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Degradation of diesel oil by microorganisms isolated from lagoons of the "Totora" wastewater treatment plant, Ayacucho

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Abstract: The degradation of oil by microbial consortia isolated from maturation ponds of the Wastewater Treatment Plant "Totora," Ayacucho, was evaluated. Samples were collected from three ponds and transported to the Environmental Microbiology Laboratory - UNSCH. The microorganisms were isolated by inoculating 10 mL of sample in Bushnell-Haas medium with 4% v/v oil, incubated at room temperature with shaking for 21 days. They were then cultured on nutrient agar and Sabouraud agar, by surface exhaustion, incubated at 37°C and 25°C, respectively. The strains were grouped into three consortia by origin (LM1, LM2, and LM3; each composed of four bacterial strains and six mold strains), with which soils containing one, four, eight, and 16% oil were bioremediated in vitro, in plastic containers with one kg of agricultural soil, with three repetitions, each treatment inoculated with 10 mL (1.5 x 10^8 CFU/mL) of each strain of the consortium, with a blank per treatment without inoculant. They were evaluated every 14 days for 70 days, measuring residual oil by agitation-centrifugation and gravimetry. The LM2 consortium achieved better results, with oil removals of 100%, 100%, 92%, and 42% in soils with one, four, eight, and 16% oil, respectively. The strains of the LM2 consortium were: LM2A-1, LM2A-2, LM2B-1, and LM2B-2 (Gram-negative bacteria), LM2A-1 and LM2B-1 (Penicillium sp.), LM2A-2 (Pithomyces sp.), LM2A-3 (Wallemia sp.), and LM2A-4 and LM2B-6 (Geotrichum sp.). The ANOVA and Duncan tests indicate that there are significant differences ($p \leq 0.05$).
Keywords: *Bioremediation, Microbial consortia, Oil biodegradation.*

1. Introduction

Hydrocarbon contamination is a global environmental issue with widespread geographical distribution, primarily caused by oil spills that significantly impact both terrestrial and aquatic ecosystems. The transport and use of crude oil and its derivatives have led to an increasing number of spill incidents, affecting soil, water, and biodiversity [1]. These spills form a layer on water surfaces, obstructing sunlight penetration and disrupting photosynthetic processes, which in turn affects primary producers and contaminates entire food chains [2]. Moreover, crude oil deposits on land and water hinder the regeneration of plant and animal life, leading to severe ecological consequences.

At the international level, various approaches have been developed to mitigate the impact of hydrocarbon pollution. Physical, chemical, and biological methods have been employed to remove contaminants and minimize environmental damage [3, 4]. Among these, bioremediation has gained increasing attention as a sustainable and cost-effective strategy. Research has demonstrated that certain microorganisms, including bacteria and fungi, can degrade petroleum hydrocarbons, converting them into less harmful substances [5]. Bacteria such as Pseudomonas, Agrobacterium, and Bacillus have

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shown promising results in breaking down aliphatic and polycyclic aromatic hydrocarbons [6]. Fungi, though not using hydrocarbons as a primary carbon source, can metabolize them through co-metabolism, with species such as *Cunninghamella elegans* and *Trametes versicolor* playing key roles in hydrocarbon degradation [6].

In Perú, hydrocarbon pollution remains a persistent problem, exacerbated by frequent oil spills along coastal and Amazonian regions. Since 1997, nearly 600 oil spills have been recorded, causing irreversible environmental damage and significantly impacting local biodiversity [7]. One of the most severe cases occurred on January 15, 2022, at the La Pampilla refinery, where approximately 12,000 barrels of crude oil were spilled into the Pacific Ocean [8]. This disaster led to the declaration of an environmental emergency, highlighting the inadequacies in both governmental and corporate responses to such crises [9]. The contamination of ecosystems and water sources poses a direct threat to communities that rely on marine and freshwater resources for their livelihood. Despite existing environmental policies, effective remediation measures remain insufficient, underscoring the urgent need for alternative biotechnological solutions.

