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Harnessing *Bacillus cereus* from Surabaya Seawater for Enhanced Diesel Fuel Bioremediation in Tropical Ocean

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Abstract

Hydrocarbon is one of the primary organic pollutants that contaminate seawater. Most hydrocarbons which pollute the sea waters of Indonesia are the constituents of diesel fuel. This condition emphasizes the need to develop a bioremediation strategy for diesel fuel pollution in the country. A successful plan for bioremediation is primarily determined by the choice of the bacterial agent, which can be isolated from the polluted seawater. Furthermore, the setup must consider the characteristics and capability of the bacterial isolates to degrade diesel fuel. This study thus aimed to analyze the potential of *Bacillus cereus* isolated from the polluted seawater of Surabaya, East Java, Indonesia, as a bioremediation agent. The results of bacterial density, pH measurements, TPH and GC-MS assays indicated that the locally isolated *Bacillus cereus* could significantly reduce the hydrocarbon concentrations. More importantly, after 14 days of bioremediation, the polluting diesel fuel was categorized as biodegradable based on the BOD:COD ratio. This study shows that *Bacillus cereus* has excellent potential for bioremediating hydrocarbon contaminants in tropical seawater.

Keywords: Aquatic ecosystem, Bioremediation, Microbial ecology, Resources, Seawater pollution

Introduction

Hydrocarbon is a dominant chemical compound representing over 90 % of the crude oil content. It possesses a unique composition of hydrogen and carbon atoms [1]. This distinctive constitution is the leading cause of why crude oil spills are difficult to degrade [2,3]. A fraction of crude oil can be processed into diesel fuel, a commonly known fuel with an approximate boiling point of 250 - 350 °C, often referred to as middle distillate [4]. Diesel fuel consists of hydrocarbon and non-hydrocarbon compounds [5]. The main hydrocarbon compounds, such as paraffin and naphthalene, are in saturated forms. Interestingly, those hydrocarbon constituents have their own characteristics that depend on the source of crude oil [6,7]. Meanwhile, the non-hydrocarbon compounds are characterized by non-metal elements, such as sulfur, nitrogen and oxygen, and metal compounds like vanadium, nickel and iron [8].

In Indonesia, as much as 90 % of energy needs for transportation is supplied by fuel oil, including diesel fuel. Its consumption is projected to increase by 3.19 % in 2025 [9], which describes the high demand for Indonesian fishermen's boats or other types of vessels that mostly use diesel engines. Bearing in mind the country's widespread use of diesel fuel by boats, it is of concern that its leakage into aquatic environments will cause pollution if not addressed sufficiently [10]. The high probability of diesel leakage from boats into the waters during transport or docking poses a harmful long-term effect on the biota of Indonesian seas.

Diesel fuel has a smaller density than water [11]. Thus, when there is a spillage, the diesel layer will form and cover the surface of the water. This will automatically prevent the penetration of sunlight and hamper oxygen diffusion. Oxygen is crucial for the respiration of sea organisms, while sunlight naturally aids photosynthesis. Moreover, if the sea organisms ingest the chemical compounds of diesel oil by accident, some of the compounds could be excreted with the feces. In contrast, some others will accumulate as fats and proteins in the body of the organisms [12]. These pollutant aggregates can be easily transferred to the foraging organism following the natural food chain [13]. It will create an endless chain of

transmission to more significant terrestrial or aquatic organisms, even to humans who consume the contaminated sea organisms [14].

Considering the severe impact of diesel fuel contamination, regardless of its concentration, rigorous studies are paramount to discover feasible and affordable technologies to solve the problem. Preferably, the technologies should have as minimal environmental side effects as possible. Various methods employing chemicals are available to tackle the contamination from diesel fuel leakage in waters, such as using organic solvents with or without surfactants [15,16]. The mixtures of solvents are collected either by mechanical absorbents to convert the spilled fuel into storable forms for a short-term solution or by physicochemical methods with the help of chemical agents [16,17]. However, those methods potentially poison aquatic organisms due to the toxic compounds resulting from the chemical reactions, thereby increasing the recovery cost [17].

One potent alternative that is safer for the environment is bioremediation, a process of environmental recovery from organic or inorganic pollutants using organisms such as bacteria [18,19]. Biodegradation of hydrocarbon compounds in diesel fuel contamination by bacteria is determined by the strain's various physical, chemical and biological characteristics [20]. In this context, isolating local bacterial strains from polluted seawater is essential. This represents a strategy to obtain the candidates of hydrocarbonoclastic bacteria. These specific strains can degrade and use hydrocarbon compounds as the source of energy [21], which will be suitable for bioremediation in certain area and conditions. The development of such a method must also be based on the characteristics and abilities of the bacterial isolates to degrade the polluting diesel fuel.

