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Original Research

Evaluation of Black Oil Biodegradation by a Consortium of Indigenous *Bacillus cereus* and *Pseudomonas aeruginosa*

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Abstract: Black oil is a refined oil product that poses a significant environmental risk. It contains complex multi-hydrocarbons that decompose slowly. Black oil remains in the environment for a long time, that cause various toxic effects. This study was focused on three aspects. First of all, the local bacteria were isolated. Then, the potential of these bacteria for degrading black oil were determined. Finally, the efficiency of bacterial consortium in degrading black oil was evaluated. Three black oil-degrading bacteria were isolated from crude oil contaminated soil, and molecularly identified by 16s rDNA sequencing as *Bacillus cereus* strain ZG.S6, *Bacillus cereus* strain ZG.S12, and *Pseudomonas aeruginosa* strain ZG.S11. Based on the measurement of optical density and chromatogram analysis, *B. cereus* strain ZG.S6, *B. cereus* strain ZG.S12, and *P. aeruginosa* strain ZG.S11 degrade black oil efficiently by reducing the number of their compounds to 10, 16, and 14, respectively. The results were compared to the complex combination of black oil (control group), which consists of 25 compounds of aliphatic and aromatic substances. The bacterial consortium demonstrated compatibility with each other. This helped them to degrade black oil more efficiently than individual strains, reducing its compounds to seven. Consequently, the consortium is a promising candidate of black oil bioremediation.

Key Words	Crude oil, *P. aeruginosa*, *B. cereus,* Bacterial community, Bioremediation.
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1. INTRODUCTION

Urbanization and industrialization have led to the generation of hazardous non-biodegradable pollutants that cause many toxic effects on human health and the environment (Bekele *et al.*, 2023). Petroleum

and its derivatives are serious compounds that significantly impact the soil, surface and groundwater, as well as the stability and diversity of the microbial population (Ja'afaru *et al.*, 2017). Therefore, there is a demand for rapid and effective methods to remove these contaminants.

Among of petroleum derivatives, black oil is one of the most hazardous heavy fuel for ecosystems and is a common pollutant in Iraq (Owaied *et al.*, 2018). It comprises heavy hydrocarbons such as resins, asphaltene, solid paraffin, long-chain alkanes, cyclic alkanes, polycyclic aromatics, unsaturated compounds, sulfuric compounds, and heavy metals (Khorasani *et al.*, 2013). Black oil is slowly degraded and it can remain in the environment for a long time. Black oil cause various toxic effects through the possibility of some components accumulating within susceptible organisms and being transferred to other levels in the food chain (Al-Wasify and Hamed, 2014).

In general, conventional remediation processes for petroleum hydrocarbons are inappropriate due to their high application costs and the hazardous intermediates they produce. Bioremediation strategies, using biological agents such as plants and microbes, are a better option for restoring contaminated sites. It ensures a simple, cheap, and eco-friendly cleanup method (Chandran *et al.*, 2020; Alsayegh *et al.*, 2021). Microorganisms are utilized primarily in bioremediation because of their rapid growth, nutritional flexibility, and ability to adapt to extreme environments, thus enhancing their function as bioremediation agents (Ayilara and Babalola, 2023).

A group of microorganisms, including many species of bacteria, yeast, and fungi, that inhabit polluted sites and have high metabolic potential are important for microbial bioremediation (Rahayu *et al.*, 2019; Chandran *et al.*, 2020). Numerous studies have demonstrated that many bacterial species are common hydrocarbon degraders (Rahayu *et al.*, 2019; Tuhuloula *et al.*, 2019; Alsayegh *et al.*, 2021). *Pseudomonas* and *Bacillus* species are recognized as proficient degraders of hydrocarbons by utilizing them as sole sources of carbon and energy (Ja'afaru *et al.*, 2017; Rahayu *et al.*, 2019; Abdal-Satter, 2020; Abena *et al.*, 2020; Faisal *et al.*, 2023). These bacteria have improved their enzymatic systems and produced surface active compounds, enabling them to utilize such pollutants and modify them into nontoxic and eco-friendly products (Owaied *et al.*, 2018; Tuhuloula *et al.*, 2019; Faisal *et al.*, 2023).

The present study aimed to estimate the possibility of a local bacterial consortium, *Bacillus cereus* strain ZG.S6, *Bacillus cereus* strain ZG.S12 and *Pseudomonas aeruginosa* strain ZG.S11, to degrade black oil.

2. MATERIALS AND METHODS

Black oil sample

A sample of black oil was collected from the Al-Doura refinery located southeast of Baghdad, Iraq. It was stored in a dark, tightly sealed bottle, then transported to the laboratory and kept in a cool, dark place until use.

Isolation and Identification of black oil-degrading bacteria

A selective enrichment method, using mineral salt medium (MSM) pH 7, enriched with 2% (v/v) black oil, was used to isolate black oil-degrading bacteria from sample of soil contaminated with crude oil. The soil sample was obtained from a depth of (10-20 cm) from an electrical generator in Baghdad, Iraq. MSM contained (g/l): KH_2PO_4 (1.0), K_2HPO_4 (1.0), NaCl (1.0), $CaCl_2$ (0.05), $(NH_4)_2$ SO_4 (1.0), $MgSO_4.7H_2O$ (0.5), $FeCl_3$ (0.002), and Yeast extract (0.1) (Faisal *et al.*, 2023). Bacterial isolates were molecularly identified by 16S rRNA sequencing, using universal primers 27F: 5`-AGAGTTT-GATCCTGGCTCAG-3` and 1492R: 5`-TACGGTTACCTTGTTACGACTT -3`, and compared with those of 16S rRNA genes from NCBI Gene bank. PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation-Korea. The results were received by E-mail then analyzed using genius software.

Preparation of the bacterial cultures and bacterial consortium and Estimating their ability to degrade black oil

This experiment was performed in triplicate. One milliliter of fresh culture (OD_{600} = 1.0) was inoculated individually into a 500 ml Erlenmeyer flask containing 100 ml MSM with 2% black oil, and incubated in a 150 rpm shaker incubator at 30 °C for 14 days. To prepare bacterial consortia, a 500-ml Erlenmeyer flask containing the same medium composition was inoculated with 1 ml of each fresh bacterial culture (OD_{600} = 1.0) and kept under the same conditions. Flask with the same culture media composition but no inoculums was used as a control. After 14 days of incubation, bacterial growth was assessed using a UV/Vis spectrophotometer (OD_{600}), and compared to MSM as a blank (Lima *et al.*, 2020).