Given this context, this study aims to evaluate the ability of microbial consortia isolated from the Totora Wastewater Treatment Plant lagoons in Ayacucho to degrade diesel oil. The research seeks to identify and characterize hydrocarbon-degrading microorganisms as a potential alternative for the bioremediation of hydrocarbon-contaminated environments. Addressing this issue is crucial, as microbial biodegradation presents a sustainable and efficient approach to mitigating hydrocarbon pollution. The central research question guiding this study is: To what extent can microbial consortia isolated from the Totora Wastewater Treatment Plant degrade diesel oil? Answering this question will contribute to the existing body of knowledge on bioremediation and provide valuable insights into the application of microbial biotechnology for environmental restoration.

2. Theoretical Framework

2.1. Conceptual Definition and Theoretical Foundations

Hydrocarbon contamination is a significant environmental issue caused by the release of petroleum and its derivatives into ecosystems. Petroleum hydrocarbons are classified into two main categories: aliphatic hydrocarbons, which include linear and branched compounds, and polycyclic aromatic hydrocarbons (PAHs), which consist of multiple fused benzene rings [10]. Due to their complex molecular structures, PAHs are particularly resistant to degradation and persist in the environment for extended periods [11]. Microbial biodegradation is one of the most effective strategies for removing hydrocarbons from contaminated environments. Bacteria such as Pseudomonas, Bacillus, and Agrobacterium play a key role in degrading aliphatic and aromatic hydrocarbons by utilizing these compounds as their primary carbon and energy sources [6]. Unlike bacteria, fungi rely on cometabolism to degrade hydrocarbons, meaning they require an additional carbon source to metabolize petroleum derivatives efficiently [12]. Research has demonstrated that fungal species like Trametes versicolor and Cunninghamella elegans exhibit PAH degradation capabilities, making them suitable candidates for bioremediation applications [13].

2.2. Previous Studies and Quantitative Findings

Numerous studies have reported the effectiveness of microbial consortia in hydrocarbon degradation. For instance, Medina, et al. [14] demonstrated that indigenous microbial populations in petroleum-contaminated soils in Venezuela achieved a biodegradation rate of up to 79% within 60 days under optimal conditions. Similarly, Bacosa and Inoue [15]found that microbial communities enriched from tsunami sediments in Japan degraded 65% of PAHs within 28 days. Another study by del Refugio Castañeda-Chávez, et al. [16] reported that diesel degradation efficiency by a microbial consortium reached 72.3% under controlled laboratory conditions. These findings highlight the potential of microbial biotechnology for hydrocarbon bioremediation. However, degradation rates vary depending on environmental factors such as temperature, oxygen availability, and microbial diversity [17]. In

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Peru, Llenque Díaz [18] identified hydrocarbon-degrading heterotrophic bacteria from petroleum-contaminated soils in Trujillo, emphasizing the importance of local microbial strains in bioremediation strategies.

2.3. General and Specific Theoretical Perspectives

Several theoretical models explain the biodegradation of hydrocarbons. One of the most widely accepted is the Biochemical Pathway Theory, which describes how microbial enzymes, such as oxygenases and peroxidases, catalyze the breakdown of hydrocarbons into intermediate compounds that are further metabolized into CO_2 and water [19]. A microbial consortium can perform complex functions that individual populations would be unable to achieve. Moreover, the interaction among its members not only enhances long-term stability but also provides greater resistance to environmental fluctuations [20].

Additionally, the Environmental Bioavailability Theory states that the rate of hydrocarbon degradation is influenced by the chemical properties of the pollutant, as well as environmental conditions such as pH, temperature, and nutrient availability [21]. These theories provide a comprehensive understanding of the factors that influence the efficiency of microbial hydrocarbon degradation, supporting the rationale for studying microbial consortia in bioremediation.

2.4. Theoretical Relevance

The review of literature demonstrates that microbial bioremediation is a promising strategy for mitigating petroleum contamination. Bacterial and fungal species capable of hydrocarbon degradation have been extensively studied, with varying degrees of efficiency reported in different environmental settings. Previous studies have established the role of microbial consortia in enhancing bioremediation processes, emphasizing the necessity of optimizing environmental conditions for maximum degradation efficiency. However, there are still gaps in knowledge regarding the specific microbial interactions that enhance degradation rates and the applicability of laboratory findings to real-world contaminated sites. This study aims to address these gaps by evaluating the hydrocarbon degradation potential of microbial consortia isolated from the Totora Wastewater Treatment Plant. By contributing new insights into the metabolic pathways and environmental conditions that influence microbial biodegradation, this research will provide valuable data for the development of sustainable bioremediation strategies.

