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Aromatic Hydrocarbons : Degrading Bacteria in the Desert Soil of Kuwait

البكتيريا المحللة للمركبات الهيدروكربونية الأروماتية في التربة الصحراوية بالكويت

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Abstract: Soil samples of different levels of oil pollutants were collected from Kuwait's Burgan Oil Field, near an oil lake. The samples represented, highly polluted (8.0% w/w), moderately polluted (2.1%-3.4%) and slightly polluted (0.5-0.8%). The aromatic fractions of the collected samples were in the range of (0.21-2.57g/100g) soil. (GC) analysis of the aromatic fractions for the resolution of the different individual (PAHs) revealed the presence of (16) different (PAHs) resolved from the aromatic fraction of the highly polluted sample (S3). (15), (14) and (13) individual (PAHs) were identified from soil samples (S5), (S2) and (S1, S4, S6) respectively. The most frequent (PAH) was indeno (1, 2, 3-c,d) pyrene (22.5%-45.11%) followed chrysene (13.6%-19.48%). Eight carcinogenic (PAHs) were resolved from the aromatic fractions of the polluted samples. Total carcinogenic (PAHs) recorded in this study were in the range of (11.53) (for S4) – (510.98) (for S3) ppm. The counts of (CFU) of aromatic degraders (AD) were in the range of (3×10^3) - (110×10^3) (CFU/g) soil (with a percent of (2.2%-69.6%)). The results show that, higher counts of (AD) were recorded from the highly polluted sample (S3), followed by the moderately polluted samples; total of (51) bacteria, that gave presumptive positive biodegradation activities, were isolated and identified. (45.1%) of them were isolated from the highly polluted sample (S3). Total of (13) different species were identified of which *Micrococcus luteus* was more frequent (23.5) followed by *Bacillus licheniformis* (19.6%) and *Bacillus subtilis* (11.8%). The three *Pseudomonas* species collectively were represented by (11.8%). Five different species proved to be of good activities, they are: *Bacillus brevis, Bacillus licheniformis, Pseudomonas aeruginosa, Pseudomonas stutzeri and Pseudomonas flourescens.* The ability of the five species and their mixture was investigated. The three *Pseudomonas spp* were able to degrade more (PAHs) than the *Bacillus spp.* On the other hand the mixed culture showed high biodegradation activities (88.6%) as compared to the pure individual cultures.

Key words: Kuwait, polluted desert soil, aromatic hydrocarbons, biodegradation, Bacteria.

المستخلص: جمعت عينات مختلفة من تربة صحراء الكويت الملوثة بدرجات متفاوتة بالنفط من منطقة حقل برقان القريبة من البحيرات النفطية ، وتمثل العينات التي جمعت ؛ عينات عالية التلوث (8 %) وعينات متوسطة التلوث (2.1% - 3.4 %) وعينات قليلة التلوث (0.5% - 0.8 %) من وزن التربة . وتم تحليل هذه العينات للتعرف على محتواها من المركبات الهيدروكربونية العطرية (الأروماتية) بواسطة كروماتوجرافيا الغاز ، ودلت النتائج على وجود ستة عشر مركباً من المركبات الأروماتية في العينة ذات التلوث العالي و خمسة عشر مركباً من المركبات الأروماتية في العينة متوسطة التلوث ، بينما وجد في العينة قليلة التلوث 14 مركباً فقط . وكانت المركبات الأروماتية الأكثر تواجداً هي: إندينو *indino* (d,c -3,2,1) بيرين *pyrene* (22.5%-45.11%)، يليه كريسيسين *chrysene* (13.6% -19.48%) وقد أمكن التعرف على ثمانية من المركبات الهيدروكربونية المسرطنة تراوحت كميتها بين (11.53 - 510,58) جزء من المليون في عينات التربة الملوثة رقمي (S4) و (S3) على التوالي. وقد تم التوصل إلى أن عدد الوحدات المكونة للمستعمرات (CFU) من البكتيريا المحللة للمركبات الهيدروكربونية الأروماتية المسرطنة تتراوح بين (3×10^3) في الجرام الواحد من التربة الملوثة ، كما دلت النتائج إلى أن التربة الأكثر تلوثاً تحتوي على عدد أكبر من الكائنات المحللة للمركبات الهيدروكربونية الأروماتية . وتم تعريف احدى وخمسين عزلة تتبع ثلاثة عشر نوعاً من البكتيريا ذات الاحتمالية الإيجابية للنشاط التحليلي ؛ (45.1%) منها عزلت من التربة الأعلى تلوثاً ، وتمثلت هذه المعزولات في *Micrococcus leuteus* (23.5%)، *Bacillus licheniformis* (19.6%)، و *Bacillus subtilis* (11.8%) ، وثلاثة أنواع من الجنس *Pseudomonas spp* (11.8%)، وكانت هناك خمسة أنواع أخرى جيدة النشاط تم دراسة قابليتها للتحليل فرادى أو مجتمعة مع بعض وثبت أن ثلاثة أنواع من *Pseudomonas spp* لديها قابلية على تحليل المركبات الهيدروكربونية بدرجة أكبر من *Bacillus spp* ، بينما أعطت المزرعة الخليط كفاءة عالية للتحلل البيولوجي (88.6 %) بالمقارنة بالمزارع المفردة.

كلمات مدخلية: الكويت، التربة الصحراوية الملوثة، المركبات الهيدروكربونية الأروماتية، التحلل البيولوجي، البكتيريا

Introduction

Aromatics, especially those possessing polyaromatic rings are recalcitrant to biodegradation. Some petroleum aromatic hydrocarbons (PAHs) are considered as hazardous, because of their mutagenic and carcinogenic activities (Kalf *et al*, 1997); (Saltiene *et al*, 2002). Significant amount of (PAHs) are contained in crude oil. Accidental spillage and improper disposal of these materials has given the rise to the contamination of soil and other sites. These contaminated sites are of concern, because of

the harmful effects of these hydrocarbons.

As a result of the Gulf oil spill caused by Iraqi forces in 1991, more than (60) million gallons of crude oil were released from the destroyed oil wells to the desert soil in Kuwait, forming more than (330) oil lakes covering an area of about (49 km^2) (Al-Awadi *et al*, 1995; Salam, 1995). Most of the soil has been pumped by the efforts of Kuwait Oil Company (KOC) leaving the lake beds heavily contaminated. Although several treatment methods, for example, (Al-Gounaim *et al,* 1992, Al-Awadi *et al*, 1996; Cho *et al*, 1997 a,b, Al-Gounaim and Diab, 1998 (a,b); Balba *et al*, 1998; Yateem *et al*, 1998; Yateem *et al*, 2000); Al-Gounaim and Diab, 2002), Diab and Al-Gounaim, 2003); Al-Gounaim *et al*, 2004), have been considered for the remediation of this polluted soils, large quantities remain unremediated in the desert of Kuwait.

This contaminated desert soil is of concern since some of the petroleum (PAHs) especially the higher condensed compounds with four or more rings and their metabolites have a variety of mutagenic and carcinogenic effects and are classified as compounds with significant human health (Kalf *et al*, 1997). Therefore, removal of such pollutants become of particular concern for the environmental protection in the State of Kuwait.

One of the most promising methods of removal of (PAHs) from contaminated environments is through biodegradation process. Microorganisms can totally degrade or partially transform (PAHs) through the action of individual microbes or inter-dependent communities (Cerniglia and Heitkamp, 1989). However, complete mineralization of high molecular weight (PAHs) can be achieved by only limited number of microorganisms (Cerniglia, 1992).

Cho *et al* (1997b) studied the biodegradation of oil pollutants in the desert soil of Kuwait, they reported that the slow rate of biodegradation of the aromatics in this type of soil was, due to the low population of the aromatic-decomposing micro-organisms. Ran and Obbard (2004) reported that microbial degradation of (PAHs) during the process of bioremediation can constrained by lack of nutrients, low bioavailability of the contaminants or scarcity of PAH-degrading microorganisms.

Hence, the need to develop practical bioremediation strategies through which PAH-degrading microorganisms are to be enriched in the polluted site is of importance.

The above review indicates that, no detailed studies were carried out on PAH-degrading bacteria in the polluted desert soil in Kuwait. Accordingly, in the present work enumeration, isolation and identification of PAH-degrading bacteria were attempted with a view on their PAH-biodegradiation activities.

Materials and Methods

(1) Collection of Soil Samples

Samples with previous history of pollution were collected from different sites of an oil lake lies at Burgan oil field, South of Kuwait City (Fig.1).

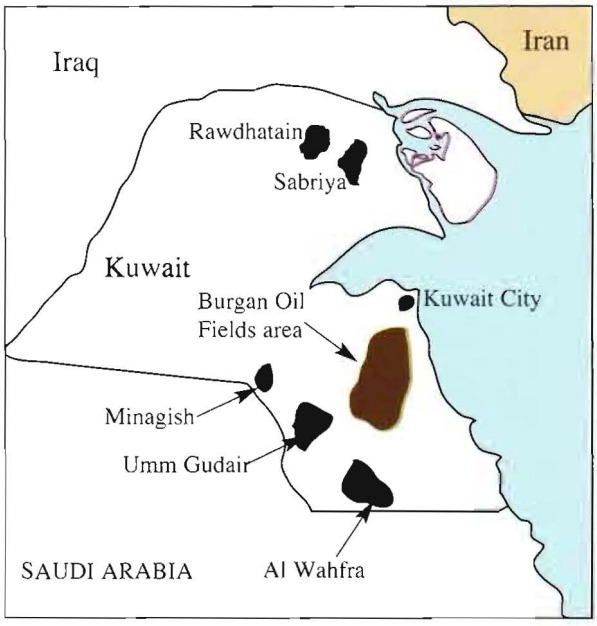

Figure (1) Major oil fields of Kuwait of which Burgan oil fields (from which the samples were collected) are indicated.

(2) Determination of the residual oil content of the collected samples.

The method of (Chaineau *et al* 1996) was used as follows:

- (16-gram) portion of each sample was mixed by a sufficient amount of anhydrous (Na_2SO_4) to remove moisture.
- The residual oil in the sample was Soxhlet extracted with chloroform for (8-19h).
- The extract was evaporated in a reweighed beaker, and the residual content was determined as (%w/w).

(3) Determination of the saturates, aromatics, resins and asphaltene fractions of each sample.

This was carried out according to the methods described by (Oudot 1984) and (Chaineau *et al* 1995) as follow:

- A known weight of the residual material was suspended in (n-hexane) and filtered through a weighed filter paper (Wattman No.1) to remove the non-soluble fraction asphaltene.
- The soluble fraction was fractionated by

liquid-solid chromatography on (100-200) mesh activated silica gell into saturates, aromatics and resins.
- The amount of each fraction was determined.
- The aromatic fraction was further resolved by (GC) analysis.

(4) (GC) analysis of the aromatic fraction for the resolution of the polycyclie aromatic hydrocarbons (PAHs).

Identification and quantification of the individual polycyclic aromatic hydrocarbons were determined using Chrompack (CP 9001) gas chromatograph equipped with (CP 9050) liquid sampler, and configured with (FID), using helium (Grade 6) as a carrier gas, with a flow rate of (1mL/min). A (CP) Sil (19CB) column (25m long x 0.32mm diameter x (0.2 um) thickness for the stationary phase) was used. Temperature programming of initial holding at (40°C) (2min.), and then heating with a rate of (10°C/min) to (295°C) (holding 12 min.) was applied. The total time of analysis was (45) minutes. Injector and detector temperatures were (250°C) and (280°C) respectively. Injection volume was (1μL) for all samples. The quantification of (PAHs) was based on application of reference standard materials (199 ppm for each), obtained from Supelco Co. Samples were run in duplicates, and the mean value was taken.

(5) Enumeration of Polycyclic Aromatic Hydrocarbons degrading microorganisms.

- Enumeration of (PAH-degraders) using the spray method

  Serials of dilutions were made from the soil samples. A (1-ml) portion of each dilution was introduced into a sterile Petri dish. A known volume of the mineral medium agar was poured into each dish. The plates were gently rotated to mix the sample with the medium. After solidification, the plates were sprayed with the (PAHs) mixture. All plates were incubated at (30°C) for a period of (25-30) days, after which the developed colonies surrounded by clear zones were counted and expressed as (CFU/g) soil. (Deziel *et al*, 1996).
- Enumeration of (PAHs-degraders) using the (MPN) method

  The simple (3) tubes (MPN) method that was described by Chaieneau *et al* (1996) was used as follows:
  - (F ml.) of the medium, (Chaineau *et al*, 1996)) was introduced into a test tube.
  - After sterilization (0.05 ml) of the aromatic fraction (in hexane) was added to each tube.
  - Serial dilutions of each sample were prepared.
  - From each dilution, three tubes each was inoculated by (1-ml) portion.
  - The inoculated tubes were incubated at (30°C) for a period of 21 days.

  Tubes in which the blue color of resazurin was changed to pink, or colorless, were considered as positive tubes. The (MPN) values were obtained from the (MPN) index of the three tubes as found in standard methods for the examination of water and waste water.

