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Bioremediation Potential of Indigenous Bacterial Isolates for Treating Petroleum Hydrocarbons-Induced Environmental Pollution

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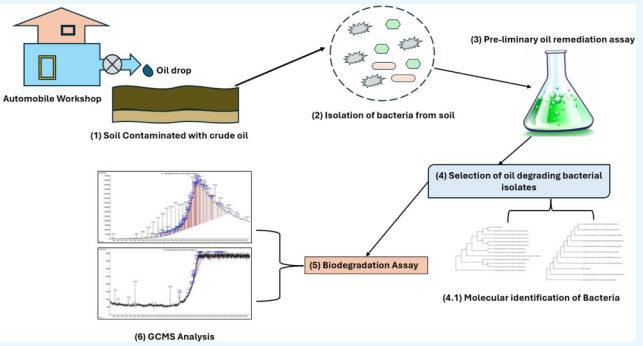
ABSTRACT: This study isolates oil-degrading bacteria from contaminated soil in automobile workshops, aiming to address environmental pollution from petroleum spills. Nineteen bacterial isolates were screened, with three isolates S4 (*Lysinibacillus macroides*), M8 (*Serratia marcescens*), and M9 (*S. marcescens*), showing significant oil degradation potency. In shake flask assays, S4 degraded 57.8%, M8 60.2%, and M9 58.1% of oil at a 5% concentration. Molecular identification through 16S rRNA gene sequencing confirmed these strains. Plate assays also supported the degradation potential, with growth zones of 38.4 ± 1.3 mm (S4), 39.7 ± 1.9 mm (M8), and 41.2 ± 1.7 mm (M9). Gas chromatography−mass spectrometry analysis of treated oil indicated the presence of hydrocarbons, phenolic compounds, and esters, suggesting microbial interactions, potentially involving the breakdown or transformation of complex hydrocarbons into simpler compounds by bacterial enzymes. These results highlight the potential of these bacterial strains, particularly S4, for the bioremediation of petroleum-contaminated sites.



1. INTRODUCTION

Petroleum is a dark, sticky, viscous liquid, consisting of complex substances known as petroleum hydrocarbons, which are composed of carbon (79.5−87.1%), hydrogen (11.5−14.8%), and oxygen (0.1−0.5%).[1] Petroleum hydrocarbons primarily contain alkanes, cycloalkanes, aromatics, and polyaromatic hydrocarbons, along with minerals and trace amounts of nonhydrocarbon components such as nitrogen, sulfur, and oxygen.[2] It has been reported that polycyclic aromatic hydrocarbons and their degradation products are toxic due to their carcinogenic nature.[3] Extensive soil pollution from petroleum hydrocarbons causes extremely hydrophobic conditions, due to which the soil becomes infertile, which results in reduced plant and microbial biomass.[4] The petroleum contamination of soil has a negative impact on human health, including skin and eye irritation, respiratory and neurological issues, as well as psychological distress.[5] Moreover, they have the potential to affect the genetic, immunological, and hormonal systems as well.[5]

Different physicochemical, biological treatment strategies, and physical methods, such as booms, skimmers, and sorbent materials, are available for the remediation of oil pollution. Booms contain oil spills to prevent their spread, and skimmers and sorbents are used to clean up the spills.[6] Chemical methods for oil spill remediation involve using dispersants to break up the oil into small droplets. Leaching and liquid extraction are common techniques for cleaning contaminated soil. It has been proven that traditional physicochemical methods are effective in removing oil spills; however, hazardous byproducts are often produced. In addition, these methods can have adverse environmental impacts and pose a potential risk to the ecosystem. Moreover, they are usually expensive and energy-intensive processes.[7]

The use of biological methods to cleanup oil spill is promising as it provides cheap and ecologically safe solutions without any negative impact on the environment.[8] Bioremediation uses microorganisms to degrade chemicals, resulting in improved environmental quality through natural processes. Techniques such as agrochemical, biotechnological, and phytomelioration help in the purification of soil along with ecological restoration.[9] In situ methods involve on-site treatment of contaminated soil through biostimulation, bioaugmentation, and phytoremediation.[10] Biostimulation promotes biodegradation through natural organisms using nutrients.[11] Bioaugmentation introduces microorganisms for

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better petroleum degradation.[12] Bioventing injects air to stimulate microbial growth, while biosparging displaces contaminants with subsurface air injection.[13] Phytoremediation uses plants for natural decontamination. Efficient degradation can be encouraged by *ex situ* bioremediation methods such as land farming, biopile composting, and the use of bioreactors. Different bacteria, including *Achromobacter*, *Acinetobacter*, and *Pseudomonas* have been used for petroleum hydrocarbon degradation.[14]

However, while there is significant research on bioremediation techniques and well-known oil-degrading bacteria, much of this work focuses on global bioremediation rates or bacteria from other environments. There is a gap in research regarding indigenous bacterial populations, specifically from environments like automobile workshop soils, which are frequently exposed to petroleum contaminants that are not often studied in-depth.

Oil-degrading bacteria were characterized both morphologically and biochemically, and their petroleum hydrocarbon degradation capabilities were assessed in the literature.[15] Traditional phenotyping techniques are laborious and subjective and do not provide the accuracy necessary for the identification of bacterial cultures. Molecular genetic methods, such as 16S rRNA gene sequencing, offer a faster and more reliable alternative, thus enabling precise microbial characterization.[16,17]

Unlike previously reported research that focuses on phenotypic characterization or global estimates of bioremediation rates, this study emphasizes the isolation and molecular identification of indigenous bacteria using 16S rRNA gene sequencing. This approach allows for more accurate identification of bacterial strains capable of degrading petroleum hydrocarbons under real-world environmental conditions. The novelty of the current study is the ability to isolate and identify indigenous oil-degrading bacterial species from polluted soils using standard and molecular methods. This study aims to fill a gap by exploring how these indigenous isolates can be used for bioremediation under stress conditions specific to automobile workshop environments, which have not been previously explored. The integration of these techniques and the primary concern for indigenous microbial populations not only identify species with a high biodegradation capacity but also offer valuable information about natural bioremediation in certain habitats. This work is important because it addresses the aforementioned gaps in an endeavor to establish how indigenous bacterial strains can be harnessed for bioremediation processes in an environmentally sustainable manner, offering an implementable solution to petroleum hydrocarbon environmental pollution.

2. MATERIALS AND METHODS

2.1. Collection of Petroleum-Contaminated Soil Sample. Soil samples were collected from the surface soil of different automobile workshops in District Kohat, Khyber Pakhtunkhwa, Pakistan (geographical coordinates: latitude 33.593954954368 11° and longitude 71.42166859022605°). The sampling was conducted at a depth of 5−10 cm to ensure the collection of surface soil, which is typically more contaminated due to direct exposure to petroleum products. The soil was selected from areas with visible oil strains or suspected contamination due to automotive activities. The collected samples were placed in presterilized plastic bags. They were then transported and stored in the Microbiology Research Laboratory, KUST, Kohat, at 25 °C for subsequent screening and examination.

2.1.1. Petroleum Hydrocarbons Source. The petroleum hydrocarbons used in the study were sourced from a local petroleum refinery in Kohat, Pakistan. This refinery processes petroleum hydrocarbons extracted from nearby oil fields, making it a representative sample of regional hydrocarbon pollution. While specific compositional details such as API gravity and sulfur content were not available, petroleum hydrocarbons were used to evaluate the hydrocarbon-degrading potential of bacterial isolates under environmentally relevant conditions.

2.2. Isolation and Identification of Bacteria from Soil Sample. For isolation of oil-degrading bacteria, 1 g of soil sample was dissolved in 100 mL of sterilized distilled water. This solution was serially diluted, yielding five dilutions from 10^{-1} to 10^{-5}. About 100 μL of the sample from each dilution was transferred to labeled nutrient agar Petri plates. These plates were then incubated at 37 °C for 24 h. After incubation, emerging colonies appearance was recorded and identified through morphological, microscopic, and biochemical techniques as per Bergey's manual of determinative bacteriology (9th edition).

2.3. Enrichment Technique for Oil-Degrading Bacteria. Oil-degrading bacteria were selected by using an enrichment technique. Bushnell-Hass broth supplemented with petroleum hydrocarbons served as the primary carbon and energy source.[18] Three treatments were prepared: (i) 100 μL of test bacterial culture in nutrient broth with 5 mL of oil, (ii) 100 μL of test bacterial culture in 5 mL of oil as a positive control, and (iii) 100 μL of test bacterial culture in 5 mL of broth as a negative control. The flasks were kept in a shaking incubator (model: Thermostable TS-20R, Daihan, Scientific, South Korea) at 120 rpm and 37 °C for 3 days. Optical density (OD) was measured at a 600 nm wavelength using a UV−vis spectrophotometer (model: MUV26-312, MA02052, USA).[19] It is well recognized that monitoring OD in the presence of hydrocarbon supplements is reliably unrealistic, since the presence of emulsion layers of oil droplets interferes with test wavelengths. Hence, besides the OD results, bacterial growth was also assessed by the colony-forming unit (CFU) method.[20]

2.4. Preliminary Oil-Degradation Assay. Selected bacterial isolates from the enrichment assay were tested for their petroleum hydrocarbon degradation ability. A 25 μL culture from each isolate was spot inoculated onto filter paper, which was then placed over 100 μL of oil. After third and seventh days of incubation at 37 °C, the diameter of the bacterial growth zone was measured. Tests were done in triplicate, and the isolate with the largest growth zone was chosen for further biodegradation studies.[21]

2.5. Biodegradation Assay. The preliminary oil degradation exhibited by nine bacterial isolates (S1, S4, S7, S10, M5, M6, M7, M8, and M9) encouraged the evaluation of their in vitro oil remediation potential through both shake flask and plate activity methods. The biodegradation assays were carried out using two levels of oil (5 and 10% concentrations). Assay flasks were assembled, one of which served as a control containing Bushnell-Hass broth without an inoculum. The rest of the flasks contained Bushnell-Hass broth, an inoculum, and different oil concentrations. Incubation was performed at 37 °C in an orbital shaking incubator set to a speed of 120 rpm for 12 days. Samples were then taken after incubation to

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Table 1. Description of Microscopy, Cultural Characteristics, and Biochemical Test Response of Bacterial Isolates[a]

isolate no.	cultural characteristics on nutrient agar media plates	gram's Rxn	fermentation			oxidase test	catalase test	citrate test	TSI test	urease test	motility test	indole test
			lactose	dextrose	sucrose							
S1	off white, opaque, medium, irregular, and flat	+ (Rods)	+	+	−	+	+	+	K/A	+	+	−
S2	off white, small, and circular	+ (Cocci)	+	A	A	−	+	+	K/A	+	+	−
S3	off white, elevated, and irregular	+ (Cocci)	+	A ±	A ±	+	+	−	K/A	+	+	−
S4	large, irregular, and off white	+ (Rods)	−	−	+	+	+	+	K/A	+	+	−
S5	orange shaded, circular, and flat	+ (Cocci)	AG	AG	AG	−	+	−	K/A	+	+	−
S6	off-white, large, elevated, and irregular	+ (Cocci)	+	+	+	−	+	−	K/A	+	+	−
S7	soft, smooth, and pale-yellow growth	+ (Rods)	+	−	−	−	+	+	K/A	+	+	−
S8	white, small, and translucent	+ (Cocci)	AG	AG	A ±	−	+	−	A/NC	+	+	−
S9	off white, small, and opaque	+ (Cocci)	+	+	+	−	+	−	K/A	+	+	−
S10	abundant. large, and off-white	+ (Rods)	A	A	A	+	+	+	K/A	+	+	−
M1	off-white, mucoid, small, and circular	+ (Cocci)	A	A	A	+	−	−	K/A	+	±	--
M2	off-white, large, irregular, and mucoid	+ (Cocci)	+	+	+	+	−	+	K/A	+	+	−
M3	white, mucoid, and abundant	+ (Cocci)	+	+	+	+	−	+	K/A	+	+	−
M4	off white, mucoid, large, and irregular	+ (Cocci)	+	+	+	+	+	+	K/NC	+	+	−
M5	white, mucoid, small, and circular	+ (Rods)	+	+	+	−	+	+	K/A	+	+	−
M6	Yellowish, mucoid, and elevated	+ (Rods)	+	+	+	+	+	+	K/A	+	+	−
M7	yellowish, mucoid, large, and irregular	+ (Rods)	+	+	+	−	+	+	K/A	+	+	−
M8	off white, filamentous, and large	- (Rods)	+	+	+	+	+	+	K/A	+	+	−
M9	off-white, small, and circular	- (Rods)	+	+	+	−	+	+	K/A	+	+	−

