ا لمجهروية الجزائرية اداليمقريطاة اليبعشة
**People's Democratic Republic of Algeria**
وزارة العتليم العلاي والثحب العلمي
**Ministry of Higher Education and Scientific Research**

**Ibn Khaldoun University, Tiaret**
**Faculty of Nature and Life Sciences**
**Department of Nature and Life Sciences**

# Dissertation

Submitted in partial fulfilment of the
requirements for the degree of

## Master of Biological Sciences

**Field:** **Nature and Life Sciences**
**Branch:** **Biological Sciences**
**Speciality:** **Cell and Molecular Biology**

Presented by:

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Title

# Isolation and characterization of bacteria and fungi with the ability to degrade petroleum hydrocarbons

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**July 2021**

# *Acknowledgments*

*We thank **ALLAH** " ﷲ " the Almighty, for having given us the courage and the patience to realize this modest work.*

*Our deep thanks and sincere gratitude are addressed to our supervisors; Miss MEKNASSI Khadidja and Mrs. AIT ABDERRAHIM Leila for their advices, encouragement, patience, competence, kindness, follow-up and the orientation from which we benefited to carry out our work.*

*Our sincere thanks go to the members of the jury*
*Mr. BENAISSA T., Mr. ACHIR M. and Mr. TAIBI K.*
*for their judicious and enriching remarks which can only improve the quality of this work.*

*We cannot omit to express our gratitude to all the teachers who have carried out the heavy task of teaching us during our school and university courses; in particular, those who lavished us their laborious knowledge in molecular and cellular biology*

*Finally, to all those who have participated directly or indirectly in the realization of this work find here the expression of our gratitude.*

*Thank you All...*

# Abstract

As the world's population grows exponentially and the ceiling of needs continues to rise, attention to the impact of the exploitation of raw materials is necessary and to the problems that have arisen. Petroleum products released into the environment affect humans, animals and plants. The consequences of pollution depend on the organisms themselves and the chemical structure of the hydrocarbons but they have immediate and irreversible effects. This forces us to intervene urgently to think about serious solutions to slow down or reduce the pollution and its effects.

This study aimed to isolate bacterial and fungal species from samples of petroleum products as well as a sample of soil contaminated with petroleum. Subsequently, a characterization of the isolates is carried out in order to select hydrocarbonoclastic microorganisms that have the ability to degrade these pollutants.

Morphological features of the isolated microorganisms are identified by mean of macroscopic and microscopic observations. Moreover, their ability to degrade gasoline and diesel is assessed through the determination of their ability to grow on media containing these two hydrocarbons. As well, whether the isolate produce or not biosurfactants was assayed through the measure of the emulsifying index $E_{24\%}$, cell surface hydrophobicity (CSH) % and the qualitative drop collapse technique (DCT).

Through this study we could isolate eight Gram positive and negative bacteria and one fungus belonging the genus Aspergillus. All the tested microbial isolates showed ability to grow on media containing gasoline and diesel in addition to their ability to produce biosurfactants with both hydrocarbons at different rates.

Taking into consideration all the obtained results, the bacteria C26 showed the best potential for use as a bioremediation agent.



**Key words:** Pollution, Petroleum hydrocarbons, gasoline, diesel, soil, hydrocarbonoclastic microorganisms, bioremediation.

# Résumé

Alors que la population mondiale croît de façon exponentielle et que le plafond des besoins ne cesse d'augmenter, il est nécessaire de prêter attention à l'impact de l'exploitation des matières premières et les problèmes qui se sont posés. Les produits pétroliers rejetés dans l'environnement affectent les humains, les animaux et les plantes. Les conséquences de la pollution dépendent des organismes eux-mêmes et de la structure chimique des hydrocarbures mais elles ont des effets immédiats et irréversibles. Cela nous oblige à intervenir en urgence pour réfléchir à des solutions sérieuses pour ralentir ou réduire la pollution et ses effets.

Cette étude vise à isoler des espèces bactériennes et fongiques à partir d'échantillons de produits pétroliers ainsi que d'un échantillon de sol contaminé par du pétrole. Par la suite, une caractérisation des isolats est réalisée afin de sélectionner des microorganismes hydrocarbonoclastes qui ont la capacité de dégrader ces polluants.

Les caractéristiques morphologiques des micro-organismes isolés sont identifiées au moyen d'observations macroscopiques et microscopiques. De plus, leur capacité à dégrader l'essence et le diesel est évaluée à travers la détermination de leur capacité à se développer sur des milieux contenant ces deux hydrocarbures. De plus, le fait que l'isolat produise ou non des biosurfactants a été testé par la mesure de l'indice d'émulsification $E_{24\%}$, de l'hydrophobie de la surface cellulaire (CSH) et de la technique qualitative de déformation goutte (DCT).

Nous avons pu isoler huit bactéries Gram positives et négatives et un champignon appartenant au genre Aspergillus. Tous les isolats microbiens testés ont montré une capacité à se développer sur des milieux contenant de l'essence et du diesel en plus de leur capacité à produire des biosurfactants avec les deux hydrocarbures à des taux différents.

Compte tenu de tous les résultats obtenus, la bactérie C26 a montré le meilleur potentiel d'utilisation comme agent de bioremédiation.



**Mots clés :** Pollution, Hydrocarbures pétroliers, essence, diesel, sol, microorganismes hydrocarbonoclastes, bioremédiation.

**الملخص**

مع نمو سكان العالم بشكل كبير واستمرار ارتفاع سقف الاحتياجات ، من الضروري الانتباه إلى تأثير استغلال المواد الخام والمشاكل التي نشأت. تؤثر المنتجات البترولية المنبعثة في البيئة على الإنسان والحيوان والنبات. تعتمد عواقب التلوث على الكائنات الحية نفسها والتركيب الكيميائي للهيدروكربونات ولكن لها تأثيرات فورية لا رجعة فيها. وهذا يدفعنا للتدخل العاجل للتفكير في حلول جادة لإبطاء أو تقليل التلوث وآثاره. هدفت هذه الدراسة إلى عزل الأنواع البكتيرية والفطرية من عينات المنتجات البترولية وكذلك عينة من التربة الملوثة بالبترول. بعد ذلك ، يتم إجراء توصيف للعزلات من أجل اختيار الكائنات الدقيقة التي لديها القدرة على تحلل هذه الملوثات. يتم تحديد السمات المورفولوجية للكائنات الدقيقة المعزولة عن طريق الملاحظات العيانية والمجهرية. علاوة على ذلك ، يتم تقييم قدرتها على تحلل البنزين والديزل من خلال تحديد قدرتها على النمو على الوسائط التي تحتوي على هذين الهيدروكربونات. كذلك ، ما إذا كانت العزلة تنتج أم لا عوامل خافضة للتوتر من .(DCT) ٪ وتقنية الانهيار النوعي(CSH) ٪ ، وكراهية سطح الخلية للماءE24 السطحي تم تقييمها من خلال مقياس مؤشر الاستحلاب خلال هذه الدراسة تمكنا من عزل ثمانية أنواع من البكتيريا موجبة وسالبة الجرام وفطر واحد من جنس الرشاشيات. أظهرت جميع العزلات الميكروبية المختبرة قدرتها على النمو على وسط يحتوي على البنزين والديزل بالإضافة إلى قدرتها على إنتاج خافض للتوتر السطحي مع كلا أفضل إمكانية C26 الهيدروكربونات بمعدلات مختلفة. مع الأخذ في الاعتبار جميع النتائج التي تم الحصول عليها ، أظهرت بكتيريا .لاستخدامها كعامل معالجة بيولوجية.

