PLOS ONE

RESEARCH ARTICLE

Anthracene biodegradation capacity of newly isolated rhizospheric bacteria *Bacillus cereus* S_{13}

Nadia Bibi[1], Muhammad Hamayun[2], Sumera Afzal Khan[1]*, Amjad Iqbal[3], Badshah Islam[3], Farooq Shah[3], Muhammad Aaqil Khan[4], In-Jung Lee[4]*

1 Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar, Pakistan, **2** Department of Botany, Abdul Wali Khan University Mardan, Mardan, Pakistan, **3** Department of Agriculture, Abdul Wali Khan University Mardan, Mardan, Pakistan, **4** School of Applied Biosciences, Kyungpook National University, Daegu, Republic of Korea

* ijlee@knu.ac.kr (IJL); shumakhan@gmail.com (SAK)

Citation: Bibi N, Hamayun M, Khan SA, Iqbal A, Islam B, Shah F, et al. (2018) Anthracene biodegradation capacity of newly isolated rhizospheric bacteria *Bacillus cereus* S_{13}. PLoS ONE 13(8): e0201620. https://doi.org/10.1371/journal.pone.0201620

Editor: Andrea Franzetti, Universita degli Studi di Milano-Bicocca, ITALY

Received: December 26, 2017

Accepted: July 18, 2018

Published: August 2, 2018

Data Availability Statement: All relevant data are within the paper.

Funding: Research was supported by the Agenda Program, Rural Development Administration, Republic of Korea (Project No. PJ01228603 to In-Jung Lee). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Biodegradation of hazardous pollutants is of immense importance for maintaining a clean environment. However, the concentration of such contaminants/pollutants can be minimized with the help of microorganisms that has the ability to degrade the toxic pollutants into non-toxic metabolites. In the current study, 23 bacterial isolates were purified from the rhizospheric soil of *Sysimbrium irio*, growing as a wild plant in the vicinity of gas filling stations in Peshawar city. The isolated strains were initially screened on solid nutrient agar and further purified by culturing it on anthracene amended mineral media (PNR). The bacterial growth and anthracene disappearance were observed by calculating optical density (OD). The isolates showed a concentration-dependent growth on anthracene amended PNR media at 30˚C and pH7. Also, an increase in bacterial OD from 0.351 to 1.80 with increased shaking speed was noticed. On the contrary, alternate carbon sources (glucose, fructose, sucrose) or nitrogen sources (KNO_3, $NaNO_3$, NH_4NO_3 and $CaNO_3$) posed inhibitory effect on bacterial growth during anthracene degradation. The recorded efficiency of anthracene degradation by the selected bacterial isolate (1.4×10^{23} $CFUmL^{-1}$ and 1.80 OD) was 82.29%, after 120 h of incubation. The anthracene was degraded to 9, 10, dihydroxy-anthracene and anthraquinone, detected through GC-MS. The efficient bacterial isolate was identified as S_{13}, a new strain of *Bacillus cereus*, using 16S rRNA analysis, showing 98% homology. The isolated bacterial strain S_{13} may be used as a potential tool for bioremediation of toxic hydrocarbons and to keep the environment free from PAH pollutants.

Background

A major portion of petroleum mixture contains fuse-ringed aromatic compounds, the poly-aromatic hydrocarbons (PAHs). The PAHs are the most abundant contaminants in the atmosphere and are kept on top of the pollutants list by US Environmental Protection Agency [1, 2]. These contaminants can be found abundant around the industrial sites, such as gas production sites and wood preservation industries, and release through automobile exhausts [3] that

Abbreviations: PAHs, Polyaromatic hydrocarbons; LMW, Low-molecular weight; 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; $MgSO_4.7H_2O$, Magnesium Sulfate Heptahydrate; $FeSO_4.7H_2O$, Iron(II) Sulfate Heptahydrate; HCl, Hydrogen chloride; KNO_3, Potassium nitrate; $NaNO_3$, Sodium nitrate; $CaNO_3$, Calcium nitrate; NH_4NO_3, Ammonium nitrate.

are consistent threats to human. PAHs along with their derivatives are the major factors causing anaemia, asthma, splenomegaly and various types of cancer in humans [4]. Some of the PAHs with low molecular weights have caused reproductive abnormalities and even death in aquatic animals [5, 6].

Anthracene is used as a signature compound for the detection of PAHs contamination, as it is an integral part of many carcinogenic PAHs. Due to its hydrophobicity and potential to bioaccumulate, it is used as model compound [7] to define factors affecting bioavailability and rate of degradation of PAHs in environment. In spite of structural identity between anthracene and phenanthrene regarding the number of aromatic rings, anthracene differs in degradation due to the hydrophobic nature [4, 8, 9]. The presences of these compounds in the environment have to be taken seriously, because of their broad toxic effects on living organisms [3, 10]. PAHs in contaminated soils must be treated to avoid any possible noxious effect on environment and human health. In this regard, microbes can play key role in degradation/bioremediation of the toxic PAHs into non-toxic compounds [4].

From the last few decades, bioremediation is getting importance day by day, because it is cheap, feasible and safe to clean the contaminated localities [6, 11]. The efficient microbial strains, unlike physical and chemical treatments can completely mineralize the PAHs present on the soil surface or soil sediments [11]. Previously, it has been discovered that certain starins of fungi, bacteria and algae can feed on the harmful PAHs and produce harmless compounds out of them [12]. Haleyur, Shahsavari (13) has demonstrated that some of the microorganisms (including, *Rhodococcus* sp., *Achromobacter* sp., *Oerskovia paurometabola*, *Pantoea* sp., *Sejongia* sp., *Microbacterium maritypicum* and *Arthrobacter equi*) exhibits catechol 1,2-dioxygenase activity. The presence of catechol 1,2-dioxygenase activity enables the above mentioned microorganisms to grow on PAHs and degrade catechol [13]. Alfaalfa, rape, vetch, mulberry and mustard rhizoremediation have been reported to grow and flourish in PAHs rich soil. In fact, this might be due to the presence of extensive population of microbes, mainly PAHs degrading bacteria around the roots of those plants [14–18], which enables them to grow normally. Also, pea straw has been found to be one of the most useful sources in decreasing PAHs concentration in the soil [19]. Metagenomic analysis proved that the effect of pea straw was indirect. In fact, pea straw has biostimulated the PAHs biodegrader (*Pseudoxanthomonas* spp. and *Alcanivorax* spp) in the soil that were lying latent prior to the addition of pea straw to the soil [19]. The ability of many microorganisms to degrade hydrocarbons [20–22] through metabolism or co-metabolism have been reported over the years [5]. However, there is still a space to discover microbial strains that can serve as a potential source to be used in bioremediation of PAHs. The present study was also focused on the exploration of prospective strains of beneficial microorganism that can degrade toxic chemicals and provide stress free environment. For this purpose research was conducted to (a) isolate Anthracene degrading rhizospheric bacteria from soil; (b) identification of potential PAHs degrading strain(s) in contaminated soils.

Methods

Nutrient agar media and mineral salts media were used for initial isolation and screening of rhizospheric bacteria. The composition of PNR and PNRG (PNR+5 mM glucose) per liter of distilled water [23, 24], is PN (20x) 50 mL used as 50 mLL^{-1}: KH_2PO_4 13.6% (wv^{-1}), $(NH_4)_2SO_4$ 2.4% (wv^{-1}), NaOH 2.5% (wv^{-1}) and R salts used as 7 mLL^{-1}, $MgSO_4.7H_2O$ 8% (wv^{-1}), $FeSO_4.7H_2O$ 0.2% (wv^{-1}), HCl 0.4% (wv^{-1}), Agar (2%) was used as solidifying agent.

Collection of samples

Sysimbrium irio was found in a wild, i.e. growing in oil contaminated soil at an operational gas filling station in Peshawar. The texture of the soil was sandy loam with pH = 7.0 ± 0.2; soil

moisture = 9.6%, maximum water holding capacity = 43.9% and electrical conductivity of 3.24 ds m^{-1}, respectively. The plants from the contaminated soil were dug as a whole, identified and placed in plastic zipper bag. The samples were immediately brought to the microbiology research laboratory at the University of Peshawar and stored at 4˚C till further processing.

Isolation of bacteria

Soil samples were sieved through 2 mm mesh to collect uniform sized sample. After grading, 1 g of soil sample was serially diluted in a distilled water and then followed the spread plate method as described by Alias S [25]. The plates were incubated at 28˚C for 3–4 days until appearance of the colonies. The developed colonies were carefully picked and further cultured in a fresh nutrient agar plates, the inoculation step was repeated till achieving the pure culture. After obtaining the pure cultures, they were stored in anthracene slants at 4˚C.

