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Isolation and characterization of polycyclic aromatic hydrocarbons (PAHs) degrading, pH tolerant, N-fixing and P-solubilizing novel bacteria from manufactured gas plant (MGP) site soils

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Abstract

Dearth of high molecular weight contaminant degradation, pH tolerance and growth limiting nutrient assimilation potentials of the selected microorganisms are some of the prime factors reasonable for the failures in field-scale bioremediation of PAH contaminated soils. Hence an effort was made in this study for the first time to identify HMW PAH degrading, N-fixing and P-solubilizing bacteria with pH tolerance from long-term manufactured gas plant site soils. Four distinct strains that could degrade both LMW and HMW PAHs were identified. Among the isolates, *Stenotrophomonas* (MTS-2) followed by *Citrobacter* (MTS-3) and *Pseudomonas* (MTS-1) were furthestmost effective in the degradation of HMW PAHs either as individual or in the presence of co-substrate (LMW PAHs). MTS-1, 2 and 3 (co)degraded model LMW PAHs, Phe (100% of 150 mg L⁻¹) and HMW PAHs Pyr (100% of 150 mg L⁻¹) or BaP (90-100% of 50 mg L⁻¹) in 3, 12-15 and 30 days, respectively and recorded the least half-life time ($t_{1/2}$) and highest biodegradation rate constants (k). One of the significant findings is the diazotrophic P-solubilization ability, acid and alkali tolerance (optimum pH = 5.0-8.0) of the HMW PAH degrading *Pseudomonas* strain MTS-1. *Stenotrophomonas* (MTS-2) was also found to be superior as it could solubilize P and tolerate acidic condition (optimum pH = 5.0-7.5) during HMW PAH degradation. Further, our study is the first evidence of diazotrophic P solubilization potential of *Agrobacterium* (MTS-4) and P-solubilizing capacity of *Citrobacter* (MTS-3) during bioremediation. Thus, the results of this study demonstrate the promising use of the newly identified PAH degraders, notably MTS-1, 2 and 3 either as individuals or as consortia as an excellent candidate in the bioremediation or phytoremediation of PAH contaminated soils.

Graphical abstract

Keywords

Bioremediation, bacteria, PAHs, Benzo[a]pyrene, MGP soil, Diazotrophic phosphate solubilizer

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one class of toxic environmental pollutants that have aroused much public concern owing to its accumulation in the environment due to a variety of anthropogenic activities and potential deleterious effects on human health [1]. Biological degradation represents the key route for ecological recovery of contaminated sites with low molecular weight (LMW) PAH compounds containing three or less fused benzene rings [2]. However the success of microbial remediation efforts has been limited by the failure to remove high molecular weight (HMW) PAHs (compounds containing four or more fused benzene rings) that are generally recalcitrant to microbial attack [3]. Over the past 35 years of research, only a few genera of bacteria (*Rhodococcus*, *Burkholderia*, *Stenotrophomonas*, *Pseudomonas*, *Mycobacteria*, *Alcaligenes*, *Sphingomonas*) with the

ability to utilize four or more ring PAHs as sole carbon and energy sources has been reported [4-7].

Apart from the lack in the catabolic potential of the strains to degrade HMW PAHs, two factors that limit the success in field-scale bioremediation of PAH contaminated zones are the lack of nutrients in the contaminated soils and inability of the selected strains to tolerate diverse physical settings, for instance, acidic or alkaline pH [8]. These two factors abruptly affect the survival of the microorganisms that are capable of degrading the more highly condensed aromatic compounds at bench-scale level. Therefore for bioremediation to be an effective tool for the clean-up of PAH-contaminated soils, an evaluation of the wide phylogenetic spectrum of microorganisms and their functional diversity involved, and isolation of those that can overcome the nutrient limitation with pH tolerance and hence successfully degrade HMW PAHs is required.

Some of the soil organisms can facilitate the growth of plants that secrete enzymes which degrade a wide range of organic compounds which is talked about as ‘microbially assisted phytoremediation’ [9]. Such plant growth promoting soil bacteria commonly referred as ‘PGPRs’, possess few or most of the constructive abilities to fix atmospheric nitrogen (diazotroph), solubilize minerals such as phosphorous, synthesize siderophores and hormones that stimulate plant growth [10]. Most of the organisms that are isolated from uncontaminated (agricultural) soils have PGPR activity [11] and so far there are no existing reports on PAH degraders with a figure of its PGPR traits. Hence our interrogation was, ‘does organisms of long-term contaminated soils possess multi-functional potential to degrade the contaminants of high molecular weight (PAHs), endure tolerance towards wide ranging pH conditions and self-assimilate the two prime growth limiting nutrients viz., nitrogen and phosphorous by diazotrophic P solubilization abilities?’. Risks of isolates failing to survive in the contaminated sites can be fairly reduced if such promising organisms with wider functional

ability, particularly to catabolize high molecular weight pollutants, tolerate diverse pH and anabolize nutrients could be cultured from the real-contaminated sites. In this context, an attempt was made in this study for the first time to (i) isolate PAH degrading bacteria from long-term contaminated soils, (ii) identify the strains by molecular approaches, (iii) characterize their degradation potential choosing model three, four and five ring PAHs both as individuals as well as in mixtures (phenanthrene, pyrene and benzo[a]pyrene) and derive their kinetic data, (v) standardize their growth parameter under different pH and (iv) explore or quantify their nitrogen fixation and phosphate solubilization potential during bioremediation.

2. Materials and Methods

2.1. Enrichment and isolation of PAH degrading bacteria

The long-term contaminated soils collected from >150 to 200 years old manufactured gas plant (MGP) sites, Australia were used as inoculum for enrichment culture. The sites were contaminated with various concentrations of PAHs, both LMW and HMW. The physicochemical characteristics of the soils (on dry weight basis) considered for this study are summarised in Table 1. The isolation and subsequent growth studies were carried out with M9 mineral medium contained per litre: 6g Na₂HPO₄, 3g KH₂PO₄, 0.5 g NaCl, 1g NH₄Cl and 2.5 mL of goodies trace element solution [12]. Enrichment cultures were established by adding 5 g of 2mm sieved soil into 250 mL Erlenmeyer flask containing 100 mL fresh M9 medium supplemented with 100 mg L⁻¹ phenanthrene as the sole carbon (C) source. The flasks were then incubated at 25 °C on a rotary shaker (175 rpm) for a week. The enrichments were subcultured weekly into a fresh M9 medium containing the same concentration of PAHs. After several successive subcultures, the resulting enriched cultures were serially diluted and plated on M9 agar medium with phenanthrene supplied as vapour

phase in Petri-dish lids (Megharaj et al. 1997). Well grown single colonies were purified and maintained in M9 broth with 100 mg L⁻¹ each of phenanthrene and pyrene (100 mg L⁻¹) as C source.

2.2. PCR amplification of 16SrRNA, DNA sequencing and phylogenetic analysis

Genomic DNA was extracted using Ultraclean® microbial DNA isolation kit (Mo-Bio Laboratories Inc., USA) following the manufacturer's instructions. The extracted DNA was used as DNA template in polymerase chain reaction (PCR) amplification. The PCR amplification of 16S rRNA gene was carried out in 50 µL reaction mixture containing 100 ng DNA template, 1 µL each of forward and reverse primers (100 µM), 2.5 µL MgCl₂ (25 mM) and 12.3 µL PCR mixture containing PCR buffer, dNTPs and *Taq* polymerase. Highly purified sterile water was used to adjust the total volume. Universal eubacterial primers (E8F: 5'- AGTTTGATCCTGGCTCAG-3' and 1541R: 5'-AAGGAGGTGATCCANCCRCA-3') were used for 16S rRNA amplification [13]. All PCR amplifications were performed in the T100™ thermal cycler (BioRad, USA). The PCR programming for 16S rRNA consisted of a pre-heating step at 95 °C for 5 min followed by 39 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 2 min, with a final extension step of 5 min at 72 °C. The yields of all genomic DNA and PCR products were verified by gel electrophoresis using 1% agarose gel with ethidium bromide at 70V for 30 min and the specific bands were visualized under UV trans-illuminator (Bio-Rad, USA). The PCR products were purified using an Ultraclean® PCR clean-up kit (Mo-Bio Laboratories Inc., USA). DNA sequencing analysis was performed at South Path and Flinders Sequencing Facility, Flinders Medical Centre, Adelaide using ABI 3130 Sequencer (Applied Biosystems, USA). The initial sequence analysis was performed using BlastN. Phylogenetic analysis was carried out with MEGA 5.0 using neighbour-joining test [14].