**الكلمات المفتاحية:** التلوث ، الهيدروكربونات البترولية ، البنزين ، الديزل ، التربة ، الكائنات الدقيقة الهيدروكربونية البلاستيكية ، المعالجة الحيوية.

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# INTRODUCTION

# Introduction

Petroleum hydrocarbons are important energy resources used by the petrochemical industry and in our daily life. At the same time, they are major pollutants of the environment (Al-Wasify and Hamed 2014). Besides, the presence of various kinds of automobiles and machinery vehicles has caused an increase in the use of motor oil which spillage contaminates our natural environment. Moreover, with increasing demands of fossil fuel energy; extensive exploration of natural sources has caused a number of large-scale accidental spills of crude oil and resulted in environmental disasters (Abioye et al. 2010). In fact, large accidents involving oil tankers, rigs and pipelines have attracted public attention to the fate of petroleum hydrocarbons in various environments (Montagnolli,et al. 2014).

Traditional methods of cleaning oil pollution are confined to physical and chemical processes. These methods are expensive, time-consuming and less effective. Biological methods can have the edge over these treatments in removing oil spills. Bioremediation technology is safe, economical, more efficient and reliable (Ghanem et al. 2016).

In 1946, Claude U. Sable recognized that microorganisms have the potential to use hydrocarbon as the sole source of carbon and energy, and discovered that these organisms are widely distributed in nature. Fungi, for instance, are capable of degrading hydrocarbons in motor oil to some extent, but they take longer to develop than their bacterial counterparts (Gopinath et al. 2015).

In this perspective, this study aimed to isolate bacterial and fungal species from samples of petroleum products as well as a sample of contaminated soil with petroleum. Subsequently, a characterization of the isolates is carried out in order to select the hydrocarbonoclastic microorganisms that have the ability to degrade petroleum pollutants.

# LITERATURE REVIEW

# Literature review

## 1. Crude oil or petroleum

### 1.1. Definition and composition

Crude oil is a yellow-to-black sticky substance found as a liquid in underground geologic formations and remains a liquid when brought to the surface. It is a complex mixture of hydrocarbons that are mainly grouped into paraffins (e.g. alkanes), olefins (e.g. alkenes), naphthenes (e.g. cycloalkane) and aromatics (e.g. benzenes) in different proportions. Heavy crude oil contains significant amounts of complex hydrocarbons, such as polynuclear aromatics (PNA) (e.g. polycyclic aromatic hydrocarbons - PAH), alkyl-aromatics, heteroatoms and metal contents, which are more difficult to process. Common hetero-atoms in hydrocarbons are sulfur, oxygen, nitrogen, and metallic atoms. Inorganic salts of sodium chloride, magnesium chloride and other mineral salts are also accompanied with crude oil from field-wells either owing to formation water or water and chemicals injected during drilling and production operations (Al-Sayegh et al. 2016). Furthermore, oil from different areas around the globe varies considerably in their composition and physical properties (Montagnolli, et al. 2014).

The name Petroleum covers both crude oils and petroleum products that are made up of refined crude oil and include liquefied petroleum gases, aviation gasoline, motor gasoline, kerosene, fuel oil, petrochemical feedstocks, lubricants, waxes, asphalt and road oil (Jukić 2013).

### 1.2. Toxicity

Exploration, transportation, and consumption of oil products causes a continuous release of hydrocarbons into pristine areas through improper disposal or leakage in storage systems. It is estimated that over 2 million tons are lost each year by inadequate oil handling (Montagnolli, et al. 2014).

Petroleum has the potential to elicit multiple types of toxic effects. It can cause acute lethal toxicity, sub-lethal chronic toxicity or both depending on the exposure, dosage, and the organism exposed. Some components of petroleum have the potential to bioaccumulate within susceptible aquatic organisms and can be passed by trophic transfer to other levels of the food chain (Al-Wasify and Hamed 2014).

When used oil is accidentally or deliberately released into the environment, it can cause serious problems to both the biotic ecosystem and abiotic ecosystem, such as carcinogenicity and mutagenicity. The release of untreated or waste oil into estuaries, lakes and ponds causes

immediate and obvious problems for animals and plants. In addition, prolonged exposure to polycyclic aromatic hydrocarbons at high levels can develop liver or kidney disease and high risk of cancer (Arnoux 2002; Gopinath et al. 2015).

According to The International Tanker Owners Pollution Federation 2012, the environmental impact mechanisms involved in an oil spill are as follows:

- physical engulfment with impact on physiological functions;
- chemical toxicity with lethal or sublethal effects or deterioration of cellular functions;
- ecological alterations, mainly the loss of key organisms of a community and the proliferation of opportunistic species within the affected habitats;
- indirect effects, such as loss of habitat or shelter resulting in the elimination of ecologically important species.

### 1.3. Impact of the pollution on the environment

When petroleum hydrocarbons are released through a spill or leak into the environment, they migrate down through soils, becoming adsorbed to the soil particles until they reach groundwater where they will dissolve in water, float on the water surface or sink to the bottom of a water aquifer (Kurnaz and Büyükgüngör 2016).

Regarding oil spill in the sea, it spreads rapidly to form oil slicks on the sea surface (Garrett et al. 2003). The oil is dispersed through the surface and beneath it while the volatile compounds are released into the atmosphere. Various weathering processes occur as well, including dissolution, photo oxidation, and microbial degradation (Montagnolli, et al, 2014). Although a substantial fraction of petroleum is removed by evaporation, a portion of petroleum inflow to marine environs is dispersed into the water column, consequently affecting the local marine biota (Gopinath et al. 2015). Oil pollution in water environment have been a major threat to the ecosystem and human being through the transfer of toxic organic materials including polycyclic aromatic hydrocarbons (PAHs) into the food chain (Al-Wasify and Hamed 2014). Only one litre of used engine oil is enough to contaminate one million gallons of freshwater (Abioye et al. 2010).

