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Isolation and identification of some bacterial isolates from soil contaminated with crude oil and Testing Their Effectiveness

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Abstract:

Bacterial strains were isolated from oil-contaminated soil, in 2018, these isolates were **identified**, and with the aim of finding out the ability of these isolates to degrede the oil compounds, the color change of medium which added to it isolates was read by the method of Pacto Bushnell Hans. Then the change in the petroleum compounds was read by gas chromatography, for the most effective isolates.

The nine isolated bacterial showed different degrees of color change, and the isolates (Pseudomonas, Bacillus, Micrococcus) outperformed the color change amount (78, 78, 77) %, respectively, compared to the control, and the three isolates together showed the best color change of 90.7. % Compared to the control, and the results of separating materials by means of gas chromatography showed that there were changes in the nature of the materials present in the crude oil (control: without isolates) compared to those present in the crude oil That disintegrated by means of the three single isolates. The three isolates together showed a greater ability to change the nature of the materials present in the crude oil. Which confirms the effectiveness of each isolate separately and the ability of these isolates together to degerde crude oil.

Keywords: Bacteria, Biodegradation, Crude Oil, Hydrocarbons, Synergic.

Introduction:

Soil contamination with oil contaminants is a common problem in oil extraction and refining regions around the world. Despite the fact that the application of modern technologies of oil extraction, refining and transportation has contributed to reducing oil contamination to a certain degree, this problem remains one of the major environmental problems in large parts of the globe[1] . In Syria, the problem of oil contamination was a limited phenomenon in the regions where the oil fields are prevailing, eastern and northeastern regions until 2011, where begen the attacks on oil installations in the eastern and midland governorates started and significantly increased in 2013, Where was sabotaging various equipment at the stations, blowing up pipelines and digging wells randomly, and burning and refining oil by primitive environment-polluting means, The steady increase in soil contamination with oil derivatives has resulted in environmental concerns for those interested in environmental protection[2] ,This causes a low soil fertility, which in turn implies low agricultural productivity and reduced source of livelihood in the affected areas[3,4] .

Oil is a viscous, flammable and naturally-formed liquid under the earth's surface. It pollutes the environment and causes ecosystem disturbances and health damages to organisms[5]. The risk of oil-contaminated soil is due to the inability of some of its compounds to dissolve and volatilize, these compounds are in the form of polycyclic aromatic hydrocarbon chains[6], they remain and cause large pollution to the environment after any oil leakage[7], besides its non-biodegradability and toxicity to soil microorganisms[8]. Reference[9] believes that the need to clean crude oil on contaminated sites has become a major environmental issue. Reference[10] considered that biodegradation has become a priority for scientific research as it aims to safely and quickly remove these contaminants from soils. Reference[11] confirmed that the final success of biodegradation depends on the nature of microorganisms which are in direct and physical

contact with the biodegradable substance, and that the bio-treatment of soils contaminated with crude oil hydrocarbons is linked to the ability of organisms to consume hydrocarbons.

In the past, organisms were used for biodegradation[12]. Bio-remediation is preferable to physiochemical remediation methods as it is simple, easy to be implemented and its ability to produce a mass[13].

Reference[14-18] treated oil- contaminated soils with common microorganisms or ones isolated from oil-contaminated sites. The ability of these microorganisms for biodegradation was tested in vitro. The bacteria *Pseudomonas aeruginosa, Micrococcus spp, Corynebacterium spp*. and *Pseudomonas spp*. were isolated. These microorganisms gradually reduced the concentrations of polycyclic hydrocarbon compounds due to their ability to survive in such soils to develop a certain enzymatic and physiological response allowing them to use the hydrocarbon compounds present in oil as an alternative to carbon. Thus, when they consume carbon, they had already broken down these long bonds and converted them into simple substances easy to be degraded. These organisms differ by environmental-contaminating conditions and the contaminants. Therefore, due to the contamination of some Syrian lands with oil and its random and unsound refining; oil leakages resulting from transport; and adverse impact on soil and plant, there is a need to obtain isolates from our local environment able to treat oil- contaminated soil.

Materials and Methods:

Materials:

- Crude Oil : a sample of crude oil was brought from banyas station, sterilized with filtration paper of 0.45 μm holes and kept in a cool place for next use.

Oil-contaminated:

• **Soil sample collection**:

Composite samples from three oil-contaminated soils at depth 0-15 cm, were collected from the following places:

- Banyas refinery in Tartous
- Homs refinery in Homs
- Agricultural soil from Azbakiya Gas Station on Baghdad Street in Damascus.

Soil samples were well mixed, excluding stones and foreign objects. Then, they were sieved using a 2 mm sieve and kept in a cool place for analysis.

• **Isolation of Bacteria**:

-Direct method as per[19,20] Under septical condition, 1 g of oil-contaminated soil was taken. 0.01 g of /Tween80/ was added to sample homogeneity, then added to 9 ml tubes of sterilized distilled water by serial dilution and cultured on Petri dishes with NA (Nutrient Agar) Later, incubated at 35±2 ˚C. The developed isolates were re-cultured until pure ones obtained. The bacteria ones were preserved by deep freezing.

- Bacto Bushnell –Hans broth method: broth method Bacto Bushnell –Hans was used for isolation and is composed of $MgSO_4$ (0.2 g l-1)، $CaCl_2$ (0.02 g l-1)، KH_2PO_4 (1g l-1)، K_2HPO_4 (1 g l-1)، FeC_{l2} (0.05 g l-1) and NH_4NO_3 (1 g l-1 and dissolved with 1000 ml of distilled water. Then, 50 ml of the previous liquid medium was added in a 250 ml conical flask and autoclave-sterilized. Under septical conditions, 1% of crude oil (volume/volume) was added as a sole carbon source, and then Tween80 (0.1%) and 1 g of oil-contaminated soil as an expected soil microorganisms source degrading hydrocarbon compounds present in oil (in three conical flasks) was added 0.1 ml on each culture was spread to agar, placed on a shaker at 35±2 ˚C for 21 days. This process was repeated several times until pure colonies were obtained, and the cultures were kept at the same previous stages.

• **Characterization of Bacteria**:

Bacteria identified according to the comparison with the characteristics contained in the Bergey's manual [12] The bacterial genera were identified. The isolates were first diagnosed based on the morphological characteristics of colonies on culture media, including size, edge, height and colour. The biochemical tests (Gram, Spores, catalase, oxidase, Motility test, IMVC (Indole test, Methyl red test, fogues Proskauer test, Citrate utilization test, Ureas), Sugar fermentation test (Sucrose, Lactose and Glucose). The most effective isolates have been characterized by slices API.

• **Verification of The Ability of Isolates for Oil Degradation**:

- Bacto Bushnell – Hans[15,21,22]

The previous Bacto Bushnell – Hans's medium was prepared and dissolved with 1000 ml of distilled water, and then 1% of crude oil (volume to volume), 0.1% of Tween80 for homogeneity and 0.008% of 2 – 6 dichlorophenol indophenol in a powder form were added to the 50 mL liquid medium in a standard 250 mL conical flask. The resulting fungal and bacterial isolates were then inoculated conical flasks: each flask with one isolate and one flask as a control isolation chamber. The conical flasks were placed in a shaker 180 rpm for 7 days. Then, 5 ml from pattern each flask was taken and their absorption was studied by spectrophotometer at a 600 nm. The chromatic change from blue to colorless refers to the ability of

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microorganisms for oil degradation.

• **Test of The Most Effective Isolates Using Gas Chromatography**:

-Degradation of crude oil compounds added isolate-induced medium was demonstrated using gas chromatography with column type HP-5MS Phenyl Methyl SiloxHP-5MS 5% Methyl Silo3, 30 m long and
dimensions 30 m × 250 µm × 0.25 µm. Oven temperature was set to rise from 80 to 220 ˚C at average 5 ˚C min-1., from 220 to 240 ˚C at an average 5 ˚C min-1. and from 220 to 260 ˚C at average 80 ˚C min-1. for one minute with delay time of dissolvent 150 second. Helium was used as a carrier phase with average total flow 19.5 ml min1. and 1 µ of each sample was injected as mentioned below. The analyses were performed at Bio-chemical Lab at Faculty of Sciences of Damascus University.

