JKAU: Mar. Sci., **Vol. 32** No. 2 pp: 13-35 (2022 A.D.)
DOI :10.4197/Mar.32-2.2

Bioremediation of Contaminated Soil with Crude Oil Using New Genus and Species of Bacteria

Ahmed Mohamed Taher and **Ibrahim Omar Saeed**

Department of Biology, Collage of Science, University of Tikrit / Salah Al-Din, Iraq

ahmed.m.taher@st.tu.edu.iq

Abstract. In this study, the ability of bacteria presents in soils contaminated with crude oil to degrade petroleum hydrocarbons at the study sites (Al-Fatha and AL-Qayyarah) is investigated. Ten bacterial isolates are isolated from the soils of the study area, collected from different areas of Al-Fatha near the North Refineries in Salah-Din Governorate and AL-Qayyarah Refinery in Nineveh Governorate. These bacterial isolates show uneven growth in the mineral medium prepared with crude oil. Three new bacterial genera and two species are obtained, one of which belongs to the genus *Bacillus* and the other to the genus Exiguobacterium. The new bacterial isolates are recorded in the National Center for Biotechnology Information (NCBI) for the first time with the following numbers:(LC596402, LC596403& LC596406) for the genus (AM-I-1, AM-I- 2, and AM-I-5), respectively and (LC596404, LC596405) for the species (AM-I-3 and AM-I-4), respectively. The results show the ability of the isolates (AM-I-1, AM-I-2, AM-I-3, AM-I-4, and AM-I-5) to consume Polyaromatic Hydrocarbons (PAHs) compounds in the mineral medium prepared with crude oil with different concentrations and degrees, temperatures and pH conditions. The optimum conditions are pH 7, temperature 40°C and 2% crude oil concentration with a period of 12 days. The bacteria (AM-I-3) has outperformed the rest of the bacterial isolates with a consumption of (PAHs) compounds of 86.5%, followed by bacterial isolates and their mixture with a consumption rate of (84.42%, 78.19%, and 75.20%) (AM-I-1, AM-I-4, & mix of isolates), respectively.

Keywords: Bioremediation, Crude oil, Biodegradation.

1. Introduction

Knowing the composition, concentration, and properties of crude oil is one of the most important factors that help determine the appropriate and correct ways to treat environments contaminated with it (Wang *et al*., 2017).

Crude oil is a complex mixture of a large number of hydrocarbon and non-hydrocarbon compounds and different chemical elements. There is a difference in the chemical composition of crude oil according to type and quantity, but the common feature that binds most of the compounds included in the composition of the oil is the presence of carbon and hydrogen atoms. It can be said that the differences in the properties of the oil are due to the effective ability of the carbon element to combine with other elements and form simple or complex compounds (Speight, 2017).

There are multiple ways to treat pollution with crude oil, including chemical methods, where hydrocarbon pollutants are dissolved using chemicals and organic solvents added to the contaminated soil. However, this method has disadvantages and is costly in addition to requiring high energy and may leave harmful deposits and unwanted materials in the soil

(Koul and Taak, 2019). Regarding the physical methods that include the use of materials that can absorb hydrocarbon pollutants, these methods are also harmful to the environment and are of high cost. The biological treatment method is considered one of the most correct and successful methods that promise to treat soil contaminated with crude oil and its derivatives. This method is of low cost compared to the above-mentioned methods, harmless to the environment, transforming complex compounds into simpler compounds using microorganisms, and its final results are harmless to the environment being in the form of carbon dioxide and water vapour (Sari *et al*., 2018).

Molecular diagnosis of microbiology is considered one of the approved and high-accuracy methods in the knowledge of genera and species compared to other methods of diagnosis. The 16S rRNA technology has become one of the basic criteria for classifying bacteria due to the difficulty of obtaining genetic mutations. This gene has regions of high change between bacterial species with the independent sequence for different bacterial species and has been relied upon in studying the evolutionary and genetic relationships between different types of bacteria (Abdullah and Mahdi, 2016).

Over the years, many environmental factors including chemical and biological ones have led to the fragmentation and breakage of rocks. As a result, the soil is formed which is regarded as the crumbling surface layer of the earth's surface (Sangeetha *et al.*, 2017).

Recently, soil pollution has attracted widespread and significant attention as this problem has negative impacts on human, animal, and plant health and may have an impact on the economy on a wider scale (Steffan *et al*., 2018). The harmful effects of crude oil on agricultural soils can be observed in the apparent changes in the organic composition of the soil and mineral elements (phosphorous and nitrogen), Cation Exchange Capacity (ECE), and pH, in addition to the increase in the concentrations of heavy metals (Moses and Uwah, 2015).

As a condition, if the crude oil remains mixed and entangled with the soil, it causes massive damage to it in terms of quality and impedes the growth of plants and biodiversity. It alters the physical and chemical properties of the soil and causes increased levels of toxic substances such as radioactive and non-radioactive aliphatic compounds, aromatic compounds and heavy metals such as cadmium, vanadium, nickel, cobalt, and iron. (Marinesco *et al*., 2017).

There is a clear and important effect of heavy metals with petroleum hydrocarbons on the activity and numbers of bacteria that live in contaminated soil as their numbers decrease clearly. However, over time these microorganisms adapt to the polluted environment and use hydrocarbons as an energy source by increasing the secretion of an enzyme (hydrogenase and oxygenase) (Klimek *et al*., 2016).

Since they have enzymes that degrade petroleum hydrocarbons within their cellular metabolism, the bacteria participate in the *bio-geochemical cycle* and play a major role in the degradation of organic pollutants and the absorption of heavy metals in polluted environments. This feature has made it adapt to cruel environmental conditions and compete with other microorganisms and with each other (Velázquez-Fernández and Muñiz-Hernández, 2014). Martin (2002) also confirms the existence of hostile relationships between prokaryotes, such as primary consumption, predation, or competition, which allows some of the prokaryotes to prey on other prokaryotes

that compete with them for the same source of energy and food.

Deng and Wang (2016) confirm in their study the existence of relationships between the types of bacteria that live in the soil within the microbial community. These relationships may be positive, synergistic, or negative, depending on the nutritional substrate, as the changeable substrate such as glucose fuels negative and competitive relationships on the power source.

Bacteria that consume oil hydrocarbons are spread in different environments and can be isolated from agricultural soils and aquatic environments. However, the bacteria that consume oil hydrocarbons are of a small percentage compared to other microorganisms in these environments when not exposed to contamination of oil or its derivatives compared to their large percentage in the micro-organism community. In addition, there is also the diversity of its ability to consume petroleum hydrocarbons in polluted environments. The percentage of bacteria consuming petroleum hydrocarbons in non-polluted environments is estimated to be (0.01-0.1%), while its percentage reaches (60-100%) of the total heterotrophic bacteria in environments contaminated with oil and its derivatives (Jassim, 2016).

EL Hanafy *et al*. (2015) have studied the efficiency of the bacterial isolates *Nitratireductor sp,* and *Pseudomonas sp* in the degradation of petroleum hydrocarbons of polluted soils in the industrial zone (Al-Yanbu) in the Kingdom of Saudi Arabia near the Red Sea. The results show that the bacterial isolates (*S3 P. aeruginosa*), (*S4 p. Aeruginosa*), (*S5 Pseudomonas Sp*) and (4b Nitratireductor Sp) have degraded petroleum hydrocarbons by 66%, 65%, 95% and 70%, respectively, during two weeks treatment at 30 °C (Mojarad *et al*., 2016). The following bacterial genera (*Enterobacter cloacae*, *Enterobacter hormaechei*, *Pseudomonas stutzeri*) have the ability to remove petroleum hydrocarbons from soils and oil-contaminated waters so that these species can degrade hydrocarbons by 65.48%, 48.48%, 67.43%, respectively, when contamination of 5% by kerosene as the sole source of carbon. Also, the genera (*Pseudomonas stutzeri* and *Enterobacter hormaechei*) can analyze 12.98% and 54.14%, respectively, when contamination of 15% by kerosene as the sole source of carbon in 7 days.

Nafal (2020) has concluded that the mixture of *K. rosea* and *B. amyloliquefaciens* bacterial isolates has achieved a decomposition rate of 95.5% for crude oil at a concentration of 2%. Moreover, the above isolates have achieved a crude oil degradation ratio of 93.8%, and 68.9% for each isolate of *K. rosea* and *B. amyloliquefaciens*, respectively, at the same concentration of 2%.

Abdulla *et al*. (2019) have observed bacterial strains of the genus (*Bacillus cereus*) that can remove (PAHs) at different concentrations (2%, 3%, 7% and10%), where the removal rate reached 95% during treatment periods (5,10,15,20 and 30 days).

The strains of the genus *Bacillus* sp. have a high ability to resist and live in environments polluted with petroleum hydrocarbons and grow in them as the only source of energy. These strains are considered the most adapted to high levels of oil pollution because they have internal capsules that make them resist and live in cruel environmental conditions. In addition, they possess crude oil degradation enzymes more than the rest of the crude oil-degrading bacteria (Darsa *et al*., 2014; AL-Wasify and Hamed 2014).

By comparison and based on the results of gas chromatography analysis of samples of crude oil-degrading bacteria and the control sample, Al-Dhabaan (2019) has concluded that

Bacillus sp. has had the highest deterioration of petroleum hydrocarbons by (68-70%).

A study by Christova *et al*. (2019) has shown the ability of *Bacillus cereus* to deteriorate 93% of the aliphatic fraction of the hydrocarbon compounds of crude oil. Kumar *et al*. (2014), have shown through their study the ability of *Exiguobacterium* sp. to degrade petroleum hydrocarbons, especially aromatic ones, from gas chromatography results.

