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ORIGINAL PAPER

Insights into polyaromatic hydrocarbon biodegradation by *Pseudomonas stutzeri* CECT 930: operation at bioreactor scale and metabolic pathways

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Abstract Contamination of the environment with polycyclic aromatic hydrocarbons is one of the major problems facing the industrialized nations today. In this work, *Pseudomonas stutzeri* CET 930 was studied for the first time as bioremediation agent for the degradation of effluents containing phenanthrene, pyrene and benzanthracene, both individually and mixed. The promising results of degradation obtained at flask scale (92, 63 and 94 % in 7 days, respectively) marks the onset of the operation at bench scale bioreactor. The overall biotransformation of phenanthrene, pyrene and benzanthracene in batch operation mode was 95, 78 and 82 % when present individually, and 100, 98 and 100 % when carrying out the biological process in cometabolic conditions, respectively. We have demonstrated the great versatility of this strain for the degradation of structurally different contaminants, such as metal working fluids, polycyclic aromatic hydrocarbons or insecticides such as chlorpyrifos, which makes it a suitable candidate to be applied at industrial scale. In all cases, the experimental data were successfully fitted to models, which turned out to be valuable tools to classify the metabolites involved in the biodegradation process as biomass-related. Since a complete mineralization was pursued, the metabolic pathways of the studied contaminants have been proposed based on gas chromatography–mass spectrometry data.

Keywords Bioremediation · *Pseudomonas stutzeri* · Polyaromatic hydrocarbons · Bioreactor · Metabolic pathway

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants that contain at least two or more fused aromatic rings in linear, angular or cluster arrangements. They represent a large and heterogeneous group of hydrophobic organic pollutants, and their presence in the environment is caused by natural and anthropogenic events, such as forest and rangeland fires or the incomplete combustion of fossil fuels and petroleum (Haritash and Kaushik 2009).

Among the major pollutants, PAHs are widely distributed environmental contaminants that are known to exert acutely toxic effects and/or possess mutagenic, teratogenic or carcinogenic properties (Simarro et al. 2011). Due to all these reasons, the US Environmental Protection Agency (USEPA) and the Agency for Toxic Substances and Disease Registry have proposed some of them as apriority pollutants (Moscoso et al. 2012d).

Although PAHs may be removed by physical (volatilization, photolysis and adsorption), chemical (chemical oxidation, photocatalysis) or biological (biosorption or biodegradation) techniques, the latter are considered a major route for their complete degradation. Microbial activities allow the mineralization of this kind of compounds into carbon dioxide and water. The potentiality of microbes as degradation agents of several PAHs thus points to biological treatment as the major promising alternative to attenuate the environmental impact caused by PAHs (Janbandhu and Fulekar 2011). Usually, there are many factors that influence the outcome of a biodegradation process. However, the microbial agent, the chemical structure of the pollutant and the environmental conditions are the most outstanding ones (Li et al. 2008). Biodegradation pathways involve breakdown of organic

compounds, being ring fission by intracellular oxidation and hydroxylation the typical initial steps.

The efficiency of PAH biodegradation under natural conditions is limited by their poor bioavailability, as a consequence of their low aqueous solubility and high hydrophobicity, which together with their high adsorption coefficient and high thermodynamic stability make up inherent features of this kind of pollutants (Cao et al. 2009). Bearing in mind these properties, considerable efforts have been focused on the enhanced solubility of these compounds with the addition of surfactants, amphiphilic molecules that increase the solubility of hydrophobic compounds by decreasing the interfacial surface tension at the aqueous/organic interphase. In this way, Sponza and Gok (2010) reported that surfactants have been shown to enhance both biodegradation and reaction rate, and Bautista et al. (2009) have even confirmed that Tween 80, a non-ionic surfactant, was used as carbon source by a *Pseudomonas* strain, thus being considered biodegradable (Álvarez et al. Álvarez et al. 2012a, b; Ulloa et al. 2012a, b).

During the last years, a wide phylogenetic diversity of bacteria belonging to genus *Arthrobacter* (Guo et al. 2008), *Burkholderia* (Kim et al. 2003), *Pseudomonas* (Chávez et al. 2004), *Rhodococcus* (Dean-Ross et al. 2002) and *Sphingomonas* (Madueno et al. 2011), among others, have been reported to be able to degrade PAHs. More specifically, bacteria from the genus *Pseudomonas,* which is present in a large number of different natural and contaminated environments, have been the subject of a great scientific interest due to both their high degree of physiological and genetic adaptability and their efficient capacity to aerobically degrade a wide range of aromatic compounds. From previous results of our group and information coming from the literature, *Pseudomonas stutzeri* strains have been demonstrated to be a promising microbial agent able to metabolize compounds such as PHE and PYR (Moscoso et al. 2012a; Seo et al. 2009; Kazunga and Aitken 2000). However, this is the first time that *P. stutzeri* is proposed for benzo[a]anthracene (BaA) degradation.

