International Journal of Research and Review
[Indexed, Peer-Reviewed Journal]
Volume 13; Issue: 10; October 2026
Website: www.ijrrjournal.com
Research Paper *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Isolation and Screening of BTEX (Benzene, Toluene, Ethylbenzene, and Xylene)-Degrading Bacteria from Petroleum-Contaminated Soil

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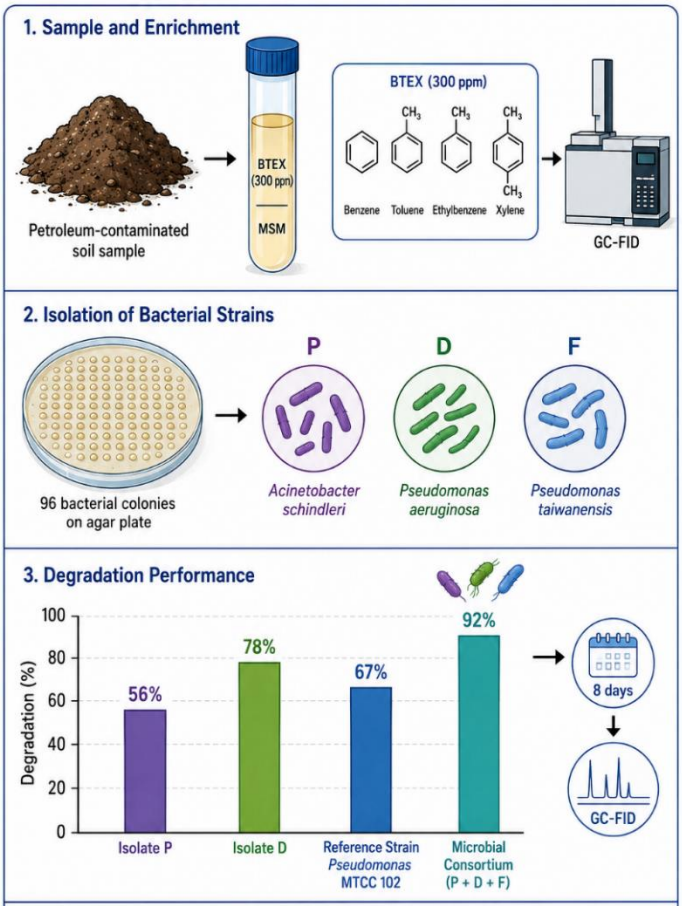
DOI: https://doi.org/10.52403/ijrr.20261032

ABSTRACT

Benzene, toluene, ethylbenzene, and xylene (BTEX) are toxic monoaromatic hydrocarbons frequently detected in petroleum-contaminated environments. Microbial biodegradation is an environmentally sustainable strategy for removing these pollutants. The present study aimed to isolate and screen indigenous BTEX-degrading bacteria from petroleum-contaminated soil samples. We used enrichment culture techniques with mineral salt medium (MSM) supplemented with BTEX as the sole carbon source. We supplied each BTEX compound at 300 ppm to evaluate microbial degradation efficiency. After enrichment and plating, we obtained 96 bacterial isolates, of which 16 showed substantial growth in BTEX-containing MSM. We selected three fast-growing isolates, designated P, D, and F, for further biodegradation analysis. Degradation experiments were conducted for 8 days, and residual BTEX concentrations were analyzed using GC-FID. All three isolates demonstrated measurable degradation, with isolate D exhibiting the highest efficiency among individual strains. Comparison with a reference strain (Pseudomonas MTCC 102) revealed compound-specific degradation behaviour, while the developed microbial consortium showed the highest overall degradation efficiency, as evidenced by higher percentage degradation and a reduced total peak area. 16S rRNA gene sequencing identified the isolates as *Acinetobacter schindleri*, *Pseudomonas aeruginosa*, and *Pseudomonas taiwanensis*. These findings highlight the superiority of microbial consortia over individual strains and demonstrate their potential for bioremediation of BTEX-contaminated environments.

Keywords: BTEX; isolation; screening; biodegradation; petroleum-contaminated soil; gas chromatography

GRAPHICAL ABSTRACT



1. INTRODUCTION

BTEX compounds- benzene, toluene, ethylbenzene, and xylene are volatile monoaromatic hydrocarbons commonly released into the environment through petroleum refining activities, fuel leakage, storage tank failures, and accidental spills.[1,2] Because of their toxicity and persistence, BTEX compounds are priority environmental pollutants and are frequently detected in petroleum-contaminated soil and groundwater systems.[3] Exposure to these contaminants poses significant risks to ecosystems and human health. Microbial biodegradation has emerged as one of the most effective and environmentally sustainable approaches for remediating hydrocarbon-contaminated environments.[4,5] Indigenous microorganisms at contaminated sites often develop metabolic pathways that enable them to utilise aromatic hydrocarbons as carbon and energy sources. These microorganisms play a crucial role in the natural attenuation of petroleum pollutants. BTEX degradation generally begins with enzymatic oxidation catalysed by monooxygenases or dioxygenases, producing intermediates such as catechol, which then undergo ring-cleavage pathways.[6] Several bacterial genera, including Pseudomonas, Bacillus, Rhodococcus, and Acinetobacter, have been reported to degrade BTEX and other petroleum-derived hydrocarbons.[7] Isolating and screening indigenous hydrocarbon-degrading microorganisms remain essential steps in identifying efficient biodegrading strains for bioremediation systems. Enrichment culture techniques using hydrocarbon-supplemented mineral salt medium are widely used to selectively isolate microorganisms capable of utilising hydrocarbons as their sole carbon source.[2] The present study aimed to isolate BTEX-degrading bacteria from petroleum-contaminated soil and evaluate their degradation potential using gas

chromatographic analysis. The efficient degraders identified in this work may serve as promising candidates for future studies on optimizing biodegradation processes and environmental bioremediation applications.

2. MATERIALS AND METHODS

2.1. Soil Sample Collection

We collected soil samples from petroleum-contaminated sites, including fuel-handling and automobile service areas. Samples were obtained from the top 10-15 cm layer using sterile tools, transferred into sterile containers, and transported to the laboratory for immediate processing.