By biological treatment, by returning bacterial strains (*Lactobacillus* sp.) and based on surfactants, it is observed that biodegradation of crude oil occurred at a rate of (75%) (Thavasi *et al*., 2011).

soil (Chandrashekar *et al*., 2014), whereas it is possible to control soil conditions and environmental factors affecting bioremediation, which are also directly related to micro-organisms in order to improve their performance and activity in destroying pollutants, there are many biotic and abiotic factors that have a direct or indirect effect on the growth and activity of micro-organisms in the soil, and among the abiotic factors, the pH, humidity, temperature, the main nutrients, which are represented by nitrogen and phosphorous, and small or scarce nutrients, which must be in concentrations that do not harm living organisms (Guo *et al*., 2010; Daldoul *et al*., 2015; Paul, 2015) and as for the biological factors that have a clear effect on microorganisms, they include the metabolic activity of each organism, the synergy and competition between them, the symbiosis between them and the plants within the environment in which these microorganisms live, the concentration of toxic substances on the growth and density of these organisms especially when in micro environments, the concentration of toxins is calculated even if it is low (Daldoul *et al*., 2015; Kumar and Gopal, 2015).

When ideal conditions are provided, such as temperatures, pH, nutrients, energy sources, oxygen, and humidity, bioremediation of soils contaminated with crude oil is very effective and increases the potential for microorganisms to remove these pollutants at high rates (Chakraborty and Das, 2014; Malscchi, 2014; Brito *et al*., 2015). Treatment methods need to be developed in proportion to the severity of the pollutants, the efficiency, and speed of treatment, in addition to the material feasibility, and the method must be easy to apply in pollution sites to ensure that pollutants do not spread and reach groundwater, and thus the food chain for humans and, other living organisms. (Daldoul *et al*., 2015; Ghori *et al*., 2015).

The bioremediation process for polluted sites includes the use of multiple technologies depending on the method of treatment. The treatment may be inside or outside of the polluted area. The treatment inside the area is meant not to transfer the soil outside the polluted location, for example, Bioventng. While treatment far from the site of contamination is drilling the soil, then transferring it to the laboratory to apply the treatment such as Biopile. Each case has its method, tools, goals and, cost, as the field of treatment that takes place inside polluted locations, has a lower cost and does not need drilling nor contain pollution by dust and it treats large areas, but it is difficult to apply in clay soils because oxygen cannot be distributed through soil layers In addition, it requires long periods to treat the contaminated site. As for treatment outside polluted sites, it is easy to manage and control and needs shorter periods. This has made it widely used as well as it treats different types of pollutants. One of its disadvantages is that it is expensive because it uses drilling and transporting contaminated soils (Gall *et al*., 2015; Assadi *et al*., 2014; Stanaszek-Tomal, 2020).

The biological treatment of soils contaminated with petroleum hydrocarbons includes a range of technologies and these technologies include Bioventing, Bioaugmentation, Biopile, and Composition (Sobosob *et al*., 2016).

The activity of microorganisms in the degradation of organic pollutants depends on the amount of oxygen, the appropriate humidity, the types of nutrients, and their availability. Thus, the Bioventing method depends on the injection of quantities of oxygen at the site of contamination in the areas that are not saturated with oxygen. It also includes adding necessary nutrients for the microorganisms and providing appropriate moisture (Azubuik *et al*.,2016). This method has been –received well in treating sites contaminated with crude oil (Höhener and Ponsin., 2014).

It is the increase in the number of microorganisms or their enzymes at the site of contamination, on it, this method depends on adding strains and certain types of microorganisms that have the superior ability to analyze oil pollutants or add special enzymes that contribute to biological treatment (Herrero and Stukey, 2015). The success or failure of this method depends on several factors that must be known, and these factors are biotic and abiotic. Biotic includes selecting and testing strains of microorganisms that can degrade pollutants as well as compete for successful competition with the original microorganisms present in the soil. It may sometimes be done for the genetic modification of these microorganisms. Abiotic factors include the ph factors, chemical properties of the soil, and the composition, types, and concentrations of pollutants (Hernández-Adame *et al*., 2021).

When ideal conditions are provided, such as temperatures, pH, nutrients, energy sources, oxygen, and humidity, bioremediation of soils contaminated with crude oil is very effective and increases the potential for microorganisms to remove these pollutants at high rates (Chakraborty and Das, 2014; Malscchi, 2014; Brito *et al*., 2015).

The growth of micro-organisms is affected by biotic and abiotic environmental factors and thus their metabolic activity in the degrading of organic and inorganic materials. Microorganisms appear to tolerate a wide range of environmental factors, and under the appropriate conditions, these organisms thrive and grow, and perform their tasks. On the contrary, in adverse conditions cells die and are damaged, and the degrading of organic matter decreases (Stanaszek-Tomal, 2020).

Temperature plays an important role in the biodegradation of crude oil. As Akbari (2014) notes, with lower temperatures, the rates of biodegradation decrease but increase with increasing temperatures. When the temperature increases (20-35 °C), the rate of degradation of naphthalene increases by 10 times, because temperatures affect the physical and chemical properties of hydrocarbons and the activity of microorganisms. When temperatures decrease, hydrocarbons turn from a liquid state to a solid state and reduce their solubility and thus their degradation. In addition, the lower temperatures affecting the effectiveness of bio-emulsions and the enzymatic system of microorganisms negatively affect the biodegradation process of petroleum hydrocarbons (Bisht *et al*., 2015).

One of the most important factors that affect the growth of microorganisms is the pH factor, where the micro-organisms that perform biodegradation grow at a pH of 6.5-8.5 (Farag *et al*., 2018). Bacteria that biodegrade petroleum hydrocarbons have an optimal pH of 7 (Heimann, 2017) .Srivastava *et al*. (2014) have confirmed that the maximum limits of pH negatively affect the ability of bacterial groups

to degrade petroleum hydrocarbons .Many biological activities, including biodegradation, are sensitive to pH. It has been shown that a decrease in pH increases the solubility of toxic metals. Microorganisms may use cellular resources to maintain homeostasis, or cytoplasmic conditions can change in response to changes in pH outside thecell. Changes in carbon-electron flow membrane structure, cytogenesis, and protein synthesis have enabled a small number of anaerobes to adapt to lower pH conditions (Slonczewski, 2009).

The pH can be variable and in a wide range, and this thing must be taken into consideration to improve biological treatment methods. The effect of pH is clearly shown from the processes that occur in the cell form membrane transport, processes of equilibrium of catabolism reactions, and the activity of enzymes. This is because enzymes are undoubtedly affected by pH most of the nutritional variables of bacteria prefer growth at a neutral pH to alkaline (Al-Hawash *et al*., 2018).

2. Materials and Methods

2.1 Collection of Samples

2.1.1 Soil samples contaminated with crude oil

Soil samples contaminated with crude oil were randomly collected from different areas of Salah -Din, and Nineveh governorates. Soil samples were taken from five areas contaminated with oil spills from the al-Fatha area, Fig. 1 (Salah-Din -Din Governorate, located about (63,2) km away from the city of Tikrit, and five other different sites from Al-Qayyarah Refinery Fig. 2 in Nineveh Governorate, located about (60) km from the city centre of Mosul. Samples are put in sterile polyethene bags and the required information (sample number, date, weight) was recorded on them and transferred to the laboratory and kept in the refrigerator at a temperature of 4 °C until use.

2.1.1 Crude oil samples

Crude oil samples were obtained from the oil fields of Qayyarah Refinery, where crude oil samples were taken after extracting them from the wells and before any treatment was carried out on them. They were placed in sterilized and opaque bottles. The required information was written on them and transferred to the laboratory to be kept in the laboratory refrigerator at a temperature of 4 °C until use in experiments

2.2 Identify and Diagnose Bacteria

2.2.1 Phenotypic diagnosis

The phenotypic characteristics of the developing colonies relied on the nutrient agar media in terms of texture, shape, size, height, colour and, odour in the diagnosis of bacterial isolates (Logan, 2009).

2.2.2 Microscopic diagnosis

A Loop campaign was taken from the developing colonies and spread in a circular motion on a glass slide after being heat-fixed and stained with a cream stain (Collee *et al*., 1996), depending on the chemical and physical properties of the cell wall and using an oily lens of 100x strength, the shapes of bacteria were identified (Soni, 2013).

2.3 Molecular Biology Identification

2.3.1 Detection of genetic sequences

After the completion of the DNA extraction process and the PCR process, the samples were sent to Macrogen (South Korea), where 20 µl of each sample was placed in an Eppendorf tube and 50 µm of the primers were placed in other Eppendorf tubes to determine the genetic sequence of the bacterial isolates, depending on the Genetic Analyzer 3130 device. Equipped from Japanese Hitachi company.

The gene sequences were matched with data available at the National Center for Biotechnology Information (NCBI).

2.4 Biodegradation Assays

2.4.1 Primary screening

To check the ability of bacteria to degrade the crude oil, a solid MSM was prepared and provided with 1% crude oil. The medium was inoculated with a lube campaign filled with bacteria from each bacterial isolate in the centre of the agar plate, and all dishes were incubated at 37 °C for 24-48 hours. After selecting the isolates capable of degradation, the crude oil, they were grown again on the medium of solid mineral salts MSM, to verify the most efficient isolates, this is done by forming a clearing area by spraying the crude oil ether solution (10% v/v) uniformly on the surface of the solid MSM dishes, as the diethyl ether evaporates immediately and a layer of crude oil remains on the surface of the agar. The previously obtained pure bacterial isolates were planted by spreading them on a 1 cm diameter circle in the middle of the solid mineral salt dishes with three replications per isolation. The dishes were incubated at a temperature. 37 °C for 24-144 hours. The bacterial isolates that gave the largest diameter were selected (Santhini *et al*., 2009).

2.4.2 Secondary screening

Growth efficiency of isolates in liquid medium containing crude oil as a sole carbon source:

In a beaker containing 50 ml of liquid MSM provided 1% of crude oil, it was inoculated with bacterial isolates selected from the primary screening and incubated for 12 days at 37 °C in a shaker incubator (150 rpm). Cell growth turbidity was measured by spectrophotometer, at a wavelength of 600 nm (Kumar *et al*., 2006).