(6) Detection of the ability of the isolated bacteria to degrade (PAHs).

Based on the method described by, Deziel *et al* (1996) biodegradation of (PAHs) was detected as follows:
- The isolated bacteria were plated on the mineral medium agar supplemented with (0.1 gram) yeast extract per liter and 1.0 gram glucose/L.
- The plates were sprayed with the aromatic fraction dissolved in pentane.
- The plates were incubated at (30°C) for a period of (21) days.
- Presumptive PAH-degraders were distinguished by formation of a clearing zone or coloration around the colonies.
- Sprayed plate experiments were performed in duplicates.
- The results were expressed as

(++) = good activity.
(+) = moderate activity and
(±) = weak activity.

Colonies showing good activities were isolated, purified and identified.

(7) Identification of the isolated bactria

Identification of the pure bacterial cultures showing good presumptive activities was carried out using (BBL Crystal, Gram Positive ID Kit and Gram negative ID Kit (Becton Dickinson UK Ltd, GB-Oxford OX4 3LY).

(8) Selection, of the most active PAH-degrading bacteria, and evaluation of their biodegradation capacity.

- Bacterial suspension of each purified bacterial culture was prepared, as described by White *et al* (1998).
- (One ml) of each bacterial suspension (10^8 cells) was inoculated to (150ml) conical flask, containing (25ml) of sterilized culture medium (Chaineau *et al*, 1996), and over laid with (100mg) of the purified sterilized aromatic fraction of the crude oil.
- The flasks were incubated at (30°C) for a period of (21d) on a rotary shaker, operated at (100rpm).
- Activity was indicated by measuring the absorption due to optical density of the turbidity and/ or emulsification of the hydrocarbon in the liquid phase of the culture at (450nm).
- Culture showing values of (1-10) in the absorption scale were selected for their quantitative biodegradation activities.

The hydrocarbon in the liquid culture was extracted by chloroform. The chloroform extract was evaporated to dryness, and then the residue was suspended in benzene and was reduced to (1ml) under stream of nitrogen. (1µl) of this extract was used for (GC) analysis by the method described before.

Results and Discussion

In this study, (6) desert soil samples with previous history of pollution were collected from different sites of an oil lake lies at Burgan Oil field, South of Kuwait City (See. fig.1). The results of hydrocarbon contents of each sample are found in Table (1). It can be seen from these results that one sample (S3) was heavily polluted with oil (8.0% w/w), three samples (S1, S2, S5) were moderately polluted (2.1-3.4% w/w) and two samples (S4, S6) were slightly polluted (0.5 and 0.8% w/w). sample (S7) was a control nonpolluted sample.

When the residual pollutants of each sample was fractionated (Table 1), it was found that the aromatic fraction, as compared to the other fractions, was of higher percentage in all samples (S1-S6). With increasing the oil pollutants, this fraction increased from 0.21g/100g in S4 to 2.57g/100g (in S3).

The same trend of results was observed for the levels of the saturated fraction in these soil samples. It was reported by other investigators that hydrocarbon concentrations in the environment vary widely, depending on the proximity of the contaminated site to the production source, the level of industrial development and the mode(s) of hydrocarbon transport (Kanaly and Harayma, 2000).

Residual (PAHs) contents of the polluted samples were quantified by (GC-FID) analysis. (16 PAHs) were resolved from the aromatic fraction of S3, (Table 2) of which indeno (1,2,3-c,d) pyrene was more frequent (45.11%), than the other (PAHs), this was followed by chrysene (19.48%). The above two compounds are considered among the carcinogenic (PAHs) (Saltiene *et al*, 2002).

From sample (S5), 15 PAH compounds were resolved, of which indeno (1,2,3- c,d) pyrene was more frequent (30.84%). This was followed by flouranthene (19.8%) and benzo(b) flouranthene (11.58%). On the other hand, (14 PAHs) were resolved from the aromatic fraction of (S2), of which indeno (1,2,3- c,d) pyrene was represented by a high amount of (22.79%) of the total (PAHs). Chrysene and benzo(ghi) perylene followed this compound (18.12%) and (17.57%) respectively). In this type of soil pyrene is represented by (14.05%). Nearly the same trend of results was observed in (PAHs) content of (S1). The above results show that indeno (1,2,3-c,d) pyrene and chrysene were more frequent in most of the studied soil samples, this was followed by benzo (a) flouranthene. All of the above (PAHs) were of the carcinogenic compounds (Saltein *et al*, 2002; Knopp *et al*, 2000).

