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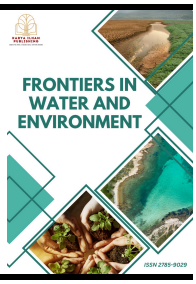

A Biological Approach to Marine Oil Spill Remediation using *Bacillus cereus*

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ABSTRACT

Oil pollution in marine environments is a major global environmental concern, largely driven by increasing oil demand and intensive maritime activities. This study aimed to evaluate the potential of *Bacillus cereus* for the biodegradation of crude oil and engine oil in seawater as an environmentally friendly approach to oil spill remediation. A laboratory-based biodegradation experiment was conducted using *Bacillus cereus* isolated from the Ranau Hot Spring, Sabah. Biodegradation assays were performed in eight experimental sets containing 10% and 15% (v/v) concentrations of crude oil and engine oil, with an incubation period of 30 days. Biodegradation efficiency and rate were determined by comparing the initial and final concentrations of crude oil and engine oil, while the degradation of aliphatic hydrocarbons was assessed by comparing chromatographic peak areas before and after treatment using Gas Chromatography–Mass Spectrometry (GC–MS). The results demonstrated that *Bacillus cereus* achieved the highest biodegradation efficiency in 10% (v/v) engine oil, which was 1.76-fold higher than crude oil and 2.98-fold higher than for 15% (v/v) crude oil. Similarly, the highest biodegradation rate was recorded for 10% (v/v) engine oil, being 2.17-fold higher than that for 10% (v/v) crude oil and 3.77-fold higher than for 15% (v/v) crude oil. GC–MS analysis revealed that aliphatic hydrocarbon compounds in engine oil were more extensively degraded than those in crude oil. Overall, this study highlights the strong potential of *Bacillus cereus* as an effective bioremediation agent, particularly for engine oil contamination in marine environments.

1. Introduction

Global oil output and its distribution have increased during the 21-year period between 2000 and 2021, averaging 0.98% yearly grow. According to BP Statistical Review of World Energy 2022, the oil consumption increased by 5.3 million barrels per day in 2021. This suggests that oil extraction, refining, and seawater transportation have increased. Expanding oil processing could make oil leak

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accidents more likely to happen [1]. Oil spills in seawater can result in leaving behind oil's substances that can exist for years, impacting human health, coastal environments, and marine life [2]. Crude oil and engine oil are two different but related types of harmful hydrocarbon chemicals that can be extremely dangerous to human health and the environment when being exposed [3]. This is because these compounds are complex combinations that may adversely affect ecosystems and organisms. The toxicity level is determined by the composition of the hydrocarbon mixture, the concentration of these compounds in the environment, the duration of exposure for an organism or individual, and the exposure pathways that facilitate contact with these toxic substances [4]. There are various oil clean-up technologies available in modern world, however, bioremediation provides better practical and cost-effective alternative for conventional clean-up techniques. This is due to characteristic of bioremediation which is eco-friendly. This approach is based on utilizing the ability of biological organisms in metabolism, such as bacteria, fungi, or plants, to degrade the pollutants present in the contaminated surroundings into less harmful ones [5]. The enzymatic breakdown of contaminants by living organisms is known as biodegradation, and it is one of the primary processes behind bioremediation [6].

Bioremediation method has proven the potential of its small environmental footprint and potential for long-term effectiveness in marine environments. Oil pollution tends to be challenging to mitigate due to various environmental factors such as temperature, nutrient, oxygen, pH, salinity, pressure, microbial community, and bioavailability that effecting the pollution treatments [7]. Thus, when employing microorganisms to break down the oil's compounds, this technique can become an effective strategy in cleaning the oil polluted environments [8]. Thermophilic bacteria, which can flourish in high temperatures, are among the several microbial communities that have the ability to clean-up the oil in seawater through biodegradation [9].