-Preparing samples for GCMS experiment:

The samples were prepared as per[21-23] with some modifications: the previous Bacto Bushnell – Hans broth was prepared, of which an amount of 50 ml was added to 250 ml conical flasks. The flasks were sterilized, and crude oil at 20% was added. Then, the isolates, achieving the highest chromatic change single and combined were added together as well as the control under septical conditions. The conical flasks were incubated after covering them paraffin and placed on a shaker for 21 days at 37 ˚C at speed 160 rpm. After incubation, the previous media were centrifuging for 10 minutes at speed 5000 rpm and the deposited portion was separated and excluded. The hexane (oil dissolvent) was added at 10% to separate crude oil, the medium was agitated for 5 minutes in order to separate the medium into two phases: upper phase consisting of hexane and crude oil, and the lower is the saline environment on which the bacteria was grown. The hexane was vaporized and 1 microliter of the remaining was injected gas chromatogram in.

Statistical analysis:

Statistical analyses of all treatments were carried out as they are a simple random-designed experiment of the chromatic change of treatments. For analysis, GeneStat software was used. LSD was calculated at significance 5%. Covariance factor was also calculated.

Results and Discussion:

A. Bacterial Isolated from Oil-Contaminated Soils:

Nine bacterial isolates were isolated from oil-contaminated soils, and the results of the tests were recorded in Table 1. In comparison with the characteristics of the Bergery Manual[24,25], The bathe color change of medium which added to it bacteria isolated has been noticed form blue to pink at most treatments, while it is pink or colorless in the2nd bacteria, 7th and then 9th (Bacillus, Micrococcus, Pseudomonas) which was more effective than other treatments and the control in terms of ability to degrade hydrocarbon compounds by oxidization and dissociation of these compounds and use of resulting carbon for growing and building their structures, while the control remains blue and this agrees with[21,22,30].

Table 1. Identification of bacteria

No\ test	Cell Shap	Gram	Spores	Catalase	Oxidase	Indole	Motility	Methyl red	Fogues	Citrate Citrate	Ureas uria	Glucose	Lactose	Sucrose	Identification of bacteria
1	Cocci	+	+	+	−	+	−	−		+	−	*A*	*A*	*AG*	*Staphylococcus sp*
2	rod single	+	−	+	+	−	+	+		+	+	−	−	−	*Bacillus sp*
3	Rod-shaped	−	−	+	+	+	+	−	−	+	+	*A*	*A*	−	*Aeromonas sp*
4	Coucus	−	+	+	+	+	+	−	−	+	+	*A*	*A*	*A*	*Aeromonas sp*
5	Rod-Shaped	+	+	+	+	−	−	+	−	+	+	*AG*	*AG*	*AG*	*Corynebacteriu m SP*
6	Rods	−	−	+	−	+	−	−	−	+	+	−	−	−	*Acinobacter SP*
7	Coucus cluster	+	−	+	+	−	−	+	−	−	+	*AG*	*AG*	*AG*	*Micrococcus SP*
8	Rod-Shaped	−	−	+	−	+	+	−	−	+	−	*AG*	*AG*	−	*Enterobact er SP*
9	Short rod	−	−	+	+	−	+	+	−	+	+	−	−	*AG*	*Pseudomonas SP*

(+) = positive; (-) =negative; A= Acid Production; G= Gas Production; AG= Acid and Gas Production

B. Bacto Bushnell – Hans Experiment for Single Isolates:

the color change of medium which added to it bacteria isolated has been noticed form blue to pink at most treatments, while it is pink or colorless in the 2nd bacteria, 7th and then 9th (Bacillus, Micrococcus, Pseudomonas) which was more effective than other treatments and the control in terms of ability to degrade hydrocarbon compounds by oxidization and dissociation of these compounds and use of resulting carbon for growing and building their structures, while the control remains blue and this agrees with[21,22,30]. The reading of this chromatic change of different treatments on spectrophotometer and after seven incubation days has confirmed the rise in percent absorption in the three treatments over other ones. This is shown in. Table2

