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ENHANCED OIL SLUDGE BIODEGRADATION BY BACTERIAL STRAINS ISOLATED FROM CONTAMINATED SOIL NEARBY POWER STATIONS IN IRAQ

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Abstract

Most lands surrounded electrical power stations in Iraq suffer from a significant pollution with oil sludge contaminated soil (OSCS). A series of experiments were designed to sort out such problem. Surface soil samples were collected from polluted areas surrounding a power station. Bacteria that biodegrade hydrocarbons (Petrophilic bacteria) were isolated from these soils, screened and diagnosed by chemical and biological methods. Three isolates were found *Acinetobacter radioresistens*, *Pseudomonas putida*, *Pseudomonas aeruginosa* which exhibited high degradation ability. For increasing the efficiency of biodegradation, the isolates were subjected to mutagenesis using Acridine Orange (AO). The expected genetic alteration was investigated and found that genes responsible for the biodegradation process are conferred on the chromosome. Hydrocarbons were analyzed using GC-MASS. *A. radioresistens* isolate degraded 9 compounds out of 43 before exposure to mutagenesis, while the same isolate degraded 41 compounds after mutation. The isolate *P. putida* degraded 25 compounds pre-mutation and 28 after mutagenesis. The isolate *P. aeruginosa* degraded 38 compounds out of 43 after mutagenesis. From the above results, it was concluded that the treatment of contaminated soil containing oil sludge by artificially mutated bacteria may be appropriate solution for biodegrading oil derivative spills nearby industrial constructions.

Keywords: Biodegradation, Petrophilic bacteria, OSCS, Oil sludge, Mutagenesis, Acridine Orange.

Introduction

A major part of environmental pollution is mainly attributed to the petroleum industry (Pala *et al.,* 2002). Petroleum contaminated soil causes organic pollution even in the ground water (Wang, 2003). Microorganisms are capable of degrading only a limited number of crude oil depending on the presence of metabolically diverse microbial communities (Atlas, 1981; Okerent and Ezerony, 2003). The success of bioremediation technologies applied to hydrocarbon-polluted environments is highly dependent on the biodegrading capabilities of native microbial populations or exogenous microorganisms used as inoculants (Venosa and Zhu, 2003). Oil biodegradation at the subsurface soil does not require oxygen, but needs certain essential nutrients (e.g., nitrogen, phosphorus, potassium), which can be provided to the water by dissolving minerals. Hydrocarbon biodegradation can occur over a wide range of temperatures that generally decrease the rate of biodegradation at low temperatures.

Highest rates of degradation generally occur in soil with temperature range 20-40ºC. Other factors such as the composition of the microbial population are also complicating the effect of temperature (Zhu and Venosa, 2001). Biodegradation is the main mechanism to decrease biodegradable contaminants. This technique offers low risk to polluted sites and it is an alternative with favorable cost-benefits (Grapes *et al.,* 2002). Some kinds of microorganisms are capable to degrade oil hydrocarbons and can be used as sources for carbon supply. The specificity of the degradation process is associated to the genetic potential of a particular microorganism to introduce hydrocarbons into molecular oxygen and to produce the intermediates that subsequently enter the common energy-yielding metabolic pathway of the cells (Millioli *et al.,* 2009). Random mutagenesis is an incredibly powerful tool for altering the properties of enzymes (Sarin *et al.,* 2008). Previous studies on use acridine orange in mutant, Kermani *et al.* (2010) isolated *P. aeruginosa* which is highly efficient in removing cadmium (heavy metal) up to 7 times after mutagenesis with used acridine orange. Al so Razia *et al.* (2019) improved the biodegradation efficiency in wild type isolates from different contamination sites, namely; *P. putida*, *A. radioresistens* and *A. baumannii* by 44.8, 65.7 and 55.9% respectively by using acridine orange and UV-irradiation. Mutagenesis may alter the genetic makeup of the microorganism at the molecular level towards utilizing the available carbon source accordingly, it is aimed in the current work is to biodegrade the most of hydrocarbons causing soil contamination nearby industrial establishments particularly electrical power stations.

Materials and Methods

Sample collection

Soil samples were collected randomly from oil sludge contaminated soil nearby an electrical power station in Najaf province, Iraq. Samples were collected from contaminated soil starting from the pollution center outwards by dividing the contaminated area to four directions at different periods since 25/11/2018 until 15/2/2019.