Gas chromatography–mass spectrometric (GC-MS) analysis

After 14 days of incubation, the residual black oil was collected separately from each bacterial culture and from the bacterial consortium by chloroform (3 sample:1 chloroform v/v) with continuous shaking. By using a separation funnel, the organic phase containing the residual black oil was separated from the aqueous phase, collected in a glass Petri dish, and then dried at 40–45°C. The precipitate was analyzed by a GC-MS instrument that contains a ZB-5MS capillary column (30 mm x 0.25 mm, I.D. 0.25 μm), using helium (He) as carrier gas with a flow rate of 2 ml/min. The temperatures of the injector and detector were 230 °C and 280 °C, respectively. The column was initially heated to 80 °C for 3 minutes and then raised to 280 °C for 10 minutes at a rate of 8 °C/min. First, 0.1 g of samples were dissolved in chloroform at a concentration of 100 μg/ml, and then 1 μl of sample was used with a split ratio of 10:1.

Data analysis

The computerized database structure was used to perform statistical analyses and report on the obtained data. One-way analysis of variance (ANOVA) was used, with a statistical significance level of $P<0.05$.

3. RESULTS AND DISCUSSIONS

Isolation and Identification of bacteria

Three effective black oil-degrading bacteria were isolated using a selective enrichment approach. This shows that bacteria can survive and utilize black oil in soil contaminated with petroleum derivatives. Previous studies indicate the possibility of isolating many types of bacteria from petroleum-contaminated soil that can utilize crude oil and hydrocarbon residues (Rahayu *et al.*, 2019; Abdal-Satter, 2020; Abena *et al.*, 2020; Faisal *et al.*, 2023).

The sequence analysis indicated that two isolates were classified as *Bacillus cereus,* while one was identified as *Pseudomonas aeruginosa* (Fig. 1, 2, and 3). Strains were documented in the NCBI GenBank nucleotide sequence database as *Bacillus cereus* strain ZG.S6, *Bacillus cereus* strain ZG.S12, and *Pseudomonas aeruginosa* strain ZG.S11 with accession numbers PP408317, PP408320, and PP408321, respectively.

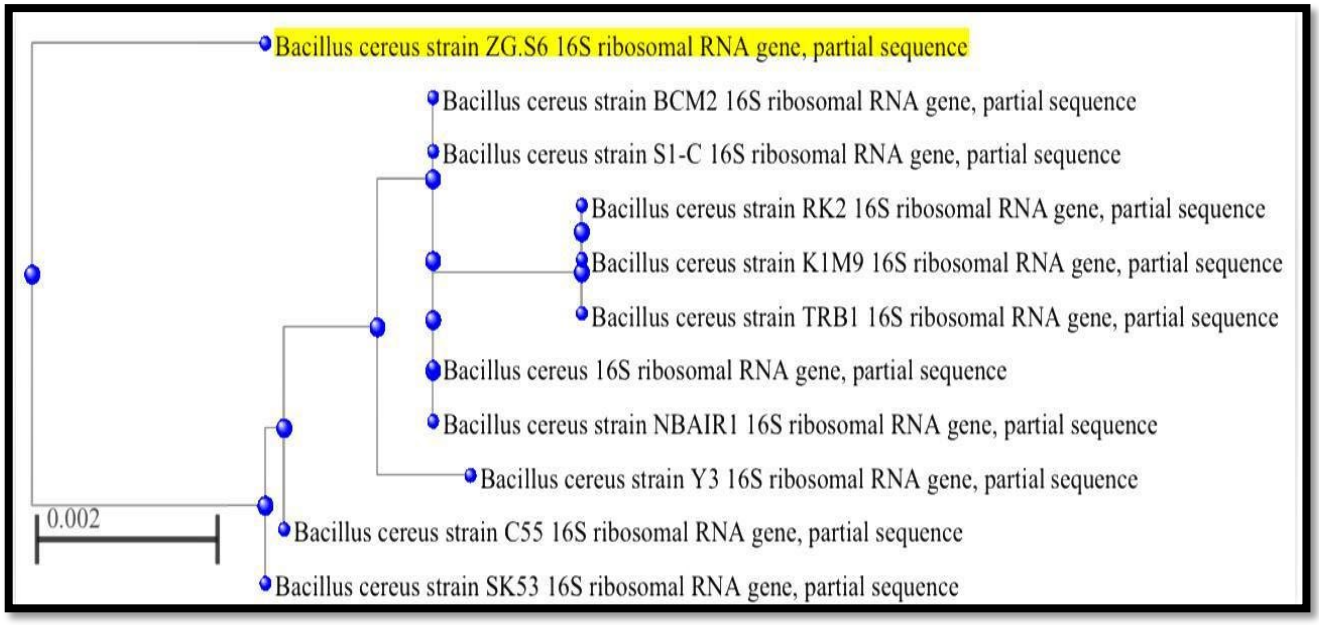

Fig. 1: *Bacillus cereus* strain ZG.S6 phylogenic tree from NCBI database.

