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Different metabolic pathways involved in anthracene biodegradation by *Brevibacillus*, *Pseudomonas* and *Methylocystis* Species

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Abstract

Background: Polycyclic aromatic hydrocarbons (PAHs) such as anthracene are one of the most toxic contaminants to our environment. Microbial biodegradation of these xenobiotics is a cost-effective technological solution. The present study aimed to recover some bacterial isolates from Beni-Suef Governorate in Egypt with high capabilities of anthracene biodegradation. The selected isolates were molecularly characterized by 16S rRNA gene sequencing, the degree of anthracene biodegradation was monitored using optical density (OD) and high-performance liquid chromatography (HPLC), PCR amplification of some selected genes encoding biodegradation of PAHs was monitored, and gas chromatography–mass spectrometry (GC–MS) analysis was applied for detecting the resulted metabolites.

Result: Three bacterial isolates were studied, the 16s rRNA sequences of the isolates showed homology of the first isolate to *Brevibacillus* sp. (94.58 %), the second isolates showed homology to *Pseudomonas* sp. (94.53%) and the third isolate showed homology to *Methylocystis* sp. (99.61 %), all isolates showed the ability to degrade anthracene. PCR amplification of some selected genes encoding biodegradation of PAHs revealed the presence of many biodegrading genes in the selected strains. Gas chromatography-mass spectrometry (GC–MS) analysis of the metabolites resulted from anthracene biodegradation in the present study suggested that more than one biodegradation pathway was followed by the selected isolates.

Conclusions: The selected strains could represent a potential bioremediation tool in solving the PAHs problem in the Egyptian environment with a clean and cost-effective technique.

Keywords: Biodegradation, Bioremediation, *Brevibacillus*, GC–MS, HPLC, *Methylocystis*, *Pseudomonas*

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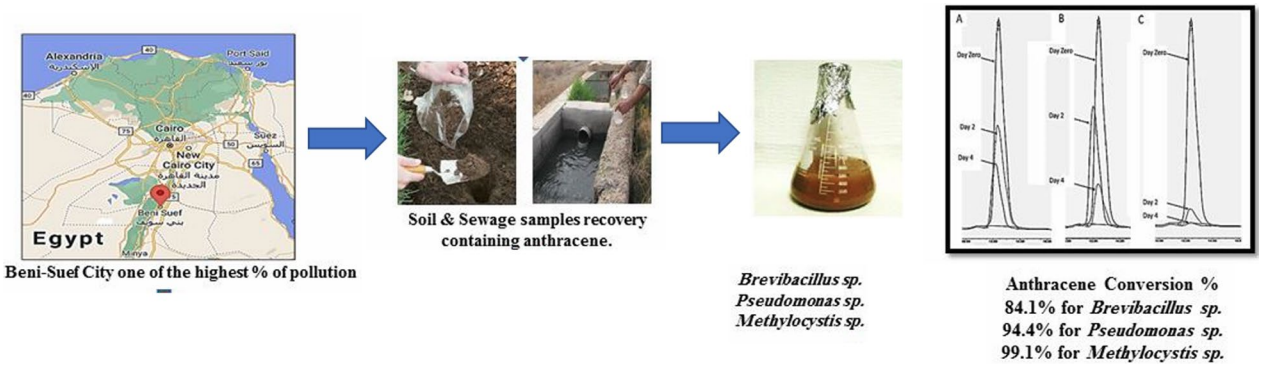
Graphical Abstract



1 Background

Polycyclic aromatic hydrocarbons (PAHs) nowadays gain a significant interest due to their environmental toxicity, biological activity, mutagenicity, and carcinogenicity [1]. They are structurally composed of fused two or more benzene rings, created naturally due to forest fires, burning coal tar, oil leakages, and solid waste incineration [2]. They are solids of different colors, with low water solubility [3]. They spread widely in the environment, especially air [4], water, and soil [5, 6]. They condense onto the particulate matter in the air that facilitates movement to distant places [7]. They have relative stability in soil and are not easily degraded [8]. They constitute a significant health hazard as medically classified as endocrine-disrupting chemicals due to their structural similarities to the real human and animal hormones that, in turn, affect their reproduction, development, metabolism, and may induce cancer [9, 10]. It was reported that high levels of PAHs in the atmosphere could cause 4–8% premature death [11].

Anthracene is used as a signature compound in PAHs biodegradation studies [12–14]. It is composed of three fused benzene rings [15]. Anthracene has been detected in vehicle exhaust fumes [16]. Anthracene as a type of PAHs is widely distributed in the environments due to many anthropogenic activities, like oil refining and the petro-chemical industry [12]. Exposure to high doses of anthracene has toxic effects on skin and tissues [17].

Anthracene has toxic effects on the growth of some eukaryotic microorganisms [18]. For example, it was investigated that anthracene toxicity induces DNA damage, intracellular oxidative stress effect and decrease in the mitochondrial membrane potential as well as cell viability to some living creatures as earthworm coelomocyte [19]. Also, accumulation of benz(a)anthracene (BaA) in soil sediments causes poisoning to typical enzymes (α-Amylase) of organisms such as *Eisenia fetida* in soil. [20]. Additionally, anthracene causes dangerous oxidative effect through the increase in the super oxide radicals in the aquatic organism mussel, *Mytilus edulis* [21]. Moreover, exposure to anthracene reduces the reproductivity of the zooplankton, *Daphnia magna* [22].

The exposure to high doses of PAHs has adverse effects on the skin and tissues [23]. So, the elimination of such toxic compounds becomes a necessity. Several bacterial and fungal species were reported in PAHs biodegradation [24–26]. Bioremediation is a clean and cheap technique that eliminates PAHs pollutants from the environment [27]. The microorganisms degrade such pollutant compounds and convert them through mono and dioxygenase enzymes to inert substances, CO_2, and water [28–30]. GC–MS analysis applied in many previous studies [31–33] is a technique used to detect the metabolites and the pathways involved in biodegradation.

The present study focused on exploring potential strains adapted in biodegrading PAHs effectively in Egypt's local areas to provide a stress-free environment. The sample location in Egypt was selected based on a recent report which proclaimed that Beni-Suef governorate area is the highest in air pollution with 10 μm in diameter particulate matter levels reached 20 folds more than the WHO limits, and 6 folds more than permissible Egyptian environmental limits, which help in the transfer of such pollutants to the water and soil [34].

2 Methods

2.1 Materials and culture media

Liquid minimum salt solution (MSM) was prepared using analytical grade chemicals as follows: 1 g K_2HPO_4, 1 g NH_4SO_4, 0.3 g $MgCl_2{\cdot}6H_2O$, 0.1 g $CaCl_2$, and 0.02 g $FeSO_4$ $0.7H_2O$ [35]. Anthracene (analytical grade) (Sigma-Aldrich Ltd.) was dissolved in methanol, then syringe-filtered, and added to MSM to a final concentration of 50 μg/mL. Agar at 1.5% was added if solid media

was required. Acetone, acetonitrile, chloroform, and water which were used in HPLC were of HPLC grade. Ethidium bromide (Fluka), yeast extract (Difco), and agar (Fisher Scientific) were also used in the study.

