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Isolation and characterisation of bacteria degrading polycyclic aromatic hydrocarbons: phenanthrene and anthracene

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Abstract: The aim was to isolate and characterise bacteria that can be used to degrade phenanthrene and anthracene. Bacteria were isolated by enrichment of contaminated soil in mineral medium. Growth profiles were assessed by colony forming units (CFU). Electron microscopy and 16S rRNA gene sequencing were employed to characterise these bacteria. Growth profiles were quantified by analysing the culture doubling time, and both the quantification of utilisation of aromatic compounds and the detection of phenanthrane metabolites were carried out by gas chromatography and mass-spectrometry. Two co-cultures of phenanthrene- and anthracene-biodegrading bacteria (PHEN-Cult and ANT-Cult, respectively) were isolated and characterised. The two co-cultures grew rapidly, reaching maximum counts of 10^{10} CFU/mL, within 2–10 days. The doubling time (dt) fell between 0.5–1.5 day (at PHEN and ANT concentrations of 1–100 ppm), making them among the most active PAH degrading microorganisms described so far. PHEN-Cult consists of two strains, *Pseudomonas citronellolis*, PHC3Z1A, and *Stenotrophomonas maltophilia*, JPHC3Z2B, while ANT-Cult is made of *Ralstonia pickettii*, JANC1A and *Thermomonas haemolytica*, JANC2B. Both co-cultures were more active at pH 7, 0–4% NaCl, and 37–40°C. They were also able to utilise naphthalene, salicylic acid and catechol. Starting with 100 ppm, within 15 days, 50–75% of PHEN and ANT were degraded, and the following were the PHEN metabolites that were identified: 3-naphthyl-allyl alcohol, phthalic acid ethyl diester, 2-hydroxybenzalpyruvic acid-methyl ester. These bacteria are appropriate for the removal of PHEN and ANT in contaminated environments, thus further studies are warranted to establish their ability to remove these PAHs in pilot and large scale.

Introduction

Phenanthrene (PHEN) and anthracene (ANT) are polycyclic aromatic hydrocarbons (PAHs) present in petroleum products, thus, are commonly found in oil-contaminated areas. These compounds consisting of three fused benzene rings, are hydrophobic and, as a result, they are absorbed by particulate materials, leading to their persistence in the environment (Desaules et al. 2008). In addition, like all PAHs, these compounds have various obnoxious effects in human, including mutagenic, carcinogenic and teratogenic effects (Bostrom et al. 2002). Several strategies, based on physical and chemical approaches, have been developed to remove these pollutants from the environments, and these approaches include coagulation, precipitation, ozonation, adsorption, ion exchange, advanced oxidation processes (Barceló and Petrovic 2008). Overall, these strategies are associated with high cost, and some of them produce secondary contaminants, thus limiting their usefulness (Barceló and Petrovic 2008). Biological approaches, based on biodegradation, can also be used to remove pollutants. They are based on the exploitation of the ability of the naturally occurring microorganisms to use pollutants as a source of carbon and energy, thus leading to the removal of pollutants. Generally, these biological approaches are relatively more cost effective and environmentally friendly than physical and chemical approaches.

Species of bacteria that can utilise PHEN as a sole source of carbon have been reported, and they include bacteria of genera of *Pseudomonas, Alcaligens, Mycobacteria, Sphingomonas* among others (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010, Nzila 2013). On the other hand, bacteria belonging to the genera of *Methylophilus, Mesorhizobium, and Terrimonas, Kurthia, Micrococcus, Deinococcus, Bacillus, Nocardia, Burkholderia, Sphingomonas* and *Pseudomonas* have been reported to efficiently degrade ANT (Haritash and Kaushik 2009, Seo et al. 2009, Bisht et al. 2010, Kanaly and Harayama 2010, Nzila 2013).

Reports on the exploitation of biodegradation (or bioremediation) to remove pollutants have generally involved the use of pure bacterial cultures. Efficient bacteria to degrade pollutants have been reported, however, the efficiency of biodegradation is generally improved with the use of 2 or more different bacterial strains. The success of biodegradation is the results of expression of efficient bacterial biochemical

pathways that lead to the complete mineralisation of pollutants. Thus, the use of more bacterial strains offers multiple and different metabolic capacities that would increase the efficiency of biodegradation process (Janbandhu and Fulekar 2011). In addition, the use of bacterial mixture has been associated with synergistic interactions among bacteria in degradation of pollutants (Kim et al. 2009). In such synergistic processes, one species removes toxic metabolites that otherwise may hinder the growth of another species, leading to a synergistic effect of the mixed bacteria (Kim et al. 2009).

Taken together, the use of 2 or more bacterial strains provides an advantage on degradation over single microbial strain. Few bacterial consortia capable of degrading PAHs have been reported (Janbandhu and Fulekar 2011, Bacosa and Inoue 2015, Komal et al. 2017)

The coastline of the Arabian Gulf is one of the most oil-contaminated areas in the world, as a result of oil extraction, storage, transportation and refinement. This area is also characterised by high temperatures, dryness and high salinity. Thus, it is conceivable that overtime, diverse groups or species of oil-degrading bacteria, with unique biochemical features, have been selected in this area (Al-Thukair and Malik 2016). This study is aimed at isolating and characterising PHEN and ANT degrading bacteria from the coastline of the Arabian Gulf that can be used in bioremediation strategies.

Material and method

Chemicals

Pyrene, ANT, PHEN, naphthalene, phthalic acid, salicylic acid (SALC), catechol (CTC), dimethyl sulphoxide (DMSO), ethyl acetate, chloroform, pyridine, N,O-bi's-trimethylsilyl acetamide, trimethylchlorosilane, formaldehyde, ethanol, acetic anhydride, sodium chloride, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, Na_2HPO_4 and $FeSO_4 \cdot 7H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO, USA) [purity> 96%]. Luria-Bertani Broth (LB) was purchased from Difco, Detroit, MI, USA.

Sample collection, enrichment and isolation of bacterial strains

The enrichment cultures were initiated with 1.0 g of contaminated soil samples in 50 mL minimum mineral medium, also known as Bushnell Hass culture medium (BH). This medium consisted of $(NH_4)_2SO_4$ (2.38 g), KH_2PO_4 (1.36 g), $CaCl_2 \cdot 7H_2O$ (10.69 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), Na_2HPO_4 (1.42 g) and $FeSO_4 \cdot 7H_2O$ (0.28 mg) per litre, and supplemented with 1000 mg/L of PHEN or ANT as sole sources of carbon; this medium was referred to as BH-PHEN and BH-ANT (when PHEN and ANT were used as a sole source of carbon respectively). Deionised water was used in the experiments. When a solute is completely dissolved, concentration in mg/L is equivalent to concentration in ppm. In the case of PAHs, since only a part is dissolved in aqueous medium, values in mg/L do not necessary equate to effective ppm, however, for the sake of comparison with previous similar work, mg/L and ppm will be used interchangeably throughout this manuscript. All the experiments were carried out in duplicate.

Enrichment experiments were conducted by incubating cultures at temperature 37°C and 120 rpm, for 2–3 weeks, followed by a transfer in a fresh BH-PHEN or BH-ANT medium

(1/10, v/v dilution), for another 2–3 weeks. After repeating the process for 4–5 times, and when the bacterial growth was ascertained, based on visual turbidity, the resulted suspensions were PHEN- and ANT-degrading co-cultures (PHEN-Cult and ANT-Cult respectively). These co-cultures were isolated and cryopreserved using 15% glycerol solution (300 μL of 50% sterile glycerol mixed with 700 μL of a bacterial suspension) at -80°C for further studies.

Thereafter, each co-culture was streaked on an agar solid plate made of 1% (wt/v) of LB rich medium. Individual colonies were isolated, and streaked again in new plates to ascertain their purity, and the resulting individual colonies were cryopreserved, in 15% glycerol solution at -80°C for further studies.

