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Molecular and metabolic characterization of petroleum hydrocarbons degrading *Bacillus cereus*

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Abstract

Hydrocarbon constituents of petroleum are persistent, bioaccumulated, and bio-magnified in living tissues, transported to longer distances, and exert hazardous effects on human health and the ecosystem. Bioaugmentation with microorganisms like bacteria is an emerging approach that can mitigate the toxins from environmental sources. The present study was initiated to target the petroleum-contaminated soil of gasoline stations situated in Lahore. Petroleum degrading bacteria were isolated by serial dilution method followed by growth analysis, biochemical and molecular characterization, removal efficiency estimation, metabolites extraction, and GC-MS of the metabolites. Molecular analysis identified the bacterium as Bacillus cereus, which exhibited maximum growth at 72 hours and removed 75% petroleum. Biochemical characterization via the Remel RapID™ ONE panel system showed positive results for arginine dehydrolase (ADH), ornithine decarboxylase (ODC), lysine decarboxylase (LDC), *o*-nitrophenyl-β-D-galactosidase (ONPG), *p*-nitrophenyl-β-D-glucosidase (βGLU), *p*-nitrophenyl-N-acetyl-β-D-glucosaminidase (NAG), malonate (MAL), adonitol fermentation (ADON), and tryptophane utilization (IND). GC-MS-based metabolic profiling identified alcohols (methyl alcohol, *o*-, *p*- and *m*-cresols, catechol, and 3-methyl catechol), aldehydes (methanone, acetaldehyde, and *m*-tolualdehyde), carboxylic acid (methanoic acid, *cis,cis*-muconic acid, cyclohexane carboxylic acid and benzoic acid), conjugate bases of carboxylic acids (benzoate, *cis,cis*-muconate, 4-hydroxybenzoate, and pyruvate) and cycloalkane (cyclohexene). It suggested the presence of methane, methylcyclohexane, toluene, xylene, and benzene degradation pathways in *B. cereus*.

Keywords: petroleum, hydrocarbons, bioremediation, degradation, *B. cereus*, pollutants, GC-MS, metabolites

Introduction

Petroleum, also referred to as rock oil, is a fossil fuel chemically composed of hydrocarbons, i.e., alkanes, cycloalkanes, olefins, aromatics, and polyaromatic hydrocarbons (PAHs) (Speight and Arjoon 2012; Arjoon and Speight 2020). The aromatics include benzene, alkylbenzenes, isopropylbenzene (cumene), nitrobenzene, benzoic acid, toluene diisocyanate (TDI), naphthalene, toluene and xylenes (Podgorski et al. 2013). It is refined to yield many valuable products, including diesel, petrol, and jet fuel, lubricating and heating oil, asphalt, waxes, natural gas, and jet fuel. These chemicals play a crucial role in the economy of a country due to their extensive use in the petrochemical industry in the production of fertilizers, plastics, synthetic fibers, insecticides, perfumes, kerosene, liquefied petroleum and natural gas, butane, propane, naphtha, naphthalene, bitumen, pesticides, nylon, paints, paraffin wax, drugs, explosives, and rubber (Mori 2023). The extensive involvement of petroleum in human civilization contributes to the oily waste produced during the processing, refining, and production but also due to spill and leakage during exploration, drilling, unplugging of wells, storage, and transportation (Ezeonu et al. 2012). An accidental petroleum spill due to pipeline breakage

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on the coast of California proved disastrous for wildlife habitats and killed fishes, birds, and algae (Ramírez-Camacho et al. 2017). The oily waste is causing devastating ecological issues like plastic pollution, species extinction, air and water pollution, global warming, natural resource depletion, and deforestation (da Silva et al. 2012; Li et al. 2020). It damages plant life by inducing oxidative stress, decreasing nutrient transportation and germination of seeds, and interfering with metabolism, physiology, and morphology (Haider et al. 2021). Human health effects exerted by total petroleum hydrocarbons (TPH) include fatigue, irritation of the stomach and throat, depression, difficulty breathing, nausea, drowsiness, headache, pneumonia, peripheral neuropathy, cancer, respiratory diseases, and even death (Adipah 2019; Abereton et al. 2023). Keeping in view these hazardous effects in addition to stability, longevity, and the tendency of bioaccumulation and biomagnification, the Environment Protection Agency (EPA) has categorized petroleum as a Persistent Organic Pollutant (POPs) (Ossai et al. 2020).

Mitigating measures taken conventionally for TPH degradation include physical methods, such as flotation, ultrasonication, incineration, electrokinetics remediation, microwave frequency heating, thermal desorption, and chemical methods comprising of synthetic detergents/surfactants, soil flushing technique, and photocatalytic removal. These conventional strategies are accompanied by some limitations like high cost, more time, greater exposure for workers to hazardous chemicals, and partial removal of pollutants (Lim et al. 2018; Wulandari and Effendi 2018; Sivagami et al. 2019; Zhao et al. 2019; Hao et al. 2020; Koe et al. 2020; Ossai et al. 2020; Liu et al. 2021a; Tran et al. 2022).

Researchers have always been trying to explore suitable, cost-effective, less time-consuming, and eco-friendly alternatives to these traditional methods. These efforts led to the emerging technique of bioremediation. i.e., the breakdown of TPH using living organisms like bacteria, fungi, and plants. Bacteria are getting attention as the best bioremediating agents because of their fast growth rate, tolerance to a wide range of temperatures (0 to 120°C) (An et al. 2023), salinity (0.3 M to 5.9 M) (Mousa et al. 2023) and pH (3 to 9) (Liu et al. 2015). On the contrary, fungi are mostly mesophilic (surviving at 25 to 30°C) (Petkova and Shilev 2023) and exhibit efficient biomass growth and sporulation only at a pH range of 5.5 to 6.5 (Gomes et al. 2018). Bacteria with efficient metabolic potential exhibit diversity and are found in the different phylum of the kingdoms/domains, i.e., Actinomycetota/Actinomycetia (*Micrococcus*), Bacteroidota/Flavobacteriia (*Flavobacterium*), Firmicutes (*Bacillus*), Proteobacteria: Pseudomonadota/Alphaproteobacteria (*Rhizobium*), Pseudomonadota/Gammaproteobacteria (e.g., *Acinetobacter*, *Pinisolibacter*, *Pseudomonas* etc. (Table I)).

These bioremediating bacteria utilize the TPH as carbon source and process them via various chemical reactions like reduction, β-oxidation, terminal oxidation, sub-terminal oxidation and ω-oxidation, dehydration, acetylation and hydroxylation, converting them into simpler compounds that can be shunt into central carbon metabolic pathway via tricarboxylic acid cycle (TCA) (Abbasian et al. 2015; Ahmed et al. 2023).

Table I
Petroleum hydrocarbons degrading bacteria reported in the literature.

Bacteria	References
Acinetobacter XS-4	Zou et al. 2023
Neorhizobium, *Allorhizobium*, *Rhizobium*, *Pararhizobium*, *Pseudomonas*, *Nocardioides*, *Simplicispira*	Eziuzor and Vogt 2023
Dehalococcoidia	Zehnle et al. 2023
Pinisolibacter aquiterrae	Bedics et al. 2022
Enterobacter	Hossain et al. 2022
Talaromyces sp.	Zhang et al. 2021
Pseudomonas pseudoalcaligenes, *Rhodococcus*	Feng et al. 2021; Chuah et al. 2022
Aquabacterium	Xu et al. 2019
Nesiotobacter exalbescens	Ganesh Kumar et al. 2019
Bradyrhizobium, *Koribacter*, *Acidimicrobium*	Jeffries et al. 2018
Sphingomonas	Zhou et al. 2016
Exiguobacterium aurantiacum	Mohanty and Mukherji 2008
Bacillus subtilis, *Alcaligenes* sp., *Flavobacterium* sp., *Micrococcus roseus*, *Corynebacterium* sp.	Adebusoye et al. 2007
Marinobacter, *Alcanivorax*, *Sphingomonas*, *Gordonia*, *Micrococcus*, *Cellulomonas*, *Dietzia*	Brito et al. 2006

Thus, ultimately, these hydrocarbons are destined for obtaining energy and assimilation into biomass. To accomplish these reactions, different bacteria have evolved different pathways for various petroleum-based hydrocarbons. Like, alkane degradation pathways, i.e., terminal oxidation, subterminal oxidation pathway, diterminal oxidation, and finnerty way pathways reported in *Alcanivorax borkumensis*, *Pseudomonas putida*, *Geobacillus thermodenitrificans* NG80-2, *Pseudomonas aeruginosa*, *Gordonia* sp. strain TY-5, *Acinetobacter* sp. and, *Falsochrobactrum tianjinense*, respectively (Hu et al. 2022; Wang et al. 2023). Additionally, petroleum aromatic compounds degradation mechanisms have also been reported in literature like o-ring cleavage and m-ring cleavage pathways, toluene 4-monooxygenase pathway (TMO), toluene 3-monooxygenase pathway (Tbu), toluene 2-monooxygenase pathway (TOM), toluene dioxygenase pathway (Tod), toluene methyl monooxygenase pathway (TOL) for toluene, salicylate pathway, gentisate pathway, procatechuic acid pathways for naphthalene, anaerobic benzene degradation pathways via methylation, hydroxylation and carboxylation, xylene degradation pathways via phenol hydroxylase, monooxygenase and dioxygenase routes, β-ketoadipic acid pathway of benzoic acid degradation and the novel pathways of toluene and naphthalene degradation involving the cyclohexene, cyclohexane, cyclohexanol and cyclohexanone formation (Reiner 1971; Denome et al. 1993; Goyal and Zylstra 1997; Abu Laban et al. 2010; Brzeszcz and Kaszycki 2018; Di Canito et al. 2018; Muccee et al. 2019; Muccee et al. 2021). Despite the significant contribution to TPH degradation, bioremediation also has limitations, such as less contaminant bioavailability and the production of more toxic compounds (Sharma 2020; Kumar et al. 2022). However, these issues can be resolved by integrating multiple bioremediation approaches and optimizing the physical conditions in the future (Salari et al. 2022).

