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Research Article

# Exploration of Indigeneous Biosurfactant-producing *Bacillus* sp. from Petroleum Hydrocarbon-contaminated Soil in Indonesia

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## Abstract

Hydrocarbonolastic bacteria are valuable for the remediation of petroleum-polluted environments. The production of biosurfactants by such bacteria significantly enhances hydrocarbon bioavailability and increases remediation efficiency. One genus of hydrocarbonolastic bacteria with the potential to produce biosurfactants is *Bacillus*, yet strain-level diversity and local adaptations remain underexplored.Tthis study aimed to isolate and characterize hydrocarbonolastic bacteria belonging to the *Bacillus* genus from soil samples collected at Geopetroleum Teksas Wonocolo, Kedewan District, Bojonegoro Regency, Indonesia, which is a historically underreported hydrocarbon site. Additionally, the capacity of *Bacillus* to synthesize biosurfactants was examined. *Bacillus* strains capable of biosurfactant production were identified via a polyphasic approach. A total of eight hydrocarbonolastic isolates from the *Bacillus* genus demonstrated biosurfactant-producing capabilities. The top three isolates, *Bacillus* sp. DIA08, *Bacillus* sp. DIA12, and *Bacillus* sp. DIA13, exhibited superior biosurfactant properties, effectively reducing the surface tension of the culture supernatant from an initial value of 72.00 mN $m^{-1}$ (distilled water) to below 20 mN $m^{-1}$ and achieving emulsification activity exceeding 50% when kerosene was used as the hydrophobic phase. Kerosene is used because it is a common substrate that is often used in emulsification index tests so that biosurfactant evaluation can be efficient and consistent and can be compared between studies. These three selected *Bacillus* sp. isolates were subjected to polyphasic identification through the integration of phenotypic and genotypic characteristics. The results revealed the presence of *Bacillus pacificus* DIA08, *Bacillus cereus* DIA12, and *Bacillus thuringiensis* DIA13. The biosurfactants produced by these three isolates show significant potential as effective agents for remediating petroleum hydrocarbon-contaminated soils. This study is the first to report *B. pacificus* DIA08 and *B. thuringiensis* DIA13, which were isolated from Indonesian soils with high biosurfactant activity, suggesting their potential as novel bioremediation agents. These findings contribute to the expansion of microbial resources for sustainable soil restoration technologies.

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## Introduction

In The demand for petroleum continues to rise alongside economic progress during the era of globalization and industrialization. In recent decades, petroleum usage has become increasingly widespread. Activities such as petroleum exploration, extraction, and transportation

frequently lead to environmental contamination, particularly soil pollution [1–4]. Moreover, this issue poses risks to human health, with effects including carcinogenic, mutagenic, teratogenic, and neurotoxic outcomes [5–6]. Recent reports indicate a yearly increase in petroleum-related soil contamination in Indonesia, especially in the Wonocolo area and on Bintan Island [7]. In response to these adverse impacts, various measures have been implemented, including the extensive use of chemical treatments, physical methods, or a combination of both. However, these applications fail to completely eliminate pollutants and often result in repeated damage to the soil ecosystem, making them appropriate only for emergency applications [8].

The biodegradation of petroleum pollutants by hydrocarbonoclastic bacteria in soil environments has emerged as a promising strategy over the past decade. In contrast, microbial bioremediation using hydrocarbonoclastic and biosurfactant-producing bacteria has emerged as a sustainable and ecologically viable alternative. Petroleum-contaminated soils harbor biosurfactant-producing bacteria that utilize hydrocarbons as a carbon source by altering the permeability of their cell membranes [9]. Biosurfactants are secondary metabolites synthesized by bacteria that reduce surface tension and facilitate the formation of microemulsions [10–13]. These compounds enhance bioremediation efficacy by increasing the bioavailability and solubility of hydrocarbon pollutants [14–15]. The *Bacillus* genus includes bacteria capable of producing potent surfactants such as lipoproteins, surfactin, fengycin, and lichenysin, which are the primary types of biosurfactants [16-19]. Currently, biosurfactant-producing bacteria can be isolated from petroleum hydrocarbon-contaminated soils; however, data on the isolation of biosurfactant-producing *Bacillus* strains in the Kedewan District, Bojonegoro Regency, East Java, Indonesia, remain limited.

This study aimed to accomplish three main objectives: (1) to isolate and screen hydrocarbonolastic bacteria of the *Bacillus* genus from petroleum hydrocarbon-contaminated soil, (2) to characterize biosurfactant production by *Bacillus*, and (3) to conduct polyphasic identification of biosurfactant-producing *Bacillus* strains.

## Materials and methods

### 1) Sampling site and collection of soil samples

Bacteria were isolated from crude oil-contaminated soil at the Teksas Wonocolo Geopetroleum site, which is located in Kedewan District, Bojonegoro Regency, East Java, Indonesia, at coordinates of 7°02'26.9"S 111°39'32.7"E. A map of the sampling location is shown in Figure 1. Crude oil-contaminated soil samples were collected from a depth of 10 cm below the surface and placed into sterile ziplock plastic bags. Upon arrival at the laboratory, the samples were stored in ice boxes and refrigerated at 4°C.