Scanning electron microscopy (SEM) analysis

SEM was carried out as described elsewhere (Oyehan and Al-Thukair 2017). Briefly, bacteria were immobilised on small microscope cover slides and then fixed in the aqueous solution of formaldehyde (5% v/v) for 12 h; thereafter, samples were dehydrated by incubation in a series of ethanol-water solutions (ethanol [v/v], 30%, 50%, 70%, 80%, 90% and 95%). The samples were then sputter-coated with gold before observation under SEM (JSM-T300, JEOL, Japan).

Species identification

The identification of bacteria species was carried out by 16S rRNA gene sequencing. The detailed methodologies of this approach have been presented elsewhere (Nzila et al. 2016).

Assessment of bacterial growth in the presence of various substrates, and effect of pH and salinity on bacterial growth

To initiate these experiments, bacteria were pre-cultured in an LB rich medium, and were centrifuged (3500 g for 15 min). The pellet was re-suspended in BH medium and centrifuged again, and the process was repeated twice, to remove the remaining rich medium. The resulting bacteria, around 10^5 CFU/mL was used to start a culture in 50 mL BH medium containing 1000 ppm or less (as this will be explicitly mentioned) of substrates. The growth was monitored by bacterial count and quantified as CFU/ml. In these experiments, non-polar substrates (PHEN, NAPH and ANT) were dissolved in dimethyl sulfoxide (DMSO), and this DMSO was evaporated before adding the culture medium. The polar substrates SALC and CTC were dissolved directly in BH medium. The growth of these bacteria in the presence of PHEN and ANT was also monitored as a function of temperature (30, 35, 37 and 40°C), salinity (0, 2, 4, and 6% of NaCl [wt/wt]) and pH (5, 6, 7 and 8).

Bacterial growth was quantified by fitting the exponential phase of the growth in the characteristic equation of bacterial growth, $Q_t = Q_o e k^t$ (Q is bacterium CFU/mL at time t, Q_o is the starting bacterial count in the culture, and k is the growth rate), using Origin 5 software (Version 9.3, Northampton, MA, USA). Thereafter, the doubling time (dt) of bacterial growth was computed as dt=ln(2)/k (in days). The lower dt, the higher the growth.

Quantification of PHEN and ANT

Analysis of residual PHEN was carried out using gas chromatography (GC) analysis (Agilent 6890N GC).

Utilisation of the substrates by microorganisms generally follows the first order kinetics, according the equation $C_t = C_o e^{-kt}$. Thus, to establish the kinetics of substrate utilisation, the same approach described earlier on growth quantification was employed, in which data were fitted in an exponential function, and the growth rate constant k was computed.

To quantify the residual PHEN or ANT, seven flask cultures (of 100 ml) were grown in the presence of 100 mg/L (100 ppm) of PHEN for PHEN-Cult and ANT for ANT-Cult, for 30 days, and one flask was collected every five days and subjected to extraction with ethyl acetate (50 mL × 2). The combined organic layers were dehydrated with calcium chloride then evaporated to dryness. The samples were then dissolved in chloroform (500 μL) prior to injection into GC-MS for quantification. The sample concentration was deduced from a standard curve that was obtained from flask containing PHEN or ANT at concentrations of 1, 5, 25, 50, 100 mg/L in the BH medium.

Detection of PHEN metabolites by GC-MS

Bacteria were grown in 1.0 L BH-PHEN medium in the presence of 100 mg/L of PHEN for 5 days. Thereafter, the medium (containing PHEN metabolites) was filtered to remove unprocessed PHEN, and the resulting filtrate was extracted with ethyl acetate (100 mL × 3). The combined organic layers were dehydrated with calcium chloride then evaporated to dryness under vacuum. The remaining residue was dissolved in 1.0 mL chloroform and divided into three portions. One part was analysed by GC-MS without further treatment. The second portion was evaporated to dryness then subjected to derivatisation with trimethylsilyl (TMS) group. This process, also known as silylation, consisted of mixing the remaining residue with pyridine (40 μL), N,O-bis-trimethylsilyl acetamide (40 μL) and trimethylchlorosilane (20 μL), followed by an incubation at 80°C for 10 min under nitrogen. The mixture was then diluted with 1.0 mL chloroform prior to its analysis by GC-MS. The third portion was evaporated to dryness and then derivatised by treatment with acetic anhydride (one drop) and pyridine (two drops) for 6 h to convert alcohols to ester prior to analysis by chiral GC-MS

GC-MS analyses were conducted using an Agilent 5975B MS attached to 6890N GC equipped with HP-5 [30 m, 0.25 mm (i.d.)] column with Helium as the carrier gas. GC analysis started with an initial temperature of 50°C held for 2 min, followed by an increase to 250°C at a rate of 5°C/min and holding time at 250°C for 30 min. The MS analysis conditions were based on an inlet temperature of 250°C and mass range of 15–550 m/z.

Statistical analyses

Statistical analyses were carried out using one-way analysis of variance (ANOVA), t-test and a simple linear regression fitting model, and the strength of linearity was assessed based on the Pearson correlation coefficient. In all tests, $p < 0.05$ was considered to be the level of significance. The software MINITAB (Version16, Coventry, UK) was employed in these analyses.

Results and discussions

Enrichment and Isolation of PHEN- an ANT-degrading bacteria

The enrichment experiments in media containing PHEN and ANT (1000 ppm) led to the isolation of 2 co-cultures of bacteria, PHEN- and ANT-degrading bacteria (PHEN-Cult and ANT-Cult respectively), within 60 days, in which cultures were passaged in a frech medium every two weeks. Figure 1 summarises the growth profile of PHEN- and ANT-Cult in the presence of PHEN and ANT respectively, as the sole source of carbon. PHEN-Cult grew steadily, with a latency period of around one week, thereafter bacteria started growing exponentially up to a maximum count of around $3 × 10^8$ CFU/mL, which was obtained after 20 days. On the other hand, ANT-degrading bacteria, ANT-Cult, harbored a slow growth rate compared to PHEN-Cult. Indeed, the maximum count was around $2.2 × 10^8$ CFU/mL and was achieved after 30 days. The computation of growth profile gave a growth rate, k, of 0.28 day^{-1} and a doubling time (dt) of 2.5 days for PHEN-Cult and a growth rate of 0.19 day^{-1} and a dt of 3.65 days for ANT-Cult, indicating a higher growth of PHEN-Cult when compared to ANT-Cult. As mentioned earlier, several consortia of bacteria have been selected by enrichnment in the presence of PAHs (including PHEN), and the following work reported those that can degrade PHEN and ANT (Janbandhu and Fulekar 2011, Bacosa and Inoue 2015, Komal et al. 2017).
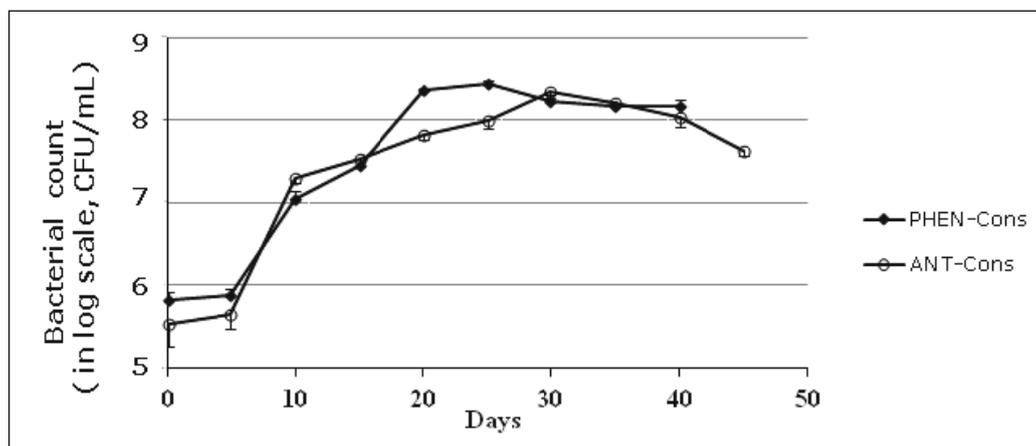
Fig. 1. Growth profiles of co-cultures ofphenanthrene (PHEN)-biodegrading bacteria (PHEN-Cult) and that of anthracene (ANT)-biodegrading bacteria (ANT-Cult) in the presence of PHEN and ANT, respectively

Isolation and morphological characterisation of individual bacteria

The next step was to isolate individual bacterial species that form these co-cultures. The streaking of co-cultures on agar plates led to the isolation of four different colonies, based on the color and the shape. These are JPHC3Z1A and JPHC3Z2B from PHEN-Cult, and JANC1A and JANC2B from ANT-Cult. The characteristics of these colonies are given in Table 1.