Enrichment and isolation

Selective enrichment technique was used for the isolation of hydrocarbon degrading bacteria in soil samples. Bushnell-Hass broth was used in this technique. For detecting bacteria, a quantity of 1 g of soil was inoculated into fifty ml of liquid BHM, dispensed in 250 ml flasks and supplemented with 1% oil sludge as a substrate. Flasks were autoclaved and incubated in a shaker incubator at 150 rpm, 30 ºC for 7 days, 1 ml from the culture was diluted using

sterilized normal saline solution to prepare serial dilutions starting from 10^{-2} to 10^{-5}, then aliquot of 0.1 ml from each dilution was spread on the BHS medium and then plates were incubated at 37 ˚C for 24-48 hrs. Bacterial colonies were selected according to Kumar *et al.* (2006); Gumma, (2007). After incubation, colonies with different morphologies (in shape, size and color) were purified by streaking method on L- agar plates, this method was repeated until pure isolated colonies were obtained and stored on slants at 4 ˚C for further experimental work (Kiyohara *et al.,*1992; Santhin *et al.,* 2009).

Screening of bacterial isolates

(1) Primary screening

Solid BHM was supplemented with 1% of oil sludge to exhibit microorganism's oil sludge degrading ability. The medium was inoculated with a loop full from each isolate in the middle of the agar plate, then incubated at 37 ˚C for 7 days depending on the type of the microorganism. The growth ability of isolates and the diameter of colonies were indicated as strong (+++), moderate (++) and weak (+), and the most active isolates were subjected for further experiments by secondary screening (Santhini *et al.,* 2009).

(2) Secondary screening

It was assessed by two methods:

(A) Formation of clear zone

An ethereal solution of oil sludge (10% v/v) was uniformly sprayed over the surface of solid BHM plates. The ether immediately vaporized and a thin layer of oil remained on the agar surface. Obtained microorganisms were cultured (loop full) by spreading technique over a 1 cm^2 area on the middle of solid medium plates, then the plates were incubated at 37 ˚C for 24 - 144 hrs. The diameter of the clear zone around colonies was measured (Kiyohara *et al*., 1992). Microorganisms which gave maximum diameter were further screened for biodegrading ability by colorimetric method according to Emitiazi *et al.* (2005) then they were divided into three categories indicated as maximum 2.0 - 3.5 cm, Moderate 1.5 - 2.0 cm and weak 1.0 - 1.5 cm.

(B) Redox indicator

Bidoia *et al*. (2010) method using DCPIP as a redox indicator was used. This method depends on adding 2,6-DCPIP to a final concentration of 10 ppm to the medium as an electron acceptor dye to test the ability of isolates to utilize the substrate by observing the color change of the indicator from blue (oxidized) to colorless (reduced). Stored isolates were reactivated for 24 hrs. using Lauria -agar medium. Aliquot of 0.5 ml from each culture was inoculated into test tubes containing 8.5 ml of liquid BHM. All tubes were supplemented with 1% (v/v) of oil sludge and 1 ml of 2,6-DCPIP indicator (Nishanthi *et al*., 2010; Bhuvaneswar *et al*., 2012). Liquid BHM medium contains 1% of oil sludge and 1 ml of 2,6-DCPIP indicator without inoculums were used as controls. The tubes were incubated at 37 ˚C for 24, 48, 72, 96, 120 or 144 hrs. at 150 rpm.

Identification of selected isolates

The diagnosis and identification was made based on the results of the first and second screening. The most active isolates were identified according to the cell morphology, arrangement and Gram stain reaction as described in Bergey's Manual of Determinative Bacteriology (Holt, 1994). Biochemical properties which were tested included, methyl red test, Voges-Proskauer test, indole, gelatin utilization test, starch utilization test, catalase, oxidase, urease producing, citrate utilization, sugars fermentation tests, motility test (Holt, 1994; Santhini *et al.,* 2009). VITEK 2 system identification test Kit was used to confirm identification of the isolates.

Acridine orange as a mutagen

This experiment was carried out to determine the concentrations of Acridine orange (AO) causing a minimum inhibitory concentration (MIC) and the optimal dose required for mutation induction. For this purpose, the strains which were diagnosed and identified were tested with various concentrations of AO. A stock solution of acridine orange (5 mg/25 ml) as a mutagenic agent was prepared. Aliquot of 10 µl was grown overnight and inoculated into test tubes containing variable concentrations of acridine orange (10, 20, 30, 40, 50, 60, 70, 80, 90 or100 µl). These test tubes were incubated at 37 °C for 24 hrs. at 100 rpm and spread over nutrient agar plates and then incubated for 24 hrs. at 37 °C. Observed colonies were randomly selected and tooth picked onto a control (Inyang *et al.,* 2017).

