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Biodegradation of Polycyclic Aromatic Hydrocarbons in Crude Oil by *Bacillus cereus* Isolate: GC-FID Analysis and PAH Characterization

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Abstract

This paper assessed the bio degradation ability of a local isolate of Bacillus cereus on crude oil polycyclic aromatic hydrocarbons (PAHs). The GC-FID analysis of the biodegraded sample (HD 5) on a ZB-5 capillary column showed that eight chromatographic peaks were observed, with the most considerable peak at 1.808 min (99.00% of total area), suggesting that there was extensive degradation of medium and heavy molecular weight hydrocarbons. HPLC analysis of 16 EPA priority PAHs in the US provided an overall residual concentration of 546.59 ppm. In comparison to medium molecular weight PAHs (4 rings, 42.32 ppm), low molecular weight PAHs (2-3 rings) had a lower mean residual concentration (28.50 ppm) and the same result confirms that lighter compounds were preferentially degraded. The results highlight the potential of using *B.cereus* as a bioremediation in the treatment of petroleum contaminated environments.

Keywords: Biodegradation, Polycyclic aromatic hydrocarbons, Crude oil, *Bacillus cereus*, GC-FID, Bioremediation, PAHs, Petroleum hydrocarbons

1. Introduction

Oil spillage is one of the most acute environmental issues facing oil-producing countries, especially Iraq, which has the fifth largest proven crude oil deposits in the world [1]. Inevitably, the exploration, extraction, transportation, and refining of crude oil result in the emission of petroleum hydrocarbons into the terrestrial and aquatic environments, which causes severe environmental degradation and can pose serious threats to human health [2]. Polycyclic aromatic hydrocarbons (PAHs) are the most troubling of all the myriad of organic compounds found in crude oil because they are persistent in the environment, not readily degraded, and have a well-known carcinogenic, mutagenic, and

teratogenic effect [3].Polycyclic aromatic hydrocarbons refer to a group of ubiquitous organic contaminants that have two or more fused aromatic rings that are arranged in linear, angular, or clustered structures. The list of priority pollutants identified by the United States Environmental Protection Agency (US EPA) includes 16 PAH compounds according to their toxicity and the rate of occurrence in polluted environments [4]. These 16 priority PAHs span between the two-ring Naphthalene (molecular weight 128.2 g/mol) up to six-ring compounds including Benzo(g,h,i)Perylene and Indeno(1,2,3-cd)Pyrene (molecular weight 276.3 g/mol). Their environmental recalcitrance and persistence is linked to the physicochemical characteristics of PAHs that include low aqueous solubility, high hydrophobicity, and high sorbability to organic matter [5].

Traditional methods of remediation of petroleum-contaminated sites, such as physical (excavation, soil washing, and thermal desorption), chemical (chemical oxidation, photocatalysis, and solvent extraction), and physicochemical (adsorption and membrane filtration) are often linked to high costs of operation, power demand, and secondary pollutants may be produced [6] Bio remediation in this regard has become an environmentally friendly, economically feasible, and technologically potential method of treatment of hydrocarbon contaminated environments. Bioremediation takes advantage of the natural metabolic properties of the microorganisms to convert or mineralize organic pollutants into less harmful or non-toxic final products such as carbon dioxide and water [7].

One of the most well-studied genera of microbes endowed with the ability to degrade hydrocarbons is the genus Bacillus, which is metabolically diverse, widespread in polluted soils, and capable of secreting biosurfactants that can increase the bioavailability of hydrocarbons [8]. A Gram-positive, rod-shaped, endosporing bacterium, Bacillus cereus, has been specifically known to be a very powerful hydrocarbon degrader that can use a wide range of petroleum hydrocarbons as sole carbon and energy sources [9]. The ability of B. cereus to form endospores contributes to a high level of resistance to unfavorable environmental factors, such as high temperatures, extreme pH levels, and high concentrations of pollutants, which increases its usability in bioremediation programs [10].

Gas Chromatography with Flame Ionization Detection (GC-FID) is one of the most powerful and commonly used analysis methods of qualitative and quantitative characterization of the petroleum hydrocarbons in the environmental samples.

The method presents high sensitivity, good reproducibility and wide dynamic range to examine volatile and semi volatile organic compounds [11]. Complementary to this, with UV/fluorescence detector, High-Performance Liquid Chromatography (HPLC) allows specific and sensitive quantification of each PAH compound, which allows detailed profiling of PAH patterns of distribution in complex petroleum matrices [12].

