ЭКОЛОГИЯ, ПОЧВОВЕДЕНИЕ И ПРИРОДОПОЛЬЗОВАНИЕ

ECOLOGY, SOIL SCIENCE AND NATURE MANAGEMENT

DOI: 10.12731/2658-6649-2025-17-6-1-1337 **EDN: KWGBLV**
UDC 579.68:628.547:504.054

Original article

ISOLATION AND IDENTIFICATION OF OIL-DEGRADING BACTERIA FROM OIL-CONTAMINATED MUDDY SOIL SAMPLES AT AUTOMOTIVE SERVICE STATIONS IN VIETNAM

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Abstract

Background. Oil pollution from vehicle maintenance and oil storage tanks at automotive service stations poses significant environmental challenges, affecting both soil and water ecosystems. Bioremediation is an effective and eco-friendly approach that utilizes microorganisms to degrade hydrocarbons in contaminated environments. Numerous indigenous bacterial species capable of hydrocarbon degradation have been studied for their potential application in pollution treatment.

Purpose. This study aimed to isolate and identify oil-degrading bacterial strains from oil-contaminated muddy soil samples collected from automotive service stations in Hanoi and Dong Nai, Vietnam. The objective was to evaluate their degradation efficiency and explore their potential application in bioremediation strategies.

Materials and methods. Four oil-contaminated muddy soil samples were collected from car wash areas and oil storage tanks in Hanoi and Dong Nai, Vietnam, in August 2024. In this study, we used methods such as: enrichment in GOST mineral medium supplemented with crude oil mixed in DO, isolation method, assessment

of oil degradation ability by gravimetric methods, OD_{600nm} measurement by UV-vis spectrophotometer, study of morphological characteristics and molecular identification of bacterial strains.

Results. From four oil-contaminated mud samples, after three enrichment cycles in a mineral medium supplemented with 5% (w/v) crude oil and diesel, sample M4 exhibited the highest oil degradation efficiency, achieving 80.12% removal after three enrichment cycles. Six representative bacterial strains were isolated on MPA agar from sample M4 and identified based on morphological and biochemical characteristics. Using molecular biological techniques, these hydrocarbon-degrading strains were identified as *Achromobacter xylosoxidans* ZB1.3 (PQ351236), *Ignatzschineria rhizosphaerae* ZB2.4 (PQ351237), *Stenotrophomonas acidaminiphila* ZB2.1 (PQ351238), *Brevundimonas diminuta* KN2.3 (PQ351239), *Aeromonas hydrophila* KN3.2 (PQ351240), and *Rhodococcus ruber* JN5.2 (PQ351241). The isolates, particularly strain JN5.2, demonstrated the ability to grow in a mineral medium supplemented with 1% oil after six days of incubation.

Conclusion. These results reveal the diversity of oil-degrading microorganisms and underscore the potential of indigenous microbial communities for self-remediation in oil-polluted environments, offering a sustainable and effective solution for environmental restoration.

Keywords: bacterial consortium; biodegradation; indigenous bacteria; oil-degrading bacterial; oil-contaminated muddy soil

For citation. Nguyen, T. K. T., Do, T. T., Tran, T. T. T., Luong, T. M., Mai, Q. T., Nguyen, T. C., Nguyen, K. T., Phan, D. D., & Ngo, C. C. (2025). Isolation and identification of oil-degrading bacteria from oil-contaminated muddy soil samples at automotive service stations in Vietnam. *Siberian Journal of Life Sciences and Agriculture*, *17*(6-1), 153-167. https://doi.org/10.12731/2658-6649-2025-17-6-1-1337

Introduction

Oil pollution in soil and water at transport units is a significant environmental issue due to the extensive use of fuels, oils, lubricants, and other petroleum-based products in operations. Contamination can occur during large-scale vehicle maintenance, refueling operations, and transportation activities, leading to accidental spills, leaks from storage tanks, and improper disposal of waste materials [1]. In soil, oil pollution reduces fertility, alters physical structure, and inhibits plant growth due to the toxic nature of hydrocarbons. In aquatic environments, oil spills can form slicks on water surfaces, disrupt ecosystems, and harm aquatic life by reducing oxygen levels and introducing toxic compounds [2]. Additionally, oil pollutants can seep into groundwater, contaminat-

ing drinking water supplies and posing long-term risks to human health and biodiversity [3].