2.3. Gene accession numbers

The nucleotide sequences of 16S rRNA gene of isolates MTS-1, 2, 3 and 4 have been deposited in GenBank under the accession numbers, KJ541831, KJ541832, KJ541833 and KJ541834 respectively.

2.4. PAH mineralization in liquid cultures

A batch experiment was conducted to determine the degradation and co-degradation potential of LMW and HMW PAHs by strains MTS-1, 2, 3 and 4. Phenanthrene (Phe), pyrene (Pyr) and benzo[a]pyrene (BaP) were chosen as the model PAH compounds. To obtain fast growth, isolates were grown in M9 media supplemented with 100 mg L⁻¹ Phe. Once the cells reached the exponential phase (2-3 days), they were harvested by centrifugation (4000 rpm for 20 min at 4 °C), washed twice with sterile distilled water and resuspended in required volume of M9 medium. The resulting suspension served as the inoculum. PAH degradation study in liquid cultures was conducted using 10 mL of broth in sterile 40 mL amber glass vials sealed with Teflon lined screw caps. One percent of the concentrated inoculum was inoculated into the medium containing individual (150 mg L⁻¹ Phe; 150 mg L⁻¹ Pyr; 50 mg L⁻¹ BaP) and mixed PAHs (100 mg L⁻¹ Phe+ 100 mg L⁻¹ Pyr; 50 mg L⁻¹ BaP + 100 mg L⁻¹ Phe; 50 mg L⁻¹ BaP + 100 mg L⁻¹ Pyr) to provide an initial cell density (OD₆₀₀) of 0.2. The inoculated tubes were incubated on a rotary shaker (175 rpm) at 25 °C in a walk-in incubator room. Controls without inoculations were also incubated under the same conditions. Samples were collected at different time intervals to determine the PAH concentration as well as to determine the bacterial growth in terms of protein content. All experiments were executed in triplicate, including controls and the mean values were taken into account.

2.5. Analytical methods

All chemicals used in the experiments were of analytical grade, obtained from Sigma-Aldrich, Australia. All solutions were prepared with Milli-Q water ($18\ \Omega\ \text{cm}^{-1}$, Milli-Q, ELGA labwater, UK). The PAH stock solutions were prepared in Dimethyl formamide and stored at $4\ ^\circ\text{C}$ in the dark. To determine the PAHs, we performed USEPA 3550B method of extraction. The liquid cultures (10 mL) were extracted by ultrasonication for 15 min (12 kHz sweep bandwidth, Soniclean Pulse Swept[®] Power Ultrasonicator, Soniclean Pty. Ltd., Australia) into 10 mL of hexane/acetone (1:1 by volume) (US EPA 1998). The extracts were concentrated under a stream of nitrogen gas into 1 mL and analysed by high-performance liquid chromatography (Agilent HPLC 1200, Japan) equipped with a UV detector set at 254 nm (EPA method 8310). A PAH reverse phase column ($4.6 \times 150\ \text{mm}$ with $5\ \mu\text{m}$ packing, Agilent Technologies Pvt. Ltd., Australia) was used with a methanol gradient from 80 to 100% at a flow rate of $1\ \text{mL min}^{-1}$. Column temperature was maintained at 30°C . External calibration was exhibited for individual PAHs using PAH standards (Supelco, Bellefonte, PA, USA). For quality control, experiments on recovery were carried out by spiking a known concentration of PAH standards (5 and $10\ \text{mg L}^{-1}$) in liquid cultures. The results showed a significant recovery of $90 \pm 4.2\%$. Periodically accuracy and precision of the calibration was checked by injecting a known standard and a blank solvent into the column for every ten samples. The detection limit of the HPLC system was $50\ \mu\text{g L}^{-1}$.

Subsequently cell growth was monitored at designated time intervals by measuring the total cell protein content using Lowry method [15] with Bovine serum albumin as the standard ($1 - 100\ \mu\text{g mL}^{-1}$). Experimental culture medium was centrifuged at 10,000 rpm for 10 min using a microcentrifuge (Sigma, Germany). The cell pellets were suspended in 1 mL of 1M NaOH and kept at $70\ ^\circ\text{C}$ for 5 min, vortexed until the cell pellets disappeared and the

solution became transparent. The 500 µL cell lysate was incubated in dark for 20 min with 700 µL Lowry's reagent, followed by 30 min incubation at 25 °C with 100 µL Folin's–Ciocalteu's reagent (diluted 1:1 by volume with MQ-water). Intensity of blue colour formed was measured at 660 nm using a Microplate Reader (SynergyTMHT, Bio-Tek).

2.6. Calculation of PAH half-life

Half-life is the length of time it takes for the concentration of a substance (PAHs) to be reduced by one-half relative to its initial level, assuming first-order decay kinetics. First order decay kinetics [16] can be explained as in Eq (1):

$$C = C_o e^{-kt} \quad (1)$$

where:

C = residual PAH content in the media at time t (mg L^{-1}),

C_o = the initial PAH content in the media (mg L^{-1}),

k = biodegradation rate constant (day^{-1}),

t = time (day).

Biodegradation rate constant (k) can be determined from the slope of plot $\ln C$ vs t .

PAHs half-life can be calculated from Eq. (2):

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

where:

$\ln 2 = 0.693$

k = biodegradation rate constant (day^{-1}).

2.7. Screening for diazotrophic PAH degraders

A preliminary experiment was conducted to test the nitrogen fixation potential of the isolates in liquid cultures. Three replicate flasks containing N-free M9 media (M9 without

NH₄Cl) supplemented with Phe + Pyr (100 mg L⁻¹each) was inoculated with each isolate to an initial OD of 0.2. Bacterial growth in terms of optical density (600 nm) was monitored for a week and the cultures that showed good growth were tested for its ability to fix N and simultaneously co-degrade Phe (100 mg L⁻¹) and Pyr (100 mg L⁻¹) in N-free M9 media following the same procedure used in the batch experiment. Triplicate samples including controls were drawn at different time interval to determine the bacterial growth in terms of protein content. On 12th day after incubation, PAH concentrations as well as the amount of total N fixed by the isolates were quantified. Prior to N fixation analysis, samples from the experimental culture medium were centrifuged at 10,000 rpm for 8 min to remove cells. Supernatants were analysed for the total N contents by dry combustion using a Trumac CN analyser (Leco[®] Corporation, Michigan, USA). The amount of total N fixed was obtained by deducting the total N content of the inoculated samples from the corresponding control.

2.8. Evaluation of PAH degraders for P-solubilization potential

Simultaneous preliminary screening and batch tests as above were carried out to determine the P solubilizing activity of MTS-1, 2, 3 and 4. P-modified M9 media [soluble form of P as KH₂PO₄ and Na₂HPO₄ in M9 media were replaced by insoluble form of P as Ca₃(PO₄)₂] was used for this assay. Followed by protein analysis at periodic intervals, PAH concentrations were recorded on 12 days after incubation. Supernatants were also analysed for the soluble P contents. The 250 µL supernatant was mixed with 30 µL reactive solution (0.6% (NH₄)₆Mo₇O₂₄·4H₂O, 12.75% H₂SO₄, 1.08% C₆H₈O₆, 0.0163% K₂Sb₂(C₄H₂O₆)₂·3H₂O) and the colour change was measured at 655 nm after 10 min [17]. KH₂PO₄ was used as standard. The amount of P solubilized was obtained by subtracting the soluble P concentration of the inoculated from that of the corresponding uninoculated control culture.