As for the nonpolluted sample (S7), 7 PAH compounds only were resolved of which flourene

Table (1) Residual oil and its fractions in the different soil samples.

Oil and its Fractions	Residue (% g/100 soil)						
	S1	S2	S3	S4	S5	S6	S7
Oil	2.8 ± 0.3	3.4 ± 0.6	8.0 ± 0.4	0.5 ± 0.01	2.1 ± 0.2	0.8 ± 0.02	0.07 ± 0.01
Saturates	0.68 ± 0.09	0.82 ± 0.09	2.16 ± 0.42	0.18 ± 0.01	0.7 ± 0.01	0.28 ± 0.03	0.02 ± 0.01
Aromatics	0.70 ± 0.04	1.10 ± 0.06	2.57 ± 0.10	0.21 ± 0.003	0.8 ± 0.14	0.31 ± 0.5	0.03 ± 0.00
Resin	0.46 ± 0.03	0.47 ± 0.01	1.23 ± 0.04	0.03 ± 0.00	0.15 ± 0.05	0.04 ± 0.001	-
Asphaltene	0.63 ± 0.04	0.73 ± 0.04	1.80 ± 0.01	0.01 ± 0.00	0.30 ± 0.04	0.06 ± 0.001	-

Table (2) (GC) analysis of residual (PAHs) of each soil sample.

(PAHs)	S1		S2		S3		S4		S5		S6		S7	
	Ppm	%	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
1- Naphthalene	-	-	-	-	1.29	0.22	-	-	0.38	0.74	-	-	-	-
2- Acenaphthylene	-	-	-	-	3.76	0.66	-	-	-	-	-	-	-	-
3- Acenaphthene	-	-	0.46	0.19	2.42	0.42	0.73	4.4	0.40	0.77	0.53	1.67	-	-
4- Flourene	0.48	1.63	0.96	0.40	2.06	0.36	0.52	3.1	1.16	2.25	10.51	33.08	11.10	79.90
5- Phenanthrene	1.13	3.85	2.46	1.01	23.69	4.18	0.97	5.9	2.89	5.61	0.26	0.82	0.38	2.72
6- Anthracene	1.61	5.48	1.00	0.41	9.03	1.60	1.54	8.8	0.84	1.63	0.37	1.16	0.33	2.36
7- Flouranthene	0.75	2.55	21.38	8.83	5.41	0.96	0.34	2.0	10.20	19.80	2.47	7.77	0.54	3.86
8- Pyrene	0.67	2.28	34.02	14.05	30.19	5.33	0.66	4.0	0.65	1.26	7.62	23.98	0.38	2.72
9- Benzo(a)anthracene	0.81	2.75	2.48	1.02	35.95	6.35	0.80	4.8	0.35	0.68	0.51	1.61	0.63	4.51
10- Chrysene	4.00	13.62	43.87	18.12	112.30	19.48	2.47	15.10	2.03	3.94	1.04	3.27	0.62	4.40
11- Benzo(b)flouranthene	3.09	10.52	27.09	11.18	18.41	3.25	2.03	12.4	5.76	11.18	0.97	2.86	-	-
12- Benzo(k)flouranthene	0.74	2.52	4.93	2.04	4.86	0.86	1.01	6.17	5.25	10.19	0.75	2.36	-	-
13- Benzo(a)pyrene	3.10	10.55	2.83	1.17	44.24	7.82	0.78	4.77	2.65	5.14	1.83	5.76	-	-
14- Dibenzo(a,h)anthracene	0.89	3.03	2.93	1.21	9.16	1.62	1.62	9.90	0.94	1.82	3.00	9.44	-	-
15- Benzo(ghi)perylene	3.42	11.64	42.56	17.57	7.92	1.40	-	-	2.13	4.10	-	-	-	-
16- Indeno(1,2,3-c,d)pyrene	8.68	29.56	55.19	22.79	255.32	45.11	2.89	17.70	15.89	30.84	1.91	6.01	-	-
Total	29.37		242.16		566.01		16.36		51.52		31.77		13.88	

was the dominant (79.4%). It was found also that, naphthalene and acenaphthylene were absent from most of the studied soil samples, while benzo (ghi) perylene was absent from (S4) and (S6).

