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Dedications

Most of all, I thank the Almighty God, who gave me strength and health while I was doing this thesis.

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Khadidja

الملخص

تهدف هذه الدراسة إلى استغلال الأنواع الميكروبية الهيدروكربونية القادرة على تحليل الهيدروكربونات البترولية في التربة الملوثة. لهذا الغرض تم عزل كائنات حية دقيقة من عينات المنتجات البترولية والتربة الملوثة بالبترول، ثمّ توصيفها وتحديدها، وقد اختبرت قدرتها على تحليل البنزين والديزل، كل على حدا، وكذلك على إنتاج إنزيمات التحلل والمواد الحيوية الخافضة للتوتر السطحي، وأخيرا تم تحديد قدرة هذه العزلات منفردة ومجتمعة على تحليل البنزين والديزل في التربة.

إجمالاً، تمّ عزل أحد عشر بكتيريا وفطر واحد وتم تحديدها على أنها مكورة معوية دجاجية (*Enterococcus gallinarum*)، زائفة زنجارية (*Pseudomonas aeruginosa*)، عصوية رقيقة (*Bacillus subtilis*)، راكدة بومانية (*Acinetobacter baumannii*)، مكورة دقيقة صفراء (*Micrococcus luteus*)، عصية البرانثراسيس (*Bacillus paranthracis*)، كوكيريا وردية (*Kocuria rosea*)، أنورينيباسيليس ميغيلانيس (*Aneurinibacillus migulanus*)، ستربتومايسس سنيريوروبر (*Streptomyces cinereoruber*)، ليزينباسيليس كافارني (*Lysinibacillus cavernae*)، ليزينباسيليس باكستانية (*Lysinibacillus pakistanensis*)، وفطر رشاشي أصفر (*Aspergillus flavus*).

تجدر الإشارة إلى أن جميع العزلات التي تم اختبارها نمت بمعدلات مختلفة في وسط زراعي يحتوي على البنزين والديزل كمصدر للكربون. وقد أظهرت عصية البرانثراسيس والمكورة الدقيقة الصفراء نمواً أفضل في الديزل والبنزين على التوالي. بالإضافة إلى ذلك، أظهرت جميع العزلات القدرة على الالتصاق بالهيدروكربونات، والقدرة على إنتاج مواد حيوية خافضة للتوتر السطحي مع كل من الهيدروكربونات، ولكن بمعدلات مختلفة. أظهرت النتائج التي تم الحصول عليها فيما يتعلق بإنتاج الإنزيمات المشاركة في التحلل الحيوي للهيدروكربونات البترولية أن الزائفة الزنجارية، الليزينباسيليس الباكستانية، الليزينباسيليس كافارني، الستربتومايسس السنيريوروبر، الراكدة البومانية والفطر الرشاشي الأصفرهي فقط التي تنتج إنزيم الليباز ولكن بنشاط أكبر عند الراكدة البومانية. علاوة على ذلك، لوحظ أن الفطر الرشاشي الأصفرهو الوحيد الذي ينتج إنزيم اللاكيز.

على الرغم من أن جميع العزلات أظهرت القدرة على تحليل الديزل والبنزين في التربة، إلا أن الأنورينيباسيليس الميغيلانيس والليزينباسيليس الباكستانية أظهرتا أعلى معدلات تحلل الديزل والبنزين على التوالي.

تجدر الإشارة إلى أن هذه الدراسة تتطرق لأول مرة، على حد علمنا، الى استخدام *Enterococcus gallinarum*، *Lysinibacillus pakistanensis*، *Lysinibacillus cavernae* و *Streptomyces cinereoruber* في عملية المعالجة البيولوجية.

بشكل عام، أظهرت المجموعات الميكروبية معدلات تحلل أعلى مقارنة بالعزلات وحدها. تظهر النتائج التي تم الحصول عليها الإمكانية الكبيرة للكائنات الحية الدقيقة المعزولة، منفردة ومجتمعة، كعوامل معالجة بيولوجية للتربة الملوثة بالبترول.

الكلمات الدالة

التلوث، الهيدروكربونات البترولية، الديزل، البنزين، الكائنات الحية الدقيقة الهيدروكربونية، المعالجة الحيوية، المواد الخافضة للتوتر السطحي، الاتحادات الميكروبية.

Abstract

This study aims to explore hydrocarbonoclastic microbial species capable of degrading petroleum hydrocarbons for bioremediation of polluted soils. For this purpose, microorganisms were isolated from samples of petroleum products and petroleum-contaminated soils. Subsequently, these microorganisms were characterized and then identified. Furthermore, their abilities to degrade gasoline and diesel, separately, as well as to produce degradation enzymes and biosurfactants were tested. Finally, these isolates were tested for their ability, alone and in consortia, to degrade gasoline and diesel in the soil.

Overall, eleven bacterial species and one fungus species were isolated and identified as *Enterococcus gallinarum*, *Bacillus paranthracis*, *Kocuria rosea*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Acinetobacter baumannii*, *Aneurinibacillus migulanus*, *Micrococcus luteus*, *Streptomyces cinereoruber*, *Lysinibacillus pakistanensis*, *Lysinibacillus cavernae* and *Aspergillus flavus*. It should be noted that all the isolates tested grew but at different rates on culture media containing gasoline and diesel as a carbon source with *Bacillus paranthracis* and *Micrococcus luteus* demonstrating better growth on diesel and gasoline respectively. Additionally, all isolates showed the ability to adhere to both tested hydrocarbons and an ability to produce biosurfactants at different rates.

The obtained results concerning the production of enzymes involved in the biodegradation of petroleum hydrocarbons showed that only *P. aeruginosa*, *L. pakistanensis*, *L. cavernae*, *S. cinereoruber*, *A. baumannii*, and *A. flavus* produce lipase enzyme, with a higher activity noted of *A. baumannii*. Furthermore, it was observed that only *A. flavus* produces laccase enzyme. Although all isolates demonstrated the ability to degrade diesel and gasoline in soil, *Lysinibacillus pakistanensis* and *Aneurinibacillus migulanus* showed the highest degradation rates of gasoline and diesel respectively.

This study reportes for the first time, to our knowledge, the use of *Enterococcus gallinarum*, *Lysinibacillus pakistanensis*, *Lysinibacillus cavernae* and *Streptomyces cinereoruber* in bioremediation process.

In general, the tested consortia showed higher degradation rates compared to the isolates alone. These findings demonstrate a significant potential of the isolated microorganisms as bioremediation agents, alone and in consortia.

Keywords

Pollution, petroleum hydrocarbons, diesel, gasoline, hydrocarbonoclastic microorganisms, bioremediation, biosurfactants, microbial consortia.

Résumé

Cette étude vise à exploiter les espèces microbiennes hydrocarbonoclastes capables de dégrader les hydrocarbures pétroliers dans les sols pollués. A cet effet, des microorganismes ont été isolés à partir d'échantillons de produits pétroliers et de sols contaminés par du pétrole. Ces microorganismes ont été ensuite caractérisés puis identifiés et leurs capacités à dégrader l'essence et le diesel, séparément, ainsi que de produire les enzymes de dégradation et les biosurfactants ont été testées. Enfin, l'aptitude de ces isolats à dégrader l'essence et le gasoil dans le sol, seuls et en consortiums, a été déterminée.

Dans l'ensemble, onze espèces bactériennes et une espèce fongique ont été isolées et identifiées comme étant *Enterococcus gallinarum*, *Bacillus paranthracis*, *Kocuria rosea*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Acinetobacter baumannii*, *Aneurinibacillus migulanus*, *Micrococcus luteus*, *Streptomyces cinereoruber*, *Lysinibacillus pakistanensis*, *Lysinibacillus cavernae* et *Aspergillus flavus.* Il est à noter que tous les isolats testés se sont développés, à différents taux, sur les milieux de culture contenant l'essence et le diesel comme source de carbone avec *B. paranthracis* et *M. luteus* ayant une meilleure croissance sur le diesel et l'essence respectivement. De plus, tous les isolats ont montré la capacité à adhérer aux deux hydrocarbures testés et la capacité à produire des biosurfactants mais à des taux différents. Concernant la production d'enzymes intervenant dans la biodégradation des hydrocarbures pétroliers, seuls *P. aeruginosa*, *L. pakistanensis*, *L. cavernae*, *S. cinereoruber*, *A. baumannii*, et *A. flavus* produisent la lipase avec une activité supérieure chez *A. baumannii*. De plus, *A. flavus* est la seule espèce qui a produit la laccase.

Quoique tous les isolats ont démontré la capacité de dégrader le diesel et l'essence dans le sol, *L. pakistanensis* et *A. migulanus* ont montré les taux de dégradation les plus élevés de l'essence et du diesel respectivement.

Cette étude rapporte pour la première fois, à notre connaissance, l'utilisation d'*E. gallinarum*, *L. pakistanensis*, *L. cavernae* et *S. cinereoruber* dans la bioremédiation des sols pollués par les hydrocarbures. De manière générale, les consortiums élaborés ont montré des taux de dégradation supérieurs par rapport aux isolats seuls. Ceci démontre un potentiel important des microorganismes isolés, seuls et en consortium, comme agents de bioremédiation des sols pollués par les hydrocarbures pétroliers.

Mots clés

Pollution, hydrocarbures pétroliers, diesel, essence, micro-organismes hydrocarbonoclastes, bioremédiation, biosurfactants, consortiums microbiens.

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List of abbreviations

BH	Bushnell Hass.
BTEX	Benzene, toluene, ethylbenzene, xylene.
CSH	Cell surface hydrophobicity.
DCT	Drop collapse technique.
E24	Emulsification index.
LiP	Lignin peroxidase.
MAHs	Monocyclic Aromatic hydrocarbons.
MATH	Microbial adhesion to hydrocarbons.
MSM	Minimal salts medium.
NaCl	Sodium chloride.
OD	Optical density.
PAHs	Polycyclic aromatic hydrocarbons.
PH	Petroleum hydrocarbon
TCA	Tricarboxylic acid cycle.
TPHs	Total petroleum hydrocarbons.

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Introduction

Introduction

Crude oil and petroleum fossil fuels are mixtures of hydrocarbons that formed from the remains of animals and plants that lived millions of years ago in a marine environment. Nowadays, many petroleum products are used in developed and industrialized nations for their daily lives (i.e., transportation fuels, heating and power-generating fuels) as well as to enhance the economy (Wante et al., 2021). Moreover, the consumption of the petroleum hydrocarbons is expected to rise from 85 million barrels in 2016 to 106.6 million barrels at the end of 2030. Nevertheless, usage differs by region, with 53% in the Middle East and 32% in Europe and Asia (Ambaye et al., 2022).

Petroleum product contamination of the soil is a current issue in several nations worldwide. This detrimental occurrence is well-known in Algeria and the saharan region (Belabbas et al., 2016). In addition, the petroleum refinery situated in the coastal area of the skikda region (Algeria) and wastewater discharges pose a significant pollution hazard in the mediterranean sea, and the environmental fate of the total hydrocarbons and PAHs contained in their effluents is highly concerning. Algeria's estuaries and coastal areas are not excluded concern (Belahmadi et al., 2023).

The acronym PHC (petroleum hydro-carbons) is widely used to refer to the hydrogen- and carbon-containing compounds originating from crude oil such as fuels like gasoline, diesel, kerosene, and lubricating oils or greases (Adedeji et al., 2022). PHCs can contaminate an area if they spill while being produced, transported, refined, or extracted. The volume of crude oil or petroleum products that is used today dwarfs all other chemicals of environmental and health concern. Due to the numbers of facilities, individuals, processes and the various ways the products are stored and handled, environmental contamination is potentially widespread. Spills of used engine oil from aircraft or gasoline engines have the potential to impact the environment (Gebregiorgis Ambaye et al., 2023). Additionally, diesel negatively impacts the environment when motor vehicle, oil tank, or ship accidents happen (Oyewole et al., 2019).

Petroleum is formed chemically, of a complex mixture of asphaltenes, aliphatic hydrocarbons, heterocyclic hydrocarbons, aromatic hydrocarbons, and non-hydrocarbon compounds. About 60 to 90 % of this is categorized as biodegradable (Adedeji et al., 2022).

Large amounts of oily and viscous waste are unavoidably produced by the petroleum industry activities; as a consequence, hydrocarbon-derived pollutants have numerous negative

effects on the functioning of natural ecosystems and are immunotoxicant, mutagenic, and carcinogenic to both humans and animals (Bekele et al., 2022). Also, monoaromatic hydrocarbons including benzene, toluene, and xylene are among the toxic and persistent constituents of PHCs that represent serious risks to people (You et al., 2018).

Besides, it has been shown that soil pollution with petroleum hydrocarbons can change the physicochemical characteristics of topsoil and subsoil and have a phytotoxic effect on seed germination, crop growth, and yields (Ambaye et al., 2022).

There are numerous methods for cleaning up hydrocarbon-contaminated soil, including *ex-situ* ones like soil removal, disposal and incineration, as well as *in-situ* ones like soil vapor extraction and chemical treatment. These methods can effectively degrade these contaminants, but their effectiveness is limited by their high operating expenses and production of secondary toxic substances (You et al., 2018).

Bioremediation represents an innovative method for treating pollutants. It uses microorganisms and plants to transform toxic pollutants into less harmful ones like water, carbon dioxide, and biomass without affecting the ecology in the contaminated area (Amran et al., 2022).

Compared to physical and chemical cleaning procedures, bioremediation technologies are becoming more prevalent due to their high effectiveness, cheap cost, and safe byproducts (Zhang et al., 2019).

Microbial bioremediation primarily utilizes the two techniques of biostimulation and bioaugmentation (Bekele et al., 2022). The biostimulation approach uses the addition of nutrients and carbon sources to stimulate the activity of native microbes, while the bioaugmentation technique involves the introduction of bacteria that break down hydrocarbons. Combining bioaugmentation and biostimulation technology enhances the characteristics of soil by stimulating native microorganisms as well as introducing active bacteria (Zhang et al., 2019). Exogenous microorganisms must frequently be added to promote pollutant degradation because native microorganisms degrade pollutants at a slow rate (Wei et al., 2021), and their performance usually declines when the concentration of pollutants is high (Zhang et al., 2019)

Moreover, degradation of petroleum products is carried out by a microbial consortium rather than a single species since hydrocarbonoclastic microbes rarely work individually (Selvam and Thatheyus, 2018)

Building bacterial consortiums allows researchers to take use of the synergistic effects of many strains to improve the breakdown of petroleum hydrocarbons, and the performance of these consortia can be noticeably better than that of individual bacteria (Wei et al., 2021). In addition, the use of biosurfactant-producing hydrocarbons degrading microorganisms is a

feasible strategy that can be used to accelerate the bioremediation of petroleum hydrocarbons polluted locations (Patowary et al., 2017).

In this perspective, the present study aims to exploit the potential of microbial species to degrade some petroleum products namely gasoline and diesel oil, in polluted soils throughout the isolation and characterization of microorganisms from samples of petroleum products and petroleum-contaminated soils. The isolates will be tested separately for their ability to degrade diesel oil and gasoline. Then, different associations of the isolates will be tested to determine the best degrading microbial consortium.

Literature review

Literature review

1. Petroleum hydrocarbons

1.1. Generalities

Petroleum, also called crude oil, is a word derived from the Latin words Petra and oleum, meaning rock oil. It is an extremely complex liquid mixture consisting of a blend of thousands of compounds of hydrocarbons that occurs naturally in sedimentary rocks (Wante et al., 2021). It may occur in gaseous, liquid or solid form as natural gas, crude oil or asphaltic solids, respectively. Petroleum is formed by the breaking down of large molecules of fats, oils and waxes, coming from marine organisms, leading to the formation of kerogen. The gradual decay by the effect of heat and pressure resulted in the formation of hundreds of compounds. Petroleum is composed mainly of hydrocarbons, but contains also some compounds of oxygen, nitrogen, sulfur and trace metal components (nickel, vanadium, iron, antimony, etc.) (Sui et al., 2021).

Petroleum hydrocarbons (PHs) make up between 50 and 98 percent of crude oil, depending on where it comes from, and are regarded as a critical component (Sayed et al., 2021). They arise both from the biosynthetic activity of microorganisms and plants (due to the enzymatic reduction of fatty acid molecules) and from slow geochemical processes that act on biological compounds at high temperature and pressure over prolonged geological periods (Abbasian et al., 2015).

Moreover, petroleum contains principally compounds of five to about twenty carbon atoms most of them consisting of straight chains of carbon atoms. Typically, PHs containing 1 to 4 carbon atoms occur as gases, molecules having 5 to19 carbon atoms as liquids, and molecules with 20 carbon atoms and more exist as solids (Sarikoc, 2020).

Besides, different products are obtained from the petroleum refinery with the majority of products used as fuels and energy source all over the world (Fig. 1). In addition, a fraction is used in the petrochemical industry for the production of a wide range of products such as plastics, solvents, pharmaceuticals, textiles, polymers, and new chemicals etc. (Sayed et al., 2021). Consequently, the exploration, production, maintenance, transportation, and storage of petroleum and petroleum products are the primary contributors to environmental pollution (Ahmed and Fakhruddin, 2018).

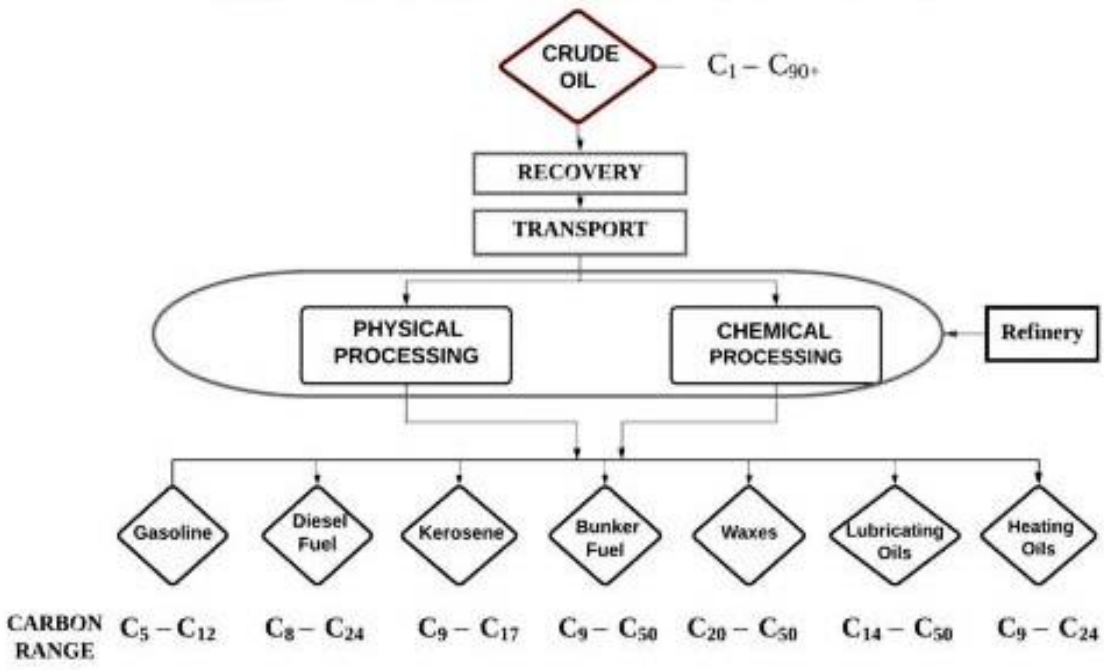


Figure 1. Pathways of petroleum hydrocarbons processing (Sayed et al., 2021).

1.2. Composition of petroleum hydrocarbons

Four classes of compounds are found in petroleum: alkanes (paraffins), cycloalkanes (naphthenes), aromatics, and heteroatomic compounds with one or more atoms of nitrogen, sulfur, and/or oxygen (Schobert 2013).

Saturates represent the major component of crude oil and petroleum products. Saturated hydrocarbons represent alkanes with the straight and branched chains of carbon atoms with the molecular formula C_nH_{2n+2} (aliphatic) (Patel et al., 2020). Aromatic hydrocarbons can be monocyclic or polycyclic polyaromatic hydrocarbons (PAHs), they possess a ring structure and are typically derivatives of benzene, C_6H_6. Those with low molecular weights tend to be more carcinogenic (Ossai et al., 2019).

1.3. Diesel

When crude oil is subjected to a distillation process between the temperatures of 200°C and 350°C at atmospheric pressure, a mixture of carbon chains with between 8 and 21 carbon atoms per molecule is formed. This fuel is also known as petro-diesel or fossil diesel. This fuel is used to power internal combustion engines like diesel engines (Demshemino et al., 2013).

Diesel oil hydrocarbons range in carbon number from 11 to 25 and between 2000 to 4000 hydrocarbons make up diesel oil, with 64 % of them being aliphatic, 2 % being olefinic, and 35 % being aromatic. According to Ahmed and Fakhruddin (2018), it is made up of four different hydrocarbon structural classes; n-alkanes or n-paraffins (saturated linear hydrocarbons),

isoparaffins and isoalkanes (branched saturated hydrocarbons), naphthenes or cycloalkanes (saturated cyclic alkanes) and aromatics. Alkanes and aromatic chemicals found in diesel oil, a complex hydrocarbon pollutant, are widely reported as soil pollutants (Palanisamy et al., 2014).

1.4. Gasoline

Gasoline is a blend of inflammable, volatile liquid hydrocarbons made from petroleum that is utilized as fuel for internal combustion engines. It is also known as gas (in the United States and Canada), petrol (in Great Britain), or benzene (in Europe) (El-Naggar, 2014).

A combination of hydrocarbons that make up gasoline typically boils at or around 180 °C and never goes over 200 °C. It includes a variety of hydrocarbons; approximately 150 hydrocarbon chemicals are commonly present in gasoline for automobiles. Depending on the source of the crude oil, the refining process, and the final specifications. The relative amounts of components might vary significantly (El-Naggar et al., 2017). The hydrocarbon components in this boiling range come into three main categories: paraffins (including cycloparaffins and branched materials), olefins, and aromatics, and have four to twelve carbon atoms in their chemical structure) (El-Naggar, 2014). Moreover, aromatics make up around 50 % of the total hydrocarbon content in gasoline. Alkanes, alkenes, and cycloalkanes are only found in trace amounts, with iso-alkanes making up roughly 35 % of the total (Ahmed and Fakhruddin, 2018).

1.5. Sources of petroleum hydrocarbon pollution

Petroleum hydrocarbon pollutants are accidentally or purposefully released into the environment by humans as a result of oil and gas exploration, production, transportation, storage; tank leaks; accidental spills during loading and unloading; ballasting and de-ballasting; bunkering; and oil tanker incidents (Fig. 2) (Ossai et al., 2019).