The four colonies were cultured in rich media, and their DNA was purified prior to 16S rRNA gene sequencing analysis. The amplified 16S rRNA genes were around 1400 nucleotides in length. Using the Basic Local Alignment Search Tool (BLAST) program for homology analysis of available 16S rRNA gene sequences in the National Center of Biotechnology Institute (NCBI) database, the following species were identified, based on the threshold of 99% homology (Table 1): JPHC3Z1A as *Pseudomonas citronellolis* (NCBI reference, *KT894554*), JPHC3Z2B as *Stenotrophomonas maltophilia* (*KT894555*), JANC1A as *Ralstonia pickettii* (*KT894552*) and JANC2B as *Thermomonas haemolytica* (*KT894553*).

As discussed earlier, PHEN-degrading bacteria have been proven to belong to several genera including, *Pseudomonas, Mycobacterium, Alcaligens, Cycloclasticus Sphingomonas, Paenibacillus, Ralstonia and Geobacillus,* among other (Haritash and Kaushik 2009, Seo et al. 2009, Bisht et al. 2010, Kanaly and Harayama 2010, Nzila 2013; Ubani et al. 2016). The mineralisation of PHEN by individual strains of *S. maltophilia* and *P. citronellolis* has been described (Mangwani et al. 2014, Oyehan and Al-Thukair 2017). Consortium of PHEN degrading bacteria consisting of *Sphingobacterium* sp.*, Bacillus cereus* and *Achromobacter insolitus* has also been reported (Janbandhu and Fulekar 2011). In this work, we confirm the ability of *S. maltophilia* and *P. citronellolis* to use PHEN as a sole source of carbon.

On the other hand, species of ANT-degrading bacteria have been shown to belong to various genera, including *Methylophilus, Terrimonas, Kurthia, Micrococcus, Deinococcus, Bacillus, Nocardia, Sphingomonas* and *Pseudomonas among others* (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010, Nzila 2013, Sydow et al. 2016, Ubani et al. 2016). The current work shows that

bacteria of *Ralstonia* and *Thermomonas* genera can also mineralise ANT.

When carrying out enrichment culture (of contaminated samples) in the presence of PHEN or ANT or any other PAHs, in general, the resulted cultures consist of 2 or more bacterial strains. For instance, a consortium of 2 bacterial strains was also reported from enrichment using ANT, PHEN and pyrene (Wu et al. 2013). In similar investigations, consortia of 3–6 bacterial strains were reported after PAHs enrichment (Janbandhu and Fulekar 2011; Bacosa and Inoue 2015; Komal et al. 2017). It is well known that most of microorganims present in soil samples are not culturable, and in addition, the use of PAHs (to select PAH-degrading bacteria only) eliminate futher a few bacteria strains that can grow *in vitro*, as a result, the number of selected and isolated bacteria are generally very low, as we have also reported in this work.

Growth as a function of PHEN and ANT concentration

The detailed observations of many investigations carried out on the biodegradation of PAHs in general and PHEN and ANT in particular indicate a range of the tested PAH concentrations falling between 1 to 1000 ppm (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010, Nzila 2013). On the other hand, it is well established that high concentrations of PAHs are associated with toxic effects against organisms, and in relation with microorganisms, the effect of these PAHs can lead to a decrease in cell growth (Bragin et al. 2016). Thus, it is important to establish the effect of PHEN and ANT concentrations on the growth of these bacteria. The growth of these co-cultures was assessed in the presence of PHEN and ANT at 1, 5, 25, 100 and 1000 mg/L, at pH 7, temperature 37°C, and in absence of salinity. PHEN-Cult showed an extremely high growth at concentrations of 1, 5 and 25 ppm, with the maximum counts of $>10^{10}$ CFU/mL, which were attained within 5 days. The lowest concentration, 1 ppm, corresponded to the highest count of 8×10^{10} CFU/mL, and was achieved after 3 days of culture only (Figure 2A). As the concentrations increase, the maximum counts decrease. For instance, the maximum count was around 10^{10} CFU/mL at 100 ppm, while this value decreased to 2.5×10^8 CFU/mL at 1000 ppm, and these values were achieved at day 13 and

Table 1. Characteristics of individual bacteria that form the co-cultures of phenanthrene-biodegrading bacteria (PHEN-Cult) and the one of anthracene-biodegrading bacteria (ANT-Cult)

	Strains names	Morphological characteristic	16S rRNA analyses and species identification	NCBI reference
PHEN-Cult	JPHC3Z1A	Pale yellow, irregular, with raised elevation and entire margin. Rode shape, size of 1.5 × 0.3 μm. Gram positive	*Pseudomonas citronellolis*	*KT894554*
	JPHC3Z2B	Pale yellow, flat, with raised elevation and entire margin. Rod shape, size of 1.1 × 0.25 μm. Gram negative	*Stenotrophomonas maltophilia*	*KT894555*
ANT-Cult	JANC1A	White, circular, with a flat elevation and entire margin.	*Ralstonia pickettii*	*KT894552*
	JANC2B	White, circular, with a convex elevation and undulate margin.	*Thermomonas haemolytica*	*KT894553*

25, respectively. Thus, there is more than two log magnitude differences between the maximum counts at 1–5 ppm and 1000 ppm. These differences were supported by the ANOVA test for single regression, showing a significant correlation between dt values and PHEN concentrations (p=0.015), with dt values of less than 0.8 day at 1–5 ppm, less than 1.5 days at 25–100 ppm, and around 2.6 days at 1000 ppm (Figure 3). This correlation can be predicted through the equation dt=2+0.23xC (R²=54.5%) [C stands for substrate concentration].

In relation with ANT-Cult, similar results were obtained, except that the maximum counts at all tested concentrations were, in general, 2–5 times lower than those observed for PHEN-Cult. For instance, at 1–5 ppm, maximum counts were at the range of 10^{10} CFU/mL (compared to $5–8 \times 10^{10}$ CFU/mL with the PHEN-Cult) (Figure 2B). The values of dt were less than 1 day at concentrations of 1–100 ppm, and they increased to around 3.1 days at 1000 ppm (Figure 3). As it is the case with PHEN-Cult, a strong correlation exists between ANT concentrations and dt values (ANOVA single regression test, p<0.001), through the linear equation dt=1.5+0.52*C (R²=93.2%).

These data confirm that the growth of the bacteria in the presence of PAHs is dependent upon the concentration of PAHs. Several reports have described the isolation of efficient PAH-degrading bacteria that can reach maximum growth in the presence of PAHs within 5–10 days or harboring dt less than 2 days (Ceyhan 2012). However, careful observation of these studies shows that, generally, concentrations of PAHs are in the range 5–100 ppm. The current data clearly showed that dt values were 1 day or less for both co-cultures at concentrations up to 100 ppm, thus, they are as fast growing PAH-degrading bacteria as the most efficient bacteria reported so far. The observation that the increase in PAHs concentrations is associated with a decrease growth is in line with many previous reports. For instance, Swaathy et al. tested the ability of the strain *Bacillus licheniformis* (MTCC 5514) to degrade ANT, at concentrations of 100, 300, 500, 750 and 1000 pm. The results showed that bacterial growth profiles were higher at 100 and 300 ppm, but significantly inhibited at concentrations > 500 ppm (Swaathy et al. 2014).