Screening of the isolated strains on solid media

The purified bacterial isolates were screened for their capability to utilize anthracene as a sole source of carbon and energy (required for biodegradation). A confirmatory spray-plate assay was used to check the efficiency of isolated bacterial strains to grow on media supplemented with anthracene [26, 27]. Anthracene was initially dissolved in acetone and sprayed on the plates containing bacterial culture. Acetone was then allowed to evaporate leaving anthracene on the surface of the plates to be digested by the bacteria.

Screening of the isolated strains in liquid media

Screening in liquid medium was performed using 250 ml flasks containing 100 ml PNR media, 10% of bacterial inoculum and 1000 mgL^{-1} anthracene. The media was incubated at 28˚C and the bacterial growth was monitored at every 24 h interval till 120 h. Spectrophotometric analysis of bacterial growth and disappearance of anthracene was observed in PNR media at 600 nm and 540 nm, respectively.

Optimization of growth conditions for the isolated strains

Different parameters, like concentration of anthracene, incubation temperature and pH of the growth media were optimized. Range of anthracene concentration was (100, 150, 500 and 1000 ppm), temperature (28, 30, 35, 40, 45 and 50˚C) and pH used were (4, 5, 6, 7, 8 and 9). Effect of shaking speed and inoculum size was quantified using speed of (0, 120, 150, 180, 200 and 220 rpm) and (0, 8, 9, 10, 11, 12, 13%). Alternate carbon source than anthracene used were different sugars, like sucrose, glucose and fructose for their effect on the growth of bacteria at the expense of selected PAH [28, 29]. The bacterial isolate S_{13} inoculum was exposed to UV-light for 15 minutes and added to media containing different concentrations of anthracene [30]. After every 24 h, one ml of culture broth was aseptically collected to check OD, while one ml was collected and stored at 4˚C to test for degradation capability. CFU was calculated at 24 h interval till 120 h in order to check the viability of the bacterial isolate. All the experiments were performed in triplicate.

Biodegradation experiment

The biodegradation experiment was performed using 250 ml flasks containing 100 ml PNR media, 10% of bacterial inoculum and 1000 mgL^{-1} anthracene dissolved in acetone. Acetone was allowed to evaporate, 100 mL media was poured to the flask containing different concentration

of anthracene and 10% bacterial inoculum was added. The flasks were incubated at 30˚C and 1 mL of sample was drawn for HPLC analysis after 24 hours interval for 5 days [31].

Extraction of anthracene for GC-MS analysis

For GC-MS analysis, Shimadzu fused silica capillary column was used. The column temperature was set to 100˚C for 1 min, 15˚C min^{-1} to 160˚C and 5˚Cmin^{-1} to 300˚C for 7min. The GC injector was held isothermally at 280˚C with a splitless period of 3 min. Helium was used as the carrier gas, at a flow rate of 1 mL min^{-1} by using electronic pressure control. The GC–MS interface temperature was maintained at 280˚C [32].

Plasmid curing, isolation and agarose gel electrophoresis

Plasmid DNA was isolated from 18–24 hours old culture grown in nutrient broth. For curing experiment the culture was exposed to high temperature of 45˚C and DNA isolation was done according to standard protocols as described earlier [33, 34].

DNA isolation, molecular identification and phylogenetic analysis of S_{13}

Isolation of genomic DNA was carried out using standard phenol/chloroform extraction protocol [35, 36]. Isolated DNA was run on agarose gel to check its purity. It was stored at -4˚C till further use [37]. Bacterial primers cloning of nearly full length 16S rDNA and sequencing were performed according to the methods described previously [37, 38]. The 16S rRNA gene sequence of the strains was analysed at NCBI (National Centre for Biotechnology Information) using BLAST tool and compared to the corresponding neighbour sequences from GenBank-NCBI database. Consensus sequence was imported into the Multalin program and multiple alignments were performed with related species (GenBank-NCBI database). Sequences were compared to those present in the data bank using blast and aligned with the ClustalW program. The results obtained were further imported into the MEGA-7 software for the construction of a phylogenetic tree using Bootstrap analysis and maximum likelihood with 500 replicates, the substitution method used was the Kimura 2-Parameter model and the statistical method used was maximum likelihood [39].

Results

Isolation of bacteria from collected samples

A total of 25 bacterial strains were isolated from rhizospheric soil samples collected from *S. irio*. Theses strains were cultured on solid and liquid media amended with anthracene.

Screening of isolated strains on anthracene amended solid media

Out of 25 bacterial isolates grown on anthracene amended nutrient agar media, 23 strains were found to utilize anthracene as a main source for energy, when cultured on PNR media amended with anthracene (Table 1).

Screening of isolated strains in liquid media

The isolates that performed best on PNR-anthracene media were further screened in liquid media. Out of 23 bacterial isolates 12 isolates having highest OD were selected for further study (Table 2). In order to confirm the bacterial growth (24 h interval till 120 h) at the expense of anthracene in liquid media, spectrophotometric analysis of bacterial growth and disappearance of anthracene was observed in PNR media at 600 nm and 540 nm, respectively (Fig 1). The results of an optimization study of parameters including anthracene concentration, temperature, pH of the media,

Table 1. Screening of bacterial isolates from *S. irio* on anthracene amended PNR media.

No	Isolate	Anthracene Concentration in ppm													
		25	50	100	200	300	400	500	600	700	800	900	1000	1100	1200
1.	S_2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
2.	S_5	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
3.	S_6	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
4.	S_7	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
4.	S_{11}	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-	-	-
5.	S_{12}	+++	+++	+++	+++	+++	+++	+++	++	+	+	+	-	-	-
6.	S_{13}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
7.	S_{15}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	+	+	-	-
8.	S_{16}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+
9.	S_{18}	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++	++	+	+
10.	S_{19}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	+
10.	S_{20}	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	++	++
11.	S_{21}	+++	++	+++	+++	+++	+++	+++	++	++	++	+	+	+	-
12.	S_{22}	+++	+++	++	+++	+++	+++	+++	+++	++	+	+	+	-	-
13.	S_{23}	+++	+++	+++	+++	+++	+++	+++	++	++	++	+	+	-	-
14.	S_{24}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-
15.	S_{30}	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	-
16.	S_{31}	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+
17.	S_{33}	+++	++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	-
18.	S_{34}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+	+
19.	S_{36}	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	-	-
20.	S_{37}	+++	+++	+++	+++	++	++	++	+	+	+	+	-	-	-
21.	S_{39}	+++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+
22.	S_{40}	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+	+	-
23.	S_{51}	+++	+++	+++	++	++	++	+	+	+	+	+	-	-	-

+++ = Rich growth

++ = Medium growth

+ = Less growth

(-) = No growth

https://doi.org/10.1371/journal.pone.0201620.t001

Table 2. Bacterial growth on anthracene after 3-days incubation in large test tube (600 ppm) PNR.

S. No	Strain	OD_{600nm}
1.	S_2	0.199
2.	S_5	0.321
3.	S_6	0.211
4.	S_{12}	0.159
5.	S_{13}	0.425
6.	S_{15}	0.200
7.	S_{19}	0.269
8.	S_{24}	0.223
9.	S_{30}	0.265
10.	S_{31}	0.169
11.	S_{34}	0.243
12.	S_{51}	0.231

https://doi.org/10.1371/journal.pone.0201620.t002

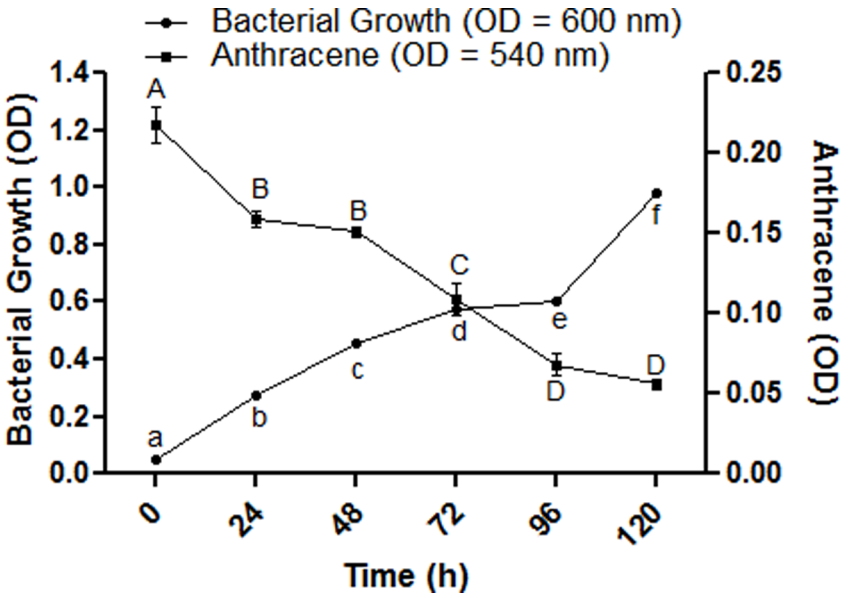

Fig 1. UV-spectrophotometric analysis of bacterial growth and anthracene disappearance. The OD of bacterial growth was observed at 600 nm; the OD of anthracene concentration was observed at 540 nm. Each data point represents the mean of triplicated data with ±S.E. The data points with similar letters are not significantly different at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0201620.g001

alternate carbon and energy source, effect of nitrate salts and UV-light, shaking speed and inoculum size are given below.