The current research was done with the main aim of assessing the biodegradation capability of a locally isolated Bacillus cereus strain on crude oil hydrocarbons with special focus on the determination of residual polycyclic aromatic hydrocarbon concentrations after bacterial treatment. This was done by combining GC-FID analysis to profile all the hydrocarbons and the quantification of the individual PAH compounds using HPLC to offer a complete evaluation of the biodegradation trends and bioremediation abilities of this bacterium.

2. Materials and Methods

2.1. Collection and Isolation of Bacterial Strain

In South of Iraq, the samples of soil were taken in petroleum polluted areas of Wasit Province. Bacteria isolation was done by the enrichment culture method in the mineral salt medium (MSM) with 1% (v/v) crude oil as the only source of carbon. Isolated strain was characterized using traditional biochemical characterization and then molecular characterization using 16S rRNA gene sequencing as isolated strain was identified as Bacillus cereus. To preserve the bacteria culture in the nutrient agar slants, it was kept at 4ºC and sub-cultured after regular intervals to maintain viability during the experimental duration.

2.2. Sample of Crude Oil and Biodegradation Experimental Design.

The Waist oil field was used to extract the Iraqi crude oil and use it as the only source of carbon and energy in the biodegradation experiment. It was done in Erlenmeyer flasks (250 mL) with 100 mL of sterile MSM supplemented with 1% (v/v) crude oil. The medium composition (g/L) was as follows: KH2PO4 (1.0), K2HPO4 (1.0), NH4NO3 (1.0), MgSO4·7H2O (0.2), CaCl2 (0.02), and FeCl3 (0.05). The pH was adjusted to 7.0 ± 0.2. The flasks were inoculated with 1 x 108 CFU/mL of B. cereus and incubated at 37 o C under aerobic conditions with constant orbital shaking at 150 rpm over a span of 21 days. Uninoculated control flasks were kept in the same conditions to consider abiotic losses.

2.3. GC-FID Analytical Conditions

Gas Chromatography with Flame Ionization Detection (GC-FID) was used to analyze the hydrocarbon mixture of the biodegraded sample of crude oil (HD 5). The instrumental parameters are summarized in Table 1.

Table1. GC-FID instrumental parameters for hydrocarbon analysis of sample HD 5

Parameter	Condition
Sample Name	HD 5
Injection Volume	1 µL
Injector Temperature	295°C
Detector (FID) Temperature	330°C
Column Type	ZB-5 Capillary Column
Oven Temperature Program	100°C (hold 2 min) → 250°C at 8°C/min
Carrier Gas Pressure	100 kPa

2.4. PAH Quantification by HPLC

The standard method EPA 8310 was followed in quantifying sixteen US EPA priority PAH compounds using High-Performance Liquid Chromatography (HPLC) with UV/fluorescence detection. Determination of the PAH levels in the biodegraded crude oil was done against calibration curves drawn with certified PAH reference standards and converted to parts per million (ppm). The 16 target analytes covered the entire PAH molecular weight, including the two ring Naphthalene, the six-ring Indeno(1,2,3-cd)Pyrene and Benzo(g,h,i)Perylene.

2.5. Statistical Analysis

Mean, standard deviation, and percentage distribution were used as descriptive statistics of the PAH concentrations. The PAH compounds were categorized into three groups according to the molecular weight: low molecular weight (LMW, 2 3 rings), medium molecular weight (MMW, 4 rings), and high molecular weight (HMW, 5 6 rings). The correlation analysis was done through Pearson correlation analysis to determine the relationship between the molecular weight and the remaining concentration of PAH.

3. Results and Discussion

3.1. GC-FID Chromatographic Profile

Figure 1 shows that the GC-FID chromatographic analysis of the biodegraded crude oil sample (HD 5) yielded eight different chromatographic peaks with a retention time range of between 1.808 and 20.347 minutes. The entire peak parameters such as retention time, peak area, area percentage, and peak height are given in table 2. The sum of the chromatographic areas was 112,743,228 units. The chromatographic profile was largely dominated by a single peak with a retention time of 1.808 minutes that made 99.00 percent of the total peak area (area = 111,626,522) and had the highest recorded intensity. This premature elution is normally explained by the solvent front or the light volatile hydrocarbons (C5-C10) that had

not been degraded or were formed as an intermediate metabolite along the biodegradation process [11]. The other seven peaks (Peaks 2-8) combined to represent a total of about 1.00 percent of the total chromatographic area; this means that B. cereus effectively metabolized all but the very small proportion of medium to heavy molecular weight hydrocarbons in the original crude oil sample.

