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Isolation and Identification of Hydrocarbon Biodegrading Bacteria from Contaminated Soil in Misrata City/Libya

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Index Terms

Biodegradation, PCR, 16S rRNA, Emulsification index (E24), Bioremediation.

Abstract

Accumulation, contamination, and non-biodegradability of hydrocarbon in the environment are a global public health and ecological concern because of their persistent toxicity. Microbial degradation is the primary and most effective natural process for removing petroleum hydrocarbon contamination from hydrocarbon-contaminated environments. 15 contaminated soil samples were collected; 6 bacterial isolates of 45 different morphological types were selected for their high growth rates in CFMM containing 2% diesel or engine oil utilizing turbidity assay. Bacteria were identified using a standard microbiological method, Phoenix using the M50 automated system, and by PCR using universal 16S rRNA primers. Emulsification activity was assessed, and the emulsification index of selected isolates was determined after 24 hr. A plasmid-curing test was performed to show whether the oil-degrading gene is plasmid-encoded or chromosomally encoded. The isolated bacteria that revealed turbidity in a tube with growth on 2% diesel or engine oil CFMM agar plates were recorded as positive in respect to the degradation of oil or diesel compared to the control. The positive PCR result for the best degradative isolates confirms that they are bacteria with high emulsification activity. The isolates are Pseudomonas mendocino, Klebsiella pneumoniae, Bacillus sp., and Klebsiella and Moraxella species. The isolates in this study could be optimized to be used as patented bioremediation agents for hydrocarbon removal.

عزل وتعريف البكتيريا محللة الهيدروكربونات بيولوجيًا من التربة الملوثة في مدينة مصراتة/ليبيا

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الكلمات المفتاحية

التحلل الحيوي، PCR، 16S rRNA، مؤشر الاستحلاب (E24)، المعالجة الحيوية.

الملخص

يشكل تراكم الهيدروكربونات وتلوثها وعدم قابليتها للتحلل البيولوجي في البيئة مصدر قلق عالمي للصحة العامة والبيئة بسبب سميتها المستمرة. التحلل الميكروبي هو العملية الطبيعية الأساسية والأكثر فعالية لإزالة التلوث بالهيدروكربونات البترولية من البيئات الملوثة بالهيدروكربونات. تم جمع 15 عينة تربة ملوثة؛ تم اختيار 6 عزلة بكتيرية من 45 نوعًا مورفولوجيًا مختلفًا لمعدلات نموها العالية في CFMM التي تحتوي على 2% ديزل أو زيت محرك باستخدام اختبار التعكر. تم التعرف على البكتيريا باستخدام الطريقة المكروبيولوجية القياسية، فينيكس باستخدام النظام الآلي M50، وعن طريق تفاعل البوليميراز المتسلسل باستخدام بادئات 16S rRNA العالمية. تم تقييم نشاط الاستحلاب، وتم تحديد مؤشر الاستحلاب للعزلات المختارة بعد 24 ساعة. تم إجراء اختبار معالجة البلازميد لإظهار ما إذا كان الجين المحلل للزيت مشفرًا بالبلازميد أو مشفرًا كروموسوميًا. تم تسجيل البكتيريا المعزولة التي كشفت عن تعكر في أنبوب مع نمو على اطباق أجار CFMM من الديزل أو زيت المحرك بنسبة 2% على أنها إيجابية فيما يتعلق بتحلل الزيت أو الديزل مقارنة بالتحكم. وتؤكد نتيجة تفاعل البوليميرز PCR المتسلسل الإيجابية لأفضل العزلات المحللة أنها بكتيريا ذات نشاط استحلاب عالي. العزلات هي Pseudomonas mendocino، وKlebsiella pneumoniae، وBacillus sp.، وأنواع Klebsiella، وMoraxella. يمكن تحسين العزلات في هذه الدراسة لاستخدامها كعوامل معالجة حيوية حاصلة على براءة اختراع لإزالة الهيدروكربون.

1. INTRODUCTION

Hydrocarbon liberation in the environment from both accident spills of oils and industrial activities is a worldwide concern. The accumulation, contamination, and non-biodegradability of hydrocarbons in the environment made them persistent toxic materials, a global public health and ecological concern (Gaur et al., 2024; Bala et al., 2022; Bekele et al., 2022; Honda and Suzuki, 2020).