Efficient growth of isolates individually and mixed in a liquid medium containing crude oil as the s sole carbon source:

In a beaker containing 100 ml liquid MSM provided 1% crude oil, it was inoculated with bacterial isolates (0.5 ml individually and 0.25 as a mixture for each isolate) incubated at 37 °C in a shaker incubator (150 rpm) for 12 days., Cell growth turbidity was measured by spectrophotometer, at 600 nm wavelength (Kumar *et al*., 2006).

2.5 Optimum Conditions for Biodegradation

2.5.1 Effect of temperature

To measure the effect of the temperature factor on the biodegradation of crude oil and to select the optimum temperature, 50 ml of sterile liquid MSM was placed in a sterile 100 ml beaker and 1% of the sterile crude oil was added by filtration, the medium was inoculated with bacterial inoculum (0.5 ml) and incubated with different temperatures (25, 30, 35, 40 m) for 12 days in a shaker incubator of 150 rpm, three replicates were made for each bacterial isolation with one for control left uninoculated. After the incubation period, the biodegradation of the crude oil for both control and bacterial isolates was determined by a spectrophotometer at a wavelength of h 600 nm (Zekri and Chaalal, 2007).

2.5.2 The effect of crude oil concentration

The same steps were followed in the effect of temperature, but the temperature was fixed at 37 ° C, with different concentrations (0.5, 1, 1.5, 2 & 3%) of crude oil incubated at 37 °C in a shaker incubator of 150 rpm for 12 days. After the incubation period, the biodegradation of the crude oil for both control and bacterial isolates was determined by spectrophotometry at 600 nm wavelength (Gupta, 2012).

2.5.3 Effect of pH

The effect of the pH on the biodegradation of crude oil was determined by preparing the relevant brine of liquid MSM with different values of pH (6, 7, 8, and 9) using HCL (0.1N) and NaOH (0.1N) after sterilizing the liquid medium with an autoclave. Inoculation with bacterial isolates (0.5 ml) while leaving one without inoculation (control), noting that the media was provided by 1% of crude oil as the only source of carbon. The inoculated and control media were incubated under the same conditions in a shaker incubator of 150 rpm for 12 days at a temperature of 37 ° C. (Yang, 2011)

Detection of the biodegradation of crude oil by gas chromatography (GC) technique:

The gas chromatography used in laboratories of the Ministry of Science and Technology to discover PAHs was Shimadzu (2014, Japan). Column isolation was performed in a DB-5 column (30 m 0.25 mm ID) covered with a 0.25 μm thick film of 5% diphenyl-poly dimethyl siloxane. Samples were injected in the split position at 280 °C.

The column temperature was initially kept at 40 °C for one minute before increasing it to 120 °C at a rate of 25 °C / min, then to 160 ° C at a rate of 10 ° C / min, and finally to 300 °C at a rate of 5 °C / min, while maintaining the final temperature (300 °C) for 15 minutes. The FID was set at 330 °C. With a constant flow rate of 5 mL/min, helium was used as the carrier gas (Zhu *et al*., 2010).

$$\% \text{ Of hydrocarbons biodegradation} = \frac{\text{total peak in control sample} - \text{total peak in teast sample}}{\text{total peak in control sample}} \times 100$$

(Venosa and Zhu.,2003).

2.5.4 Statistical analysis

The data were analyzed statistically according to the complete random design method to show the confirmation of each type of bacteria and the concentrations and the combinations between them, as well as the trend analysis of the concentrations (as levels of a quantitative factor), the differences between the averages of the factors and the combinations between them, were compared in a Duncan manner the multi-range at the level of significance (0.01) and according to this test, the values followed by the similar letters indicate that there are no significant differences at the level of significance (0.01), all statistical procedures were carried out with the help of the ready-made statistical analysis system (SAS). (Al-Zubaidy and Al-Falahy., 2016).

2.5.5 Isolation and identification of bacteria

The results of Table 1 showed the bacterial growth that was cultured on nutrient agar medium, the growth of ten bacterial isolates from soil samples contaminated with crude oil, which were collected from Al-Fatha and Qayyarah refinery areas in Salah-Din and Nineveh governorates, respectively, at dilutions from 10-1 to 10-4, Studies differ in the possibility of isolating different types of crude oil-degrading bacteria, as (Azeez and Shareef, 2012) managed to obtain six crude oil-degrading bacterial isolates from different regions of Nineveh Governorate and its dependent regions, while (Jasim, 2016)) Isolation of 44 bacterial isolates from soils contaminated with crude oil for different regions of Sulaymaniyah Governorate and using one isolate to study its efficiency in bioremediation petroleum hydrocarbons.

In areas contaminated with crude oil, many bacterial species adapt to the carbon source of crude oil and its derivatives through their enzymatic system, formation of spores, and their degradation of organic and inorganic compounds in aerobic and anaerobic conditions with the surrounding environmental factors (Yu *et al*., 2020).

The efficiency and ability of petroleum hydrocarbon-degrading bacterial isolates can

be identified through the large numbers of bacteria that are isolated from soil contaminated with crude oil, which has adapted and becomes one of the most important microorganisms that degrade petroleum hydrocarbons (Parthipan *et al.*, 2017).

2.5.6 Biochemical tests

Biochemical tests, which included the tests shown in Table 1 based on (Collee *et al*., 1996) (Macfaddin, 2013), where the test for catalase, oxidase, hemolysis, and culture was performed on McConkey's medium, mannitol medium, motility test, IMVIC group of tests, as well as TSI test (Triple Sugar Iron) and urease test, the growing isolates were diagnosed based on colony shape, colour, edge shape, interaction with gram stain and biochemical assays (Brooks *et al.*, 2013) as shown in Tables 1 & 2 and Fig. 3 & 4).

2.5.7 Molecular Diagnostics

The final diagnosis of bacterial isolates was made using DNA Sequencing technology due to the ability of this technique to diagnose genus and species with high accuracy and in less time than traditional methods of diagnosis, where special primers are used to amplify this gene, which contains specialized regions that allow the diagnosis of genus and species. This gene consists of many nucleotides (Jenkins *et al*., 2012).

16S rRNA technology is considered one of the basic criteria for classifying and identifying bacteria due to the difficulty of genetic mutations in them, and because this gene contains high change regions between bacterial species and provides an independent sequence for each bacterial species. (Abdullah and Mahdi, 2016).

2.5.8 Polymerase chain reaction PCR

The results of electrophoresis on an agarose gel of DNA samples extracted from bacterial isolates using the 16S rRNA gene primer showed that the DNA bundles were about 1200bp as shown in Fig. 5.

3. Results and Discussion

3.1 Detection of a Nucleotide Sequence

The results of samples sent to Macrogen Company in North Korea, and in comparison, with the database of the National Center for Biotechnology NCBI, showed the discovery of three new bacterial genera and two new species, and they were registered on the NCBI website for the first time with new names and given a special number called an Accession number, The new bacterial isolates were recorded in this study in the National Center for Biotechnology Information (NCBI) for the first time with the following numbers: (LC596402, LC596403& LC596406) for the genus (AM-I-1, AM-I- 2 and AM-I-5) respectively and (LC596404, LC596405) for the species (AM-I-3 and AM-I-4) respectively.

3.2 Primary Screening of Crude Oil Degradation

The results shown in Table 3 and Fig. 6 refer to the clearing area achieved by bacterial strains on MSM-Agar medium prepared with 1% crude oil. The isolate AM-I-3 attained a very strong clearing area of more than 2 cm. It is referred to as (++++), while isolates AM-I-1, AM-I-2, and AM-I-4 attained a strong clearing area of more than 1 cm and were referred to as (+++). As for isolate AM- I-5 attained an average clearing area that reached between (0.5-1) cm and was referred to as (++), while the clearing area for the rest of the bacterial isolates was weak less than 0.5 cm (+), and the clearing area indicates the ability of bacteria to grow in the medium Contaminated with crude oil and using it as a source of carbon, and according to the efficiency of bacteria in the degradation of petroleum hydrocarbons, the clearing area is attained. The results of this study were in agreement with (Nafal, 2020), (Abdelkader *et al*., 2015), and (Latha and Kalaivani, 20, which mentioned that the larger

the clearing area around the colonies growing on the medium of solid mineral salts that were sprayed with etheric oil solution and incubated for 48-24 hours at a temperature of 37 °C, evidence of crude oil decomposition, and the clearing area can be relied on to select the most efficient bacteria in crude oil biodegradation experiments and according to the clearing area attained by isolates AM-I-1, AM-I -2, AM-I-3, AM-I-4 and, AM-I-5 were selected in subsequent experiments.

3.3 Secondary Screening of Crude Oil Degradation

The efficiency of isolated growth in liquid media containing crude oil as the sole carbon source:

The results shown in Fig. 7 indicate that the optical density (OD) of bacterial isolates generally increases continuously from the third day to the twelfth day of the incubation period and that the highest optical density on the third day was (0.59) for AM-I-3 bacteria the lowest optical density was (0.19) for AM-I-5 bacteria, while the highest optical density was on the twelfth day (1.49) for AM-I-3 bacteria, and the lowest optical density for AM-I-5 bacteria attained (0.99), and this It depends on the ability of bacteria to degrade petroleum hydrocarbons, continues to grow, and adapt to live in environments polluted with crude oil. The average days were on the third day, which amounted to (0.3340), and the highest optical density of the average days was on the twelfth day, which amounted to (1.2060), and the reason for that is that when the incubation period increases, the rate of oil deterioration will increase and the growth rates of bacteria will increase with it, and it was noted that the highest intensity Optical for bacteria averages were for bacteria AM-I-3 and it was (1.0700) and that the lowest optical density for the average bacteria was for AM-I-5 bacteria and it was (0.5850), and the reason is due to the difference in the genetic content responsible for secreting crude oil-degrading enzymes. The results were consistent with what was found by (Nafal, 2020) and the results of the statistical analysis indicate that there are significant differences between the five bacterial isolates in the absorption of optical density at a significant level of $p \leq 0.01$.

