Using Biodegradation to Remove Aliphatic Compounds as Crude Oil Pollutants and Identification by Gas Chromatography

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Abstract

There are many technologies related to clean up various petroleum hydrocarbons. The biological method is one of the efficient and economical methods that depend on the ability of microbes to consume hydrocarbons as sources of carbon. The purpose of this study was to explore and define the efficiency of the fungi *Aspergillus niger* to degrade aliphatic fraction. Two strains of fungal isolation were used, the first one isolated from soil polluted with hydrocarbon for a long time and the other one isolated from soil polluted with hydrocarbon for short time. Soil of Basrah well represents the long-time exposure hydrocarbon-pollutants and soil of Dora refinery represents the short time exposure hydrocarbon-pollutants. The results show that there was a direct correlation between the efficiency of degradation and fungal-time exposure to the hydrocarbon-pollutants. It was shown that the efficiency of fungal degradation of Basrah was more than that the efficiency of fungal degradation of Dora. Another study was carried depended on the comparison of biodegradation of aliphatic alone and within the complex of hydrocarbons (Crude Oil). GC technique was used to evaluate the biodegradation by study of carbon distribution and the fractions abundance.

Keywords: Biodegradation, Crude Oil, Chromatography, and Aliphatic Fractions.

استخدام التحلل الحيوي لإزالة المركبات الاليفاتية للملوثات النفطية وتشخيصها بواسطة كروماتوغرافيا الغاز

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الخلاصـــة

هناك العديد من التقنيات التي تتعلق بإزالة المركبات الهيدروكربونية النفطية، تعد التقنيات البيولوجية واحدة من طرائق المعالجة الكفؤة والاقتصادية والتي تعتمد على قدرة الاحياء المجهرية على استهلاك المركبات الهيدروكربونية كمصدر للكاربون الذي تحتاجه في عملياتها الأيضية. الغرض من هذا البحث هو دراسة كفاءة الفطر *Aspergillus niger* لتحليل المقاطع الاليفاتية فقط عندما يكون هذا الفطر متأقلم في تربة ملوثة بالمركبات الهيدروكربونية لفترة زمنية طويله جدا وتتم مقارنة كفاءته مع فطر متأقلم في تربة ملوثة بالمركبات الهيدروكربونية لفترة زمنية قصيرة. اعتبرت تربة بئر في محافظة البصرة عينة الفطر المتعرض للملوثات الهيدروكربونية لفترة زمنية طويلة اما تربة مصفى الدورة في محافظة بغداد فتمثل عينة للفطر المتعرض للملوثات الهيدروكربونية لفترة زمنية قصيرة. بينت الدراسة العلاقة المباشرة بين كفاءة التحلل البايولوجي وفترة التعرض للملوثات الهيدروكربونية. حيث تزداد قابلية التحلل البايولوجي للفطر المعرض للملوثات الهيدروكربونية المختلفة لفترة اطول (فطر معزول من تربة محافظة البصرة) أكثر من الفطر المعرض للملوثات الهيدروكربونية لفترة قصيرة نسبيا (فطر معزول من تربة الدورة/ محافظة بغداد). تضمن البحث دراسة اخرى تعتمد على مقارنة التحلل الحيوي للمقطع الاليفاتي عندما يكون بمفرده وعندما يكون ضمن تركيبة النفط الخام. اعتمدت تقنية غاز الكروماتوغرافيا في تقييم التحلل الحيوي عن طريق دراسة التوزيع الكربوني ووفرة المقطع الاليفاتي.

الكلمات المفتاحية: التحلل الحيوي، النفط الخام، كروماتوغرافيا الغاز والمركبات الاليفاتية.