Table 2. Results of reading color changes of Bacto Bushnell on a spectrophotometer

Bacteria name	%Absorption rate
Staphylococcus sp	54 de
Bacillus sp	77.33 a
Aeromonas sp	39.67 f
Aeromonas sp	45.67 ef
Corynebacterium SP	64.33 bc
Acinobacter SP	67.67 bc
Micrococcus SP	78 a
Enterobact er SP	56.66 cd
Pseudomonas SP	78 a
control	1h
L	8.6
CV	6.9

Synergy of isolates:

The three bacterial combined isolates (*Bacillus*, *Micrococcus* & *Pseudomonas*) showed an increase in the chromatic change (colourless), and the absorption rate was up to 90.7%, the highest percentage.

A.Biodegradation of bacteria Single and Synergic Isolates Using Gas Chromatography*:*

The results of the biodegradation of single and combined bacteria using gas chromatography: The results of the biodegradation of bacterial isolates demonstrated the ability of single and combined isolates to degrade the hydrocarbon bonds of crude oil differently. This is illustrated by the change in the nature of substance involved in the composition of crude oil in the three treatments separately and collectively as compared to the control.(Table 3).

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Table 3. % of crude oil compounds and % of change in bacterial treatments.

Crude oil Csubstances	Control%	Synergic bacteria* %	Pseudo monas %	Bacillus %	Micrococ cus 7%
1-Octadecene	**0.9**	**5.1**	**3.76**	**0**	0
Benzene	**6.4**	**3.9**	**7.4**	**2.3**	0
1-Docosene	**3.8**	**5.3**	**0**	**0**	0
E-15-Heptadecenal	**6.3**	**0**	**6.1**	**0**	0
Hexadecane GAS	**6.03**	**0.8**	**1.8**	**2.7**	2.3
Hexadecane, tetramethyl	**0**	**0.8**	**0.77**	**0**	0
Hexadecane $$	**1.9**	**1.5**	**6.2**	**2.7**	0
Eicosane $$ n-Eicosane	**4.7**	**5.3**	**0**	**3.1**	1.6
Eicosane GAS	**4.7**	**0**	**3.5**	**0.8**	1.6
Octadecane (CAS)	**1.5**	**0.9**	**0.8**	**0.8**	1.19
Hentriacontane	**4.06**	**0**	**0.7**	**0**	0
Decane	**0.9**	**1.5**	**1.1**	**1.7**	1.9
Benzeneethanamine	**1.1**	**0**	**0**	**0**	0
(16S)-18,19-Dihydro-Sitsirikine-	**2.4**	**0**	**0**	**0**	0
Lauric acid	**3.6**	**0**	**0**	**0**	0
Nonadecane	**2.4**	**0**	**2.2**	**1.05**	1.7
Nonane,	**0.7**	**0**	**0**	**0**	1.8
Octadecane	**3.3**	**0**	**0**	**0**	1.1
Oxalic acid	**1.5**	**0**	**0.7**	**0.8**	0
$CH_3(CH_2)1_3CH_3$	**0.6**	**1.6**	**0**	**0**	0
Pentanal	**0.98**	**0**	**0**	**0**	0
Phenol	**0.8**	**0**	**0.9**	**2.2**	1.9
Silane	**0.7**	**0**	**1.4**	**0**	1.4
Sulfurous acid	**3.3**	**0**	**0**	**0**	1.3
2,6-dimethyl-	**0.7**	**0**	**0**	**0**	0
Tridecane	**1.7**	**0**	**1.7**	**0**	1.5
TRICOSANE	**0.9**	**0**	**0**	**1.6**	0
Triacontane	**0.7**	**0**	**0**	**0**	0
Total materials present in control	**28**	**18**	**13**	**17**	16
% of completely changed materials	**100**	**64.3**	**46.4**	**60.71**	57.1

*Synergic bacteria main three bacteria Micrococcus, Bacillus, Pseudomonas together.