The amount of natural crude oil seepage was estimated to be 600,000 metric tons per year with a margin of error of 200,000 metric tons per year (Sahu et al., 2020). As a result of petrochemical industrial effluent discharge, fugitive emissions, subterranean pipeline bursts, war and political unrest, sabotage, and natural calamities, both the terrestrial and marine habitats are negatively impacted (Ossai et al., 2019).

The main source of hydrocarbon contamination in the soil is oil spills. In Europe, 3.5 million sites are thought to have been contaminated by petroleum. About 4.8 million hectares of soil in China may contain more petroleum than is safe (Sui et al., 2021)

In 2020, it is estimated that approximately 1000 tons of petroleum hydrocarbon oil will be wasted due to ship discharges alone. Over 140 significant spills totalling more than 7 million tons of hydrocarbons have been spilled into the environment (Sayed et al., 2021).

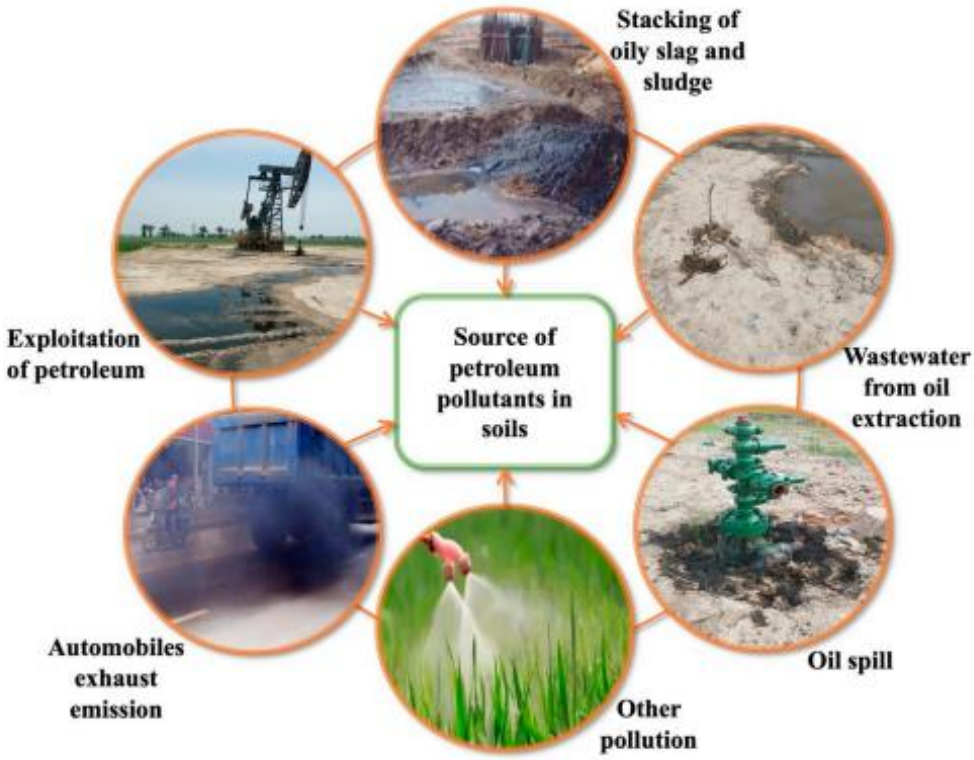


Figure 2. Main sources of petroleum hydrocarbons pollution (Sui et al., 2021).

1.6. Toxicity of petroleum hydrocarbons

When introduced into the environment, petroleum compounds have an impact on both abiotic and biotic components including microbes, plants, animals, and humans. As a result, soil pollution from these compounds is a serious issue because petroleum products' components have an impact on all living things (Ziółkowska and Wyszkowski, 2010).

The toxicity of PHs belonging to the same family and belonging to a comparable type tends to rise with decreasing molecular weight. Larger molecules are often less harmful than smaller ones. The toxicity varies by family for hydrocarbons with identical molecular weights. Alkanes, alkenes, cycloparaffins, aromatics, and polyaromatic hydrocarbons have the highest levels of toxicity in general (Pathak and Mandalia, 2012).

1.6.1. Human and animals

Petroleum hydrocarbon contamination can lead to a variety of toxicological health issues in both people and animals, such as haematotoxicity, carcinogenicity, genotoxicity, mutagenicity, teratogenicity, neurotoxicity, immunotoxicity, nephrotoxicity, hepatotoxicity, cardiotoxicity, and ocular toxicity (Ossai et al., 2019). Depending on the type of exposure and the organism exposed, the various compounds of crude petroleum oil can cause a variety of harmful impacts,

such as sub-lethal chronic toxicity, acute lethal toxicity, or both (Patowary et al., 2017). Also, the chemical structure, composition, and characteristics of the constituent fractions, as well as the route of exposure, the amount of exposure, and the time of contact, all play a role in how poisonous this substance is (Sayed et al., 2021).

Because of their great resistance to numerous techniques of bioconversion and their distinctive chemical stability, PAHs are thought to be crucial environmental pollutants (Patowary et al., 2017). In addition, used motor lubricating oils contain aliphatics and PAHs, which may lead to long-term risks like mutagenicity and carcinogenicity (Ibeto and Nwuga, 2018; Sharma, 2019).

For extent, some of the smaller compounds found in gasoline such as benzene, toluene, and xylene can affect the human central nervous system and can even be lethal if exposures are high enough. Moreover, n-hexane for example, causes a condition known as "peripheral neuropathy," which is characterized by numbness in the legs and feet and, in severe forms, paralysis. Additionally, diesel and kerosene cause central nervous system weakness, mouth and stomach inflammation, and coughing difficulties (Sayed et al., 2021). Benzene, toluene, ethylbenzene, and xylene, or BTEX (benzene, toluene, ethylbenzene and xylenes), can also impair an individual's neurological system, respiratory system, liver, and kidneys (Sui et al., 2021). At high concentrations, aliphatic hydrocarbons may also have an adverse effect on the neurological system, leading to light-headedness, headaches, exhaustion, limb numbness, tremors, transient limb paralysis, and unconsciousness (Koshlaf and Ball, 2017).

Toluene can induce nausea, lethargy, headaches, and sleepiness when inhaled at levels greater than 100 parts per million (100 ppm). Leukemia incidence is reported to be increased in areas where benzene concentrations are high. The effects of prolonged exposure to polluted areas involve fatigue, respiratory issues, eye inflammation, and headaches (Sui et al., 2021). Ingestion, inhalation, or direct contact with soil pollutants can all result in death (Adipah, 2018).

A number of physiological problems can be brought on by repeated exposure to sub-lethal doses of PAHs, including liver damage, haemolytic anemia, weight loss, gastrointestinal problems, weakened immune systems, and decreased productivity (Koshlaf and Ball, 2017). Long-term exposure to high concentrations of these pollutants can raise the risk of developing cancer and damage to the bone marrow in addition to causing liver and kidney illness in humans (Obah et al., 2020).

Eye irritation, vomiting, diarrhea, confusion, skin irritation, and inflammation are some of the immediate health impacts of PAH, in addition, the lung, skin; esophagus, colon, pancreas, bladder, and female breast are a few organs that might develop tumors as a result of prolonged exposure to these pollutants (Patel et al., 2020).

1.6.2. Soil

Petroleum and petroleum products can affect negatively agricultural soils by influencing its microbiological, biochemical, and biological properties and, consequently, plant production (Gospodarek et al., 2021). They affect soil pH, moisture, total organic carbon, types of micronutrients, total nitrogen, exchangeable potassium, and enzyme activity (urease, catalase and dehydrogenase) (Ziółkowska and Wyszkowski, 2010; Sui et al., 2021). In addition, diesel oil has been shown to increase acidity of soils (Ziółkowska and Wyszkowski, 2010).

High levels of PAHs in the soil have been found to induce tumors, reproductive, development, and immunological problems in terrestrial invertebrates (Sui et al., 2021).

According to the EPA (US Environmental Protection Agency), extremely dangerous chemicals found in petroleum like benzene, toluene, ethylbenzene, xylenes, and naphthalene can have an influence on soil's physical characteristics, as well as on compaction, saturated hydraulic conductivity, and penetration resistance (Hajabbasi, 2016; Ossai et al., 2019). Moreover, total porosity, macroporosity, and bulk density of soil are all impacted by crude oil (Sahu et al., 2020).

Waste oil causes soil to lose beneficial characteristics, including fertility, water-holding ability, permeability, and binding capacity (Ibeto and Nwuga, 2018). The amount of clay in contaminated soil increases as pollutant concentrations rise, and soil impermeability and hydrophobicity increase while soil porosity decline (Sui et al., 2021).

By obstructing the flow of nutrients, oxygen, and light, petroleum hydrocarbons cause biological damage that reduces soil fertility, stunts plant development, and prevents seed germination. Where PAHs persist, the quality and productivity of the soil are decreased; rendering the area unsuitable for investment and agriculture (Koshlaf and Ball, 2017).

1.6.3. Plants

Toxic petroleum hydrocarbon pollutants may inhibit several aspects of plant development, especially germination, plant length, and yield. They have the ability to infiltrate and pass through cell membranes, reducing membrane integrity and/or causing plant cell death (Zand et al., 2010; Obah et al., 2020).

The growth and development of plants is supported by trace metal microconcentrations, however the high bioavailability of excess metal ions in soil may have an adverse effect on plant growth. In addition, PAHs can perform biotransformation and may change root morphology, which may have an impact on the uptake of water and other minerals required for normal plant growth. Increased amounts of PAHs and trace elements cause alterations to cell structure, metabolic pathway redirection, and other effects (Wante et al., 2021).

Plant root vitality has been demonstrated to increase in low concentrations of petroleum hydrocarbons (10 g/kg), while it decreases in medium and high concentrations (30 g/kg and 50 g/kg, respectively) (Sui et al., 2021).

1.6.4. Microorganisms

Petroleum hydrocarbon contamination of a site reduces the diversity and evenness of a microbial community due to the chemical compounds' direct toxicity, the sequestration of nutrients and water, the exclusion of microorganisms of vital components for growth, and difficulty adapting to the highly nonpolar conditions, which can rupture microbial cells by dissolving the cytoplasm membrane lipids (Truskewycz et al., 2019). The hydrophobicity and insolubility of hydrocarbons have harmful effects on microbial cells and limit their absorption into them (Abbasian et al., 2015). Studies have shown that gasoline-polluted soils have the most detrimental impacts (Xu et al., 2018).

Besides, pollution of the environment with petroleum derived products affects levels of heavy metals and consequently microbial activity. Also, high levels of this pollutants in the environment may prevent oil from biodegrading because of a lack of oxygen or because volatile hydrocarbons are poisonous to microorganisms (Obah et al., 2020). In fact, one of the important factors that limit biodegradation of oil pollutants in the environment is their limited availability to microorganisms. PHs bind to soil components rendering them difficult to be removed or degraded. The susceptibility of hydrocarbons to microbial degradation differs depending on their structure and composition and can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes. High molecular weight PAHs, may not be degraded at all, while the n-alkanes are the most willingly degraded and are average in length (C10-C25) (Das and Chandran 2011).

1.7. Bioremediation

Several physicochemical treatment techniques have been developed to remove contaminants from soil (Asquith et al., 2012), including chemical oxidation, stabilization/solidification, electrochemical procedures, solvent extraction, and ion exchange as a chemical treatment.

The variations in the physicochemical characteristics of the pollutants and soil, or of the contaminated and uncontaminated soil, are the basis for physical procedures such soil vapour extraction, vitrification, washing, heating, and air sparing. Thermal processes use physiochemical techniques including electrical resistance heating, steam injection and extraction, and conductive heating that are used at high temperatures (Naseri et al., 2014). Such treatments are typically costly, energy-intensive, and unsustainable in terms of their effects on the

environment, which include harm to soil structure and toxicity problems related to chemical additives (Asquith et al., 2012).

Utilizing living organisms' capacity to reduce, degrade, and/or remove toxicants from marine and terrestrial ecosystems can lessen the risk to human health while restoring the ecosystem to its pre-polluted state. The process is called bioremediation (Santos et al., 2018). Typically, for this purpose, bacteria, plants, fungi, and algae are employed (Naeem and Qazi, 2020).

Besides, many soil microorganisms (mainly bacteria and fungi) transform petroleum-derived hydrocarbons into nontoxic compounds or perform complete mineralization producing simple nonorganic substances, such as carbon dioxide and water in addition to biomass (Gospodarek et al., 2021).

Bioremediation is a cost-effective, environmentally friendly method for increasing the metabolism of organic contaminants and reducing the negative ecological effects of oil spills (Koshlaf and Ball., 2017).

. The effectiveness of bioremediation primarily depends on the microbial diversity, the contaminated areas, and the surrounding environment. The rate of degradation and microbial activity are influenced by these variables (Mahjoubi et al., 2018).

In many instances, soil bioremediation has been demonstrated to be more promising and economical procedure than other conventional methods. It employs little technology that doesn't harm the environment (Naseri et al., 2014). Because it can be applied to vast areas, it results in the complete removal of the contaminants. Ex-situ and in-situ techniques can be used for remediation (Jabbar et al., 2019). Ex-situ technique is achieved by drilling and removal of contaminated soil, including land farming, bioreactor processing, composting, bioventing and biopiling (Naeem and Qazi, 2020; Adedeji et al., 2022).

There are two basic methods to bioremediation processes, biostimulation and bioaugmentation (Amran et al., 2022).

Bioaugmentation is the systematic addition of microorganisms that specifically degrade contaminants at a polluted site and is typically used in situations when the native microorganisms are unable to function effectively, and when there are few natural populations of hydrocarbon degraders in contaminated soils. In addition, when pollutants have a harmful effect on the local microorganisms, bioaugmentation produces the appropriate results (Naseri et al., 2014; Wu et al., 2019; Udume et al., 2023). In fact, it may occasionally be necessary to add contaminant-degrading strains, consortiums, or enzyme mixtures to the soil to promote the degradation of specific contaminants (Asquith et al., 2012).

In reality, increasing the overall number of hydrocarbon-using microorganisms and their concentration in the entire soil microbial community will enhance the biodegradation rate (Naseri et al., 2014). Moreover, isolation of potent degraders from their native microbial communities and cultivating them in a laboratory setting may enhance microbial degradation (Popoola et al., 2022).

Comparing bioaugmentation to physical and chemical remediation techniques reveals that it has better practicality and economic uses (Sui et al., 2021).

Due to the high concentration of carbon constituents in hydrocarbon-contaminated soils, these soils typically contain only small levels of inorganic nutrients, especially nitrogen and phosphorus, which are important cellular basic components and hence necessary for microbial development and activity. Nutrient supplementation (biostimulation) has been used to resolve the problem and restore the C:N:P ratio to normal (Asquith et al., 2012). It is possible to stimulate the development and metabolic activity of native microbes (Udume et al., 2023). Phosphorus (P), nitrogen (N), such as ammonium, urea, and various phosphates, nitrates and carbon (C)-rich nutrients, are frequently used in biostimulation (Naseri et al., 2014). Typically, a standard formula for a biostimulation method is a C/N/P ratio of 100/10/1 (Wu et al., 2019).

Using various treatment strategies, like bioaugmentation and biostimulation, alone or in combination, is possible to accelerate the biodegradation of PHs in soil. Results of several studies showed a significant role for combined bioaugmentation and biostimulation in the bioremediation of crude oil-contaminated soil, with corresponding reductions in total petroleum hydrocarbon (TPH) of 66 % and 74 % for biostimulation and mixed bioaugmentation and biostimulation respectively (Yaman, 2020).

1.7.1. Microbial degradation of petroleum hydrocarbons

Microbial degradation is one of the primary and most important mechanism for eliminating petroleum hydrocarbon contaminants from the environment (Unimke et al., 2018). Because they require energy and carbon for growth and reproduction, native bacteria eventually breakdown or metabolize the majority of petroleum hydrocarbons found in the environment (Xu et al., 2018). Fungi, yeast, and bacteria are principally responsible for hydrocarbon degradation in the environment. However, bacteria have been shown to be the most active agents in petroleum degradation (Chandra et al., 2012; Sharma, 2019).

Furthermore, some bacterial species are described as being extremely specialized in breaking down hydrocarbons removing them from contaminated environments and are hence known as hydrocarbonoclastic bacteria (Singh et al., 2011).

Many microbial genera that degrade hydrocarbons have been identified, they include: *Pseudomonas, Nocardia, Vibrio, Corynebacterium, Candida, Arthrobacter, Rhodotorula, Brevibacterium, Flavobacterium, Sporobolomyces, Achromobacter, Bacillus, Aeromonas, Thiobacillus, Acinetobacter, Lactobacter, Staphylococcus, Penicillium, Articulosporium, Halomonas, Klebsiella, Proteus, Aspergillus, Micrococcus, Neurospora, Rhizopus, Mucor* and *Trichoderma* (Victor et al., 2020). Also, *Achromobacter, Acinetobacter, Arthrobacter, Azoarcus, Brevibacterium, Cellulomonas, Corynebacterium, Flavobacterium, Marinobacter, Micrococcus, Nocardia, Ochrobactrum, Pseudomonas, Stenotrophomaonas,* and *Vibrio* are reported as hydrocarbon destructors (Wang et al., 2019).

Hydrocarbons are recognized as the only source of nutrition for many bacteria. Petroleum-contaminated soil and water have documented distinct bacterial communities linked to several genera: *Acinetobacter, Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Brevibacterium, Corynebacterium, Dietzia, Flavobacterium, Methylobacterium, Nocardia, Sphingomonas, Pseudomonas, Rhodococcus* and *Vibrio* (Mangla et al., 2021).

Besides, *Aspergillus, Amorphoteca, Fusarium, Graphium, Neosartoria, Paecilomyces, Penicillium, Sporobolomyces* and *Talaromyces* are some of the fungi that degrade petroleum hydrocarbons, as are yeast from the genera *Candida, Pichia, Pseudozyma, Rhodotorula* and *Yarrowia* (Al-Zahrani et al., 2022).

1.7.1.1. Factors influencing the microbial degradation of petroleum hydrocarbons

- Chemical and physical state of oil

Microbial degradation of petroleum hydrocarbons largely depends on their various chemical structures and concentrations. Long-chain (C25-C40) alkanes are hydrophobic solids in nature and highly difficult to breakdown due to limited water solubility, whereas short-chain (C10-C25) n-alkanes are more hazardous than long-chain alkanes. Branched chain alkanes and cycloalkanes both decompose more slowly than typical alkanes during the process (Unimke et al., 2018; Jadhav et al., 2019).

Because polycyclic aromatic hydrocarbons are poorly soluble, they are less accessible and persist in the environment (Al-Hawash et al., 2018). However, as they are dissolved or evaporated, they become more bioavailable. Furthermore, hydrocarbons' bioavailability to the microbial system is increased by photooxidation (Sharma, 2019).

- Temperature

Temperature affects biodegradation by affecting the physical characteristics and chemical composition of the oil, as well as the activity of microbial enzymes. The temperature ranges where hydrocarbonoclastic microbes are most active dictate how enzymes are produced. In

general, enzymatic activity decreases with decreasing temperature but some microorganisms have adapted mechanisms to thrive at low temperatures by producing cold-adapted enzymes, which allow them to function more efficiently at low temperatures (Ribicic et al., 2018; Unimke et al., 2018). High temperatures between 30°C and 40°C have shown a higher rate of hydrocarbon metabolism. The highest rates of degradation occur in soil, marine, and freshwater habitats at temperatures comprised between 30°C and 40°C, 20°C and 30°C, and 15°C and 20°C, respectively (Al-Hawash et al., 2018; Sharma, 2019).

Furthermore, the effect of temperature on the physicochemical properties of oil is likely to have a stronger influence on oil-biodegradation. Low temperatures cause the oil to become more viscous with decreased solubility, dissolution, and volatilization. These factors decrease the bioavailability of oil compounds to degradative microorganisms, hence delaying the biodegradation process (Jahangeer and Kumar, 2013).

Temperature has an effect on the soil matrix, bacterial proliferation, and metabolism, all of which affect the effectiveness of biodegradation (Xu et al., 2018).

- Nutrient Availability

The successful biodegradation of hydrocarbon pollutants depends heavily on nutrients (Das and Chandran, 2011). Phosphorus, hydrogen, potassium, nitrogen, oxygen and in some cases, iron are inorganic substances that are necessary for the growth and activity of microorganisms (Sharma, 2019). Indeed, contaminated sites have high levels of carbon which lead to the rapid depletion of available nutrients during microbial metabolism. That is why it is recommended to supplement polluted environments with nutrients such as nitrogen and phosphates to stimulate the in situ microbial biodegradation. However, high concentration of nutrients especially NPK can also adversely affect the biodegradation of hydrocarbons and limit microbial biodegradation activity (Jahangeer and Kumar, 2013; Koshlaf and Ball, 2017; Al-Hawash et al., 2018). For instance, Chaillan et al., (2006) demonstrated that nutrient amendment with elevated concentration of N-urea had highly detrimental effects on the hydrocarbon degrading fungal populations due to the production of toxic concentration of ammonia gas by nitrification. Bacterial cells need about 150 g of nitrogen and 30 g of phosphorus to convert 1 kilogram of hydrocarbons (Xu et al., 2018).

- Salinity

According to Al-Hawash et al. (2018), salinity has a significant impact on bioremediation and biodegradation processes, as well as on microbial diversity and growth. In addition, some crucial enzymes involved in the process of hydrocarbon degradation are adversely affected by salinity (Ebadi et al., 2017).

The overall microbial populations are likely to be impacted by the greater concentration of sodium chloride in the soil. High NaCl concentrations cause plasmolysis, inhibition of a number of physiological processes including the production of macromolecules, as well as osmotic stress in some bacteria (Sharma, 2019).

- Oxygen

The most rapid and complete degradation of the majority of organic pollutants is performed under aerobic conditions (Das and Chandran 2011). Through the oxygenase enzyme, bacteria and fungi typically catabolize aliphatic, cyclic, and aromatic hydrocarbons in the presence of oxygen (Sharma, 2019). Utilizing oxygen as an electron acceptor is crucial for aerobic degradation processes, but due to the low air permeability in areas with petroleum oil contamination, it is frequently insufficient (Xu et al., 2018).

However, oxygen is not always limited in the upper levels of the water column in aquatic environments and in the soil its availability depends on rates of microbial oxygen consumption, the type of soil, the soil texture and structure and the pressure of utilisable substrates which can lead to oxygen reduction. Anaerobic biodegradation of petroleum hydrocarbons by microbes occurs at very low rates, and its ecological impact has generally been seen as being minimal (Unimke et al., 2018).