Introduction

Crude oil has a complicated mixture of hydrocarbons, which is divided into two types of fractions: firstly molten (soluble) saturate, aromatic, resin NOS (Nitrogen, Oxygen, and Sulfur-containing Compounds) and second type (Insoluble) asphaltene (Jain, *et al*., 2005). Saturate, which also known as alkanes consider as a main fraction of the crude oil according to its source. (Labinger and Bercaw, 2002). There are many techniques used to separate and characterize the crude oil and related materials into fractions dependent -hydrocarbon types (Aske, *et al*., 2001). A very good review is given by (Lundanes and Greibrokk, 1994) as Saturate, Aromatic, Resin and Asphaltene (SARA) is an analysis method to divide the crude oil into four fractions depended on their polarizability and polarity. Saturate consisted of nonpolar molecules, including linear, cyclic, or branched and found in three phases: gaseous (C1–C4), liquid (C5–C16) and solid (>C17). Although highly inflammable, alkanes are less reactive as organic compounds. They are highly essential for modern life, but they pose serious ecological problems when released to the environment (Fan, *et al*., 2002). Nowadays, physical, chemical, and biodegradative methods were used in oil spill removal techniques. Compositional and structural data about crude oil components used to get an effective removal strategy to reduce the damage effect on the environment (Wang, *et al*., 1994). Among many microbes, fungi have been found to have the ability to degrade alkanes and transform them to the substrates that can be easily metabolized. Researchers focus on fungi biodegradation are less comparing to bacteria (Karigar and Roa, 2011). Recently, the essential role of fungi in degrading organic materials in the ecosystem has been studied widely to prove their potential for remediating contaminated soils and water from hydrocarbon. It has been found that degradation varies with the type and size of the hydrocarbon molecule. It can be noticed that short chain alkane (C10-C24) degrades biologically faster than long chain alkanes (Mbachu, *et al*., 2016). In this study, we will compare the degradation efficiency of fungi *Aspergillus niger* which isolated from soil exposed to crude oil for very long period taken from an oil well in Basrah refinery with the other fungi isolated from soil contaminated with crude oil exposed for short time which taken from Dora refinery to prove our hypothesis.

Materials and Methods

Chemicals

Hexane and n-pentane (Both>99% Purity), toluene, chloroform, and methylene chloride (98% Purity) were purchased from Fluka (Steinheim, Germany) methanol (99% Purity) (Only Gain Land Chemical Company) and silica Gel (35-70 Mesh ASTM) Merck. Crude petroleum oil obtained from Basrah and Dora refinery.

Culture Medium of Microorganism

The culture medium used throughout this study consisted of (1g/L) anhydrous potassium hydrogen orthophosphate (K_2HPO_4); (1g/L) anhydrous potassium dihydrogen orthophosphate (KH_2PO_4); (1g/L) anhydrous sodium hydrogen orthophosphate (Na_2HPO4); (1g/L) ammonium nitrate (NH_4NO_3); (0.02g/L) calcium chloride 2-hydrate ($CaCl_2.2H_2O$); (0.2g/L) magnesium sulfate ($MgSO_4.7H_2O$); (0.05 g/L) of iron chloride ($FeCl_3$); (0.1%) Tween 80; 100 mL distal water (Kästner, *et al*., 1994).

Gas Chromatography (GC)

Each fraction was analyzed by GC (Model GC-2014 Shimadzu GC-CHROMATOGHRAPHY), using an FID detector. The column was packed SE 30 (L3m, ID 2mm). The column temperature was 80-250 ºC for 20min rated 5cc/min.

The injector temperature was 260ºC and detector temperature 270ºC. However, asphaltenes fraction has not been analyzed by GC.

Separation of Crude Oil Fractions

Samples of crude oil (Basrah and Dora) were separated into four chemical group classes for each, namely aliphatic, aromatics, resins and asphaltenes. For that, 10 mL of n-pentane was added to 1 gm of each sample of crude oil to dissolve molten fraction aliphatic, aromatic, and resins as soluble fractions and precipitate the asphaltenes as insoluble fraction as shown in Figure (1).

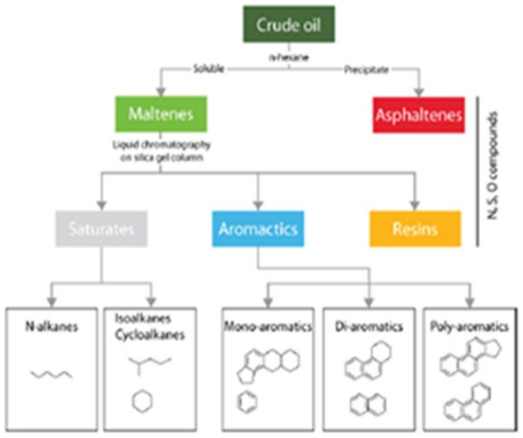

Figure (1) The Routes of Crude Oil Separation.

The soluble fractions for each sample had loaded on the top of 50.0 gm of silica gel column (35-70 mesh) (2cm × 22cm). After 5-10 min, 15mL of n-hexane was added to receive the aliphatic-pure fraction, and then dried at room temperature to produce an oily- yellow product. After that, the sample was dissolved in hexane and injected into (GC) apparatus. To obtain the aromatic fraction, 10mL of a mixture of hexane and toluene (7:3) was added to the same column then waited for 5 min to receive the aromatic fraction. The sample was dried at room temperature and weighted. The third fraction (Resin) was collected by adding 10 mL of a mixture of chloroform and methanol (1:1) on the top of the same column, waited for 5 min to receive the resin fraction then dried at room temperature and weighted. The insoluble fractions for each sample were prepared by washing the precipitation with 10 mL of n-pentane then filtrated by using a filter paper (MN#619eh). The weight of precipitation was measured after drying the sample at ambient temperature for one day. The percentages of all fractions to both samples appear in Table (1) to declare the aliphatic-pure fraction is the major fraction in both samples.

Isolation of Microorganism

The fungi *Aspergillus niger* was isolated from contaminated soil according to (Griffin, 1972). In this work two different types of habitats were available for fungal growth. The first one was related to Basrah refinery well, where a fungus suffers for long exposure time to different hydrocarbon pollutants. Which resulting in the acquisition of appropriate degradative genes. (Salam, *et al*., 2011) The second one was related to Dora refinery where a fungus was exposed to different hydrocarbon pollutants for a short time.

Biodegradation Method

Five Erlenmeyer flasks 250 mL were set up in this order:

1- Flasks 1 contained 100 mL of culture medium contained glucose (As a Source of Carbon) and three agar plugs ($1cm^2$) of *Aspergillus niger* (Control) as illustrated in Figure (2a).

2- Flasks 2 and 3 contained 1 gm of aliphatic-pure and crude oil, respectively, related to Basrah well, then added 100 mL of culture medium (Wentzel, *et al*., 2007) and three agar plugs ($1cm^2$) of Basrah *Aspergillus nger* will be added as shown in Figure (2 b and c).

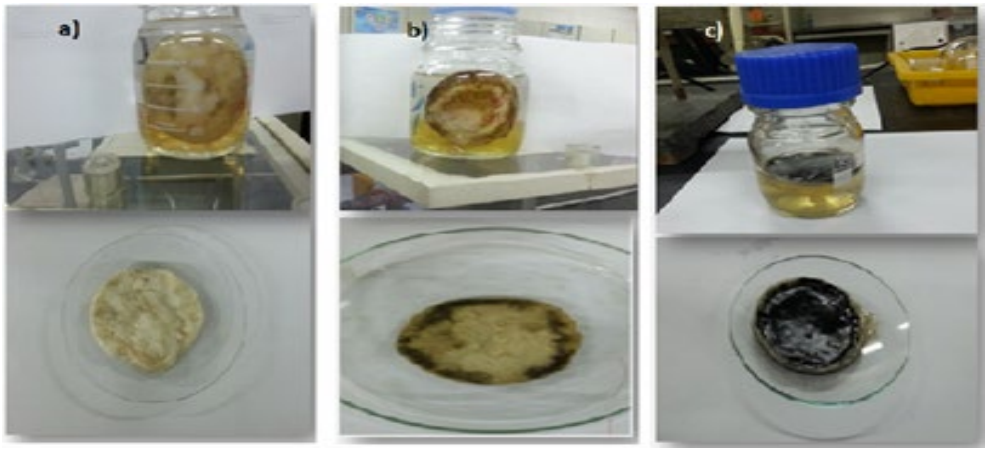

Figure (2) Photos Showing Fungi Grown on A) Control, B) Aliphatic -Pure, and C) Crude Oil.

Table (1) the Weight Percentage of Fractions in Iraqi Crude Oil.

Refinery	Aliphatic%	Aromatic%	NOS%	Asphaltene%
Basrah	58	17	12.8	12.2
Dora	42	25	18	15

3- Flasks 4 and 5: the work in two will be repeated with aliphatic-pure and crude oil of Al-Dora by using Al-Dora *Aspergillus niger*. All the above flasks incubated at (23-28 ºC) for 30 days. From flasks 2-5, aliphatic fraction was extracted by using 25 mL of pentane then loaded on the top of a silica gel column (35-70 mesh) (0.5cm × 10cm). After 5-10 minutes a calculated volume of hexane 10mL was added to receive the aliphatic fraction. The strategy of our work is shown in Figure (3).