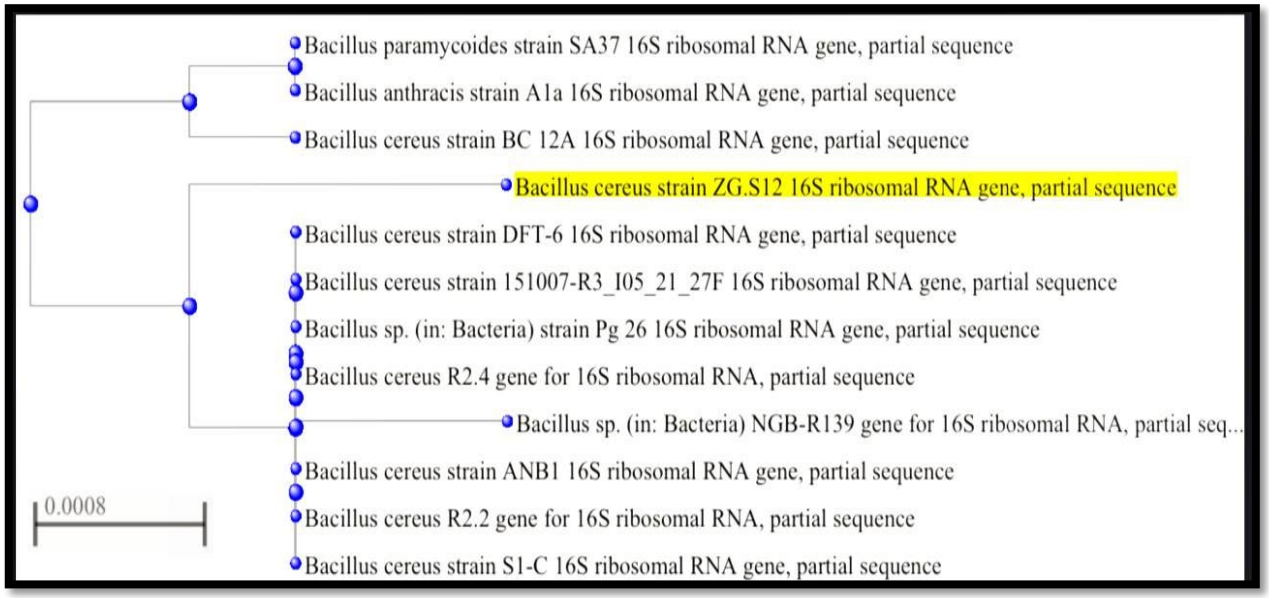

Fig. 2: *Bacillus cereus* strain ZG.S12 phylogenic tree from NCBI database.

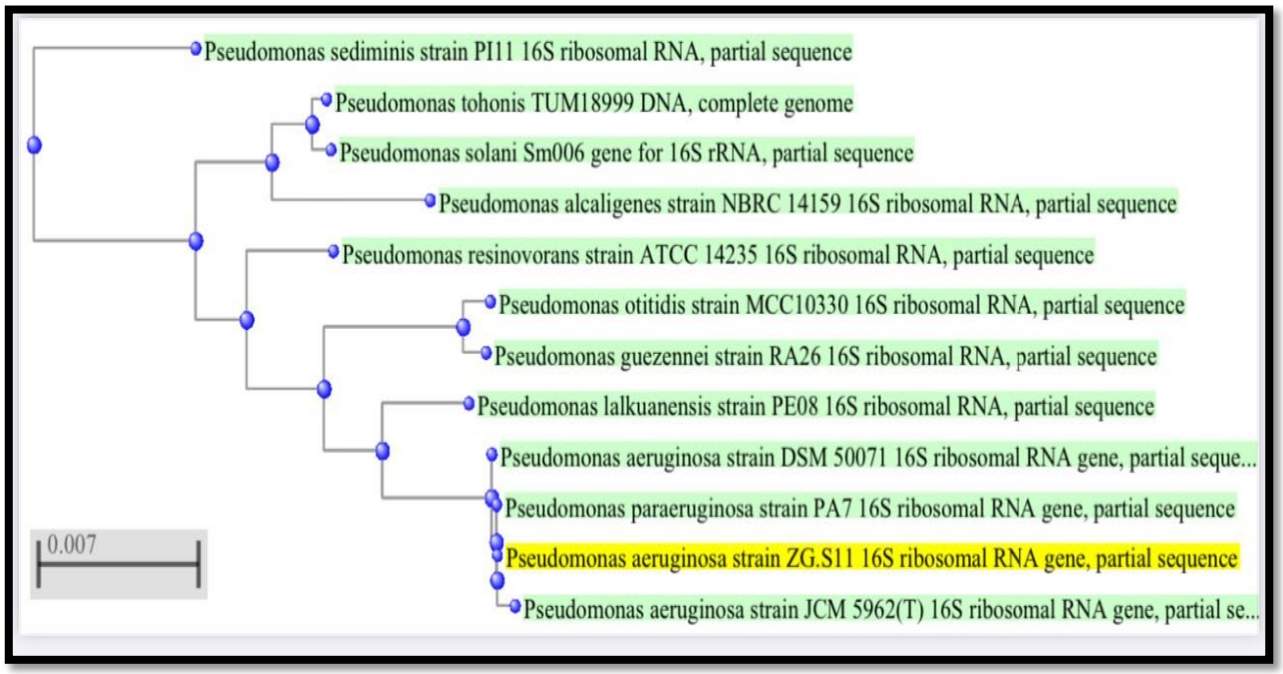

Fig. 3: *Pseudomonas aeruginosa* strain ZG.S11 phylogenic tree from NCBI database.

Bekele *et al.* (2022) explained that *Bacillus cereus* and *Pseudomonas aeruginosa* play significant roles in the environment, particularly in the decomposition of hydrocarbons and their derivatives. This is attributed to the diverse metabolic capacity of bacteria to decompose hydrocarbons and recycle metals in nature (Kebede *et al.*, 2021). Additionally, many species of bacteria can generate surface-active compounds with different chemical structures, which can minimize interfacial and surface interactions, thereby

facilitating the emulsification process of hydrocarbon contaminants and improving their bioavailability (Faisal *et al.*, 2023).

Estimating the ability of bacterial consortium to degrade black oil

By measuring the optical density of bacteria grown on MSM containing 2% black oil as a carbon and energy source, the ability of bacteria to decompose black oil was assessed. Bacterial proliferation is an indicator of their ability to degrade black oil. The variation in OD was related to the intensity of bacterial growth and the breakdown of the black oil molecular structure as a result of its utilization by bacteria as a carbon and energy source.

Through the first three days of incubation (lag phase), there was no discernible change in turbidity. This period is responsible for the bacteria adapting to their new environment in preparation for the next stage (experiential phase). After 14 days of incubation, bacterial growth reached a maximum of 1.214±0.01, 1.186±0.03, and 1.124±0.01 for *B. cereus* strain ZG.S6, *B. cereus* strain ZG.S12, and *P. aeruginosa* strain ZG.S11, respectively. The OD of the bacterial consortium recorded a significant growth intensity of 1.237±0.03, indicating an increase in the rate of black oil degradation. Table 1 showed the optical density (OD_{600}) of individual bacterial strains and their consortium after 14 days of incubation in MSM containing 2% black oil.

Table 1: The optical density (OD_{600}) of individual bacterial strains and their consortium after 14 days of incubation in MSM containing 2% black oil.

No.	Symbol	Bacterial Strain	The optical density (OD_{600}) after 14 days
1	S6	*Bacillus cereus* strain ZG.S6	1.214±0.01
2	S11	*Pseudomonas aeruginosa* strain ZG.S11	1.124±0.01
3	S12	*Bacillus cereus* strain ZG.S12	1.186±0.03
4		Consortium (S6, S11, and S12)	1.237±0.03

Data: Mean ±SEM. The experiments were performed in three replicates.