3.4 Optimal Environmental Factors for Biodegradation

Different environmental factors affect the biodegradation carried out by the bioremediation bacteria, as the more favorable these factors are, the faster the rate of degradation of oil pollutants (Farag *et al*., 2018).

3.5 Effect of Temperature

Temperature affects the composition of crude oil and its components, and thus its degradation at low temperatures, the viscosity of the oil increases, and its solubility in water decreases, thus the process of emulsification, and this leads to a delay in the biodegradation process (Ibrahim *et al*., 2017).

The results of Fig. 8 showed the growth of bacterial isolates at different temperatures (25, 30, 35 & 40 °C) and there was a gradual increase in growth with increasing temperatures, and the highest optical density was (2.32) at (40 °C) for the mixture of bacterial isolates, while the lowest optical density was (0.45) at the temperature (25 °C) for AM-I-1 bacteria, and we note that the optical density of the average temperatures is constantly increasing starting from the temperature (25 °C) degrees Celsius and was (0.5425) down to a temperature of (40 °C), where the optical density was (1.7775), and the reason for this is that the increase in temperature increases the rate of biodegradation. With the increase in activity, the rates of biodegradation increased (Kolsal *et al*., 2017), the mixture of bacterial isolates recorded the highest optical density (1.2400), and AM-I-1 bacteria recorded the

lowest level (0.7925), and the increase in optical density with increasing temperatures was significant at $p \leq 0.01$.

The optimum temperatures that microorganisms need in the degradation of petroleum hydrocarbons are within the range (30-40 °C), and the temperatures that fall outside this range, the microorganisms are in the process of either inactivity or lack of activity, low temperatures cause ineffectiveness of the system Enzymatic bacteria with a height of more than 40 °C leads to the denaturation of most bacterial enzymes, except some types of bacteria that live in extreme environments such as hot springs or frozen areas (Sihag *et al*., 2014).

3.6 Effect of pH

The pH of the medium in which the microorganisms live affects the growth of these organisms and thus the rate of degradation of petroleum hydrocarbons. In addition, a medium with a pH between (6.8-7) helps biodegradation through its effect on the movement of ions across the membranes of bacterial cells (Farag *et al*., 2018) (Pawar, 2012).

The results of Fig. 9 showed the growth of bacterial isolates in different media of pH (6, 7, 8 & 9) and that the highest optical density was at pH (7) for the mixture of bacterial isolates and it was (1.66) and the lowest optical density was at pH (6) for AM-I-1 bacteria, it attained (0.23) and with an increase in pH to 8,9 the optical density decreased, and this is consistent with the study of (Ahmed *et al*., 2011) (Hussein *et al*., 2015), where they observed the preferred pH of crude oil degrading bacteria is (7), the bacterial isolates mixture recorded the highest optical density (1.0725), and AM-I-1 bacteria recorded the lowest optical density (0.7525), and the results of the statistical analysis indicate significant differences between the bacterial isolates and their mixture at Significant level $p \leq 0.01$.

3.7 Effect of Crude Oil Concentration

The increase or decrease in the concentration of crude oil negatively affects the biodegradation process of petroleum hydrocarbons, as high concentrations cause toxicity to microorganisms, while low concentrations do not stimulate the enzymatic system of these microorganisms to carry out the biodegradation process (Ibrahim *et al.*, 2017).

The results of Fig. 10 show different growth rates in different concentrations of crude oil (0.5, 1, 1.5, 2 & 3) ml/100 ml of MSM medium for each of the bacterial isolates (AM-I-1, AM- I-3 & AM-I-4), as well as for the mixture of isolates in the incubation period (12) days, the highest growth rate of bacterial isolates was at a concentration of 2% of crude oil, where the highest reading of the optical density of the mixture of isolates was (2.011) and was the lowest value the optical density of AM-I-1 bacteria at a concentration of 3% reached (0.389), and from Table 2 we note and through the concentration rates of crude oil that the optical density at concentration (0.5) was the lowest, reaching (0.5250) and the reading began with the increase, at concentrations (1,1.5%) it became (1.3795, 1.46825), respectively, and the increase was significant, as the highest percentage of optical density at concentration (2%) was (1.70625), and the reading decreased at concentration (3%) to become (0.4525) The decrease was significant, that the increase in the concentration of crude oil causes an increase in the viscosity of the medium, which impedes the movement of nutrients as well as the transfer of oxygen molecules, and this negatively affects the activity of microorganisms and thus on my work biodegradation,

A very low drop in crude oil concentrations also has negative effects on the growth of microorganisms, and consequently,

the biodegradation rates of petroleum hydrocarbons decrease, as very low concentrations do not stimulate biodegradation enzymes in microorganisms (Roslee *et al*., 2020).

When we observe the growth rates of bacteria, we find that the optical density of a mixture of isolates (1.2960) is the highest, while the optical density of AM-I-1 bacteria is the lowest, which amounted to (0.8630), and this agrees with what was stated (Nafal, 2020), and through statistical analysis. It was found that there were significant differences in growth at probability ($P \leq 0.01$) between all bacterial isolates and their mixture and between the bacterial isolates themselves. Also, significant differences were found for the growth of bacterial isolates in different concentrations of crude oil (0.5, 1, 1.5, 2 & 3%).

3.8 Gas Chromatography (GC) Results and Biodegradation Detection

The biodegradation rate of isolated samples was measured by GC and the results of samples treated with bacteria were compared with the control sample. Polycyclic Aromatic Hydrocarbons (PAHs) are compounds that have high toxicity to humans and organisms and are considered carcinogenic (Pawar, 2012).

The results of Fig. 11 & Table 4 showed the ability of bacteria (AM-I-1) to consume polycyclic aromatic hydrocarbons for crude oil at different concentrations (0.5, 1, 1.5, 2 & 3%) and that the average of the remaining compounds (PAHs) at the concentration (0.5%), it was (1049.62ppm) with a consumption rate of 26.5%, which is considered the lowest, while the consumption of aromatic hydrocarbons increased, reaching the remainder of it (797.02, 634.07 ppm) with a consumption rate of (44.25,55.6%) at concentrations (1, 1.5%), respectively, and the highest percentage of consumption of (PAHs) compounds at the concentration was 2%, and the average of the remaining PAHs was (311.68 ppm), meaning that the percentage of consumption was 78.19%, and the percentage of consumption decreased at concentration 3% and it was (431ppm), a percentage of Consumption of 69.85%, and this is consistent with what was reached (Nafal, 2020), (Al-Dhabaan, 2019). The consumption of PAHs was significant at probability $P \leq 0.01$.

The results of Table 5 showed the ability of bacteria (AM-I-3) to consume polycyclic aromatic hydrocarbons for crude oil at different concentrations (0.5, 1, 1.5, 2 & 3%) and that the average of the remaining compounds (PAHs) at the concentration (0.5%), it was (940.69ppm), with a consumption rate of 34.19%, which is considered the lowest, while the consumption of aromatic hydrocarbons increased, and the rest of it reached (630., 473.3366ppm) with a consumption rate of (56.6,66.88 %) at concentrations (1, 1.5%), respectively, and the highest consumption rate of PAHs was at 2% concentration, and the average of the remaining PAHs was (193.013ppm), meaning that the consumption rate was 86.5% and the consumption rate decreased at 3% concentration and was (284.58ppm), with a consumption rate of 80.09%, this is consistent with what was reached (Nafal, 2020), (Christova *et al*., 2019). The consumption of PAHs was significant at probability $P \leq 0.01$.

The results of Table 6 showed the ability of bacteria (AM-I-4) to consume polycyclic aromatic hydrocarbons for crude oil at different concentrations (0.5, 1, 1.5, 2 & 3%) and that the average of the remaining compounds (PAHs) at the concentration (0.5%), it was (1016.57ppm), with a consumption rate of 28.88%, which is considered the lowest, while the consumption of aromatic hydrocarbons increased, reaching the rest of it (622.93, 513.75 ppm) with a consumption rate of (56.42, 64.05%) at concentrations (1, 1.5%), respectively, and the highest percentage of consumption of (PAHs)

compounds at concentration was 2%, and the average of the remaining PAHs was (222.67ppm), meaning that the percentage of consumption was 84.42%, and the percentage of consumption decreased at concentration 3% and it was (306.51ppm), with a consumption rate of 78.55%, and this is consistent with what was reached (Nafal, 2020), (Kumar *et al*., 2014). The consumption of PAHs was significant at probability $P \leq 0.01$.

It is evident from the Tables 4, 5, 6 & 7 that AM-I-3 bacteria are the most efficient in degrading petroleum hydrocarbons, specifically (PAHs) with a percentage of 86.5%, followed by AM-I-4 bacteria with 84.42%, then AM-I-1 bacteria at a rate of 78.19% and finally a mixture of bacterial isolates and a rate of 75.20%, The difference in the consumption of hydrocarbons and the percentage of biodegradation may be attributed to the enzymatic system of bacterial species and the way they analyze petroleum hydrocarbons, the low consumption of (PAHs) by the mixture of bacteria compared to the rest of the isolates may be caused by the presence of antagonistic relationships such as primary consumption, predation, or competition, which allows a section of prokaryotes to prey on other prokaryotes, which they compete for the same energy and food sources and predatory bacteria were probably less apt to decompose crude oil than those that were preyed (Martin., 2002).

The study (Zhou *et al*., 2020), confirmed the predatory relationship between Myxobacteria and the bacterial community that lives with it in the same environment (soil), where the Mucous bacteria produce antibodies to kill their prey that competes with it in the source of nutrition and feeds on it.

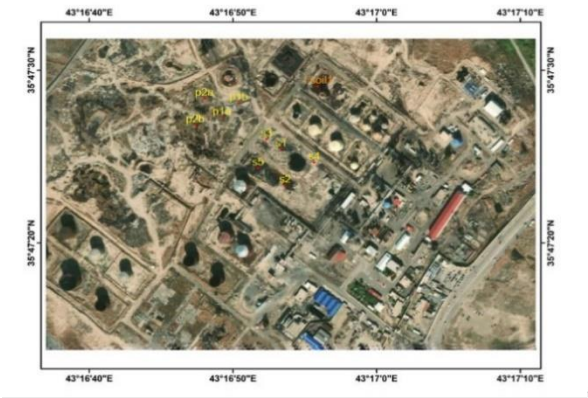


Fig. 1. AL-Fatha location.