Considering the hazardous effects associated with TPH and the need for fast-growing bacteria with new and highly efficient degradation pathways in our native unexplored area of Lahore, we initiated the present project. By exploring the petrol-contaminated soil of gasoline stations in Lahore, we might isolate petroleum-metabolizing bacteria with highly efficient removal potential and novel mechanisms. The study was not only restricted to isolation and general characterization of TPH-degrading bacteria. Rather, it was extended up to the level of metabolomics. Metabolomics profiling of TPH metabolic intermediates and products formed in bacteria on exposure to these hydrocarbons can be fruitful in deducing the confirmed bacterial pathways. Therefore, the study also targeted the comprehensive profiling of intracellular, extracellular, polar, and non-polar bacterial metabolites.

Experimental

Materials and Methods

Isolation. Soil samples were collected from gasoline stations in Lahore at Davice Road and Wahdat Road. To isolate TPH degrading bacteria, 1 g of soil sample was dissolved in the autoclaved Ringers' solution (25 ml) and kept under shaking conditions overnight (37°C, 150 rpm). After 24 hours, ringers' solution was used to make serial dilutions ranging from 10^{-1} to 10^{-7}. A minimal salt medium (MSM) supplemented with petroleum as the sole carbon source was used for isolation, serial dilutions, and spread plate method preparation. This medium comprised of $MgSO_4$ (0.2 g), $CaCl_2$ (0.02 g), K_2HPO_4 (1.0 g), KH_2PO_4 (1.0 g), NH_4NO_3 (1.0 g), $FeCl_3$ (0.05 g) and petroleum 1% v/v (Lima et al. 2019). Nutrient agar was added to MSM to solidify the medium. After spreading, petri plates were incubated at 37°C for one week. Afterward, colony morphology and response to Gram staining were analyzed. The glycerol stock of bacteria was prepared. The stock was saved –30°C.

Growth analysis. A growth curve was constructed to detect the logarithmic phase of bacterial isolate. For this task, the growth assay was performed in triplicates. Overnight-grown cultures with optical density, i.e., $OD_{600} = 1$, were used to get synchronized cultures. The bacteria were cultured in MSM supplemented with petroleum (5 ml/500 ml medium) as a carbon source under shaking at 150 and at 37°C. Midgrade gasoline with middle octane range i.e., 89–90 was used. The turbidity of cultures was determined by measuring the absorbance at regular intervals at 600 nm via a UV-Visible Spectrophotometer. To plot the growth curve, OD was plotted versus time intervals.

Estimation of petroleum removal efficiency. For quantitative estimation of the TPH removal efficiency of bacteria, a previously reported method involving the use of 2,6-dichlorophenolindophenol (DCPIP) as a redox indicator, was employed (Bilen Ozyurek and Seyis Bilkay 2017; Marchand et al. 2017; Muccee and Ejaz 2019). To perform this assay, 1% v/v of DCPIP was added to MSM containing petroleum as a carbon source. To perform assay in triplicates using synchronized cultures, the overnight grown bacteria with OD_{600} of 1 were used as inoculum. Bacteria were allowed to grow under shaking conditions (37°C, 150 rpm) for seven days. During this incubation, the culture was collected at different time intervals, i.e., 0, 24, 48, 72, 96, 120, and 144 hours. The culture was centrifuged to separate the supernatant from pelleted cells, and the supernatant was analyzed spectrophotometrically at 600 nm. The change in DCPIP color was measured in experimental flasks against the control flask (containing only MSM, petroleum, and DCPIP with no inoculum)

from this OD. This color change was directly related to the TPH oxidizing potential of bacteria.

Biochemical characterization. For biochemically characterizing the bacteria, the Remel RapID™ ONE System (Thermo Fisher Scientific, Inc., USA) panel was used. This method enabled us to detect the presence of a variety of enzymes using different reactive ingredients or substrates. Substrates were supplied in dehydrated form in reaction cavities of kit panels. Substrates used included urea, arginine, ornithine, lysine, sorbitol, aliphatic thiol, fatty acid ester, sugar aldehyde, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-β-D-galactoside, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide, malonate, proline β-naphthylamide, γ-glutamyl-β-naphthylamide, adonitol and tryptophane for URE, ADH, ODC, LDC, SBL, TET, LIP, KSF, GUR, ONPG, βGLU, βXYL, NAG, MAL, PRO, GGT, PYR, ADON, and IND test, respectively. These reaction cavities were inoculated with bacterial cells suspended in RapID™ inoculation fluid. It follows the rehydration of substrates and reaction initiation. The color change observed was compared with the differential chart provided with the kit.

Molecular characterization and phylogeny analysis. Initially, the DNA of bacteria was extracted using the organic DNA extraction method (Wright et al. 2017). DNA quantification was performed by agarose gel electrophoresis. Qualified DNA was processed for amplification of the 16S rRNA gene using forward primer AGAGTTTGATCCTGGTCAGAACGAACGCT (GC content = 48.3% and Tm = 70.4°C) and reverse primer CGTACGGCTACCTTGTTACGACTTCACCCC (GC content = 56.7% and Tm = 74.8°C). Following this, amplification was confirmed by agarose gel electrophoresis, and amplicons were sent for Sanger sequencing. The FASTA files obtained were analyzed through BLASTN (http://blast.ncbi.nlm.nih.gov/blast/Blast.cgi), and top BLAST sequence similarity was found (Chen et al. 2015). Then, to study the evolutionary relationship of present study bacteria, bacterial sequences of the 16S rRNA gene available on the NCBI database were retrieved (Schoch et al. 2020). This step followed the multiple sequence alignment via the Clustal Omega multiple sequence alignment tool (http://www.clustal.org/mbed.tgz) (Sievers and Higgins 2021). This multiple alignment was used for phylogenetic tree construction using MEGA version 11 (Tamura et al. 2021). The maximum composite likelihood neighbor-joining tree was constructed using a bootstrap value of 100.

Metabolites extraction and GC-MS analysis. Extracellular and polar, and intracellular polar and non-polar metabolites of *B. cereus* were extracted using the protocols published in (Muccee et al. 2019). Initially, bacteria were cultured and allowed to grow up to its log phase i.e., 51–72 hours, in MSM medium supplemented with midgrade petroleum (5 ml/500 ml medium) as a sole carbon source. The culturing was performed under shaking conditions at 150 rpm and 37°C. At the long phase, when maximum turbidity and OD, i.e., 1.2, were achieved, the culture was centrifuged (6,000 rpm, 10 min, 4°C) to get supernatant and cells as a source of extracellular and intracellular metabolites.

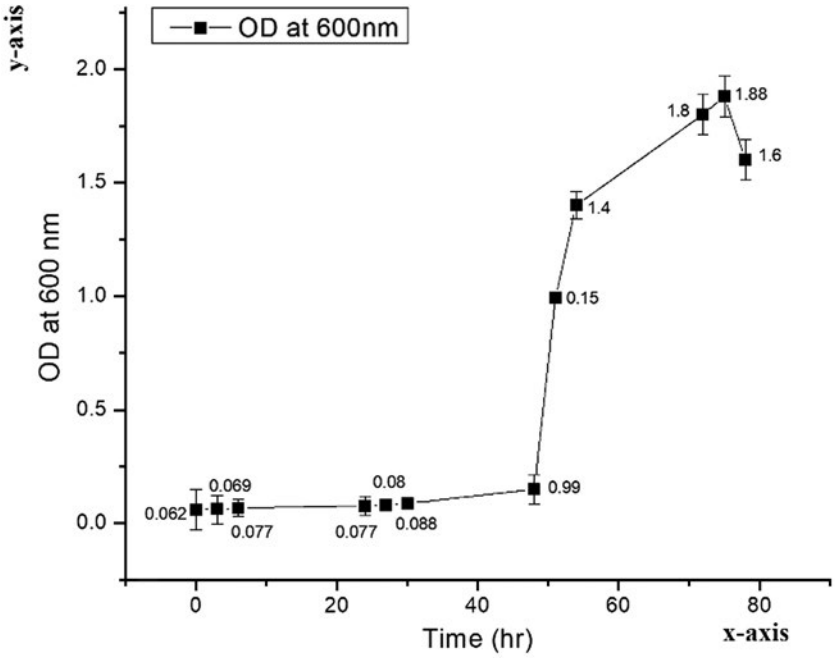
The supernatant (10 ml) was taken in a test tube and methanol (5 ml) was added, followed by centrifugation (10,000 rpm, 20 min, 4°C). Following centrifugation, methanol (3 ml) was collected from the upper surface and transferred to a glass vial. The vial was dried in an incubator at 50°C for 1 week. After drying, methanol evaporated, and bacterial extracellular metabolite extract was achieved, subjected to gas chromatography-mass spectrometry (GCMS) analysis.

To extract intracellular metabolites, the pellet was washed twice with 0.9% NaCl (1 ml) using centrifugation (4,000 rpm, 5 min, 4°C). After discarding NaCl to quench metabolism, methanol and deionized water 400 µl, were added to the washed pellet on ice. Further steps were performed on ice, during which chloroform (400 µl) was added. All the contents were transferred to Eppendorf tubes. Afterward, cells were agitated in a sonicator (4°C, 30 min) and centrifuged (10,000 rpm, 20 min, 4°C) post sonication. It resulted in the formation of three separate phases i.e., upper phase (polar metabolites source), interphase (comprising of proteins) and lower phase (non-polar metabolites source). The upper and lower phases 300 µl each, were carefully transferred to separate glass vials by moving interphase out of the way. Vials were dried in incubator to get extracts of polar and non-polar metabolites. These metabolites were sent for GCMS analysis. For GCMS analysis, already reported parameters were used (Table SI) (Sadiqi et al. 2022).