Table 1 showed variation in OD, and *B. cereus* strain ZG.S6 was the most efficient in utilizing black oil. This variation is due to different metabolic capacities of bacteria to decompose hydrocarbons (Kebede *et al.*, 2021). Furthermore, lipopeptides and glycolipides biosurfactants generated by *Bacillus* and *Pseudomonas* species can aid in the emulsification process and increase hydrocarbon bioavailability (Viesser *et al.*, 2020; Kebede *et al.*, 2021; Bekele *et al.*, 2023; Faisal *et al.*, 2023). *Bacillus* species can form spores that allow them to thrive in various environments, including hydrocarbon-polluted soils (Viesser *et al.*, 2020). Also, *Pseudomonas* species are successful in the biodegradation of petroleum

because they are less vulnerable to the toxic effects of hydrocarbons (Bilen Ozyurek and Seyis Bilkay, 2020).

GC-MS analysis

GC-MS was used to determine the residual black oil of individual bacterial cultures (*B. cereus* strain ZG.S6, *B. cereus* strain ZG.S12, and *P. aeruginosa* strain ZG.S11) and their consortium, contrasting them with the control. Table 2 shows the complicated mixture of black oil (control group), which contains 25 compounds of aliphatic and aromatic substances. Fig. 4 showed several peaks that illustrate variations between black oil compounds caused by differences in the number of carbon atoms.

Table 2: The chemical composition of black oil (control group)

NO.	RT (min)	Area %	Name	Quality	CAS Number
1	11.341	0.38	Cyclopentasiloxane, decamethyl-	80	000541-02-6
2	15.362	13.88	Dodecamethylcyclohexasiloxane	93	000540-97-6
3	16.545	0.37	Tetradecane	93	000629-59-4
4	17.894	0.19	n-Eicosane	60	000112-95-8
5	18.61	0.55	Pentadecane	96	000629-62-9
6	19.02	13.08	Dodecamethylpentasiloxane	52	000141-63-9
7	20.561	0.84	Cetane	96	000544-76-3
8	21.536	0.29	Tridecane, n-	90	000629-50-5
9	22.294	6.88	: Cyclooctasiloxane, hexadecamethyl-	59	000000-00-0
10	24.183	2.22	Octadecan	98	000593-45-3
11	25.132	3.17	Cyclononasiloxane, octadecamethyl-	90	109007-87-6
12	25.858	3.06	Nonadecane	99	000629-92-5
13	27.462	5.31	pentadecane	97	000629-62-9
14	27.68	1.85	Boldenone, di-trimethylsilyl	62	109007-87-6
15	28.998	6.73	n-Heneicosane	98	000629-94-7
16	30.461	8.18	Normal-docosane	99	000629-97-0
17	31.867	6.59	n-Tricosane	99	000638-67-5
18	33.216	6.82	Tetracosane	98	000646-31-1
19	34.518	4.92	n-Heneicosane	98	000629-94-7
20	35.769	4.03	n-Hexacosane	99	000630-01-3
21	36.972	3.28	n-Heptacosane	98	000593-49-7
22	38.13	1.98	Heptacosane, 1-chloro-	98	062016-79-9
23	39.255	1.98	n-Eicosane	95	000112-95-8
24	40.345	1.82	n-Eicosane	96	000112-95-8
25	40.791	1.58	Baccharene	53	036441-74-4

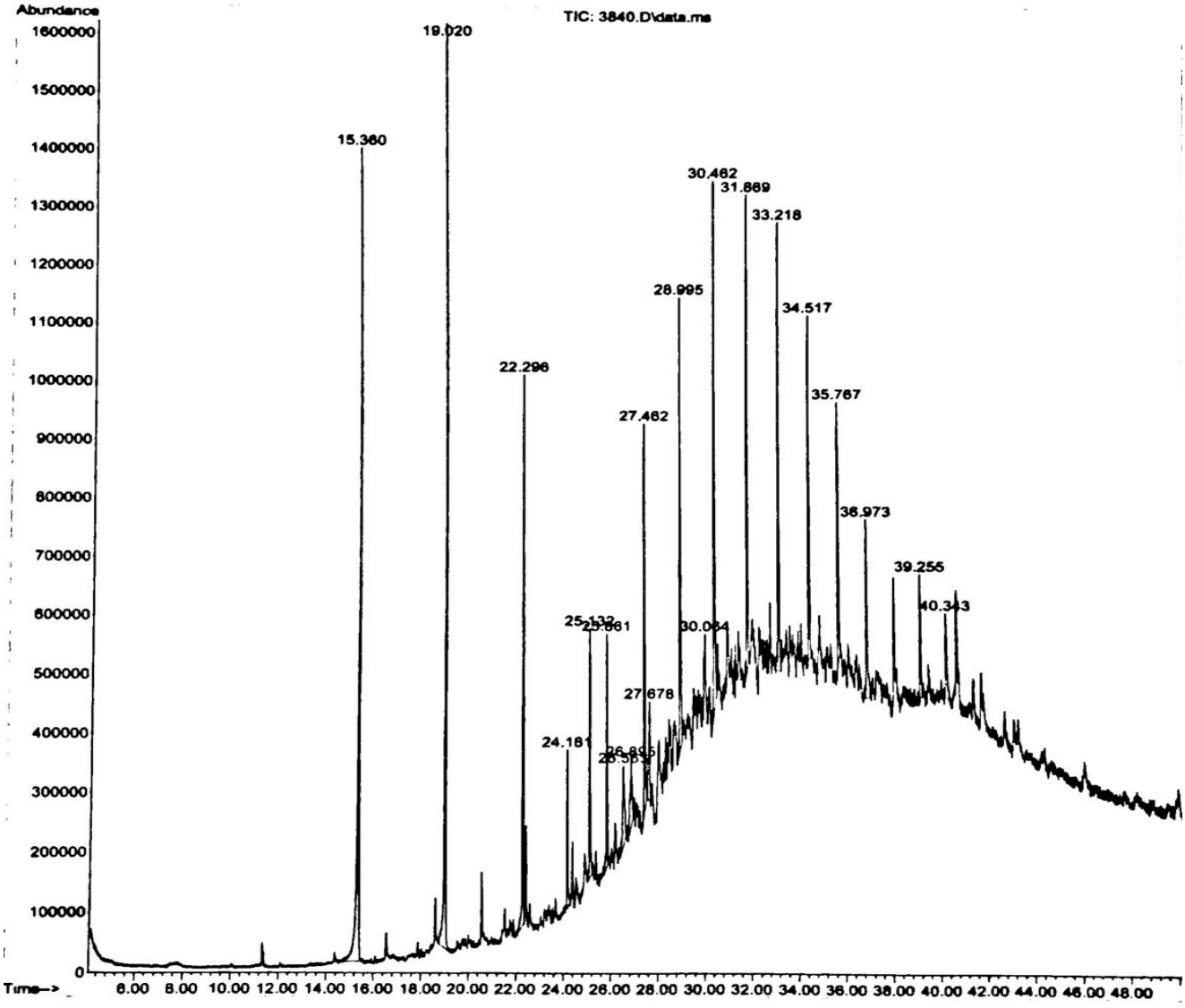

Fig. 4: Chromatogram analysis of black oil (the control group).