[a]Key: AG = acid and gas; + = positive; − = negative; ± = variable reaction; A = acid production; K = alkaline reaction; NC = no change; K/A = red/yellow; K/NC = red/no color change; A/NC = acid/no color change; and A/A = yellow/yellow.

determine the final oil contents. Viable counts on nutrient agar plates were used to determine the growth of bacteria.[22]

2.6. Gas Chromatography−Mass Spectrometry Analysis of Petroleum Oil. The samples of control and bacterial-treated petroleum hydrocarbons were collected into a sterile glass vial and then diluted using 50 mL of hexane. A separating funnel was used to extract the hydrocarbons, which were then concentrated using a rotary evaporator (model: WEV-1001L, Daihan, Scientific, South Korea) and redissolved in *n*-hexane. The hydrocarbons were extracted through *N*,*O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) to make them volatile. Gas chromatography−mass spectrometry (GCMS) analyses were done at the optimum temperature range (at 70−300 °C) for separation of compounds. Compounds were identified based on their retention times, while the relative abundance was determined by integrating peak areas in the chromatogram.[23]

2.6.1. GCMS Analysis (Supporting Information). An expanded explanation of the GCMS methodology, including the detailed process of hydrocarbon extraction, chromatogram interpretation, and compound identification and quantification, is available in the Supporting Information.

2.6.2. Determination of Hydrocarbon Degradation Using GCMS Analysis. The percentage degradation of individual hydrocarbons and the overall hydrocarbon content was calculated by comparing the GCMS spectrum of bacterial-treated samples with the control sample, i.e., peak areas in treated samples with those in the control sample, using the following formula

$$\text{degradation (\%)} = \frac{\text{peak area of control} - \text{peak area of treated sample}}{\text{peak area of control}} \times 100$$

2.7. Molecular Identification of Oil-Degrading Bacteria. Genomic DNA was extracted from bacteria by the phenol chloroform isoamyl alcohol (PCI) method.[24] The 1.5 mL sample of the bacterial culture was grown for a period of 24 h, pelleted, resuspended in TE buffer with sodium dodecyl sulfate and proteinase K, followed by incubation at room temperature. Then NaCl and CTAB/NaCl solution were added, and after incubation at 65 °C, PCI alcohol extractions were used. DNA precipitation was performed with isopropanol, washed in ethanol, and resuspended in TE buffer, while quality control of the isolated DNA was performed through gel electrophoresis followed by quantification using a spectrophotometer.[25]

For 16S rRNA gene sequencing, bacterial DNA was polymerase chain reaction (PCR) amplified using universal primers (27f: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492r: 5′-GGTTACCTTGTTACGACTT-3′). The PCR mixture containing DNA, buffer, dNTPs, and DNA polymerase were optimized for the annealing temperature and cycles. The evolutionary relationship among the isolates was reconstructed

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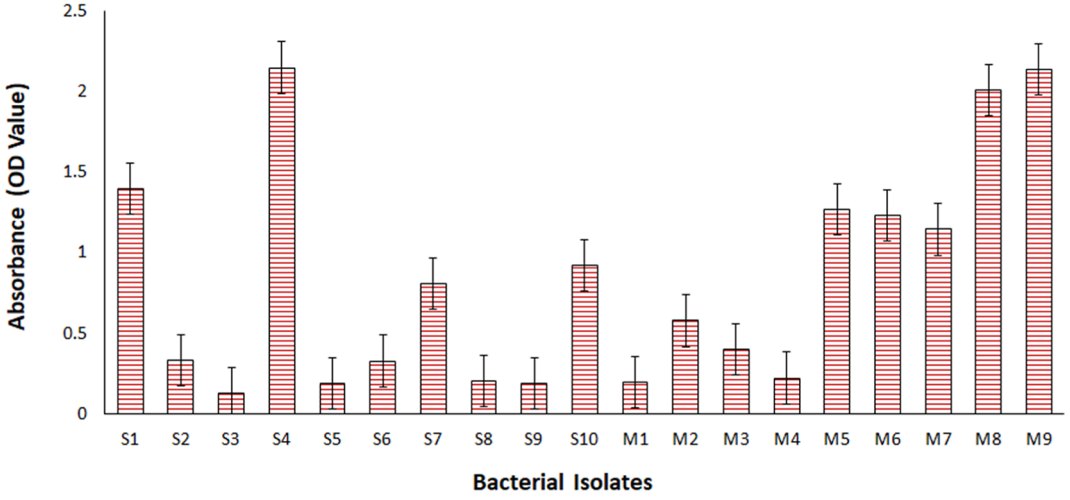

Figure 1. OD values of 19 bacterial isolates at 600 nm wavelength (P = 0.0000031).

using the maximum likelihood (ML) method under the Tamura 3-parameter nucleotide substitution model in MEGA 11 software. The sequences of 16S rRNA genes were compared with the reference sequences using Clustal W with default settings, and a phylogenetic tree was constructed with 100 bootstrap trials, which ensured the credibility of the tree constructed. There were two major reasons for selecting the Tamura 3-parameter model: base substitution rates and nucleotide frequencies vary among the sequences. Outgroup selection was carefully considered to provide accurate rooting of the tree: the H10 16S rRNA gene of *Bacillus subtilis* was used as an outgroup of the S4 bacterial isolate because of its phylogenetic difference and tolerance within the *Bacillus* genus. For the M8 and M9 bacterial isolates, the outgroup was the *Escherichia coli* strain STLIN_13 chromosome, which was appropriate from a taxonomic perspective within the *Enterobacteriaceae* family.[26]

2.8. Statistical Analysis. The experimental data were organized and analyzed using Microsoft Excel 2019. Descriptive statistics, including mean and standard deviation, were calculated for each group to summarize the data distribution and variability. To assess the statistical significance of differences between the experimental and control groups, a one-way analysis of variance (ANOVA) was performed. The ANOVA results provided the P-value, which was used to determine whether the differences observed between the groups were statistically significant. A P-value of less than 0.05 was considered indicative of significant differences. This statistical approach helped in evaluating the biological relevance of the results, ensuring that observed effects were not due to random variation.

3. RESULTS

3.1. Bacteriological Assessment of Soil Sample. In the current research study, soil samples from automobile workshops were examined using the pure culture method, resulting in the isolation of 19 distinct bacterial isolates. Microscopic analysis revealed Gram-positive rods for S1, S4, S7, S10, M5, M6, and M7, Gram-positive cocci for S2, S3, S5, S6, S8, S9, M1, M2, M3, and M4, and Gram-negative rods for M8 and M9. The biochemical test results revealed significant variations among the bacterial isolates. The oxidase test showed diverse responses, with some isolates, such as S1 and S3, testing positive, while others, including S4 and S7, were negative. The catalase test demonstrated that most isolates were positive, reflecting their ability to detoxify reactive oxygen species with a few exceptions, such as M1. Citrate utilization also varied across the isolates; for instance, S5 and S7 tested negative, whereas isolates like S4 and M3 were positive, including differences in their metabolic capabilities. The TSI test results exhibited a range of reactions, including K/A (alkaline slant/acid butt), A/NC (acid slant/no color change), and K/NC (alkaline slant/no color change), reflecting diverse fermentation and gas production patterns (see Table 1).

3.2. Preliminary Assessment of Oil-Degrading Bacterial Isolates. Among the 19 different bacterial isolates subjected to the preliminary oil-degradation assay, it was observed that nine bacterial isolates encoded as S1, S4, S7, S10, M5, M6, M7, M8, and M9 showed a positive response. Furthermore, the OD values of these nine bacterial isolates at a wavelength of 600 nm increased after 12 days of incubation, reflecting their viable growth. Specifically, S4 displayed the highest OD value (2.146), followed by M9 (2.136), M8 (2.008), S1 (1.397), M5 (1.232), M6 (1.232), M7 (1.144), S7 (0.921), and S10 (0.806), as shown in Figure 1. Moreover, a considerable difference in the bacterial growth patterns of the nine isolates was found by CFU analysis. With a CFU/mL count of around 1.72×10^9, isolate S4 had the highest level of growth. Likewise, isolates M9 and M8 had robust proliferative growth as evidenced by their high CFU values of 1.71×10^9 and 1.61×10^9 CFU/mL, respectively. The isolates S1, M5, and M6 showed moderate growth, with CFU counts ranging from 9.86×10^8 to 1.12×10^9 CFU/mL. Conversely, isolates M7, S7, and S10 showed lower CFU/ml counts of 9.15×10^8, 7.37×10^8, and 6.45×10^8, respectively, suggesting comparatively slower growth rates (Table 2).

3.3. Oil Remediation Assay. *3.3.1. Shake Flask Activity.* Shake flask experiments involved different petroleum hydrocarbon concentrations (5 and 10%). After a 12-day incubation period, the analysis revealed that three isolates (S4, M8, and M9) displayed remarkable petroleum hydrocarbon degradation capabilities when compared to the other isolates. Specifically, in the 5% petroleum hydrocarbon degradation analysis, M8 demonstrated the highest efficiency with a degradation rate of 60.2%, as confirmed by an increase in its OD value. M9 and S4 bacterial isolates also exhibited significant degradation, with

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Table 2. Estimation of Bacterial Viability and Count by CFU per mL Assay

S. no.	bacterial isolate	CFU/mL
1	S4	1.72×10^9
2	M9	1.71×10^9
3	M8	1.61×10^9
4	S1	1.12×10^9
5	M5	9.91×10^8
6	M6	9.86×10^8
7	M7	9.15×10^8
8	S7	7.37×10^8
9	S10	6.45×10^8

rates of 58.1 and 57.8%, respectively (Figure 2a). Furthermore, the analysis of 10% petroleum hydrocarbon degradation highlighted that, on the third and 12th days of incubation, M8 achieved a degradation rate of 61.5%, surpassing S4 and M8, which displayed rates of 58.1 and 55.3%, respectively (Figure 2b).

3.3.2. Plate Activity Assay for Growth of Bacterial Isolates Using Petroleum Hydrocarbons as Substrate. Plate activity assays were carried out to assess the growth of bacterial isolates under a 5% petroleum hydrocarbon concentration. The results showed that among the nine bacterial isolates, three (S4, M8, and M9) exhibited a substantial increase in their growth zones on the plates. As illustrated in Figure 3, isolate M9 displayed the largest growth zone, measuring 41.2 ± 1.7 mm, indicating a remarkable 57.3% increase in its growth rate. M8 followed closely with a growth zone of 39.7 ± 1.9 mm, signifying a 61.4% growth rate increase, while S4 displayed a growth zone of 38.4 ± 1.3 mm, corresponding to a 63.4% increase in growth rate.

