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GC-MS Analysis of Petroleum Biodegradation by Marine Bacteria Isolated from Paotere Port, Indonesia

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Abstract : This study investigated the biodegradation of petroleum hydrocarbons by marine bacterial isolates obtained from Paotere Port. The objective was to assess the efficacy of these isolates in degrading petroleum hydrocarbons using gas chromatography-mass spectrometry (GC-MS) along with emulsification index and surface tension tests. The experiments were conducted in a microcosm designed to simulate the bacteria's natural environment, incorporating sediment, natural seawater (ALN), and petroleum. Emulsification index and surface tension measurements were taken every five days. On the 30th day of incubation, a qualitative analysis using GC-MS was performed to evaluate the extent of hydrocarbon degradation. The highest emulsification index recorded was 53.84%, while the lowest surface tension observed was 22.16 dyne/cm. GC-MS chromatograms revealed significant hydrocarbon degradation, as indicated by the breakdown of carbon chains in the sample compared to the control. The bacterial isolates from Paotere Port demonstrated the capability to degrade carbon chains up to C150. These findings demonstrate the potential of petroleum-degrading bacteria from Paotere Port as effective bioremediation agents.

Keywords : Biodegradation, Marine bacteria, oil spill, oil recovery, sustainability

Introduction

Petroleum plays a crucial role globally as a primary source of energy and carbon. However, the operations of petroleum and related industries have contributed significantly to environmental pollution, particularly oil contamination.[1] Such contamination poses serious threats to both the environment and human health, especially in port areas where petroleum and its derivatives are commonly found. Petroleum is composed of complex hydrocarbons, including organic compounds such as elemental sulfur, oxygen, nitrogen, metals, and certain radionuclides. The primary components of petroleum are paraffinic hydrocarbons, saturated alicyclic hydrocarbons, and aromatic hydrocarbons, the latter of which are known to be carcinogenic.[2]

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Aliphatic and aromatic hydrocarbons constitute a significant portion of petroleum's complex composition, with many of these compounds being toxic, mutagenic, or carcinogenic.[3,4] Exposure to petroleum products, such as naphthalene, can result in adverse health effects, including skin irritation, red blood cell damage, and nephrotoxicity.[5,6] The widespread use of petroleum as a raw material for energy and chemical production, combined with tanker accidents during operations, often leads to oil spills in the environment.[7] Once released into the environment, petroleum undergoes complex transformations due to interactions with physical and biological factors. These hydrocarbon complexes are challenging to manage and can cause water pollution, which poses significant health risks to humans, particularly through the accumulation of toxic compounds in water or aquatic life.[8]

Oil pollutants pose a significant threat to human health and the environment due to their toxicity, mutagenicity, and carcinogenicity. Petroleum hydrocarbons, such as benzene, toluene, ethylbenzene, and xylene, are naturally present in gasoline and crude oil but can also accumulate on the surface of seawater and soil as a result of industrial activities, including petrochemical handling, reservoir leaks, and improper waste disposal.[9] Despite ongoing efforts, oil pollution remains a global concern, largely due to its persistent environmental impact and associated health risks. One of the primary environmental challenges associated with

petroleum residue disposal is the potential for bioaccumulation in the food chain, which poses long-term risks to both ecosystems and human health.[10] Additionally, exposure to petroleum compounds such as naphthalene can lead to adverse health effects, including skin irritation, red blood cell damage, and nephrotoxicity.[11]

Bioremediation techniques offer an effective means of eliminating carbon complexes from the environment. Among these, biodegradation has emerged as a particularly promising approach for the remediation of hydrocarbon pollutants. Biological methods have several advantages over traditional physical and chemical techniques for oil spill cleanup, primarily due to their cost-effectiveness and ecological sustainability. Bioremediation is increasingly recognized as a viable alternative for managing oil spills, avoiding the negative impacts of chemical dispersants and incineration.[12] Bioremediation involves the transformation of chemical compounds by living organisms, particularly microbes, into cell biomass, energy, and natural waste products. There are two primary types of bioremediation: biostimulation (BS) and bioaugmentation (BA). Biostimulation enhances the activity of indigenous microbial communities by adding nutrients to oil-contaminated sites, thereby accelerating the breakdown of oil. Bioaugmentation, on the other hand, involves introducing specialized oil-degrading microorganisms to the contaminated area to increase the rate of hydrocarbon degradation.[13]