Results and Discussions

Figure 4 (a and b) shows GC chromatograms of aliphatic fraction extracted from crude oil of Basrah and Aldora refineries, respectively. This fraction was before any process of biodegradation. For that was represented as a control. The peaks showed smooth and normal distribution punctuated with several high and irregular peak intensities of hydrocarbon molecules having even and odd numbers of carbon atoms. Isoalkanes and cycloalkanes (Isomers Aliphatic) showed low intensity peaks occurring among the vicinity of n- lkanes (Normal Aliphatic). There is a different carbon distribution in the crude between Basrah and Aldora. In Basrah, the carbon number distribution rangeed from C10 to C29. Usually, a carbon number of less than 10 are volatile. The loss of volatile compounds presents a problem during application of solvent evaporation during the process of sample dryness.

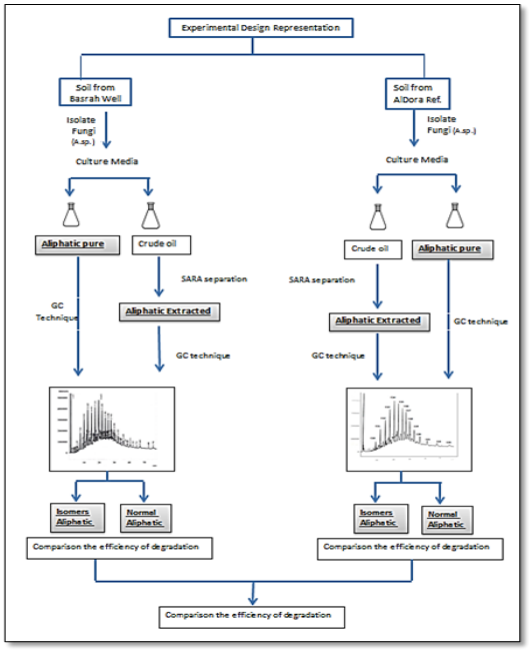

Figure (3) Analytical Flow Chart for Two Strategies of Our Work.

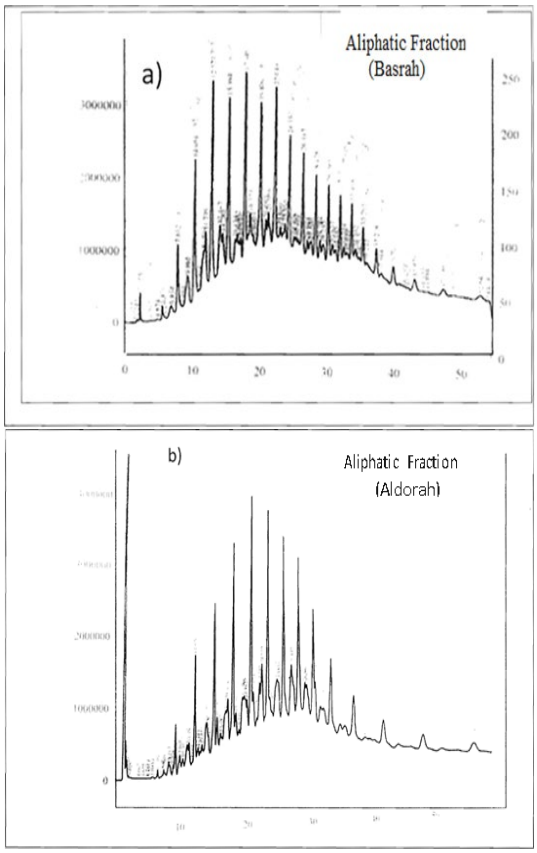

Figure (4) GC Chromatogram of Aliphatic Fraction, (A Basrah Well and B) Dora Refinery (Control)

This can be happened at some point in this process. It can be noticed that the percentage of the normal aliphatic fraction area is 49.569% of the total area, the rest of isomer aliphatic fraction which about 50.432%. While in Dora crude oil, the carbon number distribution ranged from C10 to C23 in which normal aliphatic fraction area is 45.6% of the total area and the isomer aliphatic fraction 54.26%. The percentage of normal and isomer aliphatic fraction was calculated by dividing the area of normal or isomer fraction on the total area of fractions then multiplied by one hundred. These peaks distributions were going to be under the study in the rest of this research after exposing the *Aspergillus niger* to the aliphatic fraction. The data were represented as a percentage of area (Abundance).