Results of total (PAHs) content show that the highly polluted sample (S3) contained the highest amount (566.01 ppm) as compared to (16.36 – 242.16 ppm) in the other samples. (Table 2). The non-polluted sample (S7), contained (1.88ppm) as a total (PAH). Some investigators e.g. Jones *et al*, 1989; Otte *et al*, 1994; Potter *et al*, 1999) indicated that, soil and sediments (PAH) concentration at contaminated and uncontaminated sites ranging from 1mg/kg, to over 300 g/kg.

The total carcinogenic (PAHs) recorded in this study (Table 3) were in the range of (11.53 – 510.90 ppm). Their percentages on the basis of total (PAHs) were 60.34% (for S6) – 90.28% (for S3). The more frequent (PAHs) are (in descending order) indeno (1,2,3-c,d) pyrene (10 – 50%), flouranthene (1.06 – 26), chrysene (5.42 – 23.11%), pyrene (1.69 – 39.75%); benzo(b)flouranthene (3.6-17.6%); dibenzo(ah)anthracene and benzo(a)pyrene (1.49-15.89%) and benzo(a) anthrecene.

Knopp *et al* (2000) explained that the four-ringed (PAH) chrysene and dibenzo(ah)anthrecene and the six-ringed (PAH) indeno (1,2,3-c,d) pyrene are considered by the International Agency for Research on Cancer (IARC) as carcinogenic compounds. Flouranthene was reported as the most abundant (PAH) in environmental samples was found to be cytotoxic, mutagenic and potentially carcinogenic

Table (3) Carcinogenic (PAH) compounds resolved from the aromatic fractions of the polluted desert soil in Kuwait.

(PAHs)	S1		S2		S3		S4		S5		S6	
	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%	ppm	%
1- Fluranthene	0.75	3.75	21.38	11.26	5.41	1.06	0.34	2.95	10.20	26.50	2.47	12.88
2- Pyrene	0.67	3.35	34.02	17.92	30.19	5.91	0.60	5.55	0.65	1.69	7.62	39.75
3- Benzo(a)anthracene	0.81	4.10	2.48	1.31	35.95	7.03	0.80	7.28	0.35	0.91	0.51	3.15
4- Chrysene	4.0	20.00	43.87	23.11	112.30	22.0	2.47	21.42	2.03	2.28	1.04	5.42
5- Benzo(b)fluranthene	3.09	15.46	27.09	14.27	18.4	3.60	2.03	17.60	5.76	14.97	0.97	5.06
6- Benzo(A)pyrene	3.10	15.45	2.83	1.49	44.24	8.66	0.78	6.76	2.65	6.89	1.83	9.56
7- Dibenzo(a,h)anthracene	0.89	4.45	2.93	1.54	9.16	1.79	1.62	14.05	0.94	2.44	3.00	15.65
8- Indeno(1,2,3-c,d)pyrene	6.68	33.40	15.19	29.08	255.32	49.96	2.89	25.06	15.89	41.30	1.91	9.96
Total	19.99		189.79		510.98		11.53		38.47		19.17	
%	65.95		78.37		90.28		70.48		74.67		60.34	

Table (4) Mean counts of colony forming units (CFU) of total bacteria (TB) and aromatic-degraders (AD) per gram of soil samples (S1 – S7) by using the mean probable number (MPN) and the spray (SP) methods. Percentages of (AD) are given. ± = Standard deviation.

Soil samples	Total Bacteria 10^3		Aromatic-degraders 10^3		(%) AD	
	MPN	Plate counts	MPN	SP	MPN	SP
S1	24.0 ± 1.4	15.3 ± 0.6	9.5 ± 0.5	6.3 ± 0.3	39.6	40.9
S2	88.0 ± 0.0	50.2 ± 4.3	30.0 ± 1.2	18.0 ± 1.0	34.1	35.8
S3	158.0 ± 8.4	102.3 ± 7.2	110.0 ± 6.4	60.7 ± 4.6	69.6	59.9
S4	138.2 ± 10.4	95.4 ± 4.6	3.0 ± 0.1	1.9 ± 0.2	2.2	2.0
S5	169.1 ± 16.4	88.0 ± 6.2	75.0 ± 3.8	30.1 ± 4.2	44.3	34.2
S6	57.7 ± 3.6	38.7 ± 2.2	16.0 ± 1.2	8.2 ± 3.3	27.7	21.2
S7	1020.4 ± 68.4	845.6 ± 50.1	5.0 ± 0.1	2.1 ± 0.4	0.5	0.2

(Rice *et al* 1982; West *et al*, 1986; Irvin and Martin, 1987); McElroy *et al* 1991). This compound is considered more of a potential health hazard by virtue of its abundancy than the widely studied but less abundant carcinogen benzo(a)- pyrene (Sakia *et al*, 1985; Babson *et al*, 1986; Mersh-Sunderman *et al* 1992).