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The Figs 2,3, and 4 show the disappearance of a part of bands of gas chromatography analysis and other short bands compared to the control. Fig.1 We also find differences in band length and number between the three bacterial isolates, due to the different ability of each bacterium to degrade at specific loci of hydrocarbon bonds. In addition, we noticed that the bands of combined isolates were shorter than single isolates Fig. 5 in addition to, disappearanc of bands and appearance of others, indicating changes in the nature of substances present in oil composition. These results are consistent with Table 2, as well as with the results of the chromatic reading of single and synergic isolates, where the synergic isolates gave the highest chromatic change and this coincides with [29].

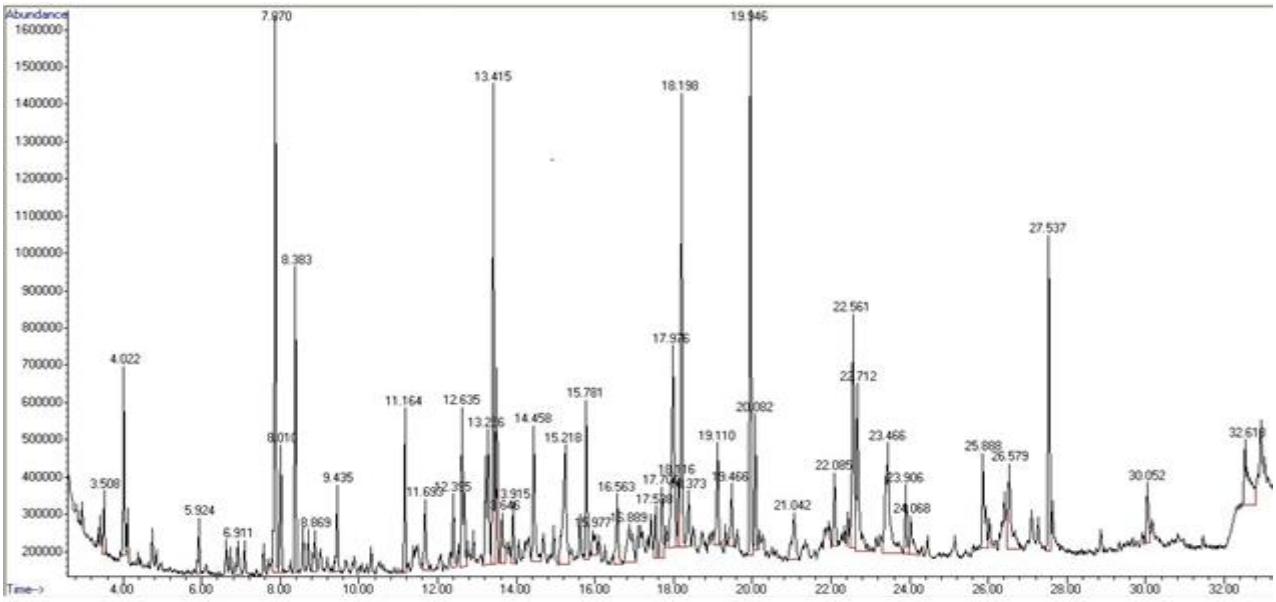

Figure 1. Results of crude oil degradation in the control.