2.2 Sampling

Soil and sewage water samples were collected from a drainage in Beni-suef Governorate (a drainage ten kilometers away from Beni-suef), Egypt. Nearly 10 gm of soil and 50–100 ml of each sewage water sample were collected in 250-ml sterile conical flasks that were stored at 4 °C until use. One gram of contaminated soil and 10 mL of sewage water samples were suspended in 50 mL MSM containing anthracene (50 µg/mL) as sole carbon source. Then, the flasks were incubated in a shaker incubator at 160 rpm for at least 20 days at 30 °C.

2.3 Bacterial isolation

A volume of 200 µL of the enrichment suspension was plated on solidified MSM-anthracene plates; control plates without anthracene were used to exclude agar-supported bacteria. The plates were wrapped in aluminum foil to prevent the photocatalytic degradation of anthracene and then incubated at 30 °C for at least 20 days. Anthracene degradation was noticed as clear zone around the bacterial colonies.

2.4 Genotypic identification

The identification of the isolates to the genus level was made using the 16S rRNA gene identification method as follows: the bacterial DNA material was extracted using a ZR Fungal/Bacterial DNA MiniPrep extraction kit (Zymo Research, Orange, CA, USA). The 16S rRNA gene was amplified using the forward primer 16F: 5 AAACTYAAAKGAATTGACGG 3 and reverse primer 4R: 5 ACGGGCGGTGTGTRC 3. The reaction mixture (25 µL) contained 12.5 µL MyTaq green Master Mix (Bioline Reagents Ltd, London, UK), 1 µL of forward primer (8 µM), 1 µL of reverse primer (8 µM), 2 µL of template DNA and 8.5 µl of sterile water. The PCR reaction was carried out using Primus 25 advanced® thermocycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) under the following conditions 5 min initial denaturation at 95 °C, followed by 36 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, 1.5 min extension at 72 °C, and a final extension step of 7 min at 72 °C [35]. Gel electrophoresis technique (Labnet's ENDURO™, Edison, NJ, USA) used for amplicon separation by using 1% (w/v) agarose gel (Invitrogen, USA) with an electric current of 90 V for 60 min, stained with ethidium bromide and visualized using UV trans-illuminator (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). PCR products were purified from agarose gel using Zymo gel extraction kit (Zymo Research, Orange, CA, USA), and the concentration of the purified amplicons was measured using Nanodrop 2000 (Thermo Fisher Scientific Waltham, MA, USA). The PCR amplicons were sent to Macrogen (Macrogen Inc., Seoul, Republic of Korea) for sequencing. Identification was made using Basic Local Alignment Search Tool (BLAST) of the obtained sequence by the NCBI database [36] in order to identify the sequences that are homologous to the sequence of interest. The obtained sequences were analyzed using MEGA-X (Molecular Evolutionary Genetics Analysis) [37] and ClustalW [38] using 15 sequences obtained from the NCBI database. The phylogenetic tree was built by neighbor-joining [39] with retrieved sequences from the NCBI database bootstrap consensus way with 100 repetitions [40].

2.5 Detection of PAHs biodegradation genes

Some genes contributing to PAHs biodegradation were screened by PCR amplification [41] using primers shown in Table 1.

2.6 Enhancement of biomass production and anthracene biodegradation

The biomass production and anthracene biodegradation of the selected isolates were investigated under different conditions. The optical density and anthracene residual concentration were monitored using a spectrophotometer (600 nm) and HPLC, respectively. A single pure colony was cultured in nutrient broth at 30 °C for

Table 1 Primers used in this study to detect the PAHs biodegrading genes

Genes	Size (bp)	Primers		References
acin GS	200	F	ATCCAACCACGTTCAAGGTC	[42]
		R	GCGATCAACAATTTTCGTCA	
tnpA	500–600	F	GCCATTGAAGGTGTCATCCG	[41]
		R	AGGTATTCCACGCCATCACG	
tadQ	400	F	ACGATGGTGCTGTTCCGCAA	[41]
		R	TATGAGGCAGGATGGTGACG	
tadQ2	400	F	AGTGCTAAGCTTTTCGTACCG TCAC	[43]
		R	GGATCCATCGCGTCTCCTAGGTTA	
tadR	400–500	F	GATGGATCCGAGGCGCTGCTC	[43]
		R	GCTCTAGACGATGCGCACCTTG	
GS2	400	F	GGCTGGATGCTGGCAGATCT	[44]
		R	AAGGTGGTTTCCATCTGGCT	
ps pass 2.3	200	F	TGGGTTTCAAGGTTGTGGAT	This study
		R	GACGTCATTCAAACCCCACT	

(bp) = base pair

24 h. A volume of 100 µL of bacterial suspension was added to different flasks to give the following conditions: (a) MSM-anthracene, static incubation at 30 °C, (b) MSM-anthracene with shaking at 160 rpm at 30 °C; [35], (c) MSM-anthracene with shaking at 160 rpm at 25 °C, (d) MSM-anthracene, yeast extract 0.1% with shaking at 160 rpm at 30 °C [45], and (e) MSM-anthracene, glucose 0.1% with shaking at 160 rpm at 30 °C [46]. All flasks contain 100 mL of MSM in a 250-mL flask and incubated for 12 days, while the starting pH was adjusted at 7 (±0.2) throughout the study. Samples were withdrawn at 24-h intervals to assay the change in the bacterial growth through the change in the optical density (OD). OD was detected using the Shimadzu UV-1280 spectrophotometer at λ600 nm. The residual anthracene was analyzed by HPLC, where the 5 mL was withdrawn at 48-h intervals, extracted with chloroform, and dried using anhydrous sodium sulfate [47]. Chloroform layer was evaporated using Rotavap, and the final residue was dissolved in 5 mL acetonitrile. The residual anthracene was detected by HPLC system equipped with Water 996 Photo Diode Array Detector (Water 2690 Alliance, USA). The HPLC gradient system was composed of acetonitrile/water as follows: 50:50 for 2 min, then, 60:40 for 3 min, followed by 70:30 for 2 min, and finally, 80:20 for 5 min. The mobile phase flow rate was 0.4 mL/min, and the UV detection wavelength was 254 nm. The injection volume was 100 µL.