Chromatogram analysis of individual bacterial strain cultures (*B. cereus* strain ZG.S6, *B. cereus* strain ZG.S12, and *P. aeruginosa* strain ZG.S11) reveals a reduction in black oil compounds compared to the control. *B. cereus* strain ZG.S6 and *B. cereus* strain ZG.S12 were able to reduce the compounds of black oil to 10 and 16, respectively (Tables 3 and 4), while *P. aeruginosa* strain ZG.S11 could reduce black oil compounds to 14 (Table 5). Figures 5, 6, and 7 illustrate the disappearance of several peaks compared to a control sample as the number of chemical components decreased. It is noteworthy that any GC reduction between the control and inoculated samples is referred to be a biodegradation process since any loss induced by non-biological factors would affect both the control and inoculated samples. This indicated that *B. cereus* strain ZG.S6, *B. cereus* strain ZG.S12, and *P. aeruginosa* strain ZG.S11 are significant black oil decomposers.

Table 3: The residual combination of black oil degraded by *B. cereus* strain ZG.S6.

NO.	RT (min)	Area %	Name	Quality	CAS Number
1	11.336	0.58	Cyclopentasiloxane, decamethyl-	83	000541-02-6
2	15.367	37.94	Cyclohexasiloxane, dodecamethyl	94	000540-97-6
3	19.025	30.38	Cyclononasiloxane, octadecamethyl-	46	013450-70-9
4	22.299	16.72	Tetracosamethyl-cyclododecasiloxane	58	018919-94-3
5	25.132	10.29	Cyclononasiloxane, octadecamethyl-	74	109007-87-6
6	30.015	1.71	Tetracosamethyl-cyclododecasiloxane	53	018919-94-3
7	30.45	0.57	Heptadecane	93	000629-78-7
8	31.862	0.58	Nonadecane	96	000629-92-5
9	32.147	0.72	Cardigin	93	109007-87-6
10	33.211	0.51	n-Eicosane	96	000112-95-8

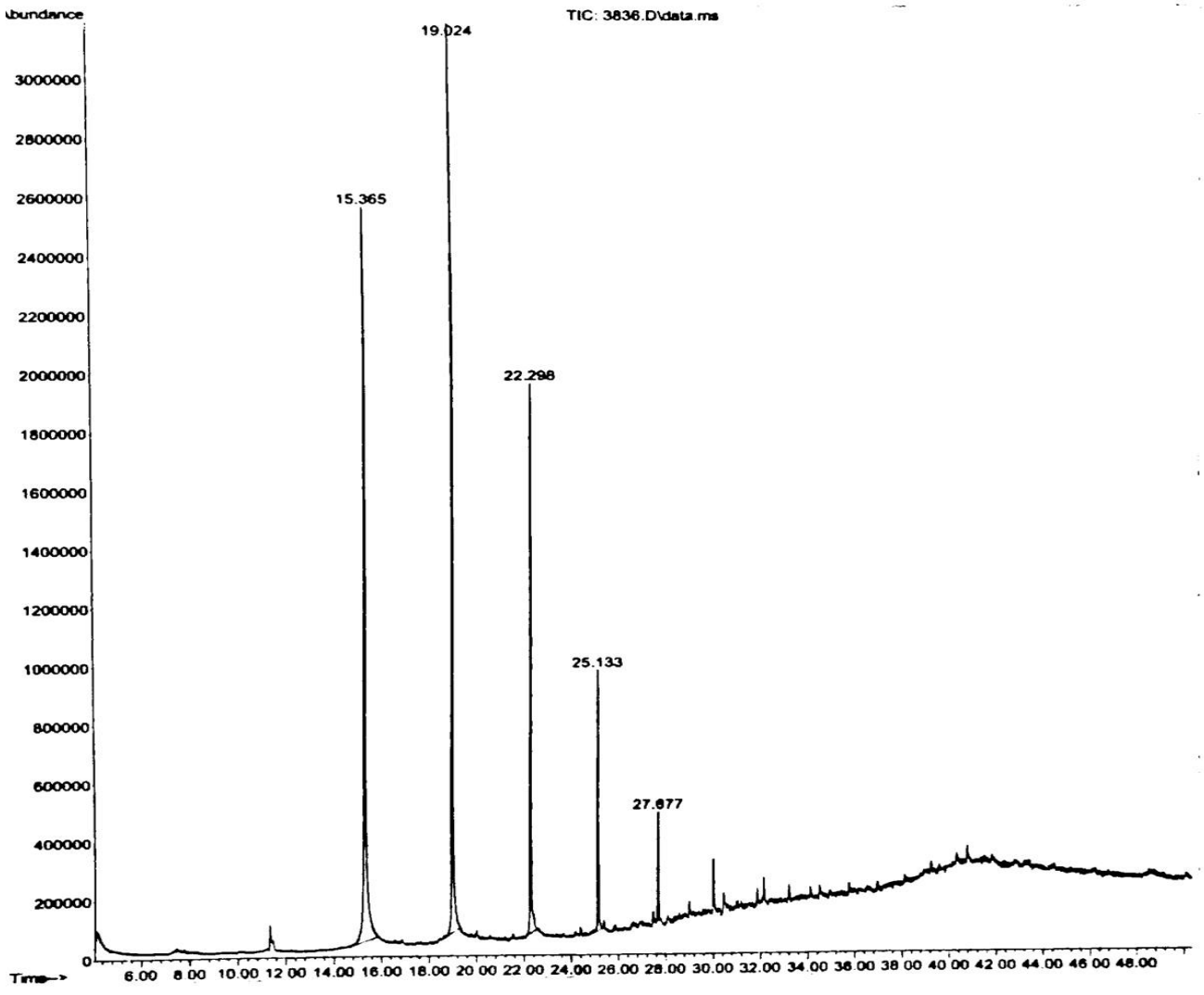

Fig. 5: Chromatogram analysis of residual black oil degraded by *B. cereus* strain ZG.S6.