Optimization of anthracene concentration and bacterial inoculum

The optimized concentration of anthracene was 1000 ppm for isolate S_{13} during this study (Fig 2A) and inoculum concentration was 10% vv^{-1} for maximum degradation as shown in Fig 2B.

Optimization of temperature, pH and shaking speed for anthracene degradation

Maximum growth of bacterial isolate S_{13} was observed at 30˚C (Fig 2C), at optimized pH 7 as shown in Fig 2D. Maximum growth was observed at 180 rpm as shown in Fig 2E.

Effect of different carbon and nitrogen sources on growth of isolated S_{13}

Both the carbon sources and nitrate salts used were inhibitory on the isolate growth except potassium nitrate, with enhanced growth of our isolate (Fig 3A and 3B).

Effect of UV-light induced mutation on anthracene utilization

The UV-light treated S_{13} isolate gave better growth results with increasing concentration of anthracene as compared to control experiment, not exposed to UV-light, as shown in Fig 3C.

CFU mL^{-1} of isolated bacteria

The viability of bacteria was determined by a CFU study of samples drawn after every 24 h intervals for 120 h as shown in Table 3. The cells showed an increase in growth from initial 1.5×10^{6} per ml to 1.4×10^{23} after 120 h.

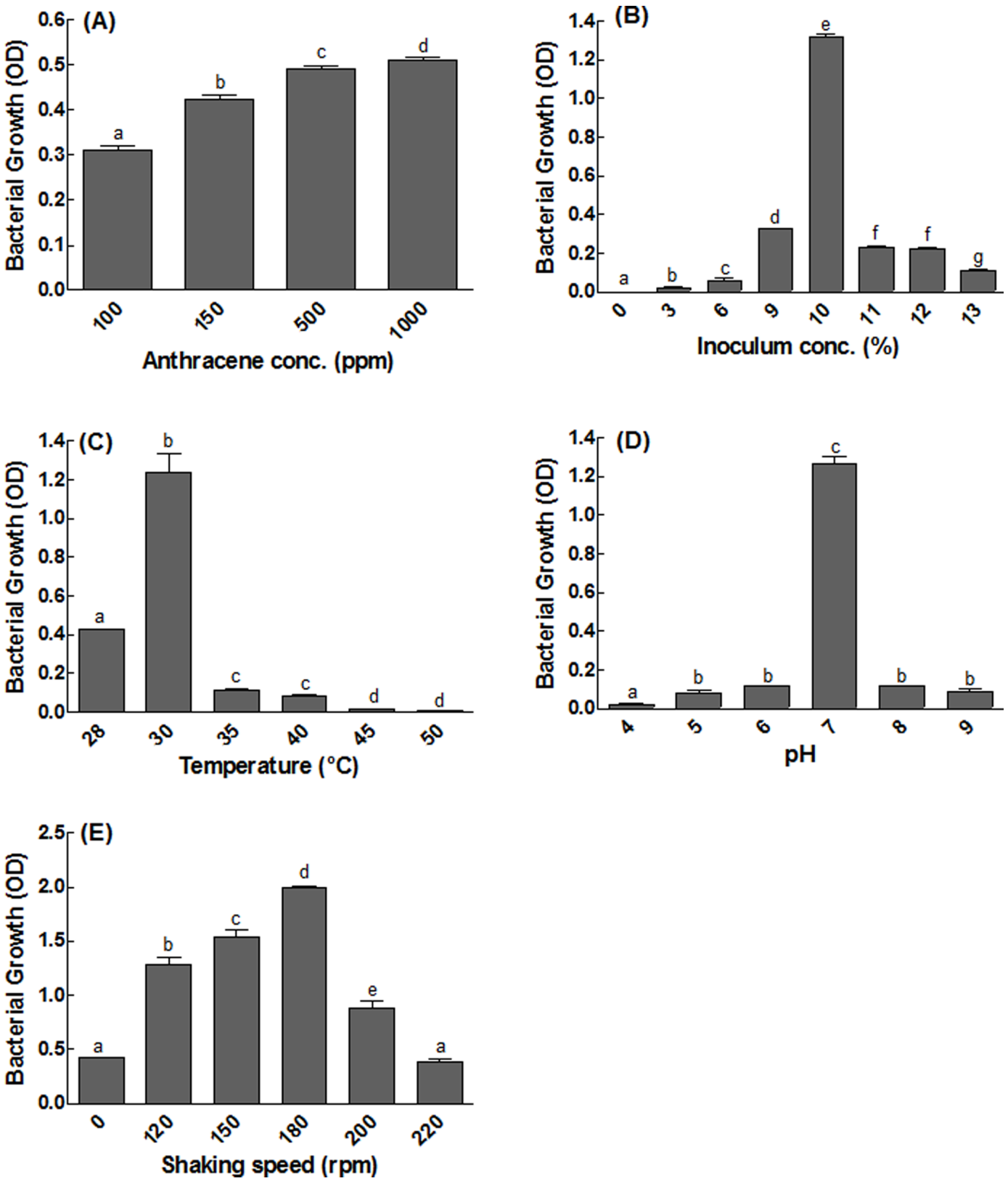

Fig 2. Optimization of conditions for the growth of bacterial isolates S_{13}. (A) represents optimization of anthracene concentration vs growth of isolate S_{13}; (B) represents optimization temperature vs growth of isolate S_{13}; (C) represents optimization of media pH vs growth of isolate S_{13}; (D) represents optimization of

agitation speed vs growth of isolate S_{13}; (E) represents optimization of inoculum concentration (%) vs growth of isolate S_{13}. Each bar represents the mean of triplicated data with ±S.E. The bars with similar letters are not significantly different at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0201620.g002

Biodegradation of anthracene

Biodegradation of anthracene and bacterial growth OD are shown in Fig 4. The isolate S_{13} degraded 82.29% anthracene in 120 h. Strain S_{13} degraded the anthracene effectively during the incubation period of 24 to 96 h, whereas the growth of the bacterial isolate reached to its maximum (OD = 1.15) at 120 h. Increase in the growth of S_{13} was observed with an OD value of 0.598–1.15 between 96–120 h at the expense of only 36.19% anthracene. Confirmation of anthracene biodegradation by bacterial isolate S_{13} was sought by GC-MS analysis. The identified products included 9, 10-dihydroxyanthracene, anthraquinone, benzene acetic acid and catechol, respectively (Fig 5).