Frequently, contaminated sites and effluents are characterized by the occurrence of complex mixtures of several pollutants which is translated into an increased complexity of the biodegradation process. Since PAHs make up a group of persistent compounds, different strategies have been tackled to succeed in their biodegradation, such as cometabolism (Pathak et al. 2008; Klankeo et al. 2009).

In this work, PHE (a three aromatic ring molecule), PYR and BaA (both with four aromatic rings) have been used as models of low molecular weight (LWM) and high molecular weight (HWM) hydrocarbons to propose a viable biodegradation strategy. After assessing the biodegradability of an effluent containing each contaminant separately, the process was approached with a mixture of the three compounds. The bioremediation strategy was scaled-up from shake flasks to stirred tank bioreactor. Up to our knowledge, this is the first time that the bacterial strain *P. stutzeri* was used for proposing a viable degradation treatment of BaA, individually and mixed with other PAHs. This research work was conducted in the University of Vigo and was completed in October 2012.

Materials and methods

Microorganism

The bacterial strain *P. stutzeri* CECT 930, used in this study was obtained from the Spanish Type Culture Collection (ATCC 17588). *P. stutzeri* was inoculated in plates containing PS medium. The composition of this medium is (per litre)as following: yeast extract 2 g, meat extract 1 g, casein peptone 5 g, NaCl 5 g and agar 15 g (pH 7.2). The plates were incubated at 26 °C for 5 days. *P. stutzeri* is a gram-negative, rod-shaped and single-polar flagellated bacterium (Lalucat et al. 2006).

Chemicals and culture media

Phenanthrene (PHE), pyrene (PYR) and benzo[a]anthracene (BaA) (purity higher than 99 %) used in degradation experiments were purchased from Sigma-Aldrich (Germany). Relevant properties of these compounds are shown in Table 1. The non-ionic surfactant Tween 80 was supplied by Merck. All chemicals used were at least reagent grade or better. PAHs stock solutions were 5 mM in acetone.

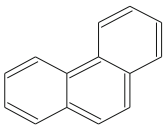
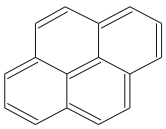
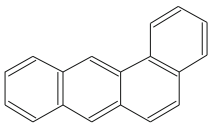
Minimal medium (MM) was used for biodegradation experiment. The medium composition was as follows (per litre): $Na_2HPO_4{\cdot}2H_2O$ 8.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g and $CaCl_2$ 14.7 mg. MM also contained trace elements (per litre) that are as follows: $CuSO_4$ 0.4 mg, KI 1.0 mg, $MnSO_4{\cdot}H_2O$ 4.0 mg, $ZnSO_4{\cdot}7H_2O$ 4.0 mg, H_3BO_3 5.0 mg, $H_2MoO_4{\cdot}2H_2O$ 1.6 mg and $FeCl_3{\cdot}6H_2O$ 2.0 mg. A concentrated stock solution containing these salts was prepared and added to the medium (2 mL/L).

Biodegradation cultures

Flask scale

Biodegradation experiments were carried out in 250-mL Erlenmeyer flasks with 50 mL of MM, containing 1 % w/v of surfactant Tween 80 and 2 % v/v of a stock solution, in order to achieve the solubilization of the PAHs (up to

Table 1 Main physical properties of used PAHs

Compound	M.F.	Structure	M.W.	B.Pt. (°C)	M.Pt. (°C)	V.P. (Pa at 25°C)	Aqueous solubility (mg/L)
PHE	$C_{14}H_{10}$		178.23	340	105.5	6.8×10^{-4}	1.20
PYR	$C_{16}H_{10}$		202.26	393	156	2.5×10^{-6}	0.077
BaA	$C_{18}H_{12}$		228.29	400	162	2.2×10^{-8}	0.010

100 μM). The non-ionic surfactant Tween 80 was chosen since non-ionic surfactants are less toxic to bacteria than anionic and cationic surfactants (Sartoros et al. 2005). Each flask, capped with cellulose stoppers, was inoculated (3 %) with actively growing cells in exponential phase (24 h). Cultures were incubated in the darkness for 7 days in an orbital shaker at 37 °C, initial pH 8.0 and 150 rpm. Samples were withdrawn at different times to monitor PAH biodegradation and cell density. All experiments were carried out in triplicate and the values shown in figures correspond to mean values with a standard deviation lower than 15 %.