2.2. Isolation of BTEX-Degrading Bacteria

We used an enrichment culture approach commonly used to isolate hydrocarbon-degrading microorganisms from contaminated environments.[1,2] After successive enrichment cycles, we plated serial dilutions and isolated morphologically distinct colonies. We obtained and preserved 96 bacterial isolates for further screening.

2.3. Primary Screening Based on Growth in BTEX-MSM

We inoculated all 96 isolates individually into MSM containing BTEX and incubated them under controlled conditions. Each BTEX compound (benzene, toluene, ethylbenzene, and xylene) was added at a concentration of 300 ppm in the medium. Growth was monitored visually and spectrophotometrically. Based on cell density and growth consistency, we selected 16 isolates with comparatively higher growth in BTEX-MSM for secondary screening.

2.4. Selection of Fast-Growing Isolates

Among the 16 selected isolates, three that showed rapid growth and higher cell density were identified as fast-growing strains and designated P, D, and F. These isolates were subjected to further biodegradation studies.

2.5. Biodegradation Experiment

We carried out biodegradation experiments in MSM supplemented with BTEX. Each BTEX compound was supplied at 300 ppm. We selected this concentration to provide sufficient substrate for microbial growth while remaining within ranges commonly used in laboratory biodegradation studies.[1,8] Flasks were inoculated with isolates P, D, and F and incubated for 8 days. Abiotic controls without bacterial inoculation were maintained in parallel. Samples were collected on day 0 and day 8 for GC analysis.

2.6. Gas Chromatography Analysis

BTEX degradation was quantified using gas chromatography (GC-FID). This analytical technique is widely used to detect and quantify volatile aromatic hydrocarbons in biodegradation studies.[8] Samples were extracted and analysed in triplicate, and peak areas for benzene (B), toluene (T), ethylbenzene (E), and xylene (X) were recorded.

2.7. Calculation of Percentage Degradation

Percentage degradation was calculated using mean GC peak area values according to the following equation:

$$\text{Degradation (\%)} = \frac{\text{Mean control peak area - Mean treated peak area}}{\text{Mean control peak area}} \times 100$$

2.8. Statistical Analysis

All GC analyses were performed in triplicate ($n = 3$). Peak area data are presented as mean ± SD. We compared isolates using one-way analysis of variance (ANOVA), and differences were significant at $p < 0.05$.

2.9. Molecular identification by 16S rRNA gene sequencing

We extracted genomic DNA from the selected BTEX-degrading bacterial isolates using a standard bacterial genomic DNA extraction protocol and amplified the 16S rRNA gene with universal primers.[9] The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). We amplified the products by PCR in a

thermal cycler under standard conditions and verified them by agarose gel electrophoresis. We purified the PCR products and sequenced them using a commercial sequencing service. We compared the sequences with reference sequences in the NCBI database using BLAST to identify the closest phylogenetic relatives of the isolates. Phylogenetic analysis confirmed the taxonomic identity of the isolates.

3. RESULTS AND DISCUSSION

3.1. Screening and Selection of BTEX-Degrading Isolates

Table 1. Growth performance of selected bacterial isolates (P, D, and F) in BTEX-supplemented mineral salt medium expressed as optical density (OD_{600}).

Isolate	P	D	F
OD_{600} (72 h)	0.49 ± 0.02	0.52 ± 0.03	0.48 ± 0.02
OD_{600} (8 days)	0.58 ± 0.03	0.63 ± 0.02	0.56 ± 0.03

We obtained 96 bacterial isolates from petroleum-contaminated soil using enrichment culture techniques in BTEX-supplemented mineral salt medium. Initial screening based on growth performance revealed that only a subset of isolates could utilise BTEX as a sole carbon source. Among these, 16 isolates exhibited comparatively higher growth, as evidenced by increased cell density in BTEX-MSM. Further screening identified three consistently growing isolates, P, D, and F, which we selected for confirmatory biodegradation analysis (Table 1 and Fig. 1). This stepwise screening approach ensured selection of robust, metabolically active BTEX-degrading candidates, consistent with previously reported isolation strategies.[2]

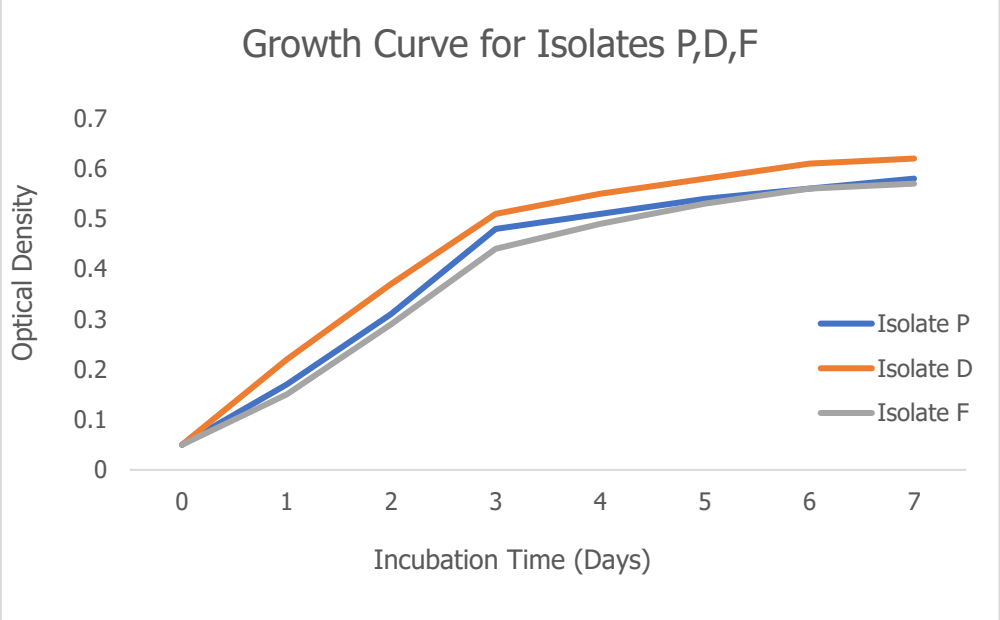


Fig. 1. Growth curves of selected bacterial isolates (P, D, and F) in BTEX-supplemented mineral salt medium over an 8-day incubation period, expressed as optical density at 600 nm (OD_{600}).

3.2. Confirmation of BTEX Degradation by GC Analysis

We used gas chromatography to confirm BTEX degradation by the selected isolates. Representative GC-FID chromatograms of control samples and cultures inoculated with isolates P, D, and F after 8 days of incubation are presented in Fig. 2. Control chromatograms showed no appreciable reduction in BTEX peak intensities over the

incubation period, indicating minimal abiotic loss. In contrast, chromatograms of inoculated samples showed a marked reduction in BTEX peak intensities, confirming active biodegradation by the bacterial isolates. Among the tested isolates, isolate D showed the most pronounced reduction in BTEX peaks, followed by isolates F and P. Benzene and toluene showed a comparatively greater reduction than ethylbenzene and xylene, suggesting preferential utilisation of simpler aromatic hydrocarbons.

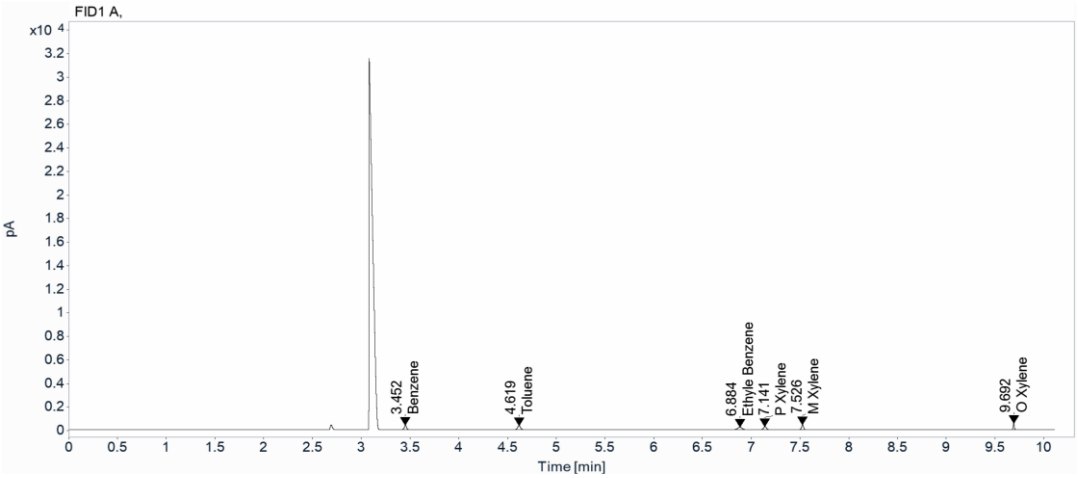


Fig. 2(a) Standard control on day 1

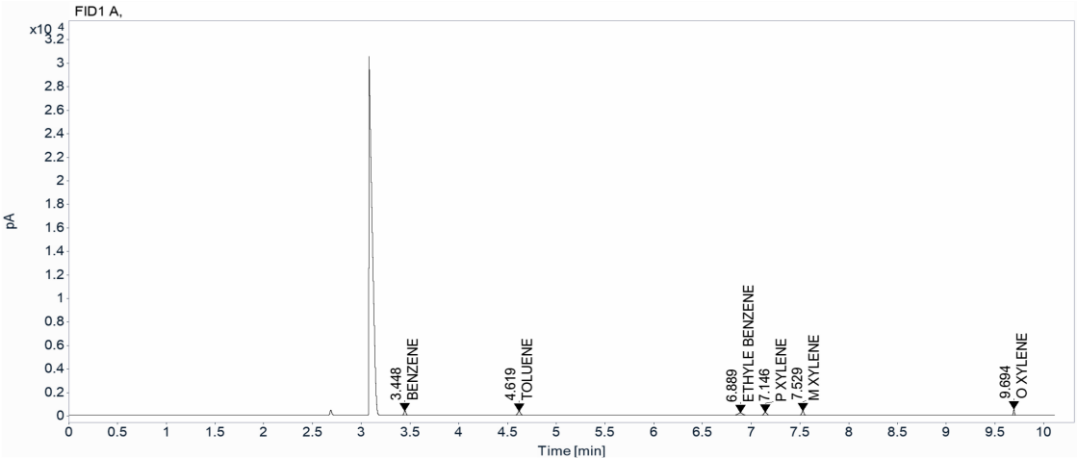


Fig. 2(b) Standard control on day 8