- pH

The pH is a very important factor in the biodegradation of petroleum hydrocarbons. In contrast to most aquatic ecosystems, soil pH can be highly variable, ranging from 2.5 in mine spoils to 11.0 in alkaline deserts (Chandra et al., 2012). However, most microbial families prefer a neutral pH (Jadhav et al., 2019). The pH of the environment influences enzyme activity as well as processes such as catalytic reaction balance and cell membrane transport (Al-Hawash et al., 2018). Through altering cell membrane transport and the capacity of microorganisms to complete their cellular activities, changes in soil pH reduce the biodegradation rate (Naseri et al., 2014). It has been demonstrated that *Burkholderia cocovenenans* degraded phenanthrene at a rate of 40 % at pH 5.5. Nevertheless, the degradation under identical conditions at neutral pH was 80 %. Additionally, several studies have discussed how effectively some microbes, such as *Pseudomonas*, may degrade hydrocarbons at an alkaline pH (Mahjoubi et al., 2018).

- Activity of water

Humidity is a necessary component of all biological mechanisms for efficient transport of nutrients and waste products into and out of microbial cells. Despite coastal environments, where water potential is favourable at close to 0.98 values, soils' water activity can range from 0.1 to 0.99 (Unimke et al., 2018). The appropriate humidity ratio will depend on whether and the kind of soil, and it can range from 30 % to 90 % in a terrestrial ecosystem where there is little water

for microbial survival (Jadhav et al., 2019). Due to its impacts on hydrocarbon bioavailability, diffusion processes, transfer of generated gases, oxygen availability in the soil, and soil toxicity level, soil humidity can affect the biodegradation rate (Naseri et al., 2014).

- Concentration of the oil

The concentration and chemical structure of hydrocarbons affect their degradation. Extremely high concentrations of petroleum hydrocarbons substantially restrict bacterial development, which results in inefficient biodegradation and even bacterial mortality (Xu et al., 2018).

Extreme environmental conditions (soil temperature below 10°C, pH below 4 and more than 9) decrease microbial activity, which diminishes the removal impact of petroleum pollutants. Generally, a pH 5.5-8.8, temperature 15-45 °C, oxygen content 10 %, low clay or silt content soil type, and C/N/P ratio of 100:10:1 are the optimum conditions for microbial remediation of oily soil (Sui et al., 2021).

1.7.1.2. Mechanisms of petroleum hydrocarbons degradation

Petroleum hydrocarbons can be biodegraded by bacteria, yeast, and fungi at different levels. Basic degradation of petroleum hydrocarbon pollutants depends on the presence or absence of oxygen (Jadhav et al., 2019). By using oxygen or nitrate, ferric iron, sulfate or other electron acceptors, the main intrinsic metabolic processes that bacteria use can take place either aerobically or anaerobically (Victor et al., 2020).

Many bacteria have been demonstrated to degrade aliphatic (alkanes, alkenes, alkynes) and aromatic hydrocarbons and use them as sole carbon and energy sources (Sierra-Garcia and de Oliveira, 2013). Understanding the metabolism of hydrocarbons in microorganisms is confronted with the chemical diversity of such compounds and their reactivities, as well as with various microbial life styles. Degradation begins by activating the hydrocarbon; since this later is an apolar, unreactive compound composed only of carbon and hydrogen. It must be first functionalized to be metabolized. Several microbial reactions of activation exist and are basically different in aerobic and anaerobic microorganisms (Sierra-Garcia and de Oliveira, 2013).

In aerobic degradation, hydrocarbon metabolism is initiated using molecular oxygen as a co-substrate in mono- or dioxygenase reactions that allow the terminal or sub-terminal hydroxylation of aliphatic alkane chains, or the mono or dihydroxylation of aromatic rings. However, in anaerobic degradation, some proposed reactions for hydrocarbon activation comprise: addition to fumarate by glycyl-radical enzymes, methylation of unsubstituted aromatics, hydroxylation with water by molybdenum cofactor containing enzymes of an alkyl

substituent via dehydrogenase, and carboxylation catalyzed by yet- uncharacterized enzymes which may actually represent a combination of the second reaction followed by the first reaction. Only the first reaction has been characterized (Fig. 3) (Sierra-Garcia and de Oliveira, 2013).

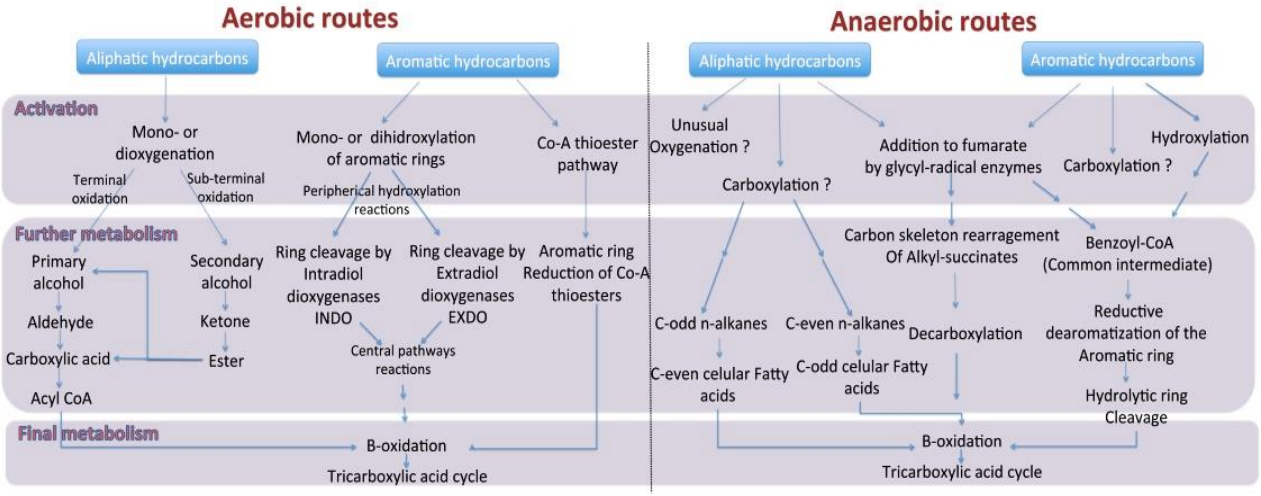


Figure 3. Pathways for aerobic and anaerobic bacterial degradation of hydrocarbon compounds (Sierra-Garcia and de Oliveira, 2013).

- Aerobic degradation

Intracellular microbial aerobic degradation of organic pollutants is an oxidative reaction where activation as well as incorporation of oxygen is catalysed by oxygenases and peroxydases (Fig. 3). Peripheral degradation mechanisms convert organic pollutants step by step into intermediates of the central intermediary metabolism like the tricarboxylic acid cycle (Victor et al., 2020). Cell biomass is formed using the central precursor metabolites such as acetyl-CoA, succinate, and pyruvate. Sugars required for various biosynthesis and growth are produced by gluconeogenesis (Fig. 4) (Das and Chandran, 2011; Unimke et al., 2018).

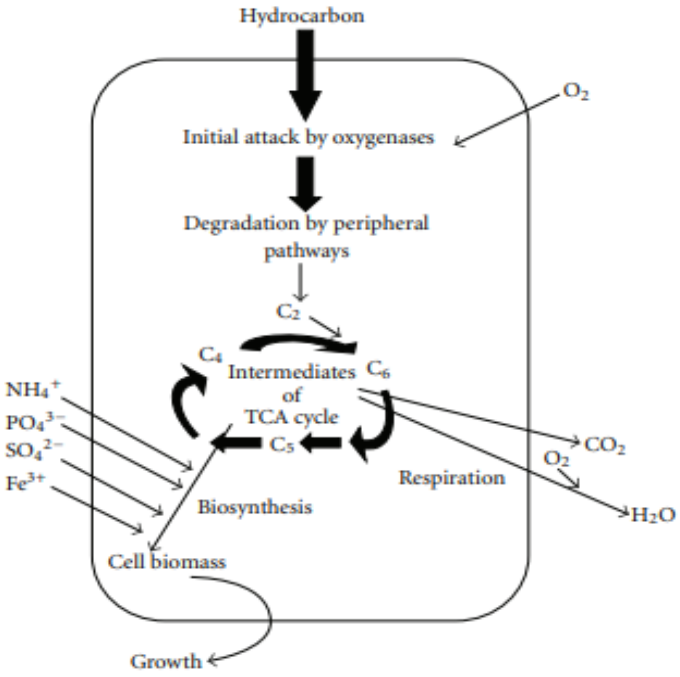


Figure 4. Main principle of microbial aerobic degradation of hydrocarbons (Victor et al., 2020).

Aerobic degradation, which primarily targets aromatic petroleum hydrocarbon contaminants, is the most effective and comprehensive method for removing them from the environment (Jadhav et al., 2019). Specific enzymes perform the degradation of petroleum hydrocarbons (table 1). Enzymes and metabolic pathways, such as alkane monooxygenase and cytochrome P450, have been considered in the degradation of petroleum hydrocarbons (Jadhav et al., 2019). In addition, other processes are employed by microorganisms such as the attachment of microbial cells to the substrates and the production of biosurfactants that act as emulsifying agents decreasing the surface tension and forming micelles that are introduced and degraded inside the cell after being encapsulated in the hydrophobic microbial cell surface (Das and Chandran, 2011).

Table 1. Enzymes participating in the degradation of petroleum hydrocarbons.

Enzymes	Substrates	Microorganisms	References
Manganese peroxidase, lignin peroxidase and laccase	PAHs (Pyrene and anthracene)	*Phanerochaete chrysosporium*, *Trametes versicolor* *Pleurotus ostreatus*	Al-Hawash et al., 2018; Novotný et al., 2004.
Laccase	Anthracene	*Pycnoporus sanguineus*	Li et al., 2014.
Cytochrome P450	Phenanthrene, fluoranthene, pyrene	*Bacillus megaterium*	Bhandari et al., 2021.

Cytochrome P450	n-alkanes	*Candida maltosa*, *Candida tropicalis*, *Candida apicola*	Chandra et al., 2012.
Cyclohexanone monooxygenase	Cyclohexane	*Acinetobacter* sp.	Abbasian et al., 2015.
Monooxygenase	Long-chain alkanes (C15 to C36)	*Geobacillus thermodenitrificans* NG80-2	Abbasian et al., 2015.
n-alkane dioxygenase	C13-C44 alkanes	*Acinetobacter* sp.	Sakai et al., 1996 ; Abbasian et al., 2015.
Alkane hydroxylases	C5-C16 alkanes	*Pseudomonas putida* GPo1	Van Beilen et al., 2003

- **Aliphatic hydrocarbons**

In general, the degradation of n-alkane is an oxidation by terminal monooxygenases/ hydroxylases leading to the corresponding alcohol which is oxidized to the corresponding aldehyde that is in turn converted into a fatty acid and then conjugated to CoA and processed by β – oxidation to generate acetyl-CoA. Short and long chains alkanes can also be oxidized by subterminal position. Both terminal and sub-terminal oxidation can coexist in some microorganisms (Ji et al., 2013). Some bacteria, including *Bacillus species*, *Rhodococcus* species, and some fungi, including *Fusarium* species and *Aspergillus* species, have the ability to oxidize n-alkanes sub-terminally (Abbasian et al., 2015).

Numerous bacteria, including *Acinetobacte*r sp. and *Rhodococcus rhodochrous* (P450oct), use their cytochrome P450 for the first activation of hydrocarbon degradation (Abbasian et al., 2015). Methane monooxygenase breaks down alkanes with short chains (C2-C4), medium n-alkanes (C5-C17) are degraded using cytochrome P450 and monooxygenases like AlkB, and long n-alkanes > C18 are thought to be degraded by a number of alkane hydroxylases (Mahjoubi et al., 2018). Moreover, *Pseudomonas* sp., contains enzymes necessary for the degradation of alkanes and polycyclic aromatic hydrocarbons, such as nahAc, catechol dioxygenase (C12O and C23O), AlkB, and cytochrome P450 (Sui et al., 2021). In addition, many microsomal Cytochrome P450 forms are involved in the ability of several yeast species to use n-alkanes and other aliphatic hydrocarbons as their primary source of carbon and energy (Das and Chandran, 2011; Chandra et al., 2012;).

In bacteria, terminal hydroxylation of n-alkanes at the beginning can be done by different enzymes, namely: propane monooxygenase (C3), different classes of butane monooxygenase (C2-C9), CYP153 monooxygenases (C5-C12), AlkB-related non-heme iron monooxigenase (C3-C10 or C10-C20), flavin-binding monooxigenase AlmA (C20-C36), flavin-dependent monooxygenase LadA (C10-C30) and copper flavin-dependent dioxygenase (C10-C30). The integral membrane non-heme iron monooxygenase (AlkB) is the best characterized one (Van Beilen and Funhoff, 2005; Sierra-Garcia and de Oliveira, 2013).

The microbial degradation of medium (C5-C11) and long (>C12)-chains alkanes has been frequently related to the presence of alkB genes. Consequently, these genes have been used as functional biomarker for the characterization of aerobic alkane-degrading microbial populations and in bioremediation experiments.

The alk system was first found to be located on the OCT plasmid of *Pseudomonas putida* GPo1. This plasmid contains two operons: alkBFGHJKL and alkST. The first operon encodes two components of the alk system, a particulate non-heme integral membrane alkane monooxygenase (AlkB) and the soluble protein rubredoxin (AlkG), in addition to other enzymes involved in further steps. The second operon encodes for a rubredoxin reductase (AlkT and AlkS), which regulates the expression of the alkBFGHJKL operon. Since this system was described, AlkB homologous have been found in many alkane-degrading bacteria and an increasing collection of alkane hydroxylase gene sequences has allowed the diversity analysis of hydrocarbon-degrading microbial populations in different ecosystems. However, comparisons of cloned alkB genes or gene fragments have showed that sequence diversity is very high, even among alkB genes within the same species. Besides, it has been observed that there is more than one alkane oxidation system exhibiting overlapping substrate ranges. Other enzyme systems for alkane degradation have not been yet elucidated (Sierra-Garcia and de Oliveira, 2013).

Four different aerobic degradation pathways of n-alkane degradation are known (Fig. 4). When fatty acids are created through terminal oxidation, they can either go into β-oxidation or be further oxygenated by fatty acid monooxygenases to create dicarboxylic acid, which is known as biterminal oxidation. The secondary alcohol or methyl acetone generated by subterminal oxidation can then be further oxidized by the subsequent Baeyer-Villiger monooxygenase and esterase to produce an alcohol and a fatty acid. Dioxygenases start the Finnerty pathway by producing n-alkyl hydroperoxides, which are then oxidized to form peroxy acids, alkyl aldehydes, and fatty acids (Fig. 5) (Singh et al., 2011; Ji et al., 2013).

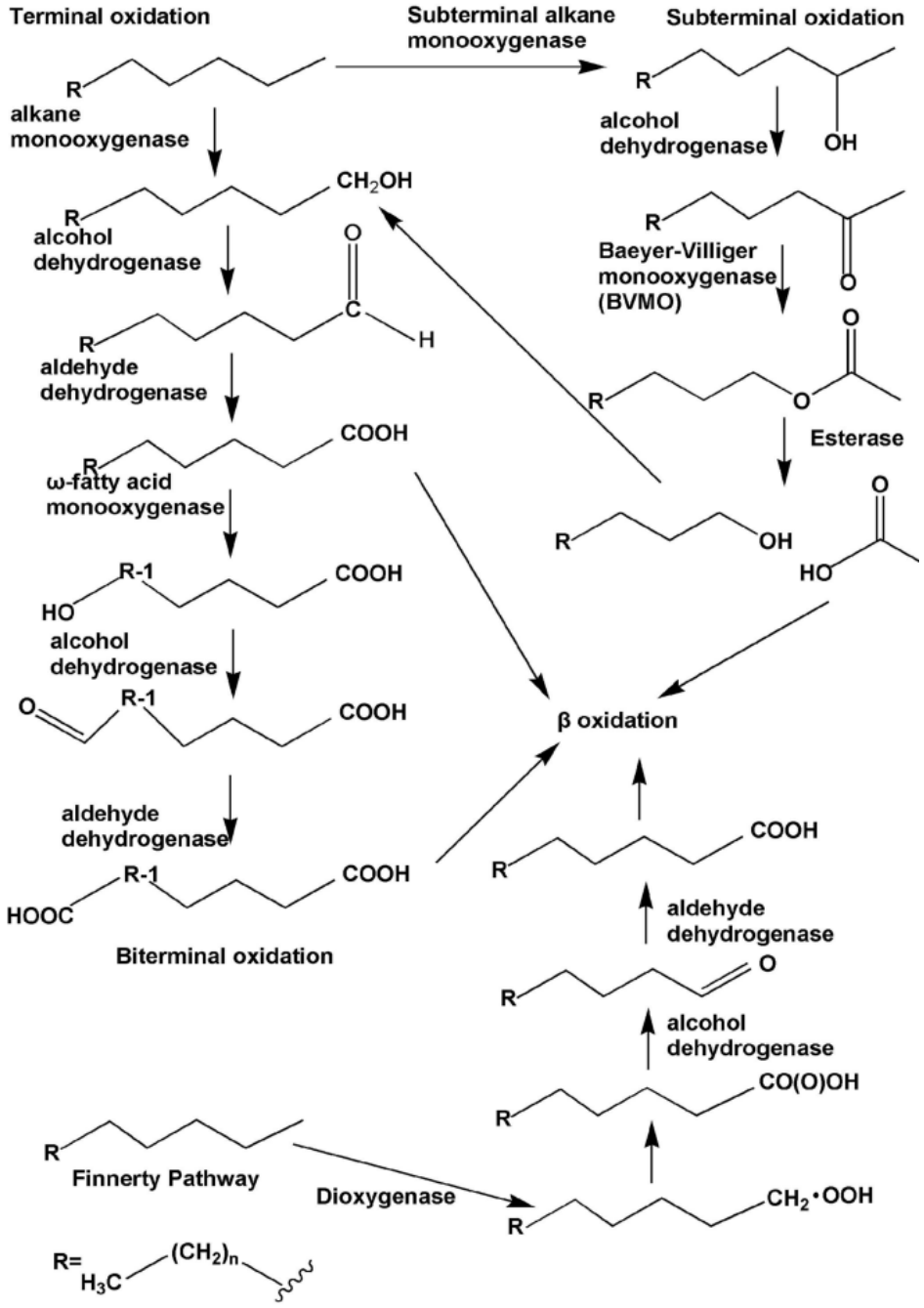


Figure 5. Aerobic pathways of n-alkane degradation (Ji et al., 2013).

- **Cycloalkane**

Branched-chain alkanes are more difficult to biodegrade than simple alkanes and alkenes, particularly l3-branched (anteiso-) hydrocarbons, quaternary compounds, and isoterpenoids (Abbasian et al., 2015). In this case, the biodegradability decreases as the number of ring structures increases. Oxidase activity converts cycloalkanes to cyclic alcohol, which is then dehydrogenated to a ketone. Cycloalkane-carboxylic acids and cycloketones are the main byproducts of cycloalkane metabolism (Kothari et al., 2014).

The cycloalkane is first oxidized by the hydroxylase to produce a cycloalkanol. Naphthenic ketone is created when the cycloalkanol removes the hydrogen. The ketooxygenase enzyme converts naphthenic ketone to caprolactone. The caprolactone ring is then broken by the insertion of an H_2O molecule whose progress results in 6-hydroxycaproic acid, which 6-hydroxycaproate dehydrogenase then further oxidizes to 6-oxohexanoic acid. Aldehyde dehydrogenase easily catalyzes the oxidation of aldehydes to produce dicarboxylic acid. To maintain the life activity of the microbes, the dicarboxylic acid is ultimately further oxidized via the tricarboxylic acid cycle (Fig. 6) (Li et al., 2019).

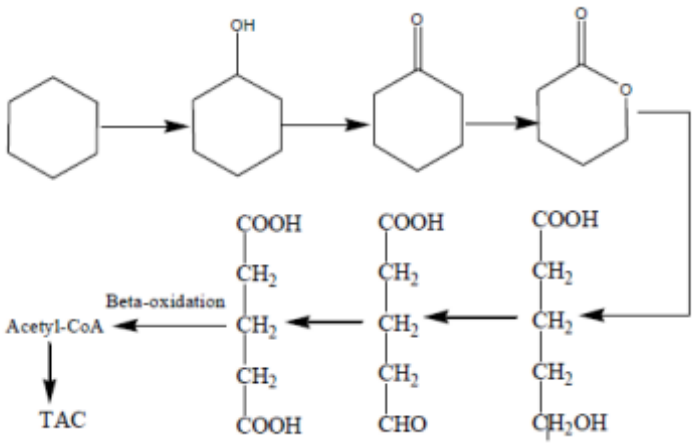


Figure 6. Process of degradation of cycloalkanes (eg. naphthenes) by petroleum-degrading bacteria (Li et al., 2019).

- **Aromatic hydrocarbons**

It is generally known that, due to their higher hazardous character, aromatic hydrocarbons are more difficult to biodegrade than short-chain alkanes and alkenes. However, under aerobic conditions, they are rapidly biodegraded by a wide variety of microorganisms, particularly bacterial and fungal species (Abdel-Shafy and Mansour, 2019).

An aromatic molecule degrades primarily through two processes: (1) ring activation, and (2) ring cleavage (Kothari et al., 2014). The process for the degradation of aromatic compounds often starts with the addition of oxygen by enzymes such as mono- and dioxygenases.

Aromatic hydrocarbons degradation comprises a wide variety of peripheral pathways that activate structurally diverse substrates into a limited number of common intermediates that are further cleaved and processed by a few central pathways to the central metabolism of the cell. Important intermediary products from this reaction include phenol, benzyl alcohol, gentisate, protocatechuate, and catechol. With the help of several oxygenase enzymes, these intermediates cause ring cleavage, which produces carboxylic acid. As a result, the biodegradation process

continues to produce acetyl-CoA and succinyl-CoA, which eventually participate in the crucial metabolic process (Fig. 7) (Abdel-Shafy and Mansour, 2019).

Bacterial degradation is initiated by members of one of the three superfamilies: the Rieske non-heme iron oxygenases (RNHO), the flavoprotein monooxygenases (FPM) and the soluble diiron multicomponent monooxygenases (SDM). Further metabolism is achieved through di- or trihydroxylated aromatic intermediates. However, activation can be carried out by CoA ligases where the formed CoA derivates are subjected to selective hydroxylation. The formed intermediates can be catalyzed by the enzymes intradiol and extradiol dioxygenases that are key enzymes in the degradation of aromatics. Other enzymes involved in aromatic compounds degradation are still being discovered. In addition, metabolic pathways and encoding genes responsible for the degradation have been characterized for many isolated bacterial strains, mainly from the Proteobacteria and Actinobacteria phyla (Sierra-Garcia and de Oliveira, 2013).

As the number of benzene rings rises, it becomes more difficult for microbes to degrade PAHs with more than four rings; co-metabolism is the only method available for their oxidative degradation.

There are numerous techniques to open and generate complex PAHs. Existing biotechnology can only be used to degrade low concentrations of polycyclic aromatic hydrocarbons since intermediate products such as trans-diols, phenols, naphthalenes, and epoxides are toxic to microorganisms (Li et al., 2019).