Biosurfactants play a crucial role in the bioremediation of oil spills in marine environments by enhancing the solubility of petroleum components and reducing the surface tension between oil and water. These microbial surfactants are also effective as antimicrobial agents. Biosurfactants, produced by microorganisms, are capable of dissolving in both organic and nonpolar solvents, as well as polar or aqueous solvents. They are categorized based on their chemical structure and the microorganisms responsible for their production. The main types include glycolipids, lipopeptides, proteins, phospholipids, polysaccharide-protein complexes, lipopolysaccharides, neutral lipids, and fatty acids. The application of biosurfactant-producing microbes shows significant potential as a facilitating agent in the degradation of hydrocarbons.[14]

Numerous studies have demonstrated that microorganisms are key contributors to the decomposition of pollutant molecules, such as hydrocarbons, in both aquatic and terrestrial environments (Table 1). For instance, Li et al. reported that *Clostridium*, *Bacillus*, and *Pseudomonas* species, which naturally produced β-cyclodextrin, resulted in 50% hydrocarbon removal from oil-contaminated soil.[15] Similarly, *Pseudomonas aeruginosa* SR17, through the production of rhamnolipid, achieved hydrocarbon removal rates ranging from 80.5% to 86.1% in crude oil-contaminated soil.[16] Prakash et al. demonstrated that *Bacillus* species, producing lipopeptide, enhanced hydrocarbon removal by up to 97% in crude oil-contaminated soil.[17] Furthermore, Pi et al. found that *Pseudomonas* LSH-7, through rhamnolipid production, achieved a 73.94% removal of hydrocarbons in marine offshore oil spill samples.[18]

Numerous studies have highlighted the significant role of microorganisms in enhancing the efficiency of hydrocarbon bioremediation. This research focuses on isolating marine bacteria from a newly identified contamination site at Paotere Port, Makassar, Indonesia, to assess their potential as effective bioremediation agents. Petroleum hydrocarbon pollution in Makassar's water bodies, particularly around Paotere Port, is predominantly linked to port activities. Oil spills resulting from ship waste discharge or cleaning operations at the port present a severe threat to marine life, such as fish, and pose health risks to humans when contaminants accumulate in seafood. The persistent oil pollution in this region has promoted the growth of hydrocarbon-degrading bacteria. While earlier studies have shown the capability of bacterial isolates from Paotere Port to degrade hydrocarbons under laboratory conditions,[19] this study aims to evaluate their efficiency in degrading oil within a controlled microcosm environment.

Experimental

A. Culture Stage

The bacterial culture used in this study was isolated by Wardhani et al. from Paotere Port, Makassar, Indonesia. To the microcosm, 40 mL of K_2HPO_4, 20 mL of $FeSO_4$, and 50 mL of petroleum were added as additional nutrients, with an additional 50 mL of petroleum serving as the carbon source. Then, 150 mL of pre-cultured bacterial isolates were inoculated into the medium (10 L ALN) and incubated for 30 days at room temperature. On day 15, additional nutrients, specifically $FeSO_4$ and K_2HPO_4, were added. Samples were collected on days 5, 10, 15, 20, 25,

Table 1. Microorganisms and Biosurfactants Used in Hydrocarbon Bioremediation.

Microorganism	Biosurfactant	Hydrocarbon Removal (%)
[15]*Clostridium*, *Bacillus*, and *Pseudomonas*	β-cyclodextrin	50
[16]*Pseudomonas aeruginosa* SR17	Rhamnolipid	80.5-86.1
[17]*Bacillus subtilis* AS2, *Bacillus licheniformis* AS3, and *Bacillus velezensis* AS4	Lipopeptide	88-97
[18]*Pseudomonas* LSH-7	Rhamnolipid	73.94%

and 30 to measure the emulsification index and surface tension. At the end of the incubation period, the samples were analyzed using Gas Chromatography-Mass Spectrometry (GC-MS).[19,20]

B. Emulsification Index Test

The emulsification index test (% EI) was conducted following the method described by Patel and Patel. Hydrocarbon-degrading bacterial cultures were grown and then centrifuged to obtain the supernatant. Two milliliters of the supernatant were placed into a test tube, followed by the addition of 2 mL of petroleum, maintaining a 1:1 ratio. The mixture was vortexed for 2 minutes and then allowed to settle for 24 hours.[21] The emulsification index was calculated using the following formula:

$$\% \text{ EI} = \frac{\text{Height of the emulsion formed}}{\text{Total height of solution}} \times 100\%$$