### 2) Isolation *of hydrocarbonoclastic Bacilus*

Bacillus strains capable of producing biosurfactants were isolated via stone mineral salt solution (SMSS) supplemented with crude oil as a carbon source following standard procedures with slight modifications [20–22]. The SMSS media used in this study contained (g $L^{-1}$) 0.05% $KH_2PO_4$, 0.1% $K_2HPO_4$, 0.05% $MgSO^4 \cdot 7H_2O$, 0.01% KCl, and 0.01% $FeSO_4$ [23]. The pH of the medium was adjusted to 7 with 1 M HCl. One gram of the soil sample was added to 100 mL of sterile distilled water in a 250-mL Erlenmeyer flask and allowed to settle. The soil suspension was then heated at 80°C for 30 min [24] to eliminate all nonspore-forming bacteria, enabling the isolation of spore-forming Bacillus strains. One milliliter of the treated suspension was inoculated into SMSS medium supplemented with 1% crude oil and 0.005% Tween 80 and incubated at 37°C for 7 days with agitation at 130 rpm. The addition of Tween 80 at low concentrations functions as a nonionic surfactant that can increase the bioavailability of hydrophobic hydrocarbon compound components, thereby increasing the growth and metabolic activity of *Bacillus* strains that degrade hydrocarbons. Following incubation, the culture was serially diluted from $10^{-4}$ to $10^{-6}$. One milliliter of each dilution was inoculated into SMSS medium agar plates via the pour plate method and incubated at 37°C for 48 hours. Bacterial colonies that developed on SMSS medium presented morphological and physiological characterristics typical of hydrocarbon-degrading bacteria, including members of the genus *Bacillus*. The isolate was presumptively categorized as *Bacillus* on the basis of its morphological and phenotypic characteristics, including gram-positive staining, rod-shaped cell morphology, and the appearance of white or cream-colored colonies on SMSS media. The isolates were subsequently transferred to fresh media until pure bacterial cultures were obtained. Definitive genotypic identification was later confirmed via 16S rRNA gene sequencing

### 3) Screening and characterization of biosurfactant-producing *Bacillus*

The biosurfactant production potential of the isolated hydrocarbonoclastic *Bacillus* strains was subsequently evaluated and characterized via three specific tests: emulsification activity, surface tension measurement, and hemolytic activity assessment. The *Bacillus* isolates were cultured in SMSS media and incubated at 30°C for 5 days with continuous agitation at 120 rpm. After incubation, the cultures were centrifuged at 8,000 rpm for 10 min at 4°C. Biosurfactant screening was conducted through both quantitative and qualitative assessments of the supernatant containing metabolites produced by the bacterial isolates. The supernatant metabolites were examined via two methods, emulsification activity (E24) and surface tension analysis, and one qualitative method, the blood hemolysis test.

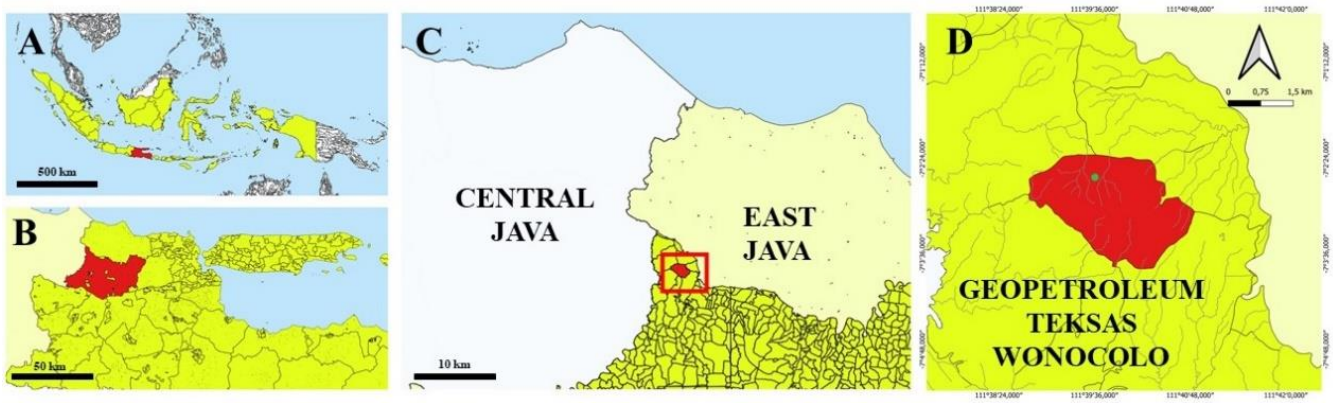


**Figure 1** Map of the sampling locations: (A) Unitary State of the Republic of Indonesia; (B) Bojonegoro Regency, East Java; (C) Kedewan District; (D) Geopetroleum Teksas Wonocolo, East Java, Indonesia

Hemolytic activity was assessed via the use of blood agar media. The *Bacillus* isolates were spot inoculated onto the agar surface via a sterile loop and incubated at 37°C for 48 hours. A clear zone around the colony indicated a positive result [25]. Emulsification activity (E24) was evaluated by adding 2 mL of the cell-free supernatant to a test tube containing 2 mL of kerosene. Kerosene is used as a hydrophobic substrate commonly used in emulsification index (E24) testing so that the evaluation of biosurfactant efficiency is consistent and comparable between studies [13]. The mixture was stirred for 2 min, and the emulsion layer was measured after 24 hours. Tween 80 was used as a positive control. The SMSS medium used for cultivation contained 0.005% Tween 80, a nonionic surfactant whose potential effects on the emulsification index (E24), surface tension, and hemolytic activity were carefully considered. Therefore, SMSS medium without inoculum but still containing Tween 80 was used as a baseline control in all tests. The emulsification index (E24) was calculated via Eq.1.

$$E_{24} = \frac{\text{Height of the emulsion layer}}{\text{Total height of mixture}} \times 100\% \qquad \text{(Eq.1)}$$

Surface tension measurements of the cell-free supernatant derived from biosurfactant production were performed via a surface/interface tensiometer and the Du-Nouy ring method. Each analysis was repeated five times to increase the reliability and precision of the collected data.

### 4) Polyphasic identification of selected biosurfactant-producing *Bacillus*

Hydrocarbonolactic *Bacillus* species capable of producing biosurfactants were identified via a polyphasic approach. This identification method involved both phenotypic and molecular characterization. Phenotypic traits were assessed through morphological, physiological, and biochemical analyses. Morphological characterization included both macroscopic and microscopic observations. Macroscopic traits included colony size, color, edge, surface, and elevation, whereas microscopic analysis was conducted via Gram staining. The physiological characteristics included motility and sulfide production. The biochemical characterization methods involved testing for catalase activity, starch hydrolysis, sugar fermentation ability, gas production, $H_2S$, indole formation, and citrate utilization. These characterizations followed the procedures outlined in the Manual for the identification of medical bacteria and Bergey's Manual of Determinitive Bacteriology [26–27].

Genotypic characterization was carried out through molecular identification of the 16S rRNA gene. The genomic DNA of the selected biosurfactant-producing *Bacillus* isolates was extracted via the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research Corp., Irvine, CA, USA) following the manufacturer's protocol. The following universal primers were used for amplification: 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) as the forward primer and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) as the reverse primer. A total of 2.5 µL of genomic DNA was combined with 25 µL of MyTaq™ HS Red Mix and 2 µL each of the forward and reverse primers. The volume was brought to 50 µL with nuclease-free water. The PCR amplification conditions included denaturation at 96°C for 45 seconds, annealing at 50–53.6°C for 60 sec, and extension at 72°C for 2 min.

The PCR products were examined via electrophoresis on 0.8% agarose gels stained with Florosafe DNA Staining BIO-5170. The results were visualized under UV light. The PCR amplification products were then sequenced. The resulting sequences were analyzed via BLAST (Basic Local Alignment Search Tool) from the National Center for Biotechnology Information through the BLASTN feature (http://www.ncbi.nih.gov/BLAST). A query coverage above 90% and the highest identity score were used to determine species-level identification, which was further verified with morphological and physiological characterization results. Following BLAST analysis, a phylogenetic tree was constructed. First, the resulting amplicons were aligned, and contigs were assembled via BioEdit Sequence Alignment Editor software for Windows. The 16S rRNA contig sequences

were then aligned with published 16S rRNA gene sequences of other bacteria available in GenBank. The partial sequence of the 16S rRNA gene was used to reconstruct the phylogenetic tree via distance matrices via the neighbor-joining model of MEGAX. Bootstrap analysis with 1000 replicates was performed to evaluate the reproducibility of the tree topology. *Escherichia coli* was included as an outgroup for phylogenetic analysis.

## Results and discussion

### 1) Isolation of hydrocarbonoclastic *Bacillus*

A total of eight hydrocarbon-degrading bacterial isolates obtained from the soil samples grew on media supplemented with crude oil. The identified isolates included *Bacillus* sp. DIA02, *Bacillus* sp. DIA03, *Bacillus* sp. DIA06, *Bacillus* sp. DIA08, *Bacillus* sp. DIA09, *Bacillus* sp. DIA11, *Bacillus* sp. DIA12, and *Bacillus* sp. DIA13. Each isolate showed the ability to degrade hydrocarbons on the basis of indirect testing in the form of the ability to grow on SMSS media with the addition of 1% crude oil as the sole carbon source and its ability to reduce surface tension and form microemulsions, as shown in Table 1. Additionally, *Bacillus* strains capable of growing on crude oil have shown potential for biosurfactant production, which enhances the solubility and dispersion of hydrocarbon compounds in the soil environment [28–29].

### 2) Screening *Bacillus* isolates for biosurfactant production

The screening results for biosurfactant-producing *Bacillus* isolates are shown in Table 1. The screening involved three methods: surface tension measurement, emulsification activity evaluation, and hemolytic activity assessment. All *Bacillus* isolates demonstrated a surface tension reduction from an initial value of 72 mN $m^{-1}$. A reduction of ≥10 mN $m^{-1}$ indicates biosurfactant production [32]. The presence of both hydrophobic and hydrophilic groups at the liquid interface reduces surface and interfacial tension [12, 33]. *Bacillus* sp. DIA03 showed the greatest reduction in surface tension, decreasing it from 72 mN $m^{-1}$ (initial surface tension of distilled water) to 13.60±0.12 mN $m^{-1}$, corresponding to an 81.1% reduction. In comparison, *Bacillus* sp. DIA12 and *Bacillus* sp. DIA13 reduced the surface tension to 23.62±0.08 mN $m^{-1}$ and 23.58±0.08 mN $m^{-1}$, respectively, corresponding to reductions of 67.2% and 67.3%. Although *Bacillus* sp. DIA03 achieved the greatest surface tension reduction, DIA12 and DIA13 exhibited greater emulsification activities, indicating distinct surfactant compositions or functional properties. However, this value does not surpass the results of other studies, where *Bacillus* species reduced the surface tension to between 25.65 and 27.29 mN $m^{-1}$ [34–35]. Since lower surface tension values indicate higher surface activity, the results obtained for *Bacillus* sp. DIA12 indicate that the biosurfactant it produces is highly efficient. This is particularly important in environmental applications such as petroleum bioremediation, where enhanced solubilization, emulsification, and mobilization of hydrophobic compounds are highly desirable [73–74]. The ability of *Bacillus* sp. DIA12 to lower surface tension to this level demonstrates its potential as a competitive biosurfactant candidate for industrial and environmental applications.

Emulsification activity refers to the ability of biosurfactants to form emulsions between two immiscible phases. This activity was evaluated over a 24-h period using the cell-free supernatant and kerosene. All the isolates except *Bacillus* sp. DIA02 exhibited emulsification activity. The biosurfactant produced by *Bacillus* sp. DIA02 is characterized as a high-molecular-weight compound that includes proteins, lipopolysaccharides, and lipoproteins [36]. This assumption is supported by previous studies reporting that members of the *Bacillus* genus, including *Bacillus cereus*, *Bacillus subtilis*, and *Bacillus licheniformis*, are capable of producing high-molecular-weight biosurfactants with emulsifying properties rather than low-molecular-weight surfactants that primarily reduce surface tension [13]. In contrast, the other isolates produced low-molecular-weight biosurfactants, mainly lipopeptides, which are characteristic of *Bacillus* species [37]. These low-molecular-weight biosurfactants, such as surfactin (a lipopeptide), possess both surface activity and emulsifying properties [38]. *Bacillus* sp. DIA12 demonstrated emulsification activity exceeding 60%, surpassing the previously reported 55% for *Bacillus mojavensis* [39]. The superior emulsification observed in the cell-free supernatant of *Bacillus* sp. DIA12 was attributed to its biosurfactant content. On the basis of these findings, it is plausible that *Bacillus* sp. DIA12, *Bacillus* sp. DIA08, and *Bacillus* sp. DIA13 produce similar macromolecular biosurfactants, although further characterization, such as gas chromatography–mass spectrometry (GC–MS) and Fourier transform infrared (FTIR) spectroscopy, is needed to confirm their chemical composition.

Hemolytic activity assessment via the blood agar lysis method involves inoculating bacterial cells onto a blood agar plate, followed by observation of clear zones in the medium [30]. Hemolytic activity is grouped on the basis of the type of hemolysis observed, namely, β-hemolysis is typically observed as a transparent zone surrounding bacterial colonies on blood agar, indicating the total breakdown of red blood cells, whereas α-hemolysis presents as a greenish halo resulting from partial erythrocyte degradation. β-Hemolytic activity is frequently employed as an indirect marker of potent biosurfactant secretion, given that biosurfactant molecules—such as glycolipids and lipopeptides—can disrupt the integrity of red blood cell membranes [75].

**Table 1** Screening of biosurfactant activity from biosurfactant-producing *Bacillus*

| Isolate | Reduce surface tension (mN/m) | % Reduction | Emulsification activity (%) | Hemolytic activity |
|---|---|---|---|---|
| *Bacillus* sp. DIA02 | 19.92 ± 0.21$^{e}$ | 72.3 | 00.00 ± 0.00$^{a}$ | β - type hemolysis |
| *Bacillus* sp. DIA03 | 13.60 ± 0.12$^{a}$ | 81.1 | 12.63 ± 2.67$^{b}$ | β - type hemolysis |
| *Bacillus* sp. DIA06 | 17.90 ± 0.15$^{d}$ | 75.1 | 00.42 ± 0.69$^{a}$ | α - type hemolysis |
| *Bacillus* sp. DIA08 | 22.97 ± 0.04$^{f}$ | 68.1 | 58.53 ± 0.75$^{d}$ | β - type hemolysis |
| *Bacillus* sp. DIA09 | 15.50 ± 0.29$^{b}$ | 78.5 | 10.46 ± 2.24$^{b}$ | α - type hemolysis |
| *Bacillus* sp. DIA11 | 17.66 ± 0.20$^{c}$ | 75.5 | 22.03 ± 11.75$^{c}$ | β - type hemolysis |
| *Bacillus* sp. DIA12 | 23.62 ± 0.08$^{g}$ | 67.2 | 65.17 ± 2.43$^{e}$ | β - type hemolysis |
| *Bacillus* sp. DIA13 | 23.58 ± 0.08$^{g}$ | 67.3 | 56.26 ± 5.41$^{d}$ | α - type hemolysis |

Furthermore, the presence of biosurfactants can be observed via hemolytic activity on blood agar plates, as biosurfactants cause erythrocyte lysis. This ability is due to their amphipathic nature, which allows them to penetrate and disrupt the lipid bilayer structure of the membrane [76–77]. Five *Bacillus* isolates, *Bacillus* sp. DIA02, *Bacillus* sp. DIA03, Bacillus sp. DIA08, Bacillus sp. DIA11, and *Bacillus* sp. DIA12, exhibited β-type hemolysis. In contrast, three isolates, *Bacillus* sp. DIA06, *Bacillus* sp. DIA09, and *Bacillus* sp. DIA13, demonstrated α-type hemolysis. The blood lysis method has been widely used for screening *Bacillus* subtilis mutants for surfactin production [30–31]. However, hemolytic activity can yield misleading positive results for partial or complete hemolysis. For example, although *Bacillus* sp. DIA13 showed partial (α-type) hemolysis, it recorded emulsification activity exceeding 50%. Therefore, relying solely on hemolytic activity does not provide a comprehensive assessment for initial biosurfactant screening.