Bioreactor scale

For the scaling up of the process, a stirred tank bioreactor (Biostat B, Braun, Germany) similar to that reported previously by our group was used (Moscoso et al. 2012a). It was filled with the medium described above containing 100 μM of each individual PAH as well as with a mixture of them (each one at 100 μM). Temperature was maintained at 37 °C by circulation of thermostatted water, and the initial pH was adjusted to 8. Bioreactor was inoculated with actively growing cells from 24-h flask cultures (3 % v/v). Air was supplied continuously at 0.17 vvm, and samples were taken regularly during the experimental period.

Analytical methods

Cell growth determination

Biomass concentration was measured by turbidimetry at 600 nm, and the obtained values were converted to grams of cell dry weight per litre using an experimental calibration curve.

PAHs analysis

PHE, PYR and BaA concentrations in the culture media were analysed by reversed-phase high-performance liquid chromatography (HPLC) equipped with a reversed-phase C8 column (150 × 4.6 mm, 5 μm-particle-size, Zorbax Eclipse) with its corresponding guard column. The HPLC system was an Agilent 1100 equipped with a quaternary pump and photodiode array UV/Vis detector (252.4 nm). Five microliters of filtered cultivation media (through a 0.45-μm Teflon filter) were injected and then eluted from the column at a flow rate of 1 mL min^{-1} using acetonitrile/water (67:33) as a mobile phase.

In order to identify intermediate compounds formed during the degradation process, aliquots of 5 mL were extracted twice with 5 mL of chloroform. The organic phase extractions were combined and dried over with anhydrous sodium sulphate (Zhao et al. 2009). one microliters of this organic phase was analysed using an Agilent GC 6850 gas chromatograph equipped with a HP5 MS capillary column (30 m × 250 μm film thickness × 0.25 mm, Agilent), operating with hydrogen as carrier gas, coupled to an Agilent MD 5975 mass spectrometer (MS). The GC injector was operated in splitless mode, and 1 μL aliquots were injected using an autosampler; GC oven was programmed to hold 50 °C for 4 min, then raise the temperature by 10 °C/min to 270 °C, which was held for 10 min. PAH degradation products were identified by comparison with the NISTS search 2.0 database of spectra.

Results and discussion

Growth and biodegradation of PAHs in cultures at flask scale

First of all, the efficiency of *P. stutzeri* for the degradation of PHE, PYR and BaA, both individually and mixed, was ascertained at small scale. As reported by Lalucat et al. (2006), this species of bacterium can grow diazotrophically, and in minimal salt medium with ammonium ions or nitrate and a single organic molecule, such as PHE, PYR and BaA, as the sole carbon and energy source.

A variety of microbial growth and biodegradation kinetic models have been developed, proposed and used by many researchers. Such models allow the calculation of the time required to reduce a contaminant to a certain concentration, the estimation of how long it will take before a selected concentration will be attained and to predict the amount of biomass production achievable at a given time.

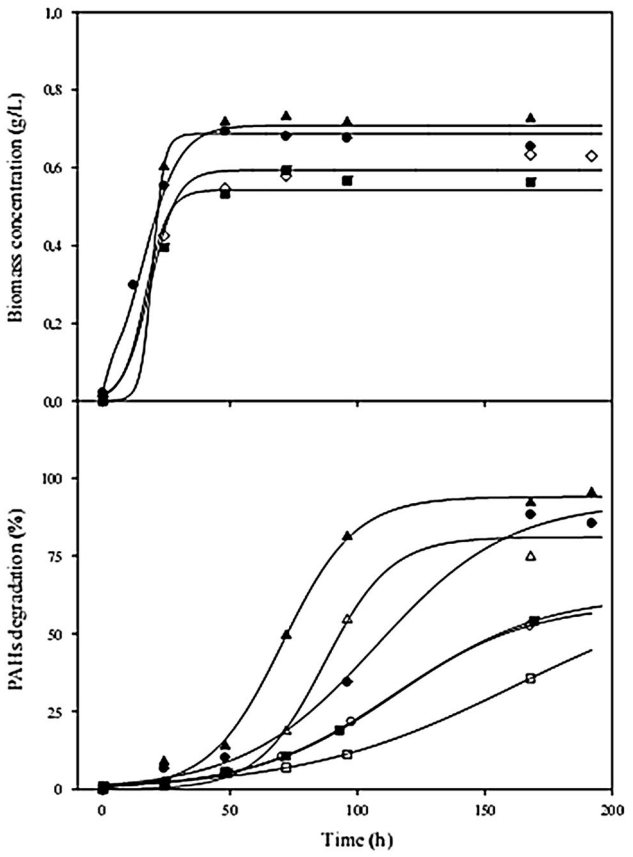


Fig. 1 Biomass concentration and PAHs degradation profiles in flask cultures of *P. stutzeri* in MM at 150 rpm and 37°C: (*circle*) PHE; (*square*) PYR, (*triangle*) BaA, (*open diamond*) PHE + PYR + BaA for biomass concentration, PAHs degradation profiles individually (*full symbols*) and mixed (*void symbols*). Experimental data are represented by *symbols* and logistic model by *solid lines*. PHE data correspond to those reported by Moscoso et al. (2012a)