### 1.4. Remediation of petroleum hydrocarbons

The petroleum mixture can be fractionated by silica gel chromatography into a saturate or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction. Several studies have been performed to determine the metabolic pathways for degradation of these compounds. The n-alkanes are generally considered the most readily degraded components in a petroleum mixture (Atlas 1981).

Mechanical removal of hydrocarbons from the environment relies on expensive, slow, and inefficient methodologies. Bioremediation technologies are an alternative to conventional methods involving excavation, landfill, pumping, treatment and addition of absorbent material. Biological treatment, or bioremediation, is a desirable alternative due to its low cost and effectiveness (Montagnolli, et al. 2014).

### 1.4.1. Bioremediation

The goal of bioremediation is to break down contaminants with microorganisms, ultimately reaching full mineralization (Montagnolli, et al. 2014).

Bioremediation of hydrocarbon pollutants is advantageous owing to the cost-effectiveness of the technology, especially when it can be carried out in situ, and the ubiquity of hydrocarbon-degrading microorganisms in the soil. Soil microbial diversity is affected by hydrocarbon perturbation, thus selective enrichment of hydrocarbon utilizers occurs (Chikere 2011).

### 1.4.2. Biodegradation

Biodegradation processes reduce petroleum hydrocarbons damage in contaminated environments. Total petroleum degradation is a result of a microbial consortium action, which is composed of different species with specific biochemical roles. Each one presents a required enzymatic mechanism capable of degrading a wide array of oil compounds (Montagnolli, et al. 2014). Biodegradation of environmental pollutants in soil is a slow process, influenced by abiotic factors and by a low abundance of indigenous pollutant-degrading microorganisms. Nutrients and oxygen supplementation, adjustment of pH and temperature (biostimulation), as well asmicrobial inoculation (bioaugmentation) were reported to increase biodegradation rates (Farber et al. 2019). The concentrations of available nitrogen and phosphorus in sea water influence hydrocarbon-degrading microorganisms' growth. By adjusting biodegradation conditions, it ispossible to stimulate oil biodegradation and turn it into a more efficient process (Montagnolli, et al. 2014).

## 1.5. Hydrocarbonoclastic microorganisms

Hydrocarbonoclastic microorganisms are involved in the mineralization of hydrocarbon pollutants in the environment. Isolated microorganisms do not usually have all the necessary enzymes to metabolize complex substrates. However, some bacterial genera found in contaminated environments have a reasonable performance during hydrocarbons biodegradation, such as Pseudomonas, Sphingomonas, Acinetobacter, Alcaligenes, Micrococcus, Bacillus, Flavobacterium, Arthrobacter, Alcanivorax, Mycobacterium, Rhodococcus, Actinobacter

(Montagnolli, et al. 2014). As well Mancera-Lopez et al. (2008), for instance, demonstrated that the three fungi Rhizopus sp., *Aspergillus sydowii* and *Penicillium funiculosum* enhanced total petroleum hydrocarbons (TPH) removal in polluted soils.

### 1.5.1. Biosurfactant

Biosurfactants are heterogeneous group of surface-active chemical compounds produced by a wide variety of microorganisms. Surface-active substances are capable of reducing interfacial tension between oil and water. Surfactants enhance solubilization and removal of contaminants thus enhancing biodegradation due to increased bioavailability of pollutants (Das and Chandran, 2011). As a result, droplets are dispersed much more efficiently in water column.

Bioremediation of areas contaminated with hydrocarbons can be achieved by bioaugmentation with biosurfactants. Biosurfactants are structurally diverse amphipathic surface-active compounds produced by a wide array of microorganism genera. As compared to their synthetic counterparts, biosurfactants are biodegradable, environmentally safe, stable under extreme conditions, and they can be produced in situ from inexpensive renewable substrates.

### 1.5.2. Biofilm

Biofilms are microbial cells attached to surfaces that communicate through quorum-sensing signals and ease their adjustment to the environment. A microbial biofilm resists mechanical stress and hydration, and can survive in nutrient-deficient conditions, as well it can overcome harsh environmental conditions, such as extreme temperatures and acidity. The attachment to a surface is the first step in biofilm formation. The topography and wettability of the surface, the bacterial cell-surface charges, and structures such as pili, fimbriae, flagella, and capsules, all influence the bacterial attachment (Farber et al. 2019).

### 1.5.3. Cell immobilization

Cell immobilization is a well-known method in biological processes for producing metabolites and for wastewater treatment. It provides a high density of microbial population and long-term bacterial survival, leading to a high biodegradation rate (Farber et al. 2019).

### 1.5.4. Genetically modified bacteria

Genetically engineered bacteria (GEM) show high degradative capacity. However, ecological and environmental concerns and regulatory constraints are major obstacles for testing GEM in the field. These problems must be solved before GEM can provide an effective clean-up process at lower cost. The combination of microbiological and ecological knowledge, biochemical

mechanisms and field engineering designs are essential elements for successful in situ bioremediation using genetically modified bacteria (Das and Chandran 2011).

### 1.6. Mechanism of petroleum hydrocarbon degradation

The most rapid and complete degradation of the majority of organic pollutants is brought about under aerobic conditions (Das and Chandran 2011). The initial intracellular attack of organic pollutants is an oxidative process and the activation as well as incorporation of oxygen is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central metabolism, for example, the tricarboxylic acid cycle (Das and Chandran 2011).

Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, acetyl-CoA, succinate and pyruvate. Sugars required for various biosynthesis and growth are synthesized by gluconeogenesis. The degradation of petroleum hydrocarbons can be mediated by specific enzyme system, the key enzyme is oxygenases (monooxygenase and peroxygenase) which play an important role in the microbial degradation of oil. Depending on the chain length, enzyme systems are required to introduce oxygen in the substrate to initiate biodegradation (Das and Chandran 2011).

#### 1.6.1. Factors influencing petroleum hydrocarbon degradation

The composition and inherent biodegradability of the petroleum hydrocarbon pollutant is the first and foremost important consideration. Among physical factors, temperature plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora, the viscosity of the oil increases, while the volatility of the toxic low molecular weight hydrocarbons are reduced, delaying the onset of biodegradation. Temperature also affects the solubility of hydrocarbons, the highest degradation rates generally occur in the range 30–40°C in soil environments, 20–30°C in some freshwater environments and 15–20°C in marine environments (Das and Chandran 2011).