3. Methodology

3.1. Study Location

This study was conducted at the Environmental Microbiology Laboratory, Academic Area of Microbiology, Faculty of Biological Sciences, National University of San Cristóbal de Huamanga (UNSCH), Ayacucho, Peru.

3.2. Preparation of Bushnell-Haas Medium with Diesel Oil

A total of 1,200 mL of Bushnell-Haas (BH) medium was prepared, containing magnesium sulfate, calcium chloride, monopotassium phosphate, dipotassium phosphate, ammonium nitrate, and ferric chloride, adjusted to pH 7 ± 0.2. The medium was distributed into six glass flasks (192 mL each) and sterilized. After cooling, 8 mL of diesel oil (4% v/v) was aseptically added to each flask.

3.3. Sample Collection and Transport

Six water samples were collected from the maturation lagoons of the "Totora" Wastewater Treatment Plant (WWTP), located in the Totora community, Jesús Nazareno district, Ayacucho. Samples were taken from three lagoons (LM1, LM2, LM3) at a depth of ± 20 cm in 750 mL glass flasks, filled to three-quarters capacity. The flasks were labeled and transported in thermal containers under ambient conditions to the laboratory.

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3.4. Isolation of Hydrocarbon-Degrading Microorganisms

Samples were homogenized and diluted in peptone water before inoculation in BH medium containing 4% v/v diesel oil. Cultures were incubated for 21 days under continuous shaking at ambient temperature. After incubation, aliquots were plated on nutrient agar for bacterial isolation (incubated at 37°C for 48 hours) and on Sabouraud agar with tetracycline for fungal isolation (incubated at 25°C for seven days). Developed colonies were subcultured for preservation.

3.5. Formation of Microbial Consortia

Microbial consortia were assembled using isolates from the same sampling locations. Three microbial consortia were created, corresponding to each lagoon: LM1, LM2, and LM3. Each consortium contained a mix of bacterial and fungal strains from its respective sources.

3.6. Preparation of Microbial Inocula

Bacterial isolates were reactivated in nutrient broth and incubated at 37°C until reaching a concentration of 1.5×10^8 CFU/mL (McFarland Standard No. 5). Fungal strains were cultivated on inclined Sabouraud agar, and spores were harvested after 7–10 days, reaching a concentration of 1.5×10^8 spores/mL. Each microbial consortium consisted of four bacterial and six fungal strains.

3.7. Soil Collection and Experimental Setup

Soil samples were collected from the park in Jesús Nazareno district, Ayacucho. The top 5 cm of the surface was cleared before excavation. The soil was sterilized, sieved (2 mm mesh), and distributed into 48 polyethylene bags (1 kg each). The sterilized soil was placed in $20 \times 20 \times 10$ cm plastic containers and contaminated with diesel oil at different concentrations (1%, 4%, 8%, and 16% w/w).

3.8. Experimental Treatments

Each microbial consortium (LM1, LM2, LM3) was evaluated for its diesel degradation capacity in soil containing different diesel concentrations (1%, 4%, 8%, 16% w/w). Each treatment included three replicates, along with control samples (sterilized soil with diesel oil but no microbial inoculation), resulting in 48 experimental units. Each unit received 100 mL of microbial culture (10 mL per strain).

3.9. Evaluation of Experimental Units

The experimental units were monitored every 14 days for a total of 70 days under ambient conditions. Soil aeration was performed manually using sterile spatulas, mixing for three minutes per session. Soil moisture was maintained by adding sterile distilled water as needed.

3.10. Determination of Soil Moisture

The gravimetric method was used to determine soil moisture content, following the protocol described by Linares [22].

3.11. Preparation of Soil Samples for Diesel Residual Extraction

From each experimental unit, 5 g of soil was collected, air-dried for 48 hours at room temperature, ground in a porcelain mortar, and placed in clean glass vials for further analysis.