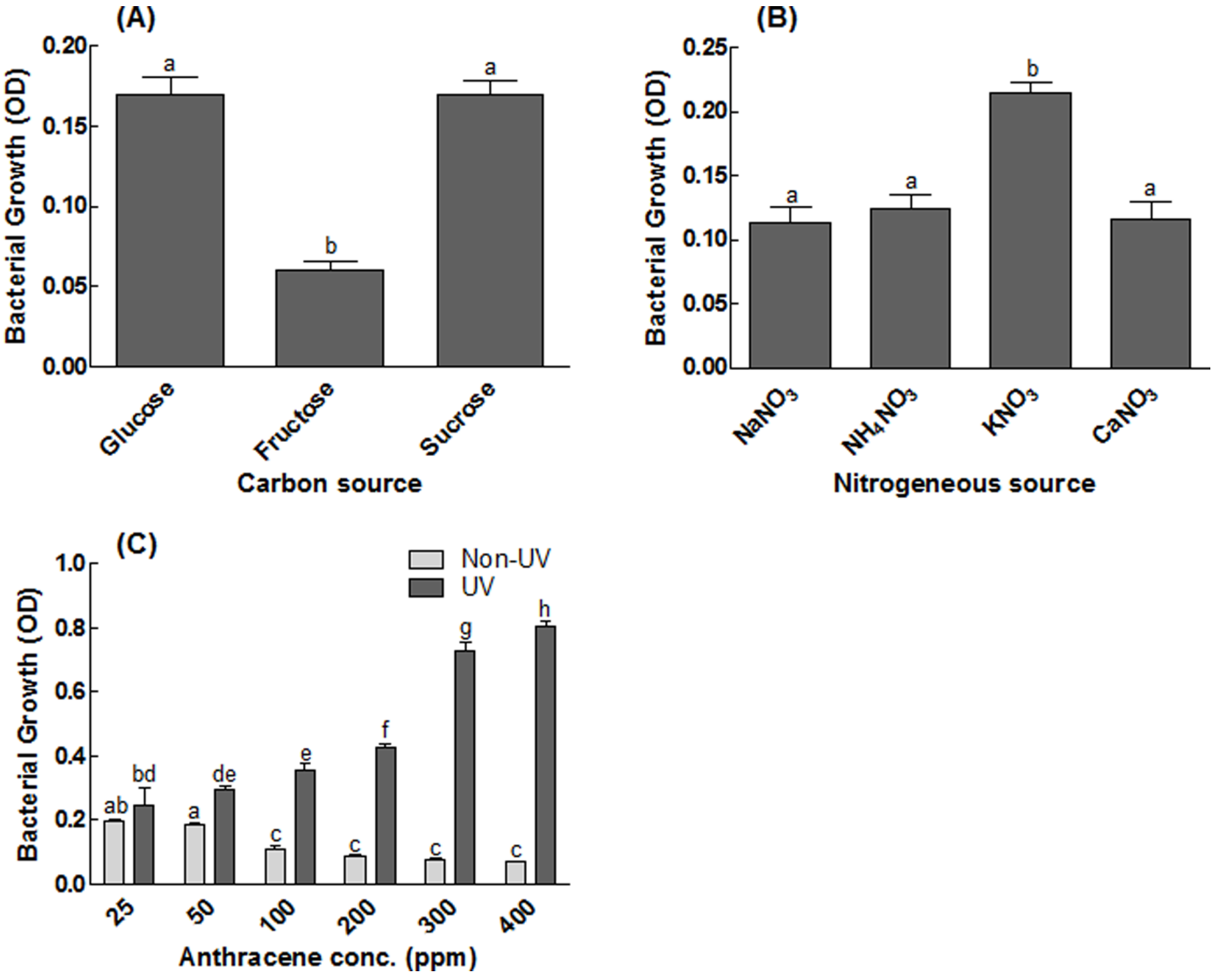

Fig 3. Effect of UV and different media on the growth of of bacterial isolate S_{13}. (A) represents the effect of different carbon sources on the growth of isolate S_{13}; (B) represents the effect of different nitrogen source on the growth of isolate S_{13}; (C) represents the effect of UV light and anthracene concentration on the growth of isolate S_{13} incubated for 96 h. Each bar represents the mean of triplicated data with ±S.E. The bars with similar letters are not significantly different at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0201620.g003

Table 3. $CFUmL^{-1}$ of the bacterium isolate S_{13}.

Time (h).	0	24	48	72	96	120
$CFUmL^{-1}$	1.5×10^{6}	2.9×10^{11}	3.8×10^{14}	1.8×10^{17}	3.2×10^{20}	1.4×10^{23}

https://doi.org/10.1371/journal.pone.0201620.t003

Plasmid curing and isolation

The results from agarose gel electrophoresis and plasmid curing suggested that anthracene degradation is certainly plasmid associated. The isolated plasmid from bacterial culture without curing treatment and gel electrophoresis indicated the presence of 7 plasmid bands of different sizes (Figs 6 and 7). Cured culture gave negative results for the plasmid presence and was unable to grow on anthracene amended media (Fig 8).

Identification of bacterial isolate S_{13}

Initially the bacterial isolate was identified biochemically. The result showed it was gram positive, motile, rod shaped, catalase and urease positive and was capable of starch hydrolysis, while it was negative for citrate, casein hydrolysis and indole production (Table 4). Colony morphology on agar plate showed off white/creamy color colonies with irregular margins, thus identified as *Bacillus sp*.

Molecular identification and phylogenetic analysis of isolate S_{13}

The strain isolated from the rhizospheric soil samples collected from *S. irio* was identified by ITS rDNA region sequencing analysis. Phylogenetic analysis was carried out through MEGA 7.0 software for the construction of a phylogenetic tree using Bootstrap analysis and maximum likelihood with 500 replicates. A total of 20 sequences were downloaded from BLAST data that were showing the maximum relatedness with our isolate. Results of BLAST search showed

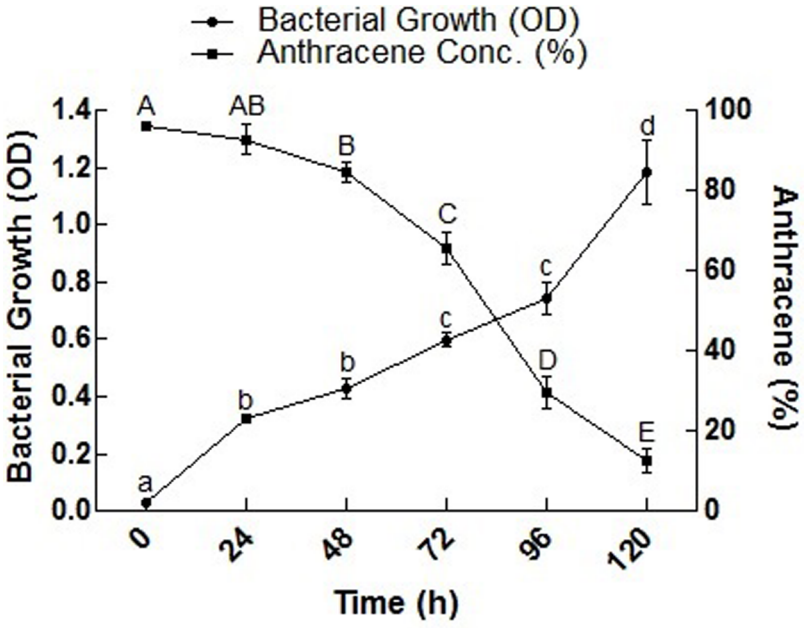

Fig 4. Anthracene disappearance by bacterial isolate S_{13}. Each bar represents the mean of triplicated data with ±S.E. The bars with similar letters are not significantly different at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0201620.g004

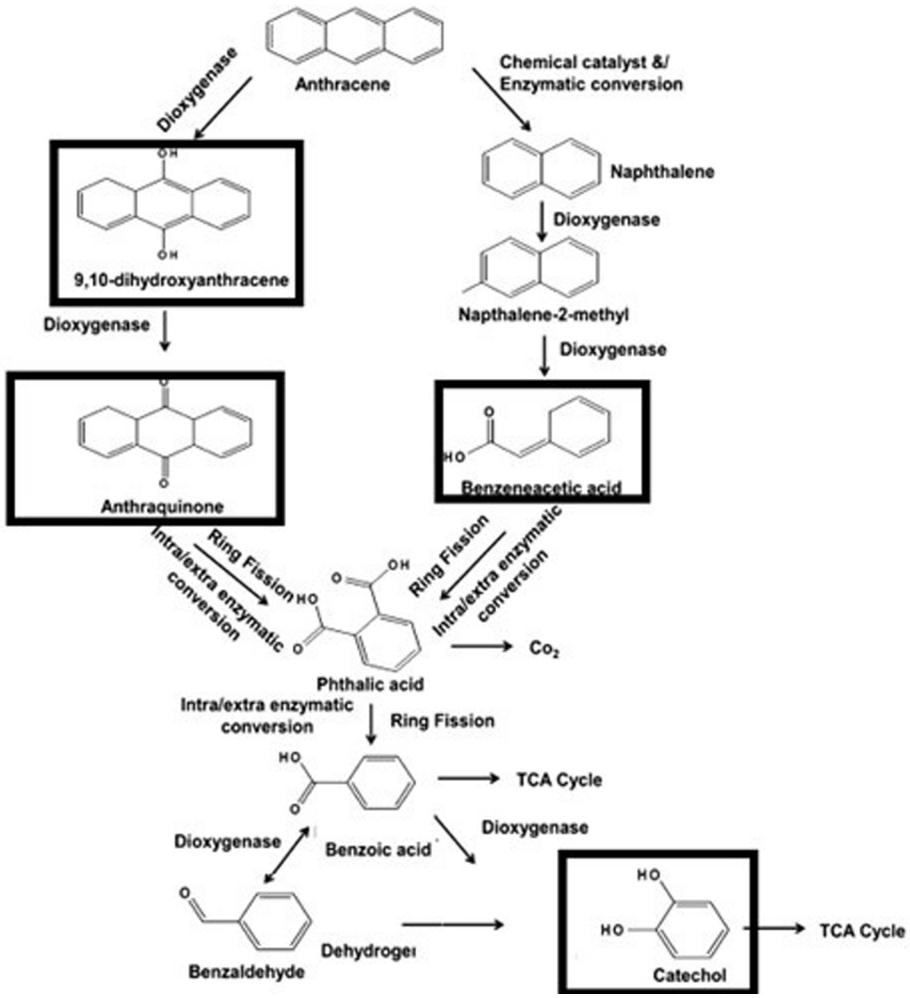

Fig 5. Biodegradation pathway of anthracene. Bold squares show the intermediates detected using GC-MS analysis of biodegraded samples by bacterial isolate S_{13}.