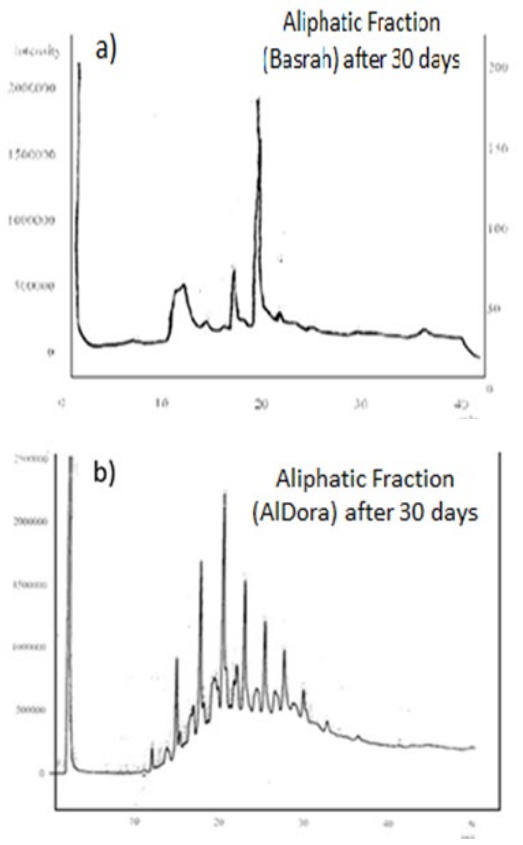

Figure (5) GC Chromatogram of Aliphatic-Pure of (A Basrah Well and B) Aldora Refinery after 30 Days of Fungal Treatment.

Figure 5 (a and b) shows the GC chromatograms of aliphatic fraction that extracted from aliphatic-pure fraction of Basrah and Dora, respectively when treated with *Aspergillus niger* for 30 days. Compared to the control, it there a significant decrease in the C-distribution, number of fractions, and the fraction abundance (Represented Area%) for both normal and isomer fractions after 30 days of fungal treatment as Illustrated in Figures (6 and 7). The efficiency of hydrocarbon degradation is shown in Table (2).

Table (2) The Efficiency of *Aspergillus niger* Degradation of Aliphatic-Pure after 30 Days.

Efficiency	Normal %	Isomer %
Basrah	89.3	99.3
Dora	60	59

The fungal efficiency for hydrocarbon degradation of Basrah was higher than Dora. The fungi isolated from Basrah well were more efficient than the fungi isolated from Dora refinery, and this confirmed our hypothesis. In Figure (6), there were completely removable to the fractions (C10 to C13 and C19 to C29) while other fractions (C15 and C16) have a very low abundance compared with the control. Fraction C14 and C17 have not changed. This might be related to some rearrangement that occurred to longer fractions which led them to transform to lower fractions. However, the isomers were removed completely except C20, which is 4 times lower than its control. In Figure (7), the fraction removal had different mechanisms confirmed that the *Aspergillus niger* isolated from Dora refinery soil was totally had different degradative gene than isolated from Basrah well soil. The other section of our work related to the studying effect of fungal efficiency between the aliphatic fraction when it was alone as an environmental pollutant or within the crude oil. Figure (8) shows the GC chromatograms of aliphatic fraction that extracted from the crude oil of a) Basrah well, and b) of Dora refinery after 30 days of fungal treatment of the crude oil. Herein, *Aspergillus niger* was expose to different type of hydrocarbons that exist within the crude oil. After 30 days, just an aliphatic fraction was extracted to be under the test. However, the polycyclic aromatic hydrocarbons (PAHs) are hard to be degradative unless under hard condition. In Figure (9), a completely different mechanism was appeared resulted in different C-distribution and abundance. Table (3) shows that there is not any fungal efficiency for hydrocarbon degradation in the *Aspergillus niger* isolated from Dora soil. This study was very vital due to its role to prove the strong relationship between the exposure time to the hydrocarbons and the genetic composition of the organism. (Salam, *et al*., 2011). In addition, it shows the importance of choosing the appropriate environments to isolate the organism.

Table (3) Efficiency of *Aspergillus niger* Degradation to Aliphatic Fraction within the Crude Oil after 30 Days.