2.9. Effect of pH on PAHs degradation by pure cultures

The effect of pH on bacterial degradation of PAHs was assessed following the same procedure used in the batch degradation experiment. Pyr (150 mg L^{-1}) was used as the model PAHs compound. To determine the effect of pH, the pH values of the culture medium were adjusted to 3, 4, 5, 6, 7, 8, 9 and 10 with predetermined amounts of filter-sterilized NaOH and HCL solutions. Samples were drawn on 30 days after incubation and the PAH degradation efficiency including growth as protein values of the cultures were observed. All the tests were performed in triplicates. Data analysis was carried out with SPSS statistical software version 20 using one-way ANOVA.

3. Results and discussion

Microbial metabolism of the most common organic contaminant encountered on urban land like PAHs is one of the research areas of great emphasis. Biological treatment of PAHs contaminated soil is a more efficient, economically reasonable and an adaptable option than physicochemical treatments because of its potential advantages, viz., lower treatment cost, greater safety, less soil disturbance and complete degradation of the pollutants [18]. The effectiveness of bioremediation is often dependent on the survival and activity of the selected microbial population or consortium [19]. PAH degrading strains can be isolated or selected from contaminated, uncontaminated or artificially spiked matrices. In comparison to the strains isolated from uncontaminated or spiked habitats, the microorganisms that are isolated from the real contaminated matrices are capable of enhancing the rate or extent of contaminant decomposition [6]. This is because, indigenous microorganisms from the long-term contaminated environments display versatile catabolic potential (metabolically capable of utilizing the contaminant as its source of carbon and energy) and are adapted to possess

several intrinsic abilities like HMW PAH degradation [20], heavy metal tolerance [12], biosurfactant production [21], etc. Such indigenous strains may represent a better strategy for field-scale bioremediation of PAHs.

One of the long-term PAHs contaminated habitats are the former MGP sites. The gas manufacturing processes at age-old MGP sites have led to the deposition of chemical residues mainly PAHs. According to Thavamani et al. [22], MGP sites are contaminated with higher concentrations of PAHs. Although, in the last decade a number of PAH degrading bacteria have been isolated from the MGP site soils [12, 23], due to legal and liability issues, a large number of gas-work sites remain unexplored. If exploited for the isolation of PAH degraders, such identified strains by virtue of its innate catabolic potential may prove to be effective in the degradation of carcinogenic HMW PAHs.

3.1. Isolation and phylogenetic characterization of MTS-1, 2, 3 and 4

Since the enrichment of microbes from environments using target contaminants as C source is a more reasonable way to acquire efficient degradation system, in this study, the PAH degrading strains were enriched and isolated from the study sites affected by long-term gas-work processes (MGP site) contaminated with varied ranges of LMW to HMW PAH fractions. Of the selected soils, S1 and S3 were near acidic, wherein S2 and S4 were far acidic (Table 1). S4 (total PAHs = 650 mg kg⁻¹) followed by S2 (total PAHs = 432 mg kg⁻¹) were highly contaminated, wherein the level of contamination was low in case of S1 and S3 that recorded total PAH concentrations of 159 and 242 mg kg⁻¹, respectively. However, in accordance to the study of Reddy et al. [24], in the evaluated MGP site soils, the ratio of HMW PAHs contamination was higher than LMW PAHs.

Several hundred dark brown bacterial colonies with clearing zones grew on M9 agar plates utilizing Phe as C source. The visible colonies appeared after 2 weeks of incubation at

room temperature (25 ± 2 °C). Of those, distinct strains from enrichment culture plates capable of utilizing PAHs rapidly were isolated. All the isolated strains were characterized as aerobic. The BLASTN search result of the near full-length 16S rRNA gene sequence (1100-1200 bp) of strains MTS-1, 2, 3 and 4 followed by evolutionary history of these four strains as inferred using the neighbour-joining method [25] showed that, MTS-1 belonged to the genus *Pseudomonas* (KJ541831) and possessed 97% similarity with *Pseudomonas multiresinivorans* (Fig. 1). MTS-2 was identified to be *Stenotrophomonas* sp. (KJ541832) which possessed a similarity of 97% with *Stenotrophomonas maltophilia*. The isolates MTS-3 and 4 were also found to be distinct bacterial species putatively identified as (100% similarity): *Citrobacter* sp. (KJ541833) and *Agrobacterium* sp. (KJ541834).

Most identified PAH degrading bacteria from MGP sites reported in the literature belonged to the genera of *Acaligenes*, *Pandorea*, *Paenibacillus* [6], *Pseudomonas* [6, 26], *Caulobacter*, *Sphingomonas* [27], *Mycobacterium* and *Gordona* [28]. Further, Nayak et al. [29] reported the isolation of *Stenotrophomonas* sp. from left-over coal fields of thermal power station. However, to our knowledge no PAH degrading strains belonging the other groups have been identified so far from coal-tar sites (Table 2). Thus, apart from MTS-1 (*Pseudomonas* sp.) and MTS-2 (*Stenotrophomonas* sp.), the isolated strains MTS-3 and 4 is the first report of PAH degrading bacterium from the genus *Citrobacter* and *Agrobacterium* identified from MGP sites. Interestingly, all the isolates were Gram-negative mostly belonging to the class Gammaproteobacteria, except *Agrobacterium*, that belong to the Alpha class. Study of Lers et al. [30] has already shown the relative abundance of Gammaproteobacteria in a coal tar contaminated soil. Probably, because of the PAH degrading capacity and better resistance to PAH toxicity, Gammaproteobacteria could survive predominantly in contaminated sites. Gammaproteobacteria can be used as a potential bioindicator for the biodegradation of low to high molecular weight PAHs [31].

3.2. Biodegradation of PAHs in M9 medium

Physicochemical properties of PAHs vary with molecular weight. For instance, aqueous solubility and volatilization of these compounds decreases with increasing molecular weight. Also as the molecular weight increases, the rate of biodegradation decreases and hence the environmental persistence of these chemicals is high. As a result PAHs differ in their behaviour, distribution and effects on biological systems in the environment. Such differences had paved way for the categorization of PAHs into two distinct groups: LMW and HMW PAHs. LMW comprise the group of PAHs with 2 to 3 aromatic (benzene) rings such as naphthalene, fluorene, phenanthrene and anthracene that had recorded acute toxicity to aquatic organisms [32]. Whereas, HMW PAHs are the compounds with 4 to 7 rings that includes chrysene, pyrene, benzo[a]pyrene, etc which are carcinogenic in most cases [33]. Most of the PAH researches are conducted using 3-ring phenanthrene as model LMW PAHs compound. Among HMW PAHs, 4-ring pyrene and 6-ring benzo[a]pyrene that are sparingly soluble and are resistant to degradation are widely used as model PAHs compounds [6]. The rate of biodegradation and the ability to degrade both LMW and HMW PAHs differs from one strain to another. Also strains that degrade LMW PAHs might fail to degrade HMW PAHs [2]. Hence, screening of bacteria with potential to degrade both LMW and HMW PAHs is of significance. In the current study, the isolated PAH degrading bacteria were initially screened for their ability to degrade the most widely used model LMW PAHs, Phe and HMW PAHs, Pyr in typical M9 medium. Then the study was extended to representative HMW PAHs of environmental concern due to its identified carcinogenicity and bioaccumulation potential, BaP [34] followed by PAH mixtures.