In this particular case, all experimental data were fitted to a logistic model (1), where A is the biomass (X, g/L) or the degradation rate (D, %) at a specific moment of the culture time t (h), A_0 and A_{max} are the initial and maximum biomass (X_0 and X_{max}, g/L) or the initial and maximum degradation rate (D_0 and D_{max}, %) and b is the maximum specific growth rate (μ, h^{-1}) or the maximum specific degradation rate (μ_D, h^{-1}).

$$A = \frac{A_{max}}{1 + e^{\left[Ln\left(\frac{A_{max}}{A_0} - 1\right) - bt\right]}} \quad (1)$$

From the data depicted in Fig. 1, and the values of the parameters presented in Table 2, it is clear that the logistic model adequately fits to the experimental data ($R^2 > 0.96$ in all cases). All the experimental data were fitted to these equations by using the SOLVER function in Microsoft EXCEL.

The analysis of the biomass parameters, listed in Table 2, allows one concluding the existence of quite higher levels of maximum biomass, similar to those reported by our group recently (Moscoso et al. 2012a) for the degradation of PHE at flask scale. In relation to the growth rate, the levels are much higher than those reported by Obayori et al. (2008) when they studied PYR biodegradation by a *Pseudomonas* species isolated from polluted tropical soils (0.024 h^{-1}). It is noticeable that the introduction of a mixture of the three PAHs leads to a decrease both in the maximum biomass levels and growth rate.

With regard to the degradation parameters (Fig. 1b; Table 2), it seems clear that quite different maximum levels are reached depending on the PAH used. Thus, while the overall biotransformation of PYR was only 63 % in 7 days, the levels corresponding to BaA and PHE reached about 94 and 92 %, respectively. This behaviour can be explained in terms of the different structure of PYR, which contrarily to the linear PHE and BaA, it is a pericondensed molecule, which stands for a higher difficulty to be accessed and consequently to be transformed. Despite the lower values, it should be remarked that the results obtained are promising since they entail a quantitative amelioration in the times required to reach the maximum, when compared with the data reported by Obayori et al. (2008) (30 days incubation period to reach similar levels of biodegradation).

Also, the introduction of a mixture containing PHE, PYR and BaA entails a reduction in the maximum levels of biodegradation of all the contaminants, as could be envisaged from the analysis of the biomass parameters. Sartoros et al. (2005) studied cometabolism of Anthracene (ANT—tricyclic aromatic hydrocarbon, similar to PHE but with a linear structure) and PYR in order to show the ability of the microbes to degrade hydrocarbons individually, indicating

Table 2 Growth and PAH biodegradation kinetic parameters defining the logistic and Luedeking and Piret-type models in cultures of *P. stutzeri* at flask and bioreactor scale

Experiment		Biomass parameters				PAHs degradation parameters				Luedeking and Piret parameters			
PAHs	Scale	X_0 (g/L)	X_{max} (g/L)	μ (h^{-1})	R^2	D_0 (%deg)	D_{max} (%deg)	μ_D (h^{-1})	R^2	D_0 (%deg)	m (%deg L/g)	n (%deg L/g/h)	R^2
PHE[a]	Flask	0.073	0.708	0.138	0.99	1.487	92.351	0.038	0.99	1.22	9.41	0.77	0.98
PYR		0.013	0.543	0.223	0.97	0.993	62.357	0.035	0.99	2.367	15.311	0.761	0.99
BaA		0.001	0.687	0.433	0.96	0.661	93.990	0.070	0.99	0	15.899	0.903	0.90
PHE-Mx		0.016	0.594	0.191	0.98	0.960	59.810	0.036	0.99	2.005	14.709	0.647	0.88
PYR-Mx						1.367	56.547	0.024	0.99	0.636	7.874	0.420	0.99
BaA-Mx						0.101	81.044	0.077	0.99	0.321	10.676	0.966	0.83
PHE[a]	Reactor	0.010	0.557	0.183	0.97	1.0	94.729	0.186	0.98	3.97	121.21	0.47	0.98
PYR		0.084	0.587	0.101	0.92	6.657	78.285	0.019	0.99	3.118	11.067	0.519	0.98
BaA		0.049	0.635	0.193	0.99	5.000	81.297	0.045	0.98	0	29.271	0.672	0.99
PHE-Mx		0.058	0.497	0.079	0.96	6.504	100.000	0.035	0.99	0.931	75.882	0.284	0.90
PYR-Mx						5.940	98.467	0.032	0.99	0.856	56.375	0.495	0.95
BaA-Mx						5.809	100.000	0.035	0.99	1.187	61.418	0.502	0.96

[a] Data reported by Moscoso et al. (2012a)

that the degradation of these two PAHs was not dependent on cometabolism (in 12 days, 100 % of PYR was transformed while ANT degradation was 80 %). According to these results, some authors suggested that the simultaneous bacteria-mediated PAH biodegradation was strongly influenced by their different bioavailabilities (González et al. 2011; Silva et al. 2009; Peng et al. 2009).