In an effort to optimise the growth conditions, the ability of these bacteria to degrade PHEN and ANT at varying pH
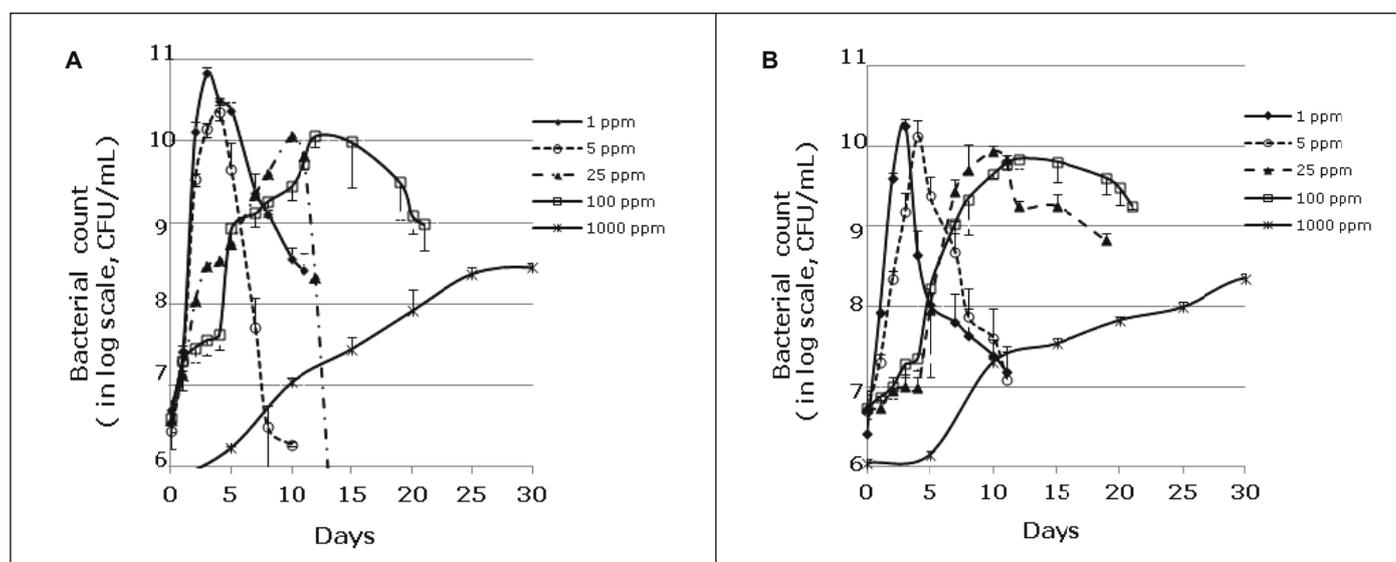
Fig. 2. Growth of co-cultures of phenantrane-biodegrading bacteria (PHEN-Cult) [**A**] and that of the anthracene-biodegrading bacteria (ANT-Cult) [**B**] as a function of phenanthrene (PHEN) and anthracene (ANT) concentrations, respectively
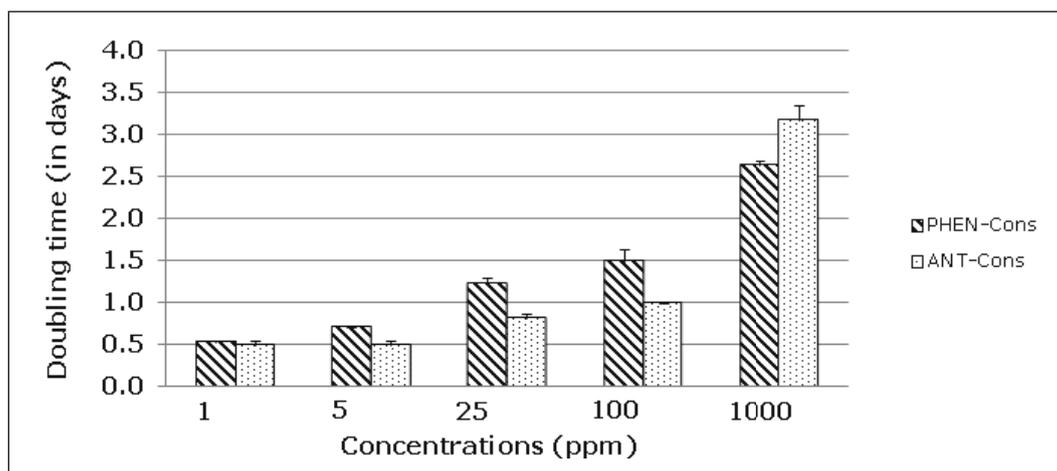
Fig. 3. Doubling time (dt, in days) of co-cultures of phenanthrene-biodegrading consortium (PHEN-Cult) and that of anthracene-biodegrading bacteria (ANT-Cult), as a function of phenanthrene (PHEN) and anthracene (ANT) concentrations, respectively

(6, 7, 8, and 9), salinity (0, 2, 4 and 6% NaCl) and temperature (30, 35, 37, 40 and 45°C) was investigated. These experiments were carried out in the presence of 1000 ppm of PHEN and ANT and the results presented below.

Effect of pH

The effect of the medium pH on the ability of bacteria to degrade PHEN and ANT was evaluated at various pHs (5, 6, 7 and 8). The PHEN-Cult grew steadily at the four tested pHs, with maximum bacterial growth falling from 2×10^8 to

3×10^8 CFU/mL, which was achieved after 20–30 days. However, the best growth was observed at pH 7, corresponding to the highest maximum count (around 3×10^8 CFU/mL), achieved after 20 days (Figure S1A). This was confirmed by the computation of dt values, showing the fast growing culture at pH 7, with dt around 2.6+0.06 days, and yet, values for the other three cultures fell between 2.93–3.18 days (Table 2). However, one-way ANOVA test showed that these differences were not statistically significant ($p=0.11$). On the other hand, the ANT-Cult showed a similar growth profile at all tested pHs,
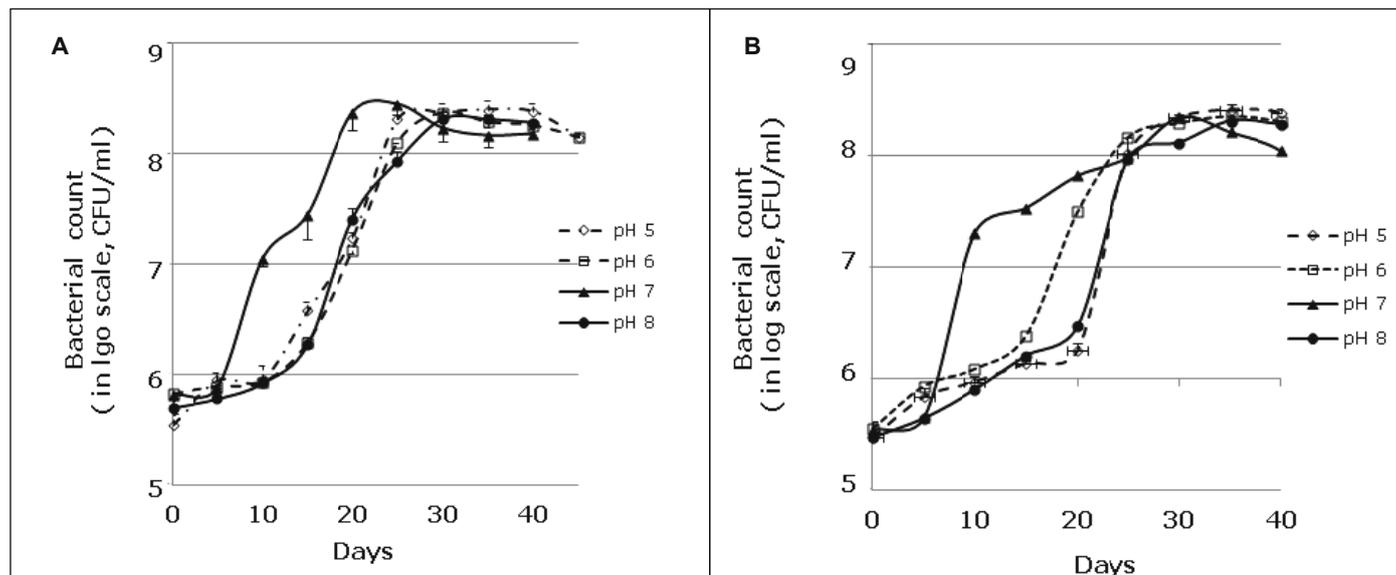
Fig. S1. Effect of pH on the growth of phenanthrene-biodegrading bacteria (PHEN-Cult) [**A**], and anthracene-biodegrading bacteria (ANT-Cult) [**B**] in the presence of 1000 ppm of phenanthrene and anthracene respectively