Seven of the (8) compounds (except flouranthene) resolved in the present study were considered by the USA, Environmental Protection Agency (EPA), to probable human Carcinogenic (PAHs). (Saltein *et al* 2002).

The above results show that the polluted desert soil of Kuwait are of concern because of the harmful effects of their (PAHs) contents. For this reason we focused on the enumeration and identification of (PAH)-degrading bacteria, with an attempt to detect their biodegradation characteristics, in order to apply these bacteria in the future for cleaning the polluted sites by bioaugemtation process.

Results of the counts of colony forming units (CFU) of (PAH-degraders) per one gram soil, are found in (Table 4). It can be seen from these results that the mean probable number (MPN) methods gave higher (CFU) than the spray method. By using (MPN) method (CFU) counts were in the range of $3x10^3$ (for S4) – $110x0^3$ (for S3) - (10^3 CFU/g) soil, this is in contrast to (2.24 - 65.65 x 10^3 CFU/g), when the spray method was used. A particular notable distinction between the (MPN) and the spray method is the easily isolation of PAH-degraders when the later method was used. These results show that the highly polluted soil (S3) was rich in (PAH-degraders) followed by the moderately polluted ones.

In this study a total of (51) bacteria presumptively PAH-degraders were isolated when the spray method was used, they were purified and identified (Table 5). The results show that (45.1%) of the total isolates were isolated from soil (S3). From the result of identification it can be seen that a total of (13) different species were identified of which *Micrococcus luteus* was more frequent (23.5%) followed by *Bacillus licheniformis* (19.6%) and

Table (5) Identification of the presumptive active bacterial isolates.

Bacterial species	S2		S3		S4		S5		S7		Total	
	No	%	No	%	No	%	No	%	No	%	No	%
Arthrobacter spp.	-	-	-	-	-	-	-	-	2	66.7	2	3.9
Bacillus breivis	1	10	3	13.0	-	-	-	-	-	-	4	7.8
Bacillus circulans	-	-	1	4.3	-	-	-	-	-	-	1	2.0
Bacillus licheniformis	-	-	6	26.1	2	40	2	20	-	-	10	19.6
Bacillus megaterium	-	-	1	4.3	-	-	-	-	-	-	1	2.0
Bacillus subtilis	-	-	1	4.3	2	40	2	20	1	33.3	6	11.8
Corynebacterium spp.	-	-	1	4.3	-	-	2	20	-	-	3	5.9
Leifsonia aquaticum	-	-	4	17.4	1	20	-	-	-	-	5	9.8
Micrococcus luteus	8	80	2	8.6	-	-	2	20	-	-	12	23.5
Pseudomonas aeruginosa	1	10	1	4.3	-	-	-	-	-	-	2	3.9
Pseudomonas stutzeri	-	-	1	4.3	-	-	2	20	-	-	3	5.9
Pseudomonas flourescens	-	-	1	4.3	-	-	-	-	-	-	1	2.0
Rothia dentacariosa	-	-	1	4.3	-	-	-	-	-	-	1	2.0
Total	10		23		5		10		3		51	

Bacillus subtilis (11.8%). On the other hand the three *Pseudomanas spp* were represented by (11.8%). Other species were in the range of (2.0-9.8%). It must be noted that the (5) *Bacillus spp* collectively were represented by (43.1%), indicating high frequency of this genus especially in the highly polluted soil (S3) in which it was represented by (52.2%). This may be due to the characteristic endospores formation of this genus which resist this difficult polluted environment.

When the above species were tested for their biodegradation activities (by measuring the absorption due to optical density of the growth turbidity and/ or emulsification of the hydrocarbon in the liquid phase), it was found that (8) isolates succeeded to give values (1-10) in the absorption scale indicating most active strains. These bacterial species were selected and quantitatively studies, singly and in a mixed culture for their ability to degrade the (PAHs) found in the crude oil. The active isolates include one strain of each of the following:

* *Bacillus brevis*
* *Bacillus licheniforms*
* *Pseudomonas aeruginosa*
* *Pseudomonas stutzeri*
* *Pseudomonas fluorescent.*

The ability of the five pure bacterial cultures and their mixture to degrade (PAH) compounds was investigated by growing the organisms in liquid culture medium, supplemented by the aromatic fraction that was extracted from (S3). From this fraction (16 PAHs) were resolved. (Table 2), i.e. at the beginning of the experiment (0-time) the (16 PAHs) were present.