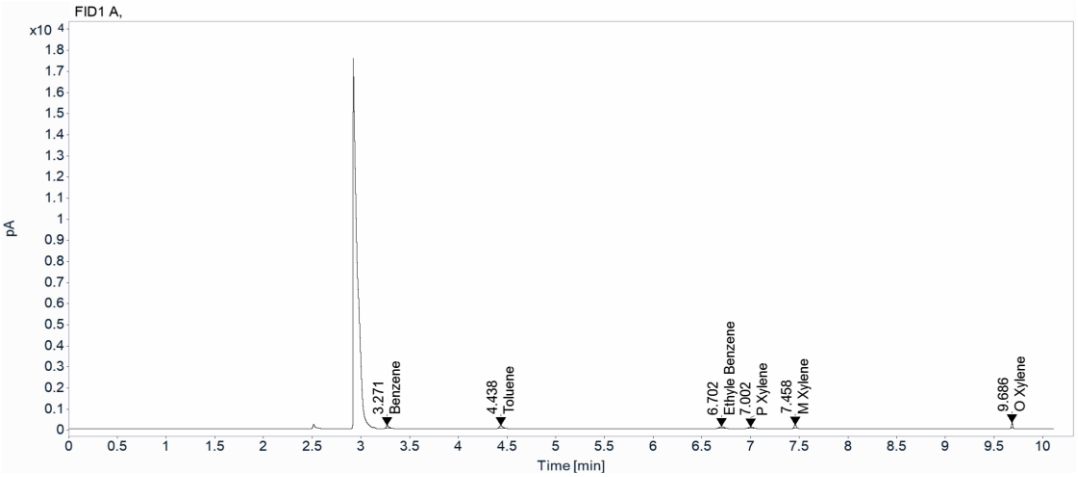


Fig. 2(c) Isolate P on day 8

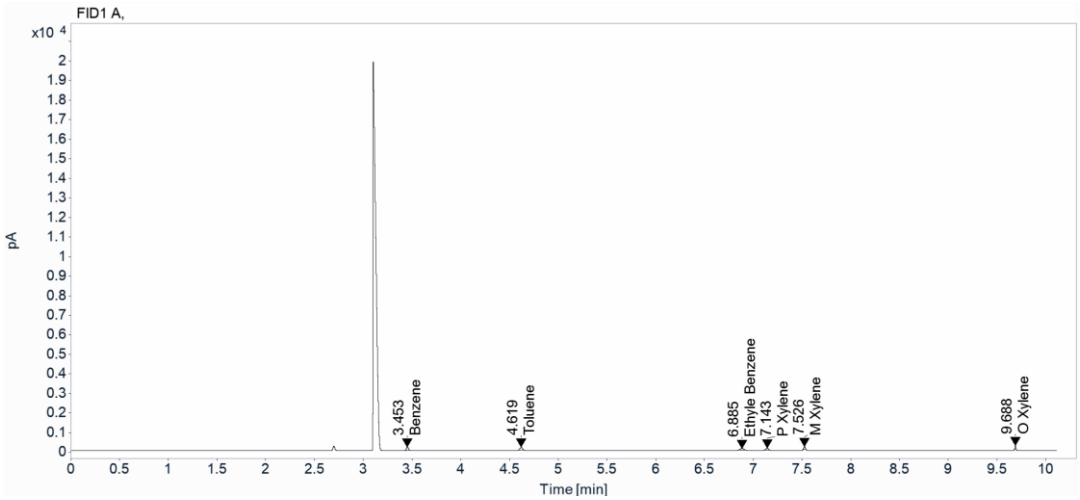


Fig. 2(d) Isolate F on day 8

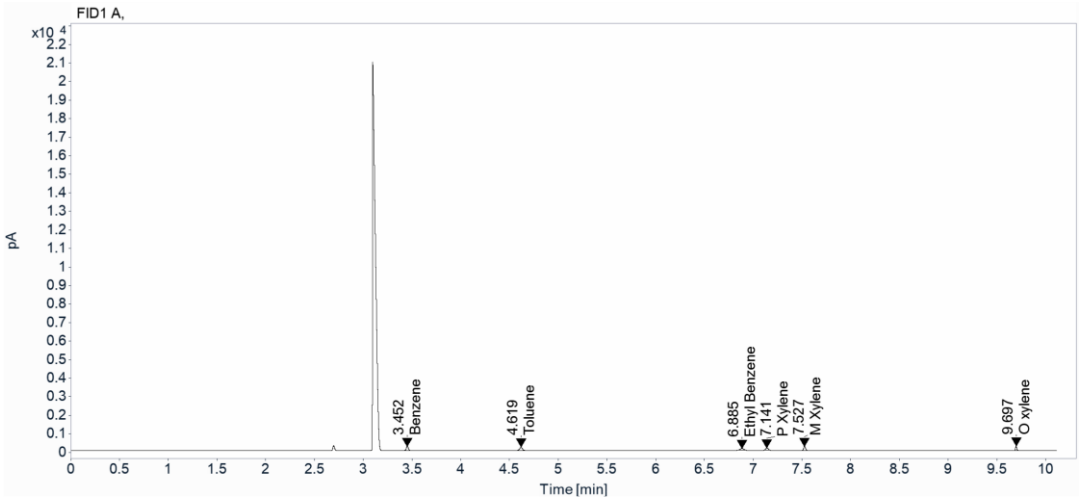


Fig. 2(e) Isolate D on day 8

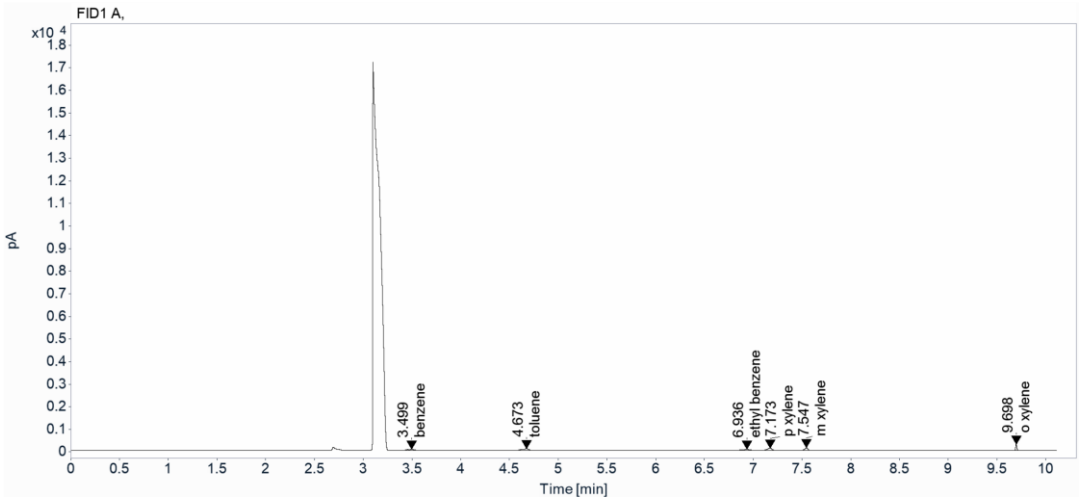


Fig. 2(f) Reference strain MTCC 102 on day 8

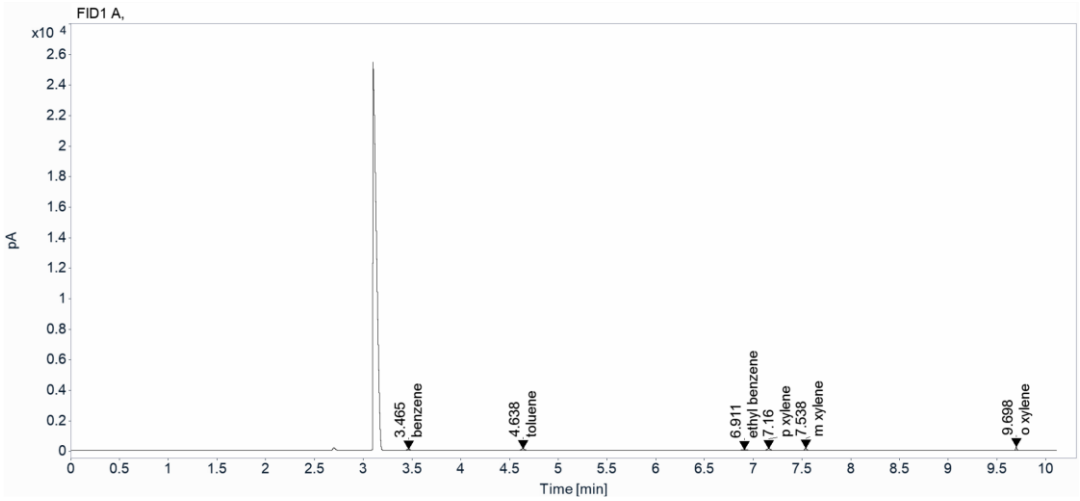


Fig. 2(g) Microbial consortium on day 8

Fig. 2. GC-FID chromatograms of BTEX compounds showing comparison between abiotic control and cultures inoculated with bacterial isolates, reference strain, and microbial consortium after 8 days of incubation.

3.3. Percentage Degradation of BTEX Compounds

Based on GC analysis, we calculated the percentage degradation of individual BTEX components using mean peak area values relative to abiotic controls (Table 3 and Fig. 4).

Table 2. Mean GC-FID peak areas (± standard deviation) of BTEX compounds in control, bacterial isolates, reference strain (*Pseudomonas* MTCC 102), and microbial consortium after 8 days of incubation.

Compound	Control Day 8 (Mean ± SD)	Isolate P (Mean ± SD)	Isolate D (Mean ± SD)	Isolate F (Mean ± SD)	MTCC 102 (Mean ± SD)	Consortium (Mean ± SD)
Benzene	535.63 ± 2.60	253.16 ± 0.34	239.96 ± 2.81	314.83 ± 1.28	162.55 ± 0.06	123.07 ± 0.33
Toluene	569.34 ± 7.41	284.43 ± 17.24	264.12 ± 0.21	312.30 ± 3.16	256.88 ± 0.03	196.70 ± 0.21
Ethylbenzene	542.98 ± 6.80	201.62 ± 3.39	183.82 ± 0.39	253.82 ± 0.42	173.40 ± 0.27	133.37 ± 0.24
p-Xylene	462.66 ± 6.62	210.73 ± 3.14	203.30 ± 0.94	203.09 ± 0.48	270.62 ± 0.09	208.44 ± 0.20
m-Xylene	484.12 ± 4.32	219.72 ± 1.34	170.99 ± 0.96	276.56 ± 0.86	197.23 ± 0.09	150.96 ± 0.30
o-Xylene	406.21 ± 3.65	188.58 ± 1.98	107.61 ± 0.37	202.72 ± 2.38	226.69 ± 0.18	174.49 ± 0.27

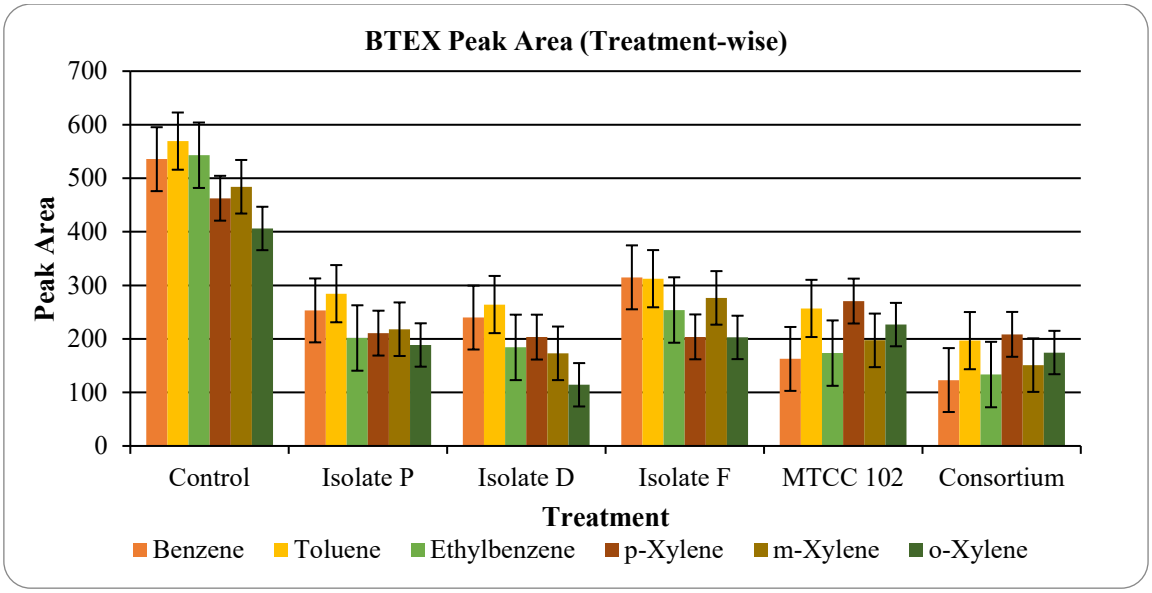


Fig. 3. Mean GC-FID peak area comparison of BTEX compounds across different treatments.

Table 3. Percentage degradation of BTEX compounds by selected bacterial isolates, reference strain, and microbial consortium after 8 days of incubation in mineral salt medium supplemented with 300 ppm of each compound.

Compound	Isolate P (%)	Isolate D (%)	Isolate F (%)	MTCC 102 (%)	Consortium (%)
Benzene	52.75 ± 1.2	55.21 ± 1.0	41.18 ± 1.5	69.65 ± 0.3	77.02 ± 1.1
Toluene	50.05 ± 1.1	53.61 ± 0.9	45.13 ± 1.3	54.87 ± 0.2	65.44 ± 1.0
Ethylbenzene	62.87 ± 1.3	66.10 ± 1.1	53.24 ± 1.4	68.07 ± 0.4	75.44 ± 1.2
p-Xylene	54.45 ± 1.0	56.04 ± 1.2	55.96 ± 1.1	41.51 ± 0.3	54.95 ± 1.3
m-Xylene	54.96 ± 1.2	64.26 ± 1.0	42.87 ± 1.6	59.27 ± 0.4	68.83 ± 1.1
o-Xylene	53.58 ± 1.1	71.86 ± 1.2	50.10 ± 1.4	44.19 ± 0.3	57.05 ± 1.2

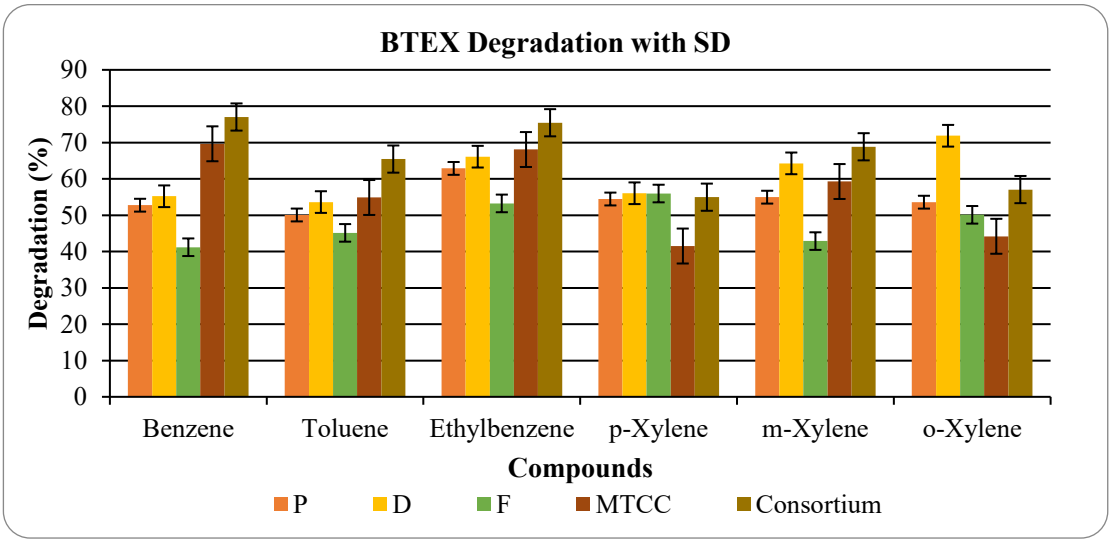


Fig. 4. Percentage degradation of BTEX compounds by bacterial isolates, reference strain (*Pseudomonas* MTCC 102), and microbial consortium after 8 days of incubation.

Based on GC-FID analysis, the percentage degradation of individual BTEX compounds was calculated using mean peak area values relative to the abiotic control (Table 3 and

Fig. 4). The results demonstrated that all selected bacterial isolates, the reference strain, and the microbial consortium were capable of degrading BTEX compounds under the experimental conditions. Among the individual isolates, isolate D showed the highest degradation efficiency across all compounds, with the highest degradation for o-xylene (71.86%), indicating superior metabolic capability for structurally complex hydrocarbons. Isolates P and F showed comparatively moderate degradation performance. The reference strain (*Pseudomonas* MTCC 102) exhibited compound-specific degradation behaviour, with higher degradation efficiency for benzene and ethylbenzene and comparatively lower degradation for xylene isomers. This pattern suggests substrate specificity and differences in enzymatic pathways involved in hydrocarbon degradation. Notably, the microbial consortium exhibited the highest degradation efficiency for most BTEX compounds, indicating enhanced biodegradation potential due to synergistic interactions among the microbial members. These interactions enable complementary metabolic pathways, facilitating more efficient, complete degradation of complex hydrocarbon mixtures. Overall, the results highlight variability in metabolic capacity among individual isolates and emphasize the advantage of microbial consortia over single strains for effective BTEX bioremediation.

Table 4. Total GC–FID peak area of BTEX compounds across treatments after 8 days of incubation.

Treatment	Total Peak Area
Control	3000.94
Isolate P	1358.24
Isolate D	1169.80
Isolate F	1563.32
MTCC 102	1287.37
Consortium	987.03

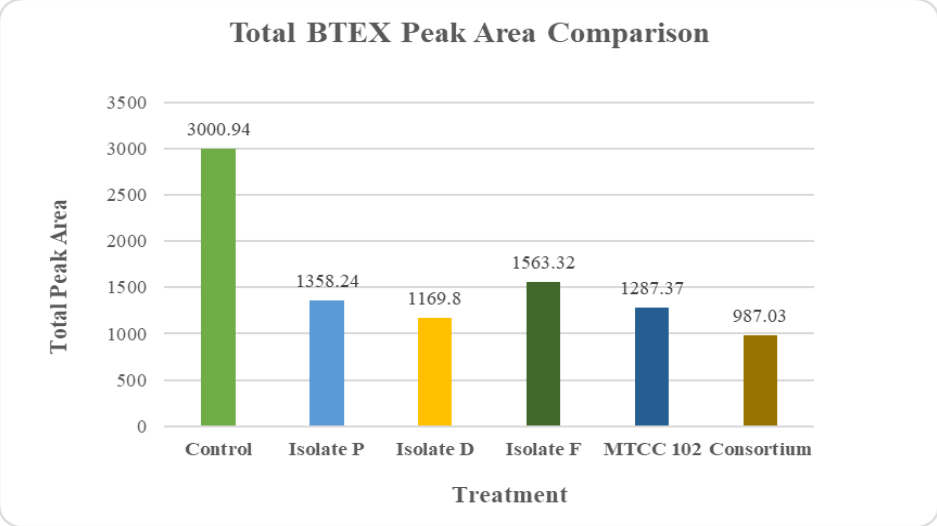


Fig. 5. Comparative total GC-FID peak area of BTEX compounds across all treatments after 8 days of incubation, indicating overall biodegradation efficiency.

We further evaluated overall degradation efficiency based on total peak area reduction, which provides a cumulative measure of BTEX removal. As shown in Fig. 5, the total peak area decreased from control to treated samples. The microbial consortium exhibited the lowest total peak area, indicating the highest overall degradation efficiency, followed by isolate D and the reference strain (*Pseudomonas* MTCC 102). Among the individual isolates, isolate D performed best, while isolates P and F showed comparatively lower degradation efficiency. The negligible reduction observed in the abiotic control confirms that the observed decrease in peak area is primarily due to microbial activity

rather than abiotic loss. The consortium's enhanced degradation is attributed to synergistic interactions among microbial members, which facilitate efficient utilisation and breakdown of complex BTEX mixtures.

3.4. Statistical Analysis and Comparative Evaluation

One-way ANOVA was applied to compare degradation performance among treatments, and differences were considered significant at $p < 0.05$. Isolate D showed significantly higher degradation efficiency than isolates P and F for all BTEX compounds, confirming its superior performance among the individual strains. The reference strain (*Pseudomonas* MTCC 102) exhibited comparable degradation for certain compounds, particularly benzene and ethylbenzene, but showed lower efficiency for xylene isomers. In contrast, the microbial consortium demonstrated a significantly higher overall degradation efficiency than all individual treatments, indicating the advantage of synergistic microbial interactions. These results highlight treatment-specific differences in BTEX degradation potential and emphasise the importance of microbial consortia in enhancing the biodegradation of complex hydrocarbon mixtures.

3.5. Molecular identification of selected BTEX-degrading isolates

We subjected the three bacterial isolates that showed rapid growth and significant BTEX degradation to molecular identification using 16S rRNA gene sequencing (Table 5). Amplification of the 16S rRNA gene yielded an approximately 1500 bp amplicon for all isolates, confirming successful PCR amplification. BLAST analysis of the sequences against the NCBI Type Strain database showed that isolate P had the highest similarity (99.65%) to *Acinetobacter schindleri*. Isolate **D** exhibited 100% sequence similarity with *Pseudomonas aeruginosa*, while isolate **F** showed 99.86% similarity with *Pseudomonas taiwanensis*. Phylogenetic analysis using the maximum-likelihood method further confirmed each isolate's clustering with its closest reference strain, validating its taxonomic affiliation (Fig. 6). These results indicate that the selected isolates belong to bacterial genera widely reported for their hydrocarbon-degrading potential.

Table 5. Molecular identification of selected BTEX-degrading bacterial isolates based on 16S rRNA gene sequence analysis

Isolate	Closest Species	Sequence Similarity (%)
P	*Acinetobacter schindleri*	99.65
D	*Pseudomonas aeruginosa*	100
F	*Pseudomonas taiwanensis*	99.86

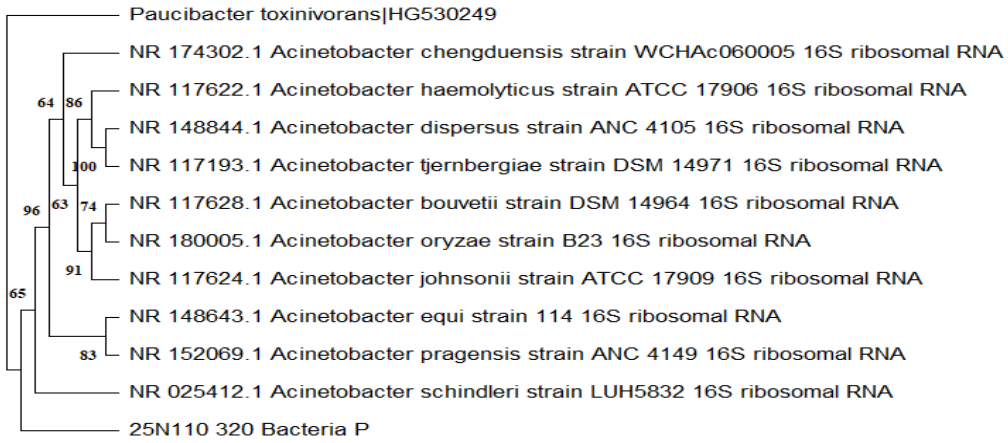


Fig. 6(a) Isolate P

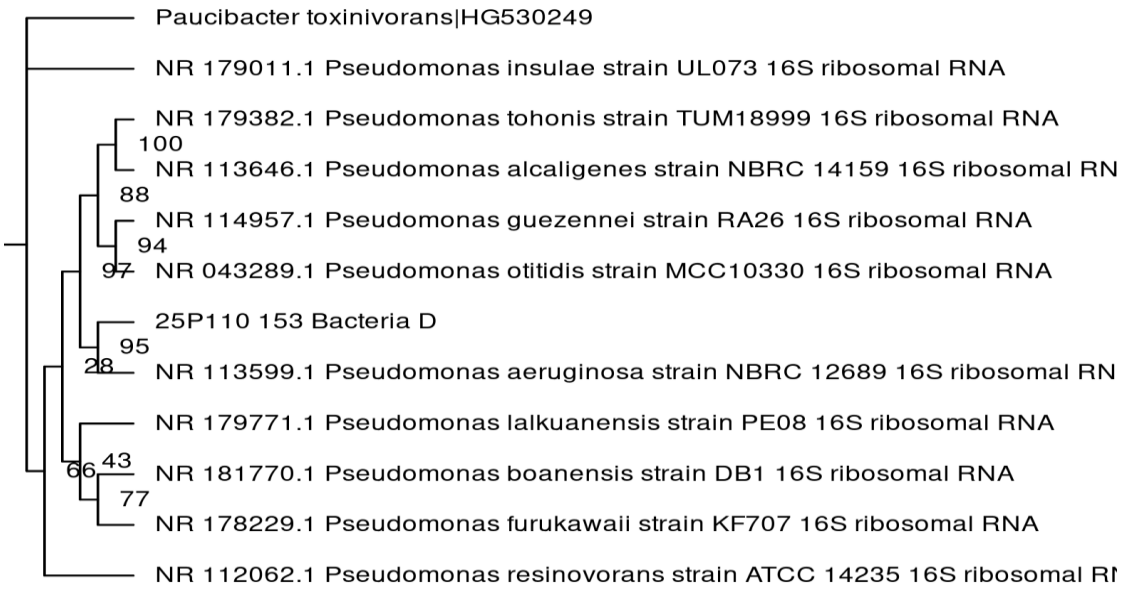


Fig. 6(b) Isolate D

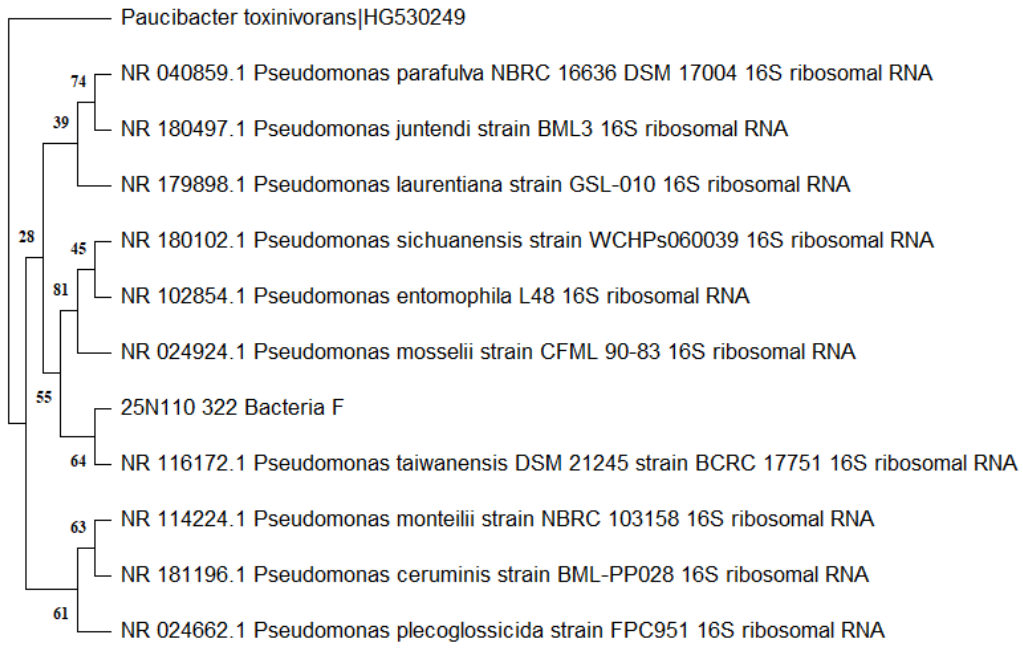


Fig. 6(c) Isolate F

Fig. 6. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship between isolates and their closest reference strains.

The successful isolation of BTEX-degrading bacteria from petroleum-contaminated soil demonstrates the adaptive capability of indigenous microbial communities in hydrocarbon-polluted environments. Microorganisms present in contaminated soils are frequently exposed to petroleum hydrocarbons and therefore develop metabolic pathways that enable them to utilise these compounds as carbon and energy sources. The enrichment-based isolation strategy used in this study effectively selected microorganisms capable of utilizing BTEX as the sole carbon source. Similar enrichment approaches have been widely reported for isolating hydrocarbon-degrading bacteria from contaminated soils.[2] All three isolates

degraded BTEX compounds, although their efficiencies varied. Such variability among bacterial isolates is common in biodegradation studies and often reflects differences in metabolic pathways, enzyme systems, and substrate transport mechanisms.[10] This study also observed preferential degradation of benzene and toluene over ethylbenzene and xylene, which has been reported and may relate to structural differences among these compounds and differences in enzymatic accessibility.[11] Molecular identification revealed that the isolates belong to bacterial genera widely known for hydrocarbon degradation. Species of the genus Pseudomonas are particularly well known for their metabolic versatility and their ability to degrade a wide range of aromatic hydrocarbons due to the presence of oxygenase enzyme systems that initiate aromatic ring oxidation.[7,12] Gas chromatography reliably confirmed BTEX degradation by the selected isolates, as shown by reduced peak areas in inoculated samples compared with abiotic controls. These analytical approaches commonly monitor hydrocarbon biodegradation in laboratory studies.[8] Overall, integrating enrichment-based isolation, growth screening, chromatographic confirmation, and molecular identification provides a robust strategy for identifying efficient BTEX-degrading microorganisms. These isolates represent promising candidates for further investigation and potential application in the bioremediation of petroleum-contaminated environments.

4. CONCLUSION

The present study successfully isolated and screened BTEX-degrading bacteria from petroleum-contaminated soil. Out of 96 initial isolates obtained through enrichment in BTEX-containing mineral salt medium, 16 isolates exhibited appreciable growth, and three fast-growing isolates (P, D, and F) were selected based on sustained cell density and degradation performance. GC–FID analysis confirmed that these isolates degrade BTEX compounds, with isolate D showing the highest degradation efficiency. Comparative evaluation with the reference strain (*Pseudomonas* MTCC 102) revealed compound-specific degradation behaviour, with higher efficiency for benzene and ethylbenzene and comparatively lower degradation of xylene isomers, thereby validating the experimental system. Notably, the microbial consortium showed the highest overall degradation efficiency, as indicated by the lowest total peak area and higher BTEX degradation percentages. This improved performance likely reflects synergistic interactions among microbial members, enabling more efficient utilization and complete breakdown of complex hydrocarbon mixtures. Molecular identification identified the selected isolates as Acinetobacter schindleri, Pseudomonas aeruginosa, and Pseudomonas taiwanensis, all well known for their hydrocarbon-degrading capabilities. Overall, the findings demonstrate that microbial consortia outperform individual strains and highlight their significant potential for bioremediation of BTEX-contaminated environments.

Declaration by Authors

Acknowledgement: None
Source of Funding: None
Conflict of Interest: No conflicts of interest declared.

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How to cite this article: Pradnya Ingale, Lolly Jain. Isolation and screening of BTEX (benzene, toluene, ethylbenzene, and xylene)-degrading bacteria from petroleum-contaminated soil. *International Journal of Research and Review*. 2026; 13(10): 356-369.
DOI: *https://doi.org/10.52403/ijrr.20261032*

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