^{abcd}

detected at the end of the assay. Although naphthalene and phenanthrene degradation was not decreased after halving *Pseudomonas* proportion, not improvement of pyrene removal was observed when actinobacteria inoculum was doubled (Fig. 3). These results suggest that the preparation of inoculum, changing the proportion of individual strains for the formulation of the mixed culture, did not affect the removal of PAHs at the end of the biodegradation process.

After these results, a mix of LMW PAH (naphthalene and phenanthrene) and HMW PAH (pyrene) was used to study the biodegradation kinetic and bioemulsifier production of C15 defined mixed culture during a period of 14 days (Fig. 4). Thus, *P. monteilii* P26, *Pseudomonas* sp. N3, *Gordonia* sp. H19, and *Rhodococcus* sp. F27 were mixed in equal proportion to formulate the *Pseudomonas*-actinobacteria defined mixed culture C15. It was observed that naphthalene (two aromatic rings PAH) was completely removed after 24 h, while phenanthrene (three aromatic rings PAH) needs three days of incubation to be completely removed by the mixed culture. The mixed culture C15 reached a maximum pyrene (HMW-PAH composed by four aromatic rings) removal value of 52% after ten days of incubation. According to the obtained results, maximum removal of pyrene was accompanied by a high bioemulsifying activity of the mixed culture (EI-24 57.5%).

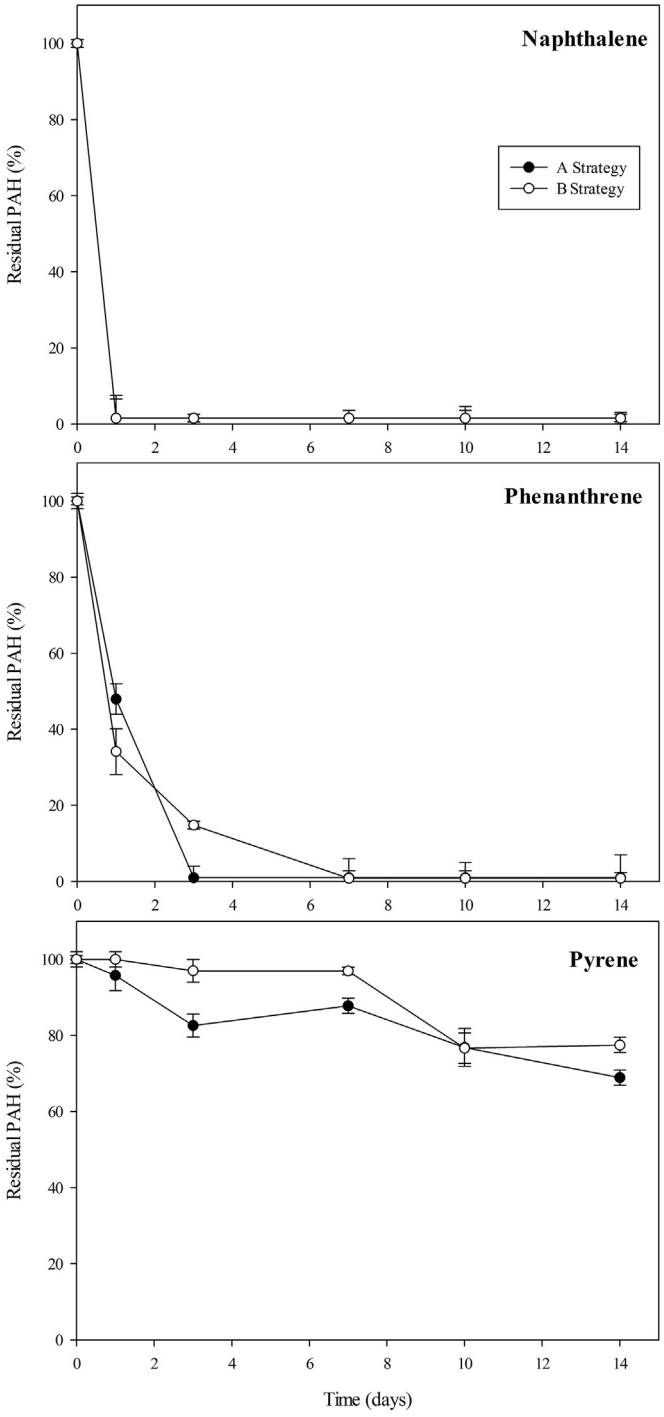

Fig. 3. Comparison of two different inoculums strategies (A and B) for defined mixed culture C15 formulation. Removal capability was determinate according to PAHs removal using a mixture of naphthalene, phenanthrene and pyrene.