Results of the biodegradation of the individual (16 PAHs) are found in table (6). The results show that the three *Pseudomanas spp* were able to degrade more of the total(PAHs (73.4-77.4%) than the two *Bacillus spp* (60.0-61.2%). The activity of *Pseudomanas* spp to biodegrade hydrocarbons was investigated by different authors (Leahy and Collwell, 1990; Gero *et al*, 1997; Kuzunga and Atiken, 2000; Okah 2003; Al-Gounaim and Diab, 2004).

On the other hand some investigators (Richard and Vogel, 1999) found that four species of the genus *Pseudomanas* when grown in liquid culture were unable to degrade the hydrocarbons found in diesel oil. In liquid cultures, metabolic byproducts like fatty acids, ketones or alcohols may accumulate and inhibit or decrease the biodegradation potential of the microorganisms (Leahy and Collwell, 1990; Chaineau *et al*, 1996).

When the five bacterial species were mixed in one culture, the results show that this mixed culture was able to degrade more of the total PAHs (88.6%) than each of the individual pure culture (60-77.4%). Another particular notable distinction between this mixed culture and the individual pure cultures in

Table (6) Bidoegradation of (PAH) compounds as a results of the activities of the different bacterial species.

(PAHs)	0-time ppm		Bidegradation (% w/w PAH)				
		B.b	B.l	P. ar	P. st	P. fl	Mixture
1- Naphthalene	1.29	81.3	69.2	84.5	90.1	89.2	95.4
2- Acenaphthylene	3.76	70.0	15.3	81.4	89.6	92.1	98.1
3- Acenaphthene	2.42	48.4	60.2	80.3	78.4	90.1	94.3
4- Flourene	2.06	50.2	45.1	48.3	49.2	53.4	80.8
5- Phenanthrene	23.69	65.4	67.2	70.3	66.4	68.2	87.6
6- Anthracene	9.03	56.3	48.5	55	60.3	58.1	80.4
7- Flouranthene	5.41	65.6	58.4	66.1	55.2	66.3	80.2
8- Pyrene	30.19	59.4	60.2	67.3	59.1	75.2	80.5
9- Benzo(a)anthracene	35.95	75.6	70.8	81.5	88.6	90.1	90.6
10- Chrysene	112.30	68.7	70.1	89.3	80.3	88.4	94.2
11- Benzo(b)flouranthene	18.41	55.6	56.4	70.8	79.9	81.2	89.7
12- Benzo(k)flouranthene	4.86	66.4	70.6	89.3	78.4	85.1	90.2
13- Benzo(a)pyrene	44.24	70.3	60.4	80.2	86.3	81.4	92.4
14- Dibenzo(ah)anthracene	9.16	66.3	60.6	90.4	85.2	89.3	94.1
15- Benzo(ghi)perylene	7.92	45.1	38.2	59.3	60.2	55.2	80.1
16- Indeno(1,2,3-c,d)pyrene	255.02	34.2	40.3	60.4	79.2	75.4	88.2
Total	566.01	61.2	60.0	73.4	74.2	77.4	88.6

(B.b), *Bacillus brevis,* (B.l), *Bacillus leicheniformis,* (P.ar), *Pseudomonas aeruginos a,* (P.st), *Pseudomonas stutzeri,* (P.fl), *Pseudomonas flooures cens.*

this biodegradation study (Table 6) is the great efficiency of the mixed culture to degrade more amounts of the carcinogenic (PAH) compounds: flouranthene (80.2%), benzo(a)anthracene (90.6%), chrysene (94.2%), benzo(b)flouranthene (89.7%), benzo(a)pyrene (92.4%), dibenzo(ah)anthracene (94.1%) and indeno(1,2,3-c,d)pyrene (88.2%). Richard and Vogel (1999) found that *Pseudomonas flourescens* (two strains) and *Achromobacter anthropi* utilized 10.4%, 12.3% and 64.1% respectively, while their mixed culture was able to utilize (90%) of the hydrocarbons of the deisel oil. (Bahatnagar and Fathepure (1991), explained that the collective metabolism of mixed cultures of microorganism may result in an enhanced (PAHs) utilization since intermediary bioremediation products from one organisms may serve as substrate for catabolism and growth of others.

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