Results and Discussion

Isolation of oil sludge degrading bacteria

In this study soil samples from nearby an electrical power station in Najaf province, Iraq were considered an important source for native bacterial capability to mineralize oil sludge hydrocarbons existed in oil contaminated sites (Sepahi *et al.,* 2008; Teli *et al.,* 2013). Results indicated that 76 bacterial isolates abundant in the southern side represents 40.2% for all site directions, these bacterial populations were randomly collected from contaminated soil starting from the pollution center outwards by dividing the contaminated area to four directions. The number of obtained isolates depended on the direction of the site as shown in Fig. 1.

Results showed that the highest number of isolates was abundant in the southern side with 27 isolates. According, there was considered a good source for bacterial isolates that capable of utilizing oil sludge.

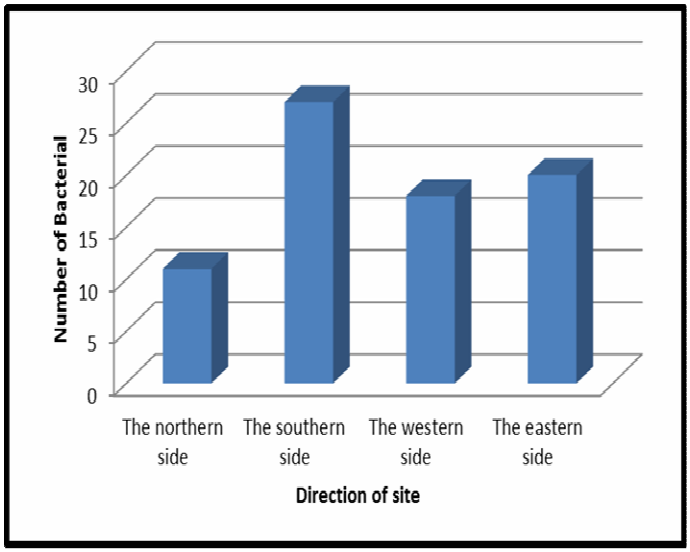

Fig. 1 : Places and numbers of isolates found in soil contaminated samples at different zones surrounded Al-Haydaraia electric power station contaminated with oil sludge.

The results of the present study are in agreement with the finding of previous studies (Gumaa, 2007; Sadoon, 2009) who reported the presence of different colony types of microorganisms that recovered on agar plates. There was an indication that longer contamination time resulted in a greater number of microorganisms. Phenotypic examination of the recovered bacteria revealed that they belong mainly to the genus *Pseudomonas* spp., *Enterobacter* spp. and *Acinetobacter* spp. Gumaa (2007) isolated 102 bacterial isolates from different soil samples contaminated with crude oil and other petroleum derivatives. Three of them were found to have good ability to utilize crude oil and hydrocarbon residues.

Primary screening

Sixty-one isolates out of 76 exhibited the highest ability for oil sludge biodegradation (Table 1). The diameter of colonies recorded 1-1.8 cm in 3 isolates while recorded 0.5-0.9 cm in 31 isolates and a diameter of <0.4 cm was represented in 27 isolates. Different efficiencies for degradation depended on the ability of isolated microorganisms in degrading hydrocarbons. Similar observation was reported by Santhini *et al.* (2009).

Table 1 : Growth of bacterial isolates expressed as a diameter of colonies showing their ability to degrade oil sludge.

Growth status	Diameter of colonies cm	Number of isolated bacterial
Strong	1-1.8	3
Moderate	0.5-0.9	31
Weak	<0.4	27

The efficiency of isolates to grow and utilize crude oil was evaluated by their growth capability on solid BHM supplemented with 1% of crude oil (Panda *et al*., 2013). The isolates which grow on the agar plates were affirmed as hydrocarbon degraders (Afuwale and Modi, 2012). The solid BHM plates supplemented with crude oil as a sole carbon source were utilized to examine the growth potential of purified bacterial isolates (Emitiazi *et al.,* 2005).