Efficiency	Normal %	Isomer %
Basrah	90	98
Dora	0	0

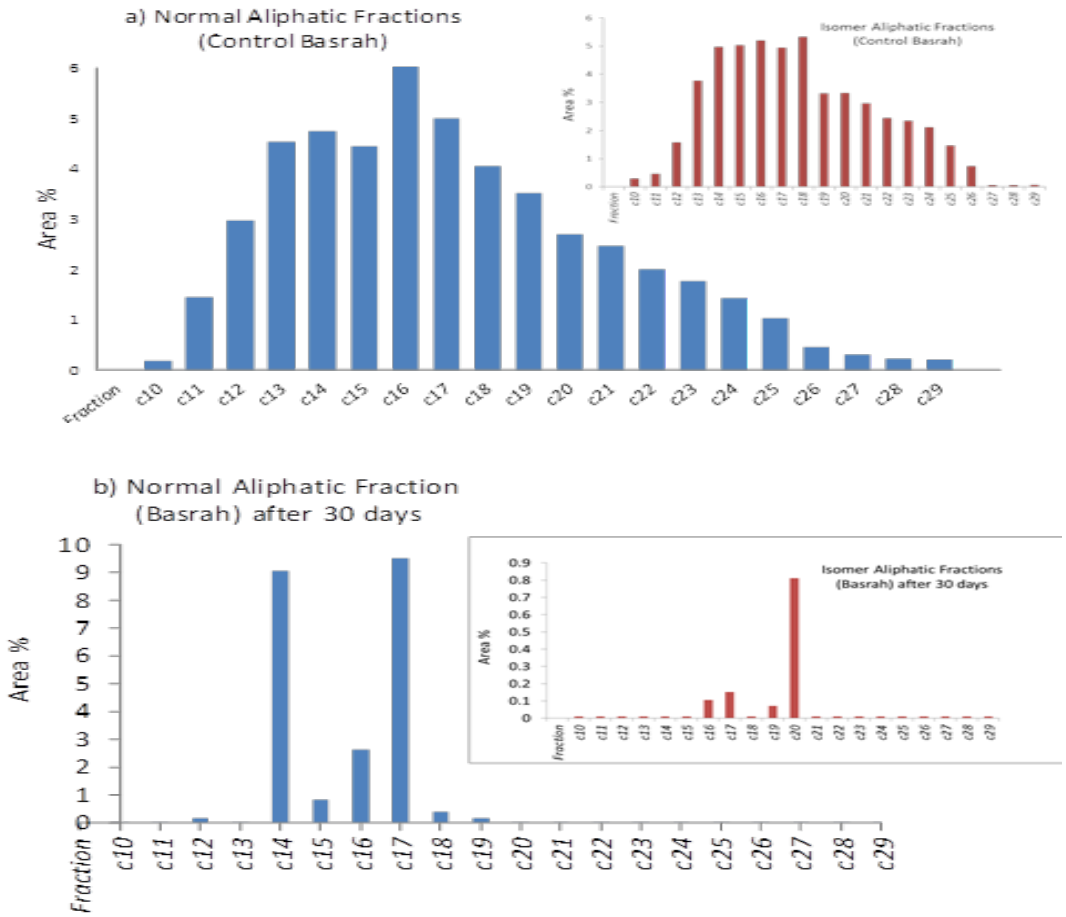

Figure (6) Normal Aliphatic Fraction C-Distribution of Aliphatic-Pure from Basrah Well. The Insets Represents the Isomer Fraction. (A Control, B) after 30 Days of Biodegrade.