2.7 GC–MS analysis

The metabolites formed during anthracene biodegradation by the selected isolates were qualitatively analyzed by GC–MS spectroscopic analysis. The bacterial isolates in MSM-anthracene were incubated in a shaking incubator at 160 rpm for 4 days at 30 °C. The samples were withdrawn at 48 h intervals and exposed to liquid–liquid extraction using chloroform (1:1 v/v) followed by addition of anhydrous sodium sulfate for drying [47]. The GC–MS analysis was done using Trace GC Ultra-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG-5MS (30 m × 0.25 mm × 0.25 µm film thickness). The column oven temperature was initially held at 100 °C and then increased by 15 °C /min to 160 °C and then increased to 300 with 5 C/min. The injector and MS transfer line temperatures were kept at 280 °C. Helium was used as a carrier gas at a constant flow rate of 1 mL/min. The solvent delay was 3 min, and diluted samples of 3 µL were injected automatically using Autosampler AS3000 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 40–500 in full scan mode. The ion source was set at 200 °C. The metabolites were putatively identified by comparing their retention times (Rt) and mass spectra with Wiley registry® and Pesticide's mass spectral database.

3 Results

3.1 Anthracene biodegradation on solid media

Observation of bacterial growth and zone of clearance indicated possible anthracene-degrading activity. Twenty isolates (9 soil and 11 sewage) were recovered initially; however, three isolates were selected for further experiments based on the maximum anthracene degradation activity. One isolate was recovered from sewage water named PM1, and the two other isolates were recovered from soil named BM1 and MM1.

3.2 Isolates identification using 16S rRNA sequencing

The sequences of the PCR products of the amplification of 16S rRNA genes of the selected isolates were compared to the Genbank database through the NCBI's nucleotide blast tool. The isolates' 16S rRNA sequences and the closely related sequences from NCBI were aligned, and the phylogenetic trees were built. The isolates genus and the sequences of the most closely similar strains were identified. BM1 isolate was identified as *Brevibacillus* sp. Its 16S rRNA sequence showed high similarity (99.5%) to *Brevibacillus panacihumi* strain DCY35 (Fig. 1a). Also, PM1 isolate was molecularly assigned as *Pseudomonas* sp. showing 89.2% similarity to *Pseudomonas canadensis* strain 2–92 (Fig. 1b). Additionally, MM1 isolate was identified as *Methylocystis* sp. It displayed 87.7% similarity to *Methylocystis heyeri* strain H2 (Fig. 1c).

3.3 Enhancement of biomass production and anthracene biodegradation

The spectrophotometric analysis of the bacterial growth of all selected strains using different culture conditions revealed an increase in the OD by time (Fig. 2). Interestingly, the shaking conditions at either 25 °C or 30 °C incubation were superior to stagnant incubation conditions. The lag phase time for the three isolates was reduced by supplementing the media with 0.1% yeast extract or 0.1% glucose. The highest growth level of *Methylocystis* isolate was at 30 °C shaking condition near to that of 0.1% yeast extract addition.

Figure 2 also shows the biodegradation of the anthracene by the selected strains by time. Shaking at 30 °C and 25 °C significantly decreased the lag phase in the case of *Brevibacillus* sp. and *Methylocystis* sp., respectively. *Methylocystis* sp. showed a high biodegradation rate, as noticed by the anthracene concentration drop to 7.67% at day 2 after shaking incubation at 25 °C. Notably, on day

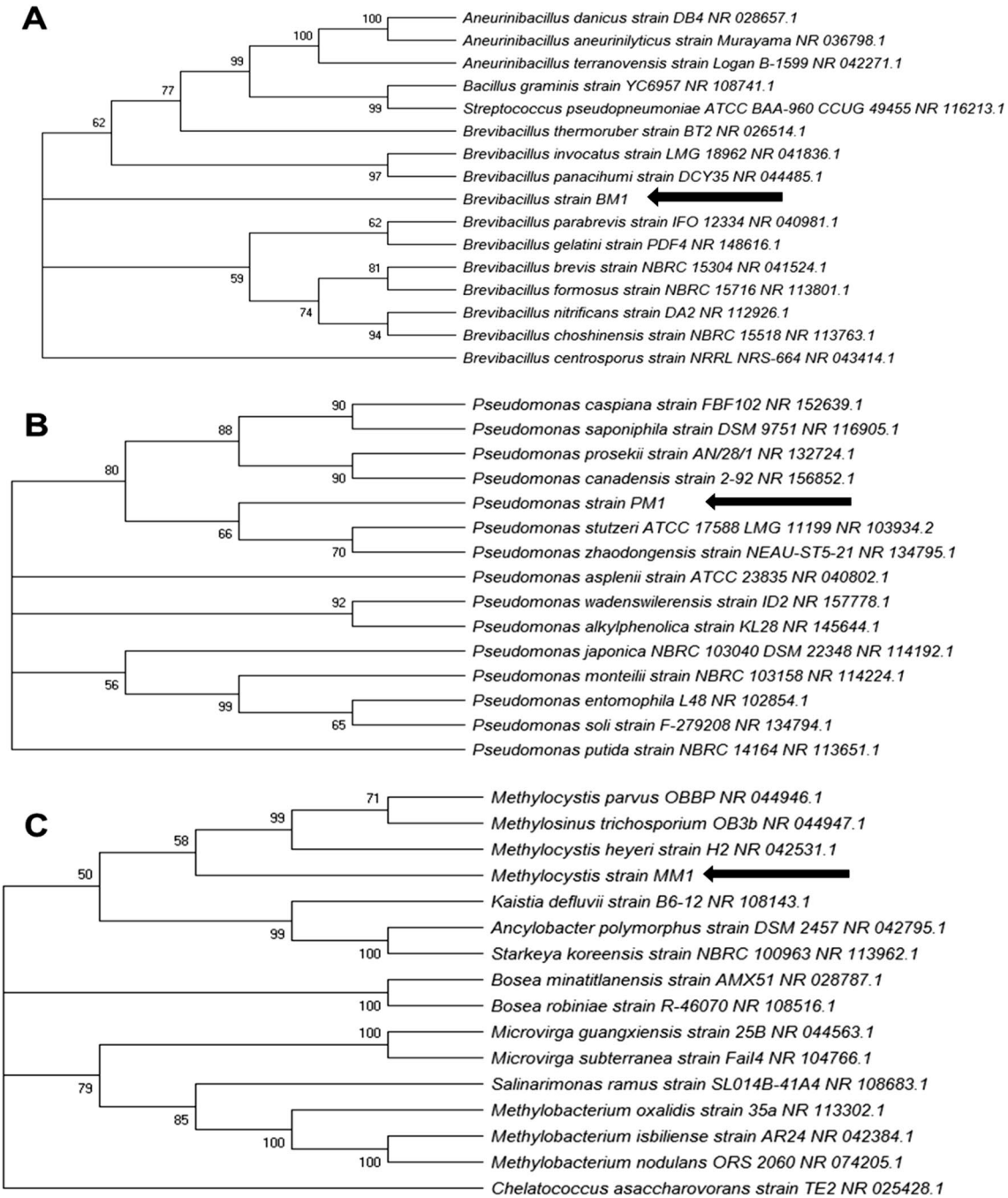


Fig. 1 Phylogenetic tree profiling of the 16SrRNA gene sequences obtained from the three isolates BM1, PM1, and MM1, to their nearest sequences. **a** *Brevibacillus* sp. **b** *Pseudomonas* sp. **c** *Methylocystis* sp. The Phylogenetic tree was built by neighbor-joining [39] with retrieved sequences from the NCBI database bootstrap consensus way with 100 repetitions [40] using MEGA X across computing platforms. The evolutionary distances were computed by the Maximum Composite Likelihood method [48]. This analysis involved 15–16 nucleotide sequences. Evolutionary analyses were conducted in MEGA X [37]

4 and day 6 for *Methylocystis* sp. using MSM-anthracene medium supplemented with 0.1% glucose at 30 °C, the residual anthracene concentration reached 0.28% and 0.09%, respectively.