Water is an important to the process of biodegradation as a mediator that transports metabolic substances between cells and as waste transporter. Water in soil also affects soil aeration status, nature and amount of soluble materials, osmotic pressure and the pH (Sims et al. 1990).

Nutrients are very important ingredients for successful biodegradation of hydrocarbon pollutants especially nitrogen, phosphorus, and in some cases iron. Some of these nutrients could become limiting factor thus affecting the biodegradation processes (Das and Chandran 2011).

# METHODOLOGY

# Methodology

## 1. Objectives

This study aimed to isolate bacterial and fungal strains from soils contaminated with petroleum hydrocarbons and then to determine their biodegradation ability.

## 2. Materials and Methods

### 2.1. Materials

#### 2.1.1. Soil sample

A sample of soil polluted with petroleum hydrocarbons was collected in June 2020 at approximately 10 m distance from a petroleum well located in In Amenas, Illizi, south-east Algeria (Fig. 1).

**Figure 1.** Petroleum refinery (Algeria).

The sample was collected in sterile plastic bag (Fig. 2, 3) and then was transferred in a cooler to the laboratory where it was stored at 4°C prior to work.

**Figure 2.** Soil sampling.

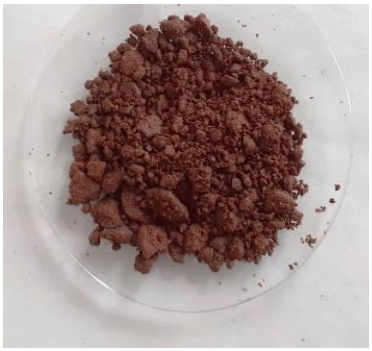


**Figure 3.** Petroleum contaminated soil sample

### 2.1.2. Petroleum samples

Samples of crude oil, condensate and slop oil (Fig. 4) were obtained from the Algerian Company SONATRACH located in Arzew, Oran (west transport region of Algeria).

The crude oil and slop oil (resulting 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 4.** Petroleum hydrocarbon samples: (a) slop, (b) crude oil, (c) condensate.

### 2.1.3. Diesel and gasoline samples

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

### 2.1.4. Chemical and culture media

Several chemicals and culture media were used:

**- Bushnell Hass Broth (BH)**

It is 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 1

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 $NH_4$ $NO_3$ and 0.05 g $FeCl_3$ (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 5.0 g NaCl, 5.0 g $KH_2PO_4$, 1.0 g $K_2HPO_4$, 1.0 g $(NH_4)2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 2.0 g $NaNO_3$, 0.02 g $FeCl_2 \cdot 4H_2O$, 0.02 g $CaCl_2$, per litre of distilled water (Obi et al. 2016).

**- Luria Bertani**

Contains ample amounts of all of the essential inorganic nutrients. The medium consists of, per litre: 10 g Tryptone, 5 g yeast extract, 5 g NaCl and 1 g Tryptophan (Morales-Guzmán et al. 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 $g.L^{-1}$, $K_2HPO_4$, 7.26 g $L^{-1}$, $KH_2PO_4$, 1.8 g $L^{-}$ MgSO4$\cdot 7H_2O$, 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 (De Angelis 2007).

## 2.2. Methods

### 2.2.1. 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. In addition, 10 g of the contaminated soil was added to the medium containing 1 % diesel and gasoline, separately, as the sole carbon source. The incubation was undertaken at 30 ºC under agitation of 170 rpm. After 5 days of incubation, a loop full of inoculum from Bushnell-Hass medium was streaked onto the nutrient agar and incubated at 30 ºC for 72 hours (Sukumar and Nirmala 2016).

Besides, the same procedure was performed using 100 mL sterilized minimal salt medium (MSM) containing 2 % (v/v) crude oil, condensate and slop. In addition, 5 g of contaminated soil was added to the medium containing as a sole carbon source diesel and gasoline (separately). The whole was then poured into a 250 mL conical flask and cultured at 35 °C and 180 rpm for 7 days. 5 mL of the first culture was transferred to 100 mL fresh enrichment medium and cultured at the same conditions. After five consecutive cycles of enrichment, the isolated bacteria were spread on the Luria Bertani agar. Thereafter, the isolated strains were conserved in 30 % (v/v) glycerol at -80 °C for further use (Wuyang et al. 2018). It must be noted that the MSM was sterilized at 121 °C for 20 min and the petroleum hydrocarbons serving as carbon source were filtered using a 0.22 mm Millipore membrane.

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.

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

Pure cultures were tested for their ability to degrade the diesel and gasoline. The test was performed in balch tubes containing 8 mL MSM supplemented with the diesel and gasoline (0.1 ml), which were inoculated by 2 mL of pure cultures of the isolates at a density of $10^8$ cells/ mL. Tubes were incubated in a shaker at 65 rpm at 30°C for 2 weeks. An abiotic control was used and consisted in the MSM supplemented with diesel and gasoline separately but devoid of microorganisms. Optical density (OD) was measured at 600 nm using Genesys 10 UVS Spectrophotometer (Nwinyi et al. 2014).

### 2.2.3. Screening of biosurfactant producing isolates

#### 2.2.3.1. Emulsification index ($E_{24\%}$)

The selection of the emulsifying bacteria was done using diesel and gasoline as a source of hydrocarbons. The emulsification index ($E_{24\%}$) was determined according to Morales-Guzmán et

al. (2017). Briefly, 2 mL of diesel and gasoline (separately) was added to a 4 mL bacterial culture propagated in Luria-Bertani medium. The mixture was shaken in a vortex for 2 min, the emulsification stability was measured after 24 h incubation at room temperature. The bacterial emulsifying effect was compared against a chemical surfactant consisting of culture medium plus Tween 80 (2:1 v/v). The $E_{24\%}$ was calculated using the following equation:

$$E24\% = \frac{Height\ of\ the\ emulsified\ layer\ mm}{Total\ height\ of\ the\ liquid\ coloumn\ mm} * 100$$

**2.2.3.2. Cell surface hydrophobicity percentage (CSH%)**

Hydrophobicity of the microbial cell suspensions was determined using MATH (microbial adhesion to hydrocarbons) assay, as a measure of their adherence to the hydrophobic hydrocarbons (Morales-Guzmán et al. 2017).