Due to their capacity to degrade PAHs, various fungal enzymes such as laccase, liP, and mnP, as well as epoxide hydrolases, cytochrome P450 monooxygenase, dioxygenases, proteases, and lipases, have received much research (Al-Hawash et al., 2018).

Naphthalene dioxygenase (NDO) is a major enzyme involved in the breakdown of several aromatic hydrocarbons from soil and aquatic ecosystems and is therefore regarded as a helpful enzyme in biodegradation (Abbasian et al., 2015)

The model P450 from *Bacillus megaterium*, CYP102A1 (P450BM3), is one of the known microbial P450s, and protein engineering studies have shown that it has the ability to oxidize PAHs, including phenanthrene, fluoranthene, and pyrene, to a combination of phenols and quinones (Bhandari et al., 2021).

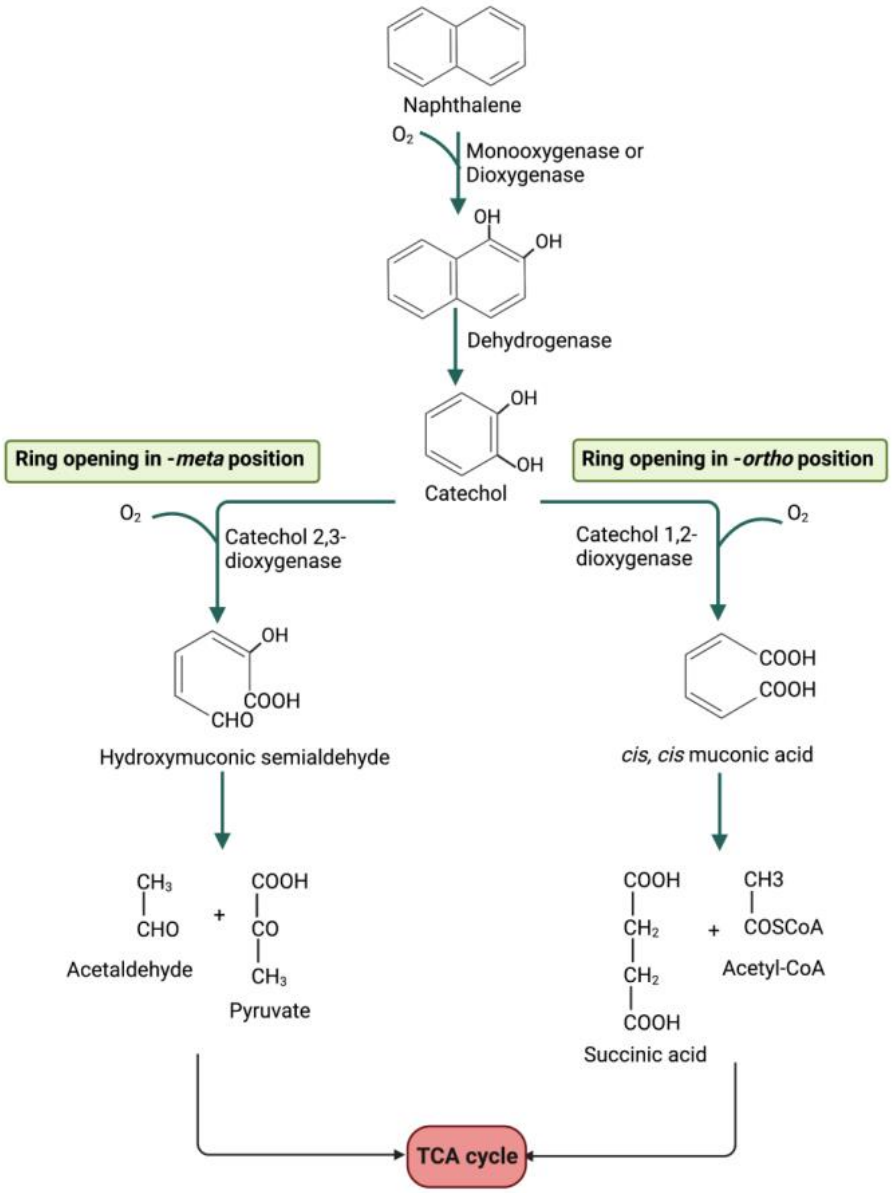


Figure 7. Biodegradation of PAHs (e.g., naphthalene) (Pandolfo et al., 2023).

- Anaerobic degradation

The microbial degradation of petroleum hydrocarbons by anaerobic microorganisms is considered as an environmentally significant method, for example, at zero oxygen concentrations, microbial degradation of aromatic benzoate, halobenzoate, and chlorophnol normally occurs (Mangla et al., 2021).

Typically, anaerobic hydrocarbon degradation occurs in deep, anoxic environments, such as natural gas and oil leaks and seeps on land or in the ocean, as well as in areas that have been contaminated by oil or its derivatives (Abbasian et al., 2015).

For both saturated and aromatic hydrocarbons, under sulfate, nitrate, metal-reducing, or methanogenic conditions, a variety of activation processes have been postulated, with fumarate addition being the best described and most frequently reported. A glycyl radical enzyme known

as benzylsuccinate synthase (BSS) or (1-methyl) alkylsuccinate synthase (ASS/MAS) catalyzes the addition of fumarate (Castro et al., 2022). Other activation methods include -independent hydroxylation on the second or third terminal C atoms (to make secondary or tertiary alcohols), carboxylation of unsubstituted carbon atoms of aromatics, hydration of alkenes' and alkynes' double and triple bonds, and reverse methanogenesis (Fig. 8) (Abbasian et al., 2015).

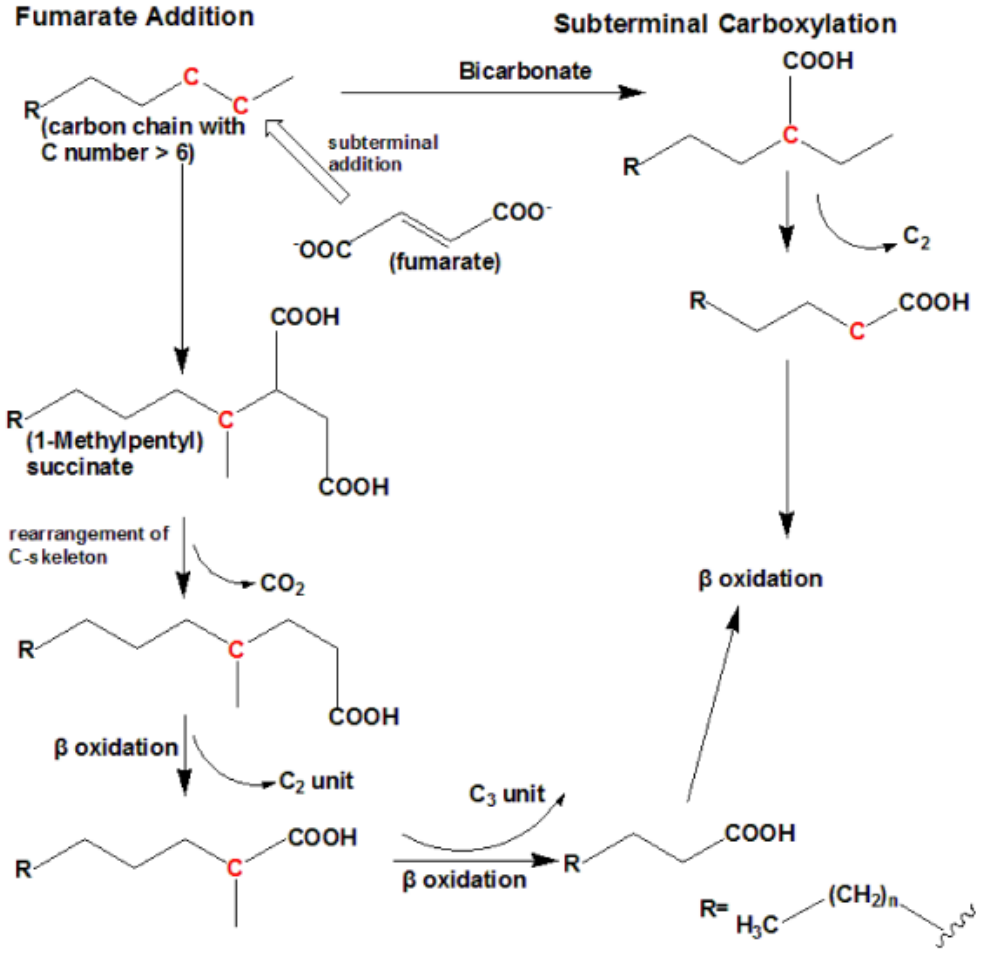


Figure 8. Anaerobic degradation pathway of alkane (Ji et al., 2013).

- **Aliphatic hydrocarbons**

Anaerobic degradation of alkanes has not been well studied presumably because greater attention has been given to BTEX compounds (benzene, toluene, ethylbenzene and xylenes) as priority pollutants. Also, the fact that anaerobic growth with n-alkanes is slow and that long chain alkanes are poorly soluble and often prevents the cultivation of cells homogeneously in the medium have made it difficult to study. However, anaerobic degradation of alkanes is important since alkanes are the major hydrocarbon components of petroleum, and some are toxic and difficult to eliminate. The two main mechanisms of anaerobic degradation of n-alkanes involve: first the initiation at the subterminal carbon of the alkane by the addition of fumarate, further reactions involve dehydrogenation and hydration. A new discovery is the anaerobic hydration

reaction, in which microbes add an H_2O molecule to the hydrocarbon to transform it into a primary alcohol. Many unsaturated alkenes and alkynes, including ethine and acetylene, can be mineralized by some bacteria, including *Nocardia rhodochrous* and *Pelobacter acetylenicus* using hydration reaction (Abbasian et al., 2015).

The presence of alkylsuccinate, metabolite originating from C3 to C11 alkanes, in oil contaminated environments indicates in situ microbial degradation of oil alkanes. The formation of alkylsuccinates is catalyzed by a strictly anaerobic glycyl radical enzyme which is named alkylsuccinate synthase or (1-methyl-alkyl) succinate synthase (Ass or Mas). The genes encoding Ass (assA) have recently been identified and are thought to be a useful biomarker for anaerobic alkane metabolism (Sierra-Garcia and de Oliveira, 2013).

The second mechanism for alkane anaerobic degradation is the carboxylation. This mechanism was proposed for the degradation of hexadecane in an alkane-degrading, nitrate-reducing consortium. Several anaerobic bacteria have been discovered that can degrade n-alkanes with six or more carbons, especially hexadecane (C16), by using sulfate or nitrate as electron acceptors (Victor et al., 2020).

However, in both cases, the hypothetical fatty acid intermediate (2-ethylalkanoate) that should result from the incorporation of inorganic carbon at C-3 of the alkane has never been detected. In addition, from an energetic point of view, the carboxylation of alkanes is not feasible under physiological conditions, unless the concentration of the fatty acid (2-ethylalkanoate) is in the micromolar order of magnitude or less. Other alternative anaerobic activation mechanisms of alkanes are proposed. For instance, the "unusual oxygenation" mechanism is used by the strain *Pseudomonas chloritidismutans* AW-1T, that is supposed to produce its own oxygen via chlorate respiration used for subsequent metabolism of alkanes. Other alternative mechanism considers that activation in the anaerobic methanogenic system may be initiated by an anaerobic hydroxylation reaction (Sierra-Garcia and de Oliveira, 2013.

- **Aromatic hydrocarbons**

The anaerobic microbial degradation of aromatic compounds is different from the aerobic pathway and is based on reductive reactions to attack the aromatic ring. The genes and process involved in such degradation are not well studied.

The most characterized anaerobic mechanism is the radical-catalyzed addition of fumarate to hydrocarbons, yielding substituted succinate derivatives, which involves the activation of several alkyl-substituted benzenes as well for n-alkanes. This mechanism is more understood in toluene. Benzylsuccinate synthase is the key enzyme in this reaction. All enzymes required for β-oxidation of benzylsuccinate are encoded by the bbs operon. Further degradation of benzoyl-CoA is accomplished via reductive dearomatization, hydrolytic ring cleavage, β-oxidation to

acetyl-CoA units and terminal oxidation to Co_2 (Sierra-Garcia and de Oliveira, 2013; Castro et al., 2022).

In addition, despite the chemical and structural similarities between the two compounds, the degradation of ethylbenzene (and probably other alkylbenzenes with carbon chain of at least 2) is different. It involves a direct oxidation of the methylene carbon via (S)-1-phenylethanol to acetophenone. Ethylbenzene is anaerobically hydroxylated and dehydrogenated to acetophone, which is then carboxyled and converted to benzoylCoA as the first common intermediate of the two pathways (Sierra-Garcia and de Oliveira, 2013).

- Biosurfactants

Biosurfactants are a heterogeneous range of surface-active chemical molecules produced by a diverse variety of microorganisms (Jahangeer and Kumar, 2013). These compounds contain amphipathic molecules that interact with fluids of various polarities (oil/water and water/oil) and have hydrophobic and hydrophilic components (Silva et al., 2014). By accumulating at the interface between immiscible fluids like water and oil or air and water, biosurfactants reduce surface and interfacial tension. These characteristics led to emulsifying, foaming, and detergency (Almansoory et al., 2014)

The biosurfactant molecule creates a new surface area at the water/oil interface by producing a monolayer of surfactant around the hydrocarbon particle with the hydrophobic tail pointed toward the liquid phase. Emulsification is made easier, and the hydrocarbon substrate's surface area increases as a result. The overall phenomena improve the solubilisation of hydrocarbons in water or water in hydrocarbons, which increases the bioavailability of pollutants for microbial degradation (Patowary et al., 2017).

Biosurfactants are classified following their microbial origin and chemical structure mainly into fatty acids, glycolipids (rhamnolipids, trehalolipids and sophorolipids), lipopeptides (surfactin), phospholipids, neutral lipides and polymeric biosurfactants (emulsan, liposan, alasan, lipomanan and other polysaccharide-protein complexes) (table 2) (Patel and Kharawala, 2022). The hydrophilic and hydrophobic moieties make up the majority of them. Acids, peptides, mono-, di-, or polysaccharides make up the hydrophilic moiety, while saturated or unsaturated fatty acids make up the hydrophobic moiety (Janaki et al., 2016).

When compared to the synthetic compounds' equivalents, biosurfactants have a number of advantages including biodegradability, low toxicity, availability of raw materials, and low cost. Environmental parameters like temperature, pH, and ionic strength tolerances do not impact many biosurfactants (Fakruddin, 2012). They are used in oil recovery and hydrocarbon bioremediation thanks to a low critical micelle concentration (Patel and Kharawala, 2022).

Table 2. Biosurfactants involved in the biodegradation of petroleum hydrocarbon.

Type of biosurfactant	Biosurfactant	Microorganism	Reference
Glycolipides	Glycolipid	*Bacillus* sp.	Chandra et al., 2012. Tabatabaee et al., 2005.
	Rhamnolipids	*Pseudomonas aeruginosa*	Soberón-Chávez et al., 2021.
	Trehalose	*Rhodococcus erythropolis*	Mahjoubi et al., 2018
	Sophorolipid	*Candida* sp.	Archana et al., 2019.
Lipopeptide	Glycolipid	*P. aeruginosa*	Sun et al., 2019.
	Surfactin	*Bacillus subtilis*	Fei et al., 2020.
	Lipopeptide	*B. subtilis*	Sharma and Pandey, 2020.

Members of various bacterial genera have been extensively examined for their capacity to increase the degradation of petroleum oil through the production of biosurfactants including *Bacillus cereus* and *Pseudomonas aeruginosa* (Fig. 9) (Jahangeer and Kumar, 2013; Janaki et al., 2016).

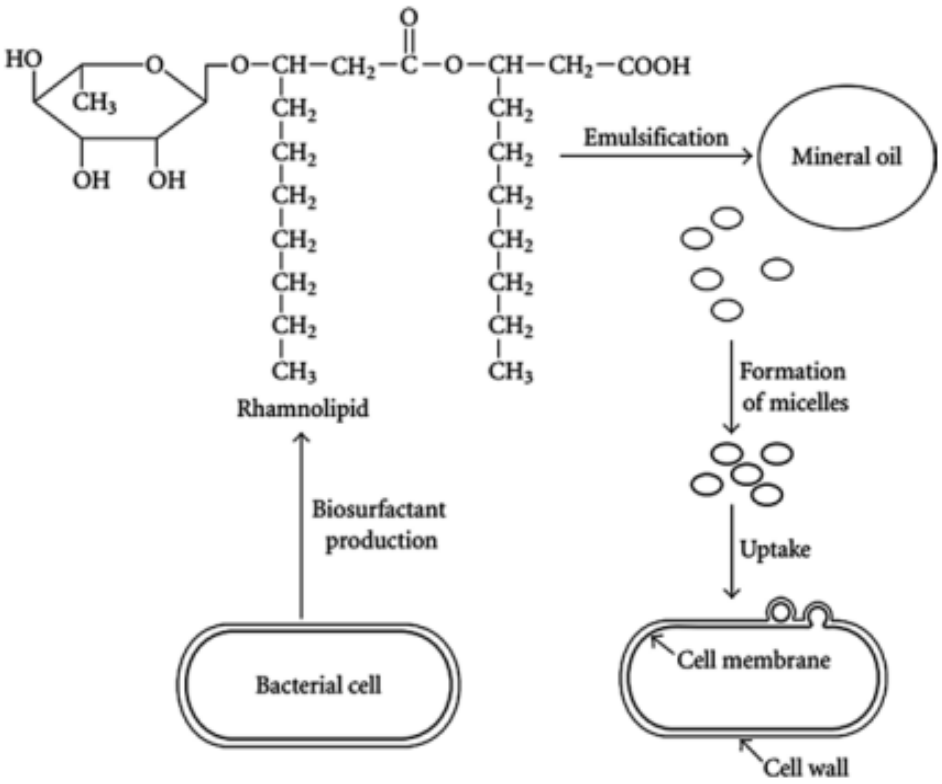


Figure 9. Participation of the biosurfactant (rhamnolipid) synthesized by *Pseudomonas* sp. in the absorption of hydrocarbons (Unimke et al., 2018).

Methodology

Methodology

1. Materials

1.1. Soil samples

Soil samples contaminated with petroleum oil (Fig. 10) were collected from different regions of Algeria near petroleum oil wells. The first sample was collected in June 2020, at approximately 10 m distance from petroleum well located in In Amenas, Illizi (south-east Algeria). Four soil samples were collected from Hassi Messaoud, Ouargla (south-east Algeria), in February 2021, where one was sampled near an oil well and three near an oil storage tank at different distances (1 and 2 meter). Three soil samples were taken in February 2021 from Oued Souf, El Oued (south-east Algeria) near a well. Another soil sample was collected from the region of Sougueur, Tiaret (north-west Algeria).

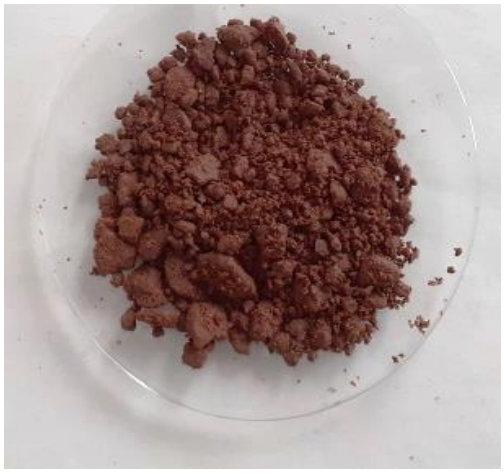


Figure 10. Petroleum contaminated soil sample.

The samples were collected in sterile plastic bags and then transferred in a cooler to the laboratory where they were stored at 4°C prior to work.

1.2. Petroleum samples

Samples of crude oil, condensate and slop (Fig. 11) were obtained from the Algerian Company SONATRACH located in Arzew, Oran (west transport region of Algeria). The crude oil and slop (that results from the sedimentation residues of crude oil), were obtained directly from the hydrocarbon storage tanks in "Arzew Crude Arrival Terminal", while the condensate, which is a liquid mixture of light hydrocarbons resulting from the condensation of certain crude natural gases, was obtained from the "Arzew Condensate Arrival Terminal".

Figure 11. Petroleum hydrocarbon samples: a) slop, b) crude oil, c) condensate.

1.3. Diesel and gasoline samples

Gasoline and diesel samples were purchased from local gas station (Tiaret, Algeria).

1.4. Chemicals and culture media

Several chemicals and culture media were used:

- **Bushnell Hass Broth (BH)**

A medium that does not contain a carbon source, formulated to evaluate the ability of microorganisms to decompose hydrocarbons by adding the tested hydrocarbon to the medium. It is also formulated for examining fuels for microbial contamination. It is prepared by mixing for one litre distilled water: 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g K_2HPO_4, 1 g KH_2PO_4, 1 g NH_4NO_3 and 0.05g $FeCl_3$ (pH 7) (Lima et al., 2020).

- **Minimal salts medium (MSM)**

Culture medium composed only of mineral salts without any source of carbon. It is used for the enrichment, isolation and growth of microorganisms with specific nutritional types. The medium consists of (per litre distilled water): 5 g NaCl, 5 g KH_2PO_4, 1 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 2 g $NaNO_3$, 0.02 g $FeCl_2 \cdot 4H_2O$, 0.02 g $CaCl_2$ (Obi et al., 2016).

- **Luria Bertani (LB)**

Contains sample amounts of all of the essential inorganic nutrients. The medium consists of, per litre distilled water: 10 g tryptone, 5 g yeast extract, 5 g NaCl and 1 g tryptophan (Morales-Guzmán, 2017).

- **Nutrient Agar**

It is a general-purpose nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth (Sagar, 2018).

- **Sabouraud Agar**

Used for the isolation and cultivation of fungi.

- **PUM-buffer solution (Phosphorus-Urea-Magnesium)**

It consists of 19.7 g/L K_2HPO_4, 7.26 g/L KH_2PO_4, 1.8 g/L $MgSO_4 \cdot 7H_2O$, pH 7.1 (Morales-Guzmán et al., 2017).

- **Phosphate buffer**

Consists of a mixture of monobasic dihydrogen phosphate and dibasic monohydrogen phosphate (1 M). Phosphates have a very high buffering capacity and are highly soluble in water.

- **Olive oil agar**

It is composed of phenol red (0.01 % w/v), olive oil (0.1 % v/v), $CaCl_2$ (0.1 % w/v), and agar (2 % w/v). pH is adjusted to 7.3 before autoclaving at 121°C for 15 min (Mahmood et al., 2017).

- **Tween 80 agar**

Contains per liter distilled water: 10g peptone, 5 g NaCl, 0.1 g $CaCl_2.2H_2O$, 20 g agar (Pham et al., 2021).