Remediation of oil-polluted environments is challenging and typically involves complex physical, chemical, and biological methods to restore ecosystem health and prevent long-term damage. Among these, bioremediation stands out as an eco-friendly and cost-effective approach, leveraging the natural capabilities of microorganisms to degrade oil pollutants [4]. Soil-dwelling bacteria capable of degrading hydrocarbons are highly diverse and abundant. They are commonly found in large concentrations in areas affected by oil pollution [5]. Zhang et al. isolated 38 bacteria by enrichment cultivation from oil-contaminated soils of an oil field in Daqing, China. Of which, 11 strains could degrade the total petroleum hydrocarbon (TPH) of diesel oil by more than 70% in 7 days [6]. Hydrocarbon-degrading bacteria play a crucial role in breaking down complex hydrocarbons into less harmful substances. The indigenous microbes such as *Achromobacter, Arthrobacter, Pseudomonas, Bacillus, Staphylococcus, Rhodococcus, Enterobacter*, ... utilize hydrocarbon contaminants as the sole carbon and energy sources based on their natural metabolic pathways [3; 7].

The effectiveness of bioremediation depends on several factors, including environmental conditions (e.g., pH, temperature, oxygen levels), the type of oil present, and the availability of essential nutrients like nitrogen and phosphorus [1]. Studies have demonstrated that introducing pre-selected strains of native microorganisms from contaminated environments can significantly enhance the cleanup process. Bento demonstrated that the effective and straightforward method for cleaning up diesel-contaminated soils using in situ bioremediation is to introduce a pre-selected strain of native microorganisms from their own surroundings [8]. Similarly, in aquatic environments, bioaugmentation with hydrocarbon-degrading bacteria has shown promise in accelerating the breakdown of oil spills and mitigating their ecological impact [9].

Given the critical role of indigenous microorganisms in bioremediation, isolating and studying their biological characteristics is essential for developing effective pollution treatment strategies. This study focuses on isolating bacterial strains capable of degrading crude oil and diesel oil from contaminated muddy soil samples collected near car washes and oil storage tanks in Hanoi and Dong Nai, Vietnam. The research aims to evaluate the degradation efficiency of these strains and identify indigenous bacteria with the potential to bioremediate oil-contaminated environments. From this, sustainable solutions can be proposed to mitigate oil pollution in both terrestrial and aquatic ecosystems.

Materials and methods

Materials and chemicals

Four oil-contaminated muddy soil samples were collected at depths ranging from 5 to 30 cm from areas near car washes and oil storage tanks at automotive service stations, where oil pollution due to discharge activities had occurred in Hanoi (Coordinates: 21.1152° N, 105.4555° E) and Dong Nai (Coordinates: 10.9080° N, 106.8832° E), Vietnam, in August 2024 (Figure 1). From each location, a minimum of three samples were collected randomly and mixed together to create a single pooled analytical sample. The samples were packed in sterile plastic bags and stored at 4 ˚C for transportation to the laboratory of Department of Biotechnology, Joint Vietnam - Russia Tropical Science and Technology Research Center.