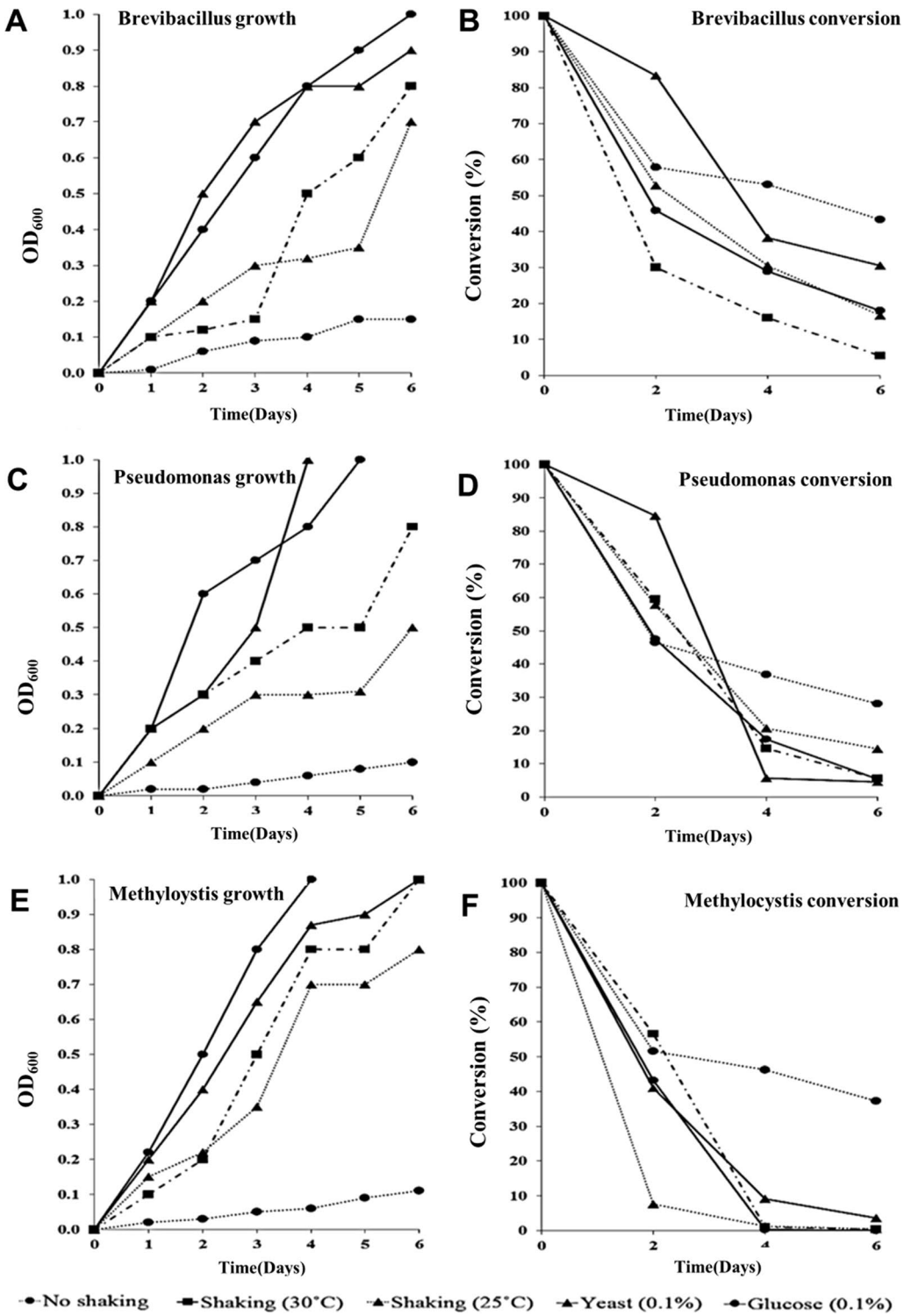


Fig. 2 Enhancement of biomass production and anthracene biodegradation; **a**, **c**, **e** the growth of the three bacterial isolates *Brevibacillus* sp., *Pseudomonas* sp., and *Methylocystis* sp., respectively, on anthracene as a sole carbon source in MSM under five different conditions, i.e., static at 30 °C, shaking at 30 °C, shaking at 25 °C, yeast augmented medium, and glucose augmented medium. The microbial growth was monitored at 600 nm OD. **b**, **d**, **f** the conversion of anthracene at different time points using HPLC for the three isolates *Brevibacillus* sp., *Pseudomonas* sp., and *Methylocystis* sp., respectively, under the same five conditions

In addition, the residual anthracene percentage using *Brevibacillus* sp. upon shaking incubation at 30 °C was 29.95%, 15.9%, and 5.43% on days 2, 4, and 6, respectively. Moreover, for *Brevibacillus* sp. in MSM-anthracene medium at 30 °C with shaking, anthracene utilization was more than that at 0.1% yeast or 0.1% glucose supplementation.

Although the biodegradation efficacy of *Pseudomonas* sp. in static condition started with 53.56% on day 2, the degradation rate slowly decreased over time. On the other hand, the residual anthracene decreased to 5.48% by *Pseudomonas* sp. after shaking incubation at 30 °C for 6 days. Similarly, in 0.1% yeast augmented media, *Pseudomonas* sp. decreased the residual anthracene at day 4 and day 6 to 5.64% and 4.53%, respectively.

Figure 3 shows the follow-up of the anthracene degradation at three different time points, day 0, day 2, and day 4 for the three selected isolates tested using shaking incubation at 160 rpm at 25 °C. As shown in Fig. 3c, *Methylocystis* sp. almost degraded all anthracene (Rt 12.4 min) used in the experiment at day 4 sample point. Noteworthy, these results were in line with the OD and HPLC results mentioned earlier.

3.4 PCR amplification of PAHs biodegradation genes

Successfully, specific genes were detected in the three selected isolates. Notably, *Methylocystis* sp. revealed the presence of *tnpA*-like gene, *tadR*-like gene, *tadQ*-like gene, *ps pass 2.3*-like gene, *GS2*-like gene, and *acin GS*-like gene, while *Brevibacillus* sp. showed presence of *tnpA*-like gene, *tadQ*-like gene, *acin GS*-like gene, *tadR*-like gene, and *tadQ2*-like gene. On the other hand, only *acin GS*-like gene was screened in *Pseudomonas* sp. using PCR amplification.