2. Methods

2.1. Enrichment, purification and isolation of hydrocarbon degrading bacteria

100 mL of Bushnell-Hass (BH) medium was used as the enrichment media with 1% (v/v) of crude oil, condensate and slop, separately. In addition, 10 g of contaminated soil was added to the medium containing 1 % diesel and gasoline, separately, as the only carbon source. The incubation was carried out at 30 °C with 170 rpm agitation. After 5 days of incubation, a loop full of inoculum from BH medium was streaked onto the nutrient agar and incubated at 30 °C for 72 hours (Sukumar, 2016).

Besides, the same procedure was performed using 100 mL of sterilized minimal salt medium (MSM) containing 2 % (v/v) crude oil, condensate, and slop, separately. In addition, 5g of contaminated soil was added to the medium, which contained diesel and gasoline (separately) as its sole carbon source.

The mixture was then placed in a 250 mL conical flask and incubated for 7 days at 35 °C and 180 rpm. 5 mL of the initial culture is transferred to 100 mL of fresh enrichment medium, and grown under the same conditions. The isolates are spread on Luria Bartani agar following five successive cycles of enrichment. The isolated strains are then stored for further use in 30 % glycerol (v/v) at -80 °C.

It must be noted that the MSM was sterilized at 121 °C for 20 minutes, and the petroleum hydrocarbons serving as the carbon source were filtered using a 0.22 mm Millipore membrane (Sun et al., 2018).

Macroscopic and microscopic observations were performed on the isolates to determine their characteristic morphological features in addition to the Gram staining to determine their cell wall type.

2.2. Growth of microorganisms on diesel and gasoline as carbon and energy source

The isolates' ability to degrade diesel and gasoline was investigated; the experiment was carried out in Balch tubes with 8 mL of MSM supplemented with the diesel and gasoline (0.1 mL), which were inoculated with 2 mL of pure cultures of the isolates at a density of 1 x 10^8 to 2x10^8 cells/mL corresponding to an optical density (OD) ranging from 0.08 to 0.1 according to Bekele et al (2022).

Tubes were incubated at 65 rpm at 30 °C in a shaker for 2 weeks. An abiotic control, consisting of MSM supplemented with diesel and gasoline, separately, but lacking microorganisms, was used. UV-Vis Spectrophotometer was used to detect optical density at 600 nm (Nwinyi et al., 2014).

2.3. Screening of biosurfactant production

2.3.1. Emulsification index (E24%)

The emulsification index (E24%) test is assessed to determine emulsifying capacity (Panjiar et al., 2015). The selection of the emulsifying bacteria was done using diesel and gasoline as sources of hydrocarbons.

The E24 % is determined according to (Morales-Guzmán et al., 2017). Briefly, 2 mL of diesel and gasoline (separately) are added to a 4 mL bacterial culture grown in Luria-Bertani medium. The mixture is shaken in a vortex for 2 minutes, and then allowed to sit at room temperature for 24 hours. The bacterial emulsifying impact is contrasted with a chemical surfactant made up of culture medium plus Tween 80 (2:1 v/v). The E24 % is calculated using the following equation:

E24 % = [Height of the emulsified layer (mm) / total height of the liquid column (mm)] x 100

2.3.2. The qualitative drop collapse technique (DCT)

In the drop collapsing test, a drop of a cell suspension is placed on an oil-coated surface. Drops containing biosurfactants collapse, whereas non-surfactant-containing drops remain stable (Tugrul and Cansunar, 2005). The use of a drop-collapse technique for the screening of

biosurfactant producing microorganisms was determined by the method of Bodour and Miller-Maier (1998).

In 96-well plates, a thin layer of diesel and gasoline were added separately (50 µL per well), and the plates were left at room temperature for 1 hour. Following that, 100 µL aliquots of the corresponding bacterial suspension were introduced and kept in triplicate. The negative control was made up of distilled water. By observing the drop's movement within the well, it was possible to identify the formation of biosurfactants.

2.4. Cell surface hydrophobicity percentage (CSH %)

As a measure of the microbes' adherence to the hydrophobic hydrocarbons, the hydrophobicity of the microbial cell suspensions was assessed using the MATH (microbial adhesion to hydrocarbons) assay (Morales-Guzmán et al., 2017).

The bacteria were grown on Luria Bertani medium. Bacterial samples were taken at the exponential phase (72 h) through three washings with the PUM buffer solution for 5 min at 8000 g. The microbial density was adjusted in the phosphate buffer solution to an optical density of 0.5 (λ 600). After being sheared for two minutes in a vortex with the equivalent bacterial suspension in 2 mL of each dilution tube along with 2 mL of each fuel (separately), the phases were allowed to separate for 15 minutes. In the aqueous phase, the absorbance A (λ 600) was measured. The following equation was used to calculate the CHS %:

$$\mathrm{CSH\%} = \left[1 - \frac{\mathrm{A_{600}\ Final}}{\mathrm{A_{600}\ Initial}}\right] \times 100$$

2.5. Screening the isolates for enzymes activity

2.5.1. Lipase assays

- Qualitative assays

- **Screening of microbial lipase production on Tween 80 agar plates**

After the preparation and autoclaving the Tween 80 agar medium, 10 mL of Tween 80 is added as a final step after the medium has cooled to 45°C. All tested microorganisms are inoculated separately on plates and incubated at 37 °C for 48 hours. The enzymatic activity is then determined by visual precipitation of calcium salts from fatty acids released from the hydrolysis reaction (Pham et al., 2021).

- **Screening of microbial lipase production on olive oil agar**

The olive oil agar plates test is a qualitative test used for the screening of extracellular lipase activity. The microbial isolates are inoculated by streaking onto the agar plates with

phenol red and then incubated at 30 °C for 24 h. Lipase-producing bacteria use phenol red agar to convert the red color on olive oil to yellow (Mahmood et al. 2017).

- Quantitative assays

The positive colonies for lipase enzyme production are inoculated on agar slants and subsequently incubated at 37 °C overnight before storing at 4 °C as stock culture for further use (Mahmood et al. 2017). The selected strains are grown in 250 mL Erlenmeyer containing 100 mL of medium (peptone 10 g/L, NaCl 5 g/L, $CaCl_2.2H_2O$ 0.1 g/L, Gum Arabica 0.2 g/L) supplemented with 2 % glucose and olive oil. The cultures are incubated at 30 °C and stirred at 100 rpm for 48h. Then cells are centrifuged at 4000 rpm for 10 min. The supernatant is subjected to the enzymatic activity test (Soares et al., 2015).

Lipase assay is determined by titrimetric method using olive oil as a substrate according to the method of Ba and Mosimileol (2020).

Olive oil (10 % v/v) is emulsified with Gum Arabic (5 % w/v) in 0.1 M potassium phosphate buffer (pH 7). 0.1 mL of enzyme is added to the emulsion and is incubated for 15 min at 37 °C. The reaction is stopped and fatty acids are extracted by the addition of 1 mL of acetone: ethanol solution (1:1).

The amounts of fatty acids liberated are estimated by titrating with 0.1N NaOH using phenolphthalein indicators until a light pink coloration appeared. The reading for the blank (with no enzyme in it) is also taken.

The lipase activity is calculated using the formula:

$$\text{Lipase activity (U/mL)} = \frac{(\text{Milliliter NaOH for sample} - \text{Milliliter NaOH for blank} \times \text{N})}{\text{Milliliter of lipase} \times \text{Reaction time}}$$

Where, N is normality of NaOH.

* One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1 µmol of fatty acids from triglycerides equivalent per minute under the assay conditions.

2.5.2. Laccase assay

- Qualitative assay

- **Screening of Laccase production**

Laccase production was screened on microbial isolates. Bacterial isolates were inoculated on nutrient agar amended with 0.01 % guaiacol, while the fungus was inoculated onto potato dextrose agar (PDA) medium supplemented with 0.1 % ABTS (2,2-azinobis-(3-ethylbenzthiazoline-6-sulfonate). Bacteria were incubated at 37 °C for 48h and the fungus at 25

°C for 72 h. The formation of brown color around bacterial colonies indicates the oxidation of guaiacol by the action of laccase (Gaur et al., 2018). Whereas, the formation of dark green to purple color around fungal colonies on medium containing ABTS indicates the presence of laccase being generated by the fungus (Bhavsar and Patil, 2017).

- Quantitative assay

The positive colonies for laccase enzyme are grown on medium containing per liter: 3 g peptone, 10 g glucose, 0.6 g KH_2PO_4, 0.001 g $ZnSO_4$, 0.4 g K_2HPO_4, 0.0005 g $FeSO_4$, 0.05 g $MnSO_4$ and 0.5 g $MgSO_4$, pH 5.5, then incubated at 30° for 12 days. Microbial growth and enzyme activity are checked regularly (Abd El Monssef et al., 2016).

The supernatants are collected every four days, centrifuged at 1487g for 10 min and filtered on Whatman filter paper. The experiment is performed in triplicate (Peraza-Jiménez et al., 2021).

The laccase activity is determined by using guaiacol as a substrate. The reddish-brown color that results from laccase's oxidation of guaiacol is utilized to calculate the enzyme activity at 450 nm using a spectrophotometer. The reaction mixture contains 1 mL of guaiacol (2 mM) and 3 mL of sodium acetate buffer (10 Mm) and 1 mL of enzyme extract (supernatant). Additionally, a blank is prepared using 1 mL of distilled water rather than the enzyme. The mixture is incubated at 30 °C for 15 min (Abd El Monssef et al., 2016)

One unit of laccase activity (U) is defined as the concentration of the enzyme required to oxidize 1 μM of substrate per minute (Binkowska et al. 2020). The laccase activity in U/mole is calculated by the formula:

$$EA = A.V/t.e.v$$

Where: EA = enzyme activity, A= absorbance, V= total mixture volume (mL), v=enzyme volume (mL), t= incubation time (min), e = Extinction coefficient of guaiacol (0.6740μM/cm).

2.5.3. Lignin peroxidase

Lignin peroxidase (LiP) was checked for in each pure colony on an agar plate containing 15 g/L agar, 5 g/L yeast extract, 5 g/L glucose, 2 g/L KH_2PO_4, 0.05 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L $CuSO_4 \cdot 5H_2O$, and 2 g Na_2HPO_4 (pH 7–7.5).

After the agar is autoclaved for 20 minutes at 121°C, methylene blue (MB) is added at a final concentration of 1000 mg/L. All of the samples are cultured for a week at 30 °C.

The agar plates supplemented with MB showing a white decolorized zone indicate the presence of an active LiP enzyme (Pham et al., 2022).

2.6. Bacterial identification

2.6.1. DNA extraction

The bacterial isolates were identified using genomic DNA extracted from pure cultures as described in Fabryová et al. (2018). First, bacterial lysates were obtained by picking colonies into tubes containing 50 µL of lysis buffer and incubating the tubes for 10 min at 95 °C. After that, tubes were centrifuged at 8.000 g for 5 min. Finally, 10 µL of the supernatant, containing genomic DNA, were transferred to a clean tube containing 90 µL of sterile distilled water.

2.6.2. PCR amplification

The 16S rRNA gene of each bacterial strain was amplified as described by Rivas et al. (2007). The amplification of 16S rDNA was performed using PCR with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), which spans nearly full-length of 16S rRNA gene, with an expected amplicon of 1000~1400 bp.

PCR of the 16S rRNA gene contained 12,5 µL NZYTaq II 2x Green Master Mix, 2,5 µL of primer 27F, 2,5 µL of primer 1492R, and 1 µL of DNA template, brought up to a final volume of 25 µL with ultra-pure water. PCR conditions were as follows: preheating at 94 °C for 5 min; 34 cycles of denaturing at 94 °C for 1 min; annealing at 54 °C for 1 min and 10 sec, and extension at 72 °C for 1 min and 30 sec, and a final extension at 72 °C for 7 min. The reaction was performed on PCR cycles (Biometra Thermal Cycler) (Poveda et al., 2019).

PCR products were visualized on a 1 % agarose gel electrophoresis for 2 h at 60 V, and the bands corresponding to the 16S rRNA gene were excised with sterile scalpels and processed using the QIAquick™ Gel Extraction Kit (QIAGEN), following manufacturer's instructions.

2.6.3. Sequences analysis

The 16S rRNA sequence was determined with a model 373A automated fluorescent-DNA sequencer (Applied Biosystem/USA). Approximate taxonomic identification of the sequences obtained from 16S rDNA of the endophytic bacterial isolates were achieved through the BlastN software (http://www.ncbi.nlm.nih.gov/BLAST/) and EzBioCloud (Yoon et al., 2017).

2.7. Fungal identification

2.7.1. DNA extraction

Genomic DNA was isolated from the fungus isolate and was used as a template for PCR. The pellet of the pure fungus isolate was collected by centrifugation at 6.000 g for 10 min and the total genomic DNA was extracted using the Gene Elute™ Plant Genomic DNA Purification

Kit (Sigma-Aldrich, Burlington, MA) according to the instructions of the manufacturer and the genomic DNA was transferred to a clean tube and stored at -20 °C.

2.7.2. PCR amplification

Polymerase chain reaction (PCR) amplifications were conducted according to Vilgalys and Hester (1990). The ITS regions of ribosomal DNA (rDNA), including 5.8S rRNA gene, were amplified by using eukaryotic universal primers, internal transcribed spacer (ITS), ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′).

PCR mixture was carried out in 25 µL reaction volume containing 1 µL of the DNA template, 10x reaction buffer (2.5 µL, [15 mM MgCl2, 100 mM Tris, 500 mM KCl]), BSA (2.5 µL, 1:10), forward primer (1 µL, 10 µM), reverse primer (1 µL, 10 µM), dNTPs (0.5 µL, 10 mM each dNTP), Taq-polymerase (0.15 µL, 5 U/µL), and DNA-free water (16.35 µL).

The thermal cycling program involved one cycle at 95 °C for 10 min, followed by 30 cycles with a denaturation step at 95 °C for 1 min, an annealing step at 50 °C for 30 s, and an extension step at 72 °C for 1 min, followed by one cycle incubation at 72 °C for 7 min. A negative control without DNA-template was included in each PCR run to exclude fungal DNA contamination. The reaction was performed on PCR cycles (Biometra Thermal Cycler) (Poveda et al., 2019).

PCR product was visualized on a 1 % agarose gel electrophoresis, stained with SYBR® Safe). Before sequencing, the PCR-product was purified using the DNA-CLEAN-UP & GEL-OUT MICRO SPIN KIT (EM28–050) from EXTRACTME® (Blirt, Gdansk, Poland) based on the manufacturer's protocol.

2.7.3. Sequences analysis

The cleaned PCR product was sequenced by a model 373A automated fluorescent-DNA sequencer (Applied Biosystem/USA). The obtained, quality-checked sequence was BLAST analyzed using available fungal DNA sequences in GenBank National Center for Biotechnology Information (NCBI) data library (http://www.blast.ncbi.nlm.nih.gov/blast) (Salgado Salomón et al. 2021).

2.8. Degradation of diesel and gasoline in soil

2.8.1. Microcosm preparation and inoculation with microbial isolates

The first step in this study was the preparation of soil for the assay by autoclaving it at 121 ºC for 15 min three time (24 h apart) to remove the indigenous microorganisms. The soil is then supplemented with 250 mg/Kg of $(NH_2)_2SO_4$ and 100 mg/Kg of K_2HPO_4 to biostimulate the growth of microbial inocula (Bento et al., 2005). The sterilized soil is distributed over 78 pots

where 39 pots are contaminated with 10 % diesel oil and the other 39 pots with 10 % gasoline (Fig. 3) (Agarry and Latinwo, 2015). Soils are subsequently inoculated with 3 mL volume of the standardized microbial suspensions (0.5 Mac Farland) (Ghazali et al., 2004). After homogenization with a sterile spatula, the mixture is incubated for 7 days at room temperature. Sterile distilled water is added every 48 hours with homogenization to maintain the humidity and O_2 level in the medium in order to allow biodegradation of the pollutants. Three repeats are made for each microbial isolate and for the control (without microbial suspension). Total petroleum hydrocarbons (TPH) degradation kinetics was followed by sampling at time intervals after 7 days, 14 days, 21 days and 28 days.

2.8.2. Extraction and determination of total petroleum hydrocarbon (TPH)

From each microcosm, one gram is taken and placed in a 100 mL flask, and then 2 mL of n-hexane is added. The mixture is agitated vigorously on a magnetic stirrer for 30 minutes to permit hexane to extract the oil out of the soil sample. The liquid-phase extract (filtrate) is then diluted by adding 1 mL of the extract to 5 mL of hexane after the solution had been filtered using a filter paper. The absorbance of the extract is measured spectrophotometrically at 400 nm using n-hexane as a blank. In order to determine the total petroleum hydrocarbons in soil, a calibration curve is made for freshly prepared diesel oil and gasoline (prepared separately) that had been diluted with n-hexane at various concentrations (Agarry and Latinwo, 2015). Percent degradation (D) is calculated using the following formula:

$$D = \frac{TPHi - TPHr}{TPHi} \times 100$$

Where TPHi and TPHr are the initial and residual TPH concentrations, respectively.

2.9. Microbial consortium preparation

Microbials consortia are formulated by mixing equal proportions of pure microbial cultures (Ghazali et al., 2004) that were previously isolated from hydrocarbon-contaminated samples. These were then tested for their ability to degrades diesel and gasoline in soil through the determination of TPH.

2.10. Statistical analysis

Results are expressed as means ± standard of deviation. All the experiments were replicated at least three times and quantitative data were subjected to analysis of variance. Comparison between groups was carried out using the test of Duncan. The significant differences between means were determined at $p < 0.05$ level.

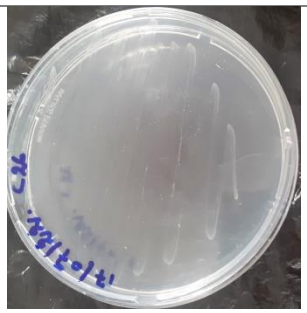
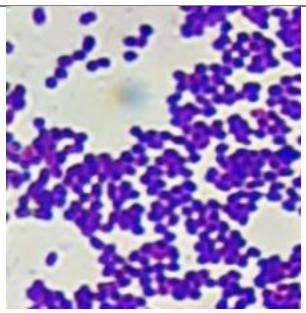
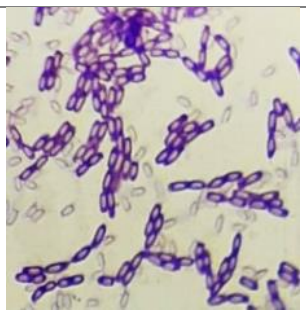
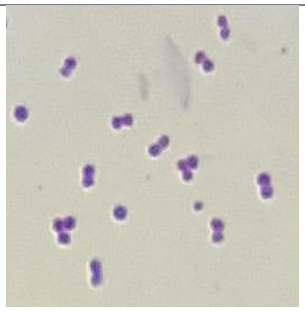
Results

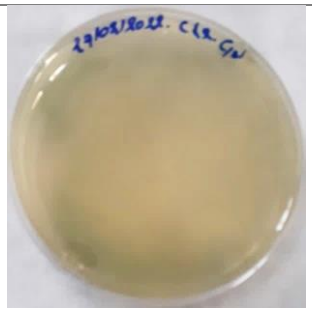
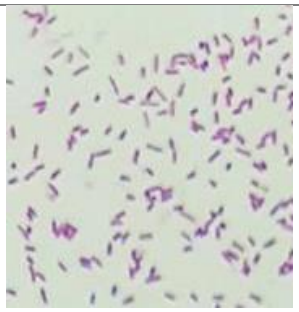
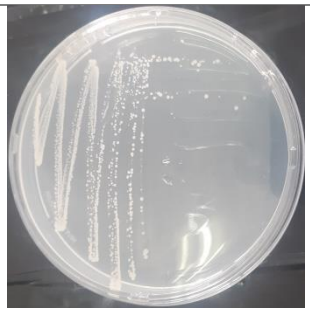
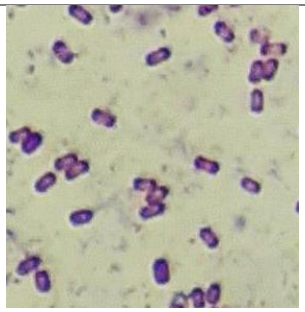
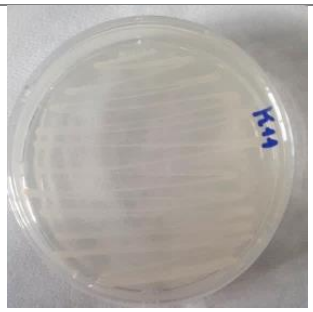
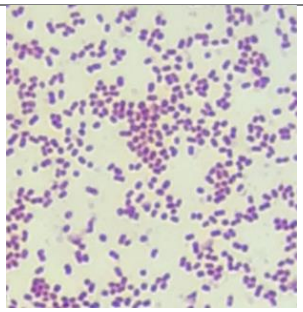
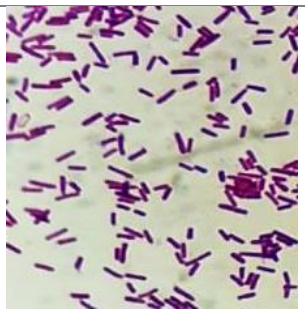
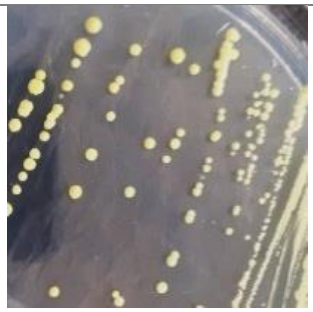
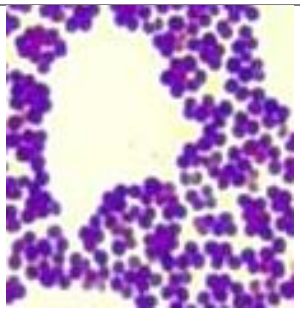
Results

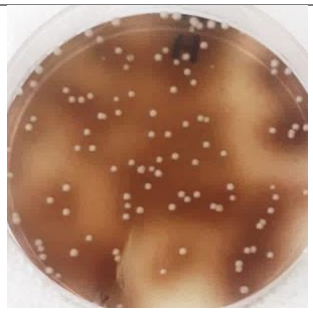
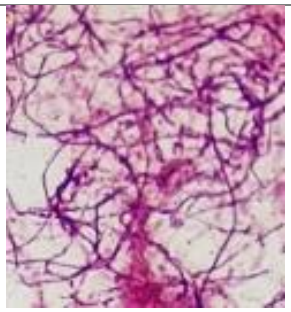
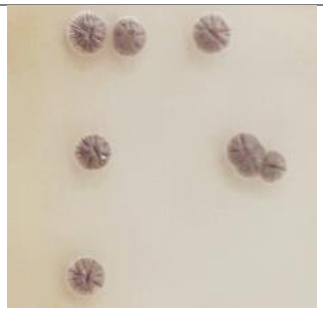
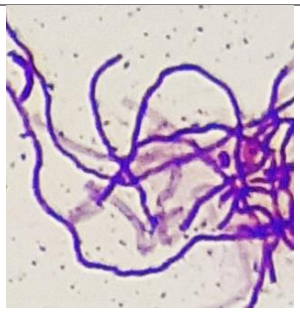
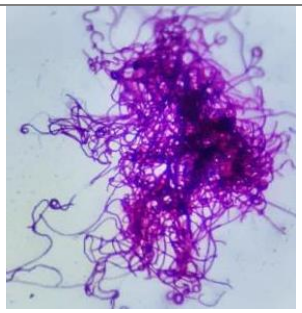
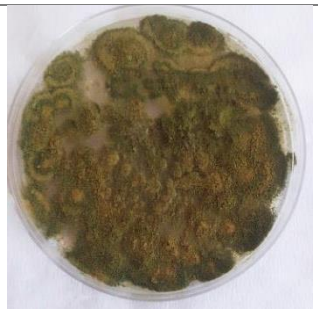
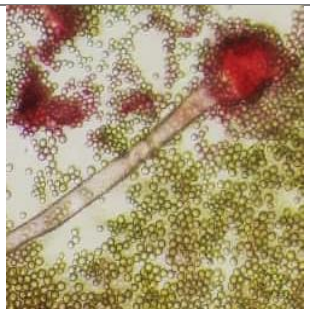
1. Identification of the microbial isolates

After the enrichment, isolation, purification and molecular identification of the obtained microbial colonies from petroleum products and contaminated soil samples, 11 bacterial and 01 fungal isolates have been characterized and identified. The macroscopic and microscopic observations are shown on table 3.