Biosurfactants play a key role in the remediation of hydrocarbon pollutants by inducing bacterial detoxification, facilitating in situ bioremediation, and promoting the solubilization and desorption of soil pollutants. Additionally, biosurfactants act as emulsifiers, aiding the use of petroleum hydrocarbons as sources of energy and carbon, thus facilitating the conversion of hydrocarbons into nonharmful alternatives [29]. The ability of biosurfactants also plays an important role in hydrocarbon bioremediation by increasing the bioavailability of hydrocarbons and facilitating the degradation of petroleum and other aromatic compounds [29, 71].

### 3) Phenotypic characterization of hydrobarconoclastic *Bacillus*

The colony morphology of all Bacillus isolates was analyzed on the basis of shape, size, color, surface, margin, form, elevation, and Gram staining. Table 2 presents the morphological characteristics of the biosurfactant-producing *Bacillus* isolates. All the isolates were confirmed to be bacilli or rod shaped, as evidenced by positive Gram staining, as shown in Table 2. The characterization results were obtained from visual observations of colonies grown in SMSS supplemented with crude oil as the sole carbon source. The isolates showed variations in colony size, which ranged from pinpoint (*Bacillus* sp. DIA06, *Bacillus* sp. DIA09, and *Bacillus* sp. DIA11) to small (*Bacillus* sp. DIA08 and *Bacillus* sp. DIA12) and moderate (*Bacillus* sp. DIA02 and *Bacillus* sp. DIA03) sizes. While most colonies appeared cream in color, two isolates, *Bacillus* sp. DIA06 and *Bacillus* sp. DIA12, presented white coloration. The surface mor-phology also varied, with punctiform features observed in *Bacillus* sp. DIA02 and DIA03, convex elevation in *Bacillus* sp. DIA06, and circular forms in the remaining isolates. All the isolates presented entire margin characteristics, except for *Bacillus* sp. DIA03, which presented undulating margins. All the isolates presented smooth surfaces. These morphological differences in bacterial colonies arise from the interaction of genetic and environmental factors that drive evolutionary adaptation and phenotypic variation. Additionally, such variations reflect bacterial adaptations to extreme environmental changes and the formation of complex microbial communities [40]. Despite belonging to the same genus, all *Bacillus* isolates presented distinct colony morphologies, including shape, size, color, surface, margin, form, and elevation, as detailed in Table 2. This variability demonstrates the phenotypic diversity present among these isolates.

### 4) Physiological and biochemical characterization of biosurfactant-producing *Bacillus*

The physiological and biochemical characterization of biosurfactant-producing *Bacillus* strains is highly important in microbiology, as it forms the basis for bacterial identification at both the genus and species levels [41]. Understanding these characteristics helps reveal the physiological traits and environmental functions of bacteria [42]. In this study, the identification of biosurfactant-producing *Bacillus* sp. isolates was carried out at the species level, as shown in Table 3. This classical approach remains important and relevant, especially for distinguishing closely related *Bacillus* sp. isolates, particularly when used in conjunction with 16S rRNA gene sequence analysis. Phenotypic evidence in the form of physiological and biochemical characterization supports molecular identification, allowing accurate taxonomic placement within the genus *Bacillus* [81].

**Table 2** Characteristics of colony morphology from biosurfactant-producing *Bacillus*

| *Bacillus* sp. | Characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Shape** | **Size** | **Color** | **Form** | **Elevation** | **Margin** | **Surface** | **Gram** |
| DIA02 | Bacilli | Moderate | Cream | Punctiform | Raised | Entire | Smooth | + |
| DIA03 | Bacilli | Pinpoint | Cream | Punctiform | Convex | Undulate | Smooth | + |
| DIA06 | Coccobacilli | Small | White | Convex | Convex | Entire | Smooth | + |
| DIA08 | Coccobacilli | Pinpoint | Cream | Circular | Convex | Entire | Smooth | + |
| DIA09 | Coccobacilli | Pinpoint | Cream | Circular | Convex | Entire | Smooth | + |
| DIA11 | Bacilli | Small | Cream | Circular | Raised | Entire | Smooth | + |
| DIA11 | Bacilli | Pinpoint | White | Circular | Raised | Entire | Smooth | + |
| DIA13 | Coccobacilli | Moderate | Cream | Circular | Raised | Entire | Smooth | + |

**Table 3** The species of biosurfactant-producing *Bacillus*

| Isolate | Species | Accession no. | Identity (%) | Query cover (%) |
|---|---|---|---|---|
| *Bacillus* sp. DIA08 | *Bacillus pacificus* strain MCCC 1A06182 | NR_157733.1 | 99.56 | 100 |
| *Bacillus* sp. DIA12 | *Bacillus cereus* strain XT421 | PQ782762.1 | 99.19 | 100 |
| *Bacillus* sp. DIA13 | *Bacillus thuringiensis* strain MSP51 | MZ666875.1 | 95.18 | 95 |

Physiological and biochemical characterization also provided initial insights into bacterial taxonomy, but the variability observed among the biosurfactant-producing bacterial isolates in Table 4 made reliable classification at the genus level difficult. Therefore, molecular identification of 16S rRNA was performed to confirm the taxonomic affiliation of the isolates accurately, as this method remains the gold standard for bacterial identification because of its high resolution and phylogenetic relevance [81]. Moreover, such traits aid in the practical application of bacteria and their metabolites for environmental remediation. For example, *Bacillus* species (*Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens*) produce secondary metabolites such as biosurfactants, which can be utilized in the remediation of petroleum hydrocarbon pollutants in soil environments [10, 13, 43, 78–79]. The physiological and biochemical features of biosurfactant-producing Bacillus isolates are presented in Table 4.