Secondary screening

A. Formation of a clear zone

Oil sludge degradation efficiency was recorded after clear zone formation on the growth medium. Results displayed in table **2** illustrated that three isolates out of **61** bacterial isolates gave maximum diameter than other ones located 2 in southern site and 1 in western site, 31 isolates were considered moderate which distributed in all directions and 27 isolates exhibited weak distribution in all directions as well. Three isolates formed maximum diameter zone on solid BHM medium, which were selected for further screening.

Table 2: Secondary screening for the most active 61 bacterial isolates after primary screening by formation of clear zone around the colonies on a selective medium.

No.	Isolate symbol	Sampling site	Biodegradation ability of isolates		
1	N-1	The northern		Moderate	
2	N-1	The northern			Weak
3	N-1	The northern		Moderate	
4	N-1	The northern		Moderate	
5	N-1	The northern			Weak
6	N-1	The northern		Moderate	
7	N-1	The northern			Weak
8	N-1	The northern			Weak
9	N-1	The northern			Weak
10	S-2	The southern		Moderate	
11	S-2	The southern	Maximum		
12	S-2	The southern		Moderate	
13	S-2	The southern			Weak
14	S-2	The southern		Moderate	
15	S-2	The southern		Moderate	
16	S-2	The southern	Maximum		
17	S-2	The southern			Weak
18	S-2	The southern			Weak
19	S-2	The southern		Moderate	
20	S-2	The southern			Weak
21	S-2	The southern			Weak
22	W-3	The western			Weak
23	W-3	The western		Moderate	
24	W-3	The western		Moderate	
25	W-3	The western		Moderate	
26	W-3	The western			Weak

27	W-3	The western			Weak
28	W-3	The western			Weak
29	W-3	The western	Maximum		
30	W-3	The western		Moderate	
31	W-3	The western			Weak
32	W-3	The western			Weak
33	W-3	The western			Weak
34	W-3	The western		Moderate	
35	W-3	The western		Moderate	
36	W-3	The western			Weak
37	W-3	The western			Weak
38	E-4	The eastern		Moderate	
39	E-4	The eastern		Moderate	
40	E-4	The eastern			Weak
41	E-4	The eastern		Moderate	
42	E-4	The eastern		Moderate	Weak
43	E-4	The eastern			Weak
44	E-4	The eastern		Moderate	
45	E-4	The eastern		Moderate	
46	E-4	The eastern			Weak
47	E-4	The eastern		Moderate	
48	E-4	The eastern			Weak
49	E-4	The eastern		Moderate	
50	E-4	The eastern		Moderate	
51	E-4	The eastern			Weak
52	E-4	The eastern		Moderate	
53	E-4	The eastern			Weak
54	E-4	The eastern		Moderate	
55	E-4	The eastern		Moderate	
56	E-4	The eastern		Moderate	
57	E-4	The eastern			Weak
58	E-4	The eastern			Weak
59	E-4	The eastern			Weak
60	E-4	The eastern		Moderate	
61	E-4	The eastern		Moderate	

Microbial growth and clearing zones were recognized on the solid medium around the microbial colonies as a proof of the ability to survive on crude oil. These results are in agreement with those obtained by Latha and Kalaivani (2012); John and Okpokwasili (2012) who reported that the largest clearing zone around bacterial isolates grown on a mineral salt medium was observed after 2 days indicating their fuel waste degradation capability.

B. Redox indicator

Results shown in table. 3 indicate that the same three isolates S-2(11), S-2(29), W-3(29) (Table 2) that nominated in the previous experiment exhibited the greatest ability to completely discolor the indicator within 24 hr, while the other 31 and 27 isolates discolored the indicator after a longer period of time extended from 48 to 144 hr. indicating a slow response to biological oxidation, and hence slow biodegradability. A rapid screening technique using a redox indicator 2, 6- DCPIP was used for further screening. The highest ability of the isolate to decolorize a liquid BH-medium with blue DCPIP indicator into colorless liquid was observed during a time period from 24 to 144 hr. as shown in the table 3.

Table 3 : Secondary screening for the most active isolates in their biodegradability of oil sludge in liquid BH-medium incubated at 37 ˚C for different periods.