Table 3. Macroscopic and microscopic observations of the microbial isolates.

Isolates	Macroscopic observation	Microscopic observation	Origin
C26 ***Enterococcus gallinarum***			Petroleum sample: condensate.
C44 ***Bacillus paranthracis***			Petroleum samples: condensate.
C50 ***Kocuria rosea***			Petroleum sample: condensate.

C52 ***Pseudomonas aeruginosa***			Soil sample (In Amenas, Illizi).
K1 ***Bacillus subtilis subsp. subtilis* str.**			Soil sample (Hassi Messaoud, Ouargla).
K11 ***Acinetobacter baumannii***			Soil sample (Hassi Messaoud, Ouargla).
K20 ***Aneurinibacillus migulanus***			Soil sample (Oued Souf, El Oued).
K31 ***Micrococcus luteus***			Soil sample (Oued Souf, El Oued).

K33 ***Streptomyces cinereoruber***			Soil sample (Hassi Messaoud, Ouargla).
S ***Lysinibacillus pakistanensis***			Soil samples (Hassi Messaoud, Ouargla.
J ***Lysinibacillus cavernae***			Soil sample (Hassi Messaoud, Ouargla)
Fungus ***Aspergillus flavus***			Soil samples (Hassi Messaoud, Ouargla).

2. Growth of microorganisms on diesel and gasoline as carbon and energy sources

After 15 days incubation of the microbial isolates in liquid medium with gasoline and diesel oil, separately, as the sole carbon source, it has been noticed that all the tested strains have the ability to grow in such media with maximal OD readings varying from 0.5 to 1.9 for the 12 isolates.

(C44) *Bacillus paranthracis* demonstrated the higher growth rate (OD=1.9, $p< 0.05$) on diesel oil, followed by (K1) *Bacillus subtilis* (OD=1.62), then the fungus *Aspergillus flavus* (OD=1.55), (C26) *Enterococcus gallinarum* (OD=1.5) and (C52) *Pseudomonas aeruginosa*

(OD=1.38) with no significant difference in growth rates between them. However, the isolate (K20) *Aneurinibacillus migulanus* demonstrated the lower growth rate (OD=0.5) (Fig. 12).

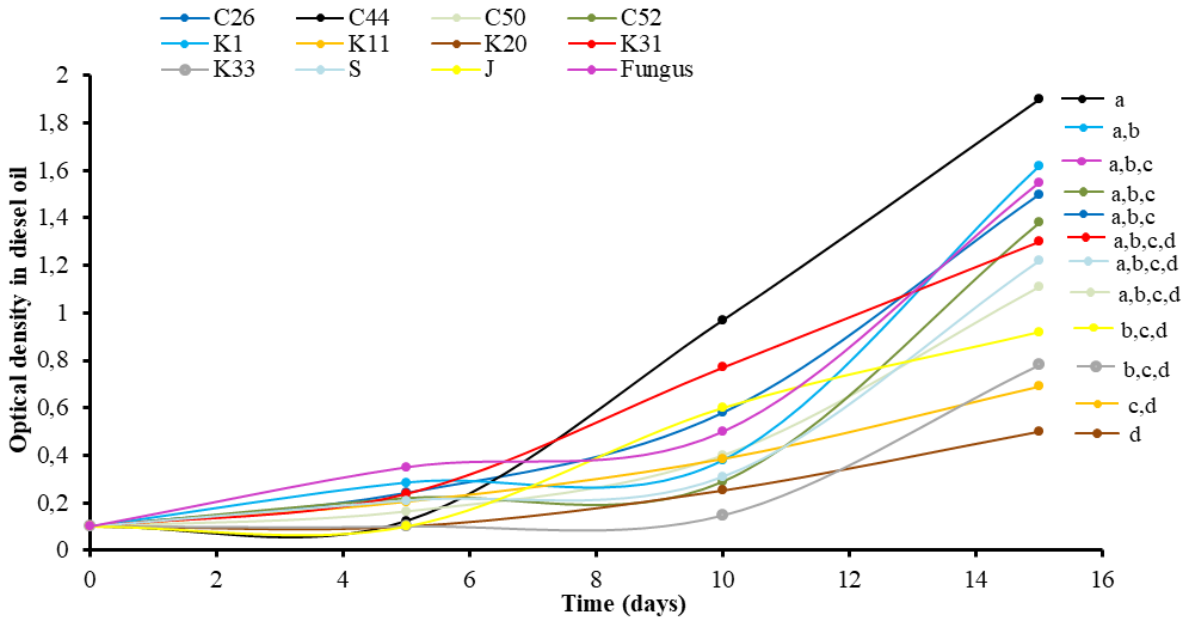


Figure 12. Growth rate of the microbial isolates in MSM supplemented with diesel oil.

Furthermore, both (K31) *Micrococcus luteus* (OD= 1.35) and the fungus *Aspergillus flavus* (OD= 1.28), showed the higher growth rates on gasoline ($p < 0.05$) with no significant difference between them. While (K20) *Aneurinibacillus migulanus* (OD= 0.28), (J) *Lysinibacillus cavernae* (OD= 0.19), (K33) *Streptomyces cinereoruber* (OD= 0.19) and (K1) *Bacillus subtilis* (DO= 0.15) revealed the lower growth rates (Fig. 13).

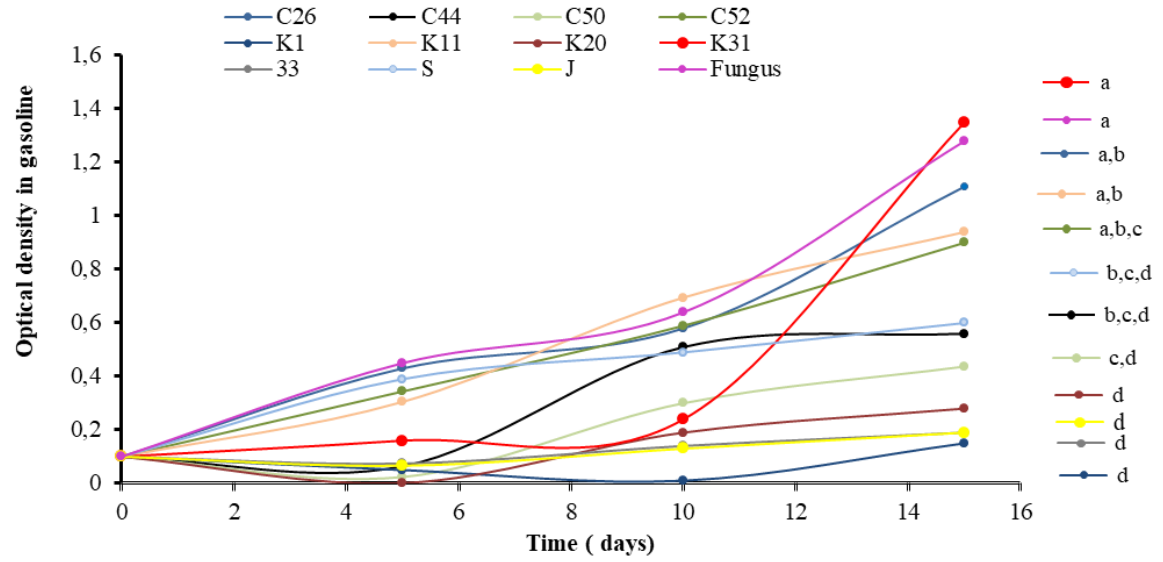


Figure 13. Growth rate of the microbial isolates in MSM supplemented with gasoline.

3. Screening of biosurfactant producing isolates

3.1. Emulsification index (E24%)

Emulsification index represents a rapid qualitative method for evaluating the emulsifying properties of biosurfactants produced by a microorganism. The obtained results regarding E24% of the selected microbial isolates are shown in figure 14.

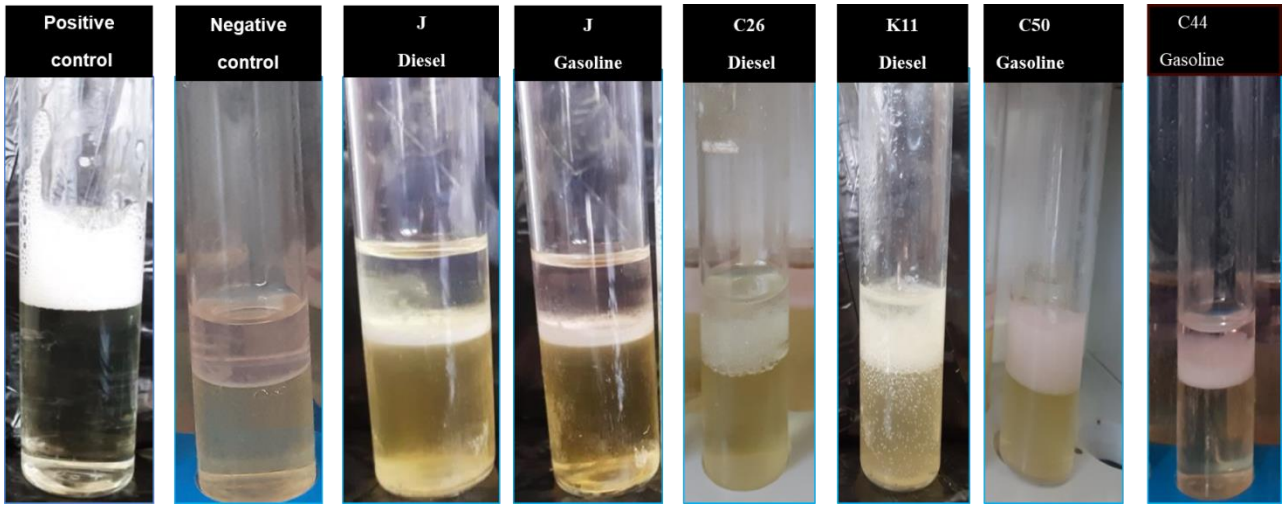


Figure 14. Isolates with emulsifying capacity on diesel and gasoline.

Among the 12 microbial isolates, 9 strains showed an emulsification capacity (E24%) on diesel containing medium.(C26) *Enterococcus gallinarum* presented the higher percentage (29.09 %, $p < 0.05$) and appears in a separated statistical group (Fig. 15) followed by (K20) *Aneurinibacillus migulanus* (19.36 %) and (K11) *Acinetobacter baumannii* (18.26 %) as homogenous statistical group.(K31) *Micrococcus luteus* (12.70 %), (J) *Lysinibacillus cavernae* (7.87 %), (C52) *Pseudomonas aeruginosa* (7.54 %), (K1) *Bacillus subtilis* (6.77 %) and *Aspergillus flavus* (4.46 %) ranked in the third homogenous statistical group. However, *Lysinibacillus pakistanensis* showed the lower percentage of emulsification (3.12 %) (Fig. 15).

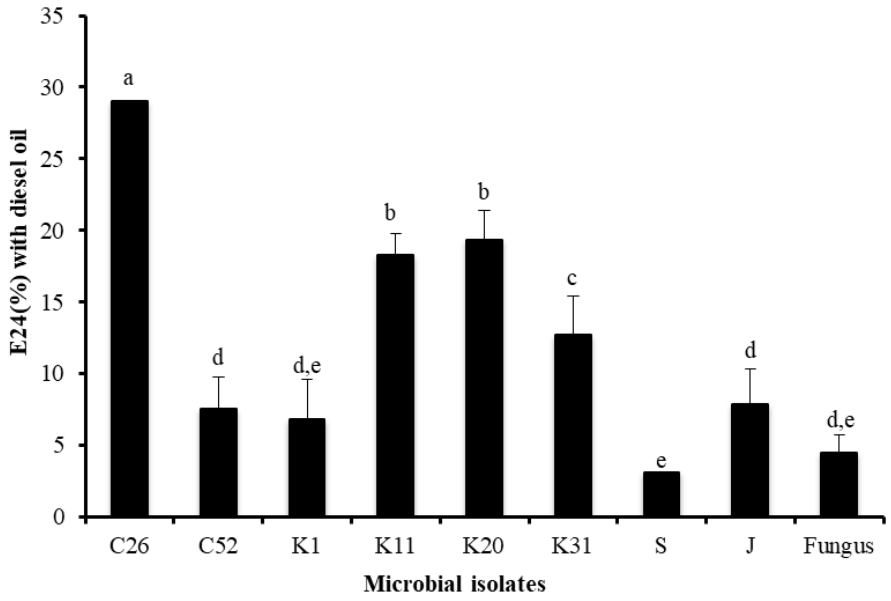


Figure 15. Emulsification index (E24%) of microbial isolates on diesel oil.

Besides, on gasoline containing medium, (C50) *Kocuria rosea* showed the higher emulsification percentage (38.61 %, $p < 0.05$), followed by (C44) *Bacillus paranthracis* (33.74 %). There was no statistically significant difference between (K31) *Micrococcus luteus* (3.73 %), the fungus *Aspergillus flavus* (3.71 %) and (C52) *Pseudomonas aeruginosa* (3.03 %), which showed the lower emulsification percentages (Fig. 16).

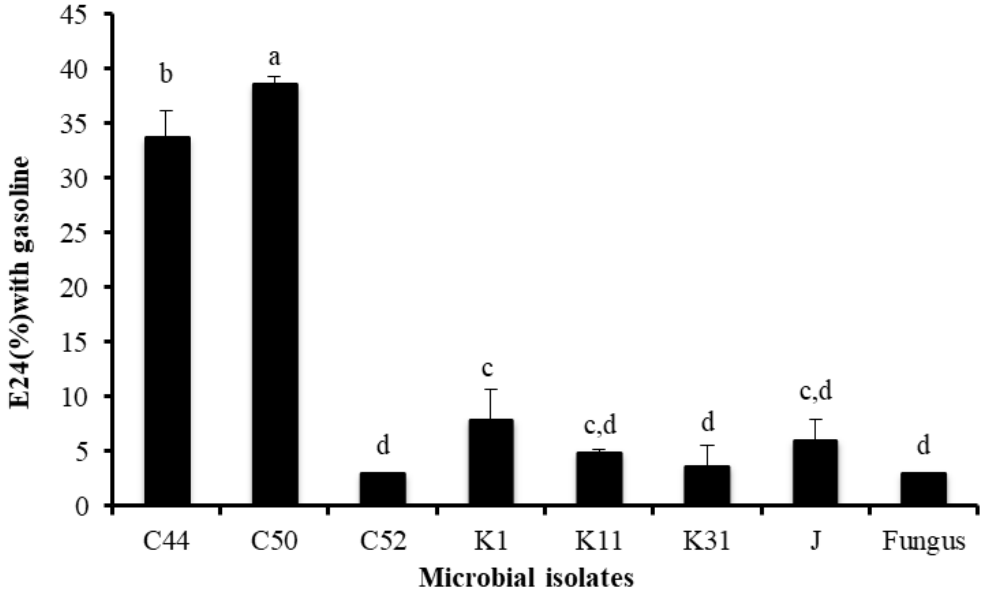


Figure 16. Emulsification index (E24%) of microbial isolates on gasoline.

3.2. Drop collapse technique (DCT)

The obtained results regarding the qualitative drop collapse technique indicate that all microbial isolates have a positive reaction which indicates that all the tested isolates may produce biosurfactants (Table 4; Fig. 17).

Table 4. Results of the qualitative drop collapse technique of the microbial isolates with diesel oil and gasoline.

Isolates	C26	C44	C50	C52	K1	K11	K20	K31	K33	S	J	Fungus
DCT(Diesel)	+	+	+	+	+	+	+	+	+	+	+	+
DCT(Gasoline)	+	+	+	+	+	+	+	+	+	+	+	+

Figure 17. Results of the drop collapse technique.

4. Cell surface hydrophobicity percentage (CSH %) of microbial isolates

All the tested microbial isolates showed their ability to adhere to the tested hydrophobic hydrocarbons (diesel and gasoline). However, a better cell surface hydrophobicity was observed with all the isolates regarding diesel, which varied between 18 % and 79 %, and between 4 % and 65.33 % regarding gasoline.

The best CSH percentage on diesel was demonstrated by (C52) *Pseudomonas aeruginosa* (79±1.41 %), followed by (K33) *Streptomyces cinereoruber* (72.66±2.30 %) and (K11) *Acinetobacter baumannii* (61±21.2 %). Moreover, (J) *Lysinibacillus cavernae* (41±4.24 %), (K1)

Bacillus subtilis (40±2 %), (C44) *Bacillus paranthracis* (37.33±3.05 %), (C26) *Enterococcus gallinarum* (36.66±4.16 %), (K31) *Micrococcus luteus* (33±12.72 %) and (K20) *Aneurinibacillus migulanus* (32.5±3.53 %) ($p < 0.05$) showed no significant difference between them, whereas (S) *Lysinibacillus pakistanensis* had the lower CSH % (18 %) (Fig. 18).

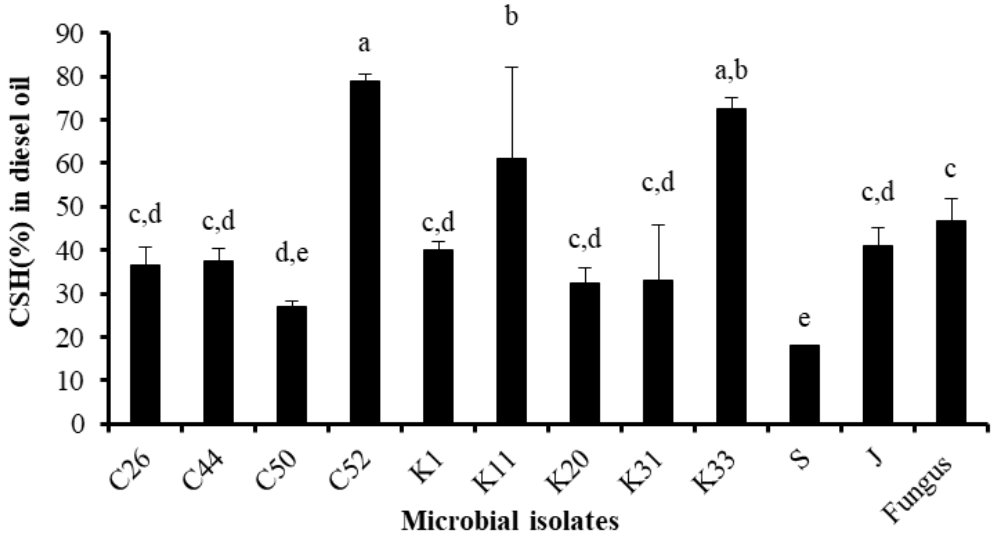


Figure 18. CSH % of microbial isolates in diesel.

Besides, (K33) *Streptomyces cinereoruber* (65.33±7.57 %) showed higher CSH % regarding gasoline, followed by (K11) *Acinetobacter baumannii* (62±14.14 %) and (C26) *Enterococcus gallinarum* (60±4.89 %), $p < 0.05$ with no significant difference between them. Whereas (S) *Lysinibacillus pakistanensis* (6 %), (K31) *Micrococcus luteus* (5 %) and (C50) *Kocuria rosea* (4 %) showed lower rates as shown on figure 19.

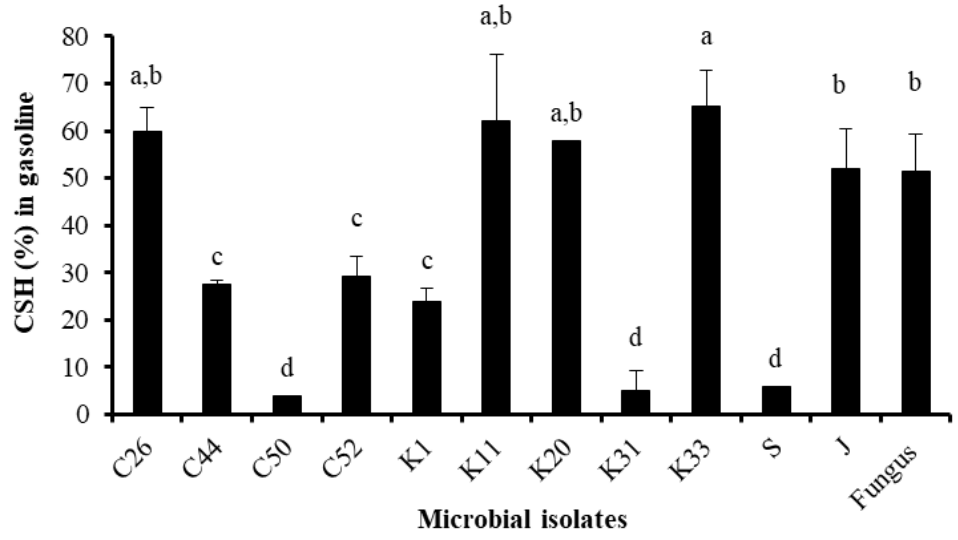


Figure 19. CSH % of microbial isolates in gasoline.

5. Screening the isolates for enzymes activity

5.1. Lipase enzyme activity

The qualitative screening method for lipase enzyme activity on olive oil agar plates supplemented with phenol red demonstrated that six isolates (C52) *Pseudomonas aeruginosa*, (K11) *Acinetobacter baumannii*, (K33) *Streptomyces cinereoruber*, (S) *Lysinibacillus pakistanensis*, (J) *Lysinibacillus cavernae* and the fungus *Aspergillus flavus*, possess positive lipase enzyme activity, whereas the other isolates do not (Fig. 20; Table 5). However, the screening method for lipase activity on Tween 80 showed that only two isolates *Pseudomonas aeruginosa* and the fungus *Aspergillus flavus* demonstrated a positive result (Fig. 21).