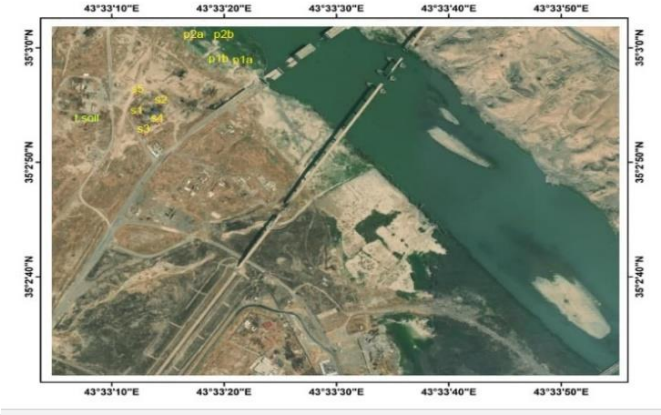


Fig. 2. Al-Qayyarah Refinery location.

Table 1. Phenotypic characteristics of bacterial isolates.

Isolates	Nature of colonies on solid nutrient medium						Gram stain	Cell pool	Cell shape
	Shape	Colour	Texture	Appearance	Hight	Edge			
AM-I-1	Circular	Yellow	Creamy	Dark	Flat	Regular	+	Tetrads	Micrococcus
AM-I-2	Circular	Bright white	Creamy	Dark	Flat	Regular	+	single	Rod
AM-I-3	Circular	light pink	Creamy	Dark	Flat	Regular	+	Single and binary	Rod
AM-I-4	Circular	Orange-yellow	Creamy	Dark	convex	Regular	-	single	Rod
AM-I-5	Irregular	Light white	smooth	Dark	umbonate	undulate	+	chain	Rod

Table 2. Biochemical tests for bacterial isolates.

No.	Tests	AM-I-1	AM-I-2	AM-I-3	AM-I-4	AM-I-5
1	Motility	-	+	+	+	-
2	Indole	-	-	-	-	+
3	Methyl red	-	-	+	+	-
4	VogesProskauer–	-	-	-	-	-
5	TSI	K\K	A\K	A\A	A\A	A\A
6	Oxidase	+	-	+	-	+
7	Catalase	+	+	+	+	-
8	Urease	-	-	-	-	+
9	Simmon Citrate	-	-	-	-	+
10	MacConkey agar	-	-	-	-	-
11	Mannitol salt agar	+\Non-F	+/F	+/Non-F	+/F	+\F
12	Blood Hemolysis	β	β	β	β	β

A, Acid; K, Alkaline; F, Fermentation; Non-F, non-Fermentation; + , positive; - , negative; β, β-Hemolytic.

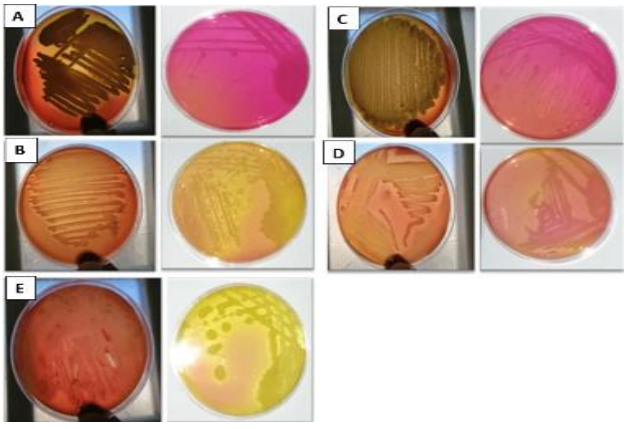


Fig. 3. Bacterial cell growth on Blood and Mannitol agar.

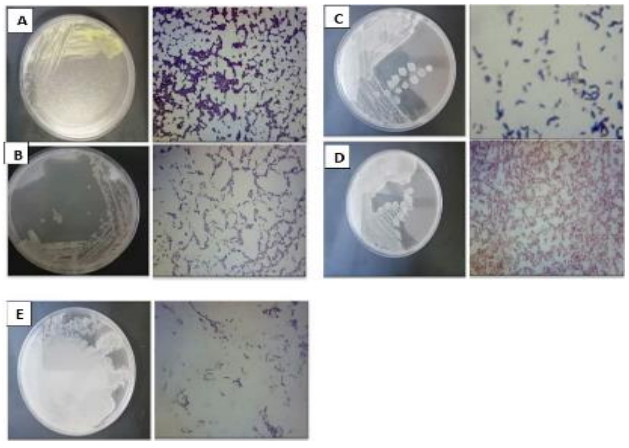


Fig. 4. Bacterial cell growth on nutrient agar and their shapes under the microscope.

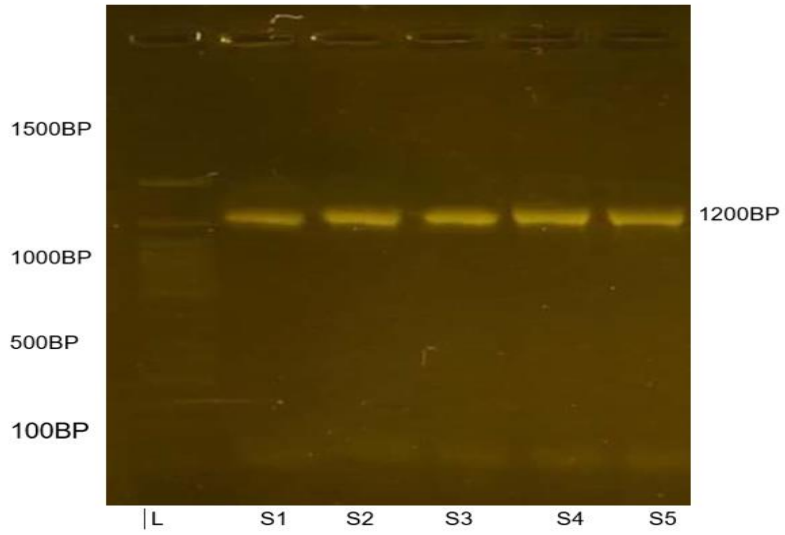


Fig. 5. Agarose gel electrophoresis shows the analysis of the PCR product for the 16S rRNA gene of bacterial isolates.

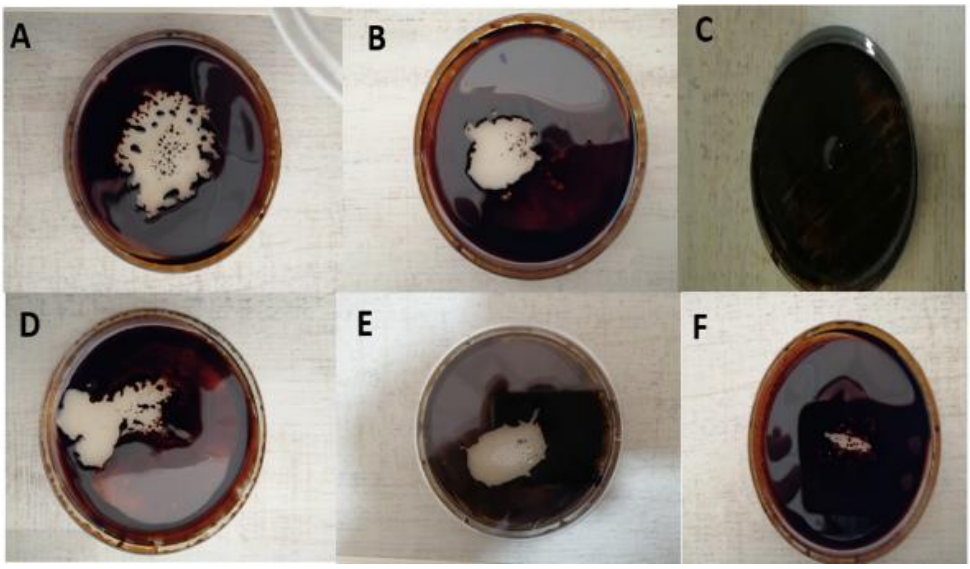
Table 3. Results of the preliminary examination of bacterial isolates and their efficiency in an analysis of crude oil, depending on the clearing area.

Isolates	The ability of bacterial isolates to grow	Diagnosed isolates
R1	++	AM-I-5
R2	+	
R3	+++	AM-I-1
R4	+++	AM-I-2
R5	+	
R6	+	
R7	+++	AM-I-4
R8	+	
R9	+	
R10	++++	AM-I-3

-Very strong=++++ the diameter of spot>2.0 cm.

-Strong = +++ the diameter of spot>1.0 cm.

- Moderate = ++ the diameter of thecolony between 0.5 - 1.0 cm. Weak = + the diameter of the spot< 5.0 cm.



A= AM-I-3 B=AM-I-1 C= Control D=AM-I-4 E=AM-I-2 F=AM-I-5

Fig. 6. Formation of the clear zone on MSM agar with an ethereal solution of crude oil (10%v/v) for bacterial isolates.