The bacterial strains were propagated in 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 8.000 g. The microbial density was adjusted to an optical density of 0.5 (A600) in phosphate buffer solution. 2 mL of the corresponding bacterial suspension were transferred to dilution tubes with 2 mL of diesel and gasoline (separately), shaken vigorously for 2 min in a vortex, and rested for 15 min to allow for the separation of phases. The optical density (A600) was measured in the aqueous phase. The CHS% was calculated using the following equation:

$$CSH\% = [1-(A600\ Final/\ A600\ Initial)] * 100$$

**2.2.3.3. The qualitative drop collapse technique (DCT)**

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). A fine layer of diesel was placed (50 µL per well) in 96-well plates and incubated at room temperature for 1 h. Subsequently, aliquots of 100 µL of the corresponding bacterial culture were added and maintained in triplicate. The biosurfactant production was detected by observing the dispersion of the drop in the well. Distilled water was used as negative control.

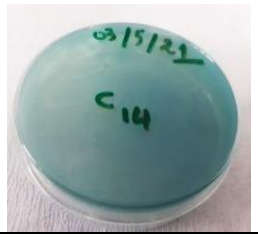
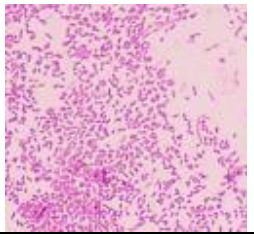
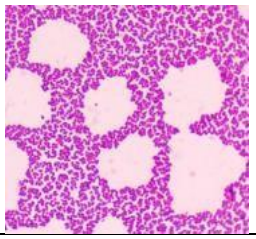
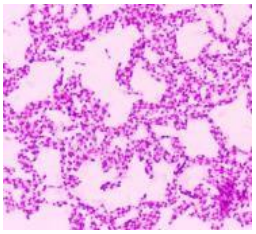
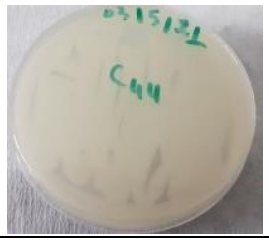
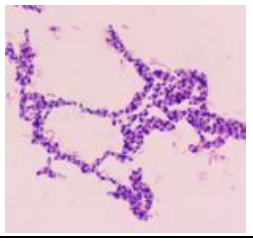
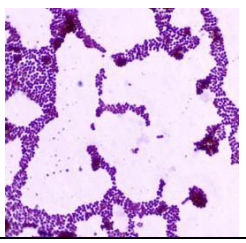
# RESULTS

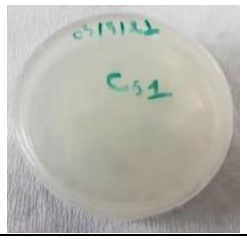
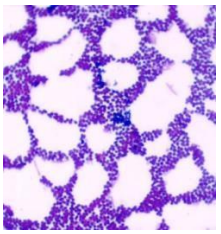
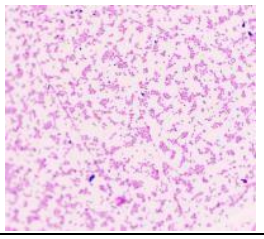
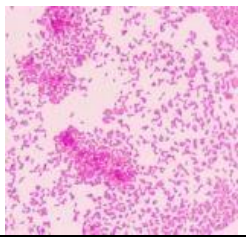
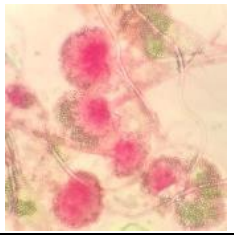
# Results

## 1. Isolated microorganisms

After the enrichment, isolation and purification of the obtained colonies on both BH and MS media from petroleum and soil samples, we obtained 08 pure bacterial isolates and 01 fungal isolate. The macroscopic and microscopic observations are shown on table 1.

**Table 1.** Macroscopic and microscopic observations of the microbial isolates.

| **Isolates** | **Macroscopic features** | | **Microscopic features** | |
|---|---|---|---|---|
| C14 | Green colony |  | - Thin short rods, in clusters<br>- Gram negative<br><br>- Suspected genus: Pseudomonas |  |
| C26 | Translucent colony |  | - Coccobacilli in clusters<br>- Gram negative<br><br>- Suspected genus: Escherichia |  |
| C31 | Whitish / Creamy colony |  | - Rods with central oval spores, in clusters<br>- Gram positive<br><br>- Suspected genus: Bacillus |  |
| C44 | Whitish colony |  | - Large rods with central oval spores, and chain<br>- Gram positive<br><br>- Suspected genus: Bacillus |  |
| C50 | White, creamy colony |  | - Diplococci in clusters<br>- Gram positive<br><br>- Suspected genus: Staphylococcus/ Micrococcus |  |

| | | | | |
|---|---|---|---|---|
| C51 | White,<br>Creamy Colony |  | - Cocci in clusters<br>- Gram positive<br><br>- Suspected genus: Staphylococcus/ Micrococcus |  |
| C52 | Green creamy colony |  | - Small rods<br>- Gram negative<br><br>- Suspected genus: Pseudomonas |  |
| C60 | Green yellow colony |  | - Small rods in cluster<br>- Gram negative<br><br>- Suspected genus: Pseudomonas |  |
| Fungus | Yellow- green colony |  | *Aspergillus* sp. |  |

We notice that apart the one fungus isolated that belong to the genus Aspergillus, the remaining eight isolates are bacteria where four are Gram negative and the other four are Gram positive.

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

After 2 weeks incubation of the microbial isolates on MSM containing either diesel or gasoline the obtained results regarding microbial growth are shown in figure 5 and 6.

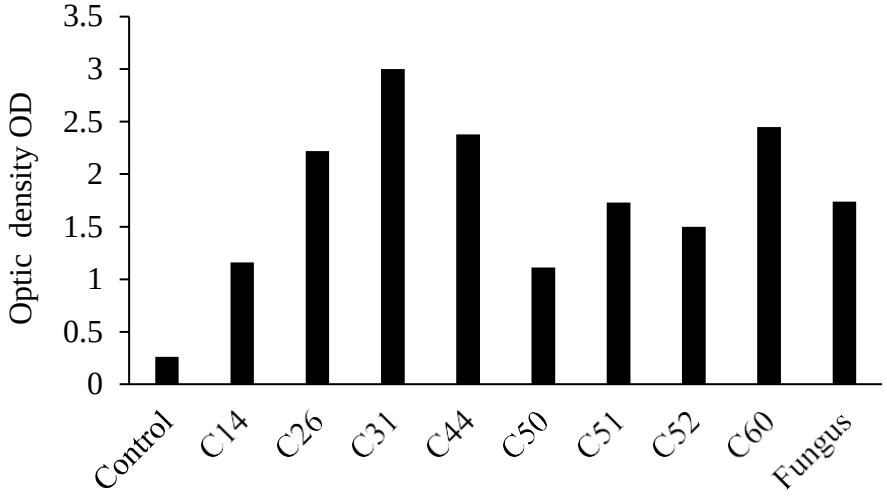



**Figure 5.** Growth of the isolated microorganisms in diesel after 15 days of incubation.

The bacterial isolates C31 showed better growth rates in the medium containing diesel oil compared to the other isolates followed by C26, C44 and C60.