C. Surface Tension Test

The surface tension test was conducted according to the method described by Lu et al. Surface tension was measured using a Du Noüy tensiometer. Hydrocarbon-degrading bacterial cultures were grown and then centrifuged for 5 minutes to obtain the supernatant. Two small petri dishes were prepared, each containing 5 mL of artificial seawater (ALN), and the initial surface tension value was measured. In the first petri dish, 4 mL of the culture supernatant was added, and after allowing it to stand for a few minutes, the surface tension value (y˙) was measured as the final value. In the second petri dish, 4 mL of the control supernatant was added, allowed to stand for a few minutes, and then the surface tension value (y˙) was measured again as the final value. Each measurement was repeated five times, and the decrease in surface tension was expressed as the difference between the initial and final values in mN/m (millinewton meter).[22]

D. Biosurfactant Extraction

To extract the biosurfactant, 25 mL of bacterial culture was prepared, followed by the addition of 50 mL of chloroform ($CHCl_3$), 50 mL of methanol, and 0.5 N KOH. The mixture was then refluxed for 4-5 hours, after which it was allowed to cool and subsequently filtered. The refluxed sample was filtered using a Buchner funnel, and the filtrate was subjected to separation using a separating funnel. This separation process was repeated three times with 10 mL of chloroform each time. The resulting extract was then treated with magnesium sulfate ($MgSO_4$) and evaporated to dryness using a rotary evaporator.

E. Qualitative Analysis of Biodegradability Using Gas Chromatography-Mass Spectrometry (GC-MS)

The ability of bacteria to break down carbon chains can be determined by qualitative testing using gas chromatography-mass spectrometry (GC-MS) (GCMS-QP2010 Ultra Shimadzu). Qualitative analysis was carried out using GC Clarus 600 plus an Elite-5MS column. A total of 1 mL of the extracted sample was injected into the column, with the injector temperature set at 280°C and the detector temperature at 290°C. Helium gas was used as a carrier gas, maintained at a constant flow rate. The sample then underwent electron impact ionization, causing the sample molecules to fragment into smaller components, which were then detected by a mass spectrometer. The resulting data are presented in the form of a chromatogram.[23]

Results and Discussion

At the end of the incubation period, noticeable changes were observed in the growth medium. The transition in the medium's appearance, from clear to cloudy brown, indicates bacterial growth activity. This color change suggests that the bacteria were able to decompose and utilize the hydrocarbon compounds present in petroleum as a carbon source.

The results of the emulsification index test, as depicted in Figure 1, demonstrate the formation of an emulsion layer.

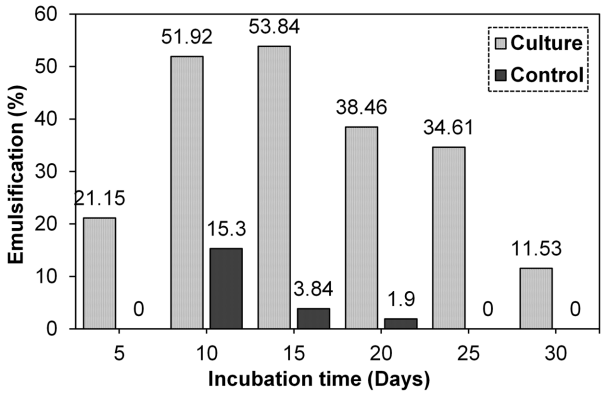

Figure 1. Emulsification index results.

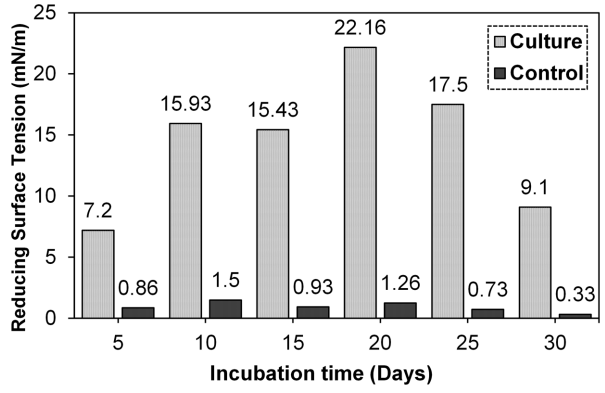

Figure 2. Surface tension reduction results.