https://doi.org/10.1371/journal.pone.0201620.g005

highest sequence similarity (98%) between the bacterial isolate S_{13}, capable of anthracene utilization as carbon and energy and *Bacillus cereus* RNS-1, *Bacillus cereus* strain LP20-03. The strain also showed 87% similarity with *Bacillus thurengensis* strain 13. On the basis of sequence homology and phylogenetic analysis, the isolated bacterial strain was identified as S_{13} strain of *B. cereus* (Fig 9).

Discussion

Polycyclic aromatic hydrocarbons are the main concern for the world environment that causes great damage to humans, plants and animals wellbeing. In the current study, we have identified an isolate S_{13} from rhizospheric soil samples collected from *S. irio* with high activity against anthracene. Furthermore, the observed bacterial isolate had achieved higher growth at an increased level of anthracene from 100–1000 ppm. Similar results have been reported in the past where higher growth was attained by the bacterial strain in a medium enriched with anthracene [31]. Other factors that can be detrimental to bacterial growth and activity to digest PAHs include temperature, pH, aeration and the presence of nutrients in the medium.

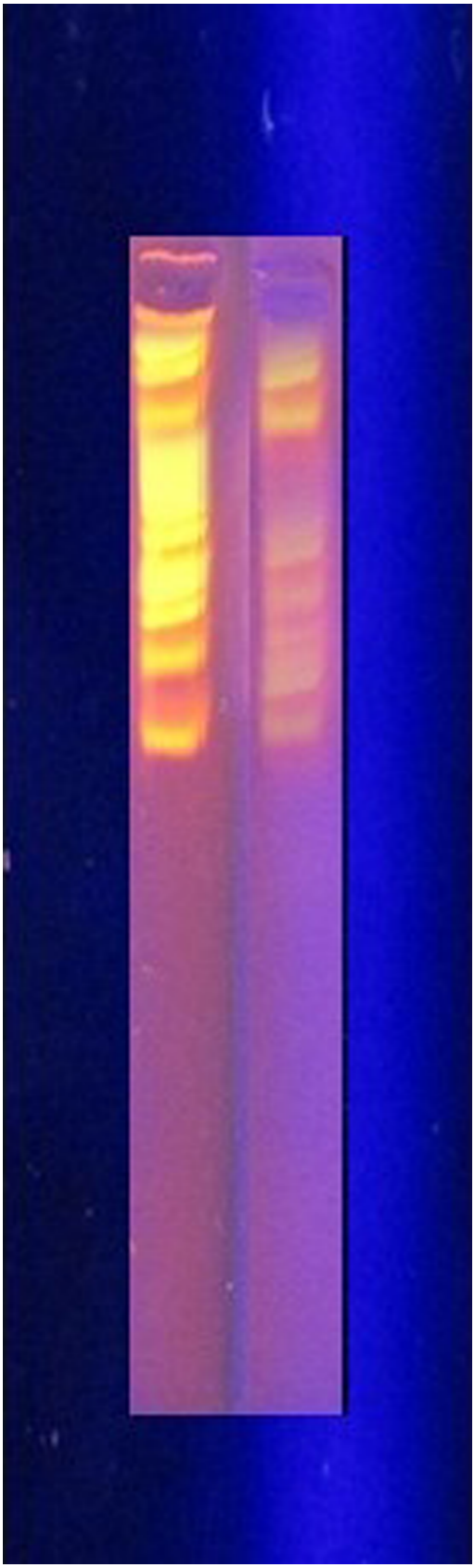

Fig 6. Plasmid bands from bacterial isolate S_{13}. 1 Kb ladder is on the left side, whereas S_{13} plasmid is on the right side of the figure.

https://doi.org/10.1371/journal.pone.0201620.g006

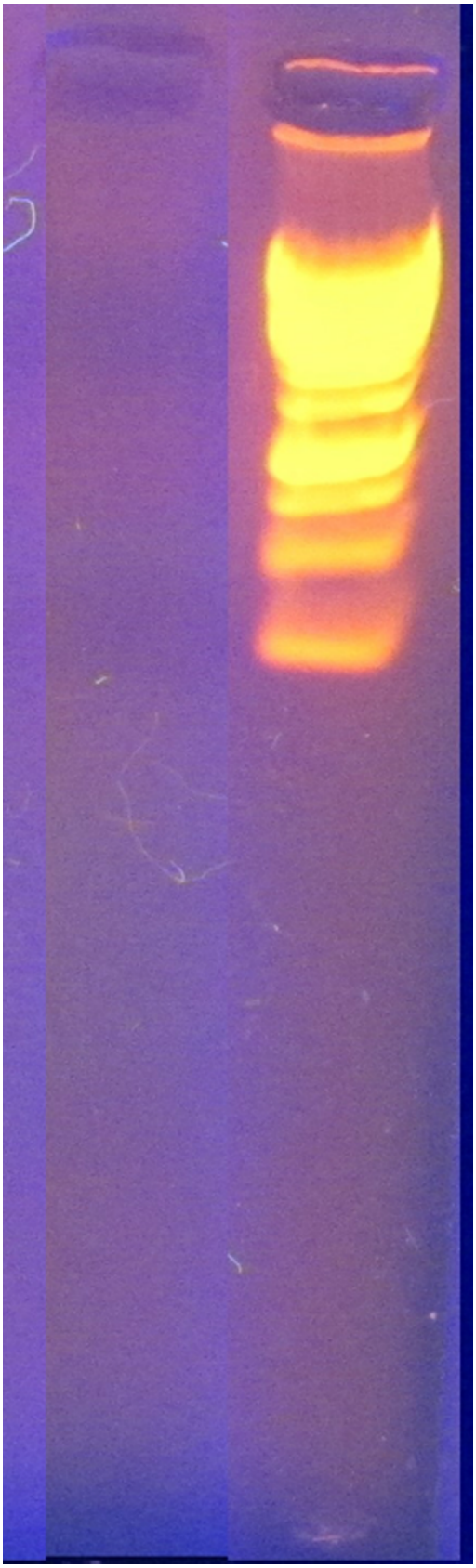

Fig 7. Cured plasmid sample from bacterial isolate S_{13} against 1 Kb ladder. No band can be seen (Left).

https://doi.org/10.1371/journal.pone.0201620.g007

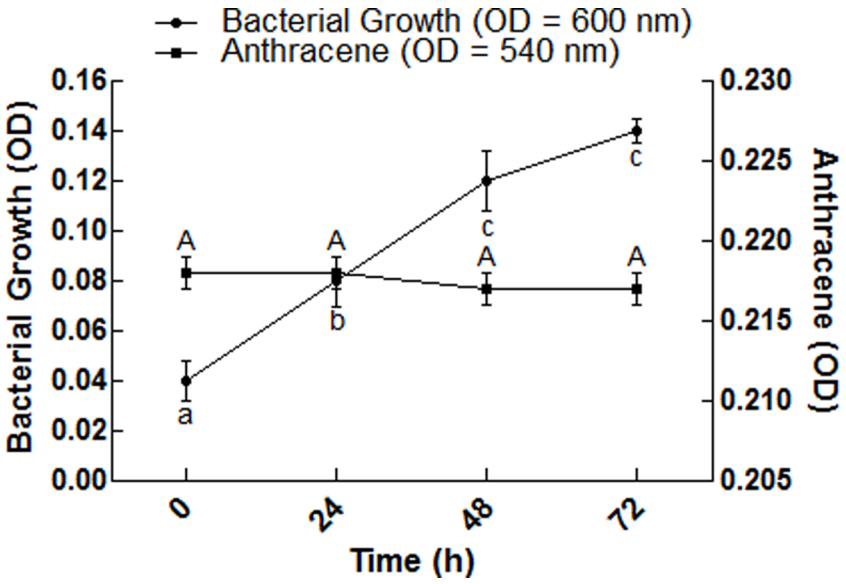

Fig 8. Degradation of anthracene by bacterial isolate S_{13} after plasmid curing. The OD of bacterial growth was observed at 600 nm; the OD of anthracene concentration was observed at 540 nm. Each data point represents the mean of triplicated data with ±S.E. The data points with similar letters are not significantly different at $P < 0.05$.