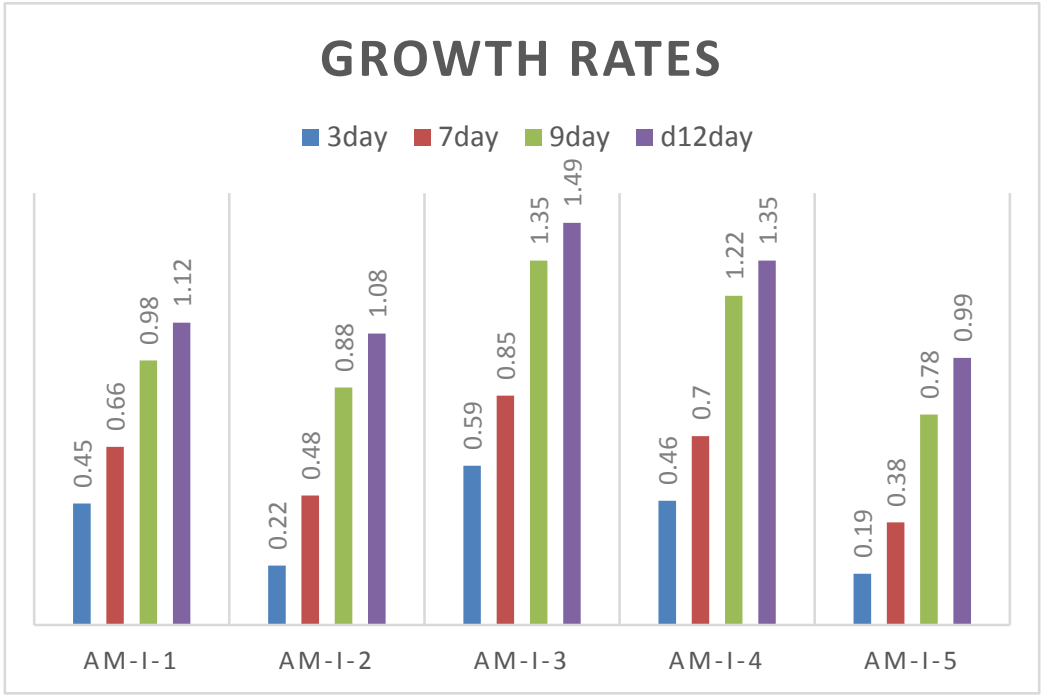


Fig. 7. The Growth of bacterial isolates in conjunction with the incubation period.

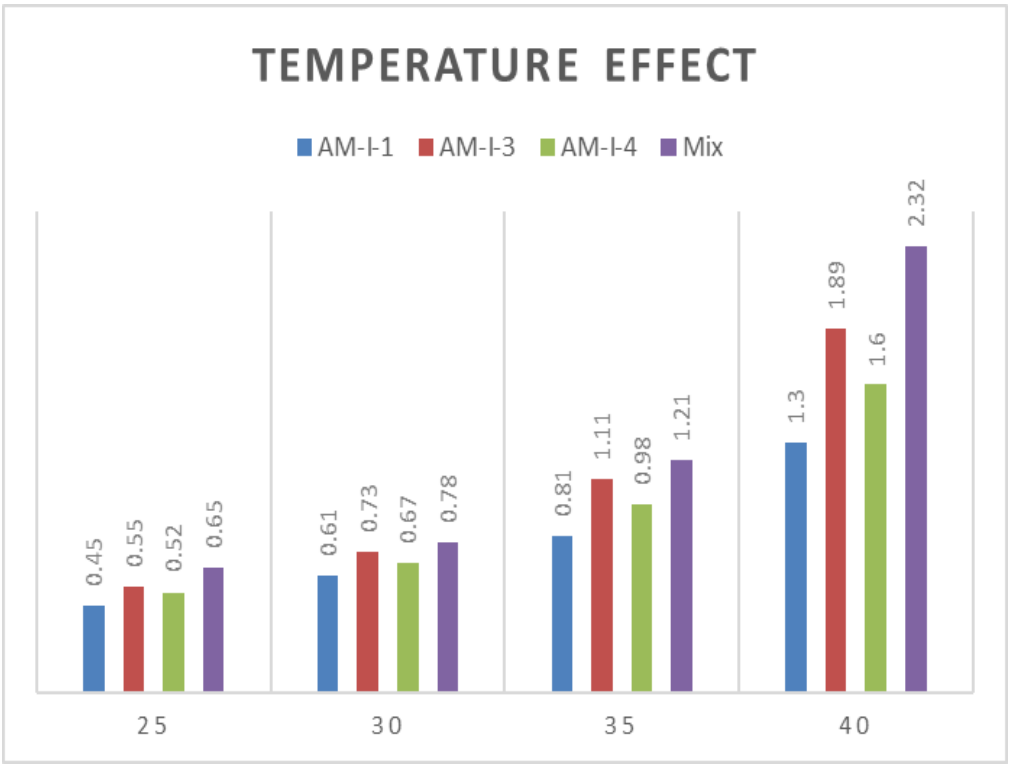


Fig. 8. Effect of temperature on biodegradation.

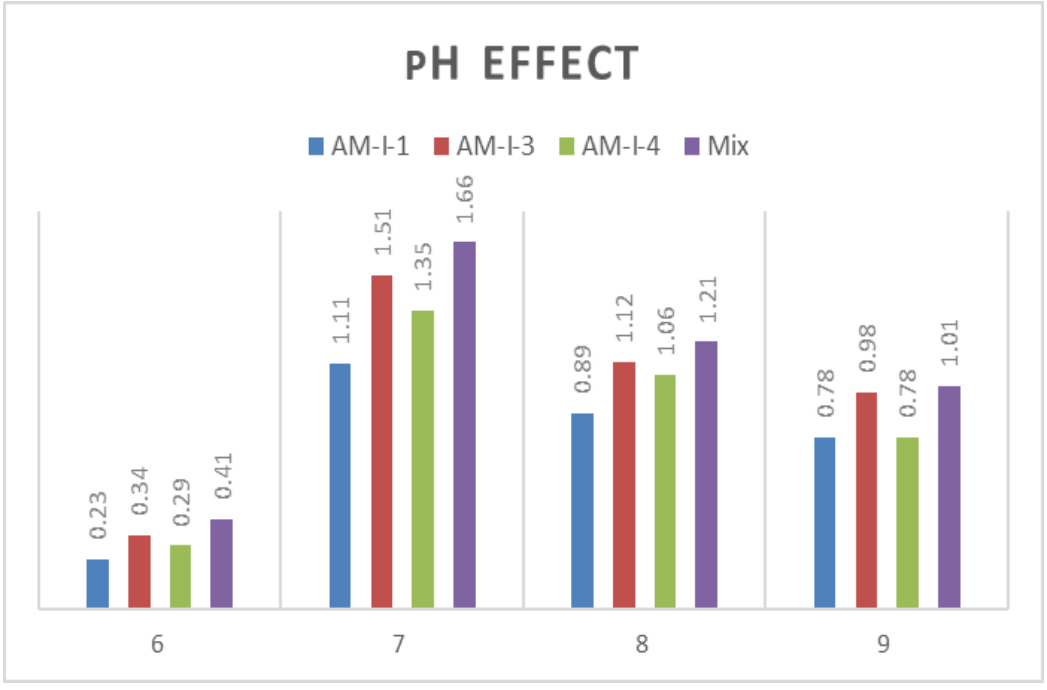


Fig. 9. Effect of pH on biodegradation.

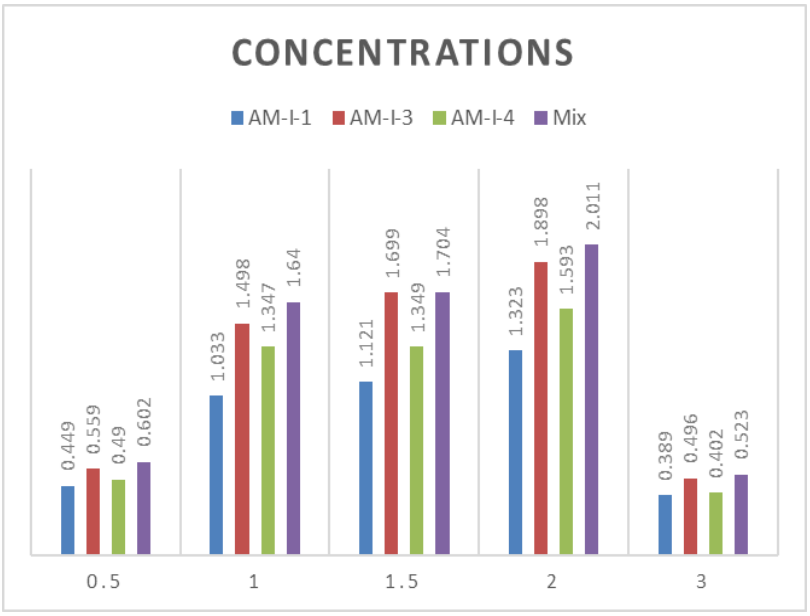
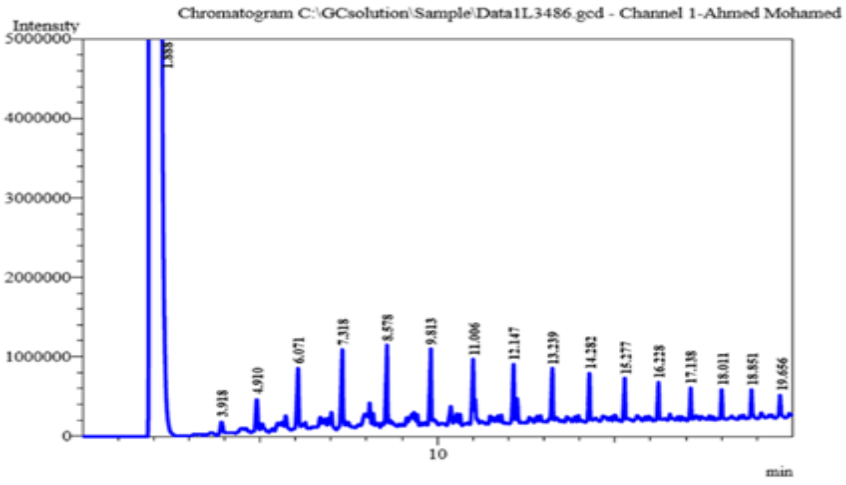


Fig. 10. Effect of crude oil concentration on biodegradation.