3.2.1. (Co)degradation of model LMW and HMW PAHs – as individuals and mixtures

All three PAHs, Phe (150 mg L^{-1}), Pyr (150 mg L^{-1}) and BaP (50 mg L^{-1}) were utilized as a sole C source by the identified strains MTS-1, 2, 3 and 4 (Fig. 2i, ii, iii). Among the selected genera, all were able to completely degrade the LMW PAHs, Phe in 3 days (Fig. 2i). In case of HMW PAHs, *Stenotrophomonas* achieved a maximum of 100% Pyr and 95% BaP degradation in 11 and 30 days, respectively followed by a proportional increase in its growth as measured in terms of protein (Fig. 2ii, iii). Both *Pseudomonas* and *Citrobacter* were also effective in degrading Pyr (100%) and BaP (82-95%) in 12-15 and 30 days, respectively. However, *Agrobacterium* recorded the lowest HMW PAHs degradation. This suggests a wide distribution of PAH degraders with varying degrees of PAH mineralization ability in the MGP sites. PAH disappearance was interpreted as biodegradation, because the decrease in PAH concentration exceeded the amount that disappeared from the abiotic control and is accounted in the presented data.

A substantial improvement in the rate of PAH mineralization by axenic bacterial cultures occurred when LMW PAHs was supplied as a co-substrate. Fig. 2iv and v revealed that when 3-ring Phe (100 mg L^{-1}) was supplied as a co-substrate, both Pyr (100 mg L^{-1}) and BaP (50 mg L^{-1}) degradation proceeded at a relatively rapid phase with an exponential increase in growth compared to Pyr and BaP alone. Thus 90-100% of Pyr (in 8 days) and 87-100% of BaP degradation (in 30 days) was observed with no remaining Phe. This was 1.3-fold more than the degradation rate observed with single HMW PAH containing cultures. Further, when 4-ring Pyr (100 mg L^{-1}) was the co-substrate, BaP (50 mg L^{-1}) degradation delayed with 65% degradation occurring during the same period in case of *Agrobacterium* (Fig. 2vi). However about 90-95% BaP with no remaining Pyr followed by a 2.4-fold increase in total cell protein was achieved in 30 days by *Pseudomonas*, *Stenotrophomonas* and *Citrobacter* sp. Our study stands as a proof that Phe is a better inducer of catabolic

enzymes during HMW PAH degradation. Observed enhancement in Pyr and BaP degradation with a co-substrate is because the LMW PAH compounds act as an inducer of catabolic enzymes during bacterial degradation of HMW PAHs, which promotes rather than inhibiting HMW PAHs degradation [6]. Further, Yuvar et al. [35] had stated that degradation efficiency improves when LMW and HMW PAHs are present together compared to present individually.

Previous studies reporting the PAH degradation potential of pure cultures isolated from contaminated soils are summarized in Table 2. It is important to stress that the available literature principally focuses on the isolation and characterization of a LMW PAH degrading bacteria or consortium (combination of two or more strains). Therefore published data are limited to compare the PAH degradation potential of the identified pure cultures (individual bacteria) particularly towards HMW PAHs degradation and as PAH mixtures. A *Pseudomonas* strain isolated from oil contaminated soil completely degraded 40 mg L⁻¹ Phe in 7 days. However, with Pyr (40 mg L⁻¹) as a sole C source the PAH mineralization was only 35% [36]. *Pseudomonas* strain from similar source studied by Kumar et al. [37] showed a total degradation of 43% Phe (200 mg L⁻¹) and 50-55% Pyr (200 mg L⁻¹) after 14 days. BaP as well as PAH mix degradation ability of single *Pseudomonas* sp. has not been reported so far. However in the current study, *Pseudomonas* strain MTS-1 exhibited a superior LMW and HMW PAH (co)degradation, during the exponential growth phase and performed relatively better than the strains reported in the literature. Similar results were observed during PAH degradation by *Citrobacter* (MTS-3) and *Agrobacterium* (MTS-4) strains. Only one study each was available to compare the results of MTS-3 [38] and MTS-4 [39] as can be seen in Table 2.

In case of *Stenotrophomonas*, Juhasz et al. [5] reported that *Stenotrophomonas maltophilia* (VUN 10,003) was capable of degrading 50 mg L⁻¹ BaP (80 %) in the presence of Pyr (250 mg L⁻¹) after 63 days with a concurrent increase in protein from 7 to 50 µg mL⁻¹.

The high degradative capacity of individual LMW and HMW PAHs by *Stenotrophomonas* demonstrated in this study (MTS-2) is found to be superior to those reported elsewhere [5, 20, 40]. Such a reasonably higher degradation rate of the isolate is mainly due to the repeated subculturing of the organism for more than a year providing PAH mix as C source which might have stimulated the culture's biodegradation efficacy in due course.

3.2.2. Biodegradation kinetics of PAHs by indigenous pure cultures

Kinetic analysis is a key factor for understanding the bioremediation speed and is needed for the development of efficient clean up strategy [41]. First-order kinetic model (Eq. 1) fitted to the biodegradation data helped to determine the rate of PAHs biodegradation by genetically diverse identified pure cultures. The biodegradation rate constant (k) and PAHs half-life ($t_{1/2}$) for the different strains are presented in Table 3. In general, highest is the k , fastest is the rate of biodegradation and lowest is $t_{1/2}$. It could be seen from Table 3 that among the four identified strains, three of them namely, *Stenotrophomonas* (MTS-2) followed by *Pseudomonas* (MTS-1) and *Citrobacter* (MTS-3) were fastest in degrading LMW PAHs (Phe) and recorded k values of 1.45-1.47 day⁻¹ and half-life equal to less than a day. As the molecular weight of the PAHs increased, the biodegradation rate of those three strains dropped and the PAHs half-life was prolonged to 1.5-2 and 24-27 days for Pyr and BaP, respectively. This is because as the number of benzene ring increases, the microbe has to expend more time and energy in cleaving the rings and utilizing the aromatics as its C source. More also, when MTS-1, 2 and 3 cultures containing HMW PAHs were amended with a co-substrate, the PAHs half-life (Pyr in the presence of Phe = 1 day; BaP in the presence of Phe = 9-15 days; BaP in the presence of Pyr = 17-21 days) reduced than when present individually. *Stenotrophomonas* (MTS-2) was the ideal strain that recorded the highest biodegradation rate constant values during degradation and co-degradation of both

LMW and HMW PAHs. The variation in the rate constants and half-lives observed among the strains may be due to two main reasons: firstly, genetic – presence of the catabolic genes that code for a key enzyme responsible for the mineralization of PAHs compounds [42]. For instance, *nahAc* (naphthalene dioxygenase) gene codes for the enzyme that not only degrades naphthalene, but also mediates degradation of other PAHs compounds [43]. Secondly, the microbes may or not possess special physiological properties viz., biosurfactant production that might increase the solubility, improve the transportation of hydrophobic hydrocarbon substrates to cells and subsequently enhance the utilization or uptake of PAHs as C source [44].

3.3. Novel acid- to alkali-tolerant HMW PAH degraders

Over the pH range evaluated (pH = 3-10), the ability of the microbes to grow on PAHs contaminated media was influenced by pH (Fig. 3). Results indicated that strain MTS-1 (*Pseudomonas*) was acid- to alkali-tolerant which was supported by an increase in the bacterial growth concomitant (data not shown) with a decrease in PAH concentration (Fig 4a). PAH analysis after 30 days revealed that MTS-1 could remove around 92, 100, 100 and 85% of 150 mg L⁻¹ Pyr at pH 5, 6, 7 and 8, respectively. While the strain failed to perform well at pH < 5 and > 8. Strain MTS-2 (*Stenotrophomonas*) could grow between pH 5 and 7.5 and the degradation was 90, 100 and 100% at pH 5, 6 and 7, respectively (Fig 4b). Results further indicated that the biodegradation ability of *Citrobacter* and *Agrobacterium* (MTS-3 and 4) was affected either by increase or decrease in pH and both performed well only under neutral conditions (Fig 4c, d). The identified strains MTS-1 and 2 are the ones among the few PAH degraders that are known for their acid- and alkali-tolerance: *Sphingomonas* [45], *Pseudomonas* [21] [46], *Alcaligenes* [46] or *Mycobacterium* [47]. To the authors knowledge strains with optimum pH (condition where the microbe can degrade over 85% PAHs without

inhibition in its growth) range between 5 and 8 as flagged in this study has been reported to date only by Jacques et al. [21]. Jacques et al. [21] showed highest log cell number of anthracene degrading *Pseudomonas* sp. at pH 7 and the isolates showed similar growth over the range 5-9, though the proportional PAH degradation at different pH was not discussed. As shown by Wong et al. [48] though isolates might grow over wide pH range, their PAH degradation may be affected and thus the strains of the current study could be taken as an indicator of good tolerance to this factor (pH) and consequently of advantage in using those isolates for remediation of problematic soils.