Table 4: The residual combination of black oil degraded by *B. cereus* strain ZG.S12.

Number	RT (min)	Area%	Name	Quality	CAS Number
1	11.335	0.56	Cyclopentasiloxane, decamethyl-	87	000541-02-6
2	15.367	33.99	Dodecamethylcyclohexasiloxane	93	000540-97-6
3	19.025	31.17	Cyclodecasiloxane, eicosamethyl-	50	000995-82-4
4	22.299	14.18	Cyclooctasiloxane, hexadecamethyl-	64	000000-00-0
5	24.39	0.82	Isotetradecane	94	000629-59-4
6	25.132	6.83	Cyclononasiloxane, octadecamethyl-	87	000556-71-8
7	27.456	0.95	pentadecane	98	000629-62-9
8	27.674	2.78	Cyclononasiloxane, octadecamethyl	53	000000-00-0
9	28.992	1.06	Heptadecane	97	000629-78-7
10	30.02	2.24	Tetracosamethyl-cyclododecasiloxane	80	018919-94-3
11	30.455	2.27	Docosane	99	000629-97-0
12	33.211	0.72	n-Heneicosane	98	000629-94-7
13	34.513	0.60	Docosane	96	000629-97-0
14	35.763	0.65	n-Nonadecane	95	000629-92-5
15	39.245	0.56	1,3-Bis(trimethylsilyl)benzene	55	002060-89-1
16	40.786	0.63	Lupan-3-ol	46	000541-05-9

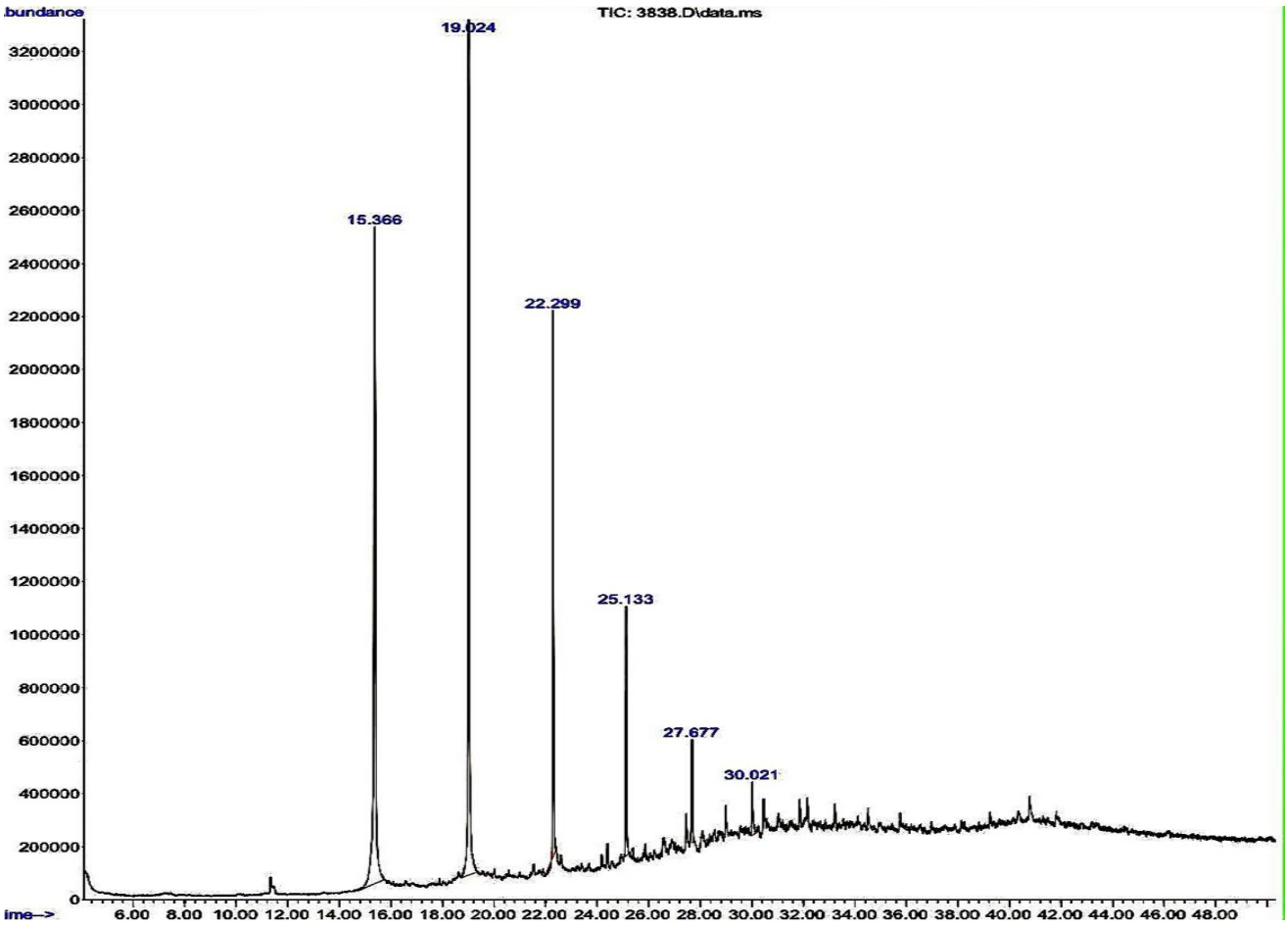

Fig. 6: Chromatogram analysis of residual black oil degraded by *B. cereus* strain ZG.S12.

Table 5: The residual combination of black oil degraded by *P. aeruginosa* strain ZG.S11.