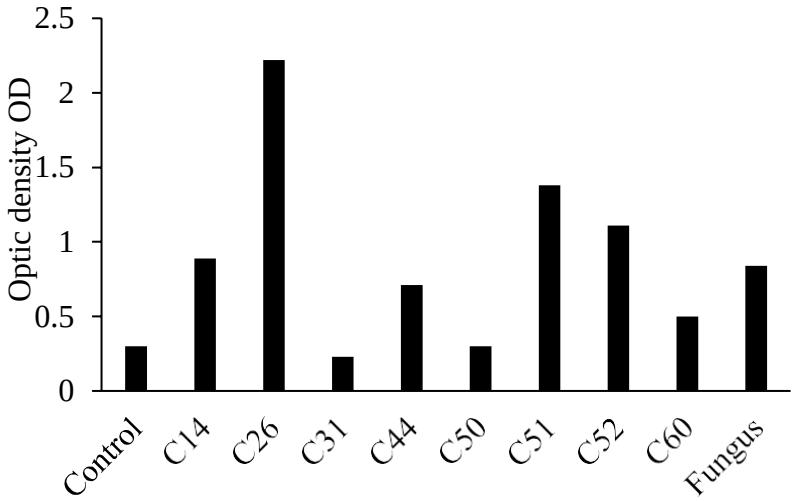



**Figure 6.** Growth of the isolated microorganisms in gasoline after 15 days of incubation.

Whereas, the bacterial isolate C26 showed better growth on gasoline containing medium followed by C51 and C52.

### 3. Emulsification index ($E_{24\%}$)

It is a qualitative and rapid method for evaluating the emulsifying properties of biosurfactants produced by a microorganism. The obtained results regarding $E_{24\%}$ of the selected microbial isolates are shown in figure 7 and 8.

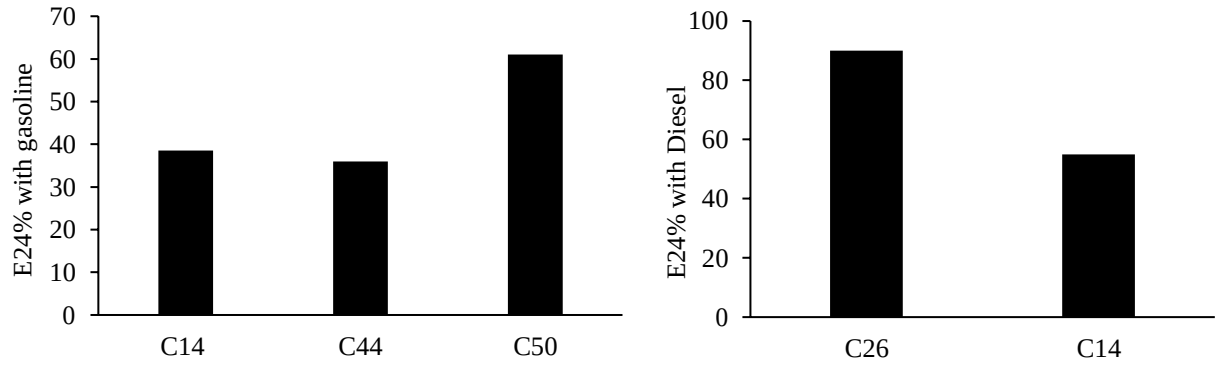



**Figure 7.** Emulsification index ($E_{24\%}$) of bacterial isolates on diesel and gasoline.

Among the 9 microbial isolates only C14, C44 and C50 showed an emulsification capacity on gasoline containing medium with C50 presenting the higher percentage (61 %). However, on diesel containing medium only C26 and C14 showed the ability to form an emulsification where C26 showed the highest emulsification index (90 %).

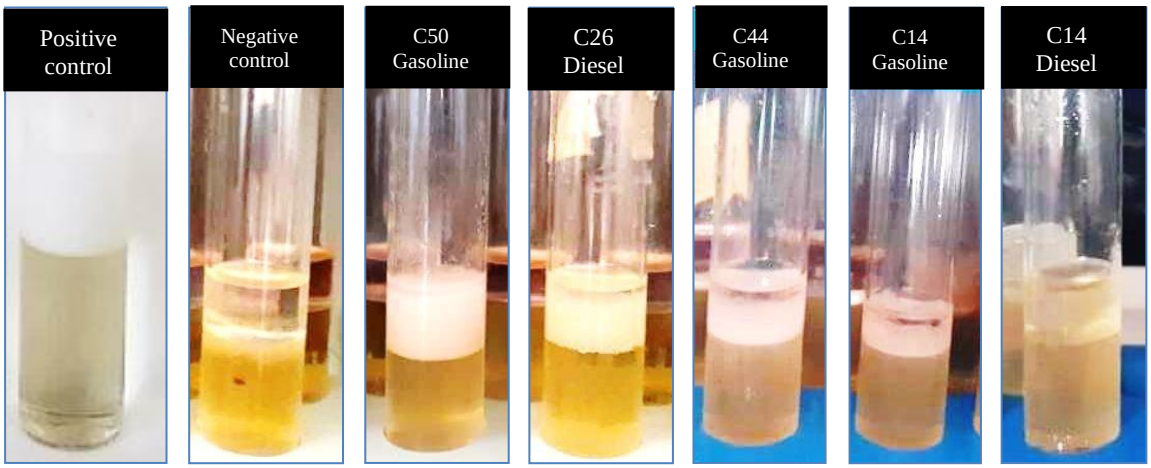



**Figure 8.** Isolates with emulsifying capacity on diesel and gasoline.

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

All the tested microbial isolates showed 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 and which varied between 24 and 46 %. C26 (46 %) and the fungus (40 %) showed the highest CSH % with diesel (Fig. 9).