In wide range of microbial communities, the ability of thermophilic bacteria, a group of microorganisms that can adapt and grow in high temperatures, able to effectively break down oil's hazardous substances especially in warm marine environments has drawn notice. Thermophilic bacteria are well known for their ability to survive in high temperature conditions, since they may flourish in temperatures as high as 45 °C [9]. Their significant metabolic pathways and special heat-stable enzymes are among their unique characteristics that make them ideal in biodegradation applications. These bacteria able to generate a variety of enzymes which catalyze the conversion of hydrocarbons into more easily metabolized substances [10]. Beside from their enzymatic properties, thermophilic bacteria also can generate surface-active molecules called biosurfactants, to improve the solubility and bioavailability of hydrophobic contaminants. This is due to these substances can be use as both carbon sources and inducers of the synthesis of biosurfactants. Thus, their abilities to break down hydrocarbons is frequently correlated with ability to generate biosurfactants. For instance, it was discovered that *Bacillus licheniformis* B3-15 produced biosurfactants with strong emulsification activities, suggesting that they may be able to improve hydrocarbon breakdown in saltwater [10].

Based on previous studied by Das and Chandran [11], reported that the rate of hydrocarbon degradation is greater at elevated temperature. This is because higher temperatures influence both the physical nature and chemical composition of the oil thus decreasing the viscosity of the oil thereby increasing its bioavailability to microbial degradation [12]. Furthermore, higher temperatures enhance the volatility of toxic, low molecular weight, short-chain alkanes by decreasing their water solubility and increasing the onset of biodegradation [11]. It may therefore be beneficial to utilise thermophilic bacteria, which naturally thrive at higher temperatures [13].

To address this gap, the present study investigates the bioremediation potential of *Bacillus cereus* by comparing its biodegradation efficiency, rate, and profile for engine oil and crude oil at different

concentration levels, and by quantifying biodegradation markers using the ratios of n-C17:Pristane, n-C18: Phytane, and Pristane: Phytane. This approach demonstrates the potential of thermophilic bacteria-based bioremediation as a sustainable and ecologically compatible strategy for marine oil contamination.

2. Methodology

2.1 Sources of Microorganisms

The thermophilic strains namely *Bacillus cereus* was obtained from Environmental Microbiology Laboratory, Faculty of Science & Technology, University Malaysia Sabah in biologically pure from by dilution plate technique. These strains were previously isolated from water samples Hot Spring, Ranau Sabah. These strains have been proven to degrade microplastic based on previous research done by Lee [14] (Table 1).

Table 1
Physical and chemical characteristic of *Bacillus cereus* used in this study

Characteristic	Condition	Image strains
Colony Morphology		
Colour	Cream	
Configuration	Irregular	
Margin	Scalloped	
Texture	Moist	
Opacity	Opaque	
Elevation	Flat	
Surface	Smooth	
Shape	Rod	
Biochemical Test		
Growth temperature	40 °C	
Gram Staining	+ve	
Motility test	+ve	

Bacillus cereus

2.2 Preparation of Culture Medium and Stock Solutions

Two main types of culture media and stock solutions were used in this study for microbial cultivation, preservation, and biodegradation assays, namely nutrient agar (NA), Ramsay broth (RB), magnesium sulphate heptahydrate solution, and glucose solution. Nutrient agar was prepared for maintaining bacterial stock cultures by dissolving 28 g of agar powder in 1 L of distilled water, autoclaving at 121 °C for 15 minutes, and dispensing into sterile petri dishes for solidification. Ramsay broth, used for microbial inoculation and biodegradation experiments, was prepared according to Zahari [15]. The magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$) stock solution was prepared by dissolving 24.647 g of powder in 100 mL sterile distilled water, filtering through a 0.2 µm syringe filter, and refrigerating at 4 °C. Similarly, the glucose ($C_6H_{12}O_6$) stock solution was prepared by dissolving 18.016 g of powder in 100 mL sterile distilled water, filtering through a 0.2 µm syringe filter, and storing at 4 °C until use.