It should be highlighted that, despite the existing reduction in the maximum degradation yields attained, the values are still very promising, since a minimum of 60 % is obtained after just 1 week of treatment.

One approach to characterize substrate biodegradation, in order to define a set of factors or variables affecting the kinetics of biodegradation and identifying an appropriate kinetic model correlating specific growth rate and PAHs removal, can be obtained by applying the model reported by Marqués et al. (1986) and exhaustively explained in previous works of the group (Deive et al. 2010).

$$D = D_0 + mX_0\left\{\frac{e^{\mu t}}{\left[1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu t})\right]}-1\right\} + n\left(\frac{X_{max}}{\mu}\right)\ln\left[1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu t})\right] \quad (2)$$

This equation allows defining biodegradation as a function of a "growth-associated" parameter (m), or/and a "non-growth-associated" parameter (n). The values obtained are presented in Table 2. It becomes patent that the biodegradation process is more dependent on the biomass production, since in all cases, m is, at least, more than nine times higher than n.

Growth and biodegradation cultures at bench scale bioreactor

After having checked the viability of the biodegradation of the selected PAHs mixture at flask scale, the study was approached at bioreactor scale. The biodegradation experiments in a 5-L bioreactor were operated in batch mode to study the scale up effect from 250-mL shake flasks. The batch was initially run for single substrate degradation study with a PAH concentration of 100 μM, to finally introduce a mixture of the three selected PAHs at 100 μM each, following the same strategy used at flask scale. The bioreactor used was fitted with a Rushton turbine driven by a DC motor, in order to provide the culture with an increased mass transfer, which could ultimately allow enhancing the bioavailability of the contaminants.

Therefore, the results of cellular concentration and biodegradation obtained after carrying out the biological reactions are shown in Fig. 2, and the kinetic parameters defining the microbiological process are also listed in Table 2. It can be checked that the levels of biodegradation reached are higher (PHE 95 %, PYR 78 and BaA 81 %) than those obtained at flask scale, thus confirming our preliminary hypothesis of a possible higher bioavailability of the pollutant. In general, these levels are higher than those reported in the literature. For instance, Trably and Patureau (2006) worked in a continuous bioreactor at an hydraulic retention time of 20 days, to achieve about 90 % of PHE biodegradation, while Nasseri et al. (2010) needed 3 weeks to reach 87 % of remediation in *Pseudomonas* cultures carried out in a slurry phase bioreactor. In the

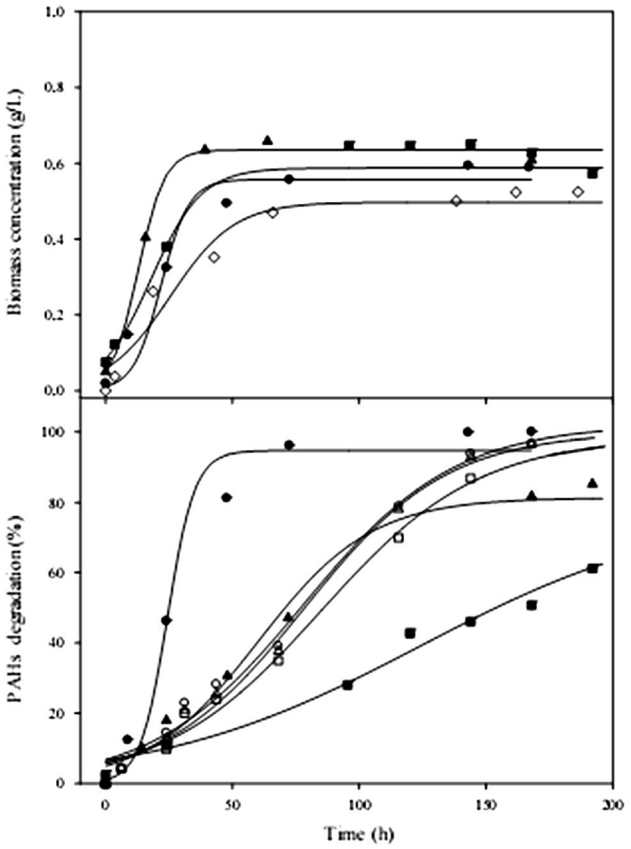


Fig. 2 Biomass concentration (**a**) and PAH degradation profiles (**b**) in bioreactor cultures of *P. stutzeri* in MM at 300 rpm, 0.17 vvm and 37°C: (*filled circle*) PHE; (*filled square*) PYR, (*filled triangle*) BaA, (*open diamond*) PHE + PYR + BaA for biomass concentration, PAHs degradation profiles individually (*full symbols*) and mixed (*void symbols*). Experimental data are represented by *symbols* and logistic model by *solid lines*. PHE data correspond to those reported by Moscoso et al. (2012a)