NO.	RT (min)	Area %	Name	Quality	CAS Number
1	11.34	0.85	Cyclopentasiloxane, decamethyl-	91	000541-02-6
2	15.367	37.80	Dodecamethylcyclohexasiloxane	94	000540-97-6
3	19.025	31.24	1,11-Dihydrogendodecamethylhexasiloxane	50	000995-82-4
4	22.299	14.17	6-Aza-5,7,12,14-tetrathiapentacene	64	000000-00-0
5	25.132	6.49	Cyclononasiloxane, octadecamethyl-	49	000556-71-8
6	27.456	0.75	Pentadecane	91	000629-62-9
7	27.674	2.80	Heptasiloxane, hexadecamethyl-	53	019095-24-0
8	28.987	0.77	Eicosane	92	000112-95-8
9	30.02	1.88	6-Aza-5,7,12,14-tetrathiapentacene	47	000000-00-0
10	30.455	0.96	Normal-heptadecane	98	000629-78-7
11	31.862	0.69	Octadecan	98	000593-45-3
12	32.147	0.53	Morphine, bis(trimethylsilyl) ether	74	109007-87-6
13	33.216	0.62	Heneicosane	96	000629-94-7
14	35.758	0.44	Docosane	95	000629-97-0

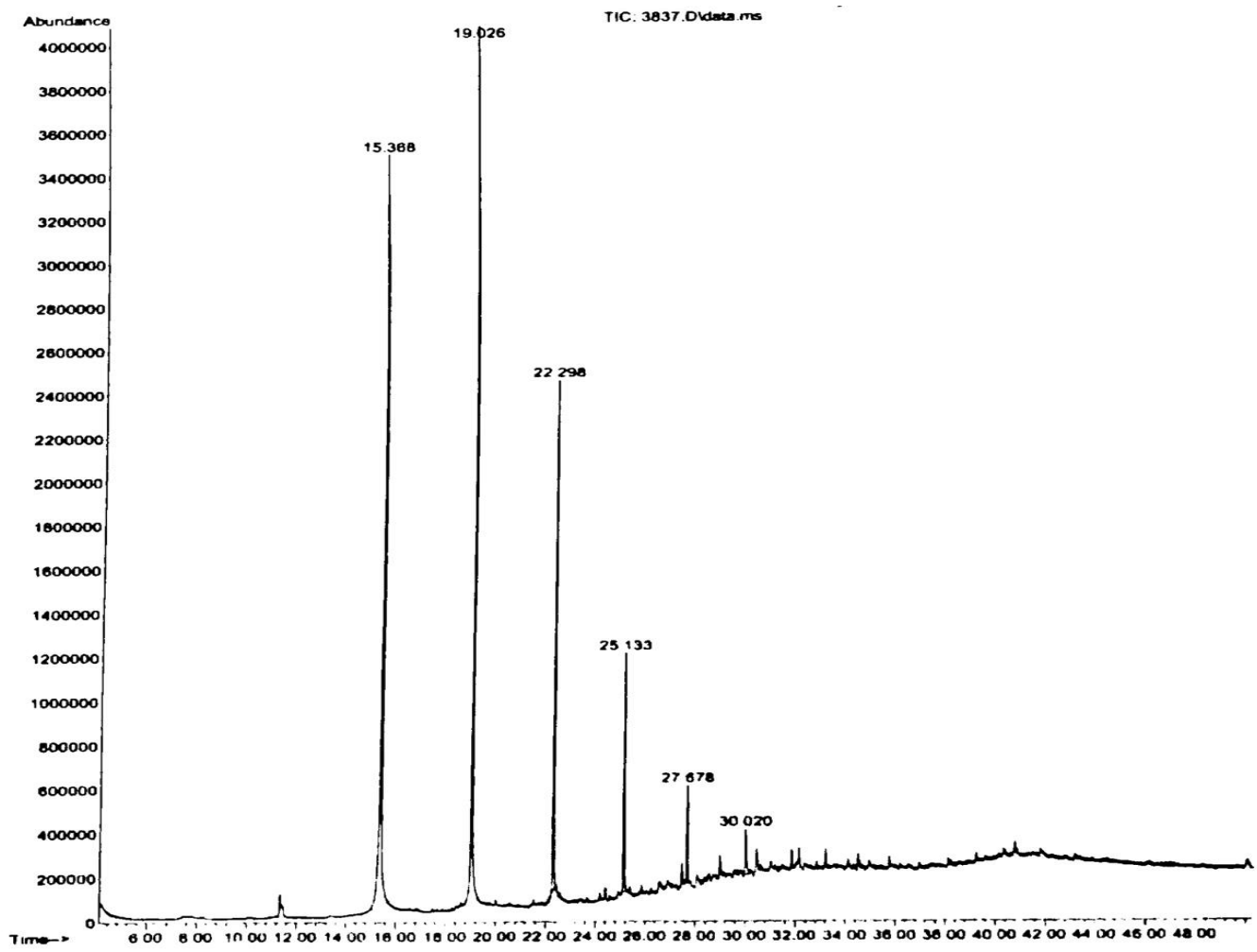

Fig. 7: Chromatogram analysis of residual black oil degraded by *P. aeruginosa* strain ZG.S11.

Comparing the chromatogram analysis of the control group, individual bacterial strains, and the bacterial consortium, the consortium showed a decrease in black oil compounds, recording seven chemicals (Table 6). Figure 8 shows the disappearance of several peaks, revealing the efficiency of the bacterial consortium in decomposing black oil, which led to a decrease in the chemical components of black oil. This indicates the compatibility of bacterial strains with each other, as they work by fragmenting aliphatic and aromatic components.

Table 6: The residual combination of black oil degraded by bacterial consortium.

NO.	RT (min)	Area %	Name	Quality	CAS Number
1	11.335	0.54	Cyclopentasiloxane, decamethyl-	83	000541-02-6
2	15.362	35.23	Dodecamethylcyclohexasiloxane	94	000540-97-6
3	19.025	33.37	Cycloheptasiloxane, tetradecamethyl-	50	000995-82-4
4	22.299	17.54	Cyclooctasiloxane, hexadecamethyl-	64	000000-00-0
5	25.132	10.67	Cyclononasiloxane, octadecamethyl-	43	000556-71-8
6	30.014	1.49	Cyclooctasiloxane, hexadecamethyl-	49	000000-00-0
7	32.147	1.12	Cyclononasiloxane, octadecamethyl-	53	109007-87-6