2.3 Preparation of Standard Inoculum and Working Volume

The preparation of the standard inoculum was carried out to ensure that the microorganisms reached an optimal growth phase prior to their transfer into the working volume for the biodegradation experiment. For this purpose, a loopful of *Bacillus cereus* was collected from a fresh overnight subculture and inoculated into a 500 mL inoculum solution. Two concentrations of carbon substrates, specifically 10% and 15% (v/v) crude oil and engine oil, were prepared for the biodegradation assays. Each working volume solution was prepared in a sterile 250 mL conical flask capped with cotton wool and wrapped with aluminum foil. The flasks containing the inoculum were subsequently incubated for 24 hours at 40 °C with agitation at 200 rpm in an incubator shaker [16]

2.4 Biodegradation Study of Crude oil and Engine oil in Seawater

The biodegradation study comprised four treatments with a 30-day incubation period. All treatments were performed in triplicate, and the average (mean) of the three independent experiments was taken as the result. The biodegradation study of crude oil and engine oil in seawater was conducted by determining the growth of microorganisms, the biodegradation rate and efficiency, and the biodegradation ratios.

2.4.1 Determination of microbial growth

Microbial growth was monitored daily throughout the 30-day incubation period. For each treatment, a 3 mL aliquot was withdrawn and transferred into a cuvette for optical density (OD) measurement. Cell density was quantified at 600 nm using a Cecil 1011 spectrophotometer. All measurements were performed in duplicate to ensure data reliability.

2.4.2 Determination of biodegradation rate and efficiency

A 1 mL oil sample was collected from each treatment during the first and final weeks of incubation. The samples were centrifuged at 600 rpm for 3 minutes to remove microbial cells prior to solvent–oil extraction for oil concentration analysis. The cell-free supernatant was subjected to solvent oil extraction following a modified Method 5520 B (APHA, 2001) [17]. The extracted residue was stored in labelled vials at 14 °C until gas chromatographic analysis.

2.4.3 Determination of Biodegradation Ratio

Following solvent oil extraction, the residues were recovered by adding 1 mL of HPLC-grade hexane and filtering through a 2 µm syringe filter. The extracts were immediately analysed by Gas Chromatography Mass Spectrometry (GC-MS). Chromatograms obtained after 4 weeks of incubation were used to assess biodegradation by calculating hydrocarbon ratios (n-C17/pristane, n-C18/phytane, and pristane/phytane) based on peak identification against the Mass Spectrometer Library. Variations in the pristane/phytane ratio served as an indicator of degradation extent, where unaltered ratios correspond to low degradation and altered ratios represent higher levels of biodegradation [18].

2.4.4 Data Analysis

The results from the biodegradation study were analysed using Microsoft Office Excel. Data collected from the biodegradation study were entered into Excel for calculating, analysing and visualization of trends.

3. Results and Discussion

3.1 Biodegradation Efficiency and Biodegradation Rate of Bacillus cereus

The biodegradation efficiency and biodegradation rate of engine oil and crude oil were monitored by comparing the initial and final concentration of extracted oil in shake flask medium for 30 days of incubation period. Figure 1 shows the biodegradation efficiency and biodegradation rate of *Bacillus cereus* in two different concentrations of engine oil and crude oil which were 10% (v/v) and 15% (v/v) respectively.