same way, Ventaka et al. (2008) investigated the use of bio slurry phase reactors to degrade PYR at 120 h of HRT, reaching similar levels of degradation as those reported in this work.

On the other hand, a final experiment was performed to check the ability of the selected strain to biodegrade a mixture of all the contaminants. Since percentages of removal for the three contaminants are higher, reaching an almost complete degradation in all cases (100 % of PHE, 98 % of PYR and 100 % of BaA), we can conclude that the hydrodynamic setting existing inside the bioreactor furthers the cometabolic degradation of the three contaminants. The values obtained are also superior to those reported by Sponza and Gok (2010), who studied the removal of PAHs from petrochemical wastewater using an aerobic stirred tank bioreactor during 25 days (74 % PHE, 60 % PYR and 37 % BaA). Regarding the advantages of this paper in comparison with previous consortia obtained from contaminated soils, the first outstanding feature that should be stressed is that this microorganism is able to efficiently carry out the biological degradation without including the typical sterilization step, which is very advantageous in terms of time consumption and bioprocess economy. Besides, we have demonstrated that the operation with this axenic culture allows a tremendous versatility, since it is able to metabolize extremely recalcitrant compounds such as Metal Working Fluids, or hydrophobic pesticides such as chlorpyrifos, as we have demonstrated very recently. (Moscoso et al. 2012b, c, 2013). More specifically, this last paper has evidenced that the microbial consortium obtained from polluted soil was almost completely unable to biodegrade the insecticide chlorpyrifos, while the cultivation of *P. stutzeri* allowed reaching values around 80 %.

Silva et al. (2009) reported that PAH cometabolization could strongly alter the final results of biodegradation in a given remediation process, since the degradation of one type of PAH can affect the degradation of other PAH. In this way, in our case, a LMW PAH such as PHE may affect the degradation of a HMW PAH, such as PYR and BaA, and vice versa, via cometabolism, induction of enzyme activities by related degradation intermediates or via competitive inhibition or toxicity (Dean-Ross et al. 2002). The metabolic cooperation by several microorganisms may result in enhanced PAH utilization, since metabolic intermediates produced by some organisms may serve as substrate for the growth of others and further studies taking into account a microbial consortium should not be discarded.

Finally, the analysis of the Luedeking and Piret parameters allow confirming the same behaviour found at flask scale, with a stronger dependence of degradation on the biomass parameter m (50 times higher than n in some cases). This behaviour is coincident with what was found in other biological processes devoted to the degradation of dyes or to the production of metabolites (Deive et al. 2009, 2010).

Identification of metabolic pathway of PAHs

It is well known that PAHs can be biodegraded by several groups of microorganisms. Among them, *P. stutzeri* stands out as an ubiquitous bacterium with a high degree of physiological and genetic adaptability (Lalucat et al. 2006). Therefore, the last step of this work consisted of elucidating the metabolic pathways followed to degrade this kind of compounds. Proven the low levels of biomass adsorption (maximum levels around 15 %), a biotransformation of the contaminants is foreseen. Usually, aerobic catabolism of PAHs involves a wide variety of peripheral degradation pathways that transform substrates into a small number of common intermediates that can be further processed by a new central pathway to tricarboxylic acid

(TCA) cycle intermediates (Peng et al. 2009; Mrozik 2003; Cerniglia 1993; Cerniglia et al. 1994).

It has long been known that the initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of the PAH to a dihydrodiol by a multicomponent enzyme system (Zhong et al. 2011). These dihydroxylated intermediates may then be processed through either an ortho cleavage type of pathway or a meta cleavage type of pathway, leading to central intermediates such as protocatechuates and catechols, which are further converted to tricarboxylic acid cycle intermediates (van der Meer et al. 1992). Hence, as reported by Cao et al. (2009), metabolites of naphthalene, such naphthalene dihydrodiols, are potentially more bioavailable and could be more toxic than naphthalene precursor, further demonstrating the importance of analysing the metabolic route for degrading the contaminants.

Previous results of our group (Moscoso et al. 2012a) allowed the elucidation of the metabolic degradation pathway of the LMW PHE. From the detection of two different products, we could demonstrate that it was degraded trough protocatechuate route instead of the salicylate one. These results will also serve to support the study of the metabolic routes followed to degrade the HMW PAHs, PYR and BaA.