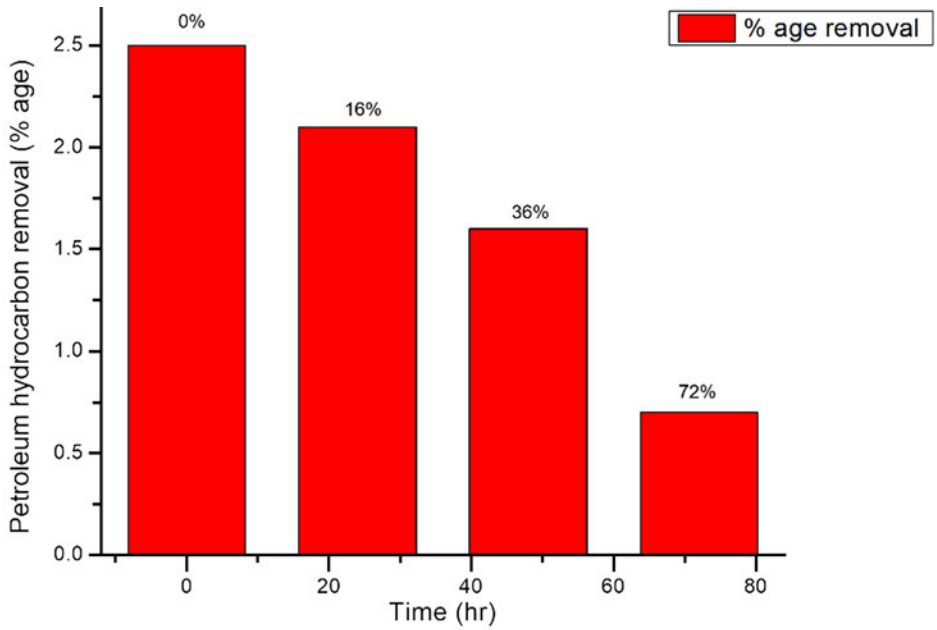
Identification of metabolic intermediates. The metabolic intermediates were identified by comparing electron ionization (EI) mass spectra and electron energies obtained in GC-MS analysis with the spectra of compounds stored in the NIST library (Margolin et al. 2020).

Results

Colony morphology and growth curve analysis. The colony morphology on agar medium was documented (Fig. S1). The bacteria were found to be Gram-positive *Bacillus* (Fig. S1). Growth analysis revealed the lag phase of 0 to 50 hours; log phase of 51 to 72 hours, static phase of 73 to 75 hours, and death phase of 76 hours onwards (Fig. 1a). Growth and removal assays were performed in triplicates.



(a) Growth of bacteria measured in terms of OD at 600 nm in MSM



(b) Petroleum hydrocarbon removal efficiency of bacterium

Fig. 1. Growth phases and petroleum hydrocarbons degradation potential analysis of *Bacillus cereus* strain sab41in present study. a) Growth curve showing lag, log, static, and death phases; b) graph showing degradation efficiency of petroleum by *B. cereus* strain sab41 estimated by measuring OD_{600} of supernatant containing DCPIP.

Assessment of petroleum removal efficiency. Oxidized DCPIP appears blue. Bacteria oxidizing the petroleum hydrocarbon generate electrons, which cause reduction of DCPIP. As it gets reduced, its color changes from blue to dark pink to light pink and finally becomes colorless. The amount of DCPIP being reduced was estimated by the decrease in absorbance of DCPIP. Absorbance measured in case of *B. cereus* was 2.5 (0 hr), 2.1 (24 hr), 1.6 (48 hr) and 0.7 (72 hr). The petroleum degradation efficiency of *B. cereus* (Fig. 1b) was estimated to be 72 ± 10.03% at 72 hr, using the following formula:

$$\%\ removal\ of\ petroleum\ hydrocarbons = \frac{OD\ (0\ hr) - OD\ (log\ phase)}{OD\ (0\ hr) \times 100}$$

Assessment of biochemical characteristics. *B. cereus* gave positive results for ADH, ODC, LDC, ONPG, βGLU, NAG, MAL, ADON, and IND. On the other



#	Biochemical test	Result
1	Urease test (URE)	-ve
2	Arginine dehydrolase test (ADH)	+ve
3	Ornithine decarboxylase test (ODC)	+ve
4	Lysine decarboxylase test (LDC)	+ve
5	Aliphatic thiol hydrolysis test (TET)	-ve
6	Fatty acid ester hydrolysis test (LIP)	-ve
7	Sugar aldehyde utilization test (KSF)	-ve
8	Sorbitol fermentation test (SBL)	-ve
9	β-nitrophenyl-β, D-glucuronidase test (GUR)	-ve
10	σ-nitrophenyl-β, D-galactosidase test (ONPG)	+ve
11	ρ-nitrophenyl-β, D-glucosidase test (βGLU)	+ve
12	β-nitrophenyl-β, D-xylosidase test (βXYL)	-ve
13	ρ-nitrophenyl-n-acetyl-β, D-glucosaminidase test (NAG)	+ve
14	Malonate fermentation test (MAL)	+ve
15	Proline β-naphthylamidase test (PRO)	-ve
16	γ-glutamyl-β-naphthylamidase test (GGT)	-ve
17	Pyrrolidonyl-β-naphthylamidase test (PYR)	-ve
18	Adonitol fermentation test (ADON)	+ve
18	Tryptophane utilization test (IDN)	+ve

Fig. 2.Biochemical characterization of *Bacillus cereus* strain sab41 using Remel RapID™ One panel.
a) Remel RapID™ One panel showing the results; b) table illustrating the biochemical tests being analyzed in the present study.

hand, the bacteria were negative for URE, TET, LIP, KSF, GUR, βXYL, GGT, and PYR. The panel with test results is shown in Fig. 2.

Molecular analysis. BLAST analysis of the FASTA sequence of present study bacteria showed top BLAST sequence similarity with *B. cereus* strain sab41. The sequence was submitted to NCBI, and an accession number was assigned, i.e., OQ954708.1.

Evolutionary relationship analysis. Phylogeny analysis revealed that present study bacteria *B. cereus* strain sab41 OQ954708.1 was closely related with bootstrap value of 100 with *Bacillus wiedmannii* strain NAF4, *Bacillus albus* strain SAB1, *B. cereus* strain EBMB1, *B. cereus* strain P627f, *Bacillus thuringiensis* strain NBAIR Bt109, *B. cereus* WK56, *Bacillus pacificus* strain WK44, *Bacillus paramycoides* strain BEFA-III, and *Bacillus tropicus* strain SAB11 (Fig. 3). It was distantly related with *Bacillus paralicheniformis* strain KJ16, *B. cereus* ATTC® 14579™ and *Bacillus licheniformis* due to a low bootstrap value of 45.

Metabolites identified through GC-MS analysis. Metabolites were identified via molecular weight comparison with previously reported intermediates and end products of TPH metabolism. By molecular weight comparison, we found twenty-one compounds belonging to alcohols, aldehydes, carboxylic acids, cycloalkanes, and cycloalkenes, etc. (Fig. 4, Fig. S2 and S3). The molecular weights, retention times, and peak areas of these metabolites are given in Table I.

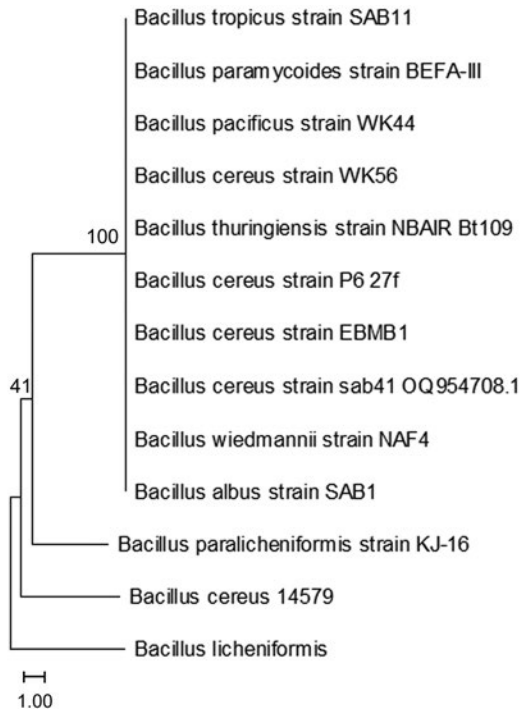


Fig. 3. Phylogenetic tree of *Bacillus cereus* strain sab41 constructed using MEGA 11 software.
A maximum composite likelihood neighbor-joining tree using a bootstrap value 100 was constructed.