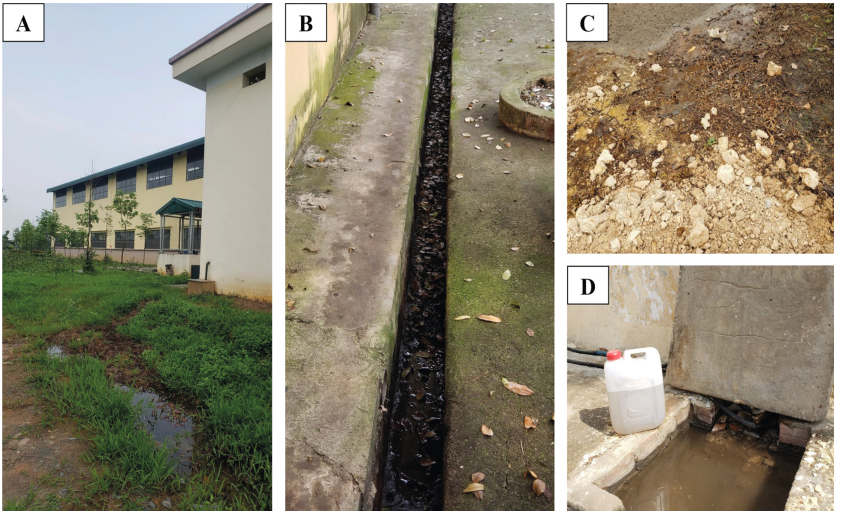

Fig. 1. Sampling locations for oil-contaminated muddy soil samples at areas near car washes and oil storage tanks in Vietnam. (A), (B), (C) HaNoi; (D) Dong Nai

Mineral salt medium (GOST 9023-74) contained the following: KNO_3 - 4 g; KH_2PO_4 - 0.5 g; Na_2HPO_4 - 1.4 g; $MgSO_4$ - 0.8 g, distilled water 1L, addition 5% crude oil mixed in DO (at a ratio of 5:95). The DO fuel used for enrichment and the petroleum products used for biodegradation experiments were collected from petroleum company (Petrolimex, Vietnam) [10].

The MPA modified medium for bacterial isolation and culture consisted of peptone 10 g/L, meat extract 5 g/L, NaCl 5 g/L, agar 20 g/L.

Analytical Methods

Enrichments and oil degradation ability of the bacterial consortium in oil-contaminated muddy soil samples

Oil-contaminated muddy soil samples were enriched in Erlenmeyer flasks containing GOST 9023-74 liquid medium supplemented with 5% crude oil mixed in DO in a ratio of 5:95 as the only energy and carbon source. The flasks were incubated on a rotary shaker at 30° C and 150 rpm for 7 days. After 7 days of incubation, the enriched culture was extracted and transferred to the same fresh medium at a ratio of 10% (v/v). After three enrichment cycles, the remaining crude oil from the culture broth was extracted by solvent extraction with n-hexane. After the solvent evaporated, the amount of residual oil was determined by gravimetric methods. The flask of mineral medium containing oil without added sample was used as control. The percentage of oil degradation was determined by the following formula [11]: Percentage of degradation = $((m_{control} - m_{residual})/m_{control})\times 100$, where $m_{control}$ is weight of the oil in control flasks after treatment (g), $m_{residual}$ is weight of the residual oil in testing flasks after treatment (g).

Isolation of oil-degrading bacterial strains

After three enrichment cycles, the total bacterial count was determined by plate counting on MPA-modified medium and incubated at 30°C for 24 hours. Single colonies with phenotypic differences (such as shape, size, color, edge, and texture of colonies, as well as cell shape and size) were streaked onto the same medium to obtain pure strains of oil-degrading bacteria. Then, the single colonies were transferred to MPA medium and stored at -20ºC in 20% glycerol for further study.

Growth conditions and crude oil removal test

The isolated bacterial strains were cultured in 500 ml Erlenmeyer flasks containing 250 ml of GOST 9023-74 medium supplemented with 1% (w/v) crude oil mixed in DO in a ratio of 5:95. The culture was incubated in a rotary shaker at 150 rpm and 30°C for 10 days. A control flask without microorganisms was maintained under the same conditions to serve as an abiotic reference. All experiments were carried out in triplicate. The growth of the isolates was regularly assessed indirectly by measuring the turbidity at OD600nm using a UV-vis spectrophotometer (Shimadzu UV-160, Japan) [12].

Morphological observation of isolated strains

The isolated colonies were incubated on MPA modified for 24-48 h and examined for morphological properties. The cell morphology of the isolated bacteria was observed under a light microscope (Zeiss Axiocam 503 Color Camera Unit) with a magnification of 1000x after Gram staining.