3.4. Diazotrophic P solubilizers in PAH remediation

Of the isolates that could degrade up to 5-ring PAHs, three of them (*Pseudomonas*, *Citrobacter* and *Agrobacterium* sp.) were able to grow in M9 media without N source and were able to solely utilize Phe and Pyr mixtures confirming their ability to fix N during PAH remediation. *Citrobacter* (MTS-3) was the most active N-fixer that could fix a highest of $235.2 \pm 4.5 \text{ mg L}^{-1}\text{N}$ in 12 days. The other PAH degraders, *Pseudomonas* and *Agrobacterium* fixed 1.5-fold lesser N compared to *Citrobacter* sp (Table 4). Ability of the strains to fix N and use PAHs as sole C source was well reflected by an increase in its protein content. Thus the significantly ($p < 0.05$) greatest degradation of 98% Phe and 90% Pyr in N-free condition by *Citrobacter* sp. resulted in a protein yield of $36.4 \mu\text{g mL}^{-1}$ in 12 days (Fig. 4a). Wherein, protein yield and degradation rate of *Pseudomonas* and *Agrobacterium* strains in N-free M9 broth were almost 2-fold lower than *Citrobacter* sp., representing MTS-3 to be one of the identified potential diazotrophic PAH degraders.

The amount of P solubilised varied significantly ($p < 0.05$) among the isolates (Table 4). In general, *Pseudomonas*, *Stenotrophomonas* and *Agrobacterium* were able to solubilize the insoluble P source utilizing PAHs (Fig. 4b). Among the PAH degraders presenting the ability to

solubilize P, *Pseudomonas* (MTS-1) followed by *Stenotrophomonas* (MTS-2) were most efficient in solubilizing 116.1 ± 3.2 and $81.5 \pm 2.5 \mu\text{g mL}^{-1}$ of total P respectively and were the two best P-solubilizing PAH degraders, that recorded 85-90% Phe and 85-75% Pyr degradation in 12 days accompanied by an 2-3 fold increase in protein concentration. Though *Agrobacterium* (MTS-4) could solubilize P during PAH degradation, its rate of P solubilization, followed by decrease in PAH concentration with relative increase in growth was not that significant compared to the other two strains. In general, *Pseudomonas* strain MTS-1 was found to be an ideal P solubilizing PAH degrader with N fixing ability.

So far, only the PGPR traits of the bacteria isolated from an uncontaminated soil that are being used as inocula for improving crop productivity has been reported. According to the findings of Mehnaz et al. [49], *Pseudomonas* sp. isolated from corn (*Zea mays*) grown in an agricultural soil was positive for N fixation and also P solubilization. *Pseudomonas* strain PGPR1 isolated from peanut (*Arachis hypogea*) growing fields was able to solubilize 48.5 mg P per 100 mL broth by 4 days [50] which is significantly less than the result of our study. de Carvalho Costa and de Melo [51] confirmed the ability of *Stenotrophomonas* (RINFA2K) isolated from cacti (*Opuntia ficus-indica*) and rhizosphere soils to solubilize P, which supports our finding. Liba et al. [52] discussed the N-fixation potential of *Stenotrophomonas* (CBMAI 458) taken from cyanobacteria-deprived lichenized fungi, however our strain did not grow in N-free condition. *Citrobacter* sp. DHRSS isolated from rhizosphere of sugarcane [53] showed P solubilization ranging from 54 ± 0.3 to $331 \pm 0.6 \mu\text{g mL}^{-1}$ on different C sources (sucrose, fructose, glucose and maltose), but our strain MTS-3 showed negative for its P solubilisation test, but proved positive for N-fix assay recording substantially higher N fixation activity during PAH degradation than other identified genera. The result thus signifies that there exists functional diversity within the organism belonging to the same group. Kavinde [54] stated that *Agrobacterium* is a diazotrophic bacterium, however not any

information supported its P solubility and ours was the first observation of the diazotrophic P solubilization potential of *Agrobacterium* strain though it exhibited a moderately low functional activity than rest of the identified genera.

4. Conclusion

This paper describes the first evidence of enhanced HMW PAH degradation and the presence of diazotrophic phosphate solubilization potential with acid- and alkali-tolerance of a strain MTS-1 belonging to the genus *Pseudomonas*. Higher P solubilization ability with acid-tolerance of MTS-2 (*Stenotrophomonas* sp.) and N-fixation potential of MTS-3 (*Citrobacter* sp.) besides the exceptionally high rate of LMW and HMW PAH biodegradation are also the first reports. Thus, these new strains could be an excellent candidate for application in the PAH bioremediation process. One of the best approaches for bioremediation of a PAH contaminated soil is to apply these strains with alike competence in the form of microbial inoculum. Furthermore, understanding the physiological abilities like biosurfactant production and metal tolerance followed by molecular identification of catabolic as well as Nif (nitrogen fixing) genes of the identified strains is crucial if successful bioremediation strategies at field-scale level are to be implemented and are thus under progress.

References

- [1] S. Kuppusamy, P. Thavamani, M. Megharaj, R. Naidu, Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by novel bacterial consortia tolerant to diverse physical settings—Assessments in liquid-and slurry-phase systems, *Int. Biodeter. Biodegr.* 108 (2016) 149-157.

- [2] S. Kuppusamy, P. Thavamani, M. Megharaj, R. Naidu, Bioaugmentation with novel microbial formula vs. natural attenuation of a long-term mixed contaminated soil – treatability studies in solid-and slurry-phase microcosms, *Water Air Soil Pollut.* 227 (2016) 1–15.
- [3] S. Kuppusamy, P. Thavamani, M. Megharaj, Y.B. Lee, R. Naidu, Kinetics of PAH degradation by a new acid-metal-tolerant *Trabulsiella* isolated from the MGP site soil and identification of its potential to fix nitrogen and solubilize phosphorous, *J. Hazard. Mater.* 307(2016) 99-107.
- [4] S. Kuppusamy, P. Thavamani, M. Megharaj, K. Venkateswarlu, R. Naidu, In-situ remediation approaches for the management of contaminated sites: a comprehensive overview, *Rev. Environ. Contam. Toxicol.* 236 (2016) 1–115.
- [5] A.L. Juhasz, G. Stanley, M. Britz, Metabolite repression inhibits degradation of benzo [a] pyrene and dibenz [a, h] anthracene by *Stenotrophomonas maltophilia* VUN 10,003, *J. Ind. Microbiol. Biotechnol.* 28 (2002) 88-96.
- [6] P. Thavamani, M. Megharaj, R. Naidu, Bioremediation of high molecular weight polyaromatic hydrocarbons co-contaminated with metals in liquid and soil slurries by metal tolerant PAHs degrading bacterial consortium, *Biodegradation* 23 (2012) 823-835.
- [7] S. Boonchan, M.L. Britz, G.A. Stanley, Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures, *Appl. Environ. Microbiol.* 66 (2000) 1007-1019.
- [8] S.M. Bamforth, I. Singleton, Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions, *J. Chem. Technol. Biotechnol.* 80 (2005) 723-736.
- [9] S. Kuppusamy, P. Thavamani, M. Megharaj, R. Naidu, Bioremediation of natural polyphenol rich green wastes: A review of current research and recommendations for future directions, *Environ. Technol. Innov.* 4 (2015) 17-28.