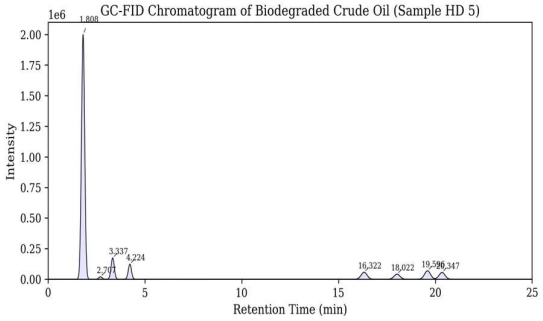


Figure 1. GC-FID chromatogram of biodegraded crude oil sample HD 5 treated with *B. cereus*. Numbers indicate retention times (min) of resolved peaks.

Table 2. GC-FID peak table for biodegraded crude oil sample HD 5

Peak	Ret. Time (min)	Area	Area %	Height
1	1.808	111,626,522	99.0095	2,000,000
2	2.707	32,096	0.0285	19,764
3	3.337	326,368	0.2895	174,280
4	4.224	202,993	0.1800	123,578
5	16.322	173,430	0.1538	57,053
6	18.022	79,808	0.0708	41,301
7	19.596	143,135	0.1270	69,086
8	20.347	158,876	0.1409	55,129
Total	—	112,743,228	100.0000	—

An examination of the minor chromatographic peaks (Peaks 2–8) provides valuable insight into the residual hydrocarbon fractions that resisted complete biodegradation (Figure 2). Peaks 2–4, eluting between 2.707 and 4.224 minutes, likely correspond to light aromatic hydrocarbons or short-chain aliphatic compounds. In contrast, Peaks 5–8, appearing at higher retention times (16.322–20.347 minutes), represent heavier hydrocarbon fractions, potentially including high molecular weight alkanes, cycloalkanes, or polycyclic aromatic hydrocarbons [12]. The persistence of these higher retention time peaks, albeit at very low abundances, suggests that certain recalcitrant hydrocarbons partially withstood the biodegradation process.

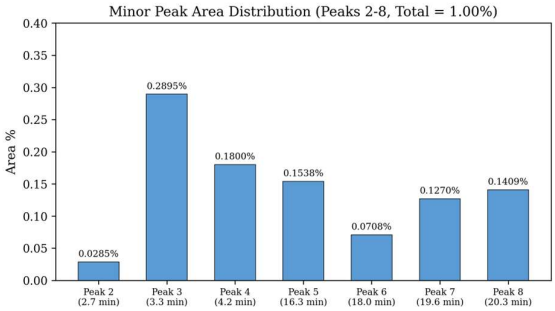


Figure 2. Distribution of minor chromatographic peaks (Peaks 2–8) representing approximately 1.00% of total GC-FID area.

3.2. Residual PAH Concentration Profile

The concentrations of the 16 US EPA priority PAH compounds detected in the biodegraded crude oil sample following treatment with *B. cereus* are presented in Table 3, together with their molecular properties and IARC carcinogenicity classifications. The total residual PAH concentration was determined to be 546.59 ppm. The residual PAH distribution profile exhibited considerable heterogeneity, with

individual compound concentrations ranging from a minimum of 8.7 ppm (Phenanthrene) to a maximum of 60.5 ppm (Indeno (1, 2, 3-cd) Pyrene).

Table3. Physicochemical properties and residual concentrations of 16 EPA priority PAHs in biodegraded crude oil