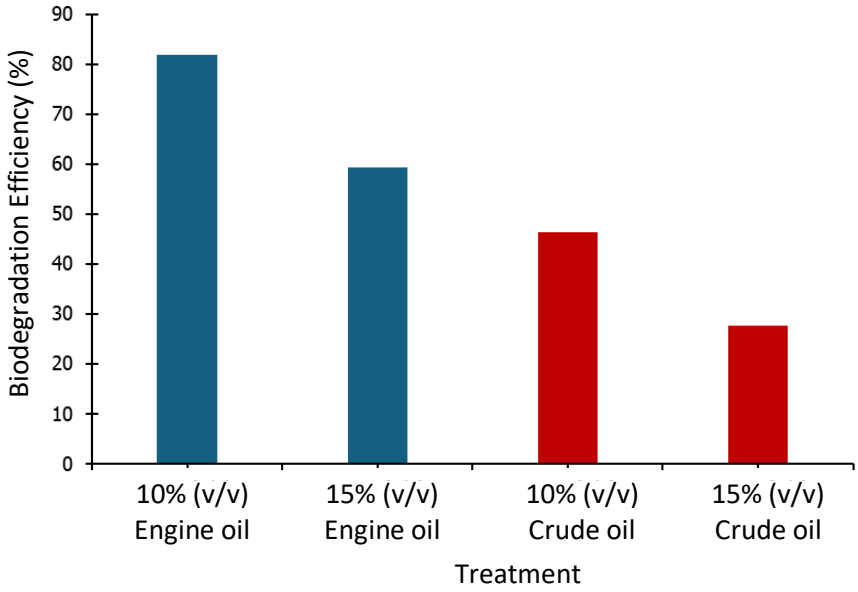

Fig. 1. Biodegradation efficiency by *Bacillus cereus* in 10% and 15% (v/v) engine oil and crude oil throughout 30 days of incubation period

After 30 days of incubation, the biodegradation efficiency of engine oil reached 80.82% and 59.12% for the 10% and 15% (v/v) treatments, respectively. In comparison, the culture of *Bacillus cereus* achieved 46.00% and 27.10% biodegradation of crude oil at 10% and 15% (v/v), respectively. For both crude oil and engine oil, an increase in concentration was associated with a reduction in biodegradation efficiency over time. Notably, complete degradation of either oil type was not observed within the 30-day incubation period. This limitation is attributed to the toxic effects of high oil concentrations on microbial cells, which reduces their growth and hydrocarbon-metabolizing activity [19]. The biodegradation performance observed in this study is consistent with previous reports. Jibo and Karamba [20], demonstrated that *Bacillus* species achieved 65% degradation of used engine oil after 8 days of incubation period at temperature 28 °C with an orbital shaker spinning at 160 rpm. Similarly, Bhurgri [21], showed that *Bacillus cereus* achieved up to 98.6% degradation of waste engine oil at a lower concentration (1%). This indicated that under optimal conditions, *Bacillus cereus* able to effectively degrade significant amounts of engine oil over time. Based on Figure 1,

engine oil at 10% (v/v) demonstrated a 21.70% higher degradation efficiency than at 15% (v/v), whereas crude oil at at 10% (v/v) exhibited an 18.19% higher efficiency than at 15% (v/v). These differences likely reflect compositional complexity, as oils contain heterogeneous mixtures of hydrocarbons with varying susceptibility to microbial attack [22]. Consequently, increasing oil concentration reduces microbial degradation capacity [19].

A similar trend was observed in the biodegradation rate (Figure 2). After 30 days, engine oil at 10% (v/v) showed the highest degradation rate (79.61 mg/L/day), followed by 15% (v/v) (62.17 mg/L/day). Crude oil at 10% (v/v) demonstrated a rate of 36.60 mg/L/day, compared to 21.12 mg/L/day at 15% (v/v). The results showed that a lower oil concentrations impose less toxicity on microbial cells, thereby enhancing their metabolic activity toward hydrocarbon substrates to microorganisms, allowing them to thrive and metabolize the available hydrocarbons more rapidly [19].

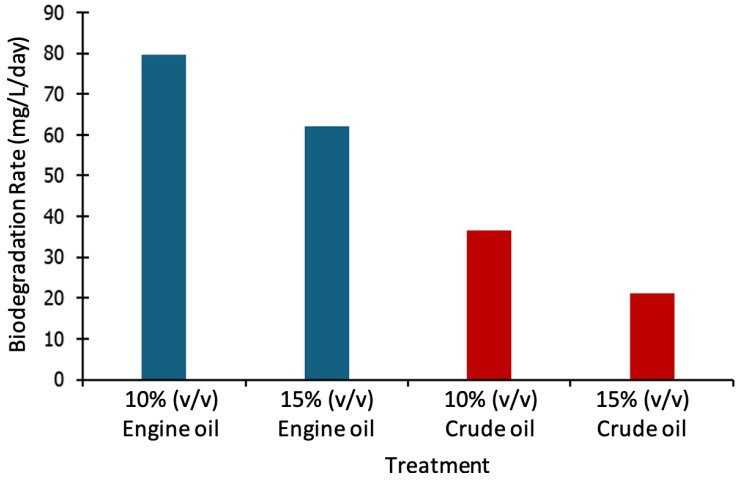

Fig. 2. Biodegradation rate by *Bacillus cereus* in 10% and 15% (v/v) engine oil and crude oil throughout 30 days of incubation period