Molecular identification of isolates by 16S rRNA sequencing

The isolated strains were identified based on partial sequence analysis of the 16S rRNA gene. Extraction of genomic DNA from the bacterial isolates was done using Kit ZR Fungal/Bacterial DNA MiniPrepTM (Zymo Research, UK) according to the manufacturer's instructions. 16S rRNA genes were amplified by PCR using the universal primer pairs 27F (5′-AGAGTTTGATCATGGCT-CAG-3′, forward primer) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′, reverse primer). The product of PCR was sequenced by 1st Base Laboratories Sdn. Bhd., Malaysia. The 16S rRNA sequencing data were compared with the corresponding sequences of the strains registered on GenBank using the BLAST tool on NCBI (http://www.ncbi.nlm.nih.gov/). The Neighbor-Joining phylogenet-ic tree was constructed using MEGA X software based on 16S rRNA sequences

Data analysis

The results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical data processing was performed using Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA).

Results and discussion

Enrichment and selection of oil-degrading microbial consortia

After each enrichment in liquid mineral medium supplemented with crude oil mixed with DO, the oil degradation ability of the four samples was evaluated using the gravimetric method. The results in Figure 2 showed that sample M4 degraded the oil most effectively, followed by M2, M1, and M3, respectively.

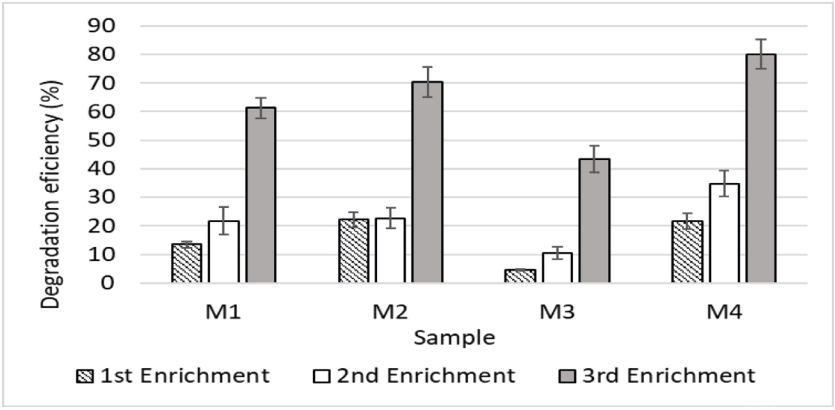

Fig. 2. Percentage of crude oil removal measured by gravimetric after each 7- day enrichment cycles

The indigenous bacteria gradually adapted to the mineral environment containing oil and utilized it as a nutrient-rich carbon source for growth, leading to a gradual increase in the amount of degraded oil over the three enrichment cycles. The results indicated that the best oil degradation performance was observed in the sample enriched for the third time. In the final enrichment, the microbial consortium in the samples degraded between 43.25% and 80.12% of the added oil. Sample M4 was chosen for further studies because it exhibited the highest oil degradation efficiency (80.12%).

Isolation and evaluation of oil-degrading ability of single bacterial strains

After a series of three further subcultures, the microbial population of sample M4 was determined by isolation on MPA medium. The bacterial density in the sample reached approximately 2.1×10^{11} CFU/mL, indicating the rapid growth of oil-degrading bacterial populations. This result is similar to the study by Tuyen et al. (2022), which found that the density of microorganisms in oil-contaminated soil samples collected in Hanoi after three enrichment cycles ranged from 9.3×10^{10} to 1.1×10^{12} CFU/mL [13].

From the third enrichment of sample M4, nine bacterial strains were isolated on MPA medium. Based on morphological characteristics, these nine strains were classified into six groups, including six representative strains with high abundance. These six strains were labeled as ZB1.3, ZB2.4, ZB2.1, KN2.3, KN3.2, and JN5.2 (Figure 3). The relatively large number of isolates after enrichment indicates the richness and diversity of oil-degrading microorganisms in sample M4.