Table 2. Doubling time (dt, in days) of bacteria as a function of pH, temperature and salinity

	Conditions	Doubling time (days) [± standard deviation]	
		PHEN-Cult	**ANT-Cult**
pH	pH 5	2.93+0.1	3.3.4±0.31
	pH 6	3.21±0.08	3.26±0.05
	pH 7	2.62+0.06	3.16+0.17
	pH 8	3.18±0.36	3.40±0.16
Temperature	30°C	2.42±0.02[a]	2.28±0.21[b]
	35°C	2.82±0.01[a]	2.66+0.14
	37.5°C	2.63±0.04	3.16±0.17[b,c]
	40°C	2.67+0.13	1.87+0.26[c,d]
	45°C	–	2.96+0.20[d]
Salinity (NaCl)	0	2.77±0.17[f]	3.16±0.17[g]
	2%	2.09±0.16[f]	3.03±0.18
	4%	2.38±0.0	2.45±0.17[g]
	6%	6.59+0.24[e]	3.97±0.08[e]

Temperature ([a,b,c,d]): In PHEN-Cult, dt at 30°C versus 35°C was statiscally significant ([a]). Likewise, in ANT-Cult, dt differences were also significant when comparing 30°C versus 37.5°C ([b]), 37.5°C versus 40°C ([c]), and 40°C versus 45°C ([d]).

Salinity([e,f,g]): The difference of dt at salinity 6% ([e]) was statistically significant with any of the tested salinity in both PHEN-Cult (p<0.001) and in ANT-Cult ((p<0.001). Statistical significant differences (p< 0.05) were also observed when comparing salinity 0 versus 2% ([f]) in PHEN-Cult and salinity 0 versus 4% in ANT-Cult (g[b]).

which was also similar to that of the PEHN-Cult (Figure S1B). As summarised in Table 2, the dt fell between 3.16–3.40 days, and differences between these dt were not statistically significant (Anova test, *p=0.67*). Thus, within the tested pH, the growth profiles of co-cultures PHEN-cult and ANT-cult) were similar.

In this study, although the differences in dt values were not significant, it is of interest to note that both PHEN-Cult and ANT-Cult harbored lowest dt values at pH 7, which is in line with reports indicating that generally, oil-degrading microorganisms are active at pH values between 6–8, and that optimum degradations tend to occur at around pH 7 (Leahy and Colwell 1990).

However, both acidophile and alkalophile PHEN- and ANT-degrading bacteria have been reported. For instance, *Bacillus radius* was reported to be active in removing ANT in alkaline conditions (Ahmed et al. 2012), and in another study, higher PHEN degradation was observed at pH 6.5 than at pH 7.5 in *Mycobacterium vanbaleeni* (Kim et al. 2005), while the degradation of PHEN by *Sphingomonas* sp. GY2B was shown to be higher in alkaline conditions than in acidic conditions (Tao et al. 2009). Overall, the bacteria PHEN-Cult and ANT-Cult reported in the current study can actively grow at pH 5–8 range.

Effect of salinity

Analysis of PHEN-Cult as a function of salinity (0, 2, 4, and 6% NaCl) showed that the growth profiles were almost the same at 0, 2 and 4% salinity. The best growth was achieved at 2% salinity, with a maximum count of around 7×10^8 CFU/mL, attained at day 25. A reduction in bacterial growth was observed at higher salinity of 6% (Figure S2A). The dt values fell between 2.09 to 2.38 at 0–4% NaCl (the lowest value being at 2% NaCl), yet at 6%, this value increased to 6.59+0.24 days (Table 2). The ANOVA test indicates a pronounced effect of salinity on dt values (*p<0.001*), and detailed analysis using Tukey's pairwise test shows that values at 6% salinity were significantly higher than those at any other tested salinity (0, 2 and 4%), and in addition, dt value at 2% salinity was significantly lower than at 0% (Tukey's pairwise test). Thus,

2% was the optimum salinities while salinity higher or equal to 6% was not appropriate for bacterial growth.

The results pertaining to ANT-Cult showed that 2 and 4% NaCl were associated with maximum counts, from 2.5×10^8 to 3.5×10^8 CFU/mL, however these counts were achieved within 25 days at 2% and 20 days at 4% (Figure S2B). In absence of salinity, the maximum bacterial count was lower than that at 2 and 4% of NaCl, and in addition, it was achieved after a longer time, i.e. 40 days (Figure S2B). As it was the case with PHEN-Cult, the lowest growth was associated with 6% salinity, with a dt of 3.97±0.08 days (Table 2), and this was supported by Tukey's pairwise tests showing a significant difference of dt at salinity 6% with any of the other tested salinity (Anova test, *p=0.003*). The highest growth was associated with salinity at 4% (dt= 2.45±0.17 days) [Table 2], though the dt difference at 4% was significant when compared to that at salinity 6% and 0% only (*p=0.003*). Thus, the growth of PHEN-Cult and ANT-Cult was least supported at 6%, while their optimum conditions were 2 and 4% salinity.

Salt loving microorganism are classified depending upon the degree of their salt requirement as slight halophiles, moderate halophile, and extreme halophiles which grow optimally at 1–5% NaCl (0.2–0.85 M), 5–20% NaCl (0.85–3.4 M), and 20–30% NaCl (3.4–5.1 M), respectively (DasSarma and DasSarma 2012). Halophilic PHEN- and ANT-degrading bacteria have been reported, and these include species belonging to the genera of *Haloarchea, Martelella, Micrococcus, Marinobacter, Haloferax, Alcaligenes*, among others (Martins and Peixoto 2012, Fathepure 2014). Although the conditions of enrichment that were employed in the current study did not favor the selection of halophilic bacteria, the isolated co-cultures, PHEN-Cult and ANT-Cult, fell within the group of slight halophilic microorganisms, thus could be used in environment where salinity does not exceed 5%.

Effect of temperature

The impact of temperature (30, 35, 37, 40 and 45°C) on the growth of these bacteria was also investigated. Overall, the
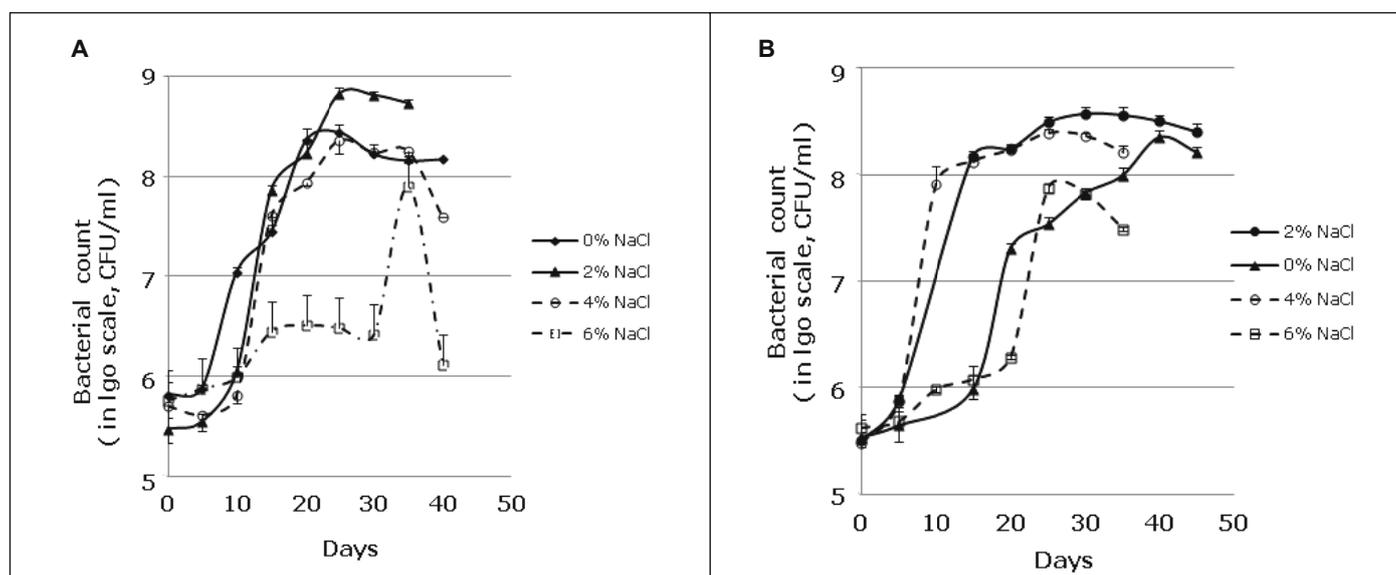
Fig. S2. Growth profile of phenanthrene-biodegrading bacteria (PEHN-Cult) in the presence of phenanthrene [**A**] and that of anthracene-biodegrading bacteria (ANT-Cult) in the presence of anthracene [**B**], as a function of salinity. The 2 substrates were used at the concentration of 1000 ppm