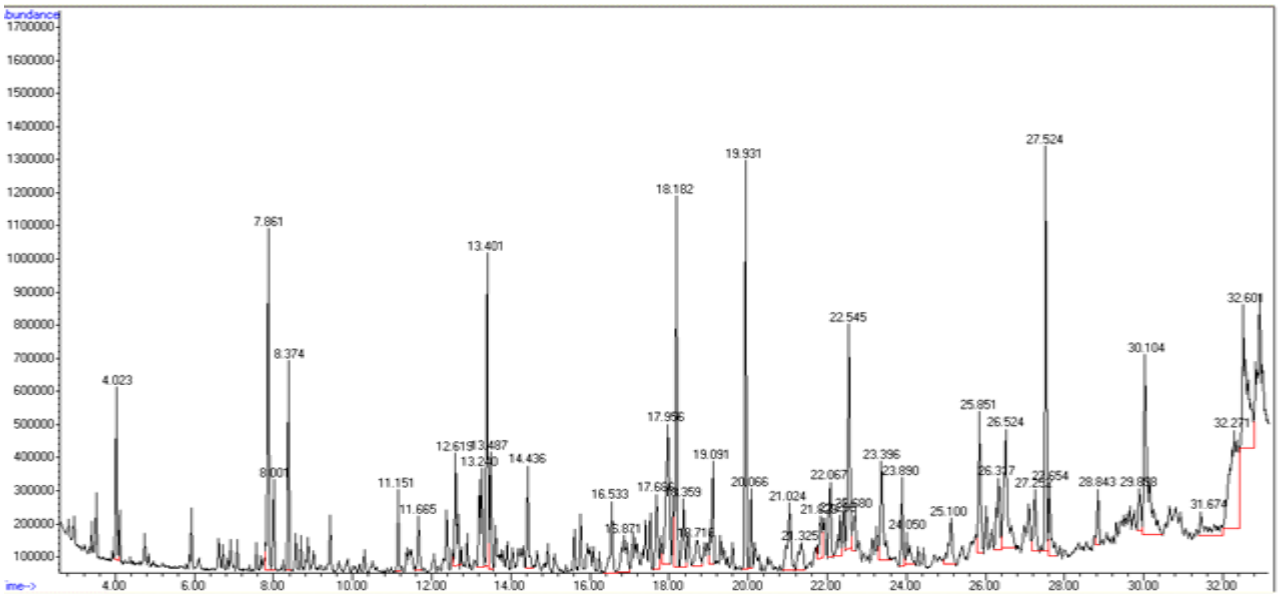

Figure 2. Results of crude oil degradation bacteria 2.

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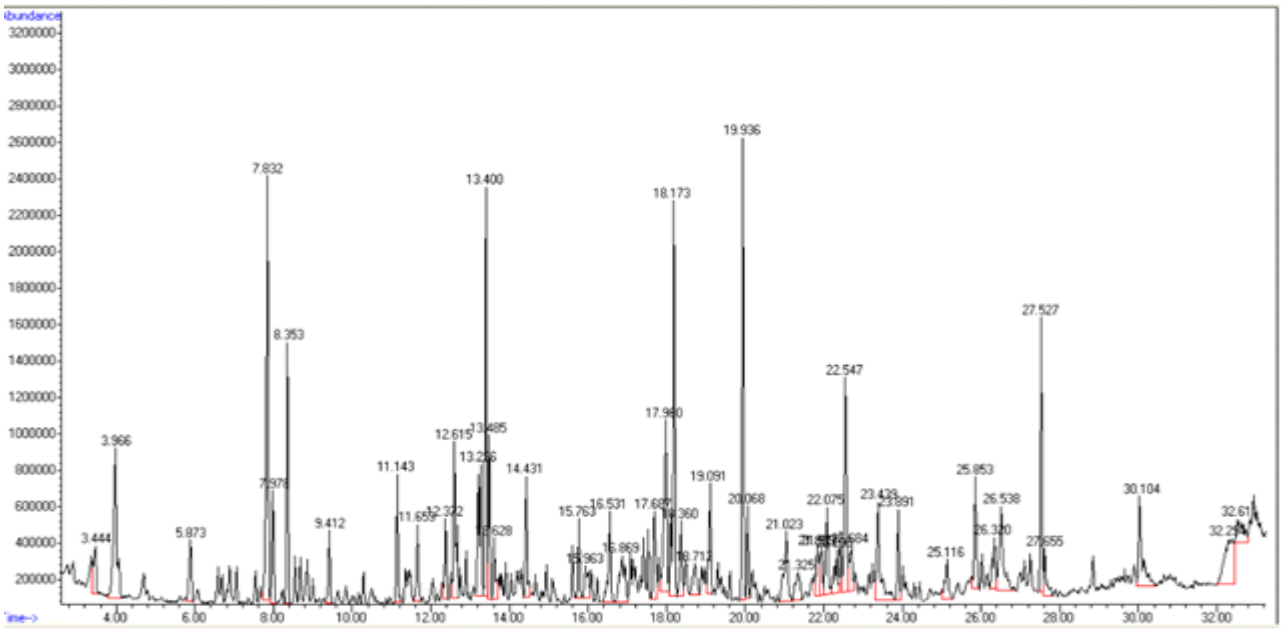