3.4. GCMS Analysis of Petroleum Hydrocarbon-Degrading Bacteria. GCMS analysis revealed that the normalized peak areas of the petroleum hydrocarbon (Mobil oil) control sample showed vast spectra of chemical compounds with different retention times and relative intensities (Figure 4). The occurrence of aromatic and saturated hydrocarbon characteristics to petroleum hydrocarbons was shown by the identification of alkanes such as p-

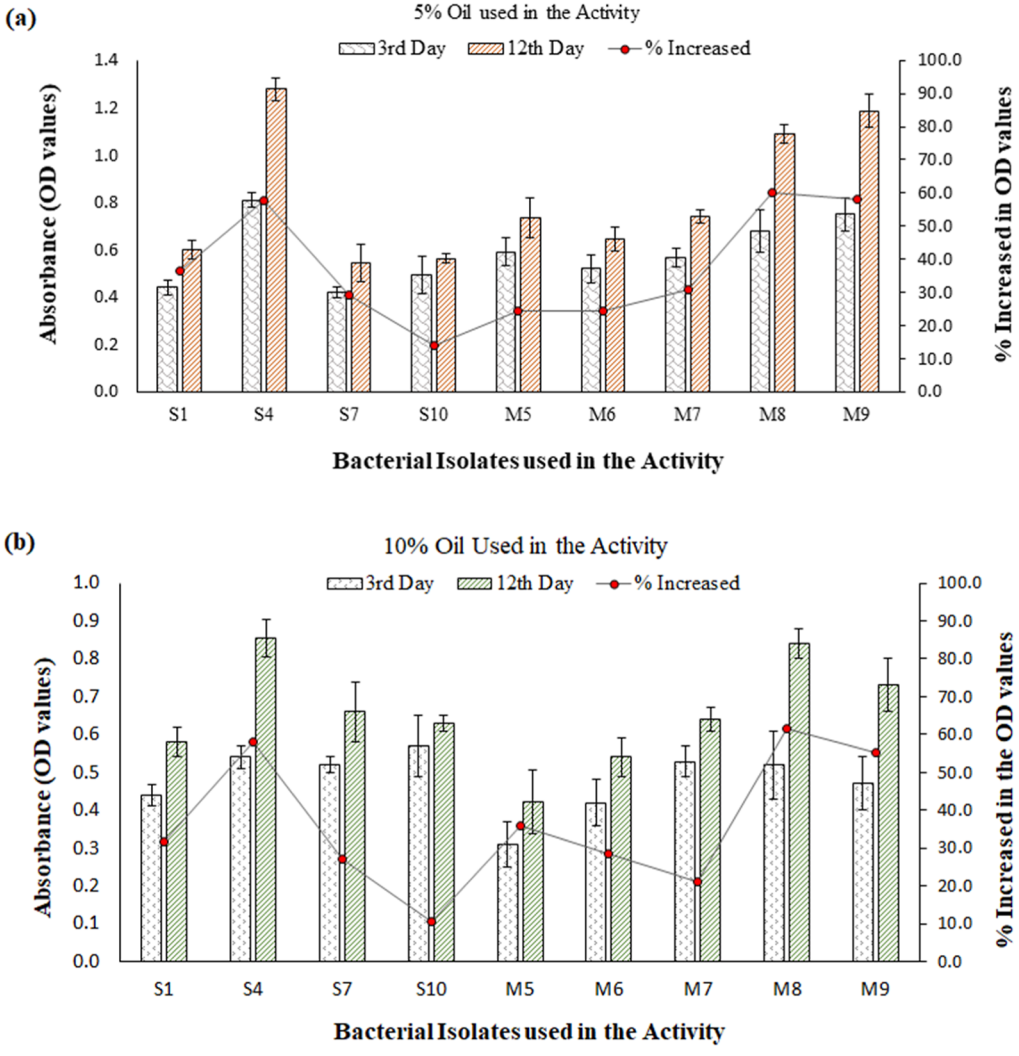

Figure 2. (a) Assessment of 5% petroleum hydrocarbon degradation analysis of bacterial isolates ($P = 0.011$) and (b) assessment of 10% petroleum hydrocarbon degradation analysis of bacterial isolates ($P = 0.000019$). Lines represent trends in degradation efficiency for individual bacterial isolates over the incubation period. The "% increase in degradation efficiency" values are relative to the initial hydrocarbon concentration at the start of the experiment and do not imply interspecies comparisons.

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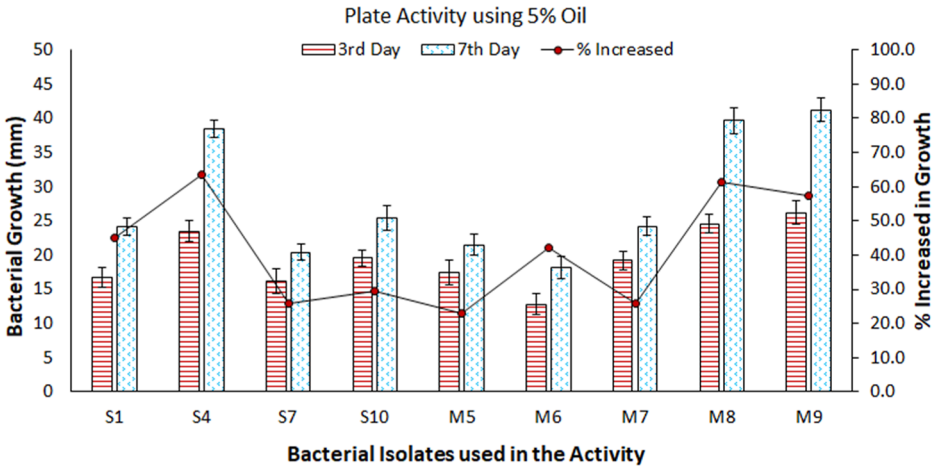

Figure 3. Assessment of growth zones of hydrocarbon-degrading bacteria ($P = 0.0028$) on plates under 5% petroleum hydrocarbon concentration. Lines represent trends in growth performance for individual bacterial isolates. The "% increase in growth zone" values are relative to the baseline growth zone measured at the start of the experiment and do not imply interspecies comparisons.

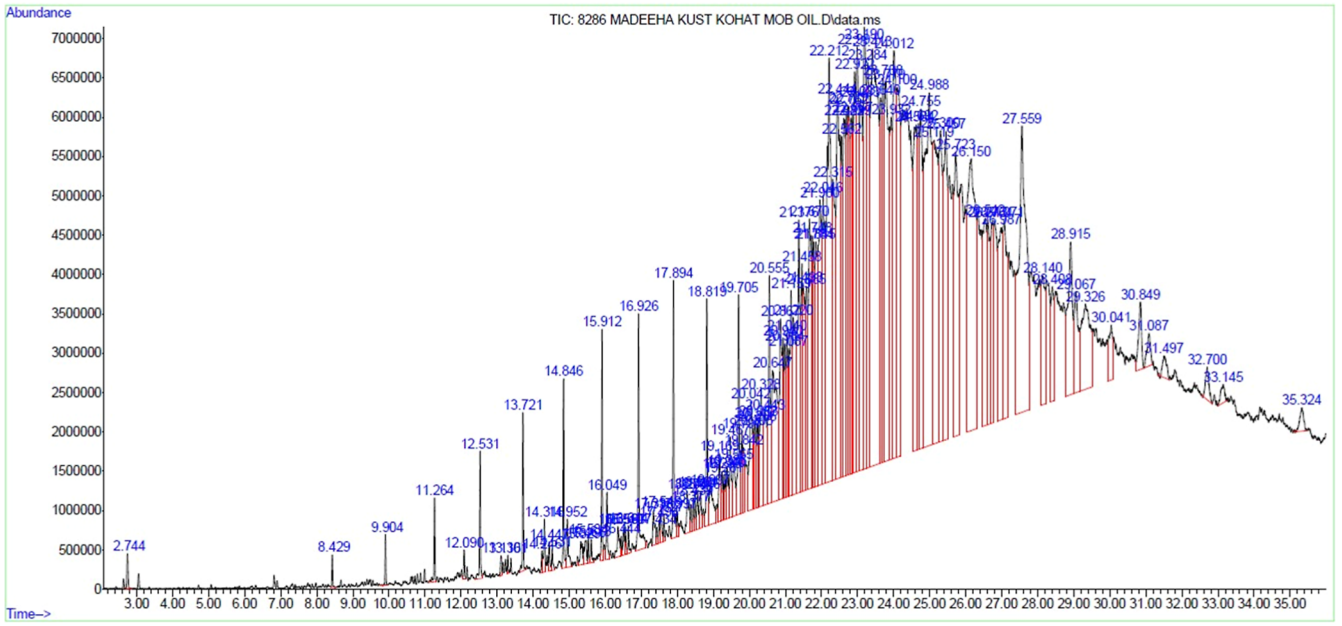

Figure 4. GCMS chromatogram of Mobil oil control sample.

xylene (peak 1), tridecane (peak 2), and tetradecane (peak 3). The intricate nature of the sample composition was further emphasized by finding substances such as hexadecane (peak 5) and heptadecane (peak7). The presence of long-chain alkanes and alkenes, such as 1-hexacosene (peak 23) and hexacosane (peak 29), was an indication that higher molecular weight hydrocarbons were present. More importantly, homohopanes and hopanes, such as 28-Nor-17.alpha.(H)-hopane (peak 30) and 17.alpha.(H),21.beta.(H)-Hopane (peak 31) have been linked to biological origins (Table 3).

The GCMS analysis of the S4 bacteria-treated oil sample revealed a broad spectrum of chemical components with specific retention time and relative intensity (Figure 5). Three major chemicals were found: sodium salicylate with an amino group (2-amino-5-methylbenzoic acid at peak 1), 4-amino-butanoic acid (peak 2), and phenylethyl alcohol (peak 3). These molecules indicate the presence of aliphatic and aromatic organic acids as well as alcohols. Of interest, fumaric acid butyl *cis*-hex-3.enyl ester (peak 4) and thymol (di*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione at peak 5) were detected in the treated sample, indicating that phenolic compounds as well as esters were present. In addition, silicon-containing compounds were observed, suggesting the existence of interaction among S4 bacteria used in treatment and oil components. These substances contained compounds such as tetrasiloxane, decamethyl (peak 20), and tris(*tert*-butyldimethylsilyloxy)arsane (peak 15), as depicted in Table 4.