- [10] B.R. Glick, Using soil bacteria to facilitate phytoremediation, *Biotechnol. Adv.* 28 (2010) 367-374.
- [11] B. Lugtenberg, F. Kamilova, Plant-growth-promoting rhizobacteria, *Annu. Rev. Microbiol.* 63 (2009) 541-556.
- [12] P. Thavamani, M. Megharaj, R. Naidu, Metal-tolerant PAH-degrading bacteria: development of suitable test medium and effect of cadmium and its availability on PAH biodegradation, *Environ. Sci. Pollut. Res.* (2013) 1-12.
- [13] D. Lane, 16S/23S rRNA sequencing, in: E. Stackebrandt, M. Goodfellow (Eds.) *Nucleic acid techniques in bacterial systematics*, Wiley & Sons, United Kingdom, 1991, pp. 115-175.
- [14] S. Kumar, K. Tamura, M. Nei, MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment, *Brief. Bioinform.* 5 (2004) 150-163.
- [15] O. Classics Lowry, N. Rosebrough, A. Farr, R. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265-275.
- [16] P. Oleszczuk, S. Baran, Degradation of individual polycyclic aromatic hydrocarbons (PAHs) in soil polluted with aircraft fuel, *Pol. J. Environ. Stud.* 12 (2003) 431-438.
- [17] J. Hernández-López, F. Vargas-Albores, A microplate technique to quantify nutrients (NO_2^- , NO_3^- , NH_4^+ and PO_4^{3-}) in seawater, *Aquacul. Res.* 34 (2003) 1201-1204.
- [18] S. Kuppusamy, P. Thavamani, M. Megharaj, K. Venkateswarlu, R. Naidu, Ex-situ remediation technologies for environmental pollutants: a critical perspective, *Rev. Environ. Contam. Toxicol.* 236 (2016) 117–192.
- [19] A. Ilyina, M. Castillo Sánchez, J. Villarreal Sánchez, G. Ramírez Esquivel, J. Candelas Ramírez, Isolation of soil bacteria for bioremediation of hydrocarbon contamination, *Vestn. Mosk. Univ. Ser. 2* (2003) 88-91.

- [20] A.L. Juhasz, G.A. Stanley, M.L. Britz, Microbial degradation and detoxification of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia* strain VUN 10,003, Lett. Appl. Microbiol. 30 (2000) 396-401.
- [21] R.J. Jacques, E.C. Santos, F.M. Bento, M.C. Peralba, P.A. Selbach, E.L. Sá, F.A. Camargo, Anthracene biodegradation by *Pseudomonas* sp. isolated from a petrochemical sludge landfarming site, Inter. Biodeter. Biodegr. 56 (2005) 143-150.
- [22] P. Thavamani, S. Malik, M. Beer, M. Megharaj, R. Naidu, Microbial activity and diversity in long-term mixed contaminated soils with respect to polyaromatic hydrocarbons and heavy metals, J. Environ. Manage. 99 (2012) 10-17.
- [23] Í.S. Silva, E.d.C.d. Santos, C.R.d. Menezes, A.F.d. Faria, E. Franciscon, M. Grossman, L.R. Durrant, Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia, Bioresour. Technol. 100 (2009) 4669-4675.
- [24] K.R. Reddy, P.R. Ala, S. Sharma, S.N. Kumar, Enhanced electrokinetic remediation of contaminated manufactured gas plant soil, Eng. Geol. 85 (2006) 132-146.
- [25] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (1987) 406-425.
- [26] H.-Y. Kahng, K. Nam, J. Kukor, B.-J. Yoon, D.-H. Lee, D.-C. Oh, S.-K. Kam, K.-H. Oh, PAH utilization by *Pseudomonas rhodesiae* KK1 isolated from a former manufactured-gas plant site, Appl. Microbiol. Biotechnol. 60 (2002) 475-480.
- [27] M.D. Jones, D.R. Singleton, D.P. Carstensen, S.N. Powell, J.S. Swanson, F.K. Pfaender, M.D. Aitken, Effect of incubation conditions on the enrichment of pyrene-degrading bacteria identified by stable-isotope probing in an aged, PAH-contaminated soil, Microb. Ecol. 56 (2008) 341-349.

- [28] M. Kästner, M. Breuer-Jammali, B. Mahro, Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH), *Appl. Microbiol. Biotechnol.* 41 (1994) 267-273.
- [29] A.S. Nayak, Y. Veeranagouda, K. Lee, T. Karegoudar, Metabolism of acenaphthylene via 1, 2-dihydroxynaphthalene and catechol by *Stenotrophomonas* sp. RMSK, *Biodegradation* 20 (2009) 837-843.
- [30] C. Lors, A. Ryngaert, F. Perie, L. Diels, D. Damidot, Evolution of bacterial community during bioremediation of PAHs in a coal tar contaminated soil, *Chemosphere* 81 (2010) 1263-1271.
- [31] M. Niepceyron, F. Martin-Laurent, M. Crampon, F. Portet-Koltalo, M. Akpa-Vinceslas, M. Legras, D. Bru, F. Bureau, J. Bodilis, *GammaProteobacteria* as a potential bioindicator of a multiple contamination by polycyclic aromatic hydrocarbons (PAHs) in agricultural soils, *Environ. Pollut.* 180 (2013) 199-205.
- [32] A.M. Willis, J.T. Oris, Acute photo-induced toxicity and toxicokinetics of single compounds and mixtures of polycyclic aromatic hydrocarbons in zebrafish, *Environ. Toxicol. Chem.* 33 (2014) 2028-2037.
- [33] E.W. Tung, N.A. Philbrook, C.L. Belanger, S. Ansari, L.M. Winn, Benzo [a] pyrene increases DNA double strand break repair *in vitro* and *in vivo*: A possible mechanism for benzo [a] pyrene-induced toxicity, *Mutat. Res-Gen. Tox. En.* 760 (2014) 64-69.
- [34] D. Liu, L. Pan, Z. Li, Y. Cai, J. Miao, Metabolites analysis, metabolic enzyme activities and bioaccumulation in the clam *Ruditapes philippinarum* exposed to benzo [a] pyrene, *Ecotox. Environ. Safe.* 107 (2014) 251-259.
- [35] S. Yuan, S. Wei, B. Chang, Biodegradation of polycyclic aromatic hydrocarbons by a mixed culture, *Chemosphere* 41 (2000) 1463-1468.

- [36] Z. Zhang, Z. Hou, C. Yang, C. Ma, F. Tao, P. Xu, Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8, *Bioresour. Technol.* 102 (2011) 4111-4116.
- [37] M. Kumar, V. León, A.D.S. Materano, O.A. Ilzins, L. Luis, Biosurfactant production and hydrocarbon-degradation by halotolerant and thermotolerant *Pseudomonas* sp, *World J. Microb. Biot.* 24 (2008) 1047-1057.
- [38] F. Li, L. Zhu, Effect of surfactant-induced cell surface modifications on electron transport system and catechol 1, 2-dioxygenase activities and phenanthrene biodegradation by *Citrobacter* sp. SA01, *Bioresour. Technol.* 123 (2012) 42-48.
- [39] O. Ben Said, M. Goñi-Urriza, M. El Bour, M. Dellali, P. Aissa, R. Duran, Characterization of aerobic polycyclic aromatic hydrocarbon-degrading bacteria from Bizerte lagoon sediments, Tunisia, *J. Appl. Microbiol.* 104 (2008) 987-997.
- [40] S. Gao, J.-S. Seo, J. Wang, Y.-S. Keum, J. Li, Q.X. Li, Multiple degradation pathways of phenanthrene by *Stenotrophomonas maltophilia* C6, *Int. Biodeter. Biodegr.* 79 (2013) 98-104.
- [41] S.E. Agarry, M.O. Aremu, O.A. Aworanti, Kinetic modelling and half-life study on enhanced soil bioremediation of bonny light crude oil amended with crop and animal-derived organic wastes, *J. Pet. Environ. Biotechnol.* 4 (2013) 2.
- [42] J. Milcic-Terzic, Y. Lopez-Vidal, M. Vrvic, S. Saval, Detection of catabolic genes in indigenous microbial consortia isolated from a diesel-contaminated soil, *Bioresour. Technol.* 78 (2001) 47-54.
- [43] C. Guo, Z. Dang, Y. Wong, N.F. Tam, Biodegradation ability and dioxygenase genes of PAH-degrading *Sphingomonas* and *Mycobacterium* strains isolated from mangrove sediments, *Int. Biodeter. Biodegr.* 64 (2010) 419-426.