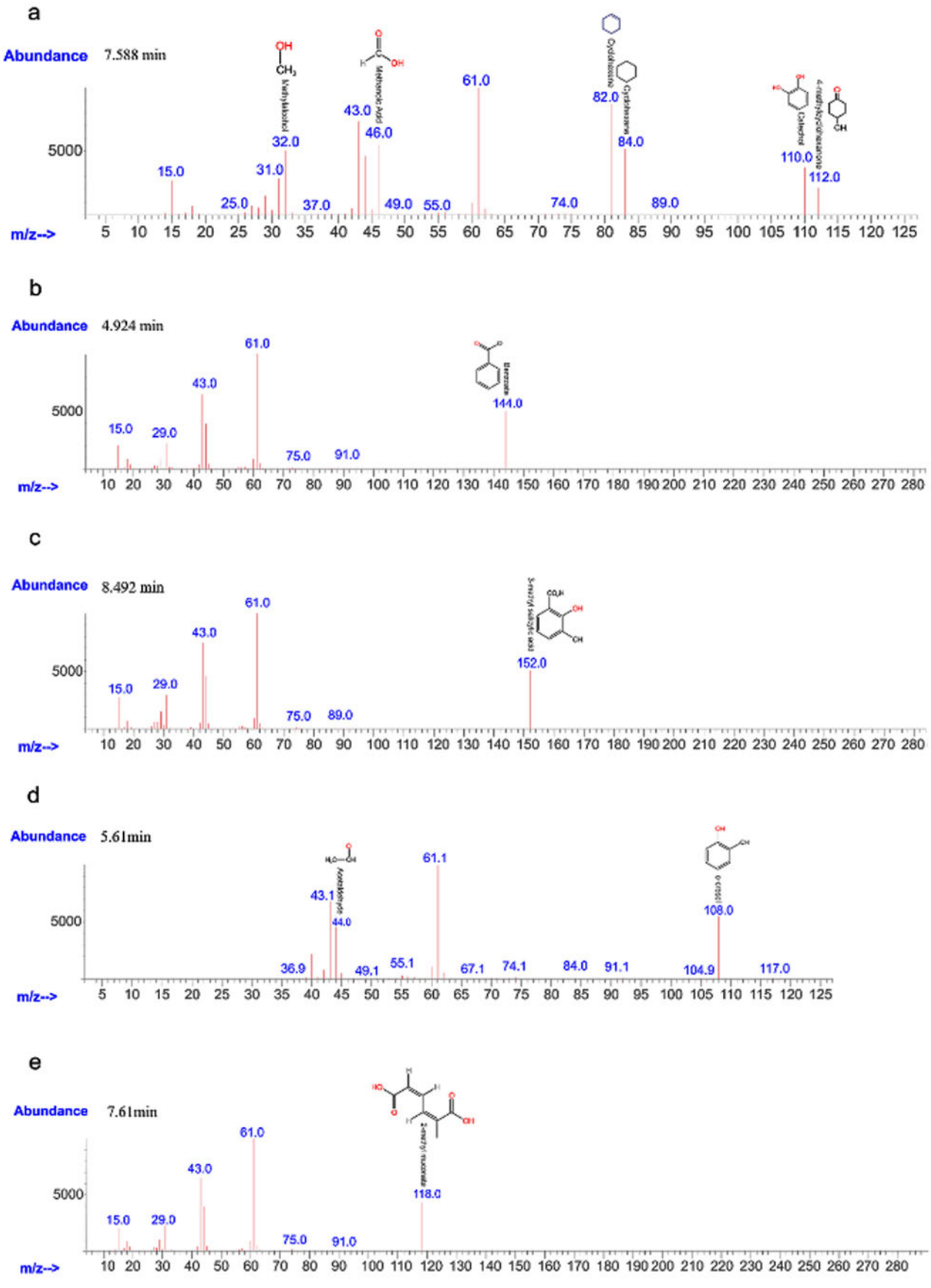


Fig. 4. GC chromatograms of *Bacillus cereus* strain sab41 showing the identified metabolites formed by degradation of petroleum hydrocarbons in the present study.

a) Peaks showing methylalcohol, methanoic acid, cyclohexene, cyclohexane, catechol, 4-methylcyclohexanone; b) peak showing benzoate; c) peak showing 3-methyl salicylic acid; d) peak showing acetaldehyde and *o*-cresol; e) peak showing 2-methylmuconate.

Discussion

The present study targeted the hydrocarbonoclastic bacterium *B. cereus* to gain insight into its petroleum hydrocarbon degradation potential. Growth analysis of *B. cereus* revealed the exponential growth starting at 51 hr and ending at 72 hr. Present findings are slightly contrary to previous reported work reporting the fastest growth rate with log phase starting at 6 hr (*Burkholderia lata* IUBP14), 24 hr (*Brevibacillus formosus* IUBP5 and *B. lata* IUBP13), 30 hr (*Brevibacillus agri* IUBP1, *B. formosus* IUBP2 and 3, *B. lata* IUBP7 and 8, *Burkholderia pyrrocinia* IUBP15), 6 hr (*Pseudomonas* sp. ZS1 and *Alcaligenes* sp. CT10) and 48 hr (*Pseudomonas* and *Bacillus* sp.) (Liang et al. 2018; Tian et al. 2018; Muccee and Ejaz 2019).

Various petroleum degraders have been biochemically characterized in previous studies and found positive for the assays like catalase test, MacConkey agar test, lactose fermentation, mannitol test, urea hydrolysis test, starch hydrolysis test, citrate test, carbohydrate utilization test, esculinase test, phosphatase test, PYR, ADH, β-D-glucosaminidase test, mannitol, sorbitol and inulin fermentation tests (Muccee and Ejaz 2019; Hossain et al. 2022). So, the present study's findings are consistent with earlier research, as *B. cereus* has also been found positive for ADH. However, this is the first study reporting ODC, LDC, ONPG, βGLU, NAG, MAL, ADON, and IND-positive petroleum degrading bacteria.

Our finding of *B. cereus* as petroleum degrading bacteria is consistent with the previous literature reporting various species of *Bacillus* as petroleum degraders like *B. cereus* ATCC® 14579™, *Bacillus tequilensis* NR104919, *Bacillus axarquiensis* NR115929, *Enterococcus faecalis* ATCC® 19433™, *B. subtilis* ATCC® 6633™, *Bacillus thuringiensis*, *Bacillus pumilus* (Bilen Ozyurek and Seyis Bilkay 2017; Tian et al. 2018; Viesser et al. 2020).

The highest petroleum removal efficiency reported in the literature is 98.245% and 85%/24 hr in *Bacillus* species and *Rhodococcus hoagii*, respectively (Al-Surrayai et al. 2009; Abdulla et al. 2019). While the lowest removal potentials during 7 days have been observed in *Pseudomonas* sp. (11.97%), *Acinetobacter* sp. (6.09%), *Enterobacter* sp. (7.46%), *B. pumilus* (23%), *Klebsiella pneumoniae* (31%), *B. tequilensis* (30%), *Enterococcus faecalis* (37%), *Enterobacter cloacae* (38%), *B. tequilensis* (48%), IUBP15 (69.5% per 6 days) and IUBP1 (41% per 6 days), HCS2 bacterial strain (80% per 21 days), (Avanzi et al. 2015; Bilen Ozyurek and Seyis Bilkay 2017; Muccee and Ejaz 2019; Hossain et al. 2022). Hence, the degradation efficiency of *B. cereus* is intermediate between the lowest and highest efficiencies of bacteria reported in the literature.

GC-MS was performed to identify the metabolites of petroleum degradation. Some part of literature supports these findings (Heider et al. 1998; Muccee et al. 2019; Liu et al. 2021b) while a few studies are not in accordance with the present work. Later investigation identified compounds pyridine 2, 3-dicarboxylic acid, three-beta-hydroxyapatite, adenosine, gentisic acid, uridine, glutathione, 9-phenanthrol, 6-methylmercaptopurine, prostaglandin A2, abietic acid and 9-fluorenone in petro-

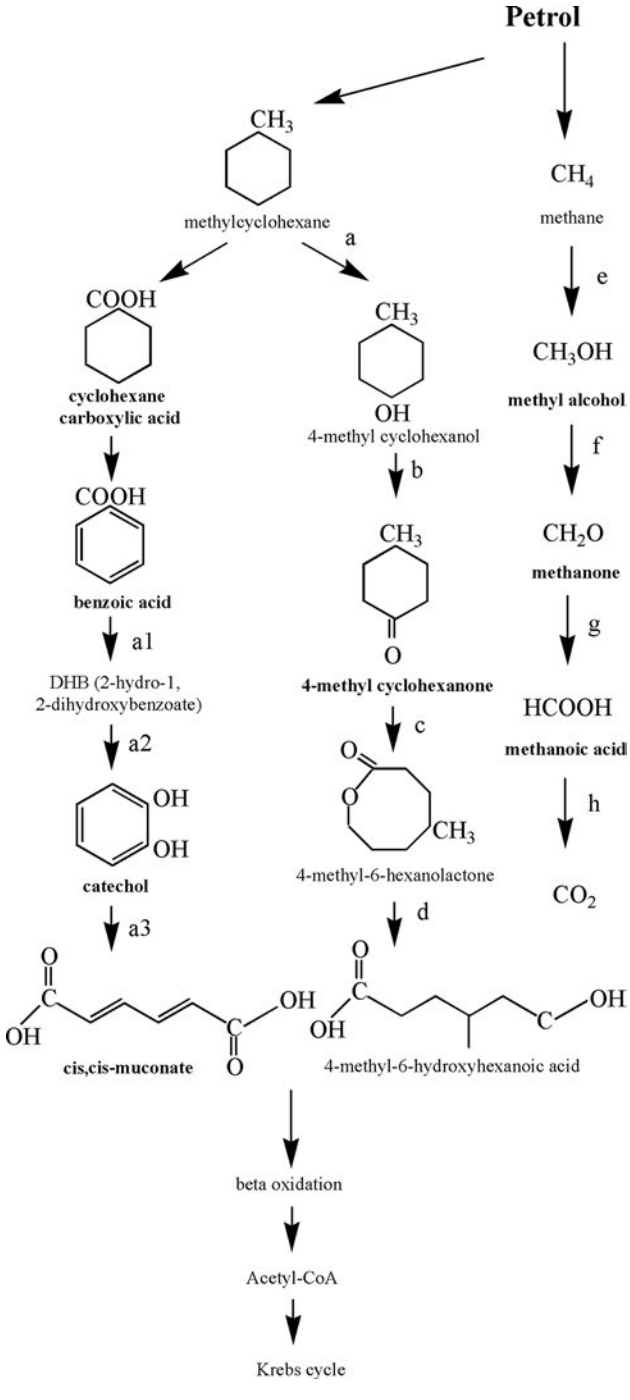


Fig. 5. Pathways identified in *Bacillus cereus* strain sab41 associated with the degradation of alkanes and cycloalkanes.

a – alkane 1-monooxygenase; b – alcohol dehydrogenase; c – cyclohexanone monooxygenase; d – 6-hexanolactone hydrolase; a1 – benzoate 1,2-dioxygenase; a2 – dihydroxybenzoate dehydrogenase; a3 – catechol 1,2-dioxygenase; e – methane monooxygenase; f – methanol dehydrogenase; g – formaldehyde dehydrogenase; h – formate dehydrogenase

leum contaminated soil comprising of *Pseudomonas*, *Pseudoxanthomonas*, *Immundisolibacter* and *Mycobacterium* via GC-TOF-MS analysis (Li et al. 2023b).