Most isolates exhibited positive motility, as evidenced by visible bacterial movement in semisolid media or the spread of bacteria from the point of inoculation [44]. This motility is crucial for the role of biosurfactant-producing *Bacillus* in remediating petroleum hydrocarbon pollutants, as it facilitates bacterial dispersion across surfaces [10, 45]. Bacillus species possess flagella that enable movement over solid substrates and promote elevated migration above the surface, a behavior referred to as swarming motility [46].

All biosurfactant-producing *Bacillus* isolates tested positive for sulfide production in both Sulfide Indole Motility (SIM) medium and Triple Sugar Iron Agar (TSIA) medium. Sulfide-producing bacteria are important in the bioremediation of petroleum-contaminated environments, as sulfide increases the solubility of certain hydrocarbons, such as phenanthrene, naphthalene, and toluene, thereby increasing their bioavailability for degradation [47]. Additionally, the catalase test results revealed positive activity in all the isolates, with bubble production ranging from weak to strong. Catalase, an enzyme that detoxifies hydrogen peroxide, is essential for bacterial survival under oxidative stress [48]. This trait allows *Bacillus* to thrive in extreme environments such as hydrocarbon-contaminated soils, and when combined with biosurfactant production, it enhances diesel biodegradation efficiency rates, exceeding 80% within 15 days [49].

The TSIA medium is used to identify the bacterial fermentation of sugars such as glucose, lactose, and sucrose [50]. Most biosurfactant-producing *Bacillus* isolates ferment only glucose. However, *Bacillus* sp. DIA13 was able to ferment all three sugars present in TSIA. Gas production was also observed in *Bacillus* sp. DIA13, followed by *Bacillus* sp. DIA09 and *Bacillus sp*. DIA12. Indole production indicates the presence of the tryptophanase enzyme [51], which contributes to bacterial adaptation in environments with petroleum hydrocarbon contamination [52]. Most isolates tested positive for indole production, except for *Bacillus* sp. DIA06 and *Bacillus* sp. DIA13. The citrate utilization test yielded positive results for three isolates: *Bacillus* sp. DIA09, *Bacillus* sp. DIA12, and *Bacillus* sp. DIA13. This test evaluates the ability of bacteria to use citrate as their sole carbon source [53]. Certain *Bacillus* species, including Bacillus subtilis and Bacillus mojavensis, are capable of utilizing citrate as a carbon source, which supports the production of biosurfactants under nutrient-limited conditions [13, 54, 80].

### 5) Identification of polyphasic-producing *Bacillus*

The screening results revealed three *Bacillus* isolates with the highest biosurfactant production: *Bacillus* sp. DIA08, *Bacillus* sp. DIA12, and *Bacillus* sp. DIA13. The molecular identification of these isolates was further pursued via 16S rRNA gene sequencing. The purity values of the genomic DNA isolated from these three isolates were 1.83, 1.85, and 1.84, respectively, indicating that the

absence of contaminants such as polysaccharides and phenols could adversely affect DNA quality [55]. The measured DNA concentrations were 59.4 ng $\mu L^{-1}$, 165.4 ng $\mu L^{-1}$, and 84.6 ng $\mu L^{-1}$, respectively. The values exceeded the minimum concentration of 20–25 ng $\mu L^{-1}$ required for bacterial PCR, thereby facilitating efficient amplification and allowing flexibility in dilution for optimization [56].

Morphological and physiological characterization of the three Bacillus isolates indicated the need to confirm their species identity through 16S rRNA analysis. The electrophoresis results for the amplified 16S RNA gene revealed bands exceeding 1,500 bp, as shown in Figure 2. The Bacillus sp. DIA08 isolate exhibited 99.56% similarity to the Bacillus pacificus strain MCCC 1A06182, as presented in Table 3. While *Bacillus pacificus* has been isolated from crude oil-contaminated soil, it has a limited capacity to degrade long-chain alkanes [57]. However, studies on its biosurfactant-producing capacity remain scarce. Therefore, the biosurfactant-producing potential of *Bacillus pacificus* DIA08 remains largely unexplored, presenting a compelling avenue for further investigation.

**Table 4** Physiological and biochemical characteristics of biosurfactant-producing *Bacillus*

| Characterization | ***Bacillus* sp.** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **DIA02** | **DIA03** | **DIA06** | **DIA08** | **DIA09** | **DIA11** | **DIA12** | **DIA13** |
| **Physiological** | | | | | | | | |
| Motility | + | + | - | + | - | - | + | + |
| Sulfide | + | + | + | + | + | + | + | + |
| **Biochemical** | | | | | | | | |
| Catalase | ++ | ++ | + | ++++ | ++++ | ++++ | ++++ | ++++ |
| Starch hydrolysis | - | - | + | + | - | + | - | + |
| TSIA (Slant) | Base | Base | Base | Base | Acid | Base | Base | Acid |
| TSIA (Butt) | Acid | Acid | Acid | Acid | Acid | Acid | Acid | Acid |
| Gas production | - | - | - | - | + | - | + | + |
| $H_2S$ | + | + | + | + | + | + | + | + |
| Indol | + | + | - | + | + | + | + | - |
| Citrate | - | - | - | - | + | - | + | + |