https://doi.org/10.1371/journal.pone.0201620.g008

Rise in temperature can affect both solubility [40] and degradation of PAHs by bacteria. For instance, high temperature can make the PAHs more soluble and bioavailable, whereas it also decreases the solubility of oxygen that can mainly affect the activity of aerobic bacteria. Therefore, most of the previous researches tend to focus on moderate temperatures rather high or low temperatures. Likewise, in the present study, high anthracene degradation has been noticed at 30˚C, which can be attributed to the optimal growth conditions of the selected strain. The optimal growth conditions can allow the MO to secrete a vast array of enzymes in the surroundings that can degrade the toxic compounds in question. Unsuitable temperatures can deter the enzyme action by blocking its access to substrate due to insolubility (low temperature) or effecting the confirmation of the enzymes (high temperature).

Similarly, all MO can perform its activity at certain pH range, i.e. minimal, maximal and an optimal pH, where at optimal pH the activity of the MO is significantly high. Any drastic changes in pH can interfere with cell wall and cell contents of the MO, thus affecting its growth and metabolism [3]. The result of this study also revealed that at pH7 the growth and activity of the bacterial isolate S_{13} was high due to balanced ionic distribution inside and outside of the cells. However, changes in pH can disturb the ionic balance and disrupt the growth and metabolism of the bacterial isolate S_{13}, resulting in low degradation of PAHs.

Shaking speed also proved to be an important factor in the aerobic degradation of PAHs that needed to be optimized in order to achieve optimum bacterial growth and degradation of the pollutants. Proper aeration has considerably improved the growth of S_{13} in the anthracene supplemented medium. Faster agitation could result in a higher degradation rate, which can

Table 4. Biochemical tests for the identification of isolate S_{13}.

Microscopy	Biochemical tests				
Rods	Gram's Test	Catalase	Starch hydrolysis	Citrate	Urease
	+ive	+ive	+ive	+ive	+ive

https://doi.org/10.1371/journal.pone.0201620.t004

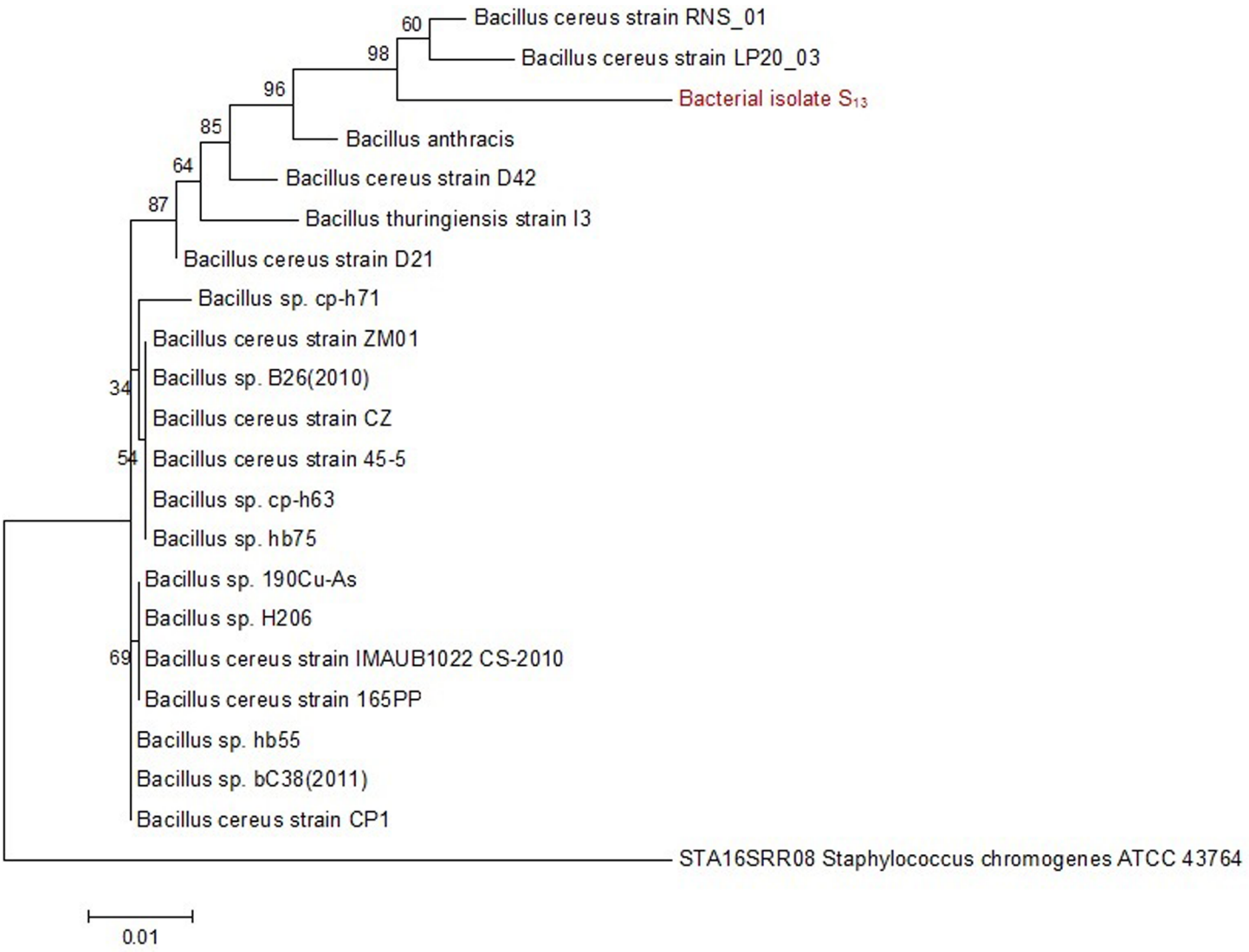

Fig 9. Phylogenetic analysis of strain S_{13}. The evolutionary history was inferred by using the Tamura-Nei model Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7.

https://doi.org/10.1371/journal.pone.0201620.g009

be credited to sufficient supply of oxygen and dissolution of solute in the solvent to be taken up by microorganism [41]. Also, the degradation of anthracene by S_{13} might be facilitated by oxygenase enzyme. The level of enzyme production and activity might enhance in the presence of molecular oxygen that can lead to a complete degradation of anthracene. Certainly, ample supply of oxygen could efficiently incorporate it enzymatically in the aromatic ring of the anthracene, a rate regulating step in the biodegradation of PAHs [26, 42, 43].

Some microorganisms can consume PAHs as a source of carbon and energy [44], but the presence of glucose in the form of additional carbon source might effect it [45]. This has been confirmed by the present study, where S_{13} failed to digest the anthracene completely as a sole carbon source in the presence of glucose, fructose and sucrose. This means that supplementation of media with any alternate carbon sources can influence the degradation of anthracene. The readily available carbon sources may negate the degradation of anthracene due to prior

assimilation, thus causing inhibition of enzymes responsible for anthracene degradation [46]. Correspondingly, supplementation of the growth media with different nitrogen sources (KNO_3, $NaNO_3$, $CaNO_3$ and NH_4NO_3) has no significant effect on growth and degradation activity of anthracene. The negative effect of different energy and nitrogen sources in this study might be linked to its role as a competitor to anthracene, making anthracene less available to be degraded.