NIST library identification probabilities helped to confirm the presence of alkane, methylcycloalkane (Fig. 5) and BTEX (Fig. 6 and 7) degradation pathways in *B. cereus* (Table II and III). Degradation pathways documented for these petroleum components are consistent with the previously reported literature i.e., methane (Kalyuzhnaya et al. 2013), methylcyclohexane

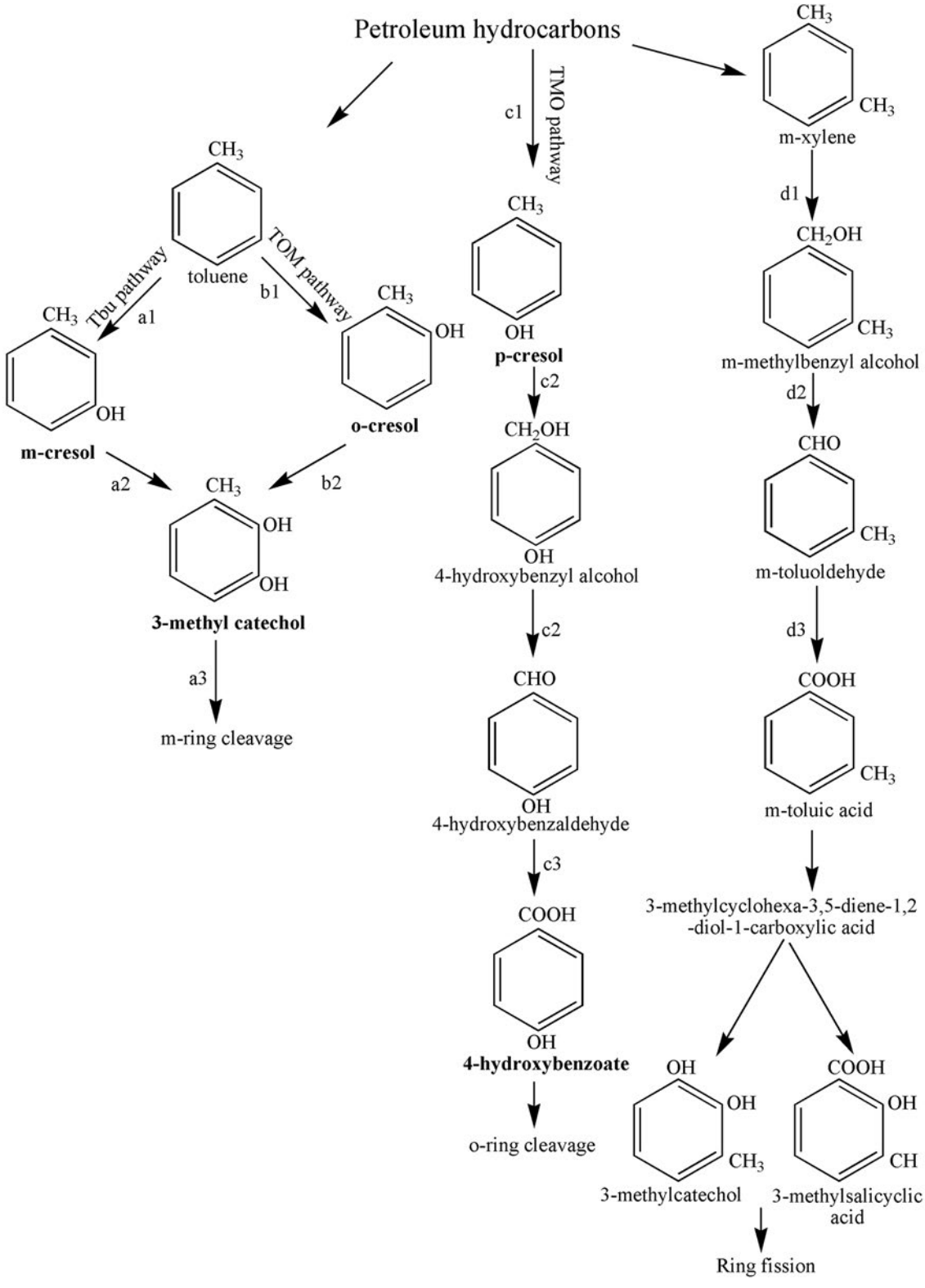


Fig. 6. Pathways identified in *Bacillus cereus* strain sab41 involved in the degradation of aromatics.

a1, a2 – toluene 3-monooxygenase; b1, b2 – toluene 2-monooxygenase; c1 – toluene 4-monooxygenase; c2 – 4-hydroxymethyl hydroxylase; c3 – 4-hydroxybenzaldehyde dehydrogenase; d1 – xylene oxidase; d2 – alcohol dehydrogenase; d3 – aldehyde dehydrogenase

Table II
Earlier reported pathways in petroleum degrading bacteria and the metabolites identified in *Bacillus cereus* strain sab41.

Petroleum hydrocarbon component	Pathway/bacterium reported in literature	Metabolites identified in present study	Reference
Methane	*Methylomicrobium alcaliphilum* 20Z	methylalcohol, methanone, methanoic acid	Kalyuzhnaya et al. 2013
Methylcyclohexane	*Pseudophaeobacter*, *Gilvimarinus*, *Pseudomonas*, *Cycloclasticus*, *Roseovarius*	cyclohexane carboxylic acid, benzoic acid, catechol, *cis,cis*-muconate	Li et al. 2023a
Toluene	*Thauera* sp. strain T1	benzoate, acetaldehyde, cyclohexene, pyruvate, cresol, 3-methyl catechol, 4-hydroxybenzoate	Heider et al. 1998; Muccee et al. 2019
	Toluene 4-monoxygenase pathway in *Pseudomonas mendocina* KR1	*p*-cresol, 4-hydroxybenzoate	Whited and Gibson 1991
	toluene 3-monooxygenase pathway in *Pseudomonas pickettii*	*m*-cresol, 3-methylcatechol	Olsen et al. 1994
Benzene	*Acinetobacter calcoaceticus*, *Rhodopseudomonas palustris*, *Pseudomonas putida* CSV86	benzoate, catechol, *cis,cis*-muconic acid, 4-hydroxybenzoate	Mackintosh and Fewson 1988; Egland et al. 1997; Basu et al. 2003
Xylene	m-xylene oxidation in *Pseudomonas* Pxy	*m*-tolualdehyde, 3-methylcatechol	Davey and Gibson 1974

Table III
Stoichiometric equations of reactions involved in formation of metabolic intermediates of petroleum hydrocarbons degradation identified in *Bacillus cereus* strain sab41

No. of reactions	Stoichiometric equations
	Methane
1	$CH_3OH \rightarrow CH_2O + 2H^+$ methylalcohol methanone
2	$CH_2O + \frac{1}{2}O_2 \rightarrow CH_2O_2$ methanone methanoic acid
	Methylcycleohexane
1	$C_7H_{12}O + \frac{1}{2}O_2 \rightarrow C_7H_{12}O_2$ methylcyclohexane cyclohexane carboxylic acid
2	$C_7H_{12}O_2 \rightarrow C_6H_5COOH + 3H_2$ cyclohexane carboxylic acid benzoic acid
3	$C_7H_6O_5 + 2H_2O \rightarrow C_6H_6O_2 + CO_2 + 2H_2O$ 2-hydro-1,2-dihydroxybenzoate catechol
4	$C_6H_6O_2 + O_2 \rightarrow C_6H_4O_4 + 2H$ *cis, cis*-muconate
	Toluene
1 (Tbu, TMO, TOM)	$C_6H_5CH_3 + O_2 \rightarrow C_7H_8O$ toluene *o-, m-, p*-cresol
2 (Tbu, TMO)	$C_7H_8O \rightarrow C_7H_8O_2$ 3-methylcatechol
3 (TMO)	$C_7H_6O_2 + \frac{1}{2}O_2 \rightarrow C_7H_6$ 4-hydroxybenzaldehyde 4-hydroxybenzoate
	Benzene
1 (pathway A)	$C_7H_5O_2^- + C_{21}H_{36}N_7O_{16}P_3S \rightarrow C_{28}H_{36}N_7O_{17}P_3S^{-4} + \frac{1}{2}O_2$ benzoate CoA benzoyl-CoA
2 (pathway B)	$C_6H_5OH + \frac{1}{2}O_2 \rightarrow C_6H_6O_2$ phenol catechol
3	$C_6H_6O_2 + O_2 \rightarrow C_6H_6O_4$ *cis, cis*-muconic acid
4 (pathway C)	$C_6H_5OH + CO_2 \rightarrow C_7H_6O_3$ phenol 4-hydroxybenzoate
	Xylene
1	$C_8H_{10}O \rightarrow C_8H_8O + 2H^+$ *m*-methylbenzyl alcohol *m*-tolualdehyde
2	$C_7H_8O_4 \rightarrow C_7H_8O_2 + O_2$ 3-methylcyclohexa-3,5-diene--1,2-diol-1-carboxylic acid 3-methylcatechol