3.5 GC–MS analysis of anthracene biodegradation

The GC–MS analysis of the metabolites detected in the crude extracts of the selected isolates incubated in MSM-anthracene for four days confirmed the anthracene biodegradation by the selected strains. Notably, *Brevibacillus* sp., *Pseudomonas* sp. and *Methylocystis* sp. degraded more than 95% of the starting concentration of anthracene. The phthalic acid (**2**) (MF; $C_8H_6O_4$) and its esters were detected as the major intermediates in the anthracene biodegradation by all selected strains suggesting that they may followed similar biodegradation pathway to degrade the anthracene as sole carbon source. Interestingly, benzoic acid (TMS derivative) (**3**) (MF; $C_7H_6O_2$) putatively identified an intermediate produced by *Brevibacillus* sp., proposing that this isolate followed this biodegradation pathway (Fig. 4). On the other hand, benzoic acid was not detected by GC–MS analysis of both *Pseudomonas* sp. and *Methylocystis* sp., suggesting

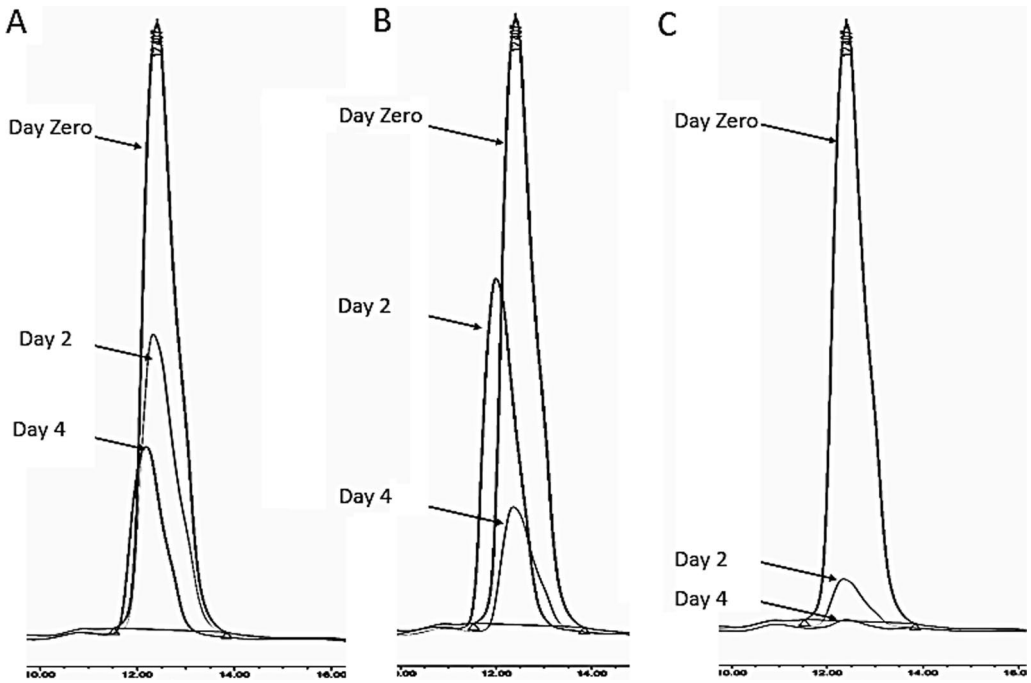


Fig. 3 Anthracene degradation curves at days 0, 2, and 4 for the selected isolates according to the HPLC analysis **a** *Brevibacillus* sp., **b** *Pseudomonas* sp., and **c** *Methylocystis* sp

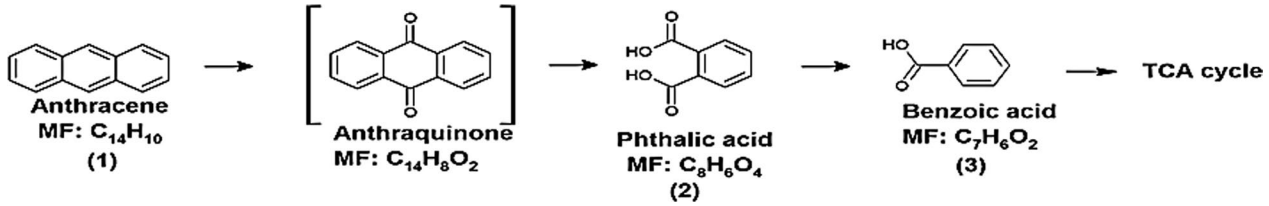


Fig. 4 Proposed pathway for anthracene biodegradation by *Brevibacillus* sp. as suggested by the GC–MS data analysis. The compound in brackets was not detected

that they either followed other biodegradation pathways or benzoic acid was further biodegraded into simpler compounds.

Moreover, six other metabolites were detected by the GC–MS analysis and were putatively identified as compounds (**4–9**) which are suggested to be intermediates in other anthracene biodegradation pathways (Fig. 5). Indeed, only compounds **4** and **8** were detected in all selected isolates, while compound **9** was identified in both *Brevibacillus* sp. and *Pseudomonas* sp. On contrast, both compounds **5** and **6** were exclusively identified by GC–MS analysis in *Brevibacillus* sp., whereas compound **7** was solely detected in *Pseudomonas* sp.

4 Discussion

PAHs were listed as priority pollutants by the US Environmental Protection Agency and the European Environment Agency [49, 50]. Human exposure to such pollutants may be done through inhalation, ingestion, and skin absorption [49]. The samples were obtained from the province of Beni-Suef, as its air quality is considered one of the highly polluted areas in Egypt [34].

PAHs removal can be adapted through different methods. However, using the biodegradation approach in PAHs removal is considered a cost-effective and environmentally clean technique [27, 51]. PAHs biodegradation capability of different microbial species was detected in several previous studies using different microorganisms including *Pseudomonas* sp. [12, 26], *Brevibacillus* sp. [24, 52, 53] and *Methylocystis* sp. (on hydrocarbons) [54, 55]. Additionally, *Bjerkandera adusta* degraded 38% of anthracene during three days of incubation [56] and 70.5% of anthracene (50 µg/mL) was degraded by *Sphingomonas* sp. KSU05 at 96 h of cultivation [14]. Surprisingly, our isolates showed a high potential to biodegrade anthracene under aerobic conditions within six days as