Anthracene is hydrophobic due to its cyclic structure and lack of highly hydrophilic hydroxyl groups (-OH) that might be the reason for the slow rate of biodegradation by the MO. The rate of bioremediation of a pollutant mainly depends on the number and nature of degrading organism, intrinsic and extrinsic factors, solvent and chemical structure of the compound to be degraded. Algae, fungi and bacteria have the capabilities to degrade PAHs into less complex substances through biotransformation mechanisms [16]. Though, MO needs to adapt the conditions first that allow the fast growth of microbial populations with the ability to degrade PAHs [47]. Additionally, bioavailability-induced adaptations are equally important for microbial populations to build an interaction with contaminants and make it more bioaccessible [48]. Similarly, the presence of other contaminants can affect the efficiency of microbial degradation, which is critical in terms of biodegradation and bioremediation. Despite well-studied phenomenon, there remains limited understanding of many fundamental aspects of plant-microbe interactions during PAHs phytoremediation [49, 50]. In the present study, it was noticed that 82.92% anthracene was degraded in 120 h in PNR media contrary to the past reports. Previously, 74.8% anthracene degradation was recorded in BSM media on the 10^{th} day of incubation, whereas the complete degradation of added anthracene to autoclaved soil by *Burkholderia* sp. has taken 20 days [10, 51]. In fact, the chemical structure and nature of a compound (including, molecular weight, water solubility and lipophobicity) and nature of MO would affect the bioaccumulation and the rate of degradation of the compound by MO. The results of this study have confirmed this argument, where bacterial isolate S_{13} has actively degraded anthracene (degradation rate = 82.29%) within six days, contrary to previous reports (89% degradation of three ring PAHs within seven weeks) [52, 53].

Conclusion

Bacillus cereus S_{13} can be used for biodegradation of anthracene, which is the main pollutants of incomplete organic combustion produced by petroleum and coal industry. We isolated novel anthracene biodegrading bacterium. Our isolate used anthracene as a sole source of carbon and it can be utilized for bioremediation of other PAHs. *Bacillus cereus* S_{13} can be a potential tool for bioremediation of toxic hydrocarbons and to keep the environment free from PAH pollutants. Though, the development of precise and effective technology for the treatment of complex PAHs mixtures is still needed.

Author Contributions

Conceptualization: Muhammad Hamayun, Sumera Afzal Khan, In-Jung Lee.

Data curation: Amjad Iqbal.

Formal analysis: Muhammad Hamayun, Sumera Afzal Khan, Amjad Iqbal.

Funding acquisition: In-Jung Lee.

Investigation: Nadia Bibi, Badshah Islam, Farooq Shah, Muhammad Aaqil Khan.

Project administration: In-Jung Lee.

Resources: Muhammad Hamayun, In-Jung Lee.

Supervision: Muhammad Hamayun, In-Jung Lee.

Writing – original draft: Muhammad Hamayun, Sumera Afzal Khan, Amjad Iqbal.

Writing – review & editing: Muhammad Hamayun, Sumera Afzal Khan, Amjad Iqbal, In-Jung Lee.

References

1. Jacques RJS, Santos EC, Bento FM, Peralba MCR, Selbach PA, Sá ELS, et al. Anthracene biodegradation by Pseudomonas sp. isolated from a petrochemical sludge landfarming site. Int Biodeter Biodegr. 2005; 56(3):143–50. http://dx.doi.org/10.1016/j.ibiod.2005.06.005.
2. Othman N HN, Talib SA. Degradation of polycyclic aromatic hydrocarbon by pure strain isolated from municipal sludge: Synergistic and cometabolism phenomenon. International Conference on Environment. 2010:86–90.
3. Nour SEG MY, Habib SA, Ali S. Evaluation of Corynebacterium variabilis Sh42 as a degrader for different poly aromatic compounds. Journal of American Science. 2010; 6(11):343–56.
4. Heitkamp MA, Cerniglia CE. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl Environ Microb. 1988; 54(6):1612–4. PubMed PMID: PMC202706.
5. Bamforth SM SI. Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. Journal of Chemical Technology and Biotechnology. 2005; 80:723–36.
6. Hickey AM, Gordon L, Dobson AD, Kelly CT, Doyle EM. Effect of surfactants on fluoranthene degradation by Pseudomonas alcaligenes PA-10. Appl Microbiol Biotechnol. 2007; 74(4):851–6. Epub 2006/11/16. https://doi.org/10.1007/s00253-006-0719-5 PMID: 17106676.
7. Wong JWC, Lai KM, Wan CK, Ma KK, Fang M. Isolation and Optimization of PAH-Degradative Bacteria from Contaminated Soil for PAHs Bioremediation. Water, Air, and Soil Pollution. 2002; 139(1):1–13. https://doi.org/10.1023/a:1015883924901
8. Sun R, Jin J, Sun G, Liu Y, Liu Z. Screening and degrading characteristics and community structure of a high molecular weight polycyclic aromatic hydrocarbon-degrading bacterial consortium from contaminated soil. Journal of Environmental Sciences. 2010; 22(10):1576–85. http://dx.doi.org/10.1016/S1001-0742(09)60292-8.
9. Lily MK BA, Bhatt KK, Dangwal K. Degradation of Anthracene by a novel strain Brachybacterium paraconglomeratum BMIT637C (MTCC 9445). International Journal of Environmental Sciences. 2013; 3(4):1242–52.
10. Dean-Ross D, Moody JD, Freeman JP, Doerge DR, Cerniglia CE. Metabolism of anthracene by a Rhodococcus species. Fems Microbiol Lett. 2001; 204(1):205–11. Epub 2001/10/30. PMID: 11682202.
11. Shahsavari E, Poi G, Aburto-Medina A, Haleyur N, Ball AS. Bioremediation approaches for petroleum hydrocarbon-contaminated environments. Enhancing Cleanup of Environmental Pollutants: Springer; 2017. p. 21–41.
12. Shokrollahzadeh SG SF. Study of Sphingopyxis isolates in degradation of polycyclic aromatic hydrocarbons. Chemical Engineering Transactions. 2012; 27:55–60.
13. Haleyur N, Shahsavari E, Taha M, Khudur LS, Koshlaf E, Osborn AM, et al. Assessing the degradation efficacy of native PAH-degrading bacteria from aged, weathered soils in an Australian former gasworks site. Geoderma. 2018; 321:110–7.
14. Morzik A PS, Labuzek S. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Polish Journal of Environmental Studies. 2003; 12:15–25.
15. van Herwijnen R, Springael D, Slot P, Govers HA, Parsons JR. Degradation of anthracene by Mycobacterium sp. strain LB501T proceeds via a novel pathway, through o-phthalic acid. Appl Environ Microbiol. 2003; 69(1):186–90. Epub 2003/01/07. https://doi.org/10.1128/AEM.69.1.186-190.2003 PMID: 12513994; PubMed Central PMCID: PMCPMC152392.
16. Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, et al. Isolation of Adherent Polycyclic Aromatic Hydrocarbon (PAH)-Degrading Bacteria Using PAH-Sorbing Carriers. Appl Environ Microb. 2000; 66(5):1834–43. PubMed PMID: PMC101420.
17. Cerniglia CE. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation. 1992; 3(2):351–68. https://doi.org/10.1007/bf00129093

18. Harayama S, Kasai Y, Hara A. Microbial communities in oil-contaminated seawater. Current opinion in biotechnology. 2004; 15(3):205–14. Epub 2004/06/15. https://doi.org/10.1016/j.copbio.2004.04.002 PMID: 15193328.

19. Koshlaf E, Shahsavari E, Aburto-Medina A, Taha M, Haleyur N, Makadia TH, et al. Bioremediation potential of diesel-contaminated Libyan soil. Ecotoxicology and environmental safety. 2016; 133:297–305. https://doi.org/10.1016/j.ecoenv.2016.07.027 PMID: 27479774

20. Milic JS BV, Ilic MV, Ali SAM, Cvijovic GDG, Vrvic MM. Bioremediation of soil heavily contaminated with crude oil and its products: composition of the microbial consortium. J Serb Chem Soc. 2009; 77:455–60.

21. Lee S-H, Lee W-S, Lee C-H, Kim J-G. Degradation of phenanthrene and pyrene in rhizosphere of grasses and legumes. J Hazard Mater. 2008; 153(1):892–8. http://dx.doi.org/10.1016/j.jhazmat.2007.09.041.

22. Joner EJ, Leyval C. Rhizosphere gradients of polycyclic aromatic hydrocarbon (PAH) dissipation in two industrial soils and the impact of arbuscular mycorrhiza. Environ Sci Technol. 2003; 37(11):2371–5. Epub 2003/07/02. PMID: 12831019.

23. Sheng-wang PAN, Shi-qiang WEI, Xin Y, Sheng-xian CAO. The Removal and Remediation of Phenanthrene and Pyrene in Soil by Mixed Cropping of Alfalfa and Rape. Agricultural Sciences in China. 2008; 7(11):1355–64. http://dx.doi.org/10.1016/S1671-2927(08)60185-6.