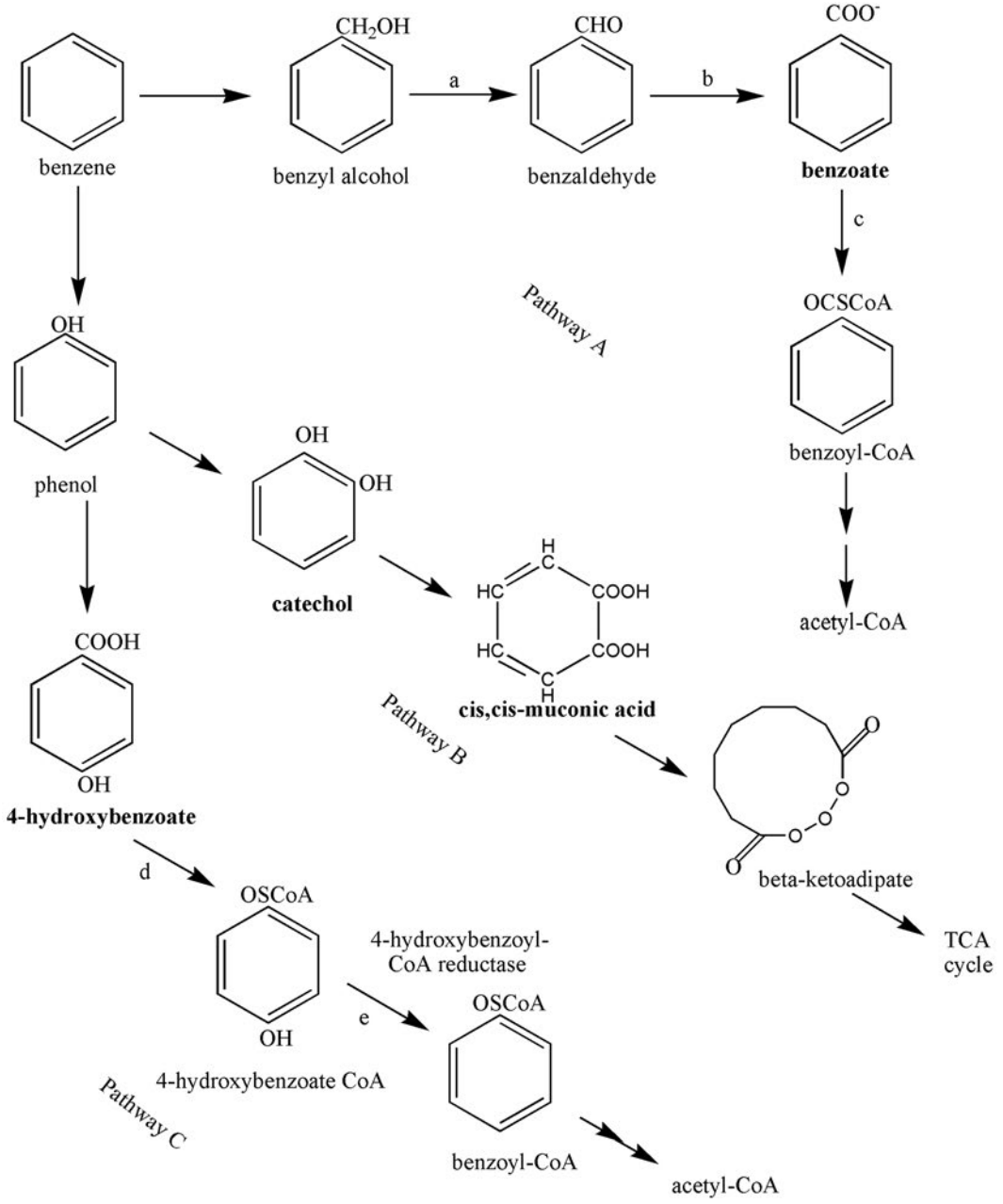


Fig. 7. Benzene degradation pathways identified in *Bacillus cereus* strain sab41 in present study via GC-MS analysis.
a – benzylalcohol dehydrogenase; b – benzaldehyde dehydrogenase; c – benzoate CoA-ligase; d – 4-hydroxybenzoate CoA-ligase; e – 4-hydroxybenoyl CoA reductase

(Li Y. et al. 2023a), toluene (Whited and Gibson 1991; Olsen et al. 1994; Newman and Wackett 1995), benzene (Marr and Stone 1961; Mackintosh and Fewson 1988; Egland et al. 1997; Basu et al. 2003; Arvind et al. 2020) and xylene (Davey and Gibson 1974).

Exploration of bacteria with novel and efficient pathways for removing petroleum hydrocarbons can play a significant role in designing engineered expression systems that may remove these xenobiotics cost-effectively and eco-friendly. For engineering eco-friendly bioremedial microbes-based systems, genes associated with the identified pathways in *B. cereus* can be isolated and cloned in a suitable expression system.

Abbreviations

ADH – arginine dehydrolase
ADON – adonitol fermentation test
DCPIP – 2,6-dichlorophenolindophenol
EPA – Environment Protection Agency
GCMS – gas chromatography mass spectrometry
GGT – γ-glutamyl-β-naphthylamidase test
GUR – β-nitrophenyl-β-D-glucuronidase test
IND – tryptophane utilization test
KSF – sugar aldehyde utilization test
LDC – lysine decarboxylase
LIP – fatty acid ester hydrolysis test
MAL – malonate fermentation test
MSM – minimal salt medium
NAG – *p*-nitrophenyl-N-acetyl-β-D-glucosaminidase

OD – optical density
ODC – ornithine decarboxylase
ONPG – *o*-nitrophenyl-β-D-galactosidase test
POPs – Persistent Organic Pollutant
PRO – proline β-naphthylamidase test
PYR – pyrrolidonyl-β-naphthylamidase test
SBL – sorbitol fermentation test
Tbu – toluene 3-monooxygenase pathway
TCA – tricarboxylic acid cycle
TDI – toluene diisocyanate
TET – aliphatic thiol hydrolysis test
TMO – toluene 4-monooxygenase pathway
Tod – toluene dioxygenase pathway
TOL – toluene methyl monooxygenase pathway
TOM – toluene 2-monooxygenase pathway
TPH – total petroleum hydrocarbons
URE – urease
βGLU – *p*-nitrophenyl-β-D-glucosidase test
βXYL – β-nitrophenyl-β-D-xylosidase

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Author contributions

Nadia Hussain: methodology, Fatima Muccee: perceived the idea, wrote manuscript and designed methodology, Muhammad Hammad: formal analysis, Farhan Mohiuddin: visualization, Saboor Muarij Bunny: validation, Hafiz, Aansa Shahab: formal analysis.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Abbasian F, Lockington R, Mallavarapu M, Naidu R. A comprehensive review of aliphatic hydrocarbon biodegradation by bacteria. Appl Biochem Biotechnol. 2015 Jun;176(3):670–699. https://doi.org/10.1007/s12010-015-1603-5

Abdulla KJ, Ali SA, Gatea IH, Hameed NA, Maied SK. Bio-degradation of crude oil using local bacterial isolates. IOP Conf Ser Earth Environ Sci. 2019;467:012173. https://doi.org/10.1088/1755-1315/467/1/012173

Abereton P, Ordinioha B, Mensah-Attipoe J, Toyinbo O. Crude oil spills and respiratory health of clean-up workers: A systematic review of literature. Atmosphere. 2023;14(3):494. https://doi.org/10.3390/atmos14030494

Abu Laban N, Selesi D, Rattei T, Tischler P, Meckenstock RU. Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. Environ Microbiol. 2010 Oct;12(10):2783–2796. https://doi.org/10.1111/j.1462-2920.2010.02248.x

Adebusoye SA, Ilori MO, Amund OO, Teniola OD, Olatope SO. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. World J Microbiol Biotechnol. 2007;23(8):1149–1159. https://doi.org/10.1007/s11274-007-9345-3

Adipah S. Introduction of petroleum hydrocarbons contaminants and its human effects. J Environ Sci Public Health. 2019;3(1):1–9.

Ahmed S, Kumari K, Singh D. Different strategies and bio-removal mechanisms of petroleum hydrocarbons from contaminated sites. Arab Gulf J Sci Res. 2023 Apr. https://doi.org/10.1108/AGJSR-09-2022-0172

Al-Surrayai T, Yateem A, Al-Kandari R, Al-Sharrah T, Bin-Haji A. The use of Conocarpus lancifolius trees for the remediation of oil-contaminated soils. Soil Sediment Contam: Int J. 2009 Apr;18(3):354–368. https://doi.org/10.1080/15320380902772661

An X, Li N, Zhang S, Han Y, Zhang Q. Integration of proteome and metabolome profiling to reveal heat stress response and tolerance mechanisms of *Serratia* sp. AXJ-M for the bioremediation of papermaking black liquor. J Hazard Mater. 2023 May 15;450:131092. https://doi.org/10.1016/j.jhazmat.2023.131092

Arjoon K, Speight JG. Chemical and physical analysis of a petroleum hydrocarbon contamination on a soil sample to determine its natural degradation feasibility. Invent. 2020 Aug;5(3):43. https://doi.org/10.3390/inventions5030043

Arvind M, Bhatt S, Nichith KR. Biodegradation of aromatics such as benzene, toluene and phenol by *Pseudomonas* strain. Eur J Environ Earth Sci. 2020;1(3). https://doi.org/10.24018/ejgeo.2020.1.3.22

Avanzi IR, Gracioso LH, Baltazar MPG, Karolski B, Perpetuo EA, Nascimento CAO. Aerobic biodegradation of gasoline compounds by bacteria isolated from a hydrocarbon-contaminated soil. Environ Eng Sci. 2015 Dec;32(12):990–997. https://doi.org/10.1089/ees.2015.0122

Basu A, Dixit SS, Phale PS. Metabolism of benzyl alcohol via catechol ortho -pathway in methylnaphthalene-degrading *Pseudomonas putida* CSV86. Appl Microbiol Biotechnol. 2003 Oct;62(5–6) 579–585. https://doi.org/10.1007/s00253-003-1305-8

Bedics A, Banerjee S, Bóka K, Tóth E, Benedek T, Kriszt B, Táncsics A. *Pinisolibacter aquiterrae* sp. nov., a novel aromatic hydrocarbon-degrading bacterium isolated from benzene-, and xylene-degrading enrichment cultures, and emended description of the genus *Pinisolibacter*. Int J Syst Evol Microbiol. 2022 Feb;72(2). https://doi.org/10.1099/ijsem.0.005229

Bilen Ozyurek S, Seyis Bilkay I. Determination of petroleum biodegradation by bacteria isolated from drilling fluid, waste mud pit and crude oil. Turk J Biochem. 2017 Dec;42(6):609–616. https://doi.org/10.1515/tjb-2017-0087