The biodegradation efficiency and rate of engine oil surpass crude oil at both concentration of 10% and 15% (v/v). This is due to the fact that both oils are consisting of different compositions. According to Al-Otibi [23], crude oil is a highly complex mixture composed of hydrocarbons, asphaltenes, cycloalkanes, alkanes, and aromatic fractions. Meanwhile, engine oil is made of base oils and performance-enhancing additives after crude oil is refined to remove numerous impurities [24]. Comparable observations have been reported by Domde [25], whereby Bacillus sp. achieved 67.8% and 65.5% degradation of engine oil within six days of incubation at 28 °C and 100 rpm, demonstrating strong bioremediation potential toward engine oil. These findings are consistent with the present study and further indicate that engine oil is more amenable to microbial degradation than crude oil. The growth of *Bacillus cereus* in this study indicates that the introduced microorganisms were capable of metabolizing hydrocarbons, with crude oil and engine oil serving as the sole carbon sources. Sustained microbial proliferation throughout the incubation period suggests the progressive utilization of oil substrates for cellular development. Microbial growth was verified through increased turbidity of the culture suspension, measured via optical density at 600 nm (OD600), while biodegradation was confirmed by a reduction in oil viscosity and a transition toward lighter coloration. Uthami and Irdawati [26] reported that increased turbidity correlates with elevated bacterial cell density and metabolic activity, whereas Bakar [27] observed that turbidity values increased in parallel with cell number over time, demonstrating a direct relationship between turbidity and microbial growth.

3.2 Biodegradation Carbon Profile of Crude Oil and Engine Oil

The percentage reduction of individual hydrocarbon constituents in both crude oil and engine oil degraded by *Bacillus cereus* was quantified based on GC–MS chromatographic data. In this study, eight saturated hydrocarbon components were identified, consistent with the classification described by Portet-Koltalo [28]. he detected hydrocarbons included heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$), nonadecane ($C_{19}H_{40}$), eicosane ($C_{20}H_{42}$), heneicosane ($C_{21}H_{44}$), docosane ($C_{22}H_{46}$), tricosane ($C_{23}H_{48}$), and tetracosane ($C_{24}H_{50}$). Based on the Figure 3, 10% (v/v) engine oil exhibited the highest overall reduction of hydrocarbon components, with the descending order of removal as follows: eicosane (87.38%) > tricosane (80.96%) > heptadecane (80.94%) > docosane (78.20%) > nonadecane (76.11%) > octadecane (75.36%) > heneicosane (71.63%) > tetracosane (68.10%). For 15% (v/v) engine oil, the descending removal pattern was: heptadecane (78.18%) > heneicosane (69.61%) > docosane (67.80%) > tetracosane (67.23%) > tricosane (66.60%) > octadecane (66.08%) > eicosane (60.58%) > nonadecane (54.64%).

In comparison, 10% (v/v) crude oil showed lower hydrocarbon reduction, ranking as follows: tetracosane (49.01%) > eicosane (35.28%) > docosane (34.06%) > heneicosane (27.20%) > heptadecane (24.81%) > tricosane (24.22%) > nonadecane (21.10%) > octadecane (17.00%). Meanwhile, the 15% (v/v) crude oil treatment exhibited a sequence of tetracosane (55.50%) > docosane (37.12%) > tricosane (36.34%) > eicosane (34.80%) > heneicosane (21.90%) > heptadecane (21.08%) > nonadecane (13.84%) > octadecane (10.51%).