PHEN-Cult grew efficiently within the temperature range of 30–40°C, with all cultures attaining a maximum growth >10^8 CFU/mL, within 20–25 days (Figure S3A). However, no growth was observed at 45°C. The lowest dt was observed at temperature 30°C (dt=2.42±0.02 day), however this value was statistically different only with that at 35°C ($p<0.022$) [Table 2], implying that the growth profile was similar at all the tested temperatures, except when growth at 30°C is compared with that at 35°C.

Interestingly, ANT-Cult could still grow at 45°C (unlike PHEN-Cult), though at a lower rate (maximum count <10^8 CFU/mL) [Figure S3B]. The computation of dt showed that the best growth was at 40°C, with a dt of 1.87+0.26 days, and the dt values at other tested temperatures fell between 2.28–3.16 days (Table 2). However, dt differences were significant only at 40°C versus 37.5°C, and 40°C versus 45°C ($p=0.007$). In addition, growth at 30°C was significantly higher than at 37.5°C ($p=0.007$). Thus, ANT-Cult is more tolerant to higher temperature than PHEN-Cult. This is not surprising since ANT-cult contains one strain of *Thermomonas* group (*T. haemolytica*), a group of meso- and thermophilic microorganisms (Wang et al. 2014). Thermophilic bacteria capable of degrading PHEN and/or ANT have been reported, and they include *Nocardia otitidiscaviarum* (Zeinali et al. 2008). The current work provides evidence that *Thermomonas* bacteria can also degrade ANT.

Substrate utilisation

The biodegradation of highly complex PAHs proceeds stepwise, by progressively reducing the number of aromatic rings. For instance, the biodegradation of PHEN first leads to derivatives of NAPH (which have 2 rings), followed by derivatives of monocyclic aromatic molecules (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010, Nzila 2013). Thus, bacteria that degrade a PAH can potentially degrade a molecule of lower molecular weight (LMW), since these bacteria express catabolite enzymes required to degrade LMW PAHs. In addition, the higher solubility of LMW compounds, compared to higher molecular weight PAH, increases their availability in the media, thus their biodegradation. As part of the current work was tested the ability of PHEN-Cult and ANT-Cult to degrade NAPH and the monocyclic aromatic molecules, SALC and CTC, the latter 2 being part of metabolites of PAH biodegradation (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010). All the substrates were tested at concentration of 1000 mg/L (1000 ppm) and the results were compared with those obtained with PHEN and ANT substrates (Figure 4). The data showed that as the number of rings decreases, bacterial growth increases, as illustrated by the decrease in the dt values, from 2.68 days for PHEN and ANT to 2.3 or less for the monocyclic aromatic SALC and CTC, and these differences were statically significant ($p=0.015$, ANOVA test for simple regression). Likewise, ANT-Cult in the presence of these aromatic compounds showed a significant decrease in the dt values as the complexity decreased, from dt of around 3 days for PHEN, ANT, and NAPH to around 2 days for the monocyclic aromatic compounds ($p=0.001$, ANOVA test for simple regression). Therefore, these bacteria can also be used to degrade compounds of molecular weights lower than PHEN and ANT, as reported in previous observations (Haritash and Kaushik 2009, Seo et al. 2009, Kanaly and Harayama 2010, Nzila 2013). In line with these observations, using PAH half-lives in contaminated soil samples, Szczepaniak et al. have recently shown a lower degradation rate of PAHs consisting of 5 rings compared to those containing < 5 rings, a clear indication that the higher the complexity of PAH, the lower the degradation rate (Szczepaniak et al. 2016).

Quantification of the utilisation of PHEN and ANT

The efficiency at which PHEN and ANT are degraded by PHEN-Cult and ANT-Cult has also been investigated. Starting with a concentration of 100 ppm, PHEN-Cult degraded 50% of PHEN after seven days; this rose to 75% after 15 days. The analysis of ANT degradation by ANT-Cult showed that about 50% and 75% of ANT were degraded after about 8 and 20 days, respectively (Figure 5). Thus, PHEN-Cult degrades
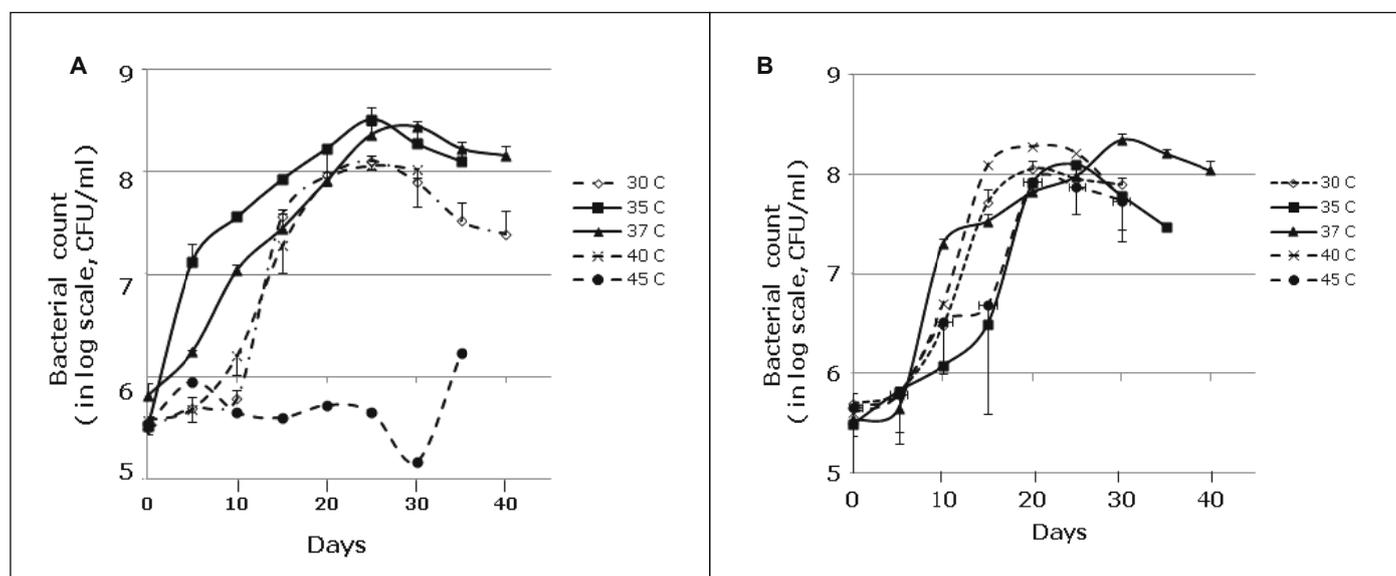
Fig. S3. Effect of temperature on the growth profiles of phenanthrene-biodegrading culture (PEHN-Cult), in the presence of 1000 ppm phenanthrene [**A**], and that of anthracene-biodegrading bacteria (ANT-Cult) [**B**], in the presence of 1000 ppm of anthracene

PHEN slightly faster than ANT-Cult degrades ANT, which is in line with the computation of k, indicating a utilisation rate of PHEN of -0.073 day^{-1} (R^2=0.98) compared to -0.055 day^{-1} (R^2=0.9) for ANT.