Brito EMS, Guyoneaud R, Goñi-Urriza M, Ranchou-Peyruse A, Verbaere A, Crapez MAC, Wasserman JCA, Duran R. Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay, Brazil. Res Microbiol. 2006 Oct;157(8):752–762. https://doi.org/10.1016/j.resmic.2006.03.005

Brzeszcz J, Kaszycki P. Aerobic bacteria degrading both *n*-alkanes and aromatic hydrocarbons: An undervalued strategy for metabolic diversity and flexibility. Biodegradation. 2018 Aug;29(4):359–407. https://doi.org/10.1007/s10532-018-9837-x

Chen Y, Ye W, Zhang Y, Xu Y. High speed BLASTN: An accelerated MegaBLAST search tool. Nucleic Acids Res. 2015 Aug;43(16):7762–7768. https://doi.org/10.1093/nar/gkv784

Chuah LF, Chew KW, Bokhari A, Mubashir M, Show PL. Biodegradation of crude oil in seawater by using a consortium of symbiotic bacteria. Environ Res. 2022 Oct;213:113721. https://doi.org/10.1016/j.envres.2022.113721

da Silva LJ, Alves FC, de França FP. A review of the technological solutions for the treatment of oily sludges from petroleum refineries. Waste Manage Res. 2012 Oct;30(10):1016–1030. https://doi.org/10.1177/0734242X12448517

Davey JF, Gibson DT. Bacterial metabolism of *para*- and *meta*-xylene: Oxidation of a methyl substituent. J Bacteriol. 1974 Sep;119(3):923–929. https://doi.org/10.1128/jb.119.3.923-929.1974

Denome SA, Stanley DC, Olson ES, Young KD. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J Bacteriol. 1993 Nov;175(21):6890–6901. https://doi.org/10.1128/jb.175.21.6890-6901.1993

Di Canito A, Zampolli J, Orro A, D'Ursi P, Milanesi L, Sello G, Steinbüchel A, Di Gennaro P. Genome-based analysis for the

identification of genes involved in *o*-xylene degradation in *Rhodococcus opacus* R7. BMC Genomics. 2018 Dec;19(1):587. https://doi.org/10.1186/s12864-018-4965-6

Egland PG, Pelletier DA, Dispensa M, Gibson J, Harwood CS. A cluster of bacterial genes for anaerobic benzene ring biodegradation. Proc Natl Acad Sci USA. 1997 Jun;94(12):6484–6489. https://doi.org/10.1073/pnas.94.12.6484

Ezeonu CS, Tagbo R, Anike EN, Oje OA, Onwurah INE. Biotechnological tools for environmental sustainability: Prospects and challenges for environments in Nigeria – A standard review. Biotechnol Res Int. 2012 May;2012:1–26. https://doi.org/10.1155/2012/450802

Eziuzor SC, Vogt C. Anaerobe isolation from denitrifying benzene-degrading enrichment culture and their capacity to mineralize benzene. bioRxiv 2023;2023.01.07.522375. https://doi.org/10.1101/2023.01.07.522375

Feng S, Gong L, Zhang Y, Tong Y, Zhang H, Zhu D, Huang X, Yang H. Bioaugmentation potential evaluation of a bacterial consortium composed of isolated *Pseudomonas* and *Rhodococcus* for degrading benzene, toluene and styrene in sludge and sewage. Bioresour Technol. 2021 Jan;320(Pt A):124329. https://doi.org/10.1016/j.biortech.2020.124329

Ganesh Kumar A, Mathew NC, Sujitha K, Kirubagaran R, Dharani G. Genome analysis of deep sea piezotolerant *Nesiotobacter exalbescens* COD22 and toluene degradation studies under high pressure condition. Sci Rep. 2019 Dec;9(1):18724. https://doi.org/10.1038/s41598-019-55115-9

Gomes E, da Silva R ,de Cassia Pereira J, Ladino-Orjuela G. Chapter 3 – Fungal growth on solid substrates: A physiological overview. In: Pandey A, Larroche C, Soccol CR, editors. Current developments in biotechnology and bioengineering. Amsterdam (The Netherlands): Elsevier B.V.; 2018. p. 31–56. https://doi.org/10.1016/B978-0-444-63990-5.00003-7

Goyal AK, Zylstra GJ. Genetics of naphthalene and phenanthrene degradation by *Comamonas testosteroni*. J Ind Microbiol Biotechnol. 1997 Nov;19(5–6):401–407. https://doi.org/10.1038/sj.jim.2900476

Haider FU, Ejaz M, Cheema SA, Khan MI, Zhao B, Liqun C, Salim MA, Naveed M, Khan N, Núñez-Delgado A, et al. Phytotoxicity of petroleum hydrocarbons: Sources, impacts and remediation strategies. Environ Res. 2021 Jun;197:111031. https://doi.org/10.1016/j.envres.2021.111031

Hao X, Chen Q, van Loosdrecht MCM, Li J, Jiang H. Sustainable disposal of excess sludge: Incineration without anaerobic digestion. Water Res. 2020 Mar;170:115298. https://doi.org/10.1016/j.watres.2019.115298

Heider J, Spormann AM, Beller HR, Widdel F. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev. 1998 Dec; 22(5):459–473. https://doi.org/10.1111/j.1574-6976.1998.tb00381.x

Hossain MF, Akter MA, Sohan MSR, Sultana DN, Reza MA, Hoque KMF. Bioremediation potential of hydrocarbon degrading bacteria: Isolation, characterization, and assessment. Saudi J Biol Sci. 2022 Jan;29(1):211–216. https://doi.org/10.1016/j.sjbs.2021.08.069

Hu M, Zhang F, Li G, Ruan H, Li X, Zhong L, Chen G, Rui Y. *Falsochrobactrum tianjinense* sp. *nov*., a new petroleum-degrading bacteria isolated from oily soils. Int J Environ Res Public Health. 2022 Sep;19(18):11833. https://doi.org/10.3390/ijerph191811833

Jeffries TC, Rayu S, Nielsen UN, Lai K, Ijaz A, Nazaries L, Singh BK. Metagenomic functional potential predicts degradation rates of a model organophosphorus xenobiotic in pesticide contaminated soils. Front Microbiol. 2018 Feb;9:147. https://doi.org/10.3389/fmicb.2018.00147

Kalyuzhnaya MG, Yang S, Rozova ON, Smalley NE, Clubb J, Lamb A, Gowda GAN, Raftery D, Fu Y, Bringel F, et al. Highly efficient methane biocatalysis revealed in a methanotrophic bacterium. Nat Commun. 2013 Dec;4(1):2785. https://doi.org/10.1038/ncomms3785

Koe WS, Lee JW, Chong WC, Pang YL, Sim LC. An overview of photocatalytic degradation: Photocatalysts, mechanisms, and development of photocatalytic membrane. Environ Sci Pollut Res Int. 2020 Jan;27(3):2522–2565. https://doi.org/10.1007/s11356-019-07193-5

Kumar A, Mallick SP, Singh D, Gupta N. Chapter 1 – Advances in bioremediation: Introduction, applications, and limitations. In: Kumar S, Hashmi MZ, editors. Biological approaches to controlling pollutants. Duxford (UK): Woodhead Publishing; 2022. p. 1–14. https://doi.org/10.1016/B978-0-12-824316-9.00003-3

Li X, Zhang F, Guan B, Sun J, Liao G. Review on oily sludge treatment technology. IOP Conf Ser Earth Environ Sci. 2020;467:012173. https://doi.org/10.1088/1755-1315/467/1/012173

Li Y, Cui Z, Luan X, Bian X, Li G, Hao T, Liu J, Feng K, Song Y. Degradation potential and pathways of methylcyclohexane by bacteria derived from Antarctic surface water. Chemosphere. 2023a Jul; 329:138647. https://doi.org/10.1016/j.chemosphere.2023.138647

Li YQ, Xin Y, Li C, Liu J, Huang T. Metagenomics-metabolomics analysis of microbial function and metabolism in petroleum-contaminated soil. Braz J Microbiol. 2023b;54:935–947. https://doi.org/10.1007/s42770-023-01000-7

Liang J, Cheng T, Huang Y, Liu J. Petroleum degradation by *Pseudomonas* sp. ZS1 is impeded in the presence of antagonist *Alcaligenes* sp. CT10. AMB Express. 2018 Dec;8(1):88. https://doi.org/10.1186/s13568-018-0620-5

Lim MW, Lau EV, Poh PE. Micro-macrobubbles interactions and its application in flotation technology for the recovery of high density oil from contaminated sands. J Petrol Sci Eng. 2018 Feb;161:29–37. https://doi.org/10.1016/j.petrol.2017.11.064

Lima SD, Oliveira AF, Golin R, Lopes VCP, Caixeta DS, Lima ZM, Morais EB. Isolation and characterization of hydrocarbon-degrading bacteria from gas station leaking-contaminated groundwater in the Southern Amazon, Brazil. Braz J Biol. 2020 Jun;80(2):354–361. https://doi.org/10.1590/1519-6984.208611

Liu JW, Wei KH, Xu SW, Cui J, Ma J, Xiao XL, Xi BD, He XS. Surfactant-enhanced remediation of oil-contaminated soil and groundwater: A review. Sci Total Environ. 2021a Feb;756:144142. https://doi.org/10.1016/j.scitotenv.2020.144142

Liu Y, Tang H, Lin Z, Xu P. Mechanisms of acid tolerance in bacteria and prospects in biotechnology and bioremediation. Biotechnol Adv. 2015 Nov;33(7):1484–1492. https://doi.org/10.1016/j.biotechadv.2015.06.001

Liu Y, Wu J, Liu Y, Wu X. Biological process of alkane degradation by *Gordonia sihwaniensis*. ACS Omega. 2021b Jan;7(1):55–63. https://doi.org/10.1021/acsomega.1c01708

MacKintosh RW, Fewson CA. Benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II from *Acinetobacter calcoaceticus*. Purification and preliminary characterization. Biochem J. 1988 Mar; 250(3):743–751. https://doi.org/10.1042/bj2500743

