Characterization of crude oil-degrading bacteria in a crude oil-contaminated and uncontaminated site in Kuwait

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Abstract

The planning of cities must insure a balance between human consumption and natural resources that should be preserved and if needed should be used minimally and efficiently following plans of sustainability. The current study investigated the possibility of recycling natural waste product "plants leaves" that usually fall off, are collected, dumped and burnt. In counties like Kