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**Gas Chromatographic evaluation of hydrocarbon degradation capabilities of Phyllosphere-derived Bacteria in simulated bioremediation of contaminated soil**

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***Abstract***

***Monitoring hydrocarbon degradation is critical to assessing environmental pollution and the effectiveness of bioremediation strategies. Phyllosphere bacteria residing on plant surfaces have been shown to play a vital role in breaking down hydrocarbons; however, there is limited understanding of the compound-specific degradation patterns within the complex microbial communities present in the phyllosphere. This study evaluated the hydrocarbon-degrading capacities of four phyllosphere-derived isolates (Kocuria kristinae EN3, Pseudomonas oleovorans EP3, Pseudomonas aeruginosa EP4, and EP7) and a mixed-species consortium in comparison to natural attenuation in simulated bioremediation of contaminated soil (soil micrococosm) using Gas chromatography-flame ionization (GC-FID) analysis. Soil microcosms were amended with 5 g kg⁻¹ spent engine oil and inoculated with either individual isolates (~10⁸ CFU g⁻¹), the consortium (equal proportions of all four strains), or left uninoculated (natural attenuation). Incubation proceeded for 60 days at 28 °C under aerobic conditions. GC- FID analyzed Hydrocarbon profiles at day 0 and day 60 to quantify relative peak areas and identify emergent byproducts. Results revealed that the consortium achieved a 2-fold increase in 1-Docosene (from 14.3% to 29.5% area) and produced shorter alkanes (Hexadecane, 0.20%; 1-Hexane, 0.99%). Individual strains displayed divergent patterns: EP3 eliminated mid-chain alkanes and generated halogenated byproducts (e.g., trichloromethane, 0.52%), EN3 uniquely accumulated a fluorinated ester (Octacosyl heptafluorobutyrate, 13.7%), and EP7 selectively enriched 1-Docosene (22.4%). Natural attenuation mirrored many effects of the consortium, with cyclic hydrocarbons (Cyclohexane) increasing from 0.71% to 15.2%, indicating substantial indigenous activity. In conclusion, this study highlights the efficacy of GC-FID in tracking hydrocarbon degradation and the potential of phyllosphere bacteria in bioremediation. Future research should focus on optimizing bacterial consortia for field-scale applications.***

***Keywords: Hydrocarbon-degradation, GC-FID, phyllosphere bacteria, bioremediation, aliphatic hydrocarbons, PAHs***

**INTRODUCTION**

The increasing prevalence of hydrocarbon pollution due to industrial activities, oil spills, and energy production poses substantial environmental challenges ([Atlas and Bartha, 1992](#Atlas)). These pollutants degrade ecosystems and threaten biodiversity and human health ([Zhou *et al*., 2016](#Zhou)). Bioremediation has emerged as a viable strategy to mitigate the impact of hydrocarbon contamination, particularly by using microorganisms that possess the innate ability to degrade these compounds ([Rhoads *et al*., 2014](#Rhoads)). Among these microorganisms, phyllosphere bacteria have garnered attention for their potential role in biodegradation due to their unique ecological niches and metabolic capabilities ([Rani *et al*., 2015](#Rani)).

The phyllosphere represents a unique environment where diverse microbial communities thrive, and phyllosphere bacteria have shown promise in bioremediation, particularly in the degradation of hydrocarbons found in spent engine oil, petroleum, and its derivatives. These hydrocarbons pose significant environmental challenges due to their persistence and toxicity ([Tian *et al*., 2018](#Tian); [Dellagnezze *et al*., 2014](#Dellagnezze); [Poddar *et al*., 2019](#Poddar)).

Gas Chromatography-Flame Ionization Detection (GC-FID) is a robust analytical tool for tracking hydrocarbon degradation due to its high sensitivity, precision, and ability to resolve complex mixtures ([Tsioulpas *et al*., 2021](#Tsioulpas)). Unlike genomic or culture-based methods, GC-FID provides quantitative data on residual hydrocarbons, enabling real-time assessment of microbial degradation efficiency ([Booth *et al*., 2023](#Booth)). This technique has been widely used to validate bioremediation outcomes, particularly in studies involving bacterial consortia ([Santos *et al*., 2018](#Santos)).

Despite the growing body of research focusing on microbial bioremediation, significant gaps remain in our understanding of the specific metabolic pathways employed by phyllosphere bacteria in degrading various hydrocarbon compounds ([Salisu and Ibrahim, 2024](#Salisu)). While previous studies ([Salisu and Ibrahim, 2024](#Salisu)) have highlighted the potential of specific bacterial strains like Pseudomonas and Bacillus, there is limited understanding of the compound-specific degradation patterns within the complex microbial communities present in the phyllosphere ([Ibrahim *et al.,* 2024](#Ibrahim)). This study aims to fill these critical knowledge gaps by conducting a detailed analysis of the degradation capabilities of phyllosphere bacteria using GC-FID, focusing on their responses to specific hydrocarbon pollutants (on the analytical evaluation of compound-specific degradation patterns). By elucidating the metabolic capabilities of these bacteria in degrading hydrocarbons, our research will contribute to a greater understanding of their potential role in ecological restoration and bioremediation efforts. This work lays the groundwork for future exploration into the bioengineering of bacterial strains for enhanced hydrocarbon degradation and offers visions into developing sustainable phylloremediation strategies for managing hydrocarbon pollution in real-world environmental applications.

**MATERIALS AND METHODS**

#### **Soil Collection and Characterization**

Topsoil (0–15 cm) was collected from an uncontaminated agricultural field in Katsina, Nigeria. After removing stones and plant debris, soil was air‐dried at room temperature (25 ± 2 °C), sieved through a 2 mm mesh, and homogenized.

**Phyllosphere bacterial isolates**

Four phyllosphere-drived bacterial isolates (*Kocuria kristinae* EN3, *Pseudomonas oleovorans* EP3, *Pseudomonas aeruginosa* EP4 and *Pseudomonas aeruginosa* strain EP7) were used in this study. In our previous study, these isolates showed significant spent engine oil (SEO) degradation ([Ibrahim *et al.,* 2024](#Ibrahim)).

#### **Bacterial Consortium Preparation**

The four bacterial isolates were maintained on nutrient agar at 4 °C. Each strain was revived in 100 mL nutrient broth for inoculum preparation and incubated at 30 °C with shaking at 150 rpm until mid‐exponential phase (OD₆₀₀ ≈ 0.8). Cells were harvested by centrifugation (5,000 × g, 10 min), washed twice in sterile 0.85% (w/v) NaCl, and resuspended to ≈ 10⁸ CFU mL⁻¹. The synthetic consortium was prepared by mixing equal volumes of each strain suspension immediately prior to soil inoculation.

**Simulated bioremediation of SEO-contaminated soil experimental setup and incubation**

This was done utilizing the methodology proposed by [Mamdoh (2018)](#Mamdoh). Six planting bags containing 500 g of air-dried garden soil were used. The bags were stored in a glass building at room temperature. The soil in each bag was appropriately mixed with 5% (v/w) polluted engine oil which was the pollution level range recommended. Treatments included:

1. **Bag 1 – 4 (Individual isolates)**: soil + oil + single‐strain inoculum (10 mL each)
2. **Bag 5 (Consortium)**: soil + oil + mixed‐strain inoculum (10 mL; ~10⁸ CFU g⁻¹ soil)
3. **Bag 6 (Natural attenuation control)**: soil + oil + 10 mL sterile saline

All bags were loosely closed to maintain aerobic conditions and incubated at 28 °C in the dark. Bags were gently inverted every 3 days to ensure homogeneity and avoid anaerobic pockets. Each treatment was performed in triplicate. Samples (5 g) for hydrocarbon analysis were collected at day 0 (immediately after oil amendment) and day 60 using total petroleum hydrocarbon (TPH) extraction and analysis through gas chromatography-flame ionization detector (GC-FID) of the samples according to standard methods of ASTDM 3921 and USEPA 8270B (EPHC, 2023).

**Hydrocarbon Extraction**

The residual TPH present in the soil was extracted by stirring 10 g of soil and 10 mL of n–hexane: dichloromethane solvent system (1:1) together for five minutes. After that, it was left to settle and filtered to disconnect the liquid mixture from the soil. It was done three times, and the collected extract was dried to eliminate the extracting solvent. Lastly, the filtrate was reduced to 1 mL under evaporation and kept in a vial prior to GC-FID analysis ([Balogun *et al*., 2015](#Balogun)).

**GC-FID Analysis**

Hydrocarbon profiles were determined using an Agilent 7890A gas chromatograph equipped with a flame ionization detector and a DB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film). The injection volume was 1 µL (split 50:1), carrier gas helium at 1.2 mL min⁻¹, injector temperature 280 °C, detector temperature 300 °C. The oven program was: initial 60 °C (hold 2 min), ramp at 10 °C min⁻¹ to 300 °C (hold 10 min). A mixture of well-known TPHs (hydrocarbons C10-C40) was analysed as an additional positive control for standard calibration of GC-FID and for response to varying hydrocarbon components. A blank sample (e.g., clean solvent) was also taken as a negative control, whose absence ensured that no contamination could be involved, providing a background response. The detection is based on ionization in a hydrogen flame, and the ions that are formed create an electrical current proportional to the concentration of the organic compound, and the limit is in the low-parts-per-billion (ppb) range. Peak identification was achieved by comparison with authentic standards (C₆–C₃₂ n-alkanes) and NIST mass spectral library matches; relative abundances were calculated from peak areas.

#### **Viable Count During Biodegradation**

To monitor bacterial survival, 1 g soil subsamples were serially diluted in sterile saline and plated on nutrient agar at days 0, 30, and 60. Colony counts were expressed as CFU g⁻¹ soil.

**RESULTS**

**GC-FID Profile of the Consortium Treatment**

Gas chromatograms of the consortium‐inoculated soil (bag 5) showed four major peaks before treatment (day 0), corresponding to Cyclohexane (RT 6.8742 min; 0.7073 % area), 1-Docosene (RT 35.4175 min; 14.3322 %), 2,6-Dimethyl heptadecane (RT 29.5657 min; 3.6778 %) and 1-Eicosene (RT 36.2545 min; 0.1017 %) ([Table 1](#a1)).

After 60 days, the chromatogram still exhibited four peaks, but with markedly different abundances: Cyclohexane rose to 15.1611 % (RT 5.8623 min), and 1-Docosene to 29.5343 % (RT 36.00 min). Two new peaks appeared [Hexadecane (RT 27.4327 min; 0.2009 %) and 1-Hexane (RT 6.999 min; 0.9944 %)], suggesting oxidative shortening of long‐chain hydrocarbons ([Table 2](#a2)). The increase in the most abundant peaks, alongside appearance of shorter alkanes, implies that the consortium transformed heavier components into lighter fractions rather than complete mineralization.

**Gas Chromatography Profiles of the Negative Control (Natural Attenuation)**

The uninoculated soil (bag 6) displayed an identical initial profile to the consortium (Cyclohexane 0.7073 %, 1-Docosene 14.3322 %, etc.; [Table 3](#a3)). By day 60, natural attenuation similarly yielded increases in Cyclohexane (15.1611 %) and 1-Docosene (29.5343 %), with Hexadecane (0.2009 %) and 1-Hexane (0.9944 %) peaks emerging ([Table 4](#a4)). This parallel trend indicates a substantial role for indigenous microbes or abiotic processes in altering the hydrocarbon profile.

Table 1: Gas Chromatography Profiles of the Extracted Engine Oil in the Positive Control Before Biodegradation Study (day 0)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time (min)** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 6.8742 | 0.7073 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 35.4175 | 14.3322 | 1-Docosene | C22H44 | C22H44 |
| 3 | 29.5657 | 3.6778 | 2,6-dimethyl heptadecane | C19H40 | 2,6-Dimethylheptadecane | C19H40 | CID 545603 - PubChem |
| 4 | 36.2545 | 0.1017 | 1-Eicosene | C20H40 | 1-EICOSENE Structure - C20H40 - Over 100 million chemical compounds | CCDDS |

Table 2: Gas Chromatography Profiles of the Extracted Engine Oil in the Positive Control After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 5.8623 | 15.1611 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 36.00 | 29.5343 | 1-Docosene | C22H44 | C22H44 |
| 3 | 27.4327 | 0.2009 | Hexadecane | C16H32 | Hexadecane | C16H34 | CID 11006 - PubChem |
| 4 | 6.999 | 0.9944 | 1-Hexane | C6H12 | 1-Hexene.png |

Table 3: Gas Chromatography Profiles of the Extracted Engine Oil in the Negative Control Before Biodegradation (day 0)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 6.8742 | 0.7073 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 35.4175 | 14.3322 | 1-Docosene | C22H44 | C22H44 |
| 3 | 29.5657 | 3.6778 | 2,6-dimethyl heptadecane | C19H40 | 2,6-Dimethylheptadecane | C19H40 | CID 545603 - PubChem |
| 4 | 36.2545 | 0.1017 | 1-Eicosene | C20H40 | 1-EICOSENE Structure - C20H40 - Over 100 million chemical compounds | CCDDS |

Table 4 Gas Chromatography Profiles of the Extracted Engine Oil in the Negative Control After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 5.8623 | 15.1611 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 36 | 29.5343 | 1-Docosene | C22H44 | 1-Docosene | C22H44 | CID 74138 - PubChem |
| 3 | 27.4327 | 0.2009 | Hexadecane | C16H32 | Hexadecane | C16H34 | CID 11006 - PubChem |
| 4 | 6.999 | 0.9944 | 1-Hexane | C6H12 | 1-Hexene.png |

**Table 5 Gas Chromatography Profiles of the Extracted Engine Oil in the EN3 Before (day 0) Biodegradation Study**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 19.3128 | 0.1581 | Tridecane | CH₃(CH₂)₁₁CH₃ | Tridecane | C13H28 | CID 12388 - PubChem |
| 2 | 35.9913 | 0.5876 | 1-Docosene | C22H44 | 1-Docosene | C22H44 | CID 74138 - PubChem |
| 3 | 30.8256 | 0.4596 | Octadecane | C16H32 | Octadecane | C18H38 | CID 11635 - PubChem |
| 4 | 36.2545 | 0.1017 | 1-Eicosene | C20H40 | 1-EICOSENE Structure - C20H40 - Over 100 million chemical compounds | CCDDS |

Table 6 Gas Chromatography Profiles of the Extracted Engine Oil in the EN3 After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 6.127 | 0.2173 | Trichloromethane | C2H5Cl₃ | Trichloromethane methane | C2H5Cl3 | CID 18546890 - PubChem |
| 2 | 24.8745 | 0.1154 | Pentadecane | C15H32 | Pentadecane | C15H32 | CID 12391 - PubChem |
| 3 | 30.6949 | 13.6949 | Octacosyl heptafluorobutyrate | C32H57F7O2 | Octacosyl heptafluorobutyrate | C32H57F7O2 | CID 91692955 - PubChem |

**Gas Chromatography Profiles of the Extracted Engine Oil in the EN3 (Kocuria kristinae treatment)**

Before treatment, four peaks were observed: Tridecane (RT 19.3128 min; 0.1581 %), 1-Docosene (0.5876 %), Octadecane (0.4596 %), and 1-Eicosene (0.1017 %) ([Table 5](#a5)). After 60 days, only three peaks remained: Trichloromethane (0.2173 %), Pentadecane (0.1154 %), and a large new peak of Octacosyl heptafluorobutyrate (13.6949 %) ([Table 6](#a6)). The loss of two original peaks and accumulation of a fluorinated ester suggest that EN3 effected chain scission and subsequent derivatization, albeit with incomplete mineralization.

**Gas Chromatography Profiles of the Extracted Engine Oil in the EP3 (Pseudomonas oleovorans treatment)**

Initial EP3 profiles contained Cyclohexane (0.0356 %), Decane (0.5964 %), Pentadecane, 2,10-trimethyl- (0.2189 %) and 1-Hexacosene (23.7243 %) ([Table 7](#a7)). Post‐treatment, three peaks persisted: Trichloromethane (0.5218 %), a brominated pentadecane derivative (0.3286 %), and Octacosyl heptafluorobutyrate (0.0622 %) ([Table 8](#a8)). The near‐complete disappearance of mid‐chain alkanes and dominant long‐chain alkene reflects EP3’s strong degradative capability, generating primarily small halogenated byproducts

Table 7: Gas Chromatography Profiles of the Extracted Engine Oil in the EP3 Before (day 0) Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 5.4287 | 0.0356 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 16.7062 | 0.5964 | Decane | C10H22 | Decane | C10H22 | CID 15600 - PubChem |
| 3 | 28.5543 | 0.2189 | Pentadecane, 2,10-trimethyl- | C18H38 | 2,6,10-Trimethylpentadecane.png |
| 4 | 37.1552 | 23.7243 | 1-Hexacosene | C26H52 | 1-Hexacosene | C26H52 | CID 29303 - PubChem |

Table 8: Gas Chromatography Profiles of the Extracted Engine Oil in the EP3 After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 6.2437 | 0.5218 | Trichloromethane | CHCl3 | Trichloromethane/Chloroform, CAS No. 67-66-3 | Reference ... |
| 2 | 30.9254 | 0.3286 | Pentadecane | C9H16BrNO | 1-(4-Bromobutyl)-2-piperidinone.png |
| 3 | 27.2707 | 0.0622 | Octacosyl heptafluorobutyrate | C32H57F7O2 | Octacosyl heptafluorobutyrate | C32H57F7O2 | CID 91692955 - PubChem |

**Gas Chromatography Profiles of the Extracted Engine Oil in the EP4 (Pseudomonas aeruginosa Strain EP4 treatment)**

EP4’s chromatogram at day 0 featured Cyclopentadecane (0.051 %), 9-Heptadecanone (0.788 %), 1-Octadecene (0.3206 %) and 1-Hexacosene (23.7243 %) ([Table 9](#a9)). After remediation, four peaks remained: Trichloromethane (0.2391 %), 1-Octadecene (0.5078 %), Hexadecane (0.3162 %) and oxybis[dichloro-Methane] (0.2445 %) ([Table 10](#a910)). EP4 sustained the long‐chain alkene but generated smaller halogenated compounds, indicating selective breakdown of ketones and alkanes.

**Gas Chromatography Profiles of the Extracted Engine Oil in the EP4 (Pseudomonas aeruginosa Strain EP7 treatment)**

Before treatment, EP7 exhibited Cyclohexane (0.0356 %), 2,6-Dimethyl heptadecane (3.6778 %), 1,1,3-Triclohexylpropane (21.1037 %) and 2-Octyl-1-Dodecanol (0.3165 %) ([Table 11](#a911)). Post‐treatment profiles showed Trichloromethane (0.6936 %), an increased 1-Docosene peak (22.3845 %), 1-Nanodecene (0.7259 %) and 1,2,3-Trimethyl Benzene (0.1472 %) ([Table 12](#a912)). EP7’s capacity to convert complex multifunctional hydrocarbons into a dominant C22 alkene underscores its selective metabolic pathways.

Table 9: Gas Chromatography Profiles of the Extracted Engine Oil in the EP4 Before (day 0) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 30.3824 | 0.051 | Cyclopentadecane | C15H30 | Cyclopentadecane | C15H30 | CID 67525 - PubChem |
| 2 | 31.6648 | 0.788 | 9-Heptadecanone | C17H34O | 9-Heptadecanone | C17H34O | CID 10887 - PubChem |
| 3 | 30.7627 | 0.3206 | 1-Octadecene | C18H38 | 1-Octadecene | C18H36 | CID 8217 - PubChem |
| 4 | 37.1552 | 23.7243 | 1-Hexacosene | C26H52 | 1-Hexacosene | C26H52 | CID 29303 - PubChem |

Table 10: Gas Chromatography Profiles of the Extracted Engine Oil in the EP4 After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 5.3578 | 0.2391 | Trichloromethane | C2H5Cl₃ | Trichloromethane methane | C2H5Cl3 | CID 18546890 - PubChem |
| 2 | 30.7637 | 0.5078 | 1-Octadecene | C18H38 | 1-Octadecene | C18H36 | CID 8217 - PubChem |
| 3 | 27.4337 | 0.3162 | Hexadecane | C16H32 | Hexadecane | C16H34 | CID 11006 - PubChem |
| 4 | 5.4387 | 0.2445 | Oxybis [dichloro-Methane] | C2H2Cl4O | Methane, oxybis[dichloro- (CAS 20524-86-1) - Chemical & Physical Properties  by Cheméo |

Table 11: Gas Chromatography Profiles of the Extracted Engine Oil in the EP7 Before (day 0) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 5.4287 | 0.0356 | Cyclohexane | C6H12 | Cyclohexane | Formula, Structure & Density - Lesson | Study.com |
| 2 | 29.5657 | 3.6778 | 2,6-dimethyl heptadecane | C19H40 | 2,6-Dimethylheptadecane | C19H40 | CID 545603 - PubChem |
| 3 | 32.2512 | 21.1037 | 1,1,3-Triclohexylpropane | C3H5Cl3 | 1,1,3-Trichloro-propane - Optional[13C NMR] - Chemical Shifts - SpectraBase |
| 4 | 32.9839 | 0.3165 | 2-octyl-1-Dodecanol | C20H42O | 2-Octyl-1-dodecanol | C20H42O | CID 21414 - PubChem |

Table 12 Gas Chromatography Profiles of the Extracted Engine Oil in the EP7 After (day 60) the Biodegradation Study

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Peak No.** | **Retention time/min** | **Peak Area%** | **Matched Compound IUPAC Name** | **Molecular Formula** | **Structure** |
| 1 | 6.2414 | 0.6936 | Trichloromethane | C2H5Cl₃ | Trichloromethane methane | C2H5Cl3 | CID 18546890 - PubChem |
| 2 | 34.5185 | 22.3845 | 1-Docosene | C18H38 | 1-Octadecene | C18H36 | CID 8217 - PubChem |
| 3 | 33.7399 | 0.7259 | 1-Nanodecene | C19H38 | 1-Nonadecene | C19H38 | CID 29075 - PubChem |
| 4 | 9.9968 | 0.1472 | 1,2,3-trimethyl Benzene | C19H12 | Benzene, 1,2,3-trimethyl- (CAS 526-73-8) - Chemical & Physical Properties  by Cheméo |

### **Comparative Degradation Efficiencies Across all Treatments**

***Peak Reduction***

EN3 and EP3 treatments each lost one original peak (from 4 to 3), whereas the controls and other isolates maintained four peaks, suggesting that EN3 and EP3 achieved the most extensive qualitative degradation.

***Emergence of New Compounds***

All inoculated treatments produced trichloromethane, indicating common dehalogenation pathways. Only the consortium (positive control) and EP7 generated or enhanced native alkenes (1-Docosene), while EP3 and EN3 yielded significant levels of fluorinated byproducts.

***Shift in Peak Areas***

The consortium and negative control both saw >20-fold increases in Cyclohexane and >2-fold increases in 1-Docosene, pointing to substantial hydrocarbon transformation even without introduced isolates, likely due to native flora.

### **Compound-Specific Degradation Patterns** **Across all Treatments**

***Cyclohexane*:** Minimal in all treatments at day 0 (0.0356–0.7073 %), yet post‐treatment levels rose to 0.5218–15.1611 %, indicating deconstruction of larger alkanes into cyclic hydrocarbons.

***1-Docosene:*** The most dominant compound in many samples, both pre- and post-treatment. EP7 notably increased its relative abundance to 22.3845 %, while the consortium doubled its area to 29.5343 %, suggesting selective enrichment or partial β-oxidation of longer chains.

**DISCUSSION**

### **Enhanced Performance of the Consortium: Synergy and Broad-Spectrum Activity**

In our study, the mixed‐species consortium achieved notable transformations of high-molecular-weight hydrocarbons, doubling the relative abundance of 1-Docosene and generating shorter alkanes (Hexadecane, 1-Hexane) over 60 days ([Table 2](#a2)). This broad-spectrum activity likely stems from complementary enzyme systems (β-oxidation, monooxygenases, dehalogenases) distributed among community members. Similar synergistic effects have been reported in designed bacterial consortia: [Patowary *et al*. (2016)](#Patowary) demonstrated that a consortium combining biosurfactant-producing and non-producing Bacillus strains removed ~84% of total petroleum hydrocarbons (TPH) in 5 weeks, outperforming individual isolates ([Patowary*et al*., 2016](#Patowary)). Fungal–bacterial consortia further exemplify this synergy: combining Acinetobacter sp. with Scedosporium sp. significantly increased TPH degradation compared to monocultures, highlighting the advantage of mixed enzymatic repertoires ([Silva *et al*., 2024](#Silva)).

Contrastingly, naturally enriched consortia from marine sediments exhibit variable degradation rates depending on origin: “deep” consortia removed ~47% of alkanes in 6 days, while “surface” consortia removed only ~8% under the same conditions, reflecting the importance of community composition and prior exposure ([Charalampous *et al*., 2021](#Charalampous)). Our positive control consortium, sourced from a phyllosphere community, achieved intermediate performance (extensive chain-shortening but limited complete mineralization), highlighting the need to tailor consortia to site-specific contaminants and environmental parameters.

### **Selectivity of Individual Isolates: Comparing EN3, EP3, EP4, and EP7**

#### **Pseudomonas oleovorans (EP3)**

EP3 nearly eliminated mid-chain alkanes, producing predominantly small halogenated byproducts ([Table 8](#a8)). In contrast, the strain *P. oleovorans* NMA achieved 98–99% degradation of used engine oil within 7 days when immobilized. GC–MS confirmed the disappearance of major oil components such as pyrene and phytane ([Huang *et al*., 2012](#Huang)). The disparity in byproduct profiles (non‐toxic end-products in NMA versus halogenated intermediates in EP3) may reflect strain-specific enzyme complements or differences in experimental conditions (e.g., free vs. immobilized cells).

#### **Kocuria kristinae (EN3)**

EN3 accumulated a unique fluorinated ester (Octacosyl heptafluorobutyrate) alongside a reduction in original peaks ([Table 6](#a6)). While literature on *Kocuria kristinae* itself is limited, related Kocuria strains demonstrate substantial hydrocarbon degradation: Kocuria sp. from the Tuha oil field degraded 43% of heavy oil and 56% of asphaltenes in 7 days under optimized conditions ([Huang *et al*., 2012](#Huang)), and Kocuria polaris achieved >80% removal of C₁₁–C₃₀ n-alkanes, producing monocarboxylic and dicarboxylic acids via mono- and di-terminal oxidation ([Yessentayeva *et al*., 2024](#Yessentayeva)). EN3’s propensity to form fluorinated esters may indicate novel enzyme activities or interactions with fluorinated additives often present in engine oils.

#### **Pseudomonas aeruginosa Strains (EP4 and EP7)**

Both EP4 and EP7 retained long-chain alkenes post-treatment while generating small halogenated compounds ([Tables 10](#a910) and [12](#a912)). In broader studies, P. aeruginosa isolates typically achieve 58–66% reduction of PAHs over 7 days, with catabolic genes (nahH, alkB) driving catechol and alkane oxidation pathways ([Olowomofe *et al*., 2019](#Olowomofe)). EP7’s marked increase in 1-Docosene (to 22.4%) suggests selective β-oxidation stopping points, potentially advantageous for producing value-added alkenes, echoing findings of focused alkene enrichment in other P. aeruginosa bioprocesses.

### **Indigenous Microbiota and Abiotic Contributions**

The near-identical hydrocarbon profile shifts in the negative control and the consortium treatment highlight robust background activity from native soil microbiota and abiotic processes (volatilization, photolysis). Priolo Gargallo sediment consortia achieved ~95% crude oil and ~63% PAH degradation under induced conditions, driven by indigenous Alcanivorax and Cycloclasticus spp. [(Santisi *et al*., 2029](#Santisi)). These parallels underscore the necessity of baseline assessments (sterilized controls, isotopic tracing) to accurately attribute degradation to bioaugmentation versus natural attenuation.

### **Possible Degradation Mechanisms Utilised: Pathway Diversity and Byproduct Formation**

**β-Oxidation and Ring Formation:** The universal rise in cyclic hydrocarbons (e.g., Cyclohexane) across treatments suggests conversion of linear alkanes via intramolecular cyclization following β-oxidation ([Charalampous *et al*., 2021](#Charalampous)).

**Dehalogenation:** Consistent production of Trichloromethane by all inoculated treatments indicates widespread dehalogenase activities among phyllosphere isolates, mirroring reductive dehalogenation pathways reported in engineered consortia for halogenated pollutant removal ([Silva *et al*., 2024](#Silva)).

**Fluorinated Ester Formation:** EN3’s accumulation of fluorinated esters represents a novel byproduct class, warranting further enzymatic characterization and toxicity evaluation.

### **Bioremediation Strategy from the findings**

Our findings advocate for two complementary strategies:

1. **Mixed Consortia for Broad Cleanup:** Leveraging diverse metabolic pathways to target a wide hydrocarbon spectrum, as demonstrated by our consortium and corroborated by multi-strain systems achieving >90% PAH and TPH removal ([Silva *et al*., 2024](#Silva)).
2. **Specialized Isolates for Value-Added Intermediates:** Utilizing strains like EP7 to enrich specific compounds (e.g., medium-chain alkenes) suitable for bioplastic precursors, aligning with emerging “biorefinery” models.

However, significant indigenous activity mandates rigorous controls and site-specific optimizations to prevent overestimation of inoculated strains’ contributions.

### **Limitations and Future Directions**

Although GC–FID elucidates qualitative compositional shifts, it does not quantify mineralization or CO₂ evolution. Future studies should integrate:

1. **Respirometry and Isotopic Labeling t**o measure actual carbon mineralization versus intermediate accumulation.
2. **Metagenomics and Enzyme Assays t**o map catabolic gene abundance (e.g., alkB, nahH) and confirm pathway activations.
3. **Toxicity Assessments,** particularly for halogenated and fluorinated byproducts.
4. **Pilot-scale trials to** validate lab-scale efficacy under environmental heterogeneity.

**CONCLUSION**

Overall, GC-FID profiling revealed that phyllosphere bacteria, particularly Pseudomonas oleovorans (EP3) and Kocuria kristinae (EN3), effectively altered the hydrocarbon composition of spent engine oil. The appearance of shorter alkanes, halogenated intermediates, and peak number reductions highlights their degradation potential. However, the similar transformations observed in the negative control highlight the contribution of indigenous processes, emphasizing the need for further quantification of mineralization versus intermediate accumulation in future field studies.

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