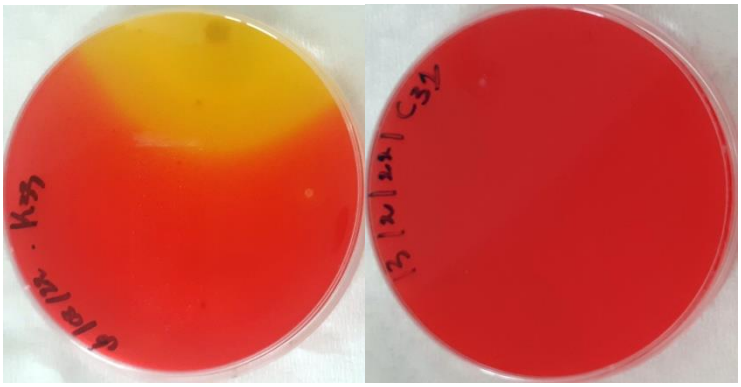


Figure 20. Positive (left) and negative (right) results in olive oil agar test for qualitative lipase enzyme activity determination.

The lipase-producing microorganisms could be easily identified on tween 80 agar due to the appearance of a crystalline halo in plates around the colonies, possibly caused by insoluble calcium salt of the fatty acid released by lipase.

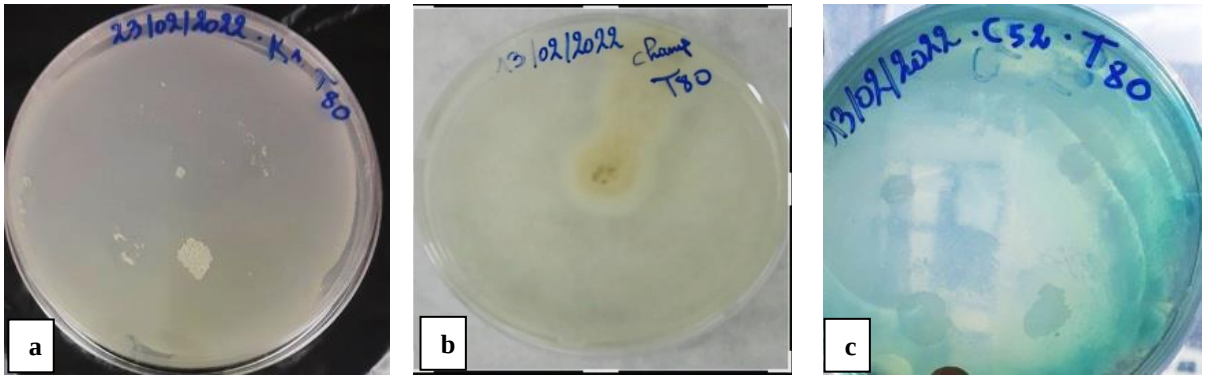


Figure 21. Tween 80 assay for lipase activity determination. a) Negative result, (b, c) positive result seen as the crystalline halo formation around the colony of *Aspergillus flavus* (b) and (C52) *Pseudomonas aeruginosa* (c) respectively.

Besides, the titrimetric assay revealed that among the six isolates,(K11) *Acinetobacter baumannii* was found positive regarding the qualitative lipase assay and had the higher lipase activity (0.81±0.16 U/mL) when compared to the others isolates (Table 5).

Table 5. Qualitative and quantitative screening of lipase activities of microbial isolates.

Isolates	Qualitative screening		Quantitative Screening
	Tween 80	Phenol Red	Enzyme activity (U/mL)
(C52) *Pseudomonas aeruginosa*	+	+	0.16±0.04
(K11) *Acinetobacter baumannii*	-	+	0.81±0.16
(K33)*Streptomyces cinereoruber*	-	+	0.31±0.03
(J) *Lysinibacillus cavernae*	-	+	0.15±0.01
(S) *Lysinibacillus pakistanensis*	-	+	0.33±0.05
Aspergillus flavus	+	+	0.16±0.03

5.2. Laccase enzyme activity

Among the microbial isolates, only *Aspergillus flavus* demonstrated positive Laccase activity, as seen through the formation of a green zone around the colony in PDA supplemented with ABTS (Fig. 22).

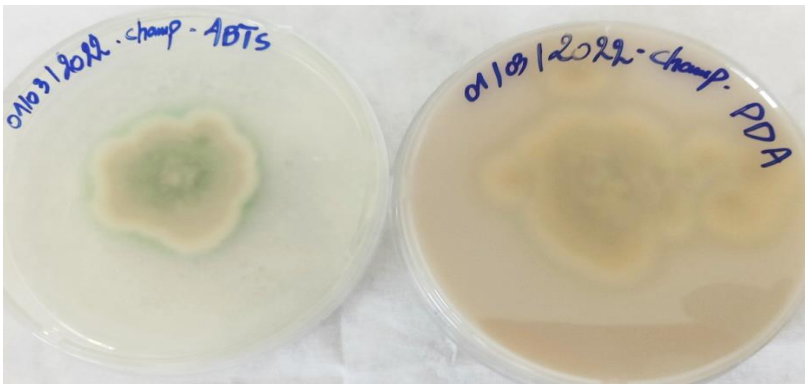


Figure 22. Laccase assay. Control (right) and positive result (left) seen as a green zone around the fungal colony.

The quantitative assay for laccase activity showed an increase of the activity over the 12 days incubation to a maximum of 0.086±0.01 U/mL (Fig. 23).

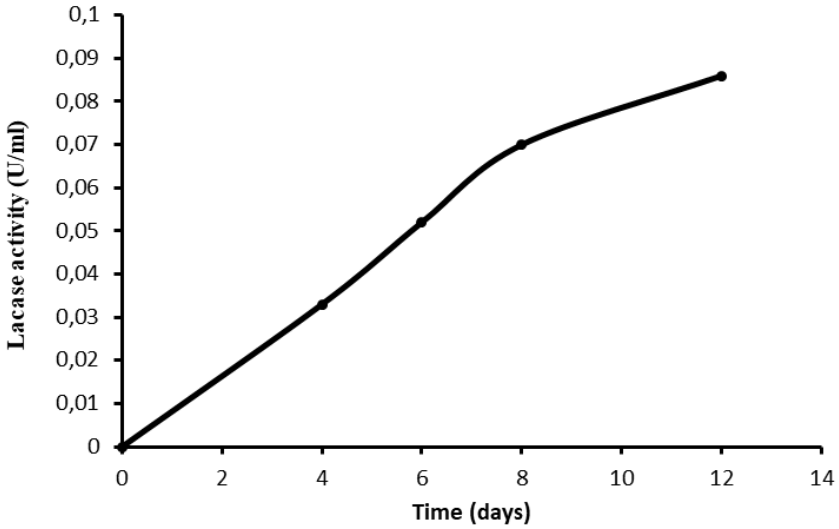


Figure 23. Laccase activity of the fungal isolate.

5.3. Lignin peroxidase enzyme activity

The qualitative screening method for peroxidase enzyme activity on the agar plates supplemented with methylene blue (MB) demonstrated that all the microbial isolates had negative results (Fig. 24).

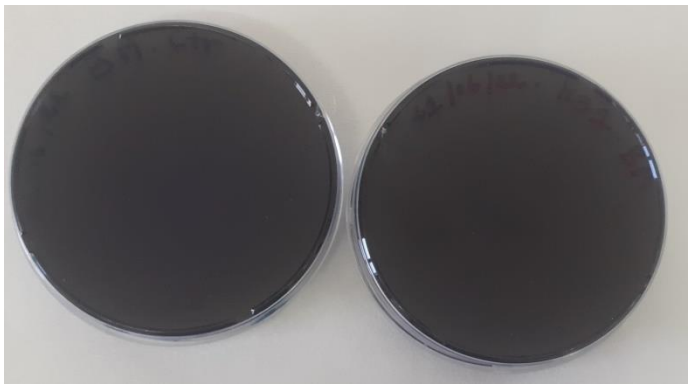


Figure 24. Lignin peroxidase activity. Control (left) and negative result (right).

6. Degradation of diesel oil and gasoline in soil

After 28 days of incubation of the microbial isolates in soils contaminated with diesel oil or gasoline, all the tested strains have demonstrated their ability to degrade both contaminants, though at different rates.

Regarding diesel oil degradation, the isolates (K20) *Aneurinibacillus migulanus* (76.08 %), (C52) *Pseudomonas aeruginosa* (73.36±2.71 %), (K33) *Streptomyces cinereoruber* (72.45±6.27 %), (J) *Lysinibacillus cavernae* (72±1.91 %) and (C50) *Kocuria rosea* (72±1.91 %), $p < 0.05$ have demonstrated higher rates of degradation, with no statistically significant difference

between them. Whereas, the strain *Acinetobacter baumannii* (9.05±3.13 %) has manifested the lower degradation rate (Fig. 25).

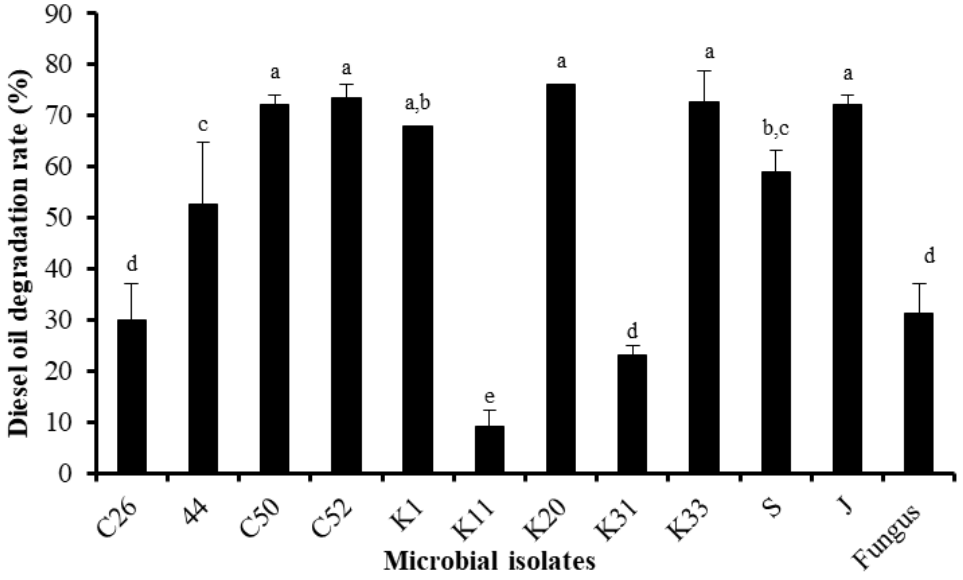


Figure 25. Percentage of degradation of diesel oil in soil by microbial isolates after 28 days.

Besides, the strain (S) *Lysinibacillus pakistanensis* (91.32±6.46 %) demonstrated the higher degradation rate regarding gasoline, while (K1) *Bacillus subtilis* (84.47±3.22 %), (J) *Lysinibacillus cavernae* (82.19 %), (C50) *Kocuria rosea* (82.19 %) and (K33) *Streptomyces cinereoruber* (79.70±9.68 %) $p < 0.05$ did not significantly differ in their rates of degradation. However, *Micrococcus luteus* (15.21±6.97 %) showed the lower degradation rate, as demonstrated in figure 26.

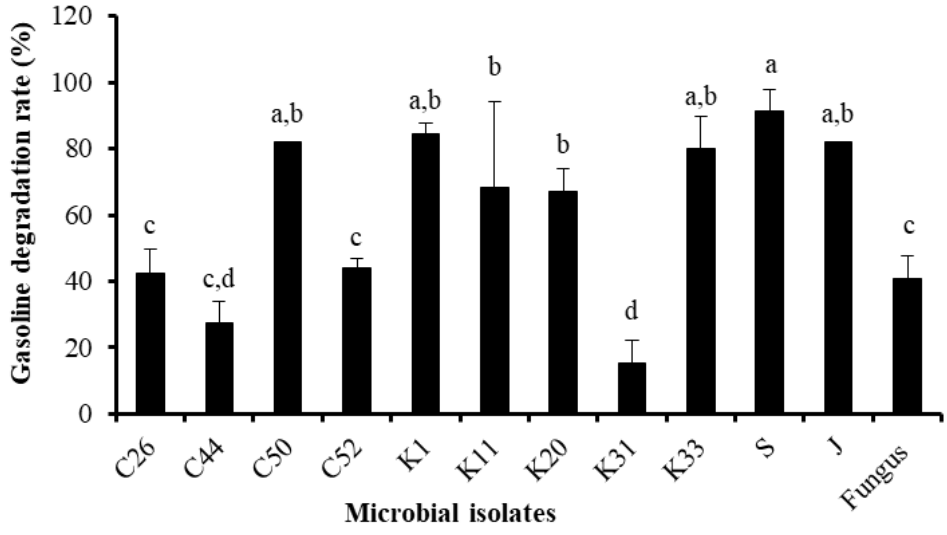


Figure 26. Percentage of degradation of gasoline in soil by microbial isolates after 28 days.

7. Rate of degradation of diesel and gasoline in soil using microbial consortia

Nine microbial consortia were prepared and tested for their ability to degrade diesel oil and gasoline. The choice of candidats microorganisms to include in the consortium was based on their ability to degrade both pollutants, their biochemical properties (biosurfactant and enzyme production), their cell wall structure (Gram positive and negative) and their type (bacteria or fungi) as follow:

Consortium A consisted of all the twelve isolates.

Consortium B consisted of all bacteria.

Consortium C consisted of Gram-positive bacteria (C26) *Enterococcus gallinarum*, (C44) *Bacillus paranthracis*, (C50) *Kocuria rosea*, (K1) *Bacillus subtilis*, (K20) *Aneurinibacillus migulanus*, (K33) *Streptomyces cinereoruber*, (S) *Lysinibacillus pakistanensis*, and (J) *Lysinibacillus cavernae*.

Consortium E consisted of Gram-positive bacteria and the fungus.

Consortium F consisted of the Gram-negative bacteria (C52) *Pseudomonas aeruginosa* and the fungus.

Consortium G consisted of (K33) *Streptomyces cinereoruber*,(S) *Lysinibacillus pakistanensis*, (J) *Lysinibacillus cavernae* and *Aspergillus flavus*.

Consortium H consisted of *Aspergillus flavus*, (S) *Lysinibacillus pakistanensis*, (C52) *Pseudomonas aeruginosa*, (C44) *Bacillus paranthracis* and (C26) *Enterococcus gallinarum*.

Consortium L consisted of *Aspergillus flavus, (K11) Acinetobacter baumannii*, (C52) *Pseudomonas aeruginosa*, (J) *Lysinibacillus cavernae* and(C26) *Enterococcus gallinarum*)

Consortium Y consisted of *Aspergillus flavus*, (K11) *Acinetobacter baumannii*, (K33) *Streptomyces cinereoruber*, (C52) *Pseudomonas aeruginosa*, (C44) *Bacillus paranthracis* and (C50) *Kocuria rosea*.

It should be noted that after testing the rates of degradation of diesel and gasoline using consortium A which consisted in all the isolates, an isolation, purification and molecular identification of microbial strains that survived was performed from the tested soil to see which isolates are still present and those who are not.

With a degradation rate of 82.72±5.58 %, the consortium A ranked first regarding the degradation of diesel ($p < 0.05$), followed by the consortium Y (73.04±9.78 %), consortium C (71.42±4.78.4 %), consortium L (67.38±3.69 %) and consortium E (61.74±4.19 %). The degradation rates of the consortia H (60.93±6.09 %) and B (60.53±1.71 %) did not differ significantly. The consortium F (35.1 %) revealed the lower degradation rate (Fig. 27).

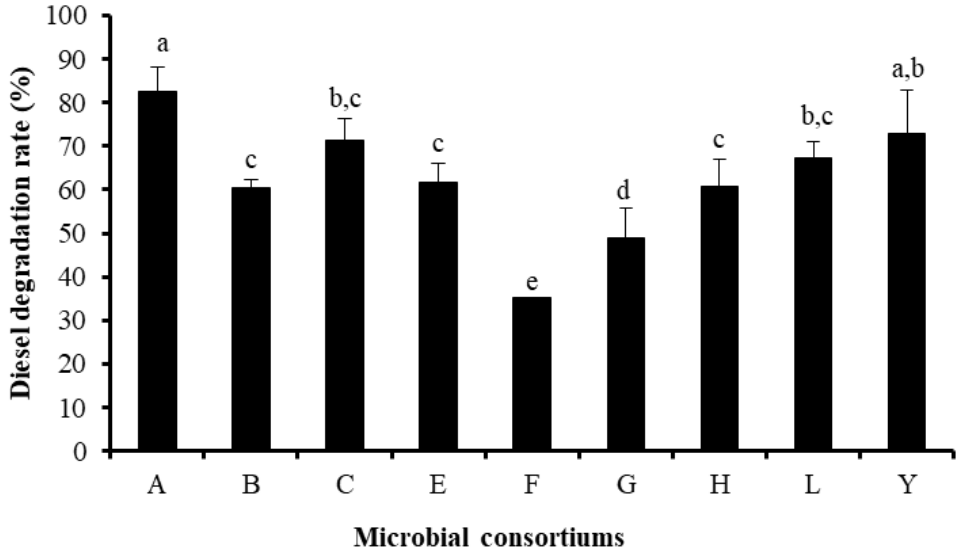


Figure 27. Degradation rate of diesel oil in soil with microbial consortiums.

In regard to gasoline, the consortia Y (93.6±3.23 %) and H (89.04±9.68 %) demonstrated higher degradation rates $p < 0.05$, with no significant difference between them. The consortia G (84.47±9.68 %), E (83.71±6.97 %) and C (82.19±13.7 %) also showed higher degradation rates, with no significant difference between them, while the consortium B demonstrated the lower degradation rate (57.07±3.23 %) (Fig. 28).

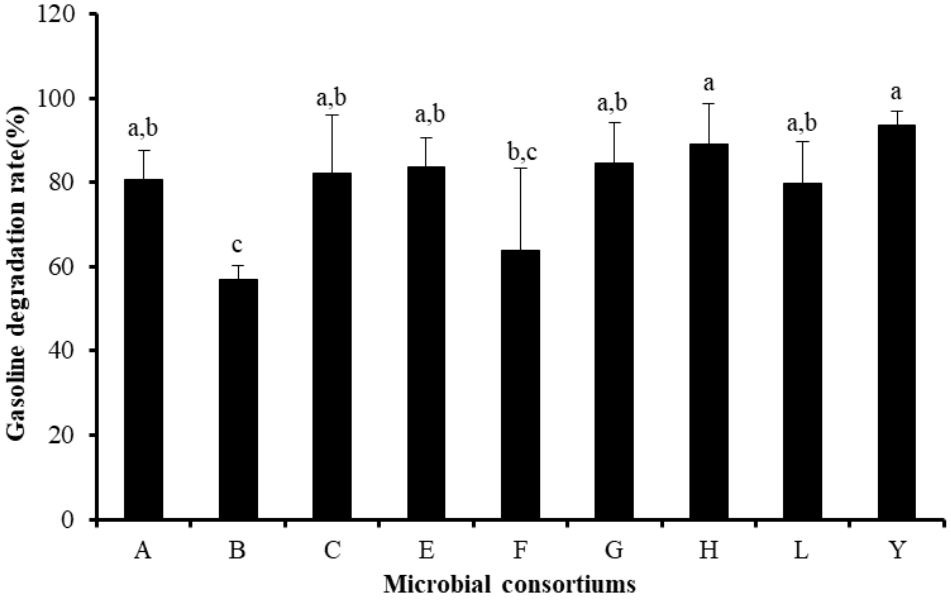


Figure 28. Degradation rate of gasoline in soil with microbial consortium.

Discussion

Discussion

Petroleum hydrocarbons are a major primary energy source for a variety of industries. These recalcitrant substances that belong to the priority pollutants category, especially in soil and water environments, are considered the most prevalent contaminants (Varjani, 2017; You et al., 2018).

The present study aimed to isolate microbial species from petroleum contaminated soils as well as from petroleum derivatives. Subsequently, their ability to grow in liquid medium containing either gasoline or diesel oil as sole source of carbon was estimated. In addition, their potential production of biosurfactants and enzymes involved in bioremediation of petroleum hydrocarbons were tested. Finaly, their ability to degrade gasoline and diesel oil in polluted soil was evaluated.

Throughout this study, 11 bacterial strains and 1 fungus were isolated and identified as *Enterococcus gallinarum*, *Bacillus paranthracis*, *Kocuria rosea*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Acinetobacter baumannii*, *Aneurinibacillus migulanus*, *Micrococcus luteus*, *Streptomyces cinereoruber*, *Lysinibacillus pakistanensis*, *Lysinibacillus cavernae* and *Aspergillus flavus.*

Moreover, all the isolates have demonstrated their ability to grow at varying degrees on media containing gasoline and diesel. In fact, bacteria and fungi have long been regarded as one of the main hydrocarbon degrading agents (Patowary et al., 2017). As the obtained isolates could grew and survived with diesel and gasoline as the only carbon source, they could be assumed to be capable of metabolizing them and resistant to their damaging effects (Ahmed et al., 2021). Indeed, the method of enrichment with the desired pollutants makes it easier to isolate only the microorganisms with the capacity for biodegradation. By simulating natural habitats, this enrichment technique increases native microorganisms' capacity for adaptation, resistance, and excellent degradation (Chaudhary et al., 2019).

A study undertaken by Zhang et al., (2010) has demonstrated that 22 different strains were able to use diesel as their only source of carbon and energy, and 11 strains were able to reduce TPHs of diesel by more than 70 % in just seven days after being obtained from enrichment cultivation of oil-contaminated soils from a Chinese oil field. Phylogenetically, 19 strains are related to *Bacillus* species.

In general, different species have been shown capable to utilize diesel constituents as a sole source of carbon and energy, such as *Bacillus subtilis* (Oyewole et al., 2019; Salmazo et al., 2023), *Bacillus cereus* (Oyewole et al., 2019), *Acinetobacter* strain CA16 (Ho et al., 2020) and

Pseudomonas aeruginosa (Bekele et al., 2022). Other bacterial species are involved in biodegradation of petroleum hydrocarbons including *Enterococcus casseliflavus* (Ozyurek and Bilkay, 2020), *Micrococcus luteus* AB023371 (Abraham et al., 2019), *Kocuria* spp. (Nafal and Abdulhay, 2020), *Kocuria sediminis* (Khalifa, 2017). *Lysinibacillu*s sp. (Ying et al., 2022), and *Streptomyces* sp. (Baoune et al., 2019). Also, *Aneurinibacillus migulanus* and *Bacillus toyonensis* isolated from the oil storage tank, were used in degradation of oily sludge (Bahmani et al., 2020).