Preliminary observations in Tanjung Perak Harbor suggested that in the occurrence of oil and grease (OG) spillage to the waters could reach 10 to 70 ppm at the highest. It was reported that even 0.01 ppm of OG can cause abnormal growth to fish species, with death being observed at 1,000 ppm [22]. This suggests a pressing need to find a suitable microbe candidate for bioremediation. To answer the need, this study aims to characterize and observe the ability of *Bacillus cereus* isolated from the polluted sea of Surabaya, East Java, Indonesia.

Materials and methods

Isolation of hydrocarbonoclastic bacteria

The seawater sample as the source of the bacteria isolates was taken from Tanjung Perak Harbor of Surabaya, East Java, Indonesia (**Figure 1**). It is one of Indonesia's most significant seaports, so that it may serve as an ideal representative for other harbors with similar conditions. The diesel fuel often leaked out from those docked vessels by accident. The polluted water sample was collected from those places. It was ensured that the polluted water sample was not ballast water from the boats.

The medium for growing the bacteria was Bushnell Haas agar spiked with 10 % diesel fuel (v/v) as the hydrocarbon source. The only bacteria that will grow in the Bushnell Haas agar is the ones capable of decomposing hydrocarbon [23]. Bacterial isolation was performed by spread-plate technique [14,20] with 10^{-8} dilutions of 1 mL polluted sample with sterile seawater. The plates were incubated at 30 °C for 120 h. After incubation, the isolated bacterial colony was streaked to Luria-Bertani (LB) agar media with the quadrant method. Identifying bacterial strains from the pure colony demonstrated that the bacteria type was *Bacillus cereus*.

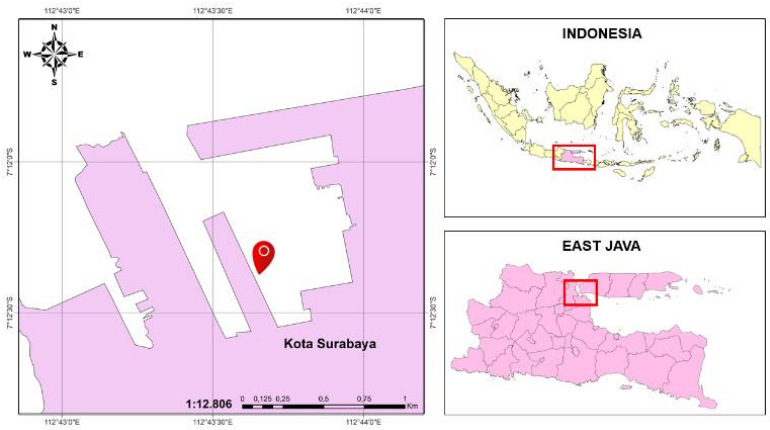

Figure 1 The map of sampling locations.

Assessment of *Bacillus cereus* ability to degrade the pollutant by Total Petroleum Hydrocarbon (TPH)

Isolates of *Bacillus cereus* (10^6 CFU/mL) were added to 10 mL LB broth containing used diesel fuel with concentrations of 15, 30 and 45 ppm. Based on our preliminary experiment, the concentrations were chosen due to the optimum bacterial growth at this range and not in lower or higher concentrations. Meanwhile, the *in-situ* data demonstrated that the pollutant concentration was 15 ppm. The culture was incubated at room temperature with shaking at 120 rpm for 24 h. The estimation of bacterial density was done daily from day 1 to 14, while the Total Petroleum Hydrocarbon (TPH) [14,21] analysis was conducted on days 0, 7 and 14.

The procedure for TPH was as follows: The LB media spiked with diesel fuel was added to a separating funnel. As many as 5 mL HCl 3 N and 60 mL n-hexane were put into the funnel. The mixture was shaken for ± 15 min and let sit until it was separated into 3 phases: Diesel fuel, n-hexane and water. The water phase was discarded, whereas the diesel fuel and n-hexane phases were filtered with filter paper pre-coated with ± 0.5 g Na_2SO_4. The filtered mixture was collected in an Erlenmeyer flask with a previously known weight. The flask was heated at 60 °C until the n-hexane evaporated, leaving only the diesel fuel. The heated flask was let cool and weighed. The amount of pure diesel fuel was calculated from the flask's weight difference between the empty and final conditions.

Measurement of pH, Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD)

The pH value of the media was measured daily with a pH meter Lutron Ph-201, electrode Pe-03, from day 1 to 14. The BOD value was analyzed by Winkler's method [24] and COD was analyzed with closed reflux spectrophotometry with a Beckman Coulter DU 700 series UV-VIS spectrophotometer. Both analyses were performed on days 0, 7 and 14.

Total bacterial density

The bacterial density count for this study was performed with a hemocytometer. The bacterial sample was 10 times diluted in distilled water and mixed with 0.4 % trypan blue with a 1:1 ratio. The bacterial count was obtained from the sum of the opaque viable cells in the 1 mm center square and the 4 corner squares. The cell concentration per mL was determined by multiplying the average count per square with the dilution factor.