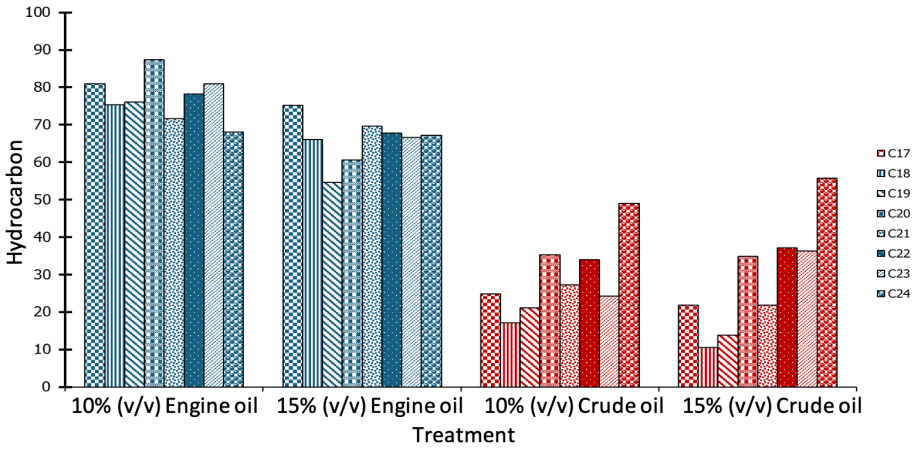

Fig. 3. Degradation percentage of individual hydrocarbon present in 10% and 15% (v/v) crude oil and engine oil by *Bacillus cereus* throughout 30 days of incubation period

Overall, the average percentage reduction of all hydrocarbon fractions for 10% (v/v) engine oil was 77.34%, followed by 65.96% for 15% (v/v) engine oil. In contrast, both 10% and 15% (v/v) crude oil treatments exhibited substantially lower mean reduction values of 29.01%. These findings demonstrate the greater susceptibility of engine oil compared to crude oil to microbial degradation by *Bacillus cereus*. According to Vinas [29] increases in peak height and area in GC–MS profiles correspond to enhanced microbial degradation of petroleum hydrocarbons, as oil degradation is associated with an increase in microbial population during the process. This suggests that engine oil may serve as a more favorable carbon and energy source for *Bacillus cereus* [30].

The biodegradation profiles of both oils at 15% (v/v) were further evaluated and visualized via GC–MS chromatograms (Figures 4 and 5). A marked reduction in the saturate fraction was observed for

both crude and engine oils across the 30-day incubation period, confirming progressive hydrocarbon degradation and active biodegradation throughout the study.