Figure 3. Results of crude oil degradation in the bacteria 7.

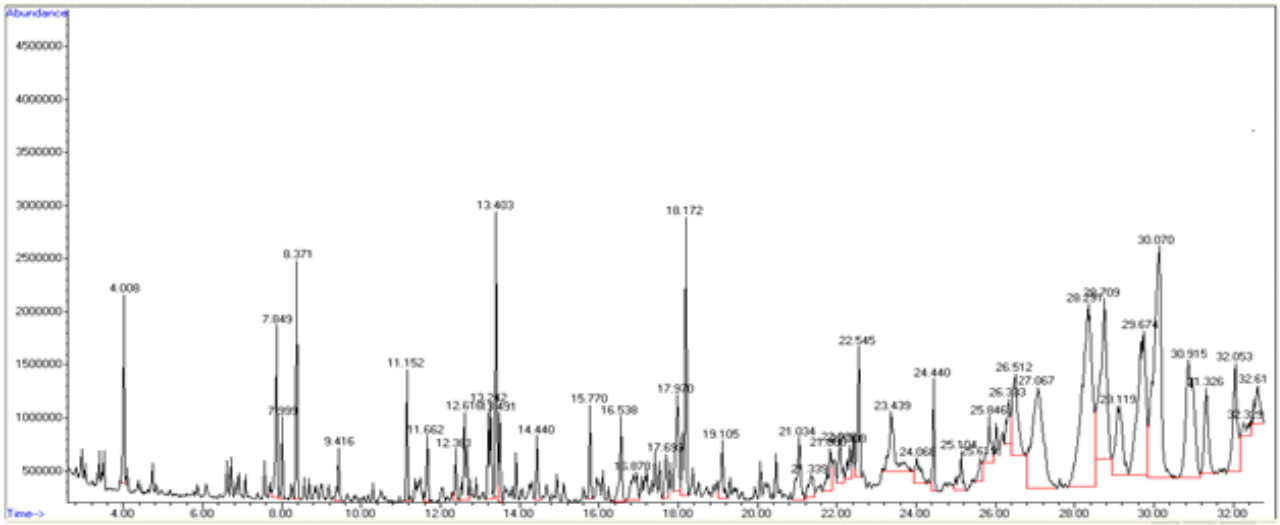

Figure 4. Results of crude oil degradation in the bacteria 9.