Gas chromatography (GC-MS)

Gas chromatography was performed on the sample before (day 0) and after bioremediation treatment (day 14). The analyzed sample was taken from the media with 15 ppm of diesel fuel. The analysis was done with the Hewlett Packard HP 5890 Series II Gas Chromatograph (Agilent Technologies) and 5890 Flame Ionization Detector (FID), equipped with preinstalled analysis software. The GC column utilized was a 30 m long glass capillary with 0.25 mm diameter. The column temperature was 80 °C. Samples were injected in 1 μL aliquot with a syringe into the injector. The pressure was 100 kPa. The sample carrier gas was helium; the flow rate used was 1.6 mL/min with an injection temperature of 300 °C.

Results and discussion

Observation of the *Bacillus cereus* growth trend

The densities of *Bacillus cereus* in the media with 15, 30 and 45 ppm of diesel fuel are depicted in **Figure 2**. The initial density used in each media was approximately 1×10^6 CFU/mL. It was shown that in each fuel concentration, the bacterial growth had already entered the exponential phase on day 1 and reached the stationary phase on day 9. At that time, the highest densities at 15, 30 and 45 ppm of fuel were 1.21×10^9, 1.26×10^8 and 1.31×10^9, respectively.

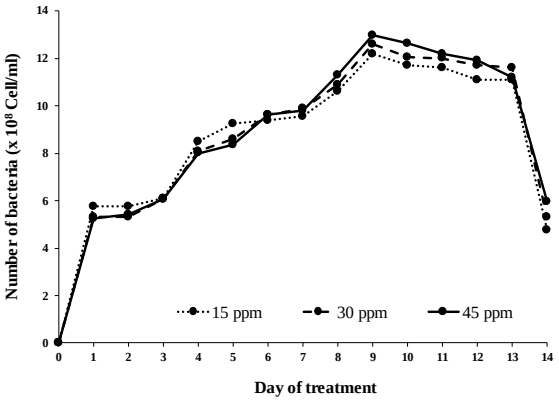

Figure 2 Total bacteria number during treatments.

The findings from this study shed light on the capabilities of *Bacillus cereus* in utilizing hydrocarbons present in diesel fuel as a primary source of nutrition, thereby facilitating its robust growth. Notably, the results showed that the highest bacterial densities were observed in media containing 45 ppm of diesel fuel, closely followed by 30 ppm, while the lowest density was recorded at 15 ppm of diesel fuel. This trend underscores this strain's ability to thrive in increasingly concentrated diesel fuel environments, implying a positive correlation between diesel fuel concentration and *Bacillus cereus* growth.

However, as day 12 approached, the bacterial density declined across all fuel concentrations. This indicates a potential depletion of essential nutrients for bacterial growth and survival, which is in line with the work of Nev *et al.* [25] that the exhaustion of key nutrients can lead to a decline in bacterial populations. It is plausible that the primary reason behind this decrease could be the depletion of hydrocarbons in the media, as the *Bacillus cereus* population exhausts this valuable source of sustenance. Furthermore, the accumulation of toxic metabolites such as alcohol might have contributed to the observed decline, as it is well-established that microbial activity can generate harmful byproducts.

Other factors contributing to the decrease in bacterial density may include the oxygen level and water supply within the media. As the *Bacillus cereus* population grows, it consumes oxygen and depletes the water supply at a rate that falls outside the range of its adaptation capabilities. This notion aligns with a previous study by Anggayasti *et al.* [26], which underscore the significance of environmental factors, such as oxygen availability, pH and water quality, in microbials and general organisms' growth and survival. Thus, the interplay of these variables forms a complex web of factors influencing the observed bacterial density trends.

The decrease in TPH value

In line to the decline of microbial number reported in **Figure 2**, **Figure 3** suggested that the depletion of hydrocarbon presumably caused *Bacillus cereus* death. In other words, the apparent decrease of bacterial density from day 9 (**Figure 2**), may be closely related to the gradual degradation of hydrocarbon supply, demonstrated by the TPH values in **Figure 4**. After day 14, the left-over diesel fuel concentrations were 2.01, 5.03 and 6.01 ppm, respectively, from the initial concentrations of 15, 30 and 45 ppm. Conversely, all control samples without the addition of *Bacillus cereus* did not experience any decrease in hydrocarbon concentration. It can be said that the use of *Bacillus cereus* in this study successfully assisted the diesel fuel bioremediation effort.

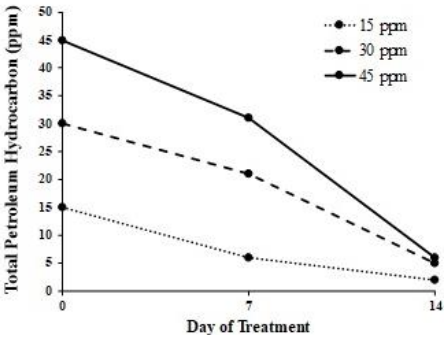

Figure 3 Total Petroleum Hydrocarbon (TPH) during bioremediation.