Peak#	Ret.Time	Area	Area%	Height	Name
1	1.888	5313767074	99.5643	576159206	
2	3.918	386468	0.0072	105670	
3	4.910	1524899	0.0286	385209	
4	6.071	1861098	0.0349	592837	
5	7.318	1640673	0.0307	655914	
6	8.578	2861921	0.0536	962105	
7	9.813	2741163	0.0514	917172	
8	11.006	2417301	0.0453	721499	
9	12.147	1390657	0.0261	532448	
10	13.239	1267269	0.0237	526683	
11	14.282	1593789	0.0299	577644	
12	15.277	1306205	0.0245	498239	
13	16.228	1139565	0.0214	439942	
14	17.138	615314	0.0115	312001	
15	18.011	975158	0.0183	365702	
16	18.851	881862	0.0165	334529	
17	19.656	650748	0.0122	247526	
Total		5337021164	100.0000	584334326	

Fig. 11. GC Solution / Sample.

Table 4. Biodegradation of AM-I-1 bacteria and percentage of PAHs removal.

Compound (ppm)	Control	AM-I-1				
		0.5%	1%	1.5%	2%	3%
Naphthalene	1568.6	1148.9	825.6	621.8	314.8	412.6
Methylnaphthalene	1789.4	1254.9	865.4	657.4	341	436.9
Acenaphthylene	1689.6	1205.3	745.8	547.9	253.6	367.5
Fluorene	923.9	658.9	510.9	417.8	241.6	356.1
Phenanthrene	1294.5	963.5	748.9	652.4	305.9	458.9
Anthracene	2586.8	1478.6	1142.2	962.4	525.9	746.5
Fluoranthene	1198.7	952.1	749.8	621.5	324.8	458.7
Pyrene	974.8	745.9	620.1	579.6	314.9	411.6
Benz[a]anthracene	1479.4	1247.9	924.9	765.4	328.9	478.3
Chrysene	2189.9	1659.9	1248.9	974.1	541.6	628.9
Benzo[b]Fluoranthene	1879.6	1528.9	1358.2	965.4	418.9	657.4
Benzo[k]Fluoranthene	869.4	652.3	512.9	420.8	208.9	257.6
Benzo[a]pyrene	124.8	95.8	86.2	71.4	52.6	66.4
Dibenz[a,h]anthracene	1478.3	1148.9	874.1	632.5	253	357.9
Benzo[ghi]perylene	1393.8	1002.5	741.5	620.7	248.9	369.7
sum	21441.5	15744.3	11955.4	9511.1	4675.3	6465
Mean of Concentration	1429.43	1049.62	797.02	634.07	311.68	431
Degradation %		26.5	44.25	55.6	78.19	69.85

Table 5. Biodegradation of AM-I-3 bacteria and percentage of PAHs removal.

Compound (ppm)	Control	AM-I-3				
		0.5%	1%	1.5%	2%	3%
Naphthalene	1568.6	985.6	745.2	598.7	214.6	325.9
Methylnaphthalene	1789.4	1058.6	689.6	541.8	247.9	336.9
Acenaphthylene	1689.6	985.4	647.8	412	205.9	324.9
Fluorene	923.9	536.9	425.8	320.8	124.8	256.9
Phenanthrene	1294.5	741.5	547.8	412.6	158.9	247
Anthracene	2586.8	1205.9	921.4	741.5	301.5	478.9
Fluoranthene	1198.7	745.6	524.8	369.8	201.5	313.6
Pyrene	974.8	514.7	425.8	300.8	147.9	188.4
Benz[a]anthracene	1479.4	1247.9	852.6	657.8	215.6	325.9
Chrysene	2189.9	1659.9	1015.4	841.5	326.9	398.7
Benzo[b]Fluoranthene	1879.6	1528.9	1099.8	745.9	278.9	412.5
Benzo[k]Fluoranthene	869.4	652.3	324.8	254.8	166.5	198.7
Benzo[a]pyrene	124.8	95.8	66.5	50.6	20.5	28.9
Dibenz[a,h]anthracene	1478.3	1148.9	624.8	417.8	124.9	199.7
Benzo[ghi]perylene	1393.8	1002.5	547.9	433.6	158.9	231.9
Sum	21441.5	14110.4	9460	7100	2895.2	4268.8
Mean of Concentration	1429.43	940.69	630.66	473.33	193.013	284.58
Degradation %		34.19	56	66.88%	86.5	80.09

Table 6. Biodegradation of AM-I-4 bacteria and percentage of PAHs removal.

Compound (ppm)	Control	AM-I-4				
		0.5%	1%	1.5%	2%	3%
Naphthalene	1568.6	998.7	787.4	635.7	255.8	348.7
Methylnaphthalene	1789.4	1147.8	720.3	599.8	296.8	365.5
Acenaphthylene	1689.6	1189.7	696.7	472.3	274.8	341.5
Fluorene	923.9	599.7	477.4	394.7	156.4	288.2
Phenanthrene	1294.5	823.6	584.9	455.4	184.7	263.5
Anthracene	2586.8	1314.5	112.3	796.8	332.5	494.5
Fluoranthene	1198.7	799.4	574.5	402.6	241.5	347.8
Pyrene	974.8	579.4	484.6	337.8	169.5	213.6
Benz[a]anthracene	1479.4	1297.4	886.9	683.4	245.6	345.9
Chrysene	2189.9	1745.6	1148.6	879.6	358.7	423.6
Benzo[b]Fluoranthene	1879.6	1624.1	1136.4	769.8	296.4	435.9
Benzo[k]Fluoranthene	869.4	688.9	365.8	282.4	189.5	213.6
Benzo[a]pyrene	124.8	103.6	75.6	59.7	33.6	40.6
Dibenz[a,h]anthracene	1478.3	1215.9	677.8	458.9	134.8	217.9
Benzo[ghi]perylene	1393.8	1120.3	614.8	477.4	169.5	256.9
Sum	21441.5	15248.6	9344	7706.3	3340.1	4597.7
Mean of Concentration	1429.43	1016.57	622.93	513.75	222.67	306.51
Degradation %		28.88	56.42	64.05	84.42	78.55

Table 7. Biodegradation of Mix of bacteria and percentage of PAHs removal.

Compound (ppm)	**Control**	**Mix of isolates**				
		0.5%	**1%**	**1.5%**	**2%**	**3%**
Naphthalene	1568.6	754.8	654.8	710.8	526.9	633.5
Methylnaphthalene	1789.4	569.8	412.9	523.6	355.8	406.8
Acenaphthylene	1689.6	745.6	524.8	589.9	452.9	541.8
Fluorene	923.9	412.6	365.9	400.5	258.9	374.5
Phenanthrene	1294.5	569.2	214.8	321.5	197.8	233.6
Anthracene	2586.8	956.8	649.8	745.9	478.9	674.1
Fluoranthene	1198.7	578.9	510.3	568.7	466.2	532.5
Pyrene	974.8	356.9	248.9	289.7	208.7	269.8
Benz[a]anthracene	1479.4	641.5	461.5	523.6	419.7	487.9
Chrysene	2189.9	1058.9	745.6	905.9	630.4	758.9
Benzo[b]Fluoranthene	1879.6	968.7	741.5	864.5	514.8	752
Benzo[k]Fluoranthene	869.4	301.5	258.9	287.9	198.7	264.1
Benzo[a]pyrene	124.8	51.6	33.6	41.8	22.9	36.9
Dibenz[a,h]anthracene	1478.3	633.6	418.9	526.9	369.7	427.9
Benzo[ghi]perylene	1393.8	549.8	326.9	477.9	214.9	337.9
Sum	21441.5	9150.2	6569.1	7779.1	5317.2	6732.2
Mean of Concentration	1429.43	610.01	437.94	518.60	354.48	448.81
Degradation %		57.32	69.36	63.71	75.20	68.60

4. Conclusion

With the ability of some bacterial species to live and adapt in soils contaminated with crude oil and consume compounds (PAHs) in different proportions and the discovery of new bacterial species and species that can degrade crude oil, there are optimum environmental conditions for the biodegradation process such as the optimum temperature is 40°C, pH 7, the optimum concentration of crude oil is 2% and the incubation period is 12 days. The presence of competitive and hostile relations between the bacterial species that live in the same environment negatively affects the treatment process.

4.1 Personal Explanation

This manuscript is the product of the work of a master's student in the College of Science at the University of Tikrit in the Republic of Iraq and I am the supervisor with the scientific title of Professor in the specialization of life technologies and bioremediation.

4.2 Pledge

I promise that this manuscript has not been previously published in a magazine and has not been submitted to any publishing house.

References

Abdelkader, TH. F; Aaref. H. H & Hummad, A. A. (2015). Testing the efficiency of hydrocarbon decomposition by bacteria isolated from soils contaminated with petroleum products. Journal of University of Anbar for Pure Science, Volume 9, Issue 3 Page 121-129.

Abdullah, R. M. & Mahdi, A.F. (2016). Identification of Pseudomonas aeruginosa from the clinical specimen by using 16S rRNA. *Iraq Acad. Sci. J*., **10**(1),: 45 - 49.

Ahmed, S. A., Syed, M. A., Arif, N. M., Shukor, M. A., & Shamaan, N. A. (2011). Isolation, identification and characterization of elevated phenol degrading Acinetobacter sp. strain AQ5NOL 1. *Australian Journal of Basic and Applied Sciences*.

Al-Dhabaan F. A. (2019). Morphological, biochemical and molecular identification of petroleum hydrocarbons biodegradation bacteria isolated from oil polluted soil in Dhahran, Saud Arabia. *Saudi Journal of Biological Sciences*, **26**(6): 1247–1252.

Al-Zubaidy, K. M. D. & AL-Falahy, M. A. H. (2016). *Principles and Procedures of Statistics and Experimental Designs*, Duhok University Press, Iraq.

Azeez, E. M. T & Shareef, A. Y. (2012). Ability of Micrococcus Isolation from soil in Biodegradation Diesel. *Rafidain Journal of Science*, **23**(8): 95-111.