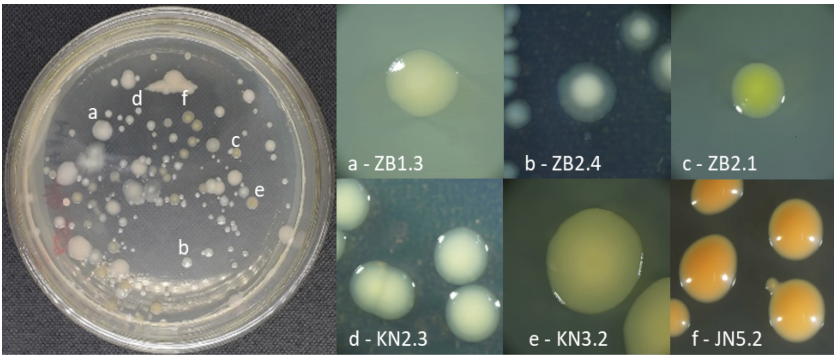

Fig. 3. Six representative bacterial strains isolated from M4 enrichment sample on MPA medium

The growth rate of the isolates in GOST 9023-74 medium containing 1% (w/v) crude oil mixed in DO in a ratio of 5:95 was determined by UV spectrometry at

OD_{600nm}. The growth curve of the strains isolated from M4 enrichment sample were depicted in Figure 4. During the incubation period, the medium gradually became turbid, indicating the growth of microorganisms. The OD_{600nm} of all the six strains on the first day was not obvious, which may be owing to adaption of the strains to the new environment. However, after three days of incubation, the OD_{600nm} of all strains exhibited a rapid increase, reaching its peak after approximately six days (OD_{600nm} values ranged from 0.2 to 1.25). This observation suggests that all six strains exclusively utilized the supplemented oil as their sole source of carbon and energy. In particular, strain JN5.2 demonstrated significantly enhanced growth, attaining a maximum OD_{600nm} value of 1.2, which was 2–3 times higher than that of the other strains. Thus, strain JN5.2 adapts well to an oil-rich environment, indicating its high potential for application in oil pollution treatment. Different bacterial strains demonstrated different growth rates in the oil-containing medium, highlighting differences in their hydrocarbon biodegradation potential. These differences could be attributed to the physiological activity of the strains, the presence of hydrocarbon-degrading enzymes, and their expression and activity [12].

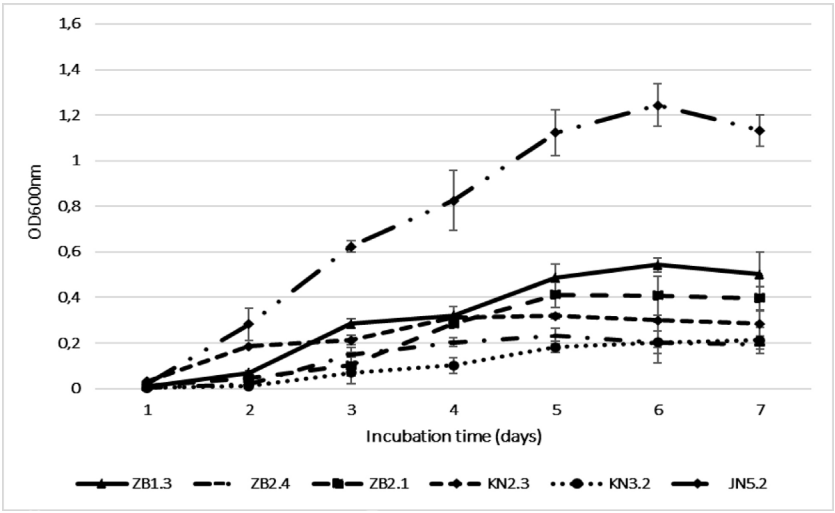

Fig. 4. The growth rates of six representative bacterial strains in mineral broth containing 1% of crude oil mixed in DO.

Morphological of oil-degrading bacteria

The six representative bacterial strains have significantly different morphological characteristics. These six strains were labeled and described as in

Table 1. The strains were creamy white or yellow in color, rod-shaped cells. 5/6 strains were Gram-negative bacteria, whereas JN5.2 was a Gram-positive bacterium (Table 1).

All bacterial strains grew well under neutral pH conditions, with a salt concentration of 0.5% and an average optimal growth temperature of approximately 30ºC. These growth parameters indicate the suitability of these strains for bacterial inoculation in the bioremediation of oil-contaminated soil and wastewater under natural ecological conditions.

Table 1.