The connection between the death rate and TPH can be explained as follows: Bacteria, including *Bacillus cereus*, employ a range of enzymes to initiate the breakdown of complex hydrocarbon compounds. Key among these enzymes are monooxygenases and dioxygenases, which play a pivotal role in cleaving the carbon-carbon bonds within aromatic rings, transforming them into simpler primary alcohols [27]. The degradation process begins with the oxidation of n-alkane of the hydrocarbon substrate by monooxygenases, converting them into alcohol derivatives such as aldehydes and fatty acids. The fatty acids are then catabolized through the β-oxidation pathway that sequentially breaks down carbon atoms into distinct fragments [28]. Therefore, the decrease of complex hydrocarbon sources, in this case diesel fuel, obviously will affect the viability of *Bacillus cereus*.

It is stated that each bacterium type may possess a unique set of enzymes and metabolic pathways, allowing it to thrive in specific hydrocarbon-rich niches [20]. *Bacillus cereus* is indicated to actively consume hydrocarbons from diesel fuel, ultimately reducing the hydrocarbon concentration. Thus, the success of bioremediation efforts, as illustrated in this study, is intimately linked to the bacterial community and its capacity to adapt to the presence of the hydrocarbon compound [26,28].

The change of pH

As previously stated, the pH values of the ecosystem can vary greatly [26] and affect the biodegradation of organic substances, including hydrocarbon, by microorganisms [29]. The analysis of pH value dynamics measured during the diesel fuel hydrocarbon degradation process is shown in **Figure 4**. In the media with an initial hydrocarbon of 15 ppm, the pH value gradually increased from 6.0 to 7.0 from day 1 to 3. The pH stayed at 7.0 through day 10 and got down to pH 6.0 on day 12 to day 14. The initial pH of 7.0 at day 0 was likely to be influenced by the pH of the media and did not necessarily reflect the biodegradation process.

For the media with 30 ppm hydrocarbon, the pH initially stayed at 7.0 before going up to 8.0 on days 5 and 6. The value returned to pH 7.0 on days 7 - 10; during days 11 - 14, the pH value became 6.0. A slightly different pattern was observed on the media with 45 ppm hydrocarbon, in which the pH went up from 7.0 to 8.0 on days 3 - 4 and subsequently returned to pH 7.0 from days 5 to 8. The value declined further to 6.0 from day 9 to 14. Bacterial biodegradation of hydrocarbon occurs within pH values of 7.0 - 8.0 [30], which describes the events during pH changes observed in this study.

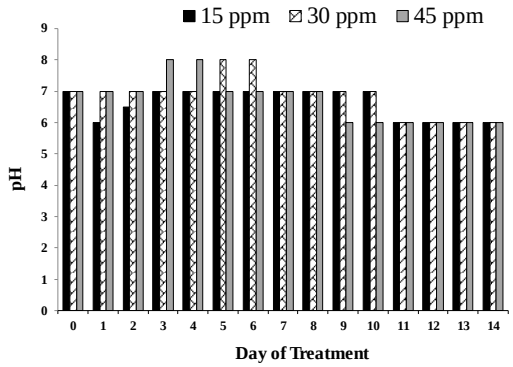

Figure 4 pH during treatments.

The dynamic changes in pH values in **Figure 4** offer valuable insights into the intricate process of diesel fuel hydrocarbon degradation. The fluctuations observed in pH, particularly the transient elevations to pH levels of 7.0 - 8.0 during the incubation period, indicate the underlying degradation of hydrocarbon compounds. These fluctuations are primarily attributed to the remarkable ability of bacteria to tolerate acidic conditions mediated by the hydrogen pump mechanism, a well-documented adaptation in microbial communities [31]. This mechanism enables bacteria to maintain pH homeostasis even in the presence of acidic metabolites generated during hydrocarbon degradation.

In addition to the bacterial homeostatic mechanisms, the pH shifts may be influenced by esters as side products of hydrocarbon degradation. These compounds can impact the surrounding pH by either buffering or contributing to the overall acidity, thereby influencing the equilibrium [32]. Moreover, the intake of protons (H^+) by bacterial cells from the environment can exert a localized reduction in pH within the immediate vicinity of the cells [18]. This phenomenon, known as proton exchange, further contributes to the intricate pH dynamics observed during hydrocarbon degradation.

The increase in biosurfactant production by bacteria, a common occurrence during the biodegradation of hydrocarbons, can directly contribute to the observed rise in pH. Biosurfactants, including rhamnolipids and surfactin, facilitate the emulsification of hydrophobic hydrocarbons and act as buffering agents, exerting an alkaline influence on the surrounding environment [33]. This phenomenon highlights the multifaceted role of bacteria in shaping their microenvironment during hydrocarbon degradation and further emphasizes the potential for enhanced biodegradation rates with an associated rise in pH.