3.12. Diesel Extraction and Residual Hydrocarbon Quantification

A total of 2 g of dry soil was mixed with anhydrous Na_2SO_4 and extracted with 5 mL of dichloromethane (99% purity). The solution was centrifuged at 150 rcf for 10 minutes, and the supernatant was collected. The extraction was repeated three times to obtain approximately 15 mL of organic extract, which was transferred to pre-weighed glass vials. The solvent was evaporated at 80°C

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for 48 hours, and the remaining residue was weighed to determine residual diesel content. The hydrocarbon concentration (HTPs) was calculated using the formula:

$$HTPs(mg/kg) = \frac{(RB - RA) \times FC}{P \times FH}$$

Where:

- *HTPs (mg/kg)*: Total petroleum hydrocarbons in dry soil.
- *RA*: Weight of empty container.
- *RB*: Weight of container with extracted hydrocarbons.
- *P*: Soil sample weight (g).
- *FH*: Moisture correction factor.
- *FC*: Correction factor (1000).

3.13. Characterization of Diesel-Degrading Microorganisms

For bacteria: Colonies were reactivated on nutrient agar and incubated at 37°C for 24 hours. Morphological characteristics (color, shape, elevation, margin) were recorded, and Gram staining was performed.

For fungi: Cultures were reactivated on Sabouraud agar, incubated at 25°C for 7 days, and examined for colony characteristics. Microcultures were prepared, and microscopic observations were performed using lactophenol-trypan blue staining at 400× magnification.

3.14. Statistical Analysis

Data was analyzed using analysis of variance (ANOVA) with a significance level of $p < 0.05$. Multiple comparisons were performed using Duncan's test. Statistical analyses were conducted using RStudio software, and results were presented in tables and figures.

4. Results and Discussion

4.1. Biodegradation of Diesel Oil in Soil by Microbial Consortia

The degradation of polycyclic aromatic hydrocarbons (PAHs) in soil is a slow process due to the native microbial flora present. Therefore, isolating and identifying microorganisms capable of degrading these compounds has become a highly effective biotechnological approach. The implementation of bioaugmentation in contaminated soils can significantly accelerate pollutant degradation [11, 23].

The use of microbial consortia for hydrocarbon fraction degradation has been widely studied, demonstrating the crucial role of microorganisms in bioremediation processes. PAHs, which are among the most toxic petroleum fractions, pose serious risks to human health [23]. The advantage of microbial consortia lies in the fact that a single microorganism may not be able to fully degrade a pollutant. Instead, the metabolic byproducts of one species can be further transformed into simpler compounds or completely degraded by other microbial species [23, 24].

4.2. Diesel Oil Removal at Different Concentrations

Figure 1 illustrates diesel oil removal by microbial consortia (LM1, LM2, and LM3) at different concentrations (1%, 4%, 8%, and 16%) and time points. The main findings include:

- 1% diesel concentration: Complete removal (100%) was achieved by day 28 in all treatments, while abiotic losses accounted for 0.205% [25].
- 4% diesel concentration: LM2 removed 100% by day 56, LM1 achieved 100% by day 70, and LM3 removed 91.75% by the end of the process. Abiotic factors accounted for 26.00% loss by day 56 and 43.13% by day 70.

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- 8% diesel concentration: LM2 removed 91.53% by day 70, while LM1 and LM3 removed 76.41% and 50.22%, respectively. Abiotic factors contributed to 24.20% removal.
- 16% diesel concentration: None of the treatments achieved 100% removal. The highest degradation was observed in LM2 (41.95%), followed by LM1 (38.57%) and LM3 (29.86%). Abiotic removal was 15.54%.