The GCMS analysis of the M8-treated oil sample displayed chemical constituents with varied percentage composition and retention times (Figure 6). The analysis of the content sample contained methoxy phenyl (peak 1), cyclohexanecarboxylic acid (peak 2), and cyclohexaneacetic acid (peak 3). The presence of phenolic compounds such as 4-(7-methyloctyl)-phenol (peak 15) and organic alcohols like 6−octaden-1-olacetate (peak 4) indicated that there were interactions between M8 bacteria and petroleum components. Additionally, the treatment was also accompanied by silicon-containing compounds represented by some of methyltris

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Table 3. Nature of Compounds with Their Relative Area of Normalization and Retention Time Present in Mobil Oil Control Sample

peak #	compounds	ret time	area	% conc.
1	p-xylene	2.744	9,647,146	0.14
2	tridecane	9.904	10,322,511	0.15
3	tetradecane	11.264	16,844,097	0.25
4	pentadecane	12.531	28,819,647	0.43
5	hexadecane	13.721	34,107,670	0.51
6	dodecane, 1,1′-oxybis	14.316	11,981,889	0.18
7	heptadecane	14.846	40,061,446	0.59
8	pentadecane, 2,6,10,14-tetramethyl	14.952	17,625,905	0.26
9	hexadecane, 2,6,10,14-tetramethyl	16.049	18139,214	0.27
10	nonadecane	16.926	59,364,736	0.88
11	octadecane, 1-chloro	17.338	15,848,123	0.23
12	1-octadecanethiol	18.49	7,616,999	0.11
13	nonadecane	18.564	11,174,659	0.17
14	heneicosane	18.819	53,548,067	0.79
15	octadecane	20.042	46,387,848	0.69
16	heneicosane	20.149	14,393,135	0.21
17	eicosane	20.206	12,020,979	0.18
18	octatriacontyl pentafluoropropionate	20.647	87,870,939	1.30
19	1-eicosene	21.22	80,194,679	1.19
20	tetracosane	21.376	109,330,520	1.62
21	docosane	21.748	100,494,707	1.49
22	Z-12-pentacosene	22.315	137,411,313	2.04
23	1-hexacosene	23.083	166,774,159	2.47
24	tricosane	23.708	102,109,567	1.51
25	nonacosane	24.584	149,814,670	2.22
26	octacosane	24.755	106,029,384	1.57
27	Z-14-nonacosene	25.3	100,831,305	1.49
28	octacosane, 2-methyl	25.457	107,386,462	1.59
29	hexacosane	26.15	79,775,383	1.18
30	28-Nor-17.alpha.(H)-hopane	27.559	151,073,565	2.24
31	17.alpha.(H),21.beta.(H)-Hopane	28.915	47,799,267	0.71
32	17.alpha.(H),21.beta.(H)-homohopane	30.849	44,396,457	0.66
33	1-pentacosanol	31.087	45,732,543	0.68

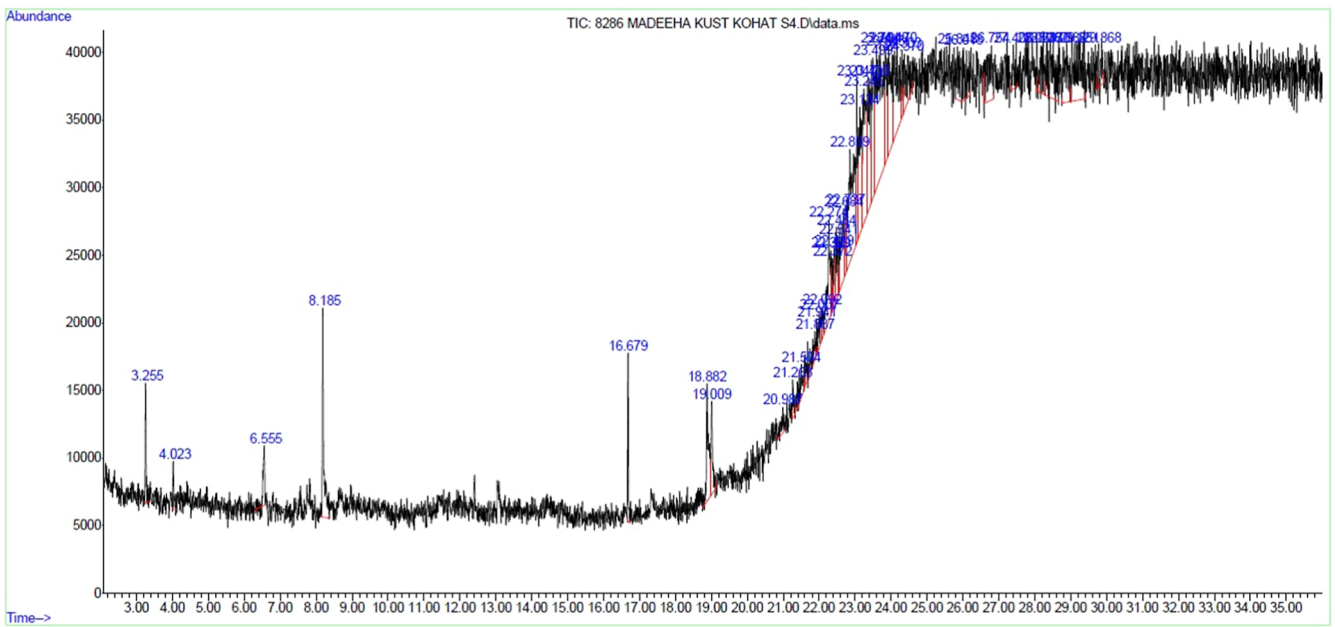

Figure 5. GCMS chromatogram of S4 bacteria-treated oil sample.

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Table 4. Nature of Compounds with Their Relative Area of Normalization and Retention Time Present in S4 Bacteria-Treated Oil Sample

peak #	compounds	ret. time	area	% conc.
1	2-amino-5-methylbenzoic acid	3.255	144,312	1.17
2	4-aminobutanoic acid	4.023	64,741	0.53
3	phenylethyl alcohol	6.555	124,662	1.01
4	fumaric acid, butyl *cis*-hex-3-enyl ester	8.185	432,119	3.52
5	di*tert*-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	16.679	203,077	1.65
6	2-chloro-5,10-dihydro-5,10-ethanophenazine	18.882	363,667	2.96
7	1,2,5-trimethylindol-6-amine	19.009	198,627	1.62
8	1-nitro-9,10-dioxo-9,10-dihydro-anthracene-2-carboxylic acid diethylamide	21.263	50,634	0.41
9	arsenous acid, tris(trimethylsilyl) ester	21.887	177,522	1.44
10	2-*tert*-butylphenol, *tert*-butyldimethylsilyl ether	22.007	98,105	0.80
11	1,1,1,3,5,5,5-heptamethyltrisiloxane	22.329	70,312	0.57
12	4-(7-methyloctyl)phenol, TMS derivative	22.419	113,047	0.92
13	1,2-bis(trimethylsilyl)benzene	22.541	62,970	0.51
14	thymol, TBDMS derivative	22.684	321,971	2.62
15	tris(*tert*-butyldimethylsilyloxy)arsane	23.701	1266456	10.30
16	1,4-bis(trimethylsilyl)benzene	26.018	218,859	1.78
17	silicic acid, diethyl bis(trimethylsilyl) ester	26.754	335,894	2.73
18	ethoxy(phenyl)silanediol, 2TMS	28.237	225,590	1.84
19	methyltris(trimethylsiloxy)silane	28.43	424,457	3.45
20	tetrasiloxane, decamethyl-	28.888	302,907	2.46
21	cyclotrisiloxane, hexamethyl-	29.868	156,036	1.27

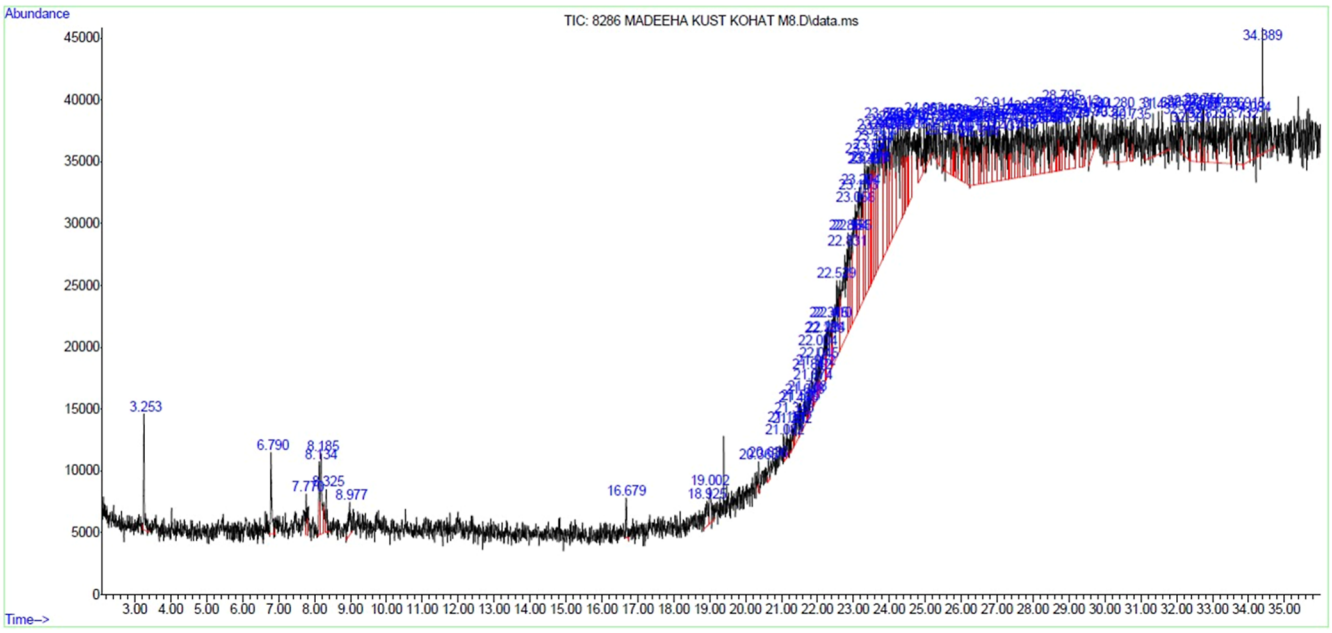

Figure 6. GCMS chromatogram of M8 bacteria-treated oil sample.

(trimethylsiloxy)silane (peak 20) and tetrasiloxane (peak 17), as shown in Table 5.

A wide range of chemical compounds was obtained for the M9-treated oil sample by GCMS analysis. Quantification of these compounds was done as the normalized peak areas, and they were characterized by their retention time and relative abundances (Figure 7). The compounds, which were detected included different chemical molecules revealing the presence of aromatic, amide, and alkene functional groups such as benzene, 1-isocyanato-2-methyl (peak 1), *N*,*N*-dimethylacetamide (peak 2), and 1-heptene, 6-methyl (peak 3). Additionally, *N*-ethyl-2-(propylsulfanyl)quinazolin-4-amine (peak 10) and carbonic acid, 2-trichloroethyl cyclohexyl ester (peak 8) suggesting that possible chemical interactions or transformations might have taken place during the treatment. Furthermore, the presence of arsenic acid, tris(trimethylsilyl) ester (peak 16), and silica acid, diethyl bis(trimethylsilyl) ester (peak 14), underlines that organosilicon or organoarsenic compounds were involved as part of treatment chemicals. Additionally, the detection of 1,2-bis(trimethylsilyl)benzene (peak 21) implies that aromatic hydrocarbons or their homologues exist (Table 6).