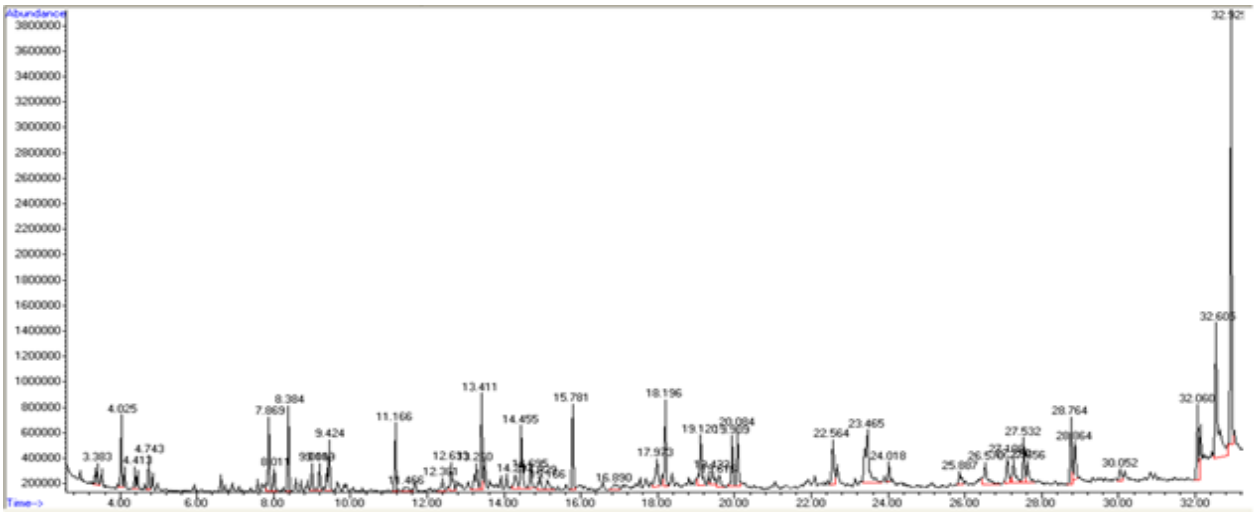

Figure 5. Results of crude oil degradation in the bacteria

Conclusion:

Nine bacterial strains different in their morphological traits and biochemical tests were isolated. They were all similar in their ability to survive in a medium in which carbon was replaced by crude oil, but they were different in their ability to degrade these compounds. The isolates 2, 7 & 9 (*Micrococcus*, *Pseudomonas*, *Bacillus*) were more effective than other isolates separately and they were synergic in their ability to make a higher chromatic change in Bushnell Hans experiment and to show a change in the composition of substances present in crude oil using gas chromatography, where they degraded the highest proportion of substances compared to the control T

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in Damascus University, Syria.

Authors' contributions:

Mohammed Said Al-Shater. and Mohammed Manhal Al Zoubi. conceived of the presented idea. Nabila Kridi developed the theory and performed the computations. Nabila Kridi verified the analytical methods. Mohammed Said Al-Shater encouraged and supervised the findings of this work. All authors discussed the results and contributed to the final m
anuscript

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عزل وتوصيف بعض العزلات البكتيرية من التربة الملوثة بالنفط الخام ودراسة فعاليته

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الخلاصة:
عزلت سلالات بكتيرية من التربة الملوثة بالنفط، في عام 2018، تم توصيف هذه العزلات، وذلك بهدف معرفة قدرة هذه العزلات على معالجة التربة الملوثة بالنفط. تمت قراءة تغير لون الوسط المضاف إليه العزلات بطريقة Pacto Bushnell Hans. كما درس التغيير في طبيعة المواد المكونة للنفط بواسطة كروماتوجرافيا الغاز للعزلات الأكثر فاعلية.
أظهرت العزلات البكتيرية التسعة المعزولة درجات مختلفة من التغير اللوني، وتفوقت العزلات (Bacillus،Pseudomonas ، Micrococcus) على باقي العزلات بكمية تغير اللون حيث بلغت النسبة المئوية للتغير اللوني (78، 78، 77) ٪ على التوالي، مقارنة مع الشاهد، وأظهرت العزلات الثلاثة مجتمعةً أفضل تغيير للون بلغ 90.7 ٪ مقارنة بالشاهد، وأظهرت نتائج فصل المواد عن طريق كروماتوغرافيا الغاز أن هناك تغيرات في طبيعة المواد الموجودة في النفط الخام (الشاهد بدون عزلات) مقارنة بتلك الموجودة في النفط الخام بدون إضافة عزلات. كما أعطت العزلات الثلاث مجتمعة أعلى نسبة تغير في تركيب المواد الداخلة في النفط الخام مقارنة بالعزلات الثلاثة المفردة. مما يؤكد فعالية كل عزلة على حدة على تفكيك النفط الخام وقدرة هذه العزلات مع بعضها(متأزرة) على تفكيك النفط الخام.

الكلمات المفتاحية: البكتيريا، التحلل الحيوي، النفط الخام، الهيدروكربونات، التآزر.