In our study, one fungus species *Aspergillus flavus* has been isolated and showed high growth rates on both gasoline and diesel oil separately. In addition, this species has demonstrated its ability to degrade both products in contaminated soils.

It should be noted that fungi have a better tolerance to hydrocarbon toxicity due to their physiology and adaptation capacity to the environmental variations (Al-Zahrani et al., 2022). The majority of fungi are xero- and osmotolerant, and they are able to grow in a variety of temperature conditions. They may also penetrate hydrocarbon-impregnated soil aggregates that may be structurally porous, anoxic, and hydrophobic (Atakpa et al., 2022).

In addition, the extracellular enzymatic systems of fungi genera allow them to degrade hydrocarbon molecules, leading to an effective break down of large compounds into small particles. They can potentially eliminate a wide range of environmental pollutants such as petroleum hydrocarbons as demonstrated in several studies (Alobaydy, 2020). Besides, *Aspergillus*, *Fusarium* and *Penicillium* are some of the fungi that degrade petroleum hydrocarbons (Al-Zahrani et al., 2022).

Fungi ramify quickly and subsequently digest the substrate. Besides, under severe environmental conditions, such as stress, their mass production is possible (Al-Zahrani et al., 2022). They have the capacity to degrade the high-molecular-weight aliphatic and aromatic compounds found in crude oil into low-toxic byproducts while continuing their normal activities (Atakpa et al., 2022).

Moreover, *Aspergillus flavus, Aspergillus versicolor, Bionectria ochroleuca*, *Penicillium chermisinum* and *Trichoderma virens* have been reported to be able to tolerate high concentrations of crude oil (Kota et al., 2014).

Several studies have examined the involvement of mycoflora in the biodegradation of various petroleum products using *Penicillium* sp. and *Aspergillus* sp. isolated from soil samples polluted with crude oil. *Aspergillus flavus* showed a rate of degradation of roughly 31.24 % and 40.80 % of diesel and gasoline, respectively, at a concentration of 10 % (Alobaydy, 2020).

In polluted soils, numerous enzymes such as laccases, transferases, and cytochrome P450 monooxygenases are used as the basic process of microflora activities (Jadhav et al., 2019).

Lipase enzyme-producing microorganisms are frequently used in the bioremediation of oil-contaminated environments (Basha, 2021).

In this study, among the twelve tested microbial isolates, six isolates showed abilities to produce lipase enzyme i.e., *Acinetobacter baumannii*, *Lysinibacillus cavernae*, *Lysinibacillus pakistanensis*, *Pseudomonas aeruginosa*, *Streptomyces cinereoruber* and *Aspergillus flavus* where *Acinetobacter baumannii* demonstrated the higher lipase activity (0.81±0.16 U/mL).

It has been reported that *Acinetobacter* species isolated from oil contaminated soil, in South Korea, were used in the synthesis of extracellular lipase enzyme (Anbu et al., 2011). In addition, researchers reported lipase production by bacteria from the genus *Acinetobacter* such as *Acinetobacter baumannii* isolated from oil contaminated soil samples which demonstrated a lipase activity at pH 8.5 and temperature of 45 °C with a value of 0.336 units/g (Parwata and Oviantari, 2016). Furthermore, *Acinetobacter baumannii* RMUTT3S8-2 showed promise as a lipase producer, especially for oily wastewater treatment with the lipase activity 216.23±3.69 U/mL (Bunmadee et al., 2022).

Besides, *Lysinibacillus* are among the genera that can be considered as lipase producer. Jigajinni and Meti (2021) reported that *Lysinibacillus macroides* FS1 is a promising alternative for the biodiesel industry as a source of biocatalyst since it can be employed as a potential bacterial source of lipase with methanol stability.

Many lipase-producing bacterial strains have been discovered in a variety of genera, including *Pseudomonas* sp. Due to lipase's capacity to utilise used motors oil as its only source of carbon and energy, *Pseudomonas* sp. may effectively degrade petroleum hydrocarbons (Sahoo et al., 2020).

It has been shown that *Streptomyces* sp. isolates have a high lipolytic activity and were used for biodiesel production (Ayaz et al., 2015). In addition, *Streptomyces exfoliates* LP10 isolated from soil collected from fuel station produced lipase at higher level compared to other isolates (6.9 U/mL) (Aly et al., 2012).

Lipase production by microorganisms is observed as a clear zone that appears around microbial colonies. Elemuo et al. (2019) reported that *Aspergillus flavus*, *Aspergillus niger*, *Verticillus* sp., *Penicillum* sp., and *Microsporum audouini*, isolated from crude oil contaminated soils, revealed good lipase producing potentials based on the magnitude of their zones of clearance. As well, *Aspergillus flavus* isolated from soils contaminated with crude oil was used for lipase production process (Osuoha et al., 2020).

In this study, only the fungus *Aspergillus flavus* showed capacity to produce laccase enzyme. Generally, laccases, peroxidases, and oxidases are three distinct extracellular fungal enzymes that catabolized hydrocarbons into excreted derivatives (Mahmud et al., 2022).

Through direct oxidoreduction to catalyze the oxidation of polyaromatic hydrocarbons (PAHs), laccase may exert a dominant influence. *Peniophora incarnate* was shown to be a potent PAH degrader by generating laccase and manganese-dependent peroxidase (Lee et al., 2015).

According to previous research on the effectiveness of halotolerant lipolytic fungi for bioremediating diesel-contaminated saline soil, the degradation was positively connected with lipase and laccase enzyme, electrical conductivity, and the soil's ability to hold water (Sajid et al., 2023).

Furthermore, biosurfactants are combinations of complex compounds like peptides, fatty acids, and polysaccharides that have the capacity to lower surface tension by solubilizing the fatty acids present in the crude oil, promoting efficient hydrocarbon exploitation by microorganisms (Parthipan et al., 2017). The addition of surfactants could significantly accelerate the petroleum hydrocarbons (PHs) degradation of crude oil which might be due to the reduced surface tension (Abraham et al., 2019; Bekele et al., 2022).

The majority of the bacteria that degrade crude oil generate extracellular biosurfactants to enhance microbial oil uptake and accelerate the process of degradation by emulsifying the hydrocarbon (Karlapudi et al., 2018). Through emulsification, the biosurfactant molecules increase the solubility of sparsely soluble hydrophobic contaminants, improving their bioavailability to the prevailing microflora (Patowary et al., 2017). Members of various bacterial genera, including *Pseudomonas, Bacillus, Acinetobacter, Alcaligenes, Rhodococcus, Corynebacterium,* etc., have been extensively investigated for their potential to increase petroleum oil breakdown through their production of biosurfactants (Patowary et al., 2017).

Emulsification activity on the diesel substrate in this study showed biosurfactant synthesis by nine isolated strains where the higher activity was observed in *Enterococcus gallinarum* (29.09 %), *Aneurinibacillus migulanus* (19.36 %), and *Acinetobacter baumannii* (18.26 %).

The obtained results regarding E24% on gasoline demonstrate that eight isolated strains may have the ability to produce biosurfactants. However, *Kocuria rosea* and *Bacillus paranthracis* showed the higher emulsification percentages (38.61 % and 33.74 % respectively). Gadakh et al. (2018) also studied the biosurfactant production from *Aneurinibacillus migulanus,* they reported an emulsification index of 16 % after 24 hours when engine oil was used as substrate, while in our study, *Aneurinibacillus migulanus* showed emulsion activity of about 19.36 % on the diesel substrate.

Pseudomonas aeruginosa strains have been found to produce mono and di-rhamnolipid type biosurfactants. *Pseudomonas aeruginosa's* rhamnolipid concentration was widely researched for its capacity to degrade hydrocarbons (Karlapudi et al., 2018).

According to Sharma et al. (2015), *Pseudomonas aeruginosa* DSVP20, or the glycolipid generated by this isolate, may play a part in the bioremediation of soil contaminated with petroleum hydrocarbons. Also, it was revealed that *Bacillus subtilis* RSL 2 could create lipopeptide as a biosurfactant and biodegrade crude oil (Sharma and Pandey, 2020).

Besides, because of their capacity of oil degradation and/or emulsion, several dominant strains, including *Bacillus* sp., can be widely exploited in the microbial enhanced oil recovery method. *Bacillus subtilis* produces a biosurfactant that emulsifies well with kerosene, hexadecane, octane, and crude oil (Wu et al., 2022).

Lysinibacillus fusiformis MK559526, which was isolated from soil found in automobile-mechanic-shop, is a viable option for the synthesis of biosurfactants and had a positive drop collapse test and an emulsification index of 65.15±0.35 % (John et al., 2021).

*Lysinibacillu*s sp. degrade fractions such as resins and asphaltenes. The glycolipid biosurfactants secreted by the strain Y316 promotes biodegradation (Ying et al., 2022).

Akbari et al. (2021) showed that the bacteria *Kocuria rosea* ABR6, which was isolated from the Esfahan oil refining company (Iran), may generate biosurfactant, which can increase crude oil recovery to 35 % and decrease the frequency of environmental contamination caused by crude oil.

The ability to produce biosurfactant was demonstrated by *Aspergillus fumigatus*, which was isolated from Malaysian crude oil sludge. This ability was revealed by the clear zone of oil (13 cm^2) and the emulsion index (3.85 %), and showed a TPH degradation of roughly 57±2 % (Othman et al., 2022).

Furthermore, all the isolates in this study have demonstrated their ability to adhere to the tested hydrophobic hydrocarbons (diesel oil and gasoline). You et al. (2018) discovered a positive correlation between hydrophobicity, emulsifiability, and degradation efficacy, and they also proved that *Pseudomonas aeruginosa's* synthesis of biosurfactants contributed to the strain's ability to degrade higher PHs.

Acinetobacter calcoaceticus SL1 produces biosurfactant, and demonstrated strong emulsifying activity for diesel oil and n-hexane, with respective values of 43.6 % and 54.5 %. Moreover, it possesses a high degree of hydrophobicity on the cell surface, which enables it to adhere and break down hydrocarbons with ease (Kwon and Song, 2017).

Biosurfactant-producing bacteria were screened utilizing a cell surface hydrophobicity assay by Tebyaniyan et al. (2014), they found a direct correlation between cell surface hydrophobicity and hexadecane degradation. In addition, Obuekwe et al. (2009) showed a significant high relationship between CSH and the capacity to degrade crude oil, as revealed by statistical analyses.

Besides, large amounts of aliphatic hydrocarbons that stay in the soil for a longer period of time could be successfully removed using a combinatorial strategy that includes bio-stimulation and bio-augmentation. In fact, nutrient amendment and bacterial inoculation increase the remediation's effectiveness (Chaudhary et al., 2019). Bioaugmentation is dependent on the exogenously supplied microorganisms, their tolerance to pollution, and their ability to survive in a polluted environment (Farber et al., 2019). In addition, the utilization of inocula composed of microbial strains or microbial consortia that have been especially adapted to the site to be decontaminated is crucial for the success of bioaugmentation treatments (Adams et al., 2015).

Study has showed that nutrient supplementation effectively accelerated the rate of diesel degradation (90 % after 35 days), demonstrating the efficiency of biostimulation (Lang et al., 2016). Also, Cai et al. (2020) showed that adding nutrients such ammonium sulfate $(NH_4)_2SO_4$, (0.43 g/Kg soil) and monopotassium dihydrogen phosphate KH_2PO_4 (0.067 g/Kg soil) to crude oil-contaminated soil activated a range of bacteria that degrade hydrocarbons. It produced lipase at the higher level of any isolate (6.9 IU/mL) (Aly et al., 2012).

In this study, after 28 days of incubation of the microbial isolates in soils contaminated with diesel oil and gasoline (10 %), separately, the isolates *Aneurinibacillus migulanus*, *Pseudomonas aeruginosa*, *Streptomyces cinereoruber*, *Lysinibacillus cavernae* and *Kocuria rosea* demonstrated the higher rates of TPH degradation regarding diesel oil. Besides, the strains *Lysinibacillus pakistanensis*, *Streptomyces cinereoruber*, *Lysinibacillus cavernae*, *Bacillus subtilis* and *Kocuria rosea* demonstrated the higher degradation rate regarding gasoline.

Numerous studies have shown that *Bacillus* sp. is dominant among microorganisms isolated from petroleum contaminated soils and plays a role in degradation of petroleum. Due to its resistant endospores, the *Bacillus* genus is highly resistant to high concentration of hydrocarbons (Ozyurek and Bilkay, 2020). Other studies have described *Lysinibacillus* sp. as powerful microbes capable of degrading hydrocarbons (Mnif et al., 2015). Several investigations have also demonstrated that *Pseudomonas* sp. is an oleophilic bacterium, which accounts for its excellent diesel degradation effectiveness. Moreover, it has a versatile metabolism, may be symbiotically related with soil, or produce a biosurfactant that enhances the utilization of diesel (Bekele et al., 2022).

The diesel removal efficiency was enhanced from 15 % (without inoculation) to 55 % in 14 days by inoculating *Pseudomonas* sp. DTF1 into diesel-contaminated soil because this bacterium may produce biosurfactants, and because of the presence of alkB gene that is related to the degradation of diesel (Yang et al., 2023). In addition, Vignesh et al. (2016) demonstrated that *Pseudomonas* spp. degraded 97.8 % of diesel.

Hernández-Santana and Dussan (2018) reported that benzene, toluene, ethylbenzene, and phenol could all be broken down by *Lysinibacillus sphaericus*. The biodegradation assays of diesel oil revealed removal rates of up to 95 % for C10-C28 hydrocarbons. In addition, the bacterium *Streptomyces* sp. has been shown to remove up to 50 % of total petroleum hydrocarbons (TPH), n-alkanes, and aromatic hydrocarbons identified in soil samples (Baoune et al., 2019). *Acinetobacter calcoaceticus* CA16 was able to grow in minimal medium with diesel as the only source of carbon. It is capable of degrading 82 to 92 % of aliphatic alkane molecules in 28 days (Ho et al., 2020). Kwon and Song (2017) also demonstrated that *Acinetobacter calcoaceticus* SL1 degrades 72.3 % of 1,000 mg/L diesel oil in 7 days and 78.0 % of 10,000 mg/L diesel oil in oil-contaminated soil during 28 days.

To completely biodegrade oil pollutants, a mixed culture of microorganisms is recommended because hydrocarbon mixtures vary significantly in their volatility, sensitivity, and solubility, and because the needed enzymes cannot be found in a single organism (Nafal and Abdulhay, 2020). In general, the combined bacterial consortia show better results due to their synergistic effects (Reddy et al., 2011). Building bacterial consortiums allows researchers to take use of the synergistic effects of many strains to improve the breakdown of petroleum hydrocarbons, and the performance of these consortia can be noticeably better than that of individual bacteria (Wei et al., 2021). Their diverse enzymatic activities, which include hydrolases, oxygenases, demethylases, dehalogenases, transferases, and oxidoreductases that can catalyze various degradation routes either aerobically or anaerobically, contribute to their effectiveness for the biodegradation and detoxification of hydrocarbon pollutants (Bekele et al., 2022). In addition, the use of biosurfactant-producing hydrocarbon degrader microorganisms is a feasible strategy that can be used to accelerate the bioremediation of petroleum hydrocarbons polluted locations (Patowary et al., 2017).

There is a lot of interest in the utilization of bacterial consortia that can break down hydrocarbons. Most experts agree that mixed cultures are more metabolically versatile than pure cultures (Mnif et al., 2015).

In this study, the obtained results have demonstrated that the different consortia tested have the ability to degrade diesel and gasoline in soil at different rates. However, the consortium A (all the twelve isolates) and B (only the bacterial isolates) demonstrated the higher rates of degradation of diesel oil while the consortium H (*A. flavus*, *L. pakistanensis*, *P. aeruginosa, B. paranthracis* and *E. gallinarum*) and Y (*A. flavus, A. baumannii, S. cinereoruber*, *P. aeruginosa*, *B. paranthracis* and *K. rosea*) demonstrated the higher degradation rates of gasoline.

The variations in biodegradation rates may be due to the wide variety of bacterial species found in different consortia (Ledezma-Villanueva et al., 2016). Furthermore, Prakash et al.

(2014) showed that a consortium consisting of *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9 and APBP1 and *Micrococcus* sp. API O4, achieved the maximum benzene and diesel degradation where, *Bacillus* sp. APHP6, *Pseudomonas* sp. APHP9 and APBP1, *Micrococcus* sp. APIO4, and the consortium resulted in a 54.8 %, 60.2 %, 40.9 %, 32.5 %, and 66.2 % decrease in benzene concentration and a decrease of 61.2 %, 68.4 %, 53.7 %, 39.3 %, and 75.4 % in diesel concentration, respectively, after 6 days of incubation as estimated by HPLC analysis.

The effectiveness of mixed culture has been reported by many researchers; biodegradation of petroleum was achieved at 82 % using a consortium consisting of *Bacillus subtilis*, *Leuconostoc mesenteroides*, *Bacillus halotolerans*, *Enterococcus casselifavus*, *Klebsiella pneumoniae* and *Pannonibacter phragmitetus* (Ozyurek and Bilkay, 2020). Besides, Abraham et al. (2019) reported that degradation of PAHs in crude oil was 46.6 % for the consortium (*Bacillus subtilis* and *Micrococcus luteus* EHSA4) while it was 19,65 % for the single bacteria *Bacillus subtilis*.

When two bacterial isolates, *K. rosea* and *B. amyloliquefaciens*, were combined to degrade sludge, the percentage of hydrocarbons degraded was at its maximum of 95.5 %. However, the individual bacteria *K. rosea* and *B. amyloliquefaciens* have demonstrated a degradation percentages of 93.8 % and 68.9%, respectively (Nafal and Abdulhay, 2020).

Also, *Pseudomonas* sp. and *Micrococcus* sp. each degrade respectively 67.57 % and 52.95 % of the diesel engine oil. However, it was found that their combination had a high ability to degrade diesel engine oil, i.e. 89.98 % after 25 days (Nikhil et al., 2013). In addition, Wei et al. (2021) demonstrated that the combination of the three strains of bacteria can achieve the effect of the synergistic removal of petroleum hydrocarbons, where after 28 days of cultivation, the degradation rates of *Pseudomonas putida*, *Acinetobacter calcoaceticus* and *Sphingomona* sp., and the bacterial consortium were 42.8 %, 48.01 %, 26.56 %, and 81.07 % respectively.

Wang et al. (2020) reported that the degradation rates of fluoranthene by *Bacillus paranthracis* YN420, *Bacillus paramycoides*, and *Bacillus paranthracis* E420 were 10.17 %, 9.19 %, and 2.9 % respectively, while the degradation rate of fluoranthene in a soil slurry of different combinations of complex bacteria was higher than that of the three strains inoculated alone.

Furthermore, petroleum hydrocarbons are efficiently degraded by the combination of a fungal strain with bacteria that produce biosurfactants. After 7 days of incubation, *Acinetobacter* sp. Y2 and *Scedosporium* sp. ZYY enhanced the degradation rate of total petroleum hydrocarbon from 23.36 % to 58.61 % (Atakpa et al., 2022).

Yuan et al. (2018) demonstrated that the cooperative breakdown of crude oil by a consortium of bacteria and fungi may be helpful for bioremediation of petroleum-contaminated sites, where the crude oil's deterioration rate rose from 61.06 % to 81.45 %.

Numerous bacterial species possessing the potential to degrade petroleum hydrocarbons have been utilized in the bioremediation process. However, during the process of practical application, a variety of problems have been discovered that slow down the effects of biodegradation. These issues include the bioavailability of pollutants, environmental limitations, metabolic restrictions and time consumption. Additionally, there are the harmful impacts of petroleum hydrocarbons, where very high concentrations of these compounds severely inhibit microbial growth, causing inefficient biodegradation or even bacterial death. Further research is needed to explore novel functional genes controlling hydrocarbon degradation pathways, and construct genetically engineered bacteria using synthetic biology technology for improved petroleum hydrocarbon degradation capabilities (Xu et al., 2018 ; Ossai et al., 2019; Chunyan et al., 2023).

Conclusion

Conclusion

The chemical industry relies heavily on petroleum as a source of energy and raw materials but the use, transportation, and refining of petroleum can contribute significantly to severe environmental damages. Microbial degradation can be considered as a key component in the clean-up strategy for petroleum hydrocarbons bioremediation.

The present study reports the identification, and the characterization of petroleum-hydrocarbons degrading capacity of eleven bacterial and one fungal species isolated from petroleum products and petroleum-contaminated soils.

Overall, all of the isolated microbial species demonstrated their ability to grow in the liquid medium MSM containing either gasoline or diesel oil as sole source of carbon with *Bacillus paranthracis*, *Micrococcus luteus* and *Aspergillus flavus* showing the higher growth rates. Moreover, all the tested microbial isolates showed ability to produce biosurfactants with both gasoline and diesel oil, at different rates, which is related to petroleum hydrocarbons degradation.

In addition, five of the isolates i.e., *Pseudomonas aeruginosa*, *Acinetobacter baumannii*,*Streptomyces cinereoruber*, *Lysinibacillus pakistanensis*,*Lysinibacillus cavernae*, and *Aspergillus flavus*, can produce lipase enzyme while only the fungal species *Aspergillus flavus* produced laccase enzyme. Furthermore, all the isolates couldn't produce peroxidase enzyme.

Besides, all the microbial isolates are able to degrade diesel oil and gasoline in the soil with *Aneurinibacillus migulanus* having the best potential in diesel oil degradation and *Lysinibacillus pakistanensis* in gasoline degradation.

It must be noted that this study reported for the first time, to our knowledge, the use of *Enterococcus gallinarum*, *Lysinibacillus pakistanensis*, *Lysinibacillus cavernae* and *Streptomyces cinereoruber* in bioremediation process of petroleum hydrocarbons.

The application of microbial consortia with nutrients supplementations (nitrogen and phosphorus) clearly improved bioremediation of diesel oil and gasoline in contaminated soils. In general, the tested consortia showed higher degradation rates compared to the isolates alone. These results indicate that the tested microbial isolates may be potential bioremediation agents, whether the whole organisms or their derived molecules namely biosurfactants and enzymes.

This finding constitutes a basis for further ‘‘*in situ*’’ studies for contaminated soils and aquatic environments.

A combinatorial approach, including biostimulation and bioaugmentation, could be used to effectively remove large quantities of petroleum hydrocarbons persisting for a longer period in the soil.

This work should be followed-up by the identification of biosurfactants produced by the microbial isolates and the enzymes involved in the process of petroleum hydrocarbons biodegradation. As well, the identification of genes implicated in the biodegradation process should be performed. Furthermore, other petroleum hydrocarbons as well as other pollutants should be tested to determine their susceptibility to biodegradation by the isolated microbial species.

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