3.5. GCMS Analysis of Hydrocarbon Degradation by Bacterial Isolates S4, M8, and M9. Using GCMS analysis, the degradation efficiency of bacterial isolates S4, M8, and M9 on petroleum hydrocarbons (Mobil oil) was examined (Figure 8). The control sample comprised a variety of aliphatic and

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Table 5. Nature of Compounds with Their Relative Area of Normalization and Retention Time Present in M8 Bacteria-Treated Oil Sample

peak #	compounds	ret. time	area	% conc.
1	methoxy phenyl	3.253	162,352	0.87
2	cyclohexanecarboxylic acid	6.79	150,037	0.77
3	cyclohexaneacetic acid	8.134	97,312	0.50
4	6-octadecen-1-ol acetate	8.185	96,997	0.50
5	alpha.-D-mannopyranoside	8.977	104,398	0.53
6	4,5-isoxazoledione	16.679	53,199	0.27
7	1,1,3,3-tetraallyl-1,3-disilacyclobutane 2	19.002	102,846	0.53
8	triazolo, benzothiazole-3(2*H*)-thione	21.718	35,786	0.18
9	2-bis(trimethylsilyl)benzene	22.045	41,848	0.21
10	5-heptamethyltrisiloxane	22.41	85,002	0.43
11	1,4-bis(trimethylsilyl)benzene	23.647	450,428	2.30
12	ethoxy(phenyl)silanediol, 2TMS	26.75	280,318	1.43
15	4-(7-methyloctyl)phenol, TMS derivative	28.022	175,853	0.90
16	silicic acid, diethyl bis(trimethylsilyl) ester	28.293	144,439	0.74
17	tetrasiloxane, decamethyl-	29.024	207,720	1.06
19	thymol, TBDMS derivative	29.733	25,359	0.13
20	methyltris(trimethylsiloxy)silane	32.829	87,603	0.45
21	arsenous acid, tris(trimethylsilyl) ester	33.45	526,719	2.69
22	cyclotrisiloxane, hexamethyl	34.084	200,165	1.02
23	tris(*tert*-butyldimethylsilyloxy)arsane	34.389	349,955	1.79

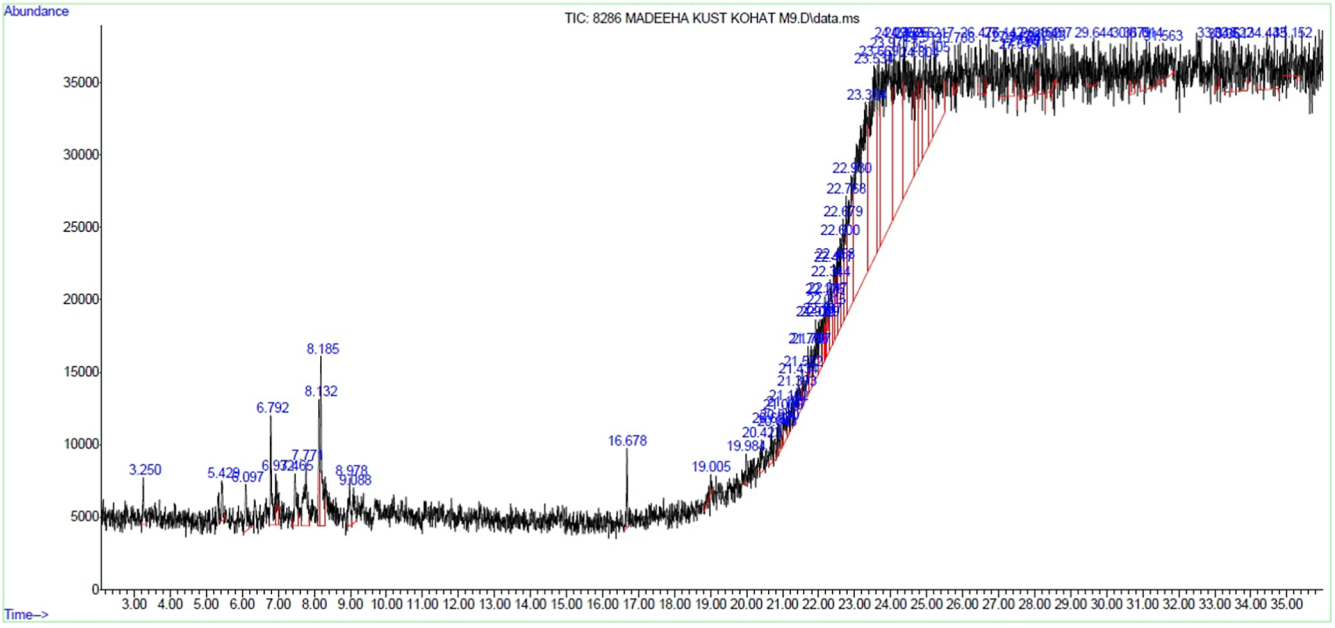

Figure 7. GCMS chromatogram of M9 bacteria-treated oil sample.

aromatic hydrocarbons, with major peaks representing compounds such as *p*-xylene (0.14%), tridecane (0.15%), hexadecane (0.51%), nonadecane (0.88%), tetracosane (1.62%), and various hopane derivatives. These compounds served as a baseline for evaluating the hydrocarbon degradation by each bacterial isolate. Isolate S4 showed significant hydrocarbon degradation, reducing many important hydrocarbons in the control sample; overall, S4 achieved 83.6% total hydrocarbon degradation. Specific compounds like hexadecane and nonadecane were nearly completely degraded, with new metabolites like fumaric acid derivatives and silyl ethers emerging in the profile.

Isolate M8 showed moderate degradation, with a total reduction of 67.4% of the hydrocarbons compared to isolate S4. Important hydrocarbons such as tridecane and hexadecane broke down, but certain substances, such as derivatives of silicic acid, remained, indicating partial degradation. Furthermore, siloxanes and trimethylsilyl derivatives were also found, suggesting incomplete hydrocarbon metabolism. On the other hand, isolate M9 revealed a poorer degrading efficiency compared to S4 and M8 isolates, with a total hydrocarbon reduction of 55.2%. While a number of peaks indicating residual chemicals such as siloxanes and cyclohexaneacetic acid persisted, several hydrocarbons underwent partial degradation.

3.6. Molecular Identification of Oil-Degrading Bacteria. Using the PCI method, the DNA of the selected isolates was isolated and visualized through gel electrophoresis (Figure 6a). Additionally, PCR amplification was conducted using 16S

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Table 6. Nature of Compounds with Their Relative Area of Normalization and Retention Time Present in M9 Bacteria-Treated Oil Sample

peak #	compounds	ret time	area	% conc.
1	benzene, 1-isocyanato-2-methyl	3.25	83,977	0.46
2	*N*,*N*-dimethylacetamide	5.429	64,233	0.35
3	1-heptene, 6-methyl	6.097	139,373	0.76
4	urea, ethyl	6.932	93,339	0.51
5	4-decen-1-ol, chlorodifluoroacetate	7.465	145,096	0.08
6	*N*-(3-aminopropyl)methanesulfonamide	7.771	251,193	1.38
7	cyclohexaneacetic acid	8.132	179,728	0.99
8	carbonic acid, 2-trichloroethyl cyclohexyl ester	8.185	320,070	1.76
9	oxazole, 4,5-dimethyl	8.978	99,118	0.54
10	*N*-ethyl-2-(propylsulfanyl)quinazolin-4-amine	9.001	360,190	0.56
11	1,4-bis(trimethylsilyl)benzene	9.268	182,060	0.59
12	1*H*-imidazole-2-methanol, 1-decyl	21.006	29,082	0.16
13	2-ethylacridine	21.572	55,164	0.30
14	silicic acid, diethyl bis(trimethylsilyl) ester	22.458	171,832	0.94
15	2-(acetoxymethyl)-3-(methoxycarbonyl)biphenylene	24.095	1465409	8.04
16	arsenous acid, tris(trimethylsilyl) ester	27.142	185,398	1.02
17	ethoxy(phenyl)silanediol, 2TMS	29.644	120,846	0.66
18	cyclotrisiloxane, hexamethyl	30.679	153,016	0.84
19	tris(*tert*-butyldimethylsilyloxy)arsane	33.522	203,351	1.12
20	methyltris(trimethylsiloxy)silane	35.152	288,358	1.58
21	1,2-bis(trimethylsilyl)benzene	22.344	162,426	0.89

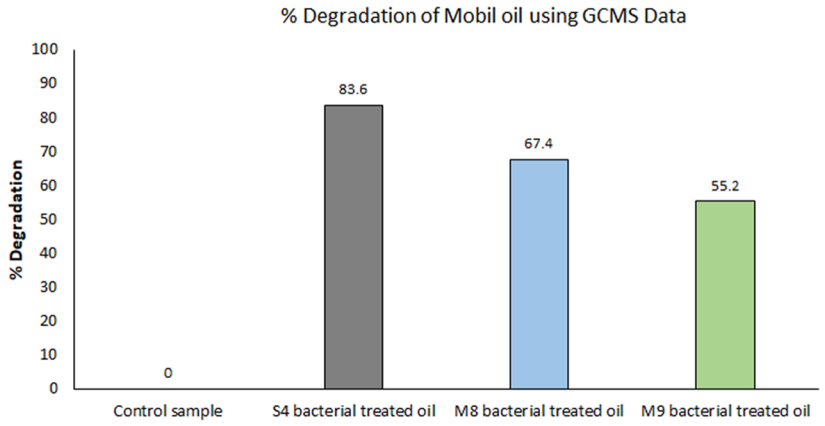

Figure 8. Assessment of hydrocarbon degradation (%) using GCMS analysis of control and bacterial-treated oil samples.

rRNA gene universal primers, and a 1.7% agarose gel was utilized to visualize the PCR amplified product for S4, M8, and M9 bacterial isolates, which was approximately 1500 bp.

Furthermore, the phylogenetic trees of the bacterial isolates S4, M8, and M9 were constructed using the Tamura 3-parameter model with ML implemented in MEGA 11 software. Bootstrap values were shown at the major nodes to indicate the stability of the tree that had been inferred. Large values of the bootstrap test at the crucial nodes reflected high levels of confidence in those particularly evolutionary relationships. The nucleotide sequences of the S4 bacterial isolate were closely related to *Lysinibacillus macroides* strain AzoM1 with a bootstrap value of 88 (Figure 9), M8 was closely related to *Serratia marcescens* strain GN03438 with a bootstrap value of 99 (Figure 10), and M9 was closely related to *S. marcescens* strain 14BL09 with a bootstrap value of 80 (Figure 11).

4. DISCUSSION

Petroleum hydrocarbon contaminants present in soil habitats have significant negative impacts on the ecosystem and constitute a substantial threat to aquatic and terrestrial life.[27,28] In the literature, different treatments have been used for the remediation of oil pollution in the environment, including physical, chemical, and biological methods.[29] Physico-chemical interventions for managing petroleum-contaminated sites often acquire significant costs and exhibit reduced efficacy. Additionally, scientists have proved that bioremediation is flexible, very stable, and can be used in different fields, including waste treatment at low cost to ensure mineralization of crude oil.[9,11−14] Bioremediation is a process that involves microorganisms to degrade environmental pollutants.[30] The present study was conducted to isolate oil-degrading bacterial strains from soil samples of automobile workshops at District Kohat, Khyber Pakhtunkhwa, Pakistan, and then characterize them through 16S rRNA gene sequencing.