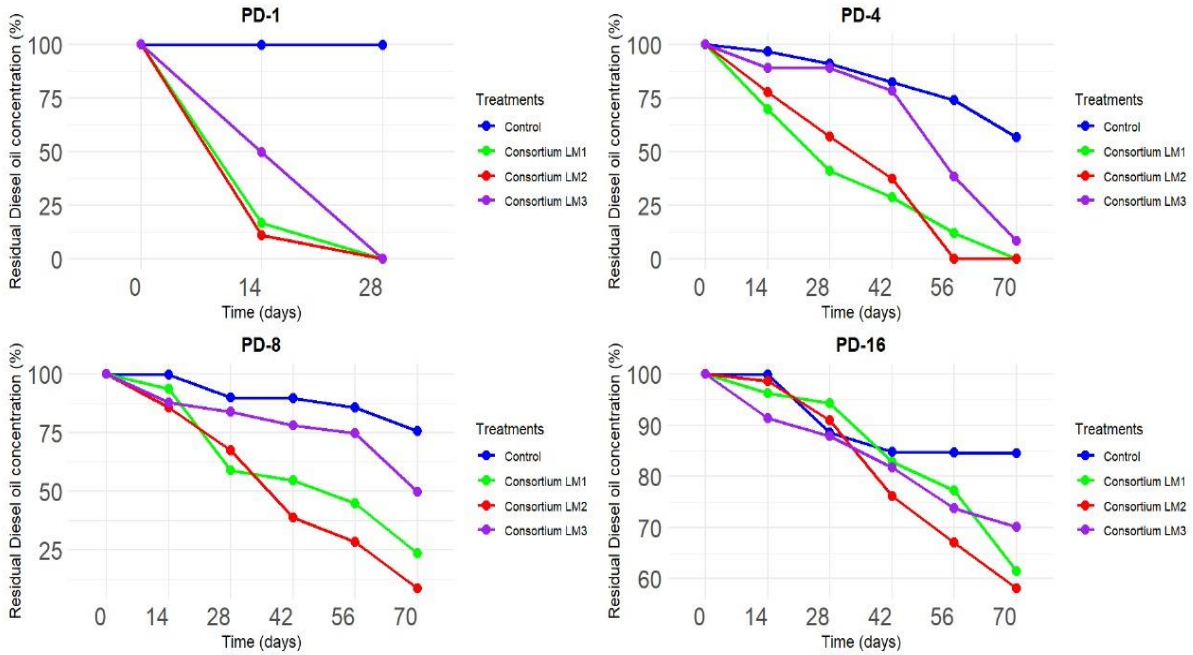

Figure 1.
Diesel oil removal by microbial consortia at different concentrations.

These results confirm that both biotic and abiotic processes contribute to diesel degradation, with abiotic losses ranging from 0.205% to 43.13% across different treatments. Similar findings have been reported by Alvaro, et al. [26] who noted that a significant proportion of monoaromatic hydrocarbons volatilize during soil aeration. Likewise, Ruberto, et al. [25] demonstrated substantial hydrocarbon loss across all remediation treatments, including abiotic controls, reporting hydrocarbon reductions of 54% to 61% within the first 10 days.

4.3. Impact of Initial Diesel Concentration on Biodegradation Efficiency

The results indicate that lower initial diesel concentrations lead to faster and more efficient removal. For 1% diesel concentration, degradation occurred rapidly, reaching 100% removal by day 28 across all treatments. These results align with Medina, et al. [14] who reported 85% petroleum removal at 30 days using fungal bioremediation. Similarly, del Refugio Castañeda-Chávez, et al. [16] demonstrated that a microbial consortium isolated from a cenote in Mexico achieved 98.47% diesel degradation from an initial concentration of 1.3% in a bioreactor with aeration.

Conversely, higher initial diesel concentrations (4%, 8%, and 16%) led to slower and less effective removal, with variations observed among consortia. These findings suggest that diesel concentration is a key abiotic factor influencing microbial degradation efficiency. Previous studies Gustafson, et al. [27]; Severin, et al. [28]; Bacosa and Inoue [15] and Bacosa, et al. [29] have highlighted that crude oil is a complex mixture of hydrocarbons (alkanes, PAHs, and polar compounds) with varying biodegradability

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and toxicity. At high concentrations, these hydrocarbons can exhibit toxic effects, inhibiting microbial growth.

Adams, et al. [30] also observed a slight increase in toxicity with higher initial hydrocarbon concentrations, although statistical significance was not confirmed. However, at the end of the experiment, toxicity levels were reduced to nearly non-toxic levels due to volatilization of semi-volatile hydrocarbons Gustafson, et al. [27] biological oxidation and mineralization of asphaltenes Flores, et al. [4]; Fernández, et al. [21] and Adams, et al. [30] and microbial humification processes [10].