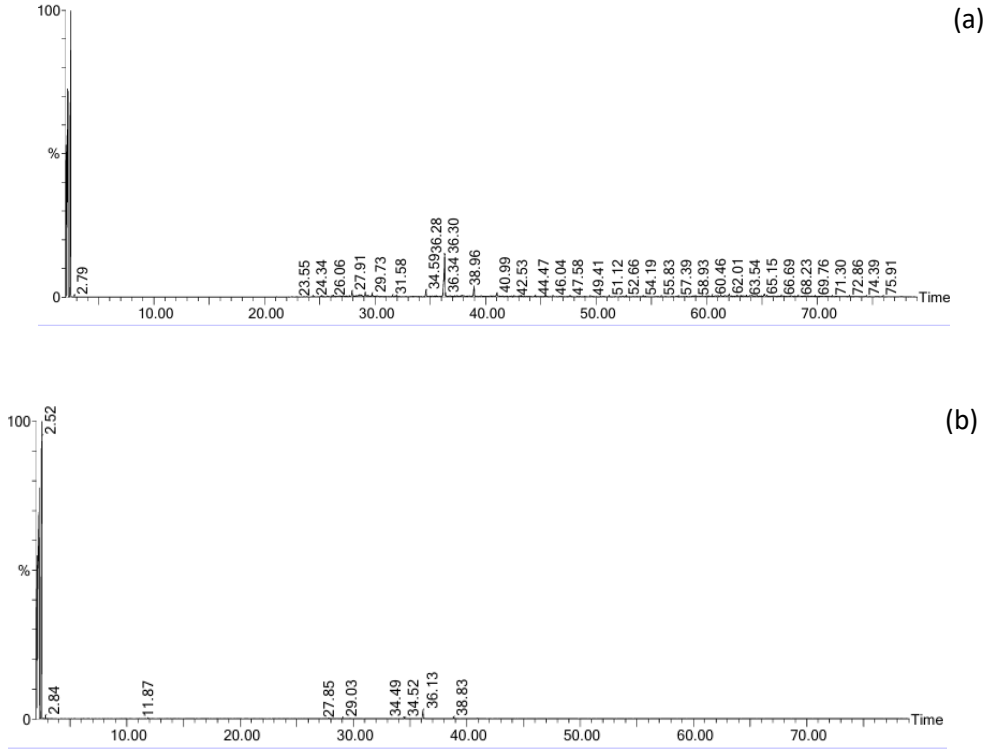

Fig. 4. Gas Chromatography profiles of *Bacillus cereus* for biodegradation of 15% (v/v) crude oil for (a) 0-day and (b) 30-day

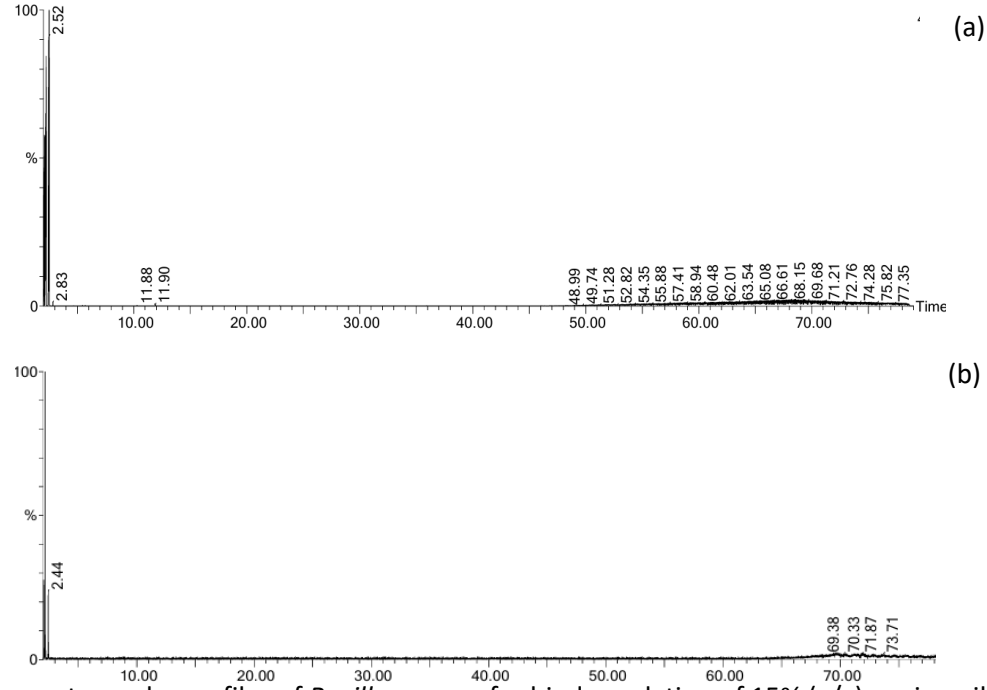

Fig. 5. Gas Chromatography profiles of *Bacillus cereus* for biodegradation of 15% (v/v) engine oil for (a) 0-day and (b) 30-day

3.3 Biodegradation Ratios

The biodegradation ratio of crude oil and engine oil can be determined through an empirical evaluation of isoprenoids pristane (Pr) and phytane (Ph). According to Rontani and Bonin [31] pristane and phytane are widely recognized biomarkers due to their relatively high chemical stability and resistance to microbial degradation compared to other alkanes. Their persistence during biodegradation processes makes them suitable indicators for evaluating the extent of hydrocarbon degradation [32]. As these biomarkers degrade more slowly and retain their structural integrity over time, changes in their relative abundance can reflect the metabolic capacity of microorganisms toward hydrocarbon substrates.