Isolates biodegradability of oil sludge using redox indicator (2,6-DCPIP)								
No.	**Isolate symbol**	**Sampling site**	**Decolonization time (hr.)**					
			24	**48**	**72**	**96**	**120**	**144**
1	N-1	The northern		+				
2	N-1	The northern					+	
3	N-1	The northern						+
4	N-1	The northern					+	
5	N-1	The northern			+			

6	N-1	The northern			+			
7	N-1	The northern						+
8	N-1	The northern					+	
9	N-1	The northern		+				
10	S-2	The southern					+	
11	S-2	The southern	+					
12	S-2	The southern		+				
13	S-2	The southern		+				
14	S-2	The southern		+				
15	S-2	The southern			+			
16	S-2	The southern	+					
17	S-2	The southern		+				
18	S-2	The southern			+			
19	S-2	The southern					+	
20	S-2	The southern		+				
21	S-2	The southern				+		
22	W-3	The western		+				
23	W-3	The western				+		
24	W-3	The western						+
25	W-3	The western					+	
26	W-3	The western						+
27	W-3	The western					+	
28	W-3	The western			+			
29	W-3	The western	+					
30	W-3	The western				+		
31	W-3	The western						+
32	W-3	The western			+			
33	E-4	The eastern				+		
34	E-4	The eastern					+	
35	E-4	The eastern				+		
36	E-4	The eastern			+			
37	E-4	The eastern				+		
38	E-4	The eastern					+	
39	E-4	The eastern						+
40	E-4	The eastern						+
41	E-4	The eastern						+
42	E-4	The eastern		+				
43	E-4	The eastern		+				
44	E-4	The eastern			+			
45	E-4	The eastern				+		
46	E-4	The eastern						+
47	E-4	The eastern				+		
48	E-4	The eastern				+		
49	E-4	The eastern						+
50	E-4	The eastern					+	
51	E-4	The eastern				+		
52	E-4	The eastern			+			
53	E-4	The eastern				+		
54	E-4	The eastern					+	
55	E-4	The eastern						+
56	E-4	The eastern						+
57	E-4	The eastern						+
58	E-4	The eastern		+				
59	E-4	The eastern		+				
60	E-4	The eastern			+			
61	E-4	The eastern				+		

Identification of selected isolates

Morphological and microscopic examination of the three isolates, which were selected according to their ability to degrade oil sludge, showed that the colonies of the relevant isolates were smooth, convex mucoid color except one of them was pale yellow. The Gram stain was Gram-negative, and their shape differs since two of them were rod-shaped, while the other one was coccoid. Biochemical tests suggested that the three isolates S-2(11), S-2(16) and W-

3(29) were closely related to *Pseudomonas putida*, *Pseudomonas aeruginosa* and *Acinetobacter radioresistens* respectively according to Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994).

Acridine orange as a mutagen

Numbers of colonies survived the mutagenesis were as follows; strain *P. putida* reached 4 colonies at the concentrations 10, 20 ug/ml, while at 20 and 30 ug/ml reached 3 colonies then reduced to 1 colony at 40 ug/ml, as for *P. aeruginosa* reached 5 colonies at 10 ug/ml, and at 30 ug/ml recorded 3 colonies while at 40 ug/ml appeared 1 colony and *A. radioresistens* strain recorded 3 colonies at concentrations 10, 20 ug/ml, while at 30 ug/ml reached 1 colony. All colonies grew well.

Detection of hydrocarbon compounds in liquid medium

This experiment was carried out to identify the ability of each single bacterial isolate before and after mutagenesis for biodegradation by using GC-mass analysis. Figure 2 explains hydrocarbon fractions in oil sludge extracted from control flask. The number of hydrocarbon fractions and hydrocarbon compounds were identified from their mass spectra, 43 compounds were obtained depending on their retention time.