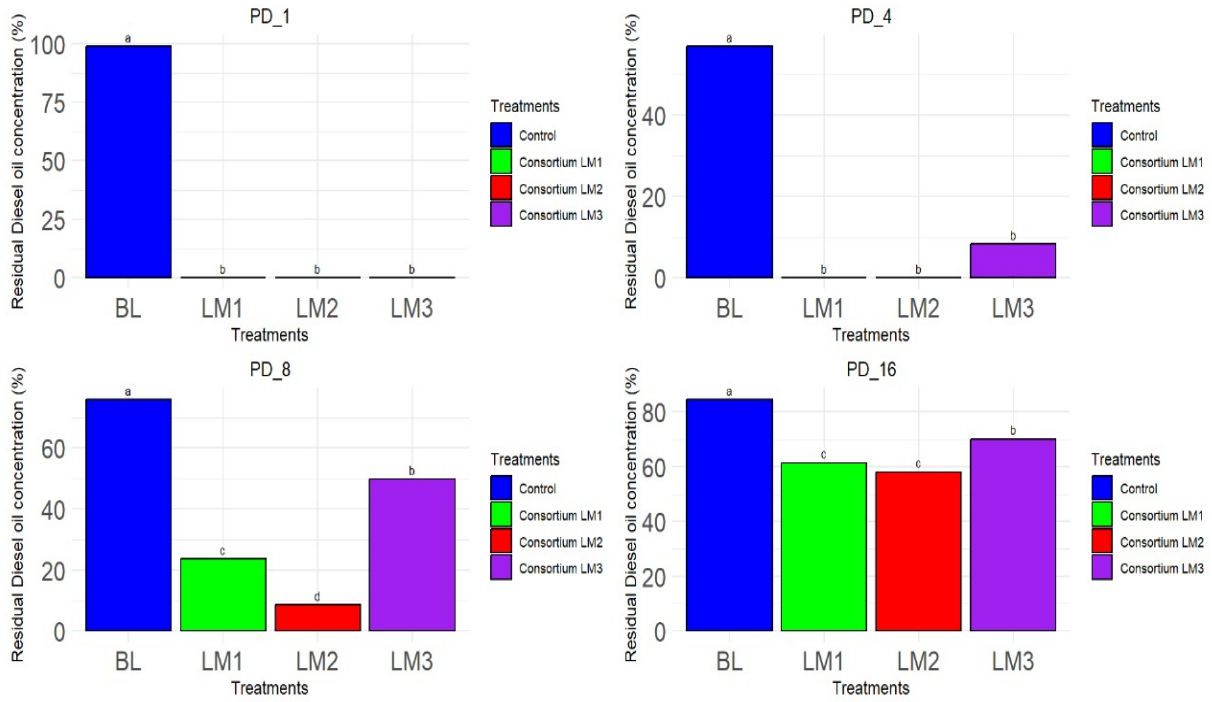

Figure 2.
Diesel Oil Removal (%) at Different Concentrations by Microbial Consortia from the "Totora" Wastewater Treatment Plant.

Figure 2 presents the percentage of crude oil removal by different microbial consortia in the treatments analyzed at day 28 for PD-1 and at day 70 for the rest (PD-4, PD-8, and PD-16). For the 1% concentration (PD-1), all three microbial consortia (LM1, LM2, and LM3) achieved 100% diesel oil removal, while abiotic factors contributed only 0.205%. Diesel oil losses due to abiotic factors in PD-4, PD-8, and PD-16 treatments ranged from 15.537% to 43.131%. For the 4% concentration (PD-4), LM1 and LM2 treatments achieved 100% removal, while LM3 reached 91.753%, resulting in net biodegradation of 56.869% for LM1 and LM2 and 48.623% for LM3. ANOVA analysis showed significant differences among treatments ($p < 0.05$), and Duncan's test confirmed differences between the treatments and the control.

For the 8% concentration (PD-8), LM1, LM2, and LM3 treatments achieved removals of 76.410%, 91.534%, and 50.215%, respectively, with net biodegradation of 52.213% for LM1, 67.337% for LM2, and 26.018% for LM3. ANOVA analysis revealed significant differences among treatments ($p < 0.05$), and Duncan's test showed differences between LM1 and LM2 compared to LM3, as well as all treatments against the control, indicating that LM1 and LM2 had superior biodegradation capacity.

For the 16% concentration (PD-16), LM1, LM2, and LM3 achieved removals of 38.573%, 41.952%, and 29.855%, respectively, with net biodegradation of 23.035% for LM1, 26.415% for LM2, and 14.317%

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for LM3. ANOVA analysis again showed significant differences among treatments ($p < 0.05$), and Duncan's test confirmed differences between LM1 and LM2 compared to LM3 and the control. These results indicate that LM1 and LM2 had superior biodegradation capacity, with LM2 showing the highest diesel oil degradation efficiency across all tested concentrations.