#	PAH Compound	Rings	MW	Category	Conc. (ppm)	IARC Class.
1	Naphthalene	2	128.2	LMW	11.4	Possible (2B)
2	Acenaphthylene	3	152.2	LMW	40.6	Not classified
3	Acenaphthene	3	154.2	LMW	35.5	Not classified
4	Fluorene	3	166.2	LMW	40.9	Not classified (3)
5	Phenanthrene	3	178.2	LMW	8.7	Not classified (3)
6	Anthracene	3	178.2	LMW	33.9	Not classified (3)
7	Fluoranthene	4	202.3	MMW	60.4	Not classified (3)
8	Pyrene	4	202.3	MMW	22.4	Not classified (3)
9	Benzo(a)Anthracene	4	228.3	MMW	54.8	Probable (2B)
10	Chrysene	4	228.3	MMW	31.7	Probable (2B)
11	Benzo(b)Fluoranthene	5	252.3	HMW	41.5	Probable (2B)
12	Benzo(k)Fluoranthene	5	252.3	HMW	30.0	Probable (2B)
13	Benzo(a)Pyrene	5	252.3	HMW	22.7	Carcinogenic (1)
14	Indeno(1,2,3-cd)Pyrene	6	276.3	HMW	60.5	Probable (2B)
15	Dibenzo(a,h)Anthracene	5	278.4	HMW	30.6	Probable (2A)
16	Benzo(g,h,i)Perylene	6	276.3	HMW	21.0	Not classified (3)
	Total	—	—	—	546.59	—

MW = Molecular Weight (g/mol); LMW = Low Molecular Weight; MMW = Medium Molecular Weight; HMW = High Molecular Weight; IARC = International Agency for Research on Cancer.

Figure 3 shows the graphical representation of the residual PAH concentrations, which are color-coded based on the molecular weight category. Five of the 16 sampled PAHs had the highest residual concentrations, including: Indeno(1,2,3-cd)Pyrene (60.5 ppm),

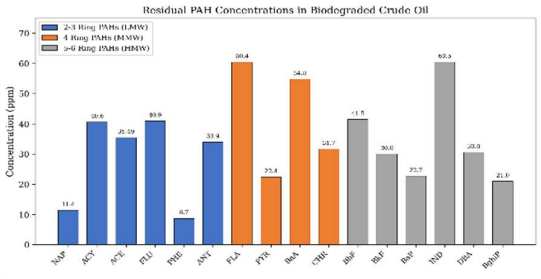


Figure 3. Leftover levels of 16 EPA priority PAH compounds in biodegraded crude oil. Blue = LMW (2–3 rings); Orange = MMW (4 rings); Gray = HMW (5–6 rings).

Fluoranthene (60.4 ppm), Benzo(a)Anthracene (54.8 ppm), Benzo(b)Fluoranthene (41.5 ppm), and Fl On the other hand the five lowest residual concentrations were: Phenanthrene (8.7 ppm), Naphthalene (11.4 ppm), Benzo(g,h,i)Perylene (21.0 ppm), Pyrene (22.4 ppm) and Benzo(a)Pyrene (22.7 ppm).

3.3. PAH Classification based on Molecular weight.

In order to explain the biodegradation trends in a more detailed manner, the 16 PAH compounds were grouped into three clusters depending on the number of aromatic rings and the molecular weight as shown in Table 4. This classification shows the specific patterns of degradation over the entire molecular weight range.

Table4. Summary of PAH concentrations classified by molecular weight category

Category	Rings	No. of PAHs	Sum (ppm)	Mean (ppm)	% of Total
LMW	2–3	6	170.99	28.50	31.3%
MMW	4	4	169.30	42.32	31.0%
HMW	5–6	6	206.30	34.38	37.7%
Total	—	**16**	**546.59**	**34.16**	**100.0%**

LMW = Low Molecular Weight; MMW = Medium Molecular Weight; HMW = High Molecular Weight.

The distribution of PAHs by ring number category and the corresponding mean concentrations are depicted in Figure 4. The LMW PAHs (2–3 rings) accounted for 31.3% of the total residual PAH concentration with a mean of 28.50 ppm, while MMW PAHs (4 rings) contributed 31.0% with a mean of 42.32 ppm. The HMW PAHs (5–6 rings) represented 37.7% with a mean of 34.38 ppm.

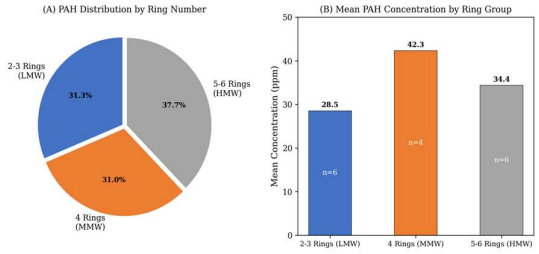


Figure4. (A) Percentage distribution of total residual PAH concentration by ring number category. (B) Mean residual concentration for each PAH molecular weight group.