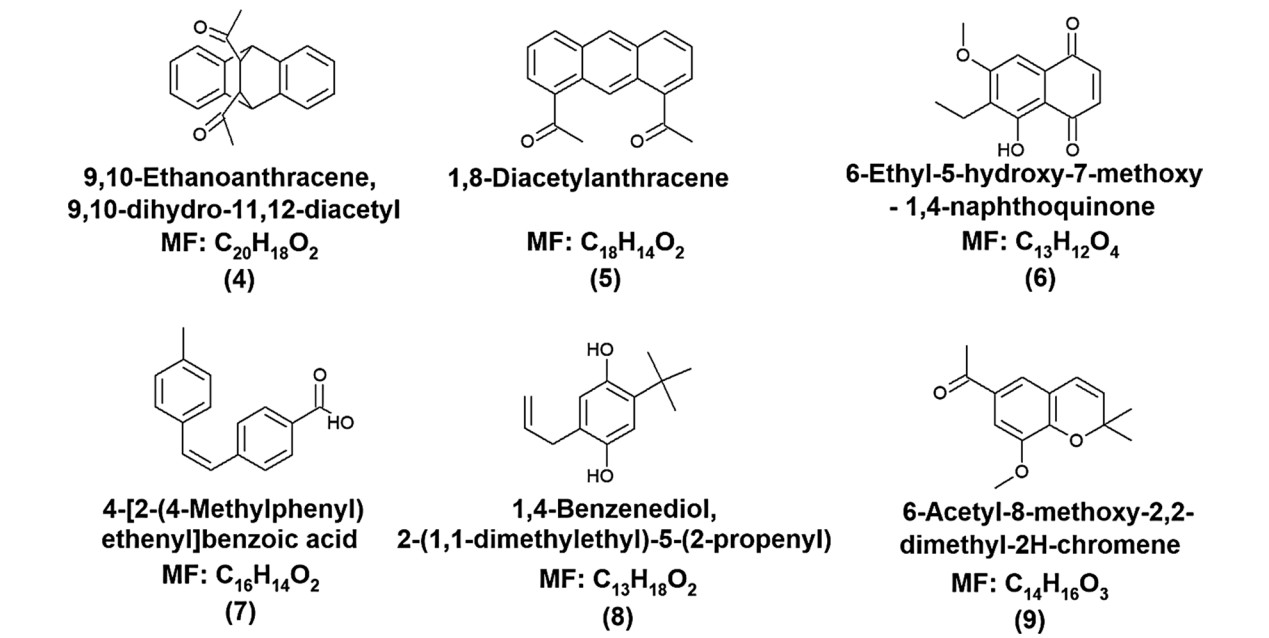


Fig. 5 Some metabolites detected by the GC–MS analysis and are suggested to be intermediates in different biodegradation pathways of anthracene by the selected isolates

94.58%, 94.53%, and 99.61% of the starting anthracene concentration were degraded by *Brevibacillus* sp., *Pseudomonas* sp., and *Methylocystis* sp., respectively.

In comparison with the literature, many pieces of literature mentioned the capabilities of *Pseudomonas sp.* [12] and *Brevibacillus sp.* [24] to degrade the anthracene. These studies match our results, although in case of *Methylocystis sp.*, there are limited pieces of literature talking about its biodegradation actions on anthracene. As shown in our study, *Methylocystis* sp. reached 99% of anthracene mineralization in 96 h showing the highest biodegradation level among the selected strains. One explanation of this finding is that the rich availability of gene clusters of degrading anthracene as mentioned earlier may stand behind the great capability of this strain to degrade the anthracene.

Performing HPLC analysis for the residual anthracene at 254 nm revealed the decrease in anthracene concentration by time with the presence of any of the three isolates. Shaking conditions increased the biodegradation activity of the three isolates, in agreement with previous studies that confirmed the benefit of the aeration factor on PAH biodegradation by *Pseudomonas* sp. [57]. Anthracene degradation of any of the three isolates incubation at 30 °C was more efficient than that at 25 °C at day 4 and may reach the double as in *Brevibacillus* sp. that case is in line with a study revealing that certain conditions including a temperature of 30.04 °C were the optimal conditions for maximum removal efficiency of phenol (99.10%) by *Candida tropicalis Z-04* [45]. *Methylocystis* sp. at day 2 at 25 °C shaking incubation degraded 92.33% of the starting anthracene concentration, while *Aspergillus fumigates* degraded only 60% of anthracene after 5 days [31]. Anthracene utilization in *Brevibacillus* sp. with 0.1% yeast or glucose was lower than that of only anthracene MSM media at 30 °C. Annadurai and his colleagues referred to that in a co-metabolic process, addition of a readily degradable secondary source of carbon (glucose) causes the normally stable compound partially degraded but not used as an energy source [58].

GC–MS analysis has been widely used in many previous studies to identify the byproducts and metabolic intermediates produced during different anthracene biodegradation pathways by various bacterial species [17, 33, 59, 60]. Therefore, it was selected in the current study to detect the different metabolites produced during the anthracene biodegradation and to suggest the proposed anthracene biodegradation pathways followed by the selected strains. In our study, the GC–MS analysis suggested that *Brevibacillus* sp. biodegraded anthracene via anthraquinone, phthalic acid and benzoic acid derivatives. Of note, many previous research showed that anthracene was biodegraded via similar pathway to ours. For example, *Bacillus thuringiensis* AT.ISM.1 degraded anthracene through anthraquinone, phthalic acid derivatives, benzoic acid derivatives, and pyrocatechol and benzaldehyde [17]. Also, another study showed that *Polyporus* sp. S133 followed similar pathway with production of anthraquinone, phthalic acid, benzoic acid, and catechol (TMS derivatives) as metabolic intermediates [61]. Additionally, *Bacillus licheniformis* MTCC 5514 and *Bacillus cereus* S_{13} followed the same pathway of anthracene biodegradation via anthraquinone, phthalic acid, benzoic acid, and catechol [33, 62].

On the other hand, only phthalic acid or its esters were detected as the major intermediates in anthracene degradation by *Methylocystis* sp. and *Pseudomonas sp.*, while neither benzoic acid nor its derivatives were identified in both isolates. Absence of these intermediates can be explained by the suggestion that the phthalic acid has been involved in another pathway [59] or it has undergone conversion into other compounds which were used in the central metabolism by these isolates [33, 62].

Moreover, some other metabolic intermediates (**4–9**) were identified by GC–MS, suggesting the presence of other biodegradation pathways of anthracene biodegradation by the selected isolates. It is well known that microorganisms can follow more than one degrading pathway for the xenobiotics to get best use of them through various pathways. This group of metabolites (**4–9**) were presented as suggested intermediates in other anthracene biodegradation pathways due to their structural similarity to other byproducts detected in anthracene biodegradation pathways other than that shown in Fig. 4 or because they were derivatives from anthracene itself which may indicate enzymatic modification of anthracene prior to its biodegradation. Also, one compound or more from this group of metabolites may be involved in other new/undiscovered pathways of anthracene biodegradation.