Discussion

Microbial communities of PAHs-contaminated soils and chronically polluted sediments are generally enriched by microorganisms able to use them as carbon and energy sources (Koutny et al., 2003; Gallego et al., 2007). In this work, bacteria recovered from selective enrichment cultures supplemented with PAH were evaluated regarding the capability of naphthalene, phenanthrene and pyrene removal in an aqueous system.

The taxonomic position of the selected strains tested was in accordance with the findings reported in previously published articles (Pizzul et al., 2006; Cao et al., 2009; Haritash and Kaushik, 2009), in which *Pseudomonas*, *Rhodococcus*, *Arthrobacter* and *Gordonia* are known as some of the main hydrocarbon-degrading bacteria isolated from contaminated soils. However, some described strains have demonstrated a highly restrictive substrate range. For example, *Rhodococcus* sp. S1 oxidizes only anthracene and 2-methylanthracene (Tongpim and Pickard, 1996) and *Nocardioides* sp. KP7 (Saito et al., 2000) grows on phenanthrene but not on naphthalene. In contrast, other bacteria exhibit a broad substrate range, including di-and tri-cyclic aromatic hydrocarbons and heterocycles. Interestingly, the substrate range can vary even between similar catabolic genes or pathways: the homologous *nah* upper pathway genes encoded on plasmids found in *P. putida* PpG7 (NAH7) and NCIB9816 have differing abilities to oxidize 2- and 3-ring PAHs (Yang et al., 1994).

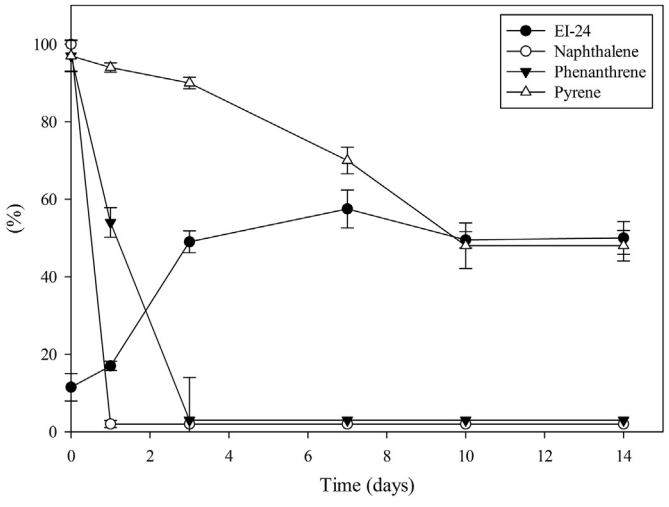

Fig. 4. Kinetics of PAHs removal and bioemulsifier production by mixed culture C15. Residual naphthalene, phenanthrene and pyrene were determined during 14 days. Bioemulsifier was determined according to EI-24. Defined mixed culture C15 is a four strains-mix (*P. monteilii* P26, *Pseudomonas* sp. N3, *Rhodococcus* sp. F27 and *Gordonia* sp. H19). Values represented are an average of triplicate samples.

On the other hand, pyrene (tetracyclic PAH), has been widely used as indicator and model compound to study HMW PAHs biodegradation (Kanaly and Harayama, 2000; Chen and White, 2004). Usually, LMW PAHs consisted in two or three aromatic rings, are readily biodegradable unlike those containing four or more aromatic rings. Several researchers demonstrated the difficulty to degrade pyrene (Zeng et al., 2010; Song et al., 2011) since HMW PAHs are regarded as recalcitrant and thermodynamically stable (Kim et al., 2007).

Although numerous studies of PAHs biodegradation have been conducted using pure bacterial cultures, the ability to degrade these chemicals may be provided through the action of many organisms acting in concert (Ghazali et al., 2004; González et al., 2011). Clearly, microorganisms may act synergistically to degrade aromatic compounds in the biodegradation of complex hydrocarbon mixtures (Trzesicka-Mlynarz and Ward, 1995; Kanaly and Harayama, 2010). All mixed cultures assayed in this work showed maximum *E* % values for naphthalene degradation, in accordance with expected results. In the same way, all mixed cultures reached 100% efficiency in phenanthrene degradation, increasing in most cases the expected value calculated from the individual strains performance. According to *E* % values, all combinations showed pyrene removal capability, although not all strains in pure culture showed that capability.