The utilisation rate of these substrates is in line with previous reports. For instance, a culture of *Pseudomonas* sp. USTB-RU was shown to remove 86% PHEN in 8 days (starting with a culture of 1000 ppm) (Masakorala et al. 2013). Using a medium containing peptone and starting with 300 ppm PHEN, *Pseudoxanthomonas* sp. DMVP2 was reported to completely metabolise PHEN within 5 days (Patel et al. 2012). Similar results were reported with the halophilic *Martelella* species AD-3 (Feng et al. 2012), a mixed culture of *Mycobacterium* sp. strain A1-PYR and *Sphingomonas* sp. strain PheB4 (Zhong et al. 2011), and a consortium of *Sphingobacterium* sp., *Bacillus cereus* and *Achromobacter insolitus* (Janbandhu and Fulekar 2011). Likewise, starting with 300 ppm ANT, *Bacillus licheniformis* (MTCC 5514) was reported to mineralise 95% of ANT within 22 days (Swaathy et al. 2014) whereas a strain of

Aspergillus fumigatus degraded 60% of ANT within 5 days (Ye et al. 2011). Comparable results were also reported elsewhere, with *Kurthia* sp. and *Bacillus circulans* (Bisht et al. 2010).

Detection of PHEN metabolites in *S. maltophilia*

PHEN metabolites by *S. maltophilia*, the most efficient strain in degrading PHEN (dt of 2.5 days compared to 3.8 days for *P. citronnelolis* at PHEN 1000 ppm) were also investigated using GC-MS, after five days of culture in the presence of PHEN as a sole source of carbon. The analysis showed a peak at retention time of 19.71 min that consisted of the following MS fragments [m/z (relative intensity): 184 (100, M$^+$), 152 (12), 139 (19), 115 (5), 92 (10)], Figure S4A. These fragments are consistent with 3-naphthyl allyl alcohol, in line with previously reported data using *Aeromonas salmonicida* subsp. *achromogenes* strain NY4 (Nie et al. 2016). Further analysis of GC-MS data showed the following fragments: [m/z (relative intensity): 222 (4, M$^+$), 177 (22), 149 (100), 121(8), 105 (8), 93 (12), 76 (24)], Figure S4B. The fragment at
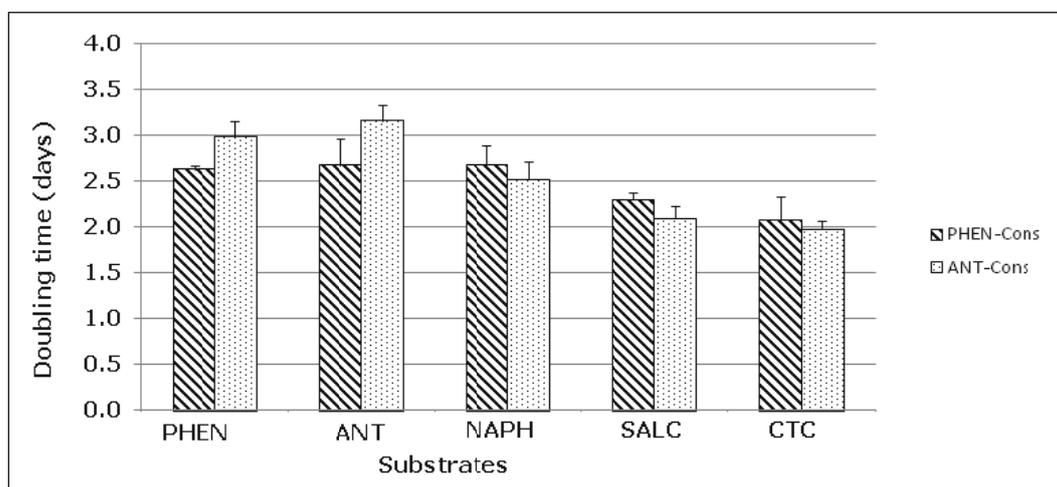
Fig. 4. Doubling time (dt) of co-cultures of phenanthrene-biodegrading bacteria (PHEN-Cult) and that of anthracene-biodegrading bacteria (ANT-Cult) in the presence of phenanthrene (PHEN), anthracene (ANT), naphthalene (NAPH), salicylic acid (SALC) and catechol (CTC)
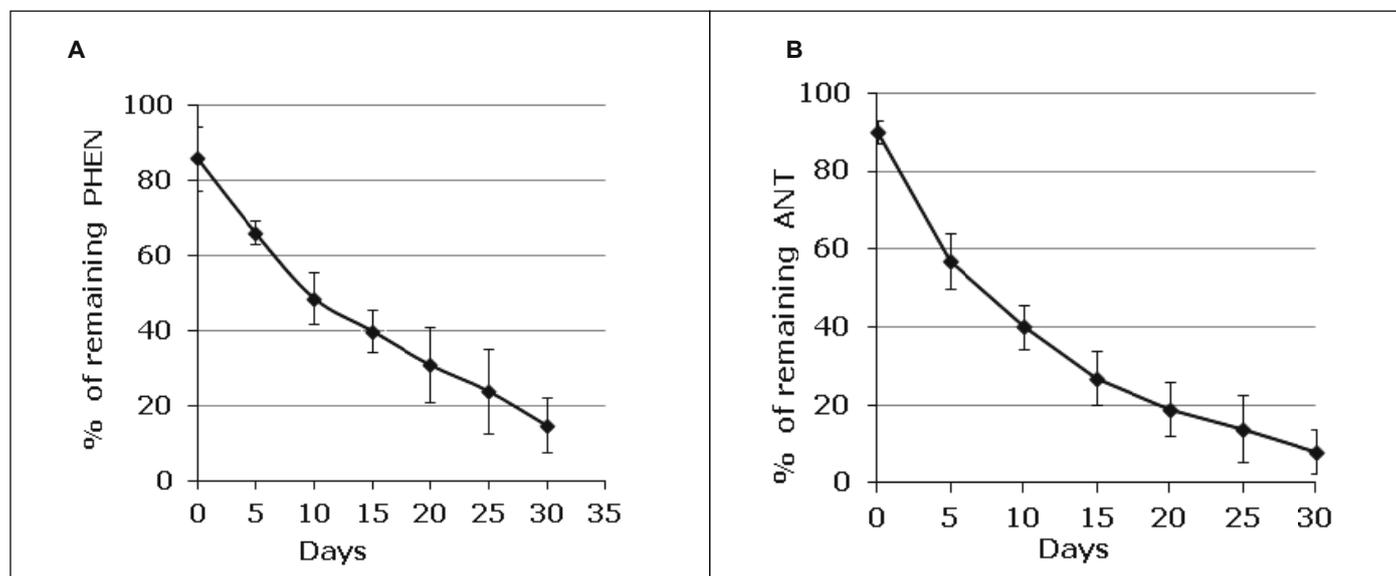
Fig. 5. Quantification of the remaining phenanthrene (PHEN) [**A**] and anthracene [**B**] in co-cultures of PHEN-biodegrading bacteria (PHEN-Cult) and ANT-biodegrading bacteria (ANT-Cult), respectively. The initial concentration of substrates were 100 ppm

m/z 149 is a characteristic peak for phthalate esters (Wang et al. 2017), and further analysis of these fragments indicated the presence of phthalic acid ethyl diester (Eibes et al. 2006). The GC-MS analysis of TMS derivatised fraction of metabolites gave a metabolite that eluted at 37.7 min which consists of the following MS fragments (Figure S5A): m/z: 278 (M^+), 263 (M^+-15, loss of CH_3), 247 (M^+-31, loss of OCH_3), 191 (M^+-87, loss of $Si(CH_3)_3$ from 263 ion), 175 (M^+-103, loss of $OSi(CH_3)_3$ from 263 ion), 147 (M^+-131, loss of $COOCH_3$