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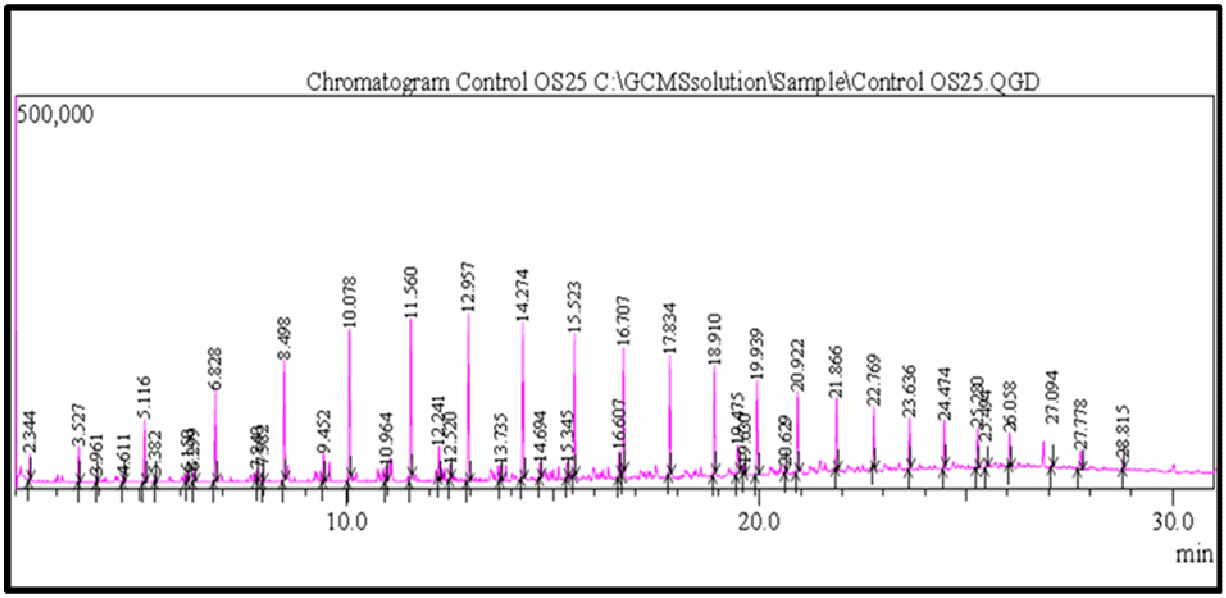
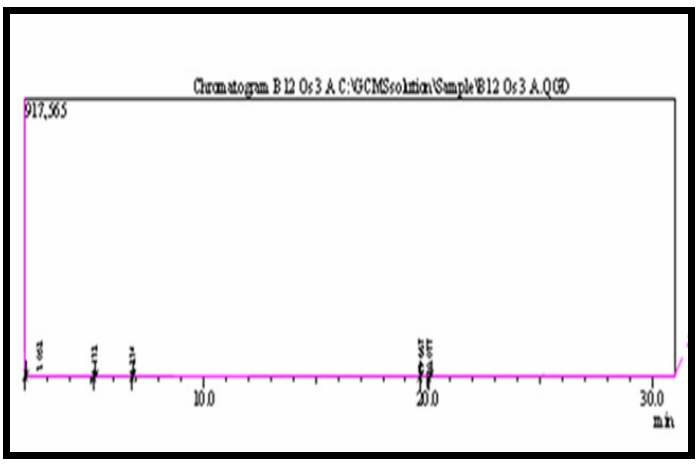


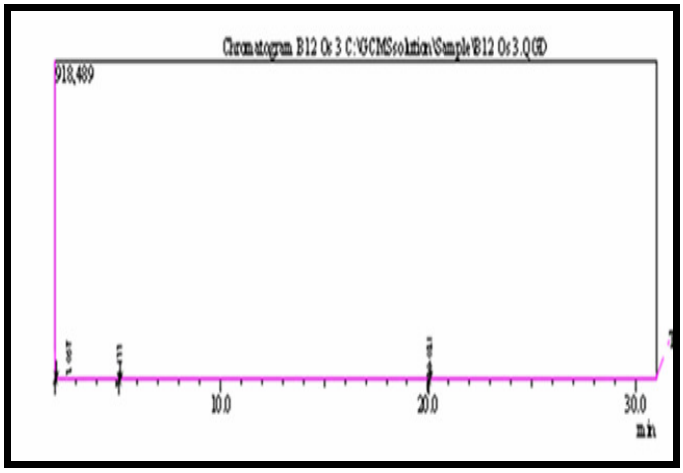
-A-

Fig. 2 : GC-mass chromatogram of oil sludge extracted from liquid BHM without inoculum (control) after 6 days of incubation at 37 °C and 150 rpm.

GC-mass chromatogram for oil sludge degradation by *P. aeruginosa*, is shown in Figure 3 indicating that there were changes in the number of peaks (hydrocarbon compounds). Peaks reduced in the chromatogram from 43 compounds to 5 before mutation and 3 remained after mutation in the biodegraded oil sludge compared with the control. The obtained results also showed that the oil sludge was affected by *P. aeruginosa* during the incubation period, because some of the hydrocarbon fractions that were detected in the control were not detected in the treated samples and the sum of peaks area for hydrocarbon fractions decreased during the incubation period.