Several researchers have studied hydrocarbon biodegradation potential of mixed cultures obtaining similar results. Thus, for example, a consortium was described by Daugulis and McCracken (2003), in which two *Sphingomonas* incubated together oxidized higher molecular weight PAH than individually. Similar results have been observed by Jame et al. (2010), who determined a significantly higher hydrocarbon degradation rate of a mixed culture of *Pseudomonas* strains when compared with pure cultures. Boonchan et al. (2000) and Machín-Ramírez et al. (2010) studied benzo(a)pyrene degradation by fungal consortia, detecting removal values higher than expected for individual species. A complete crude oil removal was obtained using defined mixed cultures of *Bacillus*, *Pseudomonas* and *Micrococcus* strains isolated from contaminated sites (Ghazali et al., 2004; Alkhatib et al., 2011).

The effectiveness of a hydrocarbon remediation process usually requires the addition of surface-active compounds (González et al., 2011). Biodegradation of PAHs is often limited due to their low bioavailability (low aqueous solubility and high sorption to soil particles), which affects its removal rate in a two-liquid-phase system, including substrate uptake and efflux (Cerniglia, 1993). Different approaches to increase the apparent PAHs solubility by treatments such as addition of synthetic surfactants or biosurfactants were done. However, these compounds could be toxic for microorganisms (Sartoros et al., 2005). The relative toxicity, low biodegradability, and low efficiency at low concentrations of synthetic surfactants reduce the potential for their application in contaminated sites (Desai and Banat, 1997). Bacteria that produce extracellular surfactants may enhance droplet formation and "pseudosolubilization" during hydrocarbons degradation, by stabilizing water-in-oil or oil-in-water emulsions and by reducing limitations due to substrate mass transport. Bioemulsifying activity increase bioavailability oils (strongly hydrophobic) by providing accessing of microorganisms at such compounds for degradation (Ron and Rosenberg, 2001).

According to our results, maximum pyrene degradation rate was obtained when the EI-24 h value of the mixed culture reached the maximum value of 57.5% (w v^{-1}). The capability of the mixed culture to produce bioemulsifying activity during PAHs removal allow us to avoid the addition of synthetic-surface-active compounds as described in previous works (Zhao et al., 2005; Bautista et al., 2009) and represents a sustainable help to improve the biodegradation process.

Moreover, PAHs are usually present in the environment as a mixture of several aromatic compounds, where each PAH has the ability to influence others affecting their bioavailability and increasing the difficulty for degradation by microorganisms (Chávez et al., 2004). The results obtained in these experiments overcome those reported by Moscoso et al. (2012) for other PAHs bioremediation process, in which pyrene removal in a mix of hydrocarbons was lower than pyrene alone.

Finally, all mixed bacterial cultures studied in this work have proven to be more efficient than individual strains for phenanthrene and pyrene removal, which suggests that strains components act synergistically in the mixed culture as a real consortium. Results from our experiment contribute to confirm the importance of interspecific interactions of microbes in mixed cultures for their metabolic cooperation (Seneviratne et al., 2008).

Conclusions

Here, we report the use of strains belonging to *Pseudomonas*, *Rhodococcus* and *Gordonia* genera in a defined mixed culture to remove a mixture of LMW and HMW PAHs in an aqueous system. Naphthalene and phenanthrene were removed from the culture medium at 100%, while between 13 and 42% of pyrene was removed, in all mixed culture tested. Removal values exceeded more than 5-times those obtained with pure culture, especially for pyrene in some cases. Degradation rate also increased considerably, indicating positive interaction between bacteria strains involved. The defined mixed culture C15 showed the best PAHs removal performance, while the bioemulsifying activity was maximum. Our results reveal that the microbial consortia of *Pseudomonas* and actinobacteria strains can be suitable to be used in PAHs bioremediation process in a valuable approach.

Up to our knowledge, there are no reports concerning PAHs removal by combining *Pseudomonas* and actinobacteria strains in a mixed culture. Further experiments will be conducted to explore the metabolic potential of the consortium C15 with other mixture of hydrocarbons and culture systems.

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