from 191 ion), 131 (M^+-147, loss of $COOCH_3$ from 175), 73 ($[Si(CH_3)_3]^+$). These fragments are consistent with the TMS derivative of 2-hydroxybenzalpyruvic acid-methyl ester, the corresponding ester of a commonly encountered metabolite in PHEN degradation 2-hydroxybenzalpyruvic acid (Pinyakong et al. 2000, Seo et al. 2009). To confirm the identity of this metabolite, GC-MS analysis for acetate derivatised metabolites was conducted under the same conditions. The analysis showed a GC peak that eluted at 38.4 min that has the
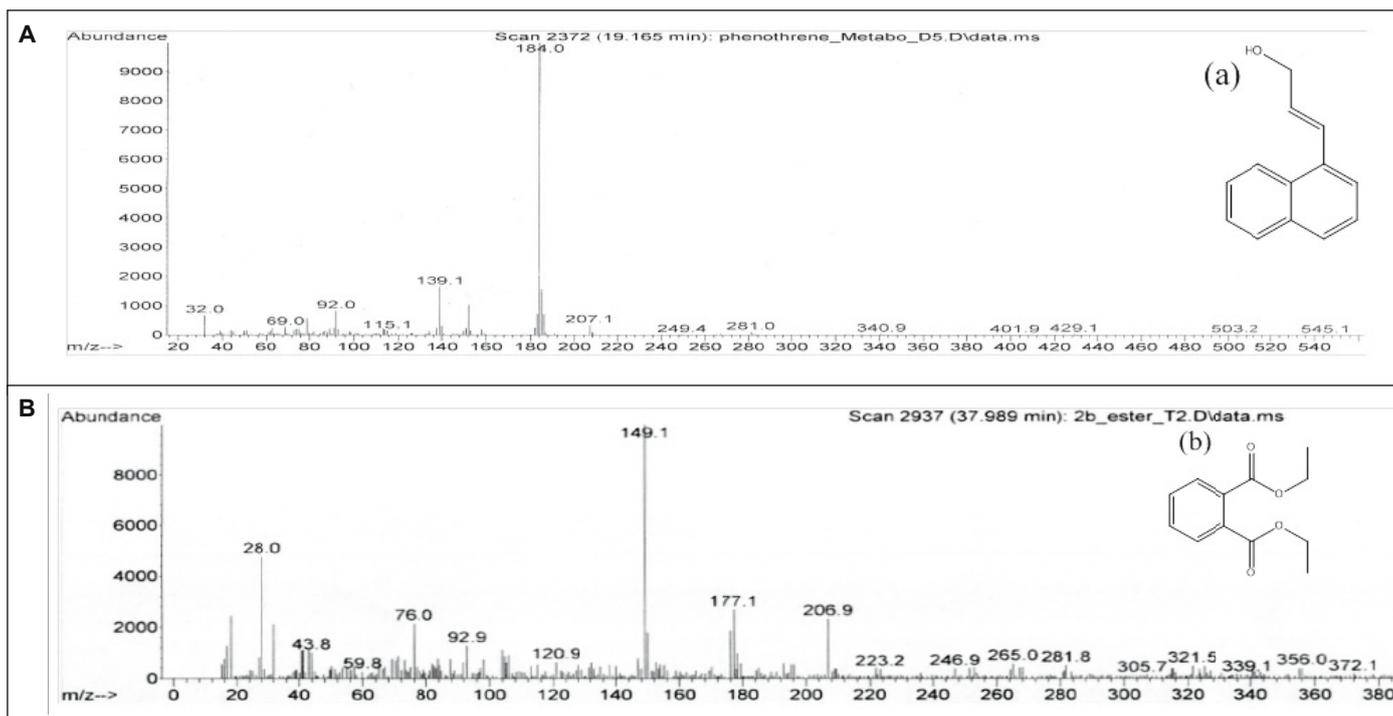
Fig. S4. Mass spectra for phenanthrene metabilites identified by GC-MS: [a] MS for 3-naphthyl allyl alcohol, [b] MS for diethylphthalate
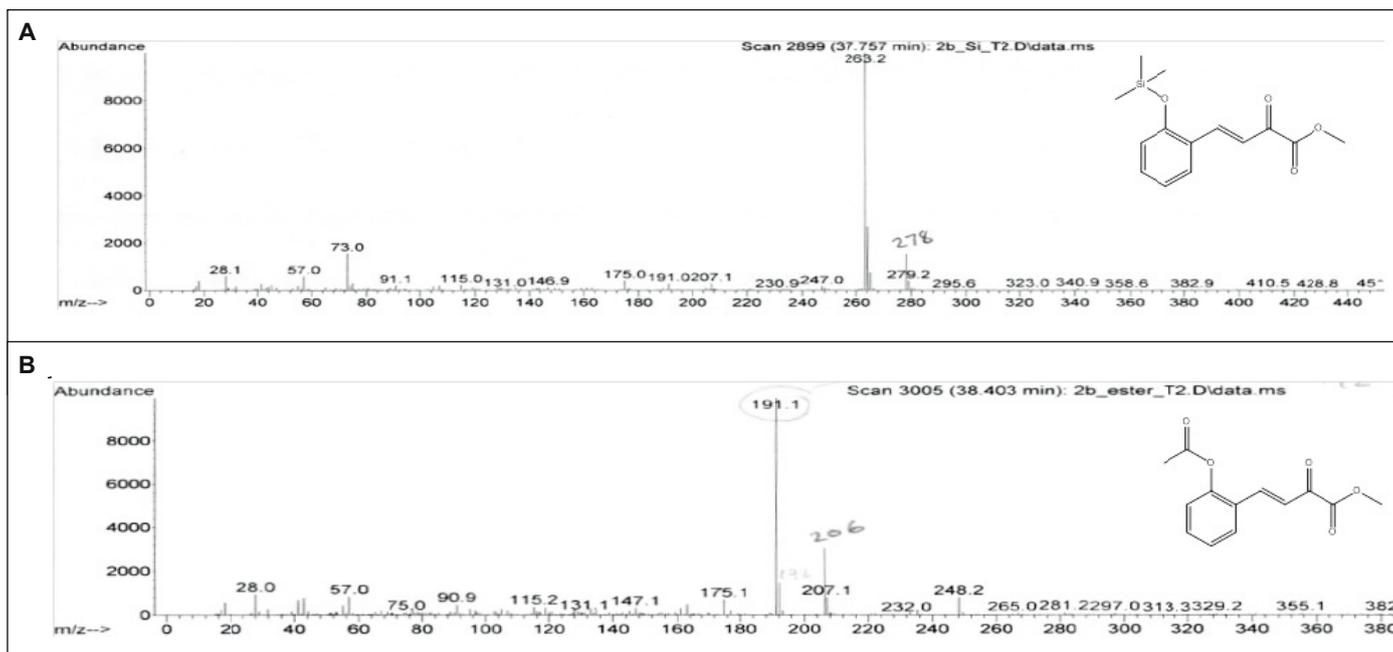
Fig. S4. Mass spectra for phenanthrene metabilites identified by GC-MS: [a] MS for 3-naphthyl allyl alcohol, [b] MS for diethylphthalate

following MS fragments (Figure S5B): 248 (M$^+$), 206 (M$^+$−42, loss of CH$_2$CO), 191 (M$^+$−57, loss of CH$_3$ from 206 ion), 175 (M$^+$−73, loss of OCH$_3$ from 206 ion), 147 (M$^+$−101, loss of COOCH$_3$ from 206). Extensive effort is currently conducted in our laboratory to identify more PHEN metabolites using *S. maltophilia*, which is crucial to propose a degradation pathway for PHEN by this strain.

Conclusion

Two co-cultures, PHEN-Cult (consisting of *P. citronellolis* and *S. maltophilia*) and ANT-Cult (made of *R. pickettii* and *T. haemolytica*), have been isolated and characterised, and are capable of degrading PHEN and ANT, respectively. These bacteria harbor dt time < 1.5 days when grown in the presence of 100 ppm or less of PHEN or ANT, making them among the most efficient PAH-degrading bacteria described so far. Thus, these bacteria are appropriate for utilisation in bioremediation of PHEN and ANT, and further studies are warranted to establish the challenge and relevance of their use in PAHs removal in pilot and large scale.

Acknowledgement

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