-B-



-BM-

Fig. 3 : GC-mass chromatogram of oil sludge extracted from liquid BHM treated with *P. aeruginosa* after 6 days of incubation which found in soil contaminated samples at different zones surrounded contaminated site. Where (B) before mutation, (BM) after mutation.

The same pattern was detected in the samples where *P. putide* grew (figure 4) since that the oil sludge was affected by *P. putida* during the incubation period by changing the number of peaks (compounds). Peaks remarkably reduced in the chromatogram from 43 compounds to 18 before mutation and 15 remained after mutation in the biodegraded oil sludge compared with the control. Some of the hydrocarbon fractions that were detected in the control were not detected in the sample and there was a reduction in other hydrocarbon fractions. At the same time, there was an appearance of new peaks, indicating the production of new intermediates during the biodegradation process, which might be due to the destruction of some long and complex carbon chains which were broken into simpler types.

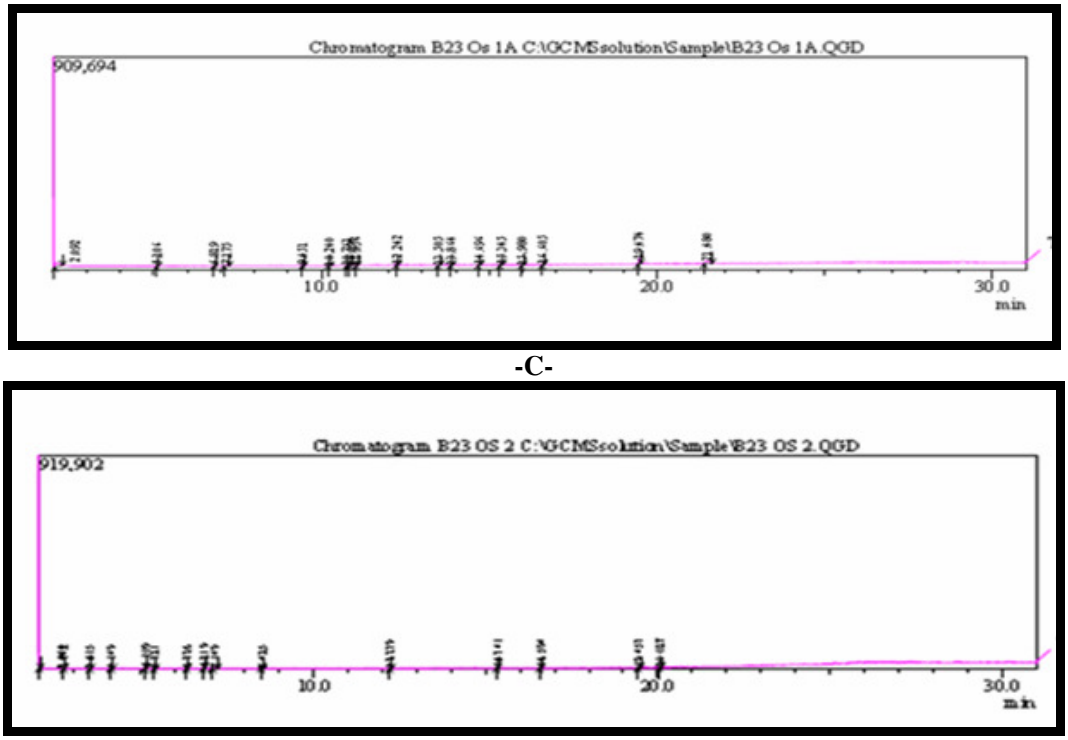


Fig. 4 : GC-mass chromatogram of oil sludge extracted from liquid BHM treated with *P. putida* after 6 days of incubation which found in soil contaminated samples at different zones surrounded contaminated site. Where (C) before mutation, (CM) after mutation.

Acinetobacter radioresistens exhibited a remarkable effect on oil sludge biodegradation compared with *P. aeruginosa* and *P. putida* isolates (figure 5) which resulted in higher biodegrading efficiency after mutation, because most of the observed hydrocarbon fractions were either changed or disappeared. Also, the results indicated that there were changes in the number and length of the hydrocarbon fraction peaks and number of peaks. Peaks remarkably reduced as shown in the chromatograms from 43 compounds to **34** before mutation and **2**remained after mutation of the biodegraded oil sludge compared with the control.