Microbial degradation is the primary and the most effective natural process for removing petroleum hydrocarbon contaminants from the environment. These microbes adapt to their surroundings, leading to mutation passing to the following generation that primes them to

break down hydrocarbon (X et al., 2018). The breakdown of a particular compound in complex hydrocarbons such as crude oil samples may be due to its bioactivities rather than its chemical structure. Hydrocarbon in the environment is primarily degraded by a variety of microorganisms, bacteria, and fungi with different biodegradation effectivities (Bala et al., 2022; Bekele et al., 2022). The ability of microbes to degrade hydrocarbons is genetically controlled by some genes or pathways (chromatid or plasmid link) (John and Okpokwasil, 2012); therefore, molecular identification of isolates is important. One of the most commonly used molecular techniques for bacterial isolates is PCR, which allowed for rapid and accurate detection using specific DNA sequences unique to the organism. 16S rRNA is highly conserved among bacteria and contains variable regions that provide specific signatures for bacterial identification (Weisburg et al., 1991; Wu et al., 2022). In this study, selected identified microbial isolates from soil contaminated by petroleum hydrocarbon were investigated for their petroleum hydrocarbon degradation activity. The selected isolates, which were identified by biochemical and molecular methods as bacteria, have hydrocarbon biodegradative activity.

2. Material and methods

2.1 Collection of soil samples:

Fifteen soil samples (each of 100 g) from three locations (Qasr Ahmed, Benghazi Street, and Kurzaz) in Misurata City, Libya, heavily contaminated with hydrocarbons (benzene and used engine oil), were collected in sterile polyethylene bags and kept at room temperature until used for isolation of hydrocarbon-degrading bacteria.

2.2 Isolation of hydrocarbon biodegrading bacteria:

The bacterial contents of hydrocarbon-contaminated soil samples were isolated by streaking soil suspension on nutrient agar and incubation for 24 h at 37°C. The ability of hydrocarbon biodegradation of isolated bacteria was carried out using the enrichment technique as described by (Makut et al. 2022). The isolate that revealed turbidity in the CFMM tube with 2% diesel or engine oil and grew on CFMM agar plates was recorded as positive in respect to the degradation of oil or diesel.

2.3 The hydrocarbon degradation potential of selected isolates:

The emulsification activity was assessed as described by (Thavasi, et al.2011) with some modification, and the emulsification index was determined after 24 h of incubation in a shaker incubator at room temperature and 135 rpm. Growth rates of selected hydrocarbon-degrading strains in CFMM containing 2% diesel were monitored for a 15-day incubation period utilizing turbidity assay. Optical density (OD600) was recorded at different intervals.

2.4 Genetic stability test:

A plasmid curing test was performed to show whether the oil degrading gene is plasmid encoded or chromosomal encoded, as described by (Sheikh, et al 2003) which depends on treatment of the bacteria with a low concentration of ethidium bromide and then retesting its ability to degrade the hydrocarbon. Colonies that were fielded to grow on 2% hydrocarbon CFMM agar were considered to have plasmid-encoding biodegrading genes.

2.5 Identification of bacterial isolates:

The isolates that revealed high biodegrading activity were chosen for biochemical identification by the BD Phoenix M50 instrument, which is an automated microbiology system that can identify Gram-positive and Gram-negative bacteria depending on the performance of many biochemical tests. In addition, a sensitivity test for all the isolates was performed using the disc diffusion method on Muller Hinton agar plates.

2.6 Amplification of bacterial 16s rRNA gene:

Bacterial genomic DNA was isolated from well-isolated single colonies that had been selected and inoculated in nutrient-rich broth at 37°C using the boiling method. A nanodrop spectrophotometer was used to evaluate the concentration and purity of extracted DNA. All DNA samples were electrophoresed on 1.5% ethidium bromide-stained agarose gel to check their integrity.

The 16s rRNA region of the rRNA genes of the isolates was amplified using the 27F (5'AGAGTTTGATCMTGGCTCAG3') and 1492R (5'TACGGYTACCTTGTTACGACTT3') primers on a mini-Amp Plus Thermocycler at a final volume of 40 µl for 30 cycles (Abellan-Schneider et al., 2021). The PCR mix included 20 µl master mix, 0.5 µl from each primer, 4 µl genomic DNA, and 15 µl deionized water. The PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds for 35 cycles, and final extension at 72°C for 5 minutes. PCR products were electrophoresed on 1.5% agarose gel in 1X TAE buffer and documented by the Gel Doc Go Imaging system. The size of bands was analyzed by comparing their position to the ZR 100 bp DNA marker.

3. Results and Discussion

3.1 Primary isolation of hydrocarbon biodegrading bacteria:

Hydrocarbon-utilizing bacteria were identified as petrophiles that, with their enzymes, oxidize the hydrocarbons and provide them as a simple carbon source for use in the ecosystem. In this study, the total contents from 15 hydrocarbon-contaminated soil samples from three regions in Misrata city were isolated, and the primary cultures revealed the presence of 45 different morphological microbes in these soil samples. The turbidity test revealed only seventeen isolates were able to utilize diesel and engine oil in CFMM with different growth rate.