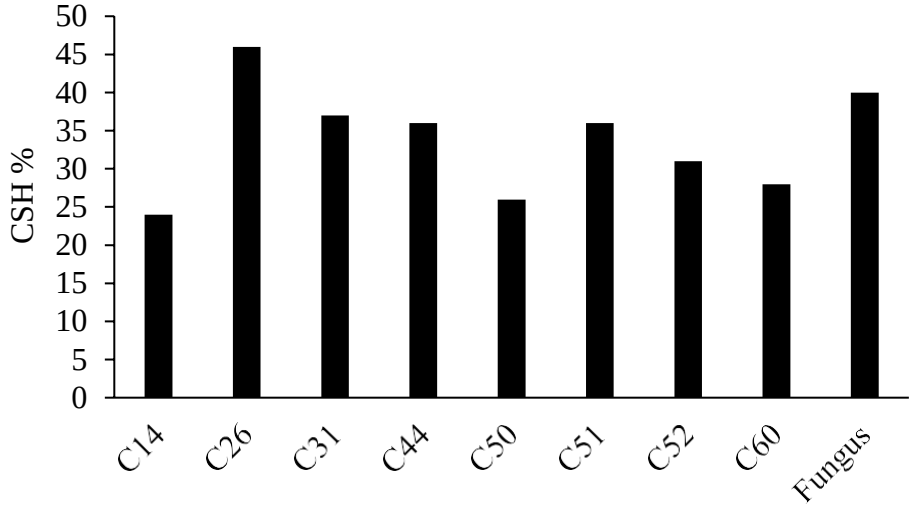



**Figure 9.** CHS % of microbial isolates with diesel.

Besides, a low cell hydrophobicity was observed with all isolates regarding gasoline, and ranged between 9 % and 79 %; C52 (79 %), C51 (63 %) and C26 (52 %) showed the highest CHS % (Fig. 10).

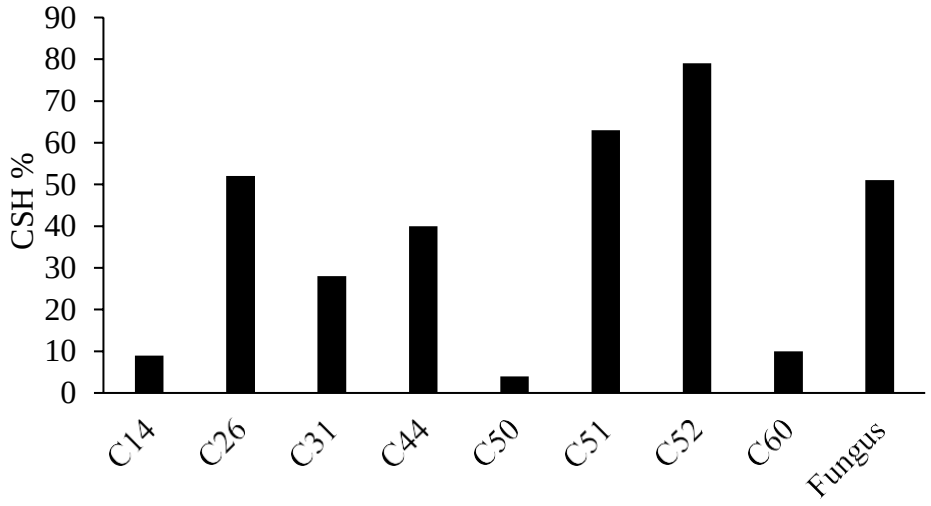



**Figure 10.** CHS % of microbial isolates with gasoline.

**5. Drop collapse technique (DCT)**

The obtained results regarding the qualitative drop collapse technique indicate that all microbial isolates have a positive reaction (Table 2 and Fig. 11) which indicates that all the tested isolates may produce biosurfactants.

**Table 2.** Results of the qualitative drop collapse technique of the microbial isolates with diesel and gasoline.

| Isolates | C14 | C26 | C31 | C44 | C50 | C51 | C52 | C60 | Fungus |
|---|---|---|---|---|---|---|---|---|---|
| DCT (diesel) | + | + | + | + | + | + | + | + | + |
| DCT (gasoline) | + | + | + | + | + | + | + | + | + |

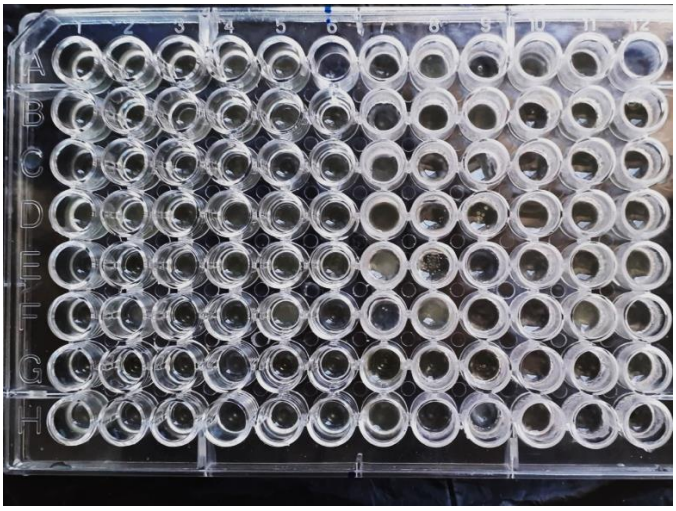


**Figure 11.** Results of the drop collapse technique.

# DISCUSSION

# Discussion

Petroleum hydrocarbons are the most common environmental pollutants (Chikere 2011). Their properties make them very toxic for living beings; that is why pollution by hydrocarbons remains a health concern that requires special attention (Soltani 2004).

In this study, 9 microbial isolates were obtained from petroleum products and polluted soil samples and were tested for their ability to degrade petroleum derived products namely gasoline and diesel.

The majority of the isolates are Gram negative and positive bacteria and only one fungus belonging to the genus Aspergillus was isolated. The predominance of bacteria over fungi can be explained by their strong ability to resist and adapt to hydrocarbons contaminated environments thanks to their variety of metabolic pathways (aerobic and anaerobic pathways). The diversity of metabolic pathways is due to the predominance of genes carried by bacterially associated plasmids in microbial communities found in hydrocarbon-contaminated soils (Hassaine 2016).

Furthermore, throughout this study we demonstrated that all the isolates have the ability to grow at varying degrees on media containing gasoline and diesel.

In fact, some of the hydrocarbon-degrading capabilities that exist in bacteria include the possession of degradation plasmids and other mobile genetic elements (Rojo 2009), the production of surfactants (Van Hamme et al. 2003) and the possession of specific catabolic enzymes such as oxygenases and hydoxylases (Atlas and Philp 2005) that appeared by new catabolic functions from selection (Abatenth et al. 2017). Besides, horizontal gene transfer is more prevalent in bacteria and has been reported as one of the primary mechanisms responsible for the evolution of enhanced hydrocarbon degradation (Chikere et al. 2011). The likelihood of mutation increases with native soil microbial populations which can improve their ability to degrade organic substances (Judith et al. 1990).