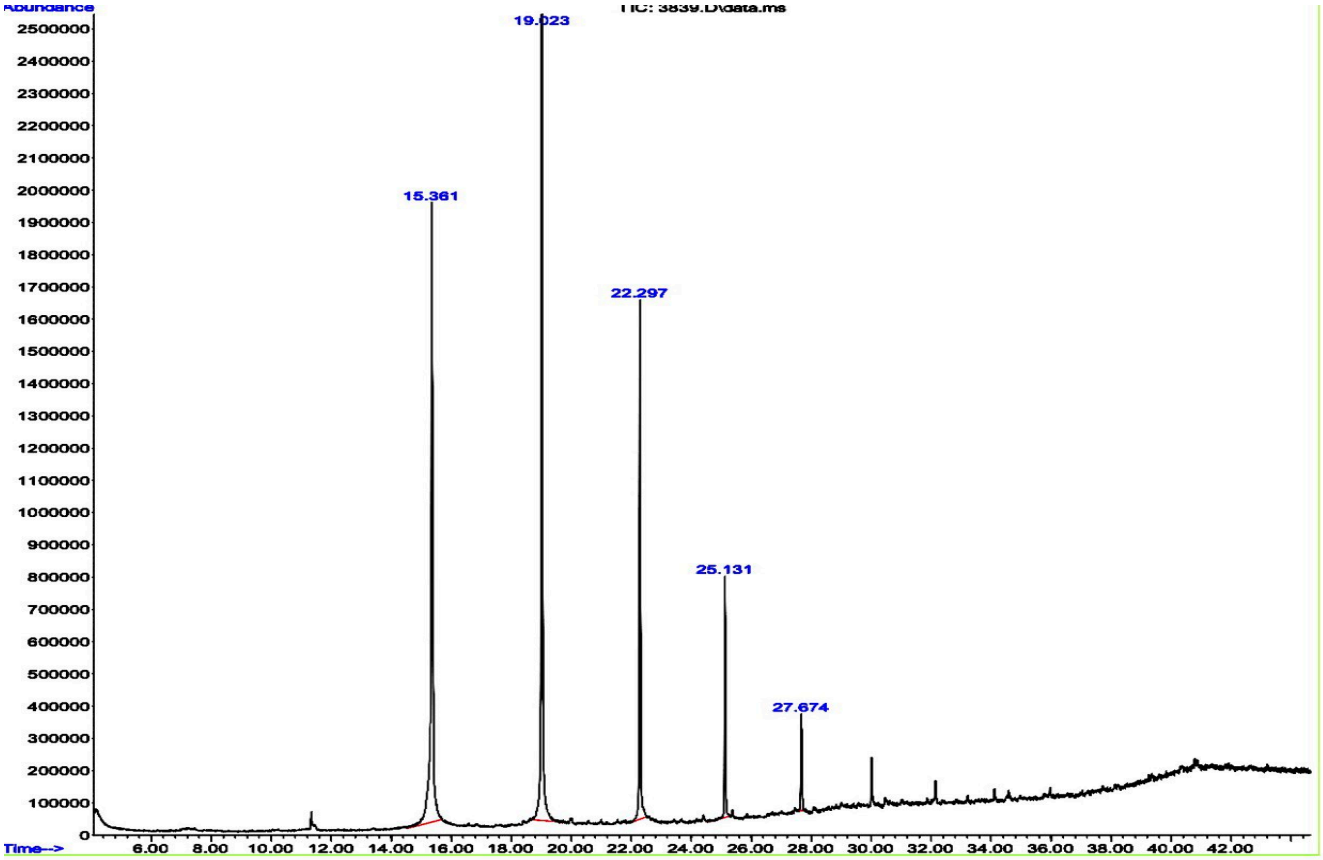

Fig. 8: Chromatogram analysis of residual black oil degraded by bacterial consortium.

Results were in accordance with Bilen Ozyurek and Seyis Bilkay (2020), who revealed the synergistic impact of a consortium containing gram-positive and gram-negative bacterial strains is more effective than a consortium containing only gram-positive or gram-negative bacteria. Tuhuloula *et al.* (2019) found that a consortium of *B. cereus* and *P. putida* at a concentration of 15% (v/v) and a bacteria ratio of 1:1 can successfully degrade oil sludge with a biodegradation percentage of 97.42%. Patowary *et al.* (2016) also found that a consortium of two *Bacillus* strains, *B. pumilus* KS2 and *B. cereus* R2, showed the best crude oil degradation, up to 84.15%. Allamin *et al.* (2020) revealed a favorable correlation between the microbial population and hydrocarbon degradation.

Bioremediation processes rely on the diverse catabolic activity of microorganisms to break down contaminants. While various microorganisms can detoxify organic compounds, bacteria are known to be the

most effictive in biodegradation. Bacteria offer several advantages, including genetic diversity, rapid reproduction, and metabolic versatility. Over millions of years, bacteria have evolved catalytic enzymes to degrade hydrocarbons, enabling them to survive in polluted environments (Bilen Ozyurek and Seyis Bilkay, 2020; Kebede *et al.*, 2021; Bekele *et al.*, 2023). Furthermore, many species of bacteria are capable of producing biosurfactants that can emulsify hydrocarbons (Viesser *et al.*, 2020; Bekele *et al.*, 2023; Faisal *et al.*, 2023). *Bacillus cereus* is known to produce surfactin (Wang *et al.*, 2024), whereas *P. aeruginosa* produces rhamnolipids (Faisal *et al.*, 2023). Rhamnolipid and surfactin increase the bioavailability of contaminants by enhancing their emulsification. The emulsification process is essential for microorganisms to take up and assimilate hydrocarbons (Viesser *et al.*, 2020).

There are few studies on the biodegradation of black oil compared with other petroleum compounds. Petroleum containing aliphatic, aromatic, and saturated compounds at different ratios is biodegraded at different rates by the same microorganism (Bilen Ozyurek and Seyis Bilkay, 2020). Various bacterial species can degrade several hydrocarbons. Each bacterial species specializes in a few hydrocarbons as distinct carbon sources, and no single bacterium can produce all enzymes essential to the degradation process (Ja'afaru *et al.*, 2017). Because an individual bacterium can metabolize a limited range of hydrocarbons, the bioremediation of complicated hydrocarbons typically requires the collaboration of many species (Patowary *et al.*, 2016). Therefore, natural microbial communities with broad enzymatic capacities can improve biodegradation processes through synergistic interactions between microbial strains, where any bacterial strain may remove the toxic metabolites produced by the metabolic activity of other microbes (Bilen Ozyurek and Seyis Bilkay, 2020). Consequently, the biodegradation of black oil necessitates a combination of different microbial strains with wide enzymatic activity.

4. CONCLUSIONS

The present study demonstrates that *B. cereus* and *P. aeruginosa*, isolated from a soil sample contaminated with crude oil, perform well in black oil biodegradation. The efficacy of the black oil degradation process varies with strains, depending on catabolic activity. The bacterial consortium was typically more effective than individual strains, degrading a considerable amount of black oil. Consequently, this bacterial consortium can be employed in contaminated soil to biodegrade black oil.

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