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Increased optical density of bacterial growth in CFMM containing 2% of diesel has been used as a medicator for the strength of biodegradation in this study (Figure 1); therefore, it is classified as biodegraded (Walaszczyk and Jasiński, 2018) and 6 isolates were selected for farther identification.

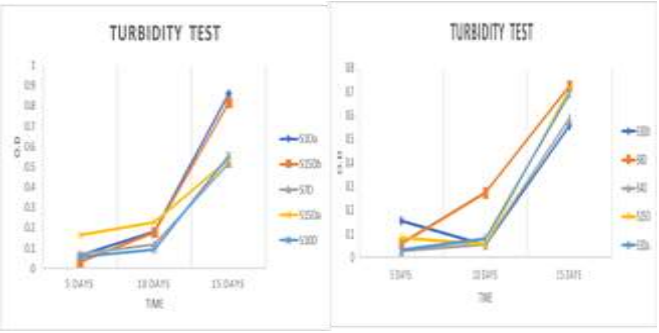


Figure 1: Measurements of some isolates' growth rate on carbon-free media with 2% diesel at different incubation times at OD600.

3.2 Hydrocarbon degradation potential of selected isolates:

The ability of bacterial strains to degrade hydrocarbon sources depends on their ability to produce appreciable biosurfactants (Thavasi et al., 2011; Walaszczyk and Jasiński, 2018; Makut et al., 2022). In this study all selected isolates showed good emulsification activity, which is the active part of the process for hydrocarbon source degradation (Figure 2:1,2). E24 % depended on isolates type fig.2:1 and emulsification activity increase by increase incubation time. Biodegradation occurs more rapidly when hydrocarbon is emulsified into small droplets by biosurfactant produced by microorganisms (Das and Chandran, 2011). Their emulsifying activity allows them to interact at the oil-water interface, enhancing the microbial uptake of petroleum hydrocarbons and utilizing them as a carbon source.



FIG. 2:1

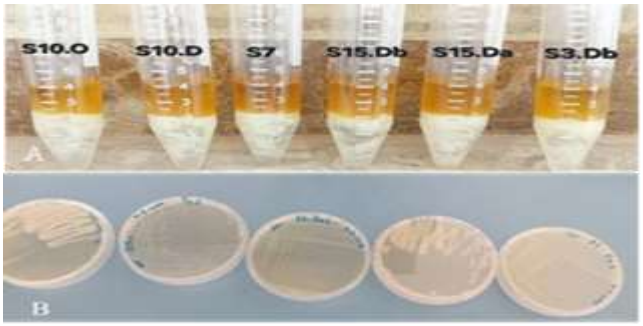


FIG.2: 2

Figure 2: Emulsification test results. FIG.2;1: isolates culture after 24h of inoculation. CFMM and diesel.fig.2.2 ; A: isolates culture after increase time off inoculation; .B: Isolates growth on nutrient agar.

*E24 (%) = (height of emulsion layer ÷ total height of liquid) × 100

3.3 Genetic stability test:

The ability of a bacterial strain to degrade hydrocarbon is genetically controlled by some gene and pathways (Varjani and Upasani, 2017). It is important to know if this activity is chromosomal linked or plasmid linked. Biodegrading activity is more stable if it is a chromosomal character that can pass from generation to generation without change. While a plasmid carries biodegradation activity, it may be lost by time because it is less stable and genotoxic, making it affected by environmental genotoxic chemicals (John and Okpokwasili, 2012). Five of 17 bio-degradative isolates did not grow in nutrient agar media after ethidium bromide treatments (genotoxic), which indicate they are plasmid carriers, and 12 isolates with positive growth show the chromosomal biodegradative ability of the tested hydrocarbon (Figure 3).

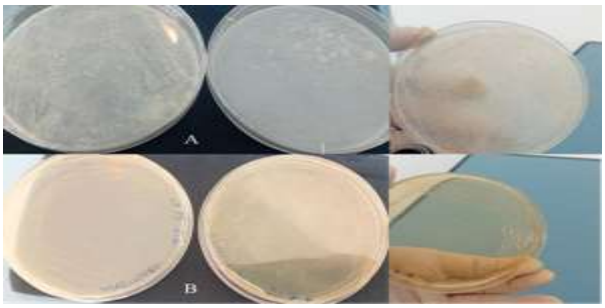


Figure 3: Biodegradative stability test: A: Isolates growth on CFMM. B: Isolate growth on nutrient media after ethidium bromide treatment.