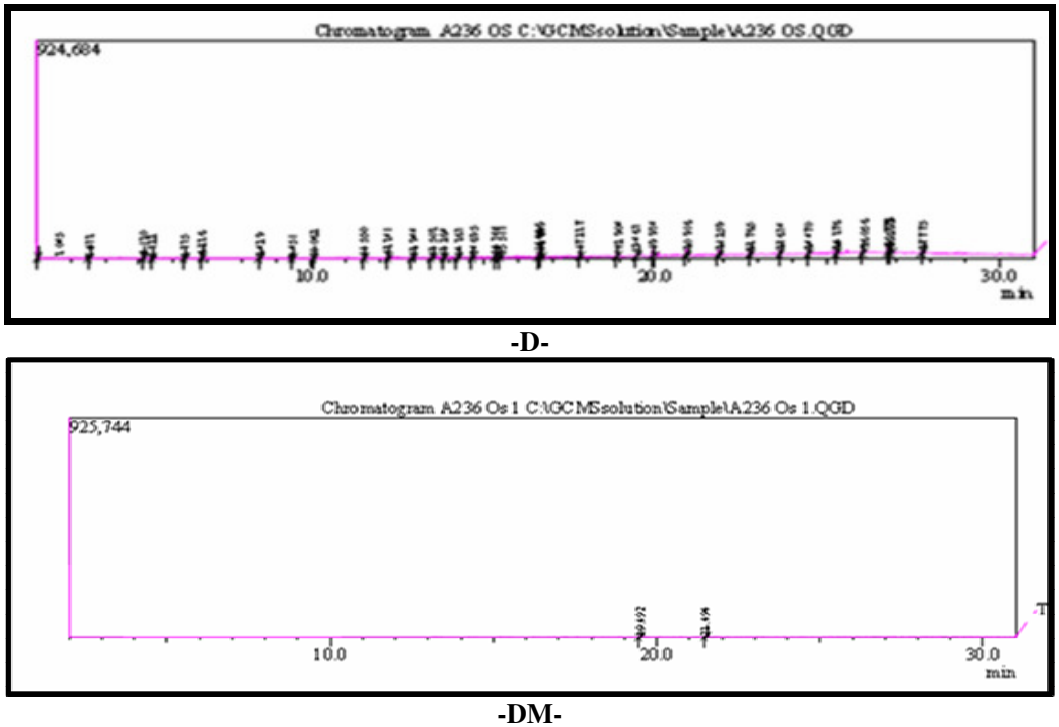


Fig. 5 : GC-mass chromatogram of oil sludge extracted from liquid BHM treated with *A. radioresistens* after 6 days of incubation which found in soil contaminated samples at different zones surrounded contaminated site. Where (D) before mutation, (DM) after mutation.

Gomes *et al*. (2010) reported that low biodegradability may be correlated with the structure of hydrocarbon compounds, most types of bacteria more easily oxidize compounds with lower molecular weight. Similarly, Latha and Kalaivani (2012) revealed that *Acinetobacter* spp. and *P. aeruginosa* isolated from crude oil contaminated sites showed maximum growth $257x10^{-6}$ cfu/ml and $248x10^{-3}$cfu/ml respectively and degradation on seventh day of incubation. These results demonstrate the biodegradation potential of these isolates. Ekpo and Udofia (2008) reported that *P. aeruginosa* and *P. putida* isolated from oil sludge showed different abilities in breaking down and utilization of crude oil when measured using GC analysis. The highest % of crude oil biodegradation was 97.2% by *P. aeruginosa*, then 82.3% by *P. putida* after **1**4 days of incubation at 37 °C. Shakibaie *et al.* (2008) mentioned in their studies that mutating *P. aeruginosa* and *P. putida* by the chemical mutagenic acridine orang enhanced the efficiency of the biodegradation more than 3 folds for zinc and cadmium in industrial waste water.

Conclusions

Contaminated soil with hydrocarbons considered as a good source for the isolation of oil sludge degrading bacteria which can then be used for bioremediation and the data presented in this paper clearly indicate that chemical mutagenesis with acridine orange enhanced the bacterial ability to biodegrade hydrocarbons and thus offers the possibility of rehabilitating the derelict lands surrounded and nearby electrical power stations so that can be planted with trees or other plants.

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