**Note:** (1) Motility: +: visible growth spread at the puncture site; -: growth not visible spread at the puncture site. (2) Sulfide: +: there is black sediment at the bottom of the medium; -: there is no black sediment at the bottom of the medium. (3) Catalase: ++++: abundant bubble formation, +++: numerous bubbles, ++: medium bubbles, +: few bubbles, -: no bubbles. (4) Starch hydrolysis: +: clear zone formed around the bacterial colony; -: no clear zone formed around the colony. (5) Triple sugar iron agar (TSIA): base (slant)/acid (butt): able to ferment glucose only; acid (slant)/acid (butt): able to ferment all three sugars, namely, glucose, sucrose, and/or fructose; and base (slant)/base (butt): unable to ferment all three sugars. (6) Gas production: +: there are cracks in TSIA media; -: there are no cracks in TSIA media. (7) $H_2S$: +: black sediment at the bottom of the sulfide indole motility (SIM) media; -: no black sediment at the bottom of the SIM media. (8) Indol: +: there is a red ring at the top of the media; -: there is no red ring at the top of the media; (9) Citrate: the media color changes from green to blue, and there is no media color change.

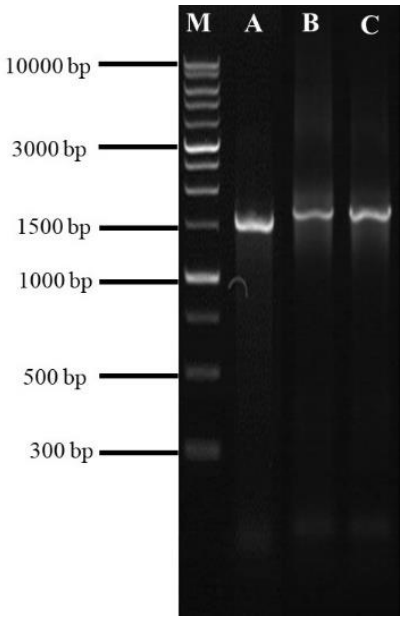


**Figure 2** Results for the 16S rRNA gene via the Basic Local Alignment Search Tool (BLAST) program.
Note: M: DNA marker, A: *Bacillus* sp. DIA08; B: *Bacillus* sp. DIA12; C: *Bacillus* sp. DIA13.

The *Bacillus* sp. DIA12 isolate showed 99.12% similarity with *Bacillus cereus* XT421, as presented in Table 3. Previous research reported that *Bacillus cereus* GX7 efficiently produced biosurfactants via water-soluble agricultural byproducts such as starch hydrolysate and wheat bran. Moreover, the biosurfactants produced significantly increased diesel oil degradation in seawater by 70.36% [58]. Furthermore, *Bacillus cereus* isolated from high-salinity soil demonstrated biosurfactant capability, enabling the removal of 60% of the oil from the oil-contaminated soil at a concentration of 3,200 ppm [59]. Local *Bacillus cereus* isolated from polluted waters of Surabaya (Indonesia) can significantly reduce hydrocarbon (diesel) concentrations after 14 days of bioremediation [60]. These findings collectively highlight the untapped biotechnological potential of *Bacillus cereus* DIA12, particularly in the sustainable remediation of hydrocarbon-polluted environments, and *Bacillus cereus* DIA12 is a promising candidate for bioremediation.

The *Bacillus* sp. DIA13 isolate exhibited 95.18% similarity to the *Bacillus thuringiensis* strain MSP51, as shown in Table 3. *Bacillus thuringiensis* (Bt) is widely recognized as a bioinsecticide because its spores contain crystalline (Cry) and cytolytic toxins (Cyt) [61–62]. However, recent studies have revealed that many *Bacillus thuringiensis* strains effectively degrade organic pollutants, including cyclic polyaromatic hydrocarbons (PAHs) and petroleum [62–63]. *Bacillus thuringiensis* strains utilize total petroleum hydrocarbons and cyclic PAHs as carbon and energy sources for synthesizing lipopeptide-type biosurfactants. For example, *Bacillus thuringiensis* strain S1 (MN180699) has biosurfactant potential that enables crude oil degradation [62, 64]. Conversely, *Bacillus thuringiensis* R116 has demonstrated efficacy in biodiesel degradation via coinoculation strategies [62, 65]. These findings highlight *Bacillus thuringiensis* DIA13 as a multifunctional microorganism, with *Bacillus thuringiensis* DIA13 emerging as a promising prospect for advanced studies in hydrocarbon bioremediation.

High-potential biosurfactant-producing *Bacillus* isolates, namely, *Bacillus pacificus* DIA08, *Bacillus cereus* DIA12, and *Bacillus thuringiensis* DIA13, were used to construct a phylogenetic tree, with *Escherichia coli* serving as the outgroup (Figure 3). Phylogenetic tree creation via the MEGAX application via the neighbor-joining method with 1,000 bootstrap replications to assess branching robustness. Phylogenetic trees help elucidate the relationships among species, provide a foundational framework for classification systems, and facilitate the investigation of the origins of new phenotypes and the mechanisms underlying biological evolution [66]. In this tree, *Escherichia coli* is positioned at the lowest branch, indicating that it is the most distantly related species among those analyzed.