There are many bacterial species that have the ability to act as efficient bioremediation agents because of their capacity to release enzymes and utilize petroleum contents as a source of energy. Bilen and Seyis-Bilkay[31] studied the organisms present

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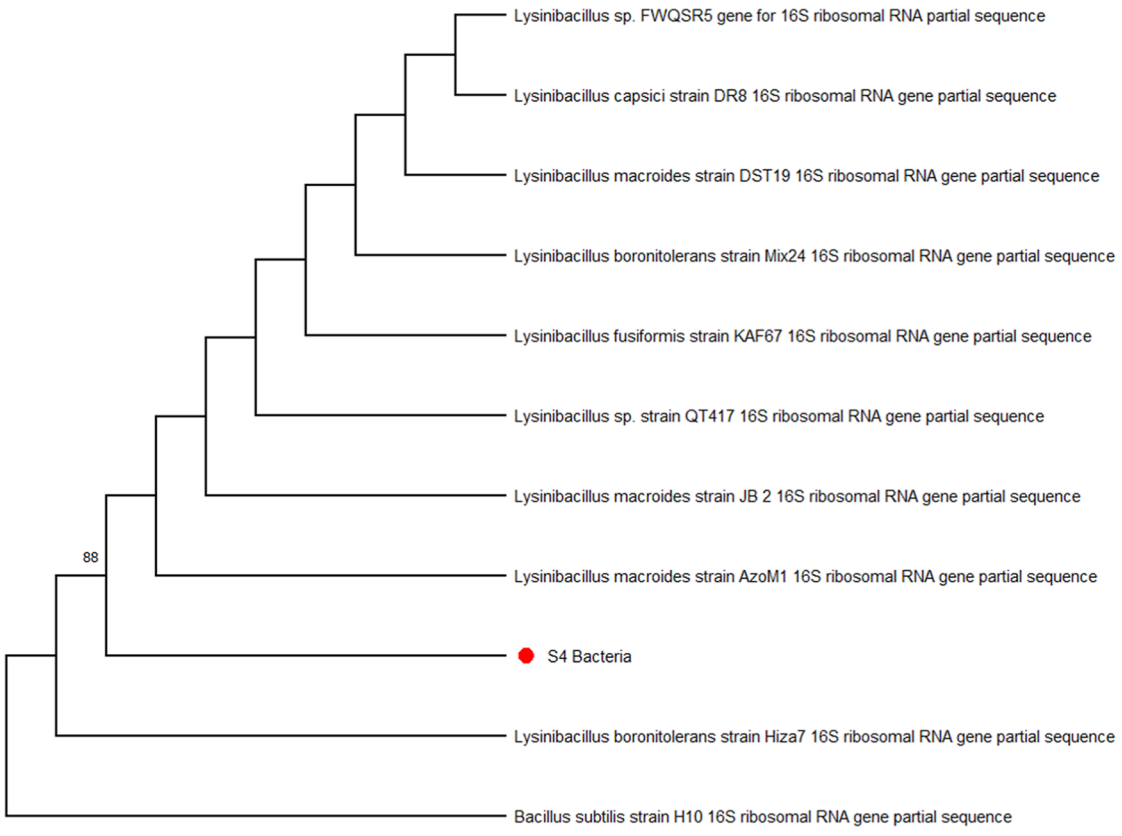

Figure 9. Phylogenetic analysis of S4 bacterial isolate using 16S rRNA gene sequence.

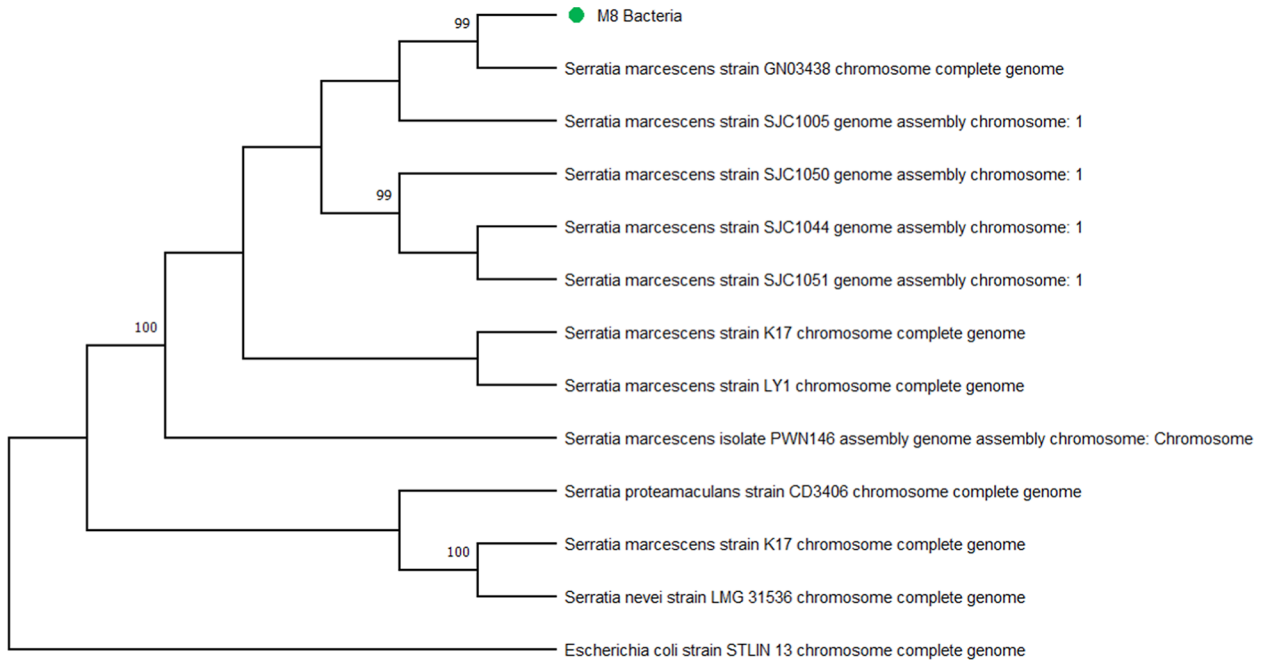

Figure 10. Phylogenetic analysis of M8 bacterial isolate using 16S rRNA gene sequence.

in oil-contaminated soil to degrade oils. Xu et al.[32] studied that most of the petroleum hydrocarbons found in their environment are degraded by indigenous bacterial populations to meet carbon and energy requirements. Ibrahim[33] stated that bacteria isolated from oil-contaminated soil have enzymatic and physiological adaptations, which enable them to utilize the hydrocarbons as a substrate, thereby allowing their survival in such an environment. Several genera of bacteria, including *Ochrobactrum*, *Bacillus*, *Pseudomonas*, *Advenella*, *Bordetella*, *Brucella*, *Achromobacter*, *Stenotrophomonas*, *Mycobacterium*, *Mesorhizobium*, *Klebsiella*, *Pusillimonas*, and *Raoultella*, have been reported to effectively participate in the degradation of petroleum hydrocarbons.[34]

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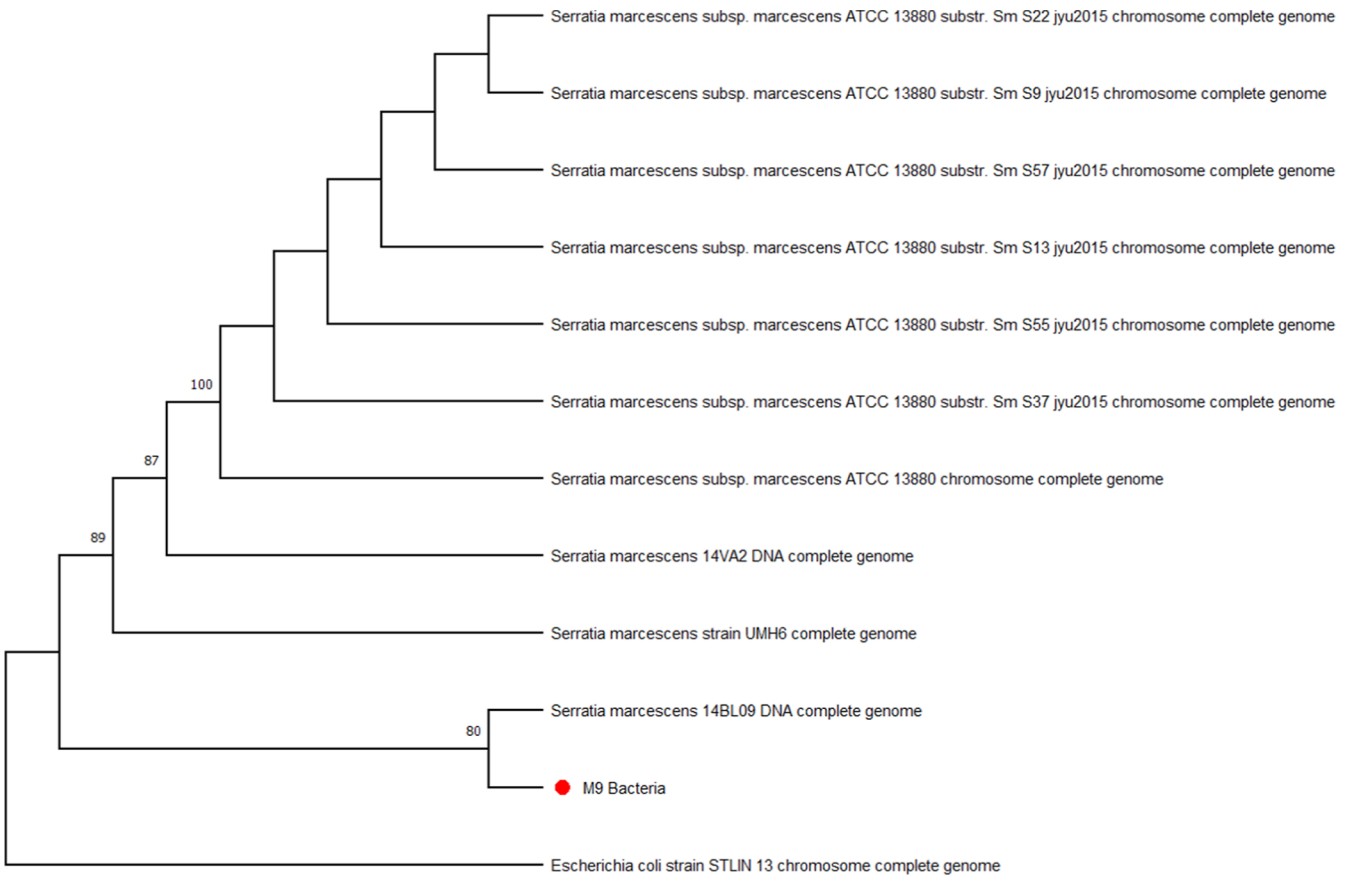

Figure 11. Phylogenetic analysis of M9 bacterial isolate using 16S rRNA gene sequence.

In this study, 19 different bacterial isolates were isolated from the soil sample by conventional culturing methods. These 19 bacterial isolates were tested for a preliminary oil degradation assay, and it was observed that nine bacterial isolates (S1, S4, S7, S10, M5, M6, M7, M8, and M9) showed significant potential for utilizing petroleum hydrocarbons. This was initially determined using OD values at 600 nm wavelength, which provided a quick overview of bacterial growth. However, recognizing the limitation of OD readings in hydrocarbon-rich environments, CFU counts (CFU/mL) were also employed for a more accurate assessment of bacterial growth. The changes in the CFU/mL of the bacterial isolates correspond to their relative growth capacity, which may be due to variances in their tolerance to environmental stress and metabolic adaptability. Based on their maximum CFU count of 1.7×10^9 CFU/mL, isolates S4 and M9 demonstrated the greatest values of metabolic activity and intense growth. These growth-promoting advantages are consistent with earlier research studies, which found that metabolic flexibility and efficient resource use are essential for development in a variety of settings.[35] Conversely, isolates with average specific adaptations showed fewer but more balanced demands for various metabolic objectives; this is seen by the moderate CFU counts found in the S1 and M6 samples, which ranged from 9.86×10^8 to 1.12×10^9 CFU/mL.[36] S7 and S10 isolates showed comparatively lower CFU counts (7.4×10^8 CFU/mL) due to inefficient or restricted metabolic pathways or a stress response. Such variations in growth capabilities highlight the significance of metabolic flexibility in petroleum hydrocarbon degradation, consistent with findings reported in earlier studies.[35,36]

In the current research study, it was observed that 9 bacterial isolates that proved maximum efficiency in the preliminary degradation assay were further subjected to different biodegradation assays. For instance, flask activity was performed using BHM broth with 5 and 10% petroleum hydrocarbon concentrations. It was observed that only 3 bacterial isolates (S4, M8, and M9) showed the highest oil degradation efficiency. Specifically, M8 bacteria displayed 60.2 and 61.5%, S4 bacteria displayed 57.8 and 58.1%, and M9 bacteria showed 58.5 and 55.3% degradation of 5 and 10% petroleum hydrocarbons, respectively. However, in the plate activity performed on BHM with 100 μL (5%) petroleum hydrocarbon concentration, it was observed that the growth of S4, M8, and M9 increased up to 63.4, 61.4, and 57.3%, respectively, using oil as a sole carbon source. Similar results were reported by Hassana et al.,[37] who found that bacterial isolates displayed 45−62% petroleum hydrocarbons degradation efficiency after 15 days of incubation. Subathra et al.[38] observed that out of 113 bacterial isolates, 15 displayed promising petroleum hydrocarbons degradation capability in the degradation assay. They further reported that *Pseudomonas* spp., showed 40% degradation, *Bacillus* spp., showed 26.67% degradation, *Micrococcus* spp., displayed 20% degradation, and *Serratia* spp., showed 6.7% degradation efficiency.