For example, compound **7** (4-[2-(4-methylphenyl) ethenyl]benzoic acid) was putatively identified as a byproduct of anthracene degradation by *Pseudomonas* sp. This compound is closely related in structure to another metabolite (1-methoxy-4-[2-(4-methylphenyl)ethenyl] benzene) detected by GC–MS analysis as intermediate during another anthracene biodegradation pathway by *Bacillus thuringiensis* in a previous study [55]. This study showed that *Bacillus thuringiensis* followed a pathway of anthracene degradation via anthracen-9(10H)-one, 1-methoxy-4-[2-(4-methylphenyl)ethenyl]benzene, benzoic acid, benzaldehyde and pyrocatechol. Additionally, the same study as well as many other previous studies identified different phenolic compounds such as phenol, catechol or pyrocatechol and their derivatives as main end products in different pathways of

microbial degradation of anthracene [55–57]. Noteworthy, a derivative of para-substituted benzenediol compound (**8**) was putatively identified as 1,4-benzenediol, 2-(1,1-dimethylethyl)-5-(2-propenyl) which is a phenolic compound detected in all selected isolates suggesting that it was one of the intermediates in anthracene biodegradation pathways by these strains. Interestingly, another old study revealed that the benzenediol itself was one of the intermediates in photocatalytic biodegradation of phthalic acid [26] which is main by-product of anthracene biodegradation in the present study.

5 Conclusions

In conclusion, this study addresses a potential solution for an area in Egypt that is considered the number-one air-polluted area that needs to be dealt with using cost-effective methods such as biodegradation. One of the recovered microbial species (*Methylocystis* sp.) showed a potential of anthracene degradation with the efficacy of 99% in 96-h incubation. Although the in vitro PAHs biodegradation abilities of the bacteria are satisfactory, several factors, either abiotic and/or biotic, should be optimized to assess these isolates' full capacities. It was also concluded from the current study that the GC–MS is a precious tool in detecting the metabolic intermediates in the biodegradation of PAHs and in assigning the proposed pathway of their biodegradation.

Abbreviations

PAHs: Polyaromatic hydrocarbons; OD: Optical density; HPLC: High-performance liquid chromatography; GC–MS: Gas chromatography–mass spectrometry; DNA: Deoxyribonucleic acid; rRNA: Ribosomal ribonucleic acid; NCBI: National Centre for Biotechnology Information; UV: Ultraviolet light; BLAST: Basic Local Alignment Search Tool; CFU: Colony-forming units; KH_2PO_4: Potassium phosphate monobasic; $(NH_4)_2SO_4$: Ammonium sulfate; NaOH: Sodium hydroxide; $MgSO_4 \cdot 7H_2O$: Magnesium sulfate heptahydrate; $FeSO_4 \cdot 7H_2O$: Iron(II) sulfate heptahydrate; HCl: Hydrogen chloride; KNO_3: Potassium nitrate; $NaNO_3$: Sodium nitrate; $CaNO_3$: Calcium nitrate; NH_4NO_3: Ammonium nitrate; MF: Molecular formula; TMS: Tetramethyl silane.

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Authors' contributions

Conceptualization: YG, AA, SA. Data curation: MM. Formal analysis: MM, YG, MS, SA. Investigation: YG, AA, MS, SA. Project administration: YG, AA, SA. Resources: MM, AA. Supervision: YG, AA, SA. Writing original draft: MM, YG, AA. Writing, review and editing: MS, SA. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

Dr Sameh AbdelGhani is a co-author of this study and Associate Editor of the journal. He declares a competing interest for this submission. He has not handled this manuscript. The rest of the authors have no conflict of interest to declare.

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References

1. Boffetta P, Jourenkova N, Gustavsson P (1997) Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes Control 8(3):444–472
2. Boehm PD (1964) Polycyclic aromatic hydrocarbons (PAHs). In: Environmental forensics. Elsevier, pp 313–337
3. Boonchan S, Britz ML, Stanley GA (1998) Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol Bioeng 59(4):482–494
4. Raiyani C (1993) Level of polycyclic aromatic hydrocarbon in ambient environment of Ahmedabad city. Indian J Environ Prot 13:206–215
5. Cerniglia CE (1993) Biodegradation of polycyclic aromatic hydrocarbons. Curr Opin Biotechnol 4(3):331–338
6. Menzie CA, Potocki BB, Santodonato J (1992) Exposure to carcinogenic PAHs in the environment. Environ Sci Technol 26(7):1278–1284
7. McVeety BD, Hites RA (1988) Atmospheric deposition of polycyclic aromatic hydrocarbons to water surfaces: a mass balance approach. Atmospheric Environment (1967). 22(3):511–536
8. Seo J-S, Keum Y-S, Li Q (2009) Bacterial degradation of aromatic compounds. Int J Environ Res Public Health 6(1):278–309
9. Birkett JW, Lester JN (2002) Endocrine disrupters in wastewater and sludge treatment processes. IWA Publishing, London
10. Bidoia ED, Montagnolli RN (2018) Toxicity and biodegradation testing. Springer, Berlin
11. WHO, *Guidelines for air quality*. 2000, World Health Organization: WHO, Geneva. (http://www.who.int/peh/).
12. Jacques RJS et al (2005) Anthracene biodegradation by *Pseudomonas* sp. isolated from a petrochemical sludge landfarming site. Int Biodeterior Biodegrad 56(3):143–150
13. Neelofur MS, Shyam P, Mahesh M (2014) Enhance the biodegradation of Anthracene by mutation from Bacillus species. BMR Biotechnol 1:1–19
14. Al Farraj DA et al (2020) Polynuclear aromatic anthracene biodegradation by psychrophilic *Sphingomonas* sp., cultivated with tween-80. Chemosphere 263:128115
15. Hurst GH (1892) *A Dictionary of the Coal Tar Colours*. https://books.google.com.eg/books/about/A_Dictionary_of_the_Coal_Tar_Colours.html?id=lY45AQAAIAAJ&redir_esc=y: Heywood and Company, p 12
16. Khillare P, Balachandran S, Hoque RR (2005) Profile of PAH in the exhaust of gasoline driven vehicles in Delhi. Environ Monit Assess 110(1–3):217–225
17. Tarafdar A, Sinha A, Masto RE (2017) Biodegradation of anthracene by a newly isolated bacterial strain, *Bacillus thuringiensis* ATISM1, isolated from a fly ash deposition site. Lett Appl Microbiol 65(4):327–334