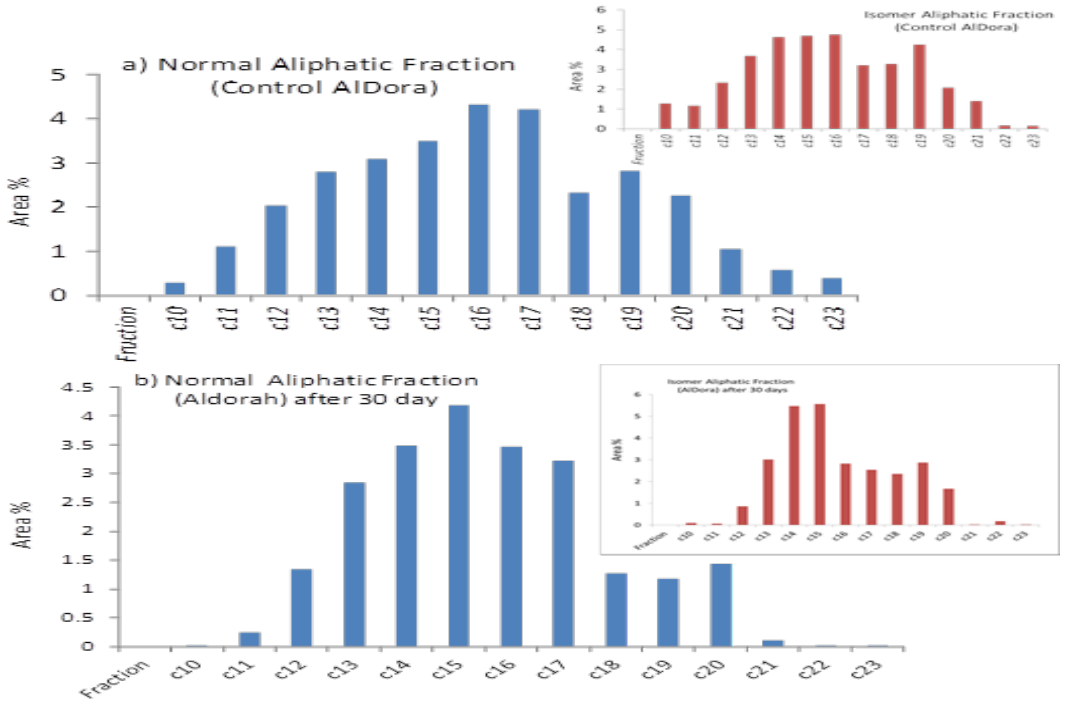

Figure (7) Normal Aliphatic Fraction C-Distribution of Aliphatic-Pure from Dora Refinery. The Insets Represent the Iisomer Fraction. (A Control, b) after 30 Days of Biodegrade.

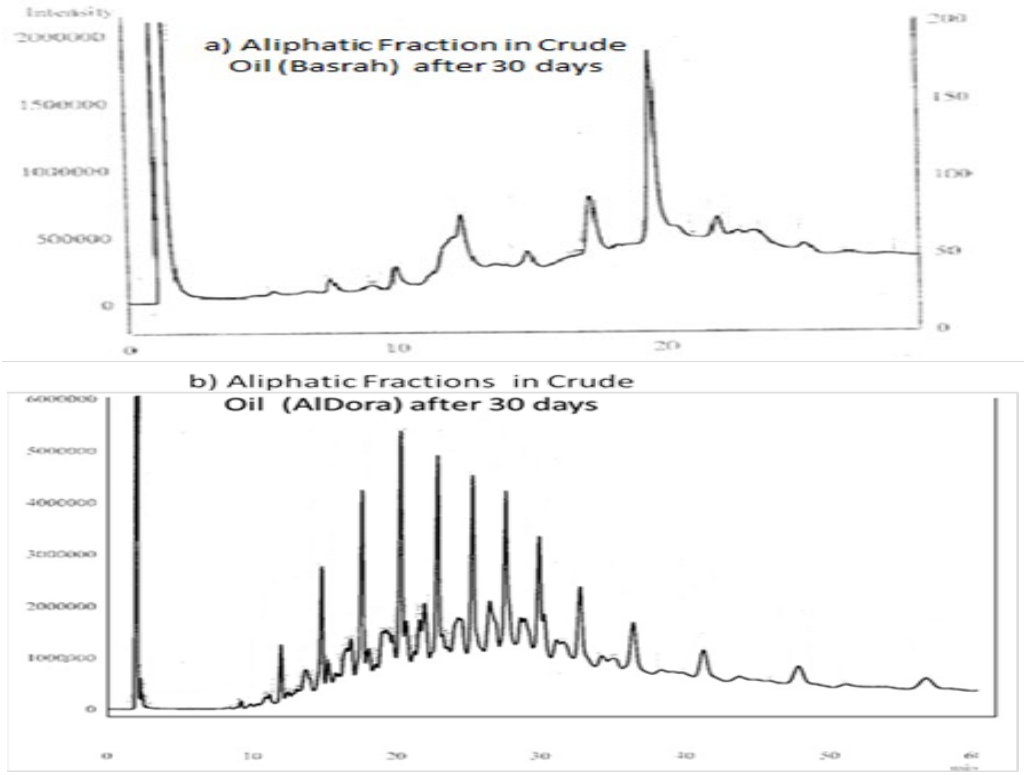

Figure (8) GC Chromatogram of Aliphatic Fraction within the Crude Oil of (A Basrah Well, and B) Dora Refinery after 30 Days of Fungal Treatment.