Besides, the obtained results demonstrated that the isolates have the ability to produce biosurfactants through the measure of their $E_{24\%}$, CSH% and DCT showing us the important role of biosurfactants in the biodegradation of petroleum products as well as making the microorganisms more resistant and selectable in hydrocarbon contaminated environments.

Bacteria despite their slow adaptation stage, are able to produce biosurfactants within the first few hours, knowing that the nature of the biosurfactants depends on the nature of the bacterial strains and the type of hydrocarbon (Chakrabarti 2012). This explains the relative difference in the growth of the isolates in the same substrate and between the two substrates (gasoline and diesel).

Although bacteria dominate over fungi in this study, several studies have shown the presence of fungi in polluted environment as well as their ability to degrade a variety of pollutants. The presence of fungi is due to the fact that they represent about 75 % of the soil biomass and can form mycelial networks over hundreds of hectares. In addition, their spores can be transferred over wide distances which makes them ubiquitous. In fact, fungi are characterized by their impressive metabolic capabilities that allow them to degrade complex natural molecules. The presence of fungi has advantages over other microorganisms in that they produce classes of enzymes that can interact with several types of polycyclic aromatic hydrocarbons with a fairly high degree of non-specific activity. Fungi are also tolerant to high concentrations of recalcitrant compounds and are able to flourish in extreme conditions (Ghanem et al. 2016). Among the fungi; Aspergillus, Candida, Cunninghamella, Fusarium, Mucor Penicillium, Phanerochaete Rhodotorula, Sporobolomyces and Trichoderma are hydrocarbon-degrading genera frequently isolated from soil. The species of this division are characterized by an abundant asexual reproduction and rapid growth that allows them to effectively colonize environments (Blackwell and Spatafora 2004; Fayeulle 2013). Fungal hyphal structures and increased surface area allow for better penetration and contact with hydrocarbons. Their extracellular enzymes, e.g., oxidases may further extend their activity into the soil (Chikere et al. 2011).

The processes of biodegradation by the microorganisms develop in general spontaneously but some require a phase more or less long of adaptation to the degradation of these various pollutants and the penetration of hydrocarbons within the cells of the microorganisms. The main ways of pollutants attack, involve either dioxygenases and mono-oxygenases (bacteria), or laccase, lignin- and manganese-peroxidase (lignolytic fungi) and/or production of oxidizing agents which will react chemically with the substrates, thus freeing the reaction of any catalytic selectivity (Johnsen et al. 2005).

Since many enzymes are not released by microbial cells, substances to be degraded must come into contact or be transported into the cells. Enzymes are generally specific to the substances they affect, so many types may be required to complete biodegradation of organic constituents (Judith et al. 1990).

For instance, the complete mineralization of PAHs into CO2 and H2O is achieved by two major successive catabolic pathways. The first one, called "peripheral" is specific of the compound or the range of degraded compounds. It produces an intermediate metabolite, often of catechol type. This enters a second pathway, called "central", common to many microorganisms, which converts the metabolite into intermediates of the Krebs cycle (Diaz 2004).

Generally, the increase in the emulsification index (E24) indicates the production of biosurfactant(s) that have the capacity to emulsify the petroleum hydrocarbons and render them more accessible for biological breakdown (Mnif et al. 2011). Biosurfactants are organic compounds with amphiphilic structure and specific functional groups conferring them properties such as wettability, micellization, surface tension lowering and formation of micro-emulsions between two different phases. Emulsions are formed when a liquid phase is dispersed as microscopic droplets in another liquid phase as a consequence of low surface tension (Amodu et al. 2014). Biosurfactants may be located inside the cells (intracellular) or secreted outside the cells (extra-cellular) (Antoniou et al. 2015). The microorganisms that degrade the hydrocarbons normally produce a variety of extracellular biosurfactants that increase the efficiency of hydrocarbon (highly hydrophobic) removal from solid surfaces by enhancing their solubility making them more bioavailable. Many biosurfactants with low molecular weight such as lipopeptides and glycolipids are effective in decreasing the surface tension, they emulsify the compounds, increase the water solubility and make the compounds more accessible for the microorganisms (Jung et al. 2010). Chakrabarti (2012) indicated that PAHs are almost removed completely in less than a month in soil contaminated sites. This is due to the strong effect of biosurfactants, which play a role in bioremediation by increasing the surface area of the substrates. In addition, biosurfactants act by changing the property of the cell surface of microorganisms; biosurfactant-producing microorganisms create their own microenvironment and promote emulsification through the release of certain compounds by various mechanisms such as quorum sensing (Cappello et al. 2007; Jung et al. 2010).

Besides, all the microbial isolates showed variations in their CSH %; higher values were obtained with gasoline where they reached 80 % for the isolate C26, 60 % and 50 % for C51 and C52 respectively. However, in diesel, CSH values did not exceed 45 % that was obtained with C26. CSH influences the direct contact of cell with hydrocarbon droplets which significantly affects the degradation of hydrophobic compounds. In addition to other physicochemical and biological parameters, CSH is an important factor for cell survival, as it controls the process of association between the cells and other surfaces which influence the rate of biodegradation (Gogra et al. 2010). Oil adhesion, pseudo-solubilization and degradation of hydrocarbons to form small droplets of oils are the sequential steps involved in the mechanisms of biodegradation. Microbial cells adhere to the drops of hydrocarbons whose size are less than that of the cells and the substrate uptake takes place by active transport or by diffusion at the point of interference between cells and hydrocarbons (Palecek et al. 2015). The degradation increases when the non-ionic surfactant exists (Itrich et al. 2015).

# CONCLUSION

# Conclusion and perspective

Petroleum hydrocarbon pollution is an alarming environmental issue, arising from industrialization. Petroleum contains many toxic and harmful components, which have a great impact on the ecological environment and can cause great harm to human beings.

Throughout this study, we were able to isolate 8 bacterial isolates and 1 fungus belonging to the genera Aspergillus from petroleum products (crude oil, condensate and slop oil) samples and soil samples taken near a petroleum well.

All the tested microbial isolates showed ability to grow on media containing gasoline and diesel in addition to their ability to produce biosurfactants with both hydrocarbons at different rates.

Taking into consideration all the obtained results, the bacteria C26 showed the best potential for use as a bioremediation agent.

This work should be followed-up by the identification of the isolates and the type of biosurfactant produced by direct sequencing of the DNA and genes implicated in biodegradation. As well, other test should be performed to identify the enzymatic machinery implicated in the biodegradation of petroleum hydrocarbons.

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