- [44] A.E.-L. Hesham, A.M. Mawad, Y.M. Mostafa, A. Shoreit, Biodegradation ability and catabolic genes of petroleum-degrading *Sphingomonas koreensis* strain ASU-06 isolated from Egyptian oily soil, *BioMed Res. Int.* 2014 (2014) 1-10.
- [45] M. Kästner, M. Breuer-Jammali, B. Mahro, Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil, *Appl. Environ. Microbiol.* 64 (1998) 359-362.
- [46] W.D. Weissenfels, M. Beyer, J. Klein, Degradation of phenanthrene, fluorene and fluoranthene by pure bacterial cultures, *Appl. Microbiol. Biotechnol.* 32 (1990) 479-484.
- [47] R. Grosser, D. Warshawsky, J.R. Vestal, Indigenous and enhanced mineralization of pyrene, benzo [a] pyrene, and carbazole in soils, *Appl. Environ. Microbiol.* 57 (1991) 3462-3469.
- [48] J. Wong, K. Lai, C. Wan, K. Ma, M. Fang, Isolation and optimization of PAH-degradative bacteria from contaminated soil for PAHs bioremediation, *Water Air Soil Pollut.* 139 (2002) 1-13.
- [49] S. Mehnaz, T. Kowalik, B. Reynolds, G. Lazarovits, Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions, *Soil Biol. Biochem.* 42 (2010) 1848-1856.
- [50] R. Dey, K. Pal, D. Bhatt, S. Chauhan, Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria, *Microbiol. Res.* 159 (2004) 371-394.
- [51] F.E. de Carvalho Costa, I.S. de Melo, Endophytic and rhizospheric bacteria from *Opuntia ficus-indica* mill and their ability to promote plant growth in cowpea, *Vigna unguiculata* (L.) Walp, *Afr. J. Microbiol. Res.* 6 (2012) 1345.
- [52] C. Liba, F. Ferrara, G. Manfio, F. Fantinatti-Garboggini, R. Albuquerque, C. Pavan, P. Ramos, C. Moreira-Filho, H. Barbosa, Nitrogen-fixing chemo-organotrophic bacteria isolated

from cyanobacteria-deprived lichens and their ability to solubilize phosphate and to release amino acids and phytohormones, J. Appl. Microbiol. 101 (2006) 1076-1086.

[53] D.K. Patel, G. Archana, G.N. Kumar, Variation in the nature of organic acid secretion and mineral phosphate solubilization by *Citrobacter* sp. DHRSS in the presence of different sugars, Curr. Microbiol. 56 (2008) 168-174.

[54] L. Kanvinde, G. Sastry, *Agrobacterium tumefaciens* is a diazotrophic bacterium, Appl. Environ. Microbiol. 56 (1990) 2087-2092.

[55] E.C. Santos, R.J. Jacques, F.M. Bento, M.d.C.R. Peralba, P.A. Selbach, E.L. Sá, F.A. Camargo, Anthracene biodegradation and surface activity by an iron-stimulated *Pseudomonas* sp., Bioresour. Technol. 99 (2008) 2644-2649.

[56] Y. Tang, X. Wang, F. Chen, S. Ma, X. Yang, Screening, identification and degradation characteristics of a dominant fluorene-degrading strain, China Environ. Sci. 30 (2010) 1086-1090.

Table 1. PAH contamination profile of the MGP site soils explored in this study for the isolation of PAH degraders.

Characteristics	S1	S2	S3	S4
pH	6.4 ± 0.7	4.4 ± 0.2	6.8 ± 0.9	3.7 ± 0.2
PAHs (mg L ⁻¹)				
Naphthalene	6 ± 2	6 ± 2	6 ± 2	6 ± 2
Acenaphthylene	10 ± 3	22 ± 11	11 ± 3	17 ± 6
Acenaphthene	8 ± 1	13 ± 4	BDL	16 ± 2
Fluorene	3 ± 0.8	10 ± 3	6 ± 3	31 ± 10
Phenanthrene	BDL	3 ± 0.6	BDL	12 ± 2
Anthracene	17 ± 5	58 ± 10	21 ± 9	24 ± 10
Fluoranthene	23 ± 6	80 ± 22	36 ± 12	182 ± 30
Pyrene	5 ± 2	15 ± 5	8 ± 2	27 ± 13
Benz[a]anthracene	9 ± 1	20 ± 5	13 ± 6	36 ± 11
Chrysene	8 ± 2	17 ± 3	10 ± 2	23 ± 6
Benzo[b]fluoranthene	18 ± 6	68 ± 15	29 ± 8	121 ± 42
Benzo[k]fluoranthene	13 ± 3	30 ± 10	17 ± 4	41 ± 12
Benzo[a]pyrene	18 ± 10	72 ± 31	71 ± 29	68 ± 33
Benzo[ghi]perylene	22 ± 6	18 ± 4	14 ± 3	45 ± 10
Total PAHs	159	432	242	650
BDL = Below detection limit in HPLC (PAHs) and ICPMS (heavy metals)				

Fig. 1. Phylogenetic dendrogram of bacteria isolated from the long-term PAH contaminated soils based on comparative analysis of 16S rRNA gene sequence data. The bootstrap values over 50% are shown and the scale bar represents 4 nucleotide substitutions per 100 nucleotides of 16S rRNA sequence.

Bacteria highlighted with a *filled diamond* = strains belonging to this study

Table 2. Reported PAH degradation by pure cultures - *Pseudomonas*, *Stenotrophomonas*, *Citrobacter* and *Agrobacterium* sp.

Isolation source	PAHs degraded	Reference
<i>Pseudomonas</i> sp.		
1. Land farm from petrochemical industries and oil refinery	72% anthracene (250 mg L ⁻¹) in 48 days	[55]
2. Oil contaminated soils	100% fluorene (40 mg L ⁻¹) in 7 days 100% phenanthrene (40 mg L ⁻¹) in 7 days 41% fluoranthene (40 mg L ⁻¹) in 12 days 35% pyrene (40 mg L ⁻¹) in 12 days	[36]
3. Petrochemical sludge landfarming site	50-70% anthracene (250 mg L ⁻¹) in 48 days 33-48% phenanthrene (250 mg L ⁻¹) in 48 days 25-37% pyrene (250 mg L ⁻¹) in 48 days	[21]
4. Oil contaminated soil	43% phenanthrene (200 mg L ⁻¹) in 14 days 50-55% pyrene (200 mg L ⁻¹) in 14 days 50-60% anthracene (200 mg L ⁻¹) in 14 days	[37]
5. Bizerte lagoon sediments	80% fluoranthene (100 mg L ⁻¹) in 7 days 75% phenanthrene (100 mg L ⁻¹) in 7 days 30% pyrene (100 mg L ⁻¹) in 7 days	[39]
6. Manufactured gas plant site soil	100% phenanthrene (150 mg L ⁻¹) in 3 days 100% pyrene (150 mg L ⁻¹) in 12 days 82% benzo[a]pyrene (50 mg L ⁻¹) in 30 days 100% pyrene (100 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 8 days 96% benzo[a]pyrene (50 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 30 days 90% benzo[a]pyrene (50 mg L ⁻¹) in the presence of pyrene (100 mg L ⁻¹) in 30 days	this study
<i>Stenotrophomonas</i> sp.		
1. Mixed contaminated	80% benzo[a]pyrene (50 mg L ⁻¹) in the presence of	[5]