The highest emulsification index was observed in the culture supernatant at T(15), with a value of 53.84%, while the control supernatant showed its best result at T(10), with an emulsification index of 15.3%. These findings indicate that the bacterial isolates from Paotere Port have a remarkable capacity to produce biosurfactants capable of emulsifying petroleum in solution. According to Damayanti et al. the biosurfactant produced by *Serratia marcescens* showed an emulsification index of 49.26%, further supporting the efficiency observed in this study.[24]

A critical aspect of hydrocarbon-degrading bacteria is their ability to emulsify hydrocarbons in solution by producing biosurfactants, which contain surface-active agents that facilitate the dispersion of hydrocarbons. The dispersion process leads to the formation of an emulsion layer in the water. The diversity of bacterial species in the environment contributes to the variety of metabolites that can degrade different types of aliphatic and aromatic hydrocarbons.[25] Bacterial biosurfactants enhance the growth of bacteria in hydrocarbon-contaminated environments by emulsifying hydrocarbons, thereby increasing their solubility and forming micelles. High-molecular-weight biosurfactants are particularly effective at stabilizing emulsions between hydrocarbons and water, thus increasing the surface area available for bacterial biodegradation.[26]

Wardhani et al. noted that bacteria capable of degrading hydrocarbons can utilize petroleum as a carbon source. These bacteria, known as hydrocarbonoclastic bacteria, thrive in environments where hydrocarbons serve as a carbon source. The petroleum clumps observed during the incubation period are a result of bacterial activity.[19] Umar et al. further explains that biosurfactants are surface-active compounds containing carbohydrates, fats, and proteins. In the culture medium, precipitates in the form of granules or lumps often adhere to the bottom of the Erlenmeyer flask due to the secretion of metabolites by bacteria, which allow them to attach to and encapsulate hydrocarbon compounds.[27]

The results after 30 days of incubation, as shown in Figure 2, indicate that the culture supernatant significantly reduces surface tension. This is evidenced by the marked decrease in surface tension, which was initially 0 mN/m without the addition of the supernatant. Upon adding the culture supernatant, the surface tension reduction reached a substantial value of 22.16 mN/m at T(20), whereas the control supernatant exhibited a much lower reduction, with a maximum value of 1.5 mN/m at T(10). These findings demonstrate that the bacterial isolates from Paotere Port are capable of producing biosurfactants, which effectively lower the surface tension between water and oil. According to Ghasemi et al. biosurfactants produced by bacteria are amphiphilic molecules, consisting of both hydrophilic and hydrophobic components. These molecules accumulate at interfaces, thereby reducing the surface and interfacial tension between aqueous solutions and immiscible phases. Biosurfactants are naturally occurring surface-active compounds synthesized by a variety of microorganisms.[28]

Several studies have demonstrated the effectiveness of bacterial biosurfactants in promoting petroleum degradation. For example, Ghorbani et al. reported a surface tension reduction of 26.8 mN/m using *Pseudomonas aeruginosa* PTCC 1340,[29] while Tripathi et al. achieved a 29 mN/m reduction with *Pseudomonas aeruginosa* IITR48.[30] Kalvandi et al. also observed a surface tension decrease of 36.5 mN/m and an emulsification index of 40.3% using *Bacillus* sp. SHA302.[31] In comparison, our study achieved a surface tension reduction of 22.16 mN/m and a higher emulsification index of 53.84%, aligning well with previous research and highlighting the effectiveness of the biosurfactants used in this study.

Biosurfactants are primary bacterial metabolites that enhance the solubility of hydrophobic compounds.[32] Hydrocarbons, which are highly hydrophobic and exhibit low water solubility, are particularly resistant to degradation. Microbes enhance the degradation of these hydrophobic compounds by releasing biosurfactants, thereby reducing interfacial tension. This process facilitates the dissolution, making them more available and easier to biodegrade. Biosurfactants are thus crucial for the bioremediation of oil spills in marine environments, as they increase the solubility of petroleum components and reduce the surface tension between oil and water.

Microbial surfactants are soluble in both organic (nonpolar) solvents and aqueous (polar) solvents, and they are categorized based on their chemical structure and microbiological source. These categories include glycolipids, lipopeptides, proteins, phospholipids, polysaccharide-protein complexes, lipopolysaccharides, neutral lipids, and fatty acids. The utilization of biosurfactant-producing microbes holds significant potential as a promoter for hydrocarbon degradation.[33]

The GC-MS analysis revealed the formation of two distinct chromatograms with different peak patterns, as shown

Table 2. Surface tension reduction and emulsification index.