Table 2
Comparison of ratios of n-C17 : Pristane, n-C18 : Phytane, Pristine : Phytane for *Bacillus cereus* throughout 30 days of incubation period

Treatment	Week	n-C17 : Pristane	n-C18 : Phytane	Pristine : Phytane
10% (v/v) Crude Oil	0	0.79	0.70	0.92
	4	0.57	0.54	0.89
15% (v/v) Crude Oil	0	0.78	0.54	0.89
	4	0.62	0.48	0.87
10% (v/v) Engine Oil	0	0.87	0.70	0.80
	4	0.66	0.40	0.46
15% (v/v) Engine Oil	0	0.78	0.68	0.76
	4	0.43	0.40	0.59

Based on Table 2, the initial n-C17/Pr ratios for crude oil at 10% and 15% (v/v) and for engine oil at 10% and 15% (v/v) were 0.79, 0.78, 0.87 and 0.78, respectively. The initial n-C18/Ph ratios for the same treatments were 0.70, 0.54, 0.70 and 0.68, respectively. The initial Pr/Ph ratios for crude oil at 10% and 15% (v/v) and engine oil at 10% and 15% (v/v) were 0.92, 0.89, 0.80 and 0.76, respectively. Throughout the 30-day incubation period, *Bacillus cereus* cultured with engine oil exhibited a greater reduction in pristane and phytane ratios compared to cultures with crude oil, indicating a higher degree of biodegradation. The greatest reduction in n-C17/Pr ratio was observed in the 15% (v/v) engine oil treatment, decreasing from 0.78 to 0.43. Conversely, the lowest reduction in n-C18/Ph ratio was observed in the 10% (v/v) engine oil treatment, decreasing from 0.70 to 0.40 after 30 days.

Significant reductions in n-C17/Pr ratios were observed in engine oil treatments at both concentrations. According to Okorondu [33], this ratio examines the concentrations of n-alkane , especially n-C17, and cycloparaffin pristane. A reduction in this ratio often suggests biodegradation, as microorganisms preferentially breakdown n-alkanes. In addition, the ratio between n-C18/Ph for engine oil in both concentrations show significant decrease compared to crude oil in both concentrations. This ratio is comparing n-C_{18} with phytane. A change in this ratio also indicates changes in hydrocarbon content due to microbial activity. Moldowan *et al*., [34] reported that pristane and phytane are derived from phytol, a chlorophyll side chain that undergoes a series of reduction, oxidation, and decarboxylation reactions, contributing to their persistence during biodegradation. Collectively, these findings suggest that *Bacillus cereus* was more effective in degrading engine oil compared to crude oil under the conditions of the present study.

Overall, all biodegradation ratios for crude oil and engine oil treatments were less than 1 (Table 2). According to Okoro and Amund [35], n-C17/Pr and n-C18/Ph ratios greater than 1 indicate that hydrocarbons present in oil-contaminated seawater have not undergone biodegradation. Therefore,

the ratios obtained in this study confirm that all treatments underwent measurable biodegradation by *Bacillus cereus*. The ratios of n-C17/pristane and n-C18/phytane are useful indicators of microbial degradation because n-C17 and n-C18 are more susceptible to environmental microbial degradation, whereas pristane and phytane are comparatively recalcitrant. Their similar volatilities make these paired ratios particularly valuable in evaluating biodegradation progression[36].

4. Conclusions

The findings of this study demonstrated that *Bacillus cereus* was capable of biodegrading both engine oil and crude oil, with greater efficiency observed at 10% (v/v) compared to 15% (v/v). Engine oil exhibited the highest biodegradation performance, achieving an efficiency of 80.82% and the highest degradation rate (79.61 mg L^{-1} day^{-1}). Hydrocarbon profiling and biomarker analyses, including the n-C17/pristane, n-C18/phytane, and pristane/phytane ratios, further confirmed active hydrocarbon degradation throughout the 30-day incubation period. Collectively, these results indicate that *Bacillus cereus* possesses considerable bioremediation potential, particularly for the treatment of engine oil contamination.

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