Morphological characteristics of six selected oil-degrading bacterial strains on MPA medium

Isolates	Morphology of colony				Gram stain	Shape of cell
	Form	Color	Elevation	Margin		
ZB1.3	Irregular	pale yellow	Flat	Smooth	-	Rods
ZB2.4	Circular	Cream	Flat	Curled	-	Rods
ZB2.1	Circular	Lemon yellow	Flat	Smooth	-	Rods
KN2.3	Circular	Cream	Raised	Smooth	-	Rods
KN3.2	Circular	Cream Yellow	Flat	Smooth	-	Rods
JN5.2	Circular	Orange	Convex	Smooth	+	Rods

Identification of oil-degrading bacteria

Based on biochemical and physiological characteristics and phylogenetic analysis, the six isolated bacterial strains were identified as belonging to 6 different bacterial genera with the identity of more than 99% homology. The 16S rRNA gene sequences of six strains were identified and submitted to NCBI databases under the GenBank accession numbers: *Achromobacter xylosoxidans* ZB1.3 (PQ351236), *Ignatzschineria rhizosphaerae* ZB2.4 (PQ351237), *Stenotrophomonas acidaminiphila* ZB2.1 (PQ351238), *Brevundimonas diminuta* KN2.3 (PQ351239), *Aeromonas hydrophila* KN3.2 (PQ351240) and *Rhodococcus ruber* JN5.2 (PQ351241), respectively (Table 2). Based on the 16S rRNA sequences of six isolates, a phylogenetic tree was constructed by the Neighbor-joining method (Figure 5).

Numerous different bacterial genera were identified indicating that the sample had a wide biodiversity. The isolation of bacteria directly from oil contaminated sites as oily sludge and soil was reported in the references. The strains isolated in sample M4 have almost been studied for their ability to degrade petroleum hydrocarbon components.

From oil-contaminated environments, many bacterial strains belonging to the genera *Achromobacter, Stenotrophomonas, Brevundimonas,...* had been isolat-

ed and studied for their ability to degrade hydrocarbons [13-15]. According to Assih's publication in 2002, *S. acidaminiphila* was mesophilic bacteria isolated from a lab-scale upflow anaerobic sludge blanket (UASB) reactor treating a petrochemical wastewater [16]. This bacterial species has also been isolated from the oil-contaminated muddy soil in Hanoi, Vietnam [13]. *B. diminuta* isolated from oil-polluted seawater has been shown to utilize diesel oil as the sole source of carbon and energy and gave a biodegradation rate of 45% over 6 days under the salinity of 3.38% (w/w) [15]. In the study of Ranjan Pandey, a bacterial strain *A. hydrophila* RP1 that produces glycolipopeptide type biosurfactant was identified and isolated for the utilization in hydrocarbon degradation and EOR [14]. Notably, the bacterial strain JN5.2 demonstrated robust growth in oil-containing environments and was identified as closely related to *R. ruber*. This species has been extensively studied for its capacity to produce biosurfactants, as documented in numerous reports, and has been widely applied in Microbial Enhanced Oil Recovery (MEOR) technology [17; 18]. The *I. rhizosphaerae* had been exclusively isolated from the rhizosphere soil of the halophytic plant *Kalidium cuspidatum* in China [19]. To date, no studies have investigated its potential for oil or hydrocarbon degradation. These findings indicate that, with the exception of *I. rhizosphaerae* strain ZB2.4, which requires further investigation, the microorganisms isolated from sample M4 exhibit significant potential for application in the remediation of oil-contaminated environments, particularly *R. ruber* strain JN5.2.

Table 2.

Identification of six strains based on 16S rRNA sequences

Isolate	Accession number/ NCBI	Identification	Identity (%)	References on oil degradation ability
ZB1.3	PQ351236	*Achromobacter xylosoxidans*	99.93%	[20]
ZB2.4	PQ351237	*Ignatzschineria rhizosphaerae*	99.86%	no study
ZB2.1	PQ351238	*Stenotrophomonas acidaminiphila*	100%	[13, 16]
KN2.3	PQ351239	*Brevundimonas diminuta*	100%	[15]
KN3.2	PQ351240	*Aeromonas hydrophila*	100%	[14]
JN5.2	PQ351241	*Rhodococcus ruber*	99.85%	[17, 18]