The smaller average residual concentration of LMW PAHs compared to the MMW PAHs is in line with the long-standing principle that the rate of microbial degradation of PAHs is usually low in the order of decreasing molecular complexity and hydrophobicity [5]. Their greater aqueous solubility, the higher bioavailability and the existence of well-characterized enzymatic pathways to degrade them in Bacillus species can explain the preferential degradation of lighter PAH compounds, including Naphthalene (2 rings, 11.4 ppm residual) and Phenanthrene (3 rings, 8.7 ppm residual) [13] Particularly, the initial oxidation of LMW PAHs is usually catalyzed by ring-hydroxylating dioxinases which introduce molecular oxygen over the aromatic ring to produce cis-dihydrodiols, which in turn leads to the ring cleavage, and full mineralization [14].

3.4. Molecular Weight–Concentration Relationship

Correlation analysis was used to determine the relationship between the residual concentrations of PAH compounds after biodegradation and the molecular weight of the compounds (Figure 5). Though there was an overall tendency of reduced degradation efficiency (i.e. increased residual concentrations) with increased molecular weight of PAHs, this correlation was not highly linear, suggesting that molecular weight on its own does not completely dictate the susceptibility of biodegradation. The observed variability in degradation efficiency is probably caused by other factors such as ring structure (linear and angular) and accessibility of bay and K-regions to enzymatic attack, and the enzymatic repertoire of B. cereus in question [15].

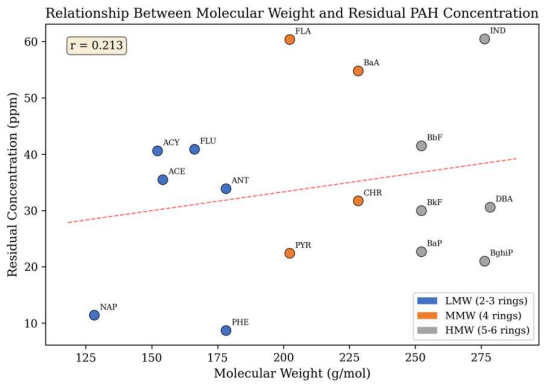


Figure5. Scatter plot illustrating the relationship between molecular weight and residual PAH concentration following biodegradation by *B. cereus*. The dashed line represents the linear trend.

3.5. Carcinogenic PAH Considerations

Interestingly, a single bacterial strain is able to attenuate partial carcinogenic PAHs, including the carcinogenic Group 1 PAH Benzo(a)Pyrene. The concerted action of two or more microbial species or the presence of growth substrates to degrade high molecular weight carcinogenic PAHs is typically caused by co-metabolic degradation [14]. The ability of B. cereus to be in a position to be able to degrade such compounds in quantifiable amounts as a single strain highlights its metabolic flexibility, and justifies its nomination to bioremediation formulations to assault petroleum-contaminated locations.

4. Conclusions

The current study thoroughly showed that a Bacillus cereus strain that was isolated in the locality had a high biodegradation capacity on crude oil hydrocarbons, especially polycyclic aromatic hydrocarbons. The key results of the present research can be summarized as follows:

The GC-FID chromatographic analysis of the biodegraded crude oil sample (HD 5) indicated that B. cereus was successful in reducing the hydrocarbon content of the crude oil with 99.00 percent of the remaining chromatographic signal in the light

hydrocarbon fraction (retention time 1.808 min), and only eight resolvable signals were present after the 21 day treatment period.

The overall residual concentration of the 16 EPA priority PAHs was 546.59 ppm and a specific trend of selective degradation of lower weight PAHs. The LMW PAHs (23 rings) had a mean residual concentration of 28.50 ppm, much lower than MMW PAHs (4 rings), at 42.32 ppm, which supported the fact that lighter PAH compounds were more susceptible to degradation by the microbes.

The bacterium was shown to be able to partially degrade even recalcitrant carcinogenic PAHs such as the Group 1 carcinogen Benzo(a)Pyrene (residual: 22.7 ppm) and therefore could have a potential use in bioremediation processes. The further research should be aimed at optimization of the biodegradation conditions, investigation of the use of the microbial consortia with B. cereus and complementary hydrocarbon-degrading species, and field-scale trials to prove the laboratory results under the environmental conditions.

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Conflict of Interest

The authors declare that there are no conflicts of interest associated with this research.

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