Towards the latter stages of the incubation period, all media exhibited a concurrent decrease in pH values, reaching a typical pH of 6.0. This decline can be attributed to various processes within the bacterial metabolism. The isolated bacteria's hydrocarbon metabolism likely generates acidic metabolites, potentially including volatile organic acids (VOA) [34]. The accumulation of these acidic intermediates, owing to the incomplete degradation of hydrocarbons, can exert a localized acidifying effect.

Additionally, the biodegradation of n-alkane components of the hydrocarbon substrates, with subsequent transformations into aldehydes and fatty acids, contributes to the observed decrease in pH [36]. These acidic metabolic byproducts lower the pH levels, emphasizing the complex interplay between microbial metabolic activities and the resultant pH dynamics during hydrocarbon degradation.

The fluctuations in pH values observed during diesel fuel degradation by *Bacillus cereus* signify the profound influence of bacterial metabolic activities on the surrounding microenvironment. These changes are a testament to the bacteria's adaptive mechanisms, buffering capacities and ability to influence pH dynamics as they metabolize hydrocarbons.

Biological Oxygen Demand (BOD)

Aerobic bacteria consume oxygen, which triggers its faster propagation and nutrient degradation. The BOD value is an indicator of the amount of oxygen that is needed to decompose organic compounds of a particular pollutant [36]; in this case, the hydrocarbons of diesel fuel, as the *Bacillus cereus* were grown in media notably spiked with that fuel. The higher the number of organic pollutants, the higher the oxygen concentration needed to decompose the pollutant. As a result, the BOD value will be high [36]. The BOD value is likely linked to pH since both factors are essential for hydrocarbon breakdowns [32].

As indicated by **Figure 5**, the starting BOD values for the media with the initial concentrations of used diesel fuel of 15, 30 and 45 ppm, respectively, were 67.6, 83.1 and 83.9 mg/L. All media uniformly showed a similar declining trend of BOD value along with the increasing incubation duration. On day 14, the final BOD values of all media with 3 different initial diesel fuel concentrations were much closer, at 5.63, 6.92 and 6.99 mg/L.

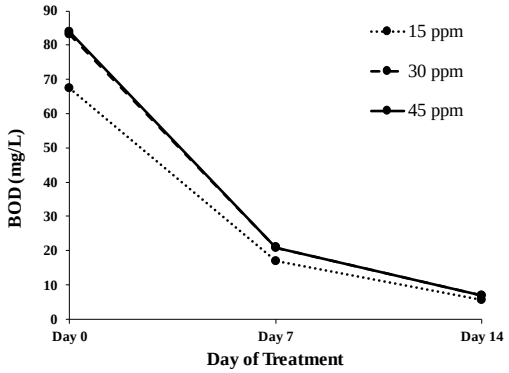

Figure 5 BOD during degradation of hydrocarbon by *Bacillus cereus*.

The declining trend in Biological Oxygen Demand (BOD) values, as illustrated in **Figure 5**, holds significant implications for the efficiency of diesel fuel bioremediation facilitated by *Bacillus cereus*. BOD, a crucial parameter in assessing the organic pollution levels in aquatic environments, reflects the dissolved oxygen microorganisms required to degrade organic matter in each sample. The observed decrease in BOD values across all media with varying initial concentrations of diesel fuel (15, 30 and 45 ppm) is a testament to the effective microbial degradation of the organic pollutant.

The reduction in BOD values can be directly attributed to the robust bacterial activity engaged in the degradation of the organic pollutant. This process involves the stepwise breakdown of hydrocarbon compounds present in diesel fuel, releasing carbon dioxide and water. Consequently, the concentration of the pollutant is significantly diminished (**Figure 2**). The pH value may also be declining as the rate of

hydrocarbon metabolism increases, up to the point where the hydrocarbon source is depleted (**Figure 5**). The striking convergence of BOD values among the media with different initial diesel fuel concentrations by day 14 reflects the remarkable efficiency of *Bacillus cereus* in converting the pollutant into less harmful end products, thereby diminishing the organic load in the medium. This convergence underscores the potential for successful bioremediation across various diesel fuel pollution levels.

The gradual sloping trend observed in BOD values from day 7 to day 14 can be intricately linked to the bacterial growth phases delineated in **Figure 2**. The *Bacillus cereus* population enters the stationary phase on day 8 and progresses to the death phase on day 12. During the stationary phase, microbial metabolic activities continue at a relatively stable rate, facilitating the continued degradation of diesel fuel. However, the death phase marks a decline in microbial activity as bacterial cells cease to replicate and the degradation processes slow down. A corresponding change in BOD values accompanies this temporal shift in microbial activity. The decline in BOD during the death phase can be attributed to the diminishing microbial population as cells perish, reducing the demand for dissolved oxygen. This intriguing trend further underscores the temporal dynamics of diesel fuel bioremediation and emphasizes the importance of considering microbial growth phases in bioremediation strategies.