factory soil	pyrene (250 mg L ⁻¹) in 63 days	
	80% dibenz[a,h]anthracene (50 mg L ⁻¹) in the presence of pyrene (250 mg L ⁻¹) in 63 days	
2. Abandoned factory site	91% phenanthrene (50 mg L ⁻¹) in 42 days 98% pyrene (50 mg L ⁻¹) in 42 days 45% fluoranthene (50 mg L ⁻¹) in 42 days 26% benz[a]anthracene (50 mg L ⁻¹) in 42 days 22% benzo[a]pyrene (50 mg L ⁻¹) in 42 days 22% dibenz[a,h]anthracene (50 mg L ⁻¹) in 42 days	[20]
3. Creosote contaminated site	100% phenanthrene (50 mg L ⁻¹) in 14 days	[40]
4. Activated sludge of coking plant	86% fluorene (40 mg L ⁻¹) in 11 days	[56]
5. Manufactured gas plant site soil	100% phenanthrene (150 mg L ⁻¹) in 3 days 100% pyrene (150 mg L ⁻¹) in 12 days 95% benzo[a]pyrene (50 mg L ⁻¹) in 30 days 100% pyrene (100 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 8 days 100% benzo[a]pyrene (50 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 30 days 95% benzo[a]pyrene (50 mg L ⁻¹) in the presence of pyrene (100 mg L ⁻¹) in 30 days	this study
<i>Citrobacter</i> sp.		
1. Contaminated soil	100% phenanthrene (80 mg L ⁻¹) in 12 days	[38]
2. Manufactured gas plant site soil	100% phenanthrene (150 mg L ⁻¹) in 3 days 100% pyrene (150 mg L ⁻¹) in 15 days 95% benzo[a]pyrene (50 mg L ⁻¹) in 30 days 100% pyrene (100 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 8 days 100% benzo[a]pyrene (50 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 30 days 90% benzo[a]pyrene (50 mg L ⁻¹) in the presence of pyrene (100 mg L ⁻¹) in 30 days	this study

<i>Agrobacterium</i> sp.		[39]
1. Bizerte lagoon sediments	75% fluoranthene (100 mg L ⁻¹) in 7 days	
	40% phenanthrene (100 mg L ⁻¹) in 7 days	
	30% pyrene (100 mg L ⁻¹) in 7 days	
2. Manufactured gas plant site soil	100% phenanthrene (150 mg L ⁻¹) in 3 days	this study
	88% pyrene (150 mg L ⁻¹) in 15 days	
	63% benzo[a]pyrene (50 mg L ⁻¹) in 30 days	
	90% pyrene (100 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 8 days	
	87% benzo[a]pyrene (50 mg L ⁻¹) in the presence of phenanthrene (100 mg L ⁻¹) in 30 days	
	65% benzo[a]pyrene (50 mg L ⁻¹) in the presence of pyrene (100 mg L ⁻¹) in 30 days	

Fig. 2. PAH mineralization by (a) *Pseudomonas* (◆), (b) *Agrobacterium* (▲), (c) *Stenotrophomonas* (●) and (d) *Citrobacter* (■) strains isolated from the long-term PAH contaminated soils. In the figure, straight lines represent the PAH degradation and dotted lines in secondary axis represents the protein concentration.

(i). Phe degradation (150 mg L^{-1})

(ii). Pyr degradation (150 mg L^{-1})

(iii). BaP degradation (50 mg L^{-1})

(iv). Co-degradation of Pyr (100 mg L⁻¹) with Phe (100 mg L⁻¹)

(v). Co-degradation of BaP (50 mg L⁻¹) with Phe (100 mg L⁻¹)

(vi). Co-degradation of BaP (50 mg L^{-1}) with Pyr (100 mg L^{-1})

Table 3. The biodegradation rate constants (k) and half-life ($t_{1/2}$) time of the model 2, 4 and 5-ring PAHs compounds by strains of this study.

	Degradation of LMW and HMW PAHs			Co-degradation of HMW PAHs in the presence of LMW PAHs		
	LMW PAHs	HMW PAHs		Pyr in the presence	BaP in the	BaP in the presence of
	2-ring Phe	4-ring Pyr	5-ring Bap	of Phe	presence of Phe	Pyr
<i>Pseudomonas</i> sp.						
k (day ⁻¹)	1.47	0.41	0.026	0.54	0.047	0.033
R ²	0.87	0.82	0.99	0.67	0.99	0.96
$t_{1/2}$ (days)	0.5	1.5	27	1	15	21
<i>Agrobacterium</i> sp.						
k (day ⁻¹)	1.22	0.06	0.015	0.12	0.03	0.014
R ²	0.93	0.97	0.98	0.95	0.99	0.98
$t_{1/2}$ (days)	0.6	12	46	6	23	48
<i>Stenotrophomonas</i> sp.						
k (day ⁻¹)	1.47	0.41	0.029	0.55	0.078	0.042
R ²	0.91	0.84	0.98	0.73	0.94	0.97
$t_{1/2}$ (days)	0.5	1.5	24	1	9	17
<i>Citrobacter</i> sp.						
k (day ⁻¹)	1.45	0.30	0.028	0.55	0.074	0.034
R ²	0.94	0.67	0.98	0.72	0.93	0.98
$t_{1/2}$ (days)	0.5	2	25	1	9	20

Fig. 3. Effect of pH on the degradation of pyrene (150 mg L⁻¹) by strains MTS-1, 2, 3 and 4. Values in the figures are the pyrene degradation data observed after 30 days of incubation. (a). *Pseudomonas* sp. strain MTS-1. (b) *Stenotrophomonas* sp. strain MTS-2. (c) *Citrobacter* sp. strain MTS-3. (d) *Agrobacterium* sp. strain MTS-4.

Table 4. N-fix and P-solubilization potential (in $\mu\text{g mL}^{-1}$) of PAH degraders isolated from MGP site soils.

PAH degraders	Diazotrophs ^a	P-solubilizers ^b
	Total N fixed	P solubilised
<i>Pseudomonas</i> sp.	149.4 $\pm$ 2.8	116.1 $\pm$ 3.2
<i>Stenotrophomonas</i> sp.	-	81.5 $\pm$ 2.5
<i>Citrobacter</i> sp.	235.2 $\pm$ 4.5	-
<i>Agrobacterium</i> sp.	177.8 $\pm$ 4.2	39.5 $\pm$ 3.7

Data are values of triplicate samples recorded at 12 days after incubation $\pm$ standard error.

^aand ^b = growth media with Phe (100 mg L⁻¹) and Pyr (100 mg L⁻¹) as C source; where, ^a = M9 without N source and ^b = M9 with insoluble P source (tricalcium phosphate).

Fig. 4. Diazotrophic P solubilizers in PAH remediation. In the figure, dotted lines represent the protein concentration and bars represents the PAH degradation. Data represents the co-degradation of Pyr (100 mg L⁻¹) with Phe (100 mg L⁻¹). (a) PAH mineralization by diazotrophs (♦ *Pseudomonas*, ▲ *Agrobacterium* and ■ *Citrobacter*) in M9 without N source. (b) PAH mineralization by P-solubilizers (■ *Pseudomonas*, ♦ *Agrobacterium* and ▲ *Stenotrophomonas*) in M9 with insoluble P source.