Brookse, Geo. F.; Carroll, Karen. C.; Butel, Janet S.; Morse, Stephen A. & Mietzner, Timothy A. (2013). *Medical Microbiology*, 26th Ed, McGraw-Hill Companies, Inc, New York:877p.

Christova, N.; Kabaivanova, L.; Nacheva, L.; Petrova, P & Stoineva, S. (2019). Biodegradation of crude oil hydrocarbons by a newly isolated biosurfactant producing strain, *Taylor & Francis*, **33**(1): 863-873.

Collee, J.G.; Fraser, A.G.; Marmion, B.P. & Simmons, A. (1996). *Mackie and McCartney, practical Medical Microbiology*, 14th Edit Churchill living stone, New York.

Farag, S.; Soliman, N. A & Adel-Fattah, Y. R. (2018). Statistical optimization of crude oil bio-degradation by a local marine bacterium isolate Pseudomonas sp. sp48., *Journal of Genetic Engineering and Biotechnology*, **16**, (2): 409-420.

Gall, J. E., Boyd, R. S. & Rajakaruna, N. (2015). Transfer of heavy metals through terrestrial food webs: a review. Environmental Monitoring and Assessment: An International *Journal Devoted to Progress in the Use of Monitoring Data in Assessing Environmental Risks to Man and the Environment*. **187**(4); 201.

Gupta, B. (2012). Isolation and characterization of naphthalene degrading bacteria .*MSC Thesis*, college of Environmental Sciences and Technology. University of chapter of Thpary, 62.

Hussein, A. A., Khudhair, S. H. & Al-Mayaly. I. K. (2015). Optimum Conditions for LDPE Strips Biodegradation by Local Bacterial Isolates, *J. Int. Environmental Application & Science*, **10**(4): 399-407.

Ibrahim, M.; Makky, E. A.; Azmi, N. & Ismail, J. (2017). Optimization parameters for mycobacterium confluents biodegradation of PAHs. *Matec Web of conference* 150.06035(2018).

Jasim, M. A. (2016). Measuring The Ability of *Bacillus* cereus Bacteria on The Biodegradation of Crude Oil in Soil Incubated at Different Temperatures and Concentrations. *Thesis (M.Sc.)*, college of science University of Tikrit, Iraq.

Jenkins, C.; Ling, C.L.; Ciesielczuk, H.L.; Lockwood, J.; Hopkins, S.; McHugh, T.D.; Gillespie, S.H. & Kibbler, C.C. (2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology*, **61**(4): 483-488.

Kolsal, F., Akbal, Z., Liaqat, F., Gök, O., Sponza, D. T. & Eltem, R. (2017). Hydrocarbon degradation abilities of psychrotolerant *Bacillus* strains. *AIMS Microbiology*, **3**(3): 467–482.

Koul, B. & Taak, P. (2019). Chemical methods of soil remediation. In: *Biotechnological strategies for effective remediation of polluted soils*. Cham, Springer, Switzerland, pp. 77-84.

Kumar, M., Leon, V., Materno, A.D. & Ilzins, O.A. (2006). Enhancement of oil degradation by co culture of hydrocarbon degrading and biosurfactant producing bacteria. *Pol. J. Microbiol*., **55** (2): 139 146.

Latha, R. & Kalaivani, R. (2012). Bacterial degradation of crude oil by gravimetric analysis. *Advances in Applied Science Research*, **3**: 2789-2795.

Logan, N.A. & De Vos, P. (2009). *Bacillus*. In; *Berge's manual of systematic Bacteriology*, **3**: 21-128.

MacFaddin, J.F. (2013). *Biochemical Tests for Identification of Medical Bacteria*. Williams & Wilkins, 441.

Martin, M. O. (2002). Predatory Prokaryotes: An emerging research opportunity, *Microbiol Biotechnol*; **4**(5):467-77.

Nafal, Dina Hasan. (2020). *Bioremediation of Polluted Soils with hydrocarbons by some Local bacterial Isolates. Thesis (M.Sc.)*, college of science University of Baghdad, Iraq.

Parthipan, P., Preetham, E., Machuca, L.L., Rahman, P.K., Murugan, K. & Rajasekar, A., Biosurfactant and Degradative Enzymes Mediated Crude Oil Degradation by Bacterium *Bacillus* subtilis A1. *Front Microbiol*. 2017 Feb **9**; 8:193.

Pawar, R.M. (2012). *The effect of soil pH on degradation of polycyclic aromatic hydrocarbons (PAHs).* University of Hertfordshire for the degree of Doctor of Philosophy: 268-270.

Roslee, A.F.A., Zakaria, N.N., Convey, P. *et al.* (2020), Statistical optimization of growth conditions and diesel degradation by the Antarctic bacterium, Rhodococcus Sp. strain AQ5−07. *Extremophiles*, **24**, 277–291 (2020).

Santhini, K.; Myla, J.; Sajani, S. & Usharani, G. (2009). Screening of Micrococcus Sp from oil contaminated soil with reference to Bioremediation, *Bot. Res. Internat*., **2** (4): 248-252.

Zhu, M.; Wang, H.; Su, H.; You, X. & Jin, W. (2010). Study on Oxidation effect of ozone on petroleum -based pollutants in water. *Modern Applied Science*, **4**(1): 6-11.

Sari, G. L., Trihadiningrum, Y. & Ni'matuzahroh, N. (2018). Petroleum hydrocarbon pollution in soil and surface water by public oil fields in Wonocolo Sub-district, Indonesia. *Journal of Ecological Engineering*. **19**(2): 184-193.

Sihag, S., Pathak, H. & Jaroli, D. (2014). Factors Affecting the Rate of Biodegradation of Polyaromatic Hydrocarbons. Int. *J. Pure App. Biosci*. **2** (3): 185-202.

Soni, A. K. (2013). Isolation and characterization of biosurfactant-producing bacteria from oil contaminated soil (*Doctoral dissertation*).

Speight, J. G. (2017). Sources and types of organic pollutants. In: *Environmental organic chemistry for engineers*. Kidlington, Oxford, United Kingdom: Butterworth-Heinemann, pp. 153-201.

Spellman, F. R. (2015). *Environmental impacts of renewable energy*. edited by Boca Raton Taylor & Francis.

Venosa, A. D. & Zhu, X. (2003). Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands. *Spill Sci. Technol. Bull*., **8**(2): 163-178.

Wang, S., Xu, Y., Lin, Z., Zhang, J., Norbu, N. & Liu, W. (2017). *The harm of petroleum-polluted soil and its remediation research*. Green Energy and Sustainable Development, Chongqing, China.

Yang, M. (2011). *Measurement of Oil in Produced Water*; K, Lee and Jerry M. Neff. Produced Water: Environmental Risks and Advances in Mitigation Technologies. (57-88). Springer Science Business Media, LLC.USA.

Yu, Y., Zhang, Y., Zhao, N., Guo, J., Xu, W., Ma, M. & Li, X. (2020) Remediation of crude oil-polluted soil by the bacterial rhizosphere community of Suaeda salsa revealed by 16SrRNA genes. *Int J Environ Res Public Health*, **17**: 1471.

Zekri, A. Y. & Chaalal, O. (2007). Effect of Temperature on Biodegradation of Crude Oil, *Energy Source*, **V** (27): 233-244.

Zhou, Y., Zhang, X., Yao, Q. & Zhu, H. (2020), Both Soil Bacteria and Soil Chemical Property Affected the Micropredator Myxobacterial Community: Evidence from Natural Forest Soil and Greenhouse Rhizosphere Soil. *Microorganisms*, **2020,** 8, 1387.

المعالجة الحيوية للتربة الملوثة بالزيت الخام باستخدام أجناس وأنواع جديدة من البكتيريا

أحمد محمد طاهر، وإبراهيم عمر سعيد

قسم الأحياء، كلية العلوم، جامعة تكريت، صلاح الدين، العراق
ahmed.m.taher@st.tu.edu.iq

المستخلص. في هذه الدراسة، تم التحقق من قدرة البكتيريا الموجودة في التربة الملوثة بالزيت الخام على تحلل الهيدروكربونات البترولية في مواقع الدراسة (الفتحة والقيارة). تم عزل عشر عزلات بكتيرية من ترب منطقة الدراسة، جمعت من مناطق مختلفة من منطقة الفتحة بالقرب من مصافي الشمال بمحافظة صلاح الدين، ومصفاة القيارة في محافظة نينوى. تظهر هذه العزلات البكتيرية نمواً غير متساوٍ في الوسط المعدني المحضر بالزيت الخام. تم الحصول على ثلاثة أجناس بكتيرية جديدة ونوعين، ينتمي أحدهما إلى جنس *Bacillus* والآخر ينتمي إلى جنس Exiguobacterium. تم تسجيل العزلات البكتيرية الجديدة في المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI) لأول مرة بالأرقام التالية: (LC596402، LC596403 & LC596406) للجنس (AM-I-1، AM-I- 2، و AM- I-5) على التوالي و (LC596404، LC596405) للأنواع (AM-I-3 و AM-I-4) على التوالي. أظهرت النتائج قدرة العزلات (AM-I-1 و AM-I-2 و AM-I-3 و AM-I-4 و AM-I-5) على استهلاك مركبات الهيدروكربونات متعددة الحلقات (PAHs) في وسط معدني محضر بالزيت الخام بتركيزات ودرجات مختلفة ودرجات حرارة وظروف pH. الظروف المثلى هي درجة الحموضة ٧ ودرجة الحرارة ٤٠ درجة مئوية وتركيز الزيت الخام بنسبة ٢٪ لمدة ١٢ يوماً. تفوقت بكتيريا (AM-I-3) على باقي العزلات البكتيرية باستهلاك مركبات (PAHs) بنسبة ٨٦,٥٪، تليها العزلات البكتيرية ومزيجها بنسبة استهلاك (٨٤,٤٢٪، ٧٨,١٩٪، ٧٥,٢٠٪). (AM-I-1 و AM-I-4 ومزيج من العزلات) على التوالي.

الكلمات المفتاحية: المعالجة الحيوية، الزيت الخام، التحلل البيولوجي.