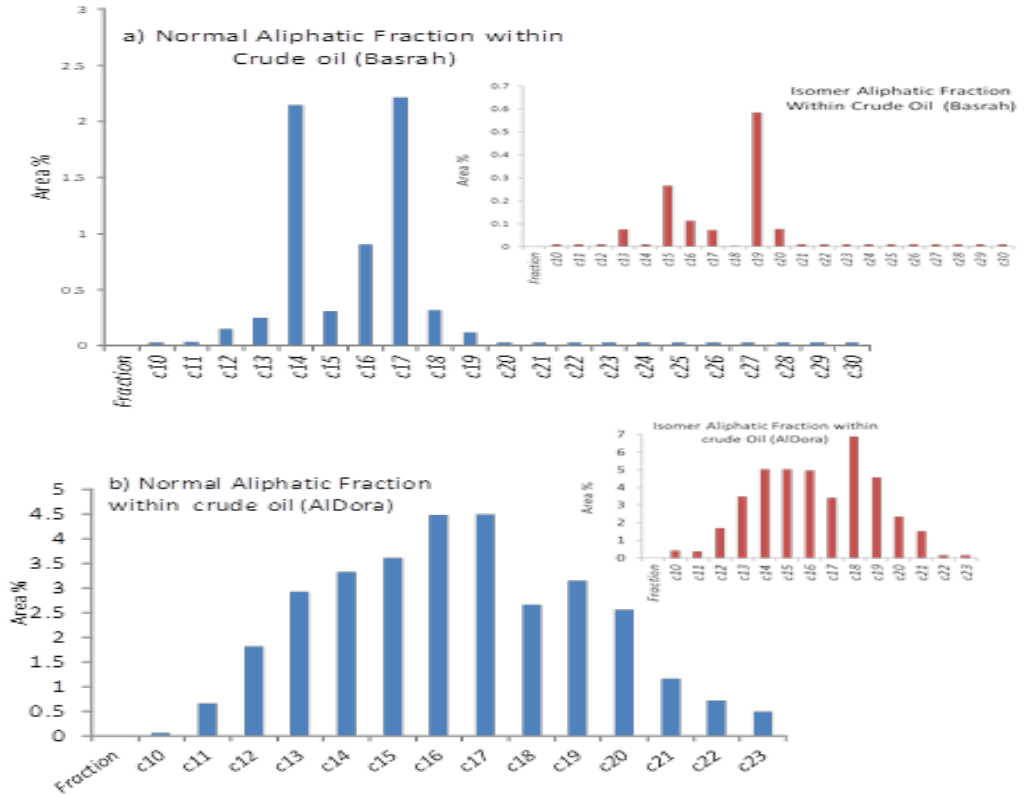

Figure (9) Normal Aliphatic Fraction C-Distribution of Aliphatic Fraction within the Crude Oil. The Insets Represent the Isomer Fraction. (A Basrah Well, B) Dora Refinery after 30 Days of Biodegrdation.

Conclusion

Our research is served in the field of fundamental and applied aspects of petroleum pollutants in the environment and their degradation by microorganisms especially by *Aspergillus niger* biodegradation methods. This work extended to previous works in the same field. Herein, aliphatic fractions degradation was evaluated by applying two strategies, the first one depended on the difference of the exposure time of fungi to the hydrocarbon. While the other strategy depended on exist of aliphatic fraction alone in the environment or within a complex of hydrocarbon (Crude Oil). In the first strategy, a direct correlation between the exposure times of fungi to the hydrocarbon with the efficiency of biodegradation was shown at the same manner, the fungi that have long exposure time kept its efficiency of biodegradation regardless on its existent in the complex hydrocarbon (Crude Oil). GC technique was applied during this study to evaluate and calculate the efficiency of biodegradation. The data obtained in this work advanced our knowledge of petroleum hydrocarbon biodegradation. Resistance in mixed culture of A. *niger* and A. fumigates isolated from soil and may make promising candidates for further investigations regarding their ability to remove petroleum hydrocarbon from contaminated environments.

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