Further, GCMS analysis provided detailed insights into the hydrocarbon degradation patterns of isolates S4, M8, and M9. The analysis revealed that S4 had the highest hydrocarbon degradation efficiency at 83%, with substantial oxidation of

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long-chain alkanes such as hexadecane and nonadecane. This degradation was associated with unique metabolic pathways, as evidenced by the accumulation of silyl ethers and fumaric acid derivatives. Isolate M8 showed a moderate degradation efficiency of 67%, with partial breakdown of hydrocarbons and the presence of siloxanes and trimethylsilyl derivatives, suggesting incomplete degradation. Meanwhile, isolated M9 exhibited a degradation efficiency of 55.2%, marked by the continued presence of cyclohexaneacetic acid and silicic acid derivatives, potentially due to enzymatic limitations or less efficient metabolic pathways. These findings corroborate earlier studies that reported similar variations in the metabolic capabilities of hydrocarbon-degrading bacteria.[39−41] A bacterial degradation study was also conducted by Mbachu et al.,[42] which reflects similar findings, and they reported that the degradation rate for *Bacillus* spp., *Acinetobacter* spp., and *Pseudomonas* spp., was 66.7, 65.47, and 58.33%, respectively. Besides this, *Corynebacterium* spp. and *Flavobacterium* spp. showed 53.57% hydrocarbon degradation capacity. They also concluded that biodegradation of engine oil showed differences in the ability of different isolates for degrading the contents of used engine oil. Similarly, Thenmozhi et al.[43] also carried out a study to assess the capability of soil bacterial strains in petroleum hydrocarbon degradation. They concluded that after 30 days of incubation, *Pseudomonas aeruginosa*, *S. marcesens,* and *Bacillus licheniformis* displayed 81, 72, and 60% degradation of petroleum hydrocarbons, respectively, under controlled conditions. Additionally, Udgire et al.[44] reported that *Bacilli* spp. had 66% degradation capacity for 1% petroleum hydrocarbons and 58% for 10% petroleum hydrocarbons. In contrast, *Enterobacter* spp. demonstrated degradation rates of 52 and 47% for 1 and 10% petroleum hydrocarbons, respectively.

Bioscavenging activity of bacterial treatment to modify the molecular profile of petroleum hydrocarbon is evidenced from comparative GCMS analysis of S4, M8, and M9 bacterial-treated samples with the control sample. It was observed that the petroleum hydrocarbon control sample (Mobil oil) contained normal hydrocarbons such as *p*-xylene, tridecane, tetradecane, etc. and the longer chain alkanes, including hexadecane and heptadecane. This was supported by biological markers such as hopanes and homohopanes that demonstrated the natural origin of petroleum hydrocarbons. Significant alterations in chemical accumulation were recorded after the application of the S4 bacterial isolate. There was an increase in the quantities of organic acids, alcohols, and esters that were detected from the GCMS analysis of the treated oil sample because hydrocarbons had been biodegraded by S4 bacterial isolates. Notably, compounds that contained silicon revealed the interaction between S4 bacteria and oil components during the bioremediation processes. Upon treatment with the M8 bacterial isolate, comparable changes in the composition of petroleum hydrocarbons were noticed. The presence of organic alcohols, cyclohexaneacetic acid, and cyclohexanecarboxylic acid confirmed microbial activity. Phenolic compounds and siloxane derivatives provide evidence that some of the components of petroleum hydrocarbons might have interacted with M8 bacteria, indicating a further microbial role in changing the composition of the oil. However, the characteristics of chemical reaction were observed after treatment of petroleum hydrocarbons with the M9 bacterial isolate. Changes in the functional groups for alkenes, amides, and aromatics were identified, which imply that hydrocarbons were degraded or transformed by M9 bacterial isolates. The presence of organosilicon and organoarsenic chemicals was a clear indication that petroleum hydrocarbons had been subjected to microbial metabolites. In general, GCMS studies demonstrated the molecular conversion of petroleum hydrocarbons after bacterial treatment; each studied strain caused specific changes closely related to microbial transformation and decay. Alnuaimi et al.[45] also used GC equipment in their study and observed that 43 different substances were found in the control sample; however, the majority of these substances were definitely absent from the sample treated with *Bacillus megaterium*. Only 20 chemical compounds were found in the treated sample, and when compared with the untreated sample, the peak area of several of these compounds also reduces.

The current study proves that the DNA extraction from bacterial isolates S4, M8, and M9, and their visualization by gel electrophoresis, allows the identification of the isolated genetic material. PCR using universal bacterial 16S rRNA primers (which amplified approximately 1500 bp fragments of the 16S rRNA gene) was used to confirm the detection of bacterial DNA for further phylogenetic analysis. The ML trees based on the Tamura 3-parameter model gave adequate insights about the phylogenetic history of these isolates. Inferred phylogenies were established with key bootstrap values that depict a high level of confidence as compared with other bootstrap values. The sequence of isolate S4 was most similar to *L. macroides* strain AzoM1 with 88% bootstrap support. *L. macroides* is known to tolerate extreme environments, and this is apparently indicative of the noted hydrocarbon-degrading potential of the S4 isolate. Additionally, both isolates M8 and M9 were closely related to *S. marcescens* strains; isolate M8 belonged to strain GN03438 with a bootstrap value of 99%, while the isolate M9 belonged to strain 14BL09 with a bootstrap of 80%. *S. marcescens* is one of the best-studied species because of its metabolic flexibility for degrading a number of environmental pollutants. Given the fact that M8 and M9 were closely related to *S. marcescens* with regard to their phylogenetic tree, we expected that they shared similar metabolic capabilities, but a slight variation was observed in their ability to degrade hydrocarbons. These variations in the degradation efficiency between M8 and M9 might be due to differences in the metabolic pathway or enzyme system, which are primitives of the same species cluster phylogenetically. Chinyelu Nkiru et al.[46] also extracted DNA of the selected bacteria, and the 16S rRNA gene sequence of the bacterial isolates was amplified via PCR and found to have a length of 1500 base pairs. Molecular analysis revealed the evolutionary link among the isolates. The results showed that the isolated bacteria were *S. marcescens* Mb4, *Lysinibacillus* spp. M2c, *Bacillus cereus* TPM-23, and *Proteus mirabilis* LS-3.

Overall, the combination of CFU data, biodegradation assays, GCMS analysis, and molecular characterization highlights the potential of the bacterial isolates S4, M8, and M9 as efficient agents for petroleum hydrocarbon remediation. These findings provide a foundation for future studies exploring genetic or environmental manipulations to enhance the hydrocarbon-degrading capabilities of bacterial strains. Moreover, the scalability of these findings could be enhanced through optimization of growth conditions and environmental parameters to ensure effective large-scale deployment in contaminated sites.

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5. CONCLUSIONS

The finding demonstrates that the soil of automobile workshops harbors diverse oil-degrading bacterial species, with 19 distinct species isolated. Preliminary oil degradation assays conducted over a 12-day incubation period revealed remarkable growth of nine bacterial isolates (S1, S4, S7, S10, M5, M6, M7, M8, and M9). Notably, subsequent shake flask tests showcased the exceptional activity of three isolates (S4, M8, and M9). Among these, M8 exhibited the highest petroleum hydrocarbon degradation efficiency, achieving an impressive 60.2% degradation of 5% petroleum hydrocarbons, followed by M9 with 58.1% and S4 with 57.8% degradation. In the 10% petroleum hydrocarbon experiment, M8 continued to excel with a degradation rate of 61.5%, surpassing S4 (58%) and M8 (55.3%). Plate activity data confirmed significant growth expansion for S4, M8, and M9 bacterial isolates. The largest growth zone was observed for M9, measuring 41.2 ± 1.7 mm, followed by M8 with 39.7 ± 1.9 mm and S4 with 38.4 ± 1.3 mm. Additionally, the GCMS study showed that isolate S4 had the highest efficiency in the breakdown of hydrocarbons at 83.6%, thus significantly decreasing major aliphatic and aromatic hydrocarbons. Isolate M8 displayed only moderate degradation of 67.4%, while isolate M9 exhibited the least efficiency, which was 55.2%, demonstrating partial degradation and lingering presence of some hydrocarbons. Also, to conform with these correct classifications, the phylogenetic tree of the 16S rRNA gene confirmed that isolate S4 was most closely related to *L. macroides* AzoM1; M8 to *S. marcescens* GN03438; and M9 to *S. marcescens* 14BL09 with robust bootstrap values. These findings provide valuable insights into the genetic underpinnings of these isolates. Furthermore, GCMS analysis of a control sample (Mobil oil) showed different chemical compounds, imprinting aromatic, and saturated hydrocarbon characteristic for petroleum hydrocarbons. Consequently, marked differences were observed in the chemical composition of S4 bacterial-treated samples with evidence of phenolic compound esters and silicon-containing compounds. In addition to this, the presence of different groups, such as aromatic, amide, and alkene groups in M8 and M9 bacterial-treated samples showed chemical transformations that may have taken place during treatment. This confirms their proficiency and capability for hydrocarbon remediation, making them promising candidates for further research in treating petroleum-induced environmental pollution through bioremediation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c06434.

GC−MS explanation for hydrocarbon analysis in control and bacterial-treated petroleum oil samples; sample preparation, derivatization using BSTFA, and GC−MS chromatogram interpretation; and identification and quantification of hydrocarbons using retention times, mass spectra, and calibration curves (PDF)

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Conceptualization and *methodology*: [Abdul Rehman and Madeeha Mehboob]; *data collection and processing*: [Madeeha Mehboob]; formal analysis and writing—original draft manuscript: [Abdul Rehman and Iffat Naz]; *critical review and editing*: [Ayesha Shuja Farooq, Baharullah Khattak, and Malik Nawaz Shuja]; and *supervision*: [Abdul Rehman]. All the authors have read and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Demirbas, A.; Balubaid, M. A.; Basahel, A. M.; Ahmad, W.; Sheikh, M. H. Octane rating of gasoline and octane booster additives. *Petroleum. Sci. Technol.* **2015**, *33* (11), 1190−1197.

(2) Alzahrani, A. M.; Rajendran, P. Petroleum hydrocarbon and living organisms. In *In Hydrocarbon Pollution and its Effect on the Environment*; Intech Open, 2019; pp 71−82. https://books.google.com.pk/books-hl=en&lr=&id=ekr9DwAAQBAJ&oi=fnd&pg=PA71&dq=.

(3) Varjani, S. J. Microbial degradation of petroleum hydrocarbons. *Biores. Technol.* **2017**, *223*, 277−286.

(4) Li, Y.; Li, C.; Xin, Y.; Huang, T.; Liu, J. Petroleum pollution affects soil chemistry and reshapes the diversity and networks of microbial communities. *Ecotoxicol. Environ. Safety* **2022**, *246*, 114129.

(5) Kuppusamy, S.; Maddela, N. R.; Megharaj, M.; Venkateswarlu, K.; Kuppusamy, S.; Maddela, N. R.; Venkateswarlu, K. Impact of total petroleum hydrocarbons on human health. In *Total Petroleum Hydrocarbons: Environmental Fate, Toxicity, and Remediation*; Springer International Publishing, 2020; pp 139−165.