Despite the increasing interest in PAH biodegradation, the metabolization of HMW PAHs is not as well understood yet. Thus, PYR, a pericondensed four ring PAH with a similar structure to several carcinogenic PAHs was often used as a model HMW PAHs for biodegradation (Zheng et al. 2007). Metabolites such as 1-hydroxy-2-naphthoic acid, phthalic acid and cinnamic acid (although their abundance is insignificant in relation to phatalic acid, which is the main compound in PHE degradation) were identified by GC-MS analyses. It can be concluded that after an initial dioxygenation steps, well described in the literature (Seo et al. 2009), PYR transforms into 3,4-dihydroxyphenanthrene, which is also an intermediate detected in PHE degradation. Therefore, it can be confirmed that PYR could be mineralized following PHE pathway, as described previously (Moscoso et al. 2012a, b, c). The schematic pathway proposed for the degradation of PYR by bacteria *P. stutzeri* is shown in Fig. 3.

However, very little is known about the bacteria able to utilize PAHs such as benzo[a]pyrene and BaA. Actually, up to our knowledge, this is the first time that the pathway for the bacterial degradation of BaA is presented. The degradation route proposed for BaA is depicted in Fig. 4, and the main metabolites identified by GC-MS data are summarized in Table 3. As it could be envisaged, the initial steps include a dioxygenation with the consequent production of 3 different diols. Depending on the position, several pathways can be followed. Diols can be formed in position 8–9 or 10–11 (Fig. 4a, b). In this case, after a few steps, compounds such as 3,4-dihydroxyphenanthrene were detected by GC-MS. Metabolites such as phenanthrol, which is a target compound detected in the PHE pathway,

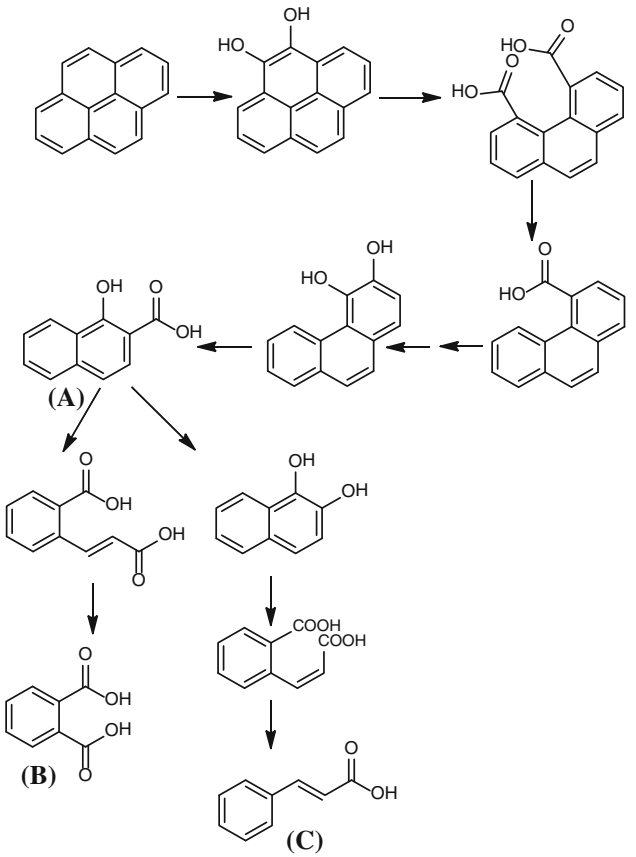


Fig. 3 Proposed metabolic pathway of PYR by *P. stutzeri* cultured at bioreactor scale. **a** 1-Hydroxy-2-naphthoic acid, **b** phthalic acid and **c** cinnamic acid

Table 3 Identification of metabolites produced during degradation of mixed PAHs (PHE + PYR + BaA) based on GCMS properties

Peak	RT (min)	*m/z* of fragment ions	Structural suggestion
1	8.26	372.3 (100), 373 (27), 374 (4)	9-Octadecenoic acid phenylmethylester
2	15.15	149 (100), 165 (87), 65 (42)	Terephthalate
3	15.98	149 (100), 177 (25)	Diethyl phthalate
4	18.46	154 (100), 125 (68)	Benzenemethanol
5	18.82	108 (100), 78 (73), 95 (73)	Benzenemethoxy
6	20.03	180 (100), 208 (98), 152 (78)	9,10-Anthracenedione (anthraquinone)
7	24.95	149 (100), 167 (44), 279 (22)	Diisooctyphtaalate