24. Ebrahimi M SM, Fallah R. Assessment of biodegradation efficiency of some isolated bacteria from oil contaminated sites in solid and liquid media containing oil-compounds. International Research Journal of Applied and Basic Sciences. 2012; 3(1):138–47.

25. Alias S A-TS, Omar M, Hussain NH. Degradation of 3-ring PAHs by S spiritovorum and C urealyticum strain under non- indigenous condition," in International congress of chemistry and environment (ICCE 2011), Port Dockson Malaysia. 2011.

26. Kumar G SR, Kumar R. Plasmid associated Anthracene degradation by Pseudomonas sp. isolated from filling station site. Nature and Science. 2010; 8:89–94.

27. Khan K NM, Arshed MJ, Asif M. Extraction and characterization of oil degrading bacteria. Journal of Applied Sciences. 2006; 6(10):2302–6.

28. Abdelhay A, Magnin J-P, Gondrexon N, Baup S, Willison J. Optimization and modeling of phenanthrene degradation by Mycobacterium sp. 6PY1 in a biphasic medium using response-surface methodology. Appl Microbiol Biot. 2008; 78(5):881–8. https://doi.org/10.1007/s00253-008-1365-x PMID: 18256822

29. Leahy JG, Colwell RR. Microbial degradation of hydrocarbons in the environment. Microbiological Reviews. 1990; 54(3):305–15. PubMed PMID: PMC372779. PMID: 2215423

30. Guo C DZ, Wong Y, Tam N. Biodegradation ability and dioxygenase genes of PAH-degrading Sphingomonas and Mycobacterium strains isolated from mangrove sediments. Int Biodeter Biodegr. 2010; 64(6):419–26.

31. Chulalaksananukul S, Gadd GM, Sangvanich P, Sihanonth P, Piapukiew J, Vangnai AS. Biodegradation of benzo(a)pyrene by a newly isolated Fusarium sp. FEMS Microbiol Lett. 2006; 262(1):99–106. Epub 2006/08/16. https://doi.org/10.1111/j.1574-6968.2006.00375.x PMID: 16907745.

32. Kiyohara H, Nagao K, Yana K. Rapid Screen for Bacteria Degrading Water-Insoluble, Solid Hydrocarbons on Agar Plates. Appl Environ Microb. 1982; 43(2):454–7. PubMed PMID: PMC241847.

33. Neelofur M SPMM. Enhance the biodegradation of Anthracene by mutation from Bacillus species. BMR Biotechnology. 2014; 1:1–19.

34. Arulazhagan P, Vasudevan N. Biodegradation of polycyclic aromatic hydrocarbons by a halotolerant bacterial strain Ochrobactrum sp. VA1. Mar Pollut Bull. 2011; 62(2):388–94. Epub 2010/10/12. https://doi.org/10.1016/j.marpolbul.2010.09.020 PMID: 20934193.

35. Mukesh KDJ AM, Devika S, Balakumaran MD, Kalaichelvan PT. Biodegradation of polycyclic aromatic hydrocarbons by Pseudomonas sp. PSS6 isolated from municipal wastes sediment. Der Chemica Sinica. 2012; 3:543–7.

36. Nnamchi CI, Obeta JAN, Ezeogu LI. Isolation and characterization of some polycyclic aromatic hydrocarbon degrading bacteria from Nsukka soils in Nigeria. International Journal of Environmental Science & Technology. 2006; 3(2):181–90. https://doi.org/10.1007/bf03325924

37. Boonchan S, Britz ML, Stanley GA. Degradation and Mineralization of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Defined Fungal-Bacterial Cocultures. Appl Environ Microb. 2000; 66(3):1007–19. PubMed PMID: PMC91936.

38. Moody JD, Freeman JP, Doerge DR, Cerniglia CE. Degradation of phenanthrene and anthracene by cell suspensions of Mycobacterium sp. strain PYR-1. Appl Environ Microbiol. 2001; 67(4):1476–83. Epub 2001/04/03. https://doi.org/10.1128/AEM.67.4.1476-1483.2001 PMID: 11282593; PubMed Central PMCID: PMCPMC92757.

39. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

40. Bhattacharya S, Das A, G M, K V, J S. Mycoremediation of congo red dye by filamentous fungi. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]. 2011; 42(4):1526–36. Epub 2011/10/01. https://doi.org/10.1590/S1517-838220110004000040 PMID: 24031787; PubMed Central PMCID: PMCPMC3768715.

41. Ye JS, Yin H, Qiang J, Peng H, Qin HM, Zhang N, et al. Biodegradation of anthracene by Aspergillus fumigatus. J Hazard Mater. 2011; 185(1):174–81. Epub 2010/10/12. https://doi.org/10.1016/j.jhazmat.2010.09.015 PMID: 20932640.

42. Macleod CJ, Semple KT. The influence of single and multiple applications of pyrene on the evolution of pyrene catabolism in soil. Environ Pollut. 2006; 139(3):455–60. Epub 2005/08/23. https://doi.org/10.1016/j.envpol.2005.06.014 PMID: 16112311.

43. Puglisi E, Cappa F, Fragoulis G, Trevisan M, Del Re AA. Bioavailability and degradation of phenanthrene in compost amended soils. Chemosphere. 2007; 67(3):548–56. Epub 2006/11/28. https://doi.org/10.1016/j.chemosphere.2006.09.058 PMID: 17125813.

44. Rugh CL, Susilawati E, Kravchenko AN, Thomas JC. Biodegrader metabolic expansion during polyaromatic hydrocarbons rhizoremediation. Zeitschrift fur Naturforschung C, Journal of biosciences. 2005; 60(3–4):331–9. Epub 2005/06/14. PMID: 15948603.

45. Fernández-Luqueño F, Marsch R, Espinosa-Victoria D, Thalasso F, Lara MH, Munive A, et al. Remediation of PAHs in a saline–alkaline soil amended with wastewater sludge and the effect on dynamics of C and N. Science of the Total Environment. 2008; 402(1):18–28. https://doi.org/10.1016/j.scitotenv.2008.04.040 PMID: 18538824

46. Sartoros C, Yerushalmi L, Béron P, Guiot SR. Effects of surfactant and temperature on biotransformation kinetics of anthracene and pyrene. Chemosphere. 2005; 61(7):1042–50. http://dx.doi.org/10.1016/j.chemosphere.2005.02.061. PMID: 16197980

47. Doick KJ, Dew NM, Semple KT. Linking catabolism to cyclodextrin extractability: determination of the microbial availability of PAHs in soil. Environ Sci Technol. 2005; 39(22):8858–64. Epub 2005/12/06. PMID: 16323787.

48. Li X, Li P, Lin X, Zhang C, Li Q, Gong Z. Biodegradation of aged polycyclic aromatic hydrocarbons (PAHs) by microbial consortia in soil and slurry phases. J Hazard Mater. 2008; 150(1):21–6. http://dx.doi.org/10.1016/j.jhazmat.2007.04.040. PMID: 17512657

49. Santos EC, Jacques RJS, Bento FM, Peralba MdCR, Selbach PA, Sá ELS, et al. Anthracene biodegradation and surface activity by an iron-stimulated Pseudomonas sp. Bioresource Technol. 2008; 99(7):2644–9. http://dx.doi.org/10.1016/j.biortech.2007.04.050.

50. Somtrakoon K, Suanjit S, Pokethitiyook P, Kruatrachue M, Lee H, Upatham S. Enhanced biodegradation of anthracene in acidic soil by inoculated Burkholderia sp. VUN10013. Curr Microbiol. 2008; 57(2):102–6. Epub 2008/04/02. https://doi.org/10.1007/s00284-008-9157-1 PMID: 18379840.

51. Simarro R, Gonzalez N, Bautista LF, Molina MC. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by a wood-degrading consortium at low temperatures. Fems Microbiol Ecol. 2013; 83(2):438–49. Epub 2012/09/12. https://doi.org/10.1111/1574-6941.12006 PMID: 22963246.

52. Swaathy S, Kavitha V, Pravin AS, Mandal AB, Gnanamani A. Microbial surfactant mediated degradation of anthracene in aqueous phase by marine Bacillus licheniformis MTCC 5514. Biotechnology Reports. 2014; 4:161–70. http://dx.doi.org/10.1016/j.btre.2014.10.004. PMID: 28626676

53. Kafilzadeh F, Khosrobak A, Jamali H. Isolation and Identification of Phenanthrene Degrading Bacteria from the Soil around Oil Company of Andimeshk and Investigation of Their Growth Kinetics. Polycyclic Aromatic Compounds. 2016; 36(1):58–71. https://doi.org/10.1080/10406638.2015.1037006