Marchand C, St-Arnaud M, Hogland W, Bell TH, Hijri M. Petroleum biodegradation capacity of bacteria and fungi isolated from petroleum-contaminated soil. Int Biodeterior Biodegrad. 2017 Jan; 116:48–57. https://doi.org/10.1016/j.ibiod.2016.09.030

Margolin Eren KJ, Elkabets O, Amirav A. A comparison of electron ionization mass spectra obtained at 70 eV, low electron energies, and with cold EI and their NIST library identification probabilities. J Mass Spectrom. 2020 Dec;55(12):e4646. https://doi.org/10.1002/jms.4646

Marr EK, Stone RW. Bacterial oxidation of benzene. J Bacteriol. 1961 Mar;81(3):425–430. https://doi.org/10.1128/jb.81.3.425-430.1961

Mohanty G, Mukherji S. Biodegradation rate of diesel range *n*-alkanes by bacterial cultures *Exiguobacterium aurantiacum* and *Burkholderia cepacia*. Int Biodeterior Biodegrad. 2008 Apr;61(3): 240–250. https://doi.org/10.1016/j.ibiod.2007.06.011

Mori R. Replacing all petroleum-based chemical products with natural biomass-based chemical products: A tutorial review. RSC Sustain. 2023;1(2):179–212. https://doi.org/10.1039/D2SU00014H
Mousa AERA. Isolation and characterization of phenol degrading bacteria from wastewater. Int J Biol Phys Chem Stud. 2023;5(2):17-24. https://doi.org/10.32996/ijbpcs.2023.5.2.3
Muccee F, Ejaz S, Riaz N, Iqbal J. Molecular and functional analysis of naphthalene-degrading bacteria isolated from the effluents of indigenous tanneries. J Basic Microbiol. 2021 Jul;61(7):627–641. https://doi.org/10.1002/jobm.202100123
Muccee F, Ejaz S, Riaz N. Toluene degradation via a unique metabolic route in indigenous bacterial species. Arch Microbiol. 2019 Dec; 201(10):1369–1383. https://doi.org/10.1007/s00203-019-01705-0
Muccee F, Ejaz S. An investigation of petrol metabolizing bacteria isolated from contaminated soil samples collected from various fuel stations. Pol J Microbiol. 2019 Jan;68(2):193–201. https://doi.org/10.33073/pjm-2019-019
Newman LM, Wackett LP. Purification and characterization of toluene 2-monooxygenase from *Burkholderia cepacia* G4. Biochemistry. 1995 Oct;34(43):14066–14076. https://doi.org/10.1021/bi00043a012
Olsen RH, Kukor JJ, Kaphammer B. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J Bacteriol. 1994 Jun;176(12):3749–3756. https://doi.org/10.1128/jb.176.12.3749-3756.1994
Ossai IC, Ahmed A, Hassan A, Hamid FS. Remediation of soil and water contaminated with petroleum hydrocarbon: A review. Environ Technol Innovation. 2020 Feb;17:100526. https://doi.org/10.1016/j.eti.2019.100526
Petkova M, Shilev S. Revealing fungal diversity in mesophilic and thermophilic habitats of sewage sludge composting by next-generation sequencing. Appl Sci. 2023;13(9):5546. https://doi.org/10.3390/app13095546
Podgorski DC, Corilo YE, Nyadong L, Lobodin VV, Bythell BJ, Robbins WK, McKenna AM, Marshall AG, Rodgers RP. Heavy petroleum composition. 5. Compositional and structural continuum of petroleum revealed. Energy Fuels. 2013 Mar;27(3):1268–1276. https://doi.org/10.1021/ef301737f
Ramírez-Camacho JG, Carbone F, Pastor E, Bubbico R, Casal J. Assessing the consequences of pipeline accidents to support land-use planning. Saf Sci. 2017 Aug;97:34–42. https://doi.org/10.1016/j.ssci.2016.01.021
Reiner AM. Metabolism of benzoic acid by bacteria: 3,5-cyclohexadiene-1,2-diol-1-carboxylic acid is an intermediate in the formation of catechol. J Bacteriol. 1971 Oct;108(1):89–94. https://doi.org/10.1128/jb.108.1.89-94.1971
Sadiqi S, Hamza M, Ali F, Alam S, Shakeela Q, Ahmed S, Ayaz A, Ali S, Saqib S, Ullah F, et al. Molecular characterization of bacterial isolates from soil samples and evaluation of their antibacterial potential against MDRS. Molecules. 2022 Sep;27(19):6281. https://doi.org/10.3390/molecules27196281
Salari M, Rahmanian V, Hashemi SA, Chiang WH, Lai CW, Mousavi SM, Gholami A. Bioremediation treatment of polyaromatic hydrocarbons for environmental sustainability. Water. 2022 Dec;14(23):3980. https://doi.org/10.3390/w14233980
Schoch CL, Ciufo S, Domrachev M, Hotton CL, Kannan S, Khovanskaya R, Leipe D, Mcveigh R, O'Neill K, Robbertse B, et al. NCBI Taxonomy: A comprehensive update on curation, resources and tools. Database. 2020 Jan;2020:baaa062. https://doi.org/10.1093/database/baaa062
Sharma I. Bioremediation techniques for polluted environment: Concept, advantages, limitations, and prospects. In: Alfonso Murillo-Tovar M, Saldarriaga-Noreña H and Saeid A, editors. Trace metals in the environment – new approaches and recent advances. London (UK): IntechOpen; 2020. https://doi.org/10.5772/intechopen.90453
Sievers F, Higgins DG. The Clustal Omega multiple alignment package. In: Katoh K, editor. Multiple sequence alignment. Methods in molecular biology, vol. 2231. New York (USA): Humana; 2021. p. 3–16. https://doi.org/10.1007/978-1-0716-1036-7_1
Sivagami K, Padmanabhan K, Joy AC, Nambi IM. Microwave (MW) remediation of hydrocarbon contaminated soil using spent graphite – An approach for waste as a resource. J Environ Manage. 2019 Jan;230:151–158. https://doi.org/10.1016/j.jenvman.2018.08.071
Speight JG, Arjoon KK. Bioremediation of petroleum and petroleum products. Hoboken (USA): John Wiley & Sons, Ltd.; 2012. https://doi.org/10.1002/9781118528471
Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis version 11. Mol Biol Evol. 2021 Jun;38(7): 3022–3027. https://doi.org/10.1093/molbev/msab120
Tian X, Wang X, Peng S, Wang Z, Zhou R, Tian H. Isolation, screening, and crude oil degradation characteristics of hydrocarbons-degrading bacteria for treatment of oily wastewater. Water Sci Technol. 2018 Dec;78(12):2626–2638. https://doi.org/10.2166/wst.2019.025
Tran HT, Lin C, Hoang HG, Bui XT, Le VG, Vu CT. Soil washing for the remediation of dioxin-contaminated soil: A review. J Hazard Mater. 2022 Jan;421:126767. https://doi.org/10.1016/j.jhazmat.2021.126767
Viesser JA, Sugai-Guerios MH, Malucelli LC, Pincerati MR, Karp SG, Maranho LT. Petroleum-tolerant rhizospheric bacteria: Isolation, characterization and bioremediation potential. Sci Rep. 2020 Feb;10(1):2060. https://doi.org/10.1038/s41598-020-59029-9
Wang M, Ding M, Yuan Y. Bioengineering for the microbial degradation of petroleum hydrocarbon contaminants. Bioengineering. 2023 Mar;10(3):347. https://doi.org/10.3390/bioengineering10030347
Whited GM, Gibson DT. Separation and partial characterization of the enzymes of the toluene-4-monooxygenase catabolic pathway in *Pseudomonas mendocina* KR1. J Bacteriol. 1991 May;173(9): 3017–3020. https://doi.org/10.1128/jb.173.9.3017-3020.1991
Wright MH, Adelskov J, Greene AC. Bacterial DNA extraction using individual enzymes and phenol/chloroform separation. J Microbiol Biol Educ. 2017 Sep;18(2):18.2.48. https://doi.org/10.1128/jmbe.v18i2.1348
Wulandari M, Effendi AJ. Effect of frequency and ratio solid liquid on ultrasonic remediation of petroleum contaminated soil. AIP Conf Proc. 2018 Sep;2014(1):020120-1–020120-7. https://doi.org/10.1063/1.5054524
Xu J, Zhang Q, Li D, Du J, Wang C, Qin J. Rapid degradation of long-chain crude oil in soil by indigenous bacteria using fermented food waste supernatant. Waste Manag. 2019 Feb;85:361–373. https://doi.org/10.1016/j.wasman.2018.12.041
Zehnle H, Otersen C, Benito Merino D, Wegener G. Potential for the anaerobic oxidation of benzene and naphthalene in thermophilic microorganisms from the Guaymas Basin. Front Microbiol. 2023 Sep;14:1279865. https://doi.org/10.3389/fmicb.2023.1279865
Zhang X, Kong D, Liu X, Xie H, Lou X, Zeng C. Combined microbial degradation of crude oil under alkaline conditions by *Acinetobacter baumannii* and *Talaromyces* sp. Chemosphere. 2021 Jun; 273: 129666. https://doi.org/10.1016/j.chemosphere.2021.129666
Zhao C, Dong Y, Feng Y, Li Y, Dong Y. Thermal desorption for remediation of contaminated soil: A review. Chemosphere. 2019 Apr; 221:841–855. https://doi.org/10.1016/j.chemosphere.2019.01.079
Zhou L, Li H, Zhang Y, Han S, Xu H. *Sphingomonas* from petroleum-contaminated soils in Shenfu, China and their PAHs degradation abilities. Braz J Microbiol. 2016 Apr;47(2):271–278. https://doi.org/10.1016/j.bjm.2016.01.001
Zou X, Su Q, Yi Q, Guo L, Chen D, Wang B, Li Y, Li J. Determining the degradation mechanism and application potential of benzopyrene-degrading bacterium *Acinetobacter* XS-4 by screening. J Hazard Mater. 2023 Aug;456:131666. https://doi.org/10.1016/j.jhazmat.2023.131666

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