These findings demonstrate that hydrocarbon removal involves both abiotic and biotic processes. The microbial consortia exhibited different degradation capacities for diesel oil components, and an inverse relationship was observed between initial diesel concentration and biodegradation efficiency, likely due to contaminant toxicity. Similar results were reported by Narváez-Flórez, et al. [31] who observed a total reduction in aliphatic hydrocarbons of 92.15% by the end of the incubation period, with bacterial cultures degrading 68.61% and abiotic factors contributing 23.54%.

The observed trend that higher diesel oil concentrations resulted in lower biodegradation rates across all three treatments (LM1, LM2, and LM3) suggests that contaminant toxicity increases at higher concentrations. Schulze and Tiehm [32] and Kulikowska and Klimiuk [33] emphasized that natural bioremediation depends on factors such as contaminant concentration, nutrient availability, and bioavailability enhancers. Similarly, Vidali, et al. [34] cited by Thapa, et al. [35] and Shukla, et al. [36] stated that optimal petroleum degradation occurs at soil concentrations of 5-10% by dry weight, with higher concentrations causing adverse effects. Lundahl and Cabridenc [37] and Vandermeulen and Lee [38] and Lal and Saxena [39] explained that petroleum hydrocarbons can disrupt microbial physiological processes, genetic mechanisms, and growth. Dillard, et al. [40] and Gold-Bouchot, et al. [41] affirmed that diesel oil contains a mixture of highly concentrated toxic compounds, while Jain and Bajpai [42] reported that petroleum contamination negatively affects soil microbes and plants. Atlas [19] further noted that oil biodegradation in marine environments is limited by the recalcitrant and toxic nature of certain petroleum components.

The microbial consortia used in this study (LM1, LM2, and LM3) consisted of different bacterial and fungal strains (four bacterial and six fungal strains). All three consortia demonstrated significant diesel oil biodegradation compared to the control ($p < 0.05$). Mohamed [43] highlighted that microbial bioremediation is an eco-friendly and sustainable solution for environmental contamination, utilizing bacteria, fungi, and algae to detoxify pollutants into less toxic byproducts. The researcher emphasized that biodegradation often requires the cooperation of multiple microbial species, as some microbes produce metabolic enzymes that directly break down pollutants, while others transform toxins into less harmful intermediates.

Similarly, Jain and Bajpai [42]; Bartha and Bossert [44] and Bajpai, et al. [45] stated that mixed microbial populations with diverse enzymatic capabilities are essential for degrading complex hydrocarbon mixtures such as crude oil and metal-contaminated soils. Additionally, Tripathi, et al. [46]; Xu, et al. [17]; Thacharodi, et al. [47] and Maqsood, et al. [48] reported that Bacillus cereus can metabolize up to 98% of n-alkanes in crude oil. Most bacterial species possess specific enzymes to degrade different petroleum components, but no single strain can degrade all crude oil components, necessitating the formation of mixed microbial consortia for simultaneous hydrocarbon degradation. Dhaker and Jain [49] and Jain, et al. [12] also confirmed that multiple microbial species participate in hydrocarbon biodegradation, as bacteria regulate enzyme synthesis to initiate degradation when needed. Many indigenous soil and water microorganisms have the ability to degrade petroleum hydrocarbons.

Duncan's statistical test revealed significant differences in diesel oil degradation capacity between LM1 and LM2 compared to LM3. These results suggest that LM1 and LM2 were more efficient, with LM2 exhibiting the highest degradation capacity across all concentrations. This difference is likely due to the distinct microbial compositions of each consortium, as each strain possesses unique genetic traits influencing its degradation capacity. Millioli, et al. [50] stated that degradation specificity is linked to a microorganism's genetic potential to introduce molecular oxygen into hydrocarbons, producing intermediates that enter the general metabolic pathway for energy production. Ochoa Carreño and Montoya Restrepo [51] further noted that microbial consortia outperform individual strains in degrading complex systems due to genetic diversity and enzymatic interactions. Similarly, Barrios-San

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Martín, et al. [52] emphasized that microbial interactions enhance hydrocarbon degradation in contaminated sites.

Table 1.
Cultural, Microscopic, and Gram Staining Characteristics of Bacterial Strains Isolated from the LM2 Microbial Consortium.