(6) Dhaka, A.; Chattopadhyay, P. A review on physical remediation techniques for treatment of marine oil spills. *J. Environ. Manage.* **2021**, *288*, 112428.

(7) Pete, A. J.; Bharti, B.; Benton, M. G. Nano-enhanced bioremediation for oil spills: a review. *ACS EST Eng.* **2021**, *1* (6), 928−946.

(8) Wolok, E.; Barafi, J.; Joshi, N.; Girimonte, R.; Chakraborty, S. Study of biomaterials for removal of the oil spill. *Arab. J. Geosci.* **2020**, *13*, 1244.
(9) Korshunova, T. Y.; Chetverikov, S. P.; Bakaeva, M. D.; Kuzina, E. V.; Rafikova, G. F.; Chetverikova, D. V.; Loginov, O. N. Microorganisms in the elimination of oil pollution consequences. *Appl. Biochem. Microbiol.* **2019**, *55*, 344−354.
(10) Sarhan, M. S.; Hamza, M. A.; Youssef, H. H.; Patz, S.; Becker, M.; ElSawey, H.; Nemr, R.; Daanaa, H. S. A.; Mourad, E. F.; Morsi, A. T.; et al. Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media−a review. *J. Adv. Res.* **2019**, *19*, 15−27.
(11) Rahmati, F.; Asgari-Lajayer, B.; Shadfar, N.; van Bodegom, P. M.; van Hullebusch, E. D. A review on biotechnological approaches applied for marine hydrocarbon spills remediation. *Microorganisms* **2022**, *10* (7), 1289−1309.
(12) Laothamteep, N.; Naloka, K.; Pinyakong, O. Bioaugmentation with zeolite-immobilized bacterial consortium OPK results in a bacterial community shift and enhances the bioremediation of crude oil-polluted marine sandy soil microcosms. *Environ. Pollut.* **2022**, *292*, 118309.
(13) Ajona, M.; Vasanthi, P. Bioremediation of petroleum contaminated soils−A review. *Mater. Today. Proc.* **2021**, *45*, 7117−7122.
(14) Paul, O.; Jasu, A.; Lahiri, D.; Nag, M.; Ray, R. R. *In situ* and *ex situ* bioremediation of heavy metals: the present scenario. *J. Environ. Eng. Landsc. Manag.* **2021**, *29* (4), 454−469.
(15) Choi, G. H.; Holzapfel, W. H.; Todorov, S. D. Diversity of bacteriocins, their classification and potential applications in combat of antibiotic resistant and clinically relevant pathogens. *Crit. Rev. Microbiol.* **2022**, *49* (5), 578−597.
(16) Hesham, A. E. L.; Mohamed, E. A.; Mawad, A. M.; Elfarash, A.; Abd El-Fattah, B. S.; El-Rawy, M. A.; El-Rawy, M. A. Molecular Characterization of Fusarium Solani Degrades a Mixture of Low and High Molecular Weight Polycyclic Aromatic Hydrocarbons. *Open. Biotechnol. J.* **2017**, *11* (1), 27−35.
(17) Sarhan, M. S.; Hamza, M. A.; Youssef, H. H.; Patz, S.; Becker, M.; ElSawey, H.; Nemr, R.; Daanaa, H. S. A.; Mourad, E. F.; Morsi, A. T.; et al. Culturomics of the plant prokaryotic microbiome and the dawn of plant-based culture media−a review. *J. Adv. Res.* **2019**, *19*, 15−27.
(18) Jayanthi, R.; Hemashenpagam, N. Isolation and identification of petroleum hydrocarbon degrading bacteria from oil contaminated soil samples. *Int. J. Novel. Trends. Pharma. Sci.* **2015**, *5* (3), 102−106.
(19) Bekele, G. K.; Gebrie, S. A.; Mekonen, E.; Fida, T. T.; Woldesemayat, A. A.; Abda, E. M.; Tafesse, M.; Assefa, F. Isolation and characterization of diesel-degrading bacteria from hydrocarbon-contaminated sites, flower farms, and soda lakes. *Int. J. Microbiol.* **2022**, *2022*, 1−12.
(20) Umar, M.; Kasim, K. A.; Zango, M. U.; Yunusa, G. H. Residual Soil Improvement Using Microbially Induced Cementation of Ureolytic Bacteria. *Platform: J. Eng.* **2024**, *8* (3), 11−17.
(21) Yalaoui-Guellal, D.; Fella-Temzi, S.; Djafri-Dib, S.; Sahu, S. K.; Irorere, V. U.; Banat, I. M.; Madani, K. The petroleum-degrading bacteria *Alcaligenes aquatilis* strain YGD 2906 as a potential source of lipopeptide biosurfactant. *Fuel* **2021**, *285*, 119112.
(22) Muhammad, R. G.; Mohammed, J. N.; Muhammad, I. L.; Hamzat, A. Stimulated bioremediation of soil contaminated with spent engine oil using organic wastes. *Sci. World. J.* **2022**, *17* (2), 308−314.
(23) Purnomo, A. S.; Rizqi, H. D.; Rafi, L. H. Culture of bacterium *Bacillus subtilis* as degradation agent for sea water remediation contaminated by petroleum. *J. Indian Chem. Soc.* **2020**, *3* (1), 53−58.
(24) Kumar, M.; Mugunthan, M. Evaluation of three DNA extraction methods from fungal cultures. *Med. J. Armed. Forces India* **2018**, *74* (4), 333−336.
(25) Khan, J. Low-Cost Methods for DNA Extraction and Quantification. *Biotechnologies and Genetics in Plant Mutation Breeding: Volume 3: Mechanisms for Genetic Manipulation of Plants and Plant Mutants*; Apple Academic Press, 2023, *49*, 137−156,
(26) Nwagwu, E. C.; Yilwa, V. M.; Egbe, N. E.; Onwumere, G. B. Isolation and identification of hydrocarbons-degrading bacteria from Panteka stream, Kaduna, Nigeria, and assessment of their potential for bioremediation. *Niger. J. Biotechnol.* **2021**, *37* (2), 84−94.
(27) Aziz, Z. S.; Jazaa, S. H.; Dageem, H. N.; Banoon, S. R.; Balboul, B. A.; Abdelzaher, M. A. Bacterial biodegradation of oil-contaminated soil for pollutant abatement contributing to achieve sustainable development goals: A comprehensive review. *Results Eng.* **2024**, *22*, 102083.
(28) Ossai, I. C.; Ahmed, A.; Hassan, A.; Hamid, F. S. Remediation of soil and water contaminated with petroleum hydrocarbon: A review. *Environ. Technol. Innov.* **2020**, *17*, 100526.
(29) Mekonnen, B. A.; Aragaw, T. A.; Genet, M. B. Bioremediation of petroleum hydrocarbon contaminated soil: a review on principles, degradation mechanisms, and advancements. *Front. Environ. Sci.* **2024**, *12*, 1354422.
(30) Zhang, S.; Zuo, X.; Wei, G.; Wang, H.; Gao, Y.; Ling, W. Immobilized Pseudomonas spp. for bioremediation of soils contaminated with emerging organic pollutants. *Appl. Soil Ecol.* **2024**, *204*, 105717.
(31) Bilen Ozyurek, S.; Seyis-Bilkay, I. Determination of petroleum biodegradation by bacteria isolated from drilling fluid, waste mud pit and crude oil. *Turkish J. Biochem.* **2017**, *42* (6), 609−616.
(32) Xu, X.; Liu, W.; Tian, S.; Wang, W.; Qi, Q.; Jiang, P.; Gao, X.; Li, F.; Li, H.; Yu, H. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. *Front. Microbiol.* **2018**, *9*, 1−11.
(33) Salisu, B.; Ibrahim, F. Microbial Bioremediation of Spent Engine Oil: Current Advances, Challenges, and Future Directions. *UMYU Scientifica* **2024**, *3* (4), 260−274.
(34) Rani, M.; Weadge, J. T.; Jabaji, S. Isolation and characterization of biosurfactant-producing bacteria from oil well batteries with antimicrobial activities against food-borne and plant pathogens. *Front. Microbiol.* **2020**, *11*, 64.
(35) Wu, M.; Feng, S.; Liu, Z.; Tang, S. Bioremediation of petroleum-contaminated soil based on both toxicity risk control and hydrocarbon removal—progress and prospect. *Environ. Sci. Pollut. Res.* **2024**, *31*, 59795−59818.
(36) Koolivand, A.; Abtahi, H.; Parhamfar, M.; Saeedi, R.; Coulon, F.; Kumar, V.; Villaseñor, J.; Sartaj, M.; Najarian, N.; Shahsavari, M.; et al. The effect of petroleum hydrocarbons concentration on competition between oil-degrading bacteria and indigenous compost microorganisms in petroleum sludge bioremediation. *Environ. Technol. Innov.* **2022**, *26*, 102319.
(37) Hassana, A.; Vincent, B. T.; Ushuji, O. D.; Yakubu, N.; Boko, U. H.; Gogo, M. F. Molecular identification of hydrocarbon degrading bacteria isolated from contaminated soil of automobile mechanic workshop in lapai, Niger State. *Int. J. Pure. Appl. Biosci.* **2019**, *7* (4), 31−37.
(38) Subathra, M. K.; Immanuel, G.; Suresh, A. H. Isolation and Identification of hydrocarbon degrading bacteria from Ennore creek. *Bioinformation* **2013**, *9* (3), 150−157.
(39) Jin, H.; Li, D.; Chen, Q.; Zhang, Y. Advances in microbial degradation of long-chain alkanes. *Biotechnol. Adv.* **2022**, *56*, 107897.
(40) Gandhimathi, R.; Kesavan, D.; Selvi, A. Biodegradation of hydrocarbon pollutants: Current challenges and future prospects. *Curr. Environ. Eng.* **2020**, *7* (3), 204−215.
(41) Davletgildeeva, A. T.; Kuznetsov, N. A. Bioremediation of polycyclic aromatic hydrocarbons by means of bacteria and bacterial enzymes. *Microorganisms* **2024**, *12* (9), 1814.
(42) Mbachu, A. E.; Onochie, C. C.; Agu, K. C.; Okafor, O. I.; Awah, N. S. Hydrocarbon degrading potentials of indigenous bacteria isolated from auto-mechanic workshops at Mgbuka-Nkpor, Nigeria. *J. Global. Biosci.* **2014**, *3* (1), 321−326.
(43) Thenmozhi, R.; Nagasathya, A.; Thajuddin, N. Studies on biodegradation of used engine oil by consortium cultures. *Adv. Environ. Biol.* **2011**, *5* (6), 1051−1057.

https://doi.org/10.1021/acsomega.4c06434
ACS Omega 2025, 10, 2501−2516

(44) Udgire, M.; Shah, N.; Jadhav, M. Enrichment, isolation and identification of hydrocarbon degrading bacteria. *Int. J. Cur. Microbiol. Appl. Sci.* **2015**, *4* (6), 708−713.
(45) Alnuaimi, M. T.; Taher, T. A.; Aljanabi, Z. Z.; Adel, M. M. High-resolution GC/MS study of biodegradation of crude oil by *Bacillus megaterium*. *Res. Crops* **2020**, *21* (3), 650−657.
(46) Chinyelu Nkiru, U.; Orsla, E. E. I.; Clarice, U. C.; Emmanuel, E. S.; Ijeoma, C. U. C. Biodegradation of crude oil-polluted Soil by Bacterial isolates from Nigeria. *Front. Environ. Microbiol.* **2019**, *5* (1), 14−28.

https://doi.org/10.1021/acsomega.4c06434
ACS Omega 2025, 10, 2501−2516