Chemical Oxygen Demand (COD)

COD value states the total oxygen needed by chemical process to oxidize all existing organic compounds in waters to become CO_2 and H_2O. **Figure 6** shows the changes in COD value during incubation. Compared to the BOD values in **Figure 5**, the COD values demonstrated much tighter values, albeit with a similar decreasing trend. The COD values on day 0 were 168.2, 170.5 and 175.3 mg/L for media with initial hydrocarbon concentrations of 15, 30 and 45 ppm, respectively. On day 7, the values ranged from 42.05, 42.62 and 43.8 mg/L, whereas on day 14, all COD values were almost similar at 8.36, 8.55 and 9.1 mg/L.

The measurement of COD provides crucial insights into the total oxygen required by chemical processes to fully oxidize all organic compounds present in water to form CO_2 and H_2O. As seen in **Figure 5**, the evolution of COD values during the incubation period represents a pivotal aspect of understanding the extent of hydrocarbon degradation by the hydrocarbonoclastic *Bacillus cereus*. Despite the similarities in the decreasing trend with the BOD values, COD values exhibit a notably tighter range, reflecting the comprehensive nature of this measurement in capturing a wide array of organic compounds.

The initial COD values, recorded on day 0, were 168.2, 170.5 and 175.3 mg/L for media with varying initial concentrations of hydrocarbons (15, 30 and 45 ppm, respectively). These values directly reflect the quantity of organic compounds, primarily hydrocarbons, present in the medium. It is well-established that higher COD values indicate a more significant organic load, underscoring the correlation between COD and the extent of hydrocarbon pollution. In this context, the observed differences in initial COD values can be attributed to the varying levels of hydrocarbon contamination in each medium, with the highest concentration demonstrating the highest initial COD value.

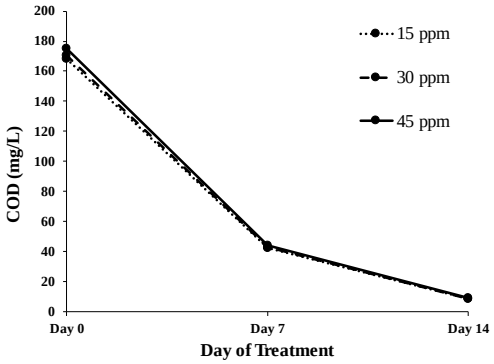

Figure 6 COD during degradation of hydrocarbon by *Bacillus cereus*.

As the bioremediation process progresses, a significant reduction in COD values becomes evident. This decline signifies the efficient decomposition of hydrocarbons and a range of other organic compounds within the medium by *Bacillus cereus*. While the primary focus is on hydrocarbon degradation, acknowledging the bacterium's capacity to digest additional compounds, such as fatty acids, is crucial. The decomposition of these alternative compounds is a pivotal contributor to the rapid decrease in COD values,

even in scenarios where the rate of hydrocarbon degradation might be comparatively slower, as depicted in **Figure 3**.

The ability of *Bacillus cereus* to target a spectrum of organic compounds for degradation showcases the versatility of this microorganism in addressing complex pollutant mixtures. Consequently, the rate of COD reduction remains consistently high, reflecting the comprehensive nature of the bioremediation process. This nuanced aspect of hydrocarbonoclastic bacteria's activity highlights the potential for these organisms to address diverse sources of organic contamination, extending their utility beyond hydrocarbon-rich environments.

Analysis of BOD:COD ratio

The ability of microorganisms, especially bacteria, to degrade hydrocarbon pollutants can be determined from the BOD:COD ratio. The tendency of the pollutant to be degraded can also be inferred from that ratio, whereby the higher the BOD:COD ratio, the more likely the pollutant to be degraded. The BOD:COD ratio during the decomposition of used diesel fuel can be seen in **Table 1**.

Table 1 The BOD:COD ratio during biodegradation of hydrocarbon by *Bacillus cereus*.

Initial concentration	Day 0	Day 7	Day 14
15 ppm	0.402	0.402	0.670
30 ppm	0.490	0.490	0.810
45 ppm	0.480	0.480	0.770

The dynamic interplay between BOD and COD ratios throughout the bioremediation process yields valuable insights into the efficacy of hydrocarbonoclastic *Bacillus cereus* in degrading complex organic compounds. As indicated in **Table 1**, the BOD:COD ratios on days 0 and 7 were relatively consistent across all 3 media, signifying the initial composition of the pollutant mixtures in the environments. This uniformity underlines the stability of the organic load and the complexity of the organic compounds, with minimal variations observed at these early stages.

However, a pivotal transition is evident on day 14 when the BOD:COD ratios experience a noteworthy increase. This observed upswing alludes to the enhanced degradation of total organic pollutants or complex organic compounds within the media, occurring primarily after the first week of bioremediation treatment. The surge in BOD:COD ratios during this period can be attributed to the significant decomposition of hydrocarbons within the initial phase of bioremediation, as evidenced in **Figure 2**. During this initial phase, the hydrocarbon content in the media was relatively high, making the complete degradation of all pollutants a challenging task. Nevertheless, as the bioremediation process advanced, the steady reduction in hydrocarbon concentration liberated the microbial community's capacity to address the broader spectrum of complex organic pollutants.

In essence, the BOD:COD ratios indicate the evolving nature of the pollutant matrix throughout the bioremediation process. The elevated BOD:COD ratios attained after 14 days of bioremediation treatment, exceeding the threshold of 0.6 [35], indicate the successful transformation of the treated diesel fuel pollutant into a biodegradable form. The ratios surpassing this threshold signify the prevalence of biodegradable organic matter, reflecting the effective removal of recalcitrant organic compounds through microbial activity. This transformation has profound implications for the potential environmental impacts of the treated media, indicating a shift from a recalcitrant, persistent pollutant profile to a more readily degradable, environmentally benign form.

GC-MS profiling of hydrocarbon compounds before and after treatment

To clarify the apparent declining trend of hydrocarbon composition within the 14-day duration of bioremediation, as shown by the TPH value (**Figure 3**) and inferred by the changes of pH, BOD and COD values (**Figures 4 - 6**), subsequently by BOD:COD ratio (**Table 1**), the ability of bacteria to decompose hydrocarbon can also be observed from the profile and the type of compounds existing in a sample before and after treatment. The obtained GC-MS chromatograms demonstrated long-chain hydrocarbon compounds' degradation before bioremediation (**Figure 7(a)**) into the ones with shorter chains after the treatment (**Figure 7(b)**). The diminishing width of the curve area in the chromatograms and the general decline of hydrocarbon concentration after treatment signified the occurrence of biodegradation of the compounds. The area changes of above 50 % are reported in this discussion.

Comparison between the GC-MS chromatograms in **Figures 7(a)** - **7(b)** showed disappearing peaks. Those peaks were identified in **Figure 7(a)** as Pentanoic acid ($C_{19}H_{30}O_3$), Tetrapetracontane ($C_{54}H_{10}$), Azulene ($C_{10}H_{16}O$) and Naphthalene ($C_{10}H_8$). Based on the calculations of the area changes, Pentanoic acid and Tetrapetracontane experienced complete degradation at 100 %. In contrast, Naphthalene and Azulene were 98 and 88 % degraded into Cyclohexanone ($C_{10}H_{16}O$) and Cyclohexane ($C_{10}H_{20}$), respectively (**Figure 7(b)**). **Figure 7(a)** further demonstrated that the peaks of Heptadecane ($C_{17}H_{36}$) and Pentatriacontane ($C_{35}H_{72}$) declined into lower intensity in **Figure 7(b)**. The short-chain hydrocarbons that correspond to, thus represent the decomposed products of those 2 compounds were 1-Octanoal,2-Butyl- ($C_{12}H_{26}O$) and Heptacosane ($C_{27}H_{56}$) (**Figure 7(b)**) with area changes of 67 and 82 %, respectively.

The decomposition of Tetrapentacontane in diesel fuel to short-chain hydrocarbons has been reported before with *Bacillus subtilis* as the bioremediation agent [37]. In this study, *Bacillus cereus* was confirmed to have a similar metabolism property. *Bacillus cereus* is mainly known to decompose Naphthalene under aerobic conditions [38], like the other *Bacillus* strains such as *Bacillus fusiformis* [40] and *Bacillus subtilis* [39]. The indication that Naphthalene was 98 % degraded after bioremediation in this study pointed out the highly efficient activity of *Bacillus cereus* as bioremediation agent.

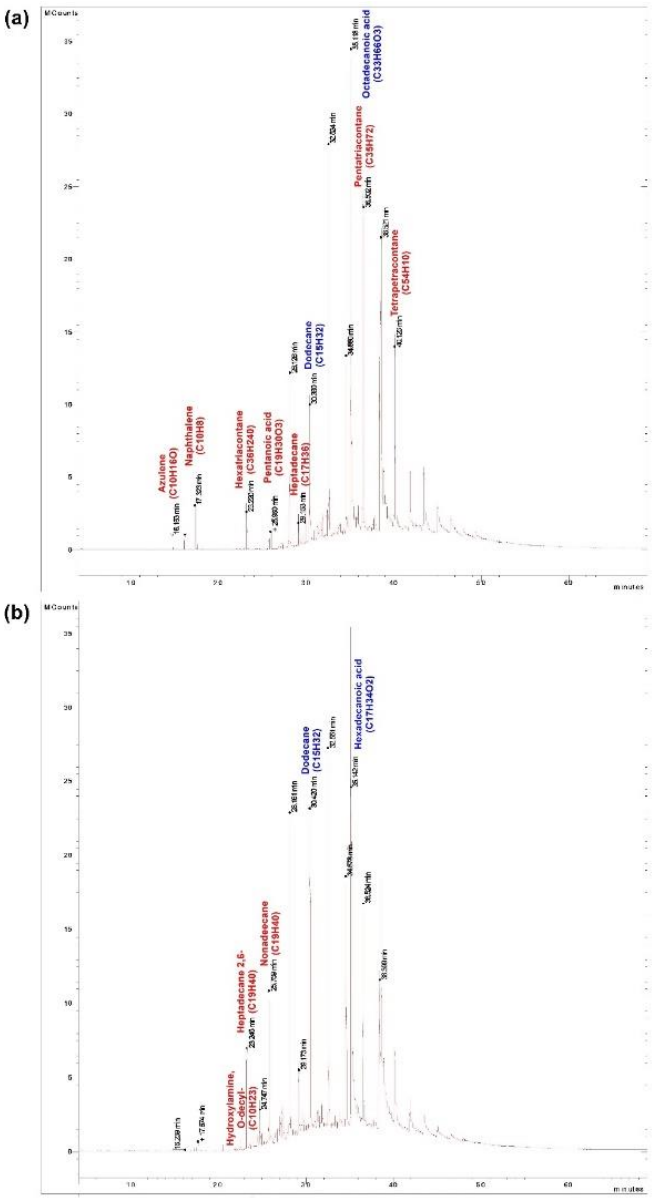

Figure 7 The results of GC-MS analysis: (a) Before the bioremediation process (day 0) and (b) After the degradation of hydrocarbon by *Bacillus cereus*. The red labels refer to hydrocarbon compounds that experience increase or decrease, whereas the blue labels refer to those that remain constant.

In contrast, new peaks formed after bioremediation treatment, as shown in **Figure 7(b)**, namely for Hydroxylamine, O-decyl- ($C_{10}H_{23}$) and Nonadeecane ($C_{19}H_{40}$). In addition, Heptadecane 2,6- ($C_{19}H_{40}$) showed rising intensity after day 14 (**Figure 7(b)**) of the bioremediation treatment with a 74 % area increase, possibly degraded from the long-chain Hexatriacontane ($C_{36}H_{240}$) in **Figure 7(a)**. Our results also indicated that Dodecane ($C_{15}H_{32}$) was detected in **Figures 7(a)** - **7(b)**. As such, the *Bacillus cereus* did not degrade Dodecane like the other hydrocarbon compounds of the diesel fuel pollutant. Thus, the compound may serve as a metabolic agent of *Bacillus cereus* that sets it apart from the other hydrocarbons.

It was also found that the peaks of Octadecanoic acid ($C_{33}H_{66}O_3$) in **Figure 6(a)** and Hexadecanoic acid ($C_{17}H_{34}O_2$) in **Figure 7(b)** did not show a remarkable change in intensity. In connection to that, a study by Lee and Nikraz [35] reported that both compounds were found in biosurfactants naturally produced by *Bacillus subtilis* A1 to remove hydrocarbons, thus helping to remediate the polluted environment. As *Bacillus* species were identified as biosurfactant producers [36], the *Bacillus cereus* in this study likely behaves similarly, hence the constant expression of Octadecanoic and Hexadecanoic acids.

The GC-MS profiling of hydrocarbon compounds before and after bioremediation underscores the remarkable adaptability and versatility of *Bacillus cereus* in transforming complex hydrocarbon mixtures. The transformation from long-chain to shorter-chain hydrocarbons, along with the formation of new compounds, highlights the comprehensive biodegradation potential of this bacterium.

Conclusions

This study sheds light on the potential of *Bacillus cereus*, isolated from the polluted sea waters of Surabaya, Indonesia, in combatting diesel fuel pollutants. Over a span of 14 days, *Bacillus cereus* exhibited robust growth by harnessing available nutrients in the media, effectively targeting and consuming the hydrocarbon pollutants derived from diesel fuel. The increase in the BOD:COD ratio is a compelling indicator of the reduction in complex organic compounds. Often resistant to decomposition, these compounds face a tangible decline during the bioremediation process. The GC-MS results further corroborate the efficacy of *Bacillus cereus* in hydrocarbon degradation by systematically transforming the compounds into shorter-chain derivatives. These findings are significant not only in the context of bioremediation but also in understanding the fundamental processes of microbial degradation of hydrocarbons. The implications of this study are far-reaching and hold promise for addressing diesel fuel contaminations in diverse tropical climates, such as in Southeast Asian countries. The robustness of *Bacillus cereus* offers a sustainable and environmentally friendly approach to combatting seawater hydrocarbon pollution. It also beckons for further exploration and investigations into alternative microbial resources. By understanding the capabilities of different microorganisms, we can harness a diverse arsenal of bioremediation agents to tackle fuel pollution problems on a global scale.

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