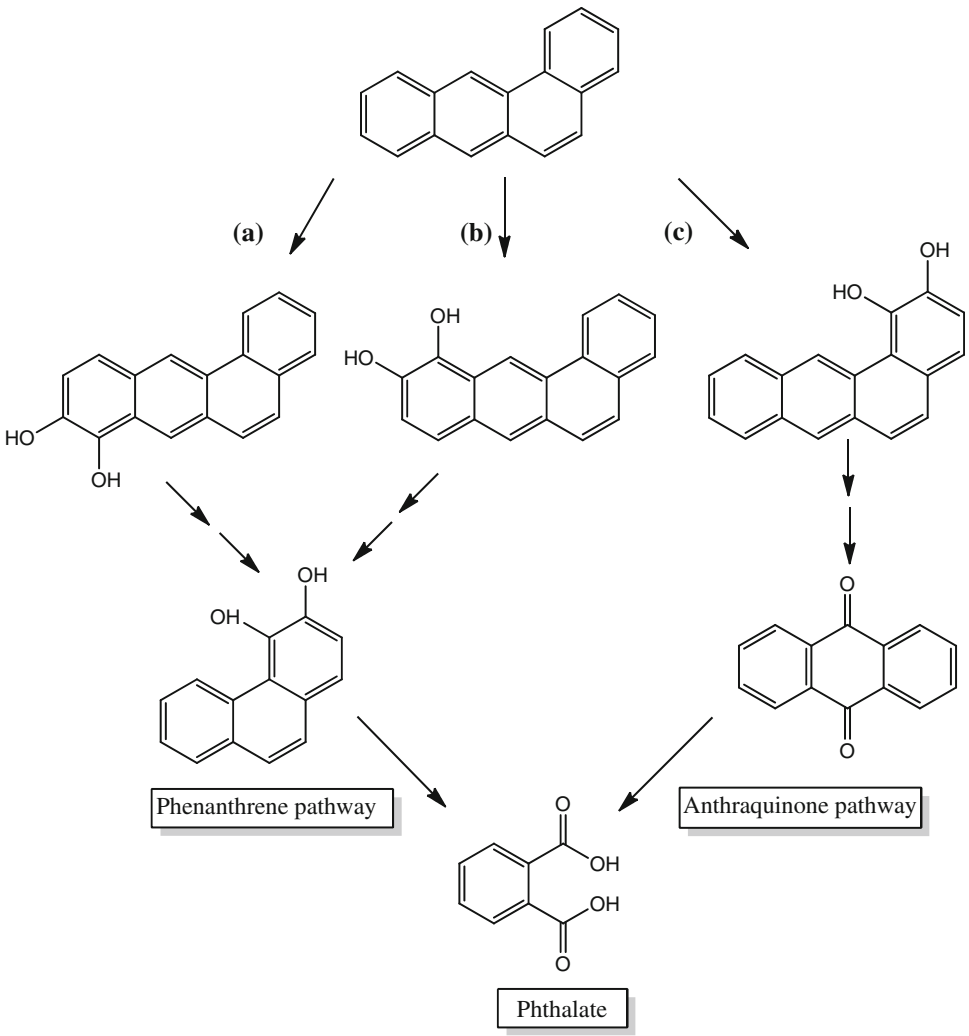


Fig. 4 Proposed metabolic pathway of BaA by *P. stutzeri* cultured at bioreactor scale

confirm these specific routes as possible pathways to degrade BaA. On the other hand, metabolites such as anthraquinone, a typical metabolite formed during the degradation of the anthracene (and more common intermediate in fungus degradation) confirm the viability of the formation of 1–2 benzanthrazenediol (Fig. 4c), which can be transformed in anthracene and subsequently in anthraquinone. This compound was also detected by HPLC data, during the first stages of the degradation process.

Conclusion

Biodegradation of different PAHs involving 3–4 rings has been successfully performed by the selected bacterium, *P. stutzeri*. Different rates of growth and degradation were reached depending on the structure of each PAH, scale used (flask vs. bioreactor) and also on the metabolic conditions. This study also confirmed the promising potential of the microorganism used to degrade mixed PAHs, given the excellent results of degradation in cometabolic conditions, which are the most probably found in real effluents. It should be noticed that this microorganism is able to efficiently carry out the biological degradation without including the typical sterilization step, which is very advantageous in terms of time consumption and bioprocess economy. Besides, we have demonstrated that the operation with this axenic culture allows a tremendous versatility, since it is able to metabolize extremely recalcitrant compounds such as metal working fluids or hydrophobic pesticides such as chlorpyrifos. The logistic and Luedeking and Piret-type models were suitably fitted to the experimental data which allowed a new insight into the kinetic characterization of the biodegradation process. Finally, GC-MS data served our goal to propose a possible pathway route to mineralize HMW PAHs such as PYR and BaA.

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