18. Bonnet J et al (2005) Assessment of anthracene toxicity toward environmental eukaryotic microorganisms: *Tetrahymena pyriformis* and selected micromycetes. Ecotoxicol Environ Saf 60(1):87–100
19. Sun K et al (2020) Anthracene-induced DNA damage and oxidative stress: a combined study at molecular and cellular levels. Environ Sci Pollut Res 27(33):41458–41474
20. Sun, K., et al., *Toxicity assessment of Fluoranthene, Benz (a) anthracene and its mixed pollution in soil: Studies at the molecular and animal levels.* Ecotoxicology and Environmental Safety, 2020. **202**: 110864.
21. Yuan M et al (2017) An integrated biomarker response index for the mussel *Mytilus edulis* based on laboratory exposure to anthracene and field transplantation experiments. Chin J Oceanol Limnol 35(5):1165
22. Holst LL, Giesy JP (1989) Chronic effects of the photoenhanced toxicity of anthracene on *Daphnia magna* reproduction. Environ Toxicol Chem Int J 8(10):933–942
23. Brown IV, Lane BP, Pearson J (1977) Effects of depot injections of retinyl palmitate on 7, 12-dimethylbenz [a] anthracene-induced preneoplastic changes in rat skin. J Natl Cancer Inst 58(5):1347–1355
24. Badis I (2016) Biodegradation of diesel and isomerate by pseudomonas aeruginosa and *Brevibacillus laterosporus* isolated from hydrocarbons contaminated soil. Adv Environ Biol 10(7):208–215
25. Chaillan F et al (2004) Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Res Microbiol 155(7):587–595
26. Whyte LG, Bourbonniere L, Greer CW (1997) Biodegradation of petroleum hydrocarbons by psychrotrophic Pseudomonas strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. Appl Environ Microbiol 63(9):3719–3723
27. Azubuike CC, Chikere CB, Okpokwasili GC (2016) Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World J Microbiol Biotechnol 32(11):180
28. Bezalel L et al (1996) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62(7):2554–2559
29. Samanta SK, Singh OV, Jain RK (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol 20(6):243–248
30. Moody JD et al (2001) Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. Appl Environ Microbiol 67(4):1476–1483
31. Ye J-S et al (2011) Biodegradation of anthracene by *Aspergillus fumigatus*. J Hazard Mater 185(1):174–181
32. Theurich J et al (1997) Photocatalytic degradation of naphthalene and anthracene: GC-MS analysis of the degradation pathway. Res Chem Intermed 23(3):247–274
33. Bibi N et al (2018) Anthracene biodegradation capacity of newly isolated rhizospheric bacteria *Bacillus cereus* S13. PLoS ONE 13(8):e0201620
34. Rights, E.I.f.P. *World Environment Day*. 2018; Available from: https://eipr.org/publications.
35. Hesham Ael L et al (2014) Biodegradation ability and catabolic genes of petroleum-degrading Sphingomonas koreensis strain ASU-06 isolated from Egyptian oily soil. Biomed Res Int 2014.
36. Altschul SF et al (1990) Basic local alignment search tool(BLAST). J Mol Biol 215(3):403–410
37. Kumar S et al (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6):1547–1549
38. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680
39. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425
40. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39(4):783–791
41. Liang Q et al (2005) Chromosome-encoded gene cluster for the metabolic pathway that converts aniline to TCA-cycle intermediates in Delftia tsuruhatensis AD9. Microbiology 151(10):3435–3446
42. Takeo M, Fujii T, Maeda Y (1998) Sequence analysis of the genes encoding a multicomponent dioxygenase involved in oxidation of aniline and o-toluidine in *Acinetobacter* sp. strain YAA. J Ferment Bioeng 85(1):17–24
43. Geng L et al (2009) Functional analysis of a putative regulatory gene, tadR, involved in aniline degradation in *Delftia tsuruhatensis* AD9. Arch Microbiol 191(7):603–614
44. Urata M et al (2004) Genes involved in aniline degradation by Delftia acidovorans strain 7N and its distribution in the natural environment. Biosci Biotechnol Biochem 68(12):2457–2465
45. Zhou J et al (2011) Optimization of phenol degradation by Candida tropicalis Z-04 using Plackett–Burman design and response surface methodology. J Environ Sci 23(1):22–30
46. Khorasani AC, Mashreghi M, Yaghmaei S (2014) Optimization of biomass and biokinetic constant in Mazut biodegradation by indigenous bacteria BBRC10061. J Environ Health Sci Eng 12(1):98
47. Lee S-Y, Lee J-Y, Shin H-S (2015) Evaluation of chemical analysis method and determination of polycyclic aromatic hydrocarbons content from seafood and dairy products. Toxicol Res 31(3):265
48. Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc Natl Acad Sci 101(30):11030–11035
49. Enzminger J, Ahlert R (1987) Environmental fate of polynuclear aromatic hydrocarbons in coal tar. Environ Technol Lett 8(1–12):269–278
50. Tobiszewski M, Namieśnik J (2012) PAH diagnostic ratios for the identification of pollution emission sources. Environ Pollut 162:110–119
51. Łebkowska M et al (2011) Bioremediation of soil polluted with fuels by sequential multiple injection of native microorganisms: Field-scale processes in Poland. Ecol Eng 37(11):1895–1900
52. Reddy MS et al (2010) Biodegradation of phenanthrene with biosurfactant production by a new strain of *Brevibacillus* sp. Biores Technol 101(20):7980–7983
53. Wei K et al (2018) Bioremediation of triphenyl phosphate by Brevibacillus brevis: degradation characteristics and role of cytochrome P450 monooxygenase. Sci Total Environ 627:1389–1395
54. Yoon S et al (2011) Constitutive expression of pMMO by Methylocystis strain SB2 when grown on multi-carbon substrates: implications for biodegradation of chlorinated ethenes. Environ Microbiol Rep 3(2):182–188
55. Kikuchi T et al (2002) Quantitative and rapid detection of the trichloroethylene-degrading bacterium *Methylocystis* sp. M in groundwater by real-time PCR. Applied microbiology and biotechnology 59(6):731–736
56. Schützendübel A et al (1999) Degradation of fluorene, anthracene, phenanthrene, fluoranthene, and pyrene lacks connection to the production of extracellular enzymes by *Pleurotus ostreatus* and *Bjerkandera adusta*. Int Biodeterior Biodegrad 43(3):93–100
57. Wald J et al (2015) Pseudomonads rule degradation of polyaromatic hydrocarbons in aerated sediment. Front Microbiol 6:1268
58. Annadurai G, Ling LY, Lee J-F (2008) Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*. J Hazard Mater 151(1):171–178
59. Van Herwijnen R et al (2003) Degradation of anthracene by *Mycobacterium* sp. strain LB501T proceeds via a novel pathway, through o-phthalic acid. Appl Environ Microbiol 69(1):186–190
60. Cui C et al (2014) Metabolic pathway for degradation of anthracene by halophilic *Martelella* sp. AD-3. Int Biodeterior Biodegrad 89:67–73
61. Hadibarata T, Khudhair AB, Salim MR (2012) Breakdown products in the metabolic pathway of anthracene degradation by a ligninolytic fungus *Polyporus* sp. S133. Water Air Soil Pollut 223(5):2201–2208
62. Swaathy S et al (2014) Microbial surfactant mediated degradation of anthracene in aqueous phase by marine *Bacillus licheniformis* MTCC 5514. Biotechnol Rep 4:161–170

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