Characteristic		**Bacteria**			
		LM2A-1	**LM2A-2**	**LM2B-1**	**LM2B-2**
Cultural	Color	cream-colored	mustard-colored	mustard-colored	cream-colored
	Shape	Circular	Circular	Circular	Circular
	Elevation	Convex	Convex	Convex	Convex
	Margin	Entire border	Entire border	Entire border	Entire border
Microscopic	Shape	Bacillus	Bacillus	Bacillus	Bacillus
Gram Staining		Negative	Negative	Negative	Negative

Table 2.
Cultural Characteristics of Fungal Strains Isolated from the LM2 Microbial Consortium.

Cultural Characteristics		**Fungi**					
		LM2A-1	**LM2A-2**	**LM2A-3**	**LM2A-4**	**LM2B-1**	**LM2B-2**
Reverse	Color	Dark green	White	White	White	Dark green	White
	Shape	*Powdery*	Filamentous	Filamentous	Filamentous	*Powdery*	Filamentous
Obverse	Color	Orange	White	White	White	Orange	White
	Shape	Irregular	Filamentous	Filamentous	Filamentous	Irregular	Filamentous

Table 3.
Identification of Fungal Strains from LM2 Through Microcultures and Pictorial Keys.

Fungi	**Genus**
LM2A-1 and LM2B-1	Penicillium sp
LM2A-2	Pithomyces sp
LM2A-3	Wallemia sp
LM2A-4 and LM2B-6	Geotrichum sp

In Tables 1, 2, and 3, the microbial strains constituting the LM2 consortium are reported. This consortium demonstrated the highest diesel oil biodegradation capacity in this experiment. The four bacterial strains are Gram-negative bacilli, while the filamentous fungi belong to the genera *Penicillium*, *Geotrichum*, *Pithomyces*, and *Wallemia*. It is widely recognized that numerous bacterial and fungal species possess the ability to degrade petroleum and its derivatives. In bioremediation studies of contaminated environments, microbial consortia have been identified as key players.

For example, Llenque Díaz [18] reported Gram-negative cocci and bacilli as hydrocarbon degraders. Araujo et al. (2006) identified both Gram-positive and Gram-negative bacilli, while Pardo-Díaz [53] recovered four bacterial strains from biopile samples (*Sps1*, *Sps2*, *Sc3*, *Sc5*) and four from agricultural soils (*ECO1P*, *ECO2P*, *Fenol2C*, *FenolP*) after 63 days of enrichment using phenol as the sole carbon source. These strains were identified as *Pseudomonas putida*, *Pseudomonas sp.*, *and Achromobacter sp.*, all *Gram-negative bacilli.*

Gómez-Reyes, et al. [13] suggested that filamentous fungi from the genus *Cladosporium* were present in microbial consortia. Mardones [54] reported that hydrocarbon-degrading fungal strains isolated from soil belonged to 15 genera, with *Penicillium* (130 strains), *Absidia* (32 strains), and *Mortierella* (29 strains) being the most abundant. Medina, et al. [14] observed fungal colonies with velvety and cottony textures, typical of molds. Meanwhile, creamy-textured colonies were characteristic of bacteria, which were not included in their study. The macroscopic and microscopic identification through Riddel's microculture technique determined that the most abundant fungal species were *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus.*

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5. Conclusions

Among the three microbial consortia (LM1, LM2, and LM3) isolated from the maturation lagoons of the Totora WWTP in Ayacucho, LM2 demonstrated the highest diesel oil biodegradation capacity. It achieved 100% removal from soil contaminated with 1% and 4% (w/v) diesel oil within 28 and 56 days, respectively, also considering abiotic removal factors.

LM1 and LM2 consortia exhibited higher removal efficiencies at 70 days from soil contaminated with 4%, 8%, and 16% diesel oil, achieving 100% removal at 4%, 76.410% and 91.534% removal at 8%, and 52.213% and 67.337% removal at 16%, respectively, also considering abiotic removal contributions. The results showed significant differences ($p < 0.05$) between the consortia and the control.

The LM2 consortium, which exhibited the highest diesel oil biodegradation capacity, consisted of four Gram-negative bacilli bacterial strains and six fungal strains: Penicillium sp. (two strains), Pithomyces sp., Wallemia sp., and Geotrichum sp. (two strains).

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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