3.4 Identification and antibiotic sensitivity test of bacteria:

The most active hydrocarbon biodegrading isolates were identified by the Phoenix M50 automated microbiology system, and they belonged to different genera mainly: Pseudomonas Mendocino, Klebsiella pneumoniae, Bacillus sp., Klebsiella, Cronobacter sakazakii, and Moraxella species. Gram stain revealed that 81% of isolates are gram-negative and highly

sensitive to most tested antibiotics, which makes them safe for human handling (Table 1). Gram-negative bacteria had high degradative potential to remove higher hydrocarbon molecules that persist in soil. Bacillus and Pseudomonas species are the most isolated and the most widely studied industry microorganisms (Widada et al., 2002; Bento et al., 2005; Althalb and Singleton, 2017), and the incidence of Gram-negative bacteria is always higher than that of Gram-positive bacteria.

3.5 Molecular identification of selected isolates:

DNA was extracted from seven of the most active bacterial isolates that showed maximum diesel-degrading capability. All the samples appeared as an intact band on the gel, indicating a good quality. Gram-positive bacteria have a thick peptidoglycan layer in their cell wall. Boiling may not effectively lyse these cells, leading to incomplete extraction of DNA (Habib et al., 2024). The partial 16S rRNA gene sequence of the selected seven isolates was amplified by using universal primers 27F and 1492R; one band was obtained at approximately 1500 bp (Figure 4). This product is evidence that our biodegradative isolates are bacterial type. PCR products will be sequenced in order to compare to the NCBI DNA sequences database using BLAST to confirm the species type and then verify the proximate phylogenetic position of these isolates

Samples	AK30	CN10	IPM10	TE10	NA30	AMC30	AX30	P1.5	VA5	Gram stain	CFMM Agar growth
S15Da (Klebsiella pneumoniae)	S	S	S	S	R	R	R	R	R	(-) gram	+
S10D (Pseudomonas mendocina)	S	S	S	S	S	S	S	R	S	(-) gram	++
S15Db (Cronobacter sakazakii complex)	S	S	S	S	S	S	R	R	R	(-) gram	+++
S10O (Bacillus spp.)	S	S	S	S	S	S	S	S	S	(+) gram	+++
S3Db (Moraxella species)	S	R		S	S	R	R	R	R	(-) gram	+++
OBD (Klebsiella pneumoniae)	R	S	S	S	S	S	S	R	R	(-) gram	+
S7 (Klebsiella species)	S	S	S	S	S	R	R	R	R	(-) gram	++

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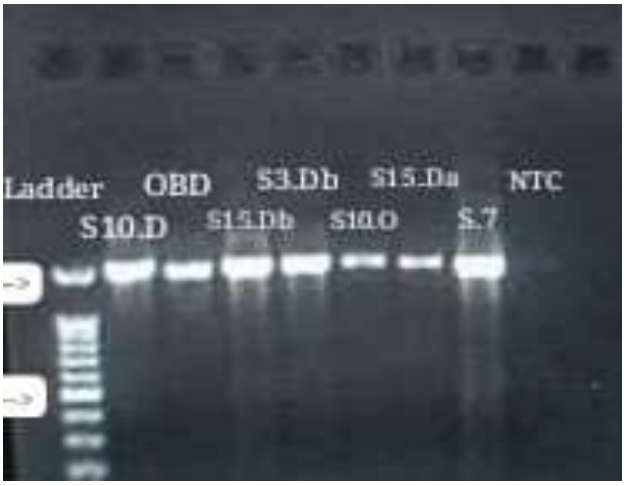


Figure 4: A: Extracted genomic DNA from selected hydrocarbon degrader bacteria. The DNA samples were visualized in 1.5% agarose gel electrophoresis and stained with ethidium bromide. B: The 16srRNA PCR product. The PCR products were visualized in 1.5% agarose gel electrophoresis. M ZR 100 bp DNA Ladder. S3Db: Moraxella species. S7: Klebsiella species. S15Db: Cronobacter sakazakii complex. S10D: Pseudomonas mendocina. S15Da: Klebsiella pneumoniae. S10 O: Bacillus spp. OBD: Klebsiella pneumoniae.

4. Conclusion and Recommendation

The study reported the presence of hydrocarbon-degrading bacteria in contaminated soils collected from three locations in Misrata. The selected isolates showed high growth density in 2% hydrocarbon CFMM with positive emulsification activity. The hydrocarbon-degrading activity was stable since it is chromosomally encoded in most of the tested isolates. Biochemical identification indicated that the active isolates belonged to several bacterial genera, including Klebsiella, Pseudomonas, Bacillus, and Cronobacter.

Further sequencing and comparison of the 16S rRNA sequences with reference sequences are recommended to confirm the taxonomic identity of these isolates and determine their phylogenetic relationships. In addition, more studies should also evaluate the degradation of different hydrocarbon compounds under diffident controlled conditions before considering the most active isolates for practical bioremediation applications.

TABLE 1: IDENTIFICATION AND SENSITIVITY TESTS OF SELECTED ISOLATES:

5. Acuanolodge

The authors wish to thank staph of Misurata university molecular research lab.

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