Organisms	Hydrocarbon	Surface tension (mN/m)	Emulsification (%)
This study	Petroleum	22.16	53.84
[29]*Pseudomonas aeruginosa* PTCC 1340	Sunfower oil	26.80	-
[30]*Pseudomonas aeruginosa* IITR48	Crude oil	29.00	-
[31]*Bacillus* sp. SHA302	Petroleum	36.50	40.30

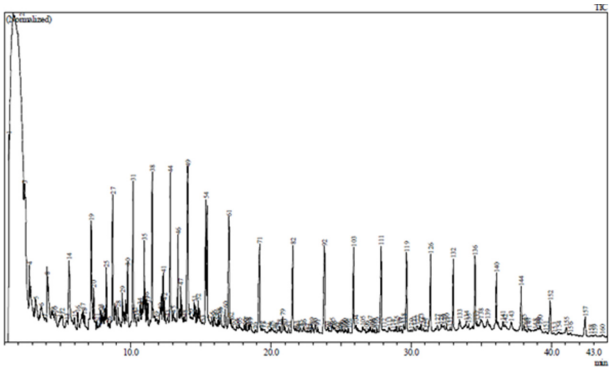

Figure 3. Initial petroleum chromatogram.

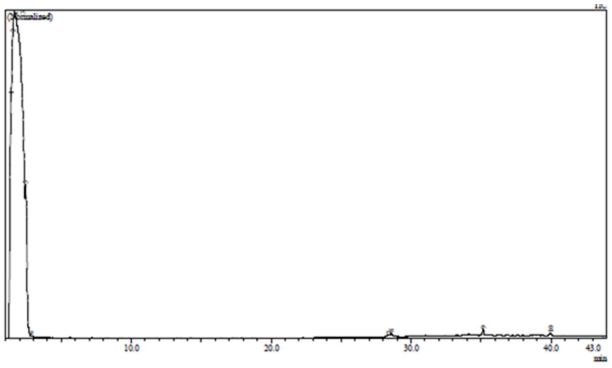

Figure 4. Petroleum chromatogram after 30 days of hydrocarbonoclastic bacteria inoculation.

in Figures 3 and 4. Figure 3 presents the control chromatogram, which indicates that all the carbon chains in petroleum remained intact. In contrast, Figure 4 displays the chromatogram of the sample, showing a significant reduction in the number of detectable carbon chains. The control chromatogram shows that carbon chains were detected up to 43.633 minutes, representing a highly complex mixture of approximately 160 carbon chains. Meanwhile, the sample chromatogram reveals that carbon chains were detected only up to 39.981 minutes, corresponding to just 10 carbon chains. This stark difference demonstrates the significant potential of the bacterial isolate obtained from Paotere Port in degrading petroleum, as it effectively broke down 150 carbon chains.

The 150 carbon chains that were degraded included alkanes, alicyclic, and aromatic hydrocarbons, while the remaining 10 undegraded carbon chains were classified as aromatic hydrocarbons. The degradation of hydrocarbons in petroleum does not occur simultaneously or uniformly; instead, it proceeds sequentially. Alkanes, particularly those with short, linear chains, are typically the first to degrade, followed by branched alkanes, cycloalkanes, and finally, aromatic compounds.[34] Aliphatic hydrocarbons, or n-alkanes, are the easiest to degrade due to their simple structure. Alkanes are the simplest type of hydrocarbons, consisting entirely of single bonds with hydrogen atoms. Hydrocarbons in the C12 to C25 range, however, require biological processes for degradation because their long chains cannot be broken down solely by physical factors.[35]

Aromatic hydrocarbons, on the other hand, are more challenging to degrade due to their benzene-based ring structure. The degradation of polycyclic aromatic hydrocarbons (PAHs) begins with the emulsification of petroleum pollutants by biosurfactants secreted by microorganisms.[36] These emulsified hydrocarbons are then adsorbed onto the surfaces of microorganisms. Once adsorbed, the hydrocarbons are transported into the microbial cell membrane through active or passive transport mechanisms. Inside the cell, the hydrocarbons undergo enzymatic reactions, facilitated by specific enzymes, which further degrade the pollutants and form emulsions.[37]

Conclusions

The emulsification index test, surface tension test, and qualitative biodegradability analysis using GC-MS demonstrate that bacterial isolates from Paotere Port possess a strong capacity for hydrocarbon degradation. This is indicated by a high emulsification index of 53.84%, a substantial reduction in surface tension to 22.16 mN/m at, and the bacteria's ability to break down carbon chains, as confirmed by GC-MS analysis. The presence of these bacteria in the petroleum-contaminated environment of Paotere harbor suggests they are highly adapted to survive under extreme conditions. In microcosm-scale experiments, these isolates exhibited significant petroleum biodegradation capability, supporting their potential for practical applications. These findings provide a foundation for the large-scale production of biosurfactants, which could be vital in addressing environmental challenges associated with oil pollution.

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