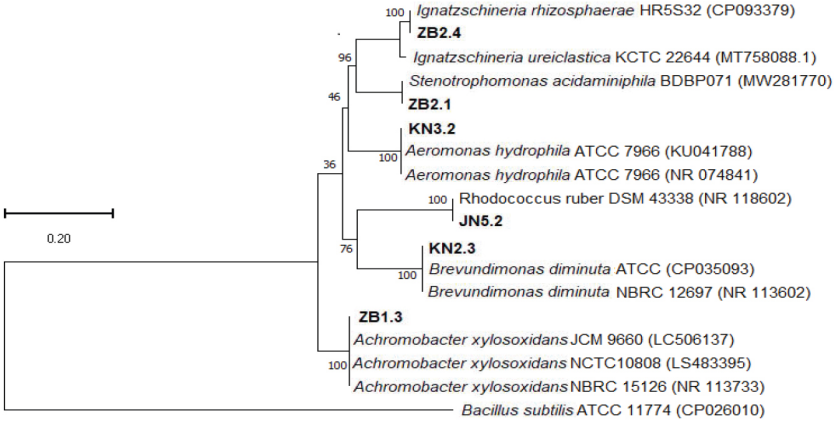

Fig. 5. Phylogenetic tree of the isolated strains based on 16S rRNA sequences using neighbour-joining method by MEGA X

The identification of indigenous microorganisms with potential biodegradation capabilities is crucial for the effective remediation of contaminated environments. The enrichment technique utilizing 5% crude oil in DO facilitated the isolation of bacteria capable of tolerating hydrocarbon residues. The selected strains exhibited enhanced adaptability to environmental stress and increased resistance to fluctuations in environmental conditions, making them promising candidates for bioremediation applications.

Conclusion

This study successfully isolated six indigenous bacterial strains with significant hydrocarbon-degrading capabilities from oil-contaminated soil near automotive service stations in Vietnam. Sample M4 showed the highest degradation efficiency, removing 80.12% of the added oil. The 16S rRNA gene sequences of the bacterial consortium were submitted to the NCBI GenBank database under the following accession numbers: *Achromobacter xylosoxidans* ZB1.3 (PQ351236), *Ignatzschineria rhizosphaerae* ZB2.4 (PQ351237), *Stenotrophomonas acidaminiphila* ZB2.1 (PQ351238), *Brevundimonas diminuta* KN2.3 (PQ351239), *Aeromonas hydrophila* KN3.2 (PQ351240), and *Rhodococcus ruber* JN5.2 (PQ351241). The strains, including *R. ruber* JN5.2, thrived in mineral environments, utilizing hydrocarbons as their sole carbon source, with optimal growth observed in 1% oil after six days. *R. ruber* JN5.2 exhibited exceptional adaptability, highlighting its bioremediation potential.

These findings emphasize the effectiveness of indigenous microorganisms in oil pollution mitigation and their promise for sustainable environmental restoration. Further research is recommended to optimize their application in large-scale bioremediation projects and explore their potential in other hydrocarbon-contaminated ecosystems.

Sponsorship information. This project is supported and implemented by the Laboratory of Microbiology - Department of Biotechnology/ Joint Vietnam - Russia Tropical Science and Technology Research Center under grant number SH.TH.01/24.

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AUTHOR CONTRIBUTIONS

Thi Kim Thanh Nguyen: study conception and design, drafting of the manuscript, and writing of the manuscript.

Thi Tuyen Do: study conception, data collection, and analysis, writing of the manuscript.

Thi Thanh Thuy Tran: sample collection and processing.

Thi Mo Luong: edit the draft of the manuscript.

Quang Tuyen Mai: sample collection and processing.

The Can Nguyen: sample collection and processing.

Khac Trinh Nguyen: sample collection and processing, editing of the draft of the manuscript.

Dinh Dai Phan: sample collection and processing.

Cao Cuong Ngo: Statistical data analysis, editing of the draft of the manuscript.

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Поступила 29.04.2025 Received 29.04.2025
После рецензирования 20.05.2025 Revised 20.05.2025
Принята 30.05.2025 Accepted 30.05.2025