The *Bacillus pacificus* DIA08 isolate closely clustered with the *Bacillus toyonensis* strain BCT-7112, suggesting a closer evolutionary relationship than the other isolates. *Bacillus toyonensis* is a member of the nonpathogenic *Bacillus cereus* group and is found in diverse environments, including deep-sea ecosystems, agricultural soils, tree cavities, and the gastrointestinal tracts of various animals. Although few studies have explored *Bacillus toyonensis* as a biosurfactant producer, this species is significant in pharmaceutical and agricultural manufacturing, healthcare, and industrial applications because of its ability to produce enzymes and antimicrobial compounds such as bacitracin and polymyxin [67–68].

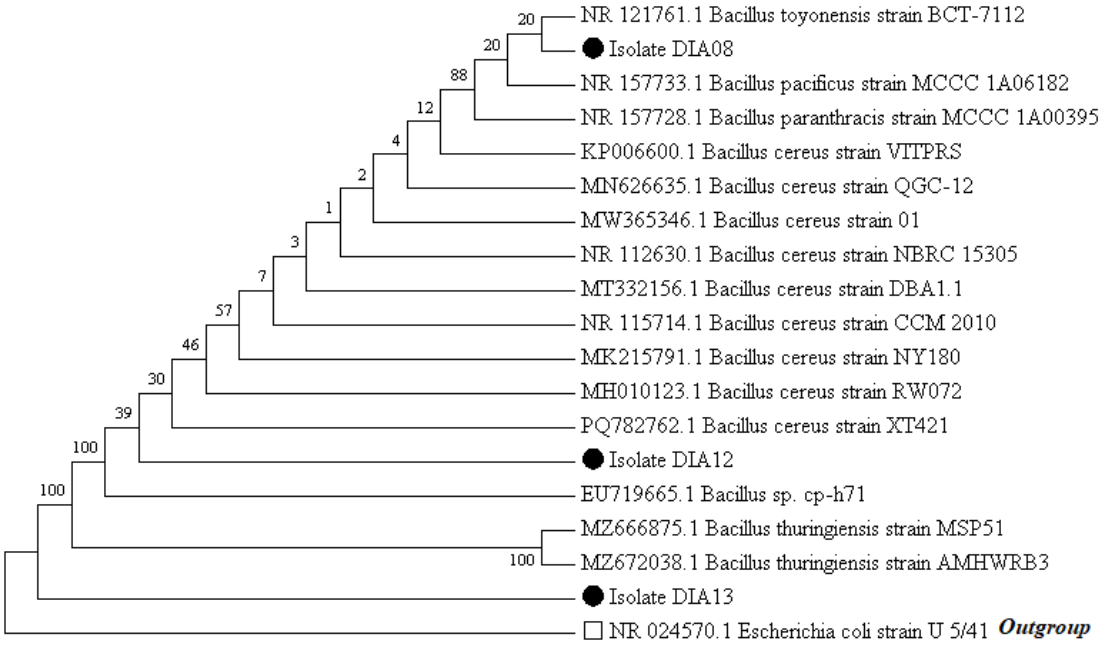


**Figure 3** Phylogenetic tree of biosurfactant-producing *Bacillus*

Similarly, *Bacillus cereus* DIA12 has a close evolutionary relationship with the *Bacillus cereus* strain XT421. While there is limited information on this strain's biosurfactant production capabilities, existing studies suggest that *Bacillus cereus* can effectively wash soil through the biosurfactants it produces, aiding in the removal of contaminants from polluted soils and sediments. In addition, *Bacillus cereus* effectively bioremediates hydrocarbons in polluted seas by reducing complex organic matter and converting hydrocarbons into shorter chains [60]. This ability is attributed to its environmentally friendly nature, low toxicity, and biodegradability. Moreover, biosurfactant adsorption to soil surfaces and complexation with metals reduce the interfacial tension, thereby facilitating the release of metals from soil particles [69].

The *Bacillus thuringiensis* DIA13 isolate exhibited a close phylogenetic relationship with *Bacillus thuringiensis* AMHWRB3. Although information about biosurfactants from *Bacillus thuringiensis* AMHWRB3 is scarce, this species produces a lipopeptide known as kurstakin, which is commonly used for remediating heavy metals in contaminated soil and water. Furthermore, kurstakin is exclusively synthesized by *Bacillus thuringiensis* and is effective in enhancing the phytoextraction process [70]. Additionally, *Bacillus thuringiensis* strain J1 has demonstrated significant potential in degrading petroleum hydrocarbon contaminants, with degradation rates ranging from 20.32% to 46.62% [62–63].

## Conclusions

Eight biosurfactant-producing *Bacillus* isolates were recovered from the soil samples, all of which demonstrated the ability to utilize crude oil as a carbon source. Among these, *Bacillus* sp. DIA08, *Bacillus* sp. DIA12, and *Bacillus* sp. DIA13 presented the highest biosurfactant activity, as indicated by marked surface tension reduction, a strong emulsification index (E24), and positive hemolytic activity. Using a polyphasic identification approach, three isolates were classified within the genus *Bacillus*: *Bacillus pacificus* DIA08, *Bacillus cereus* DIA12, and Bacillus *thuringiensis* DIA13. This is the first report of *Bacillus pacificus* DIA08 and *Bacillus thuringiensis* DIA13 from Indonesian petroleum-polluted soils, demonstrating strong biosurfactant potential. These findings highlight the underexplored microbial diversity in historically contaminated environments and introduce new microbial candidates for eco-friendly and sustainable bioremediation technologies. These isolates represent promising biotechnological resources for mitigating petroleum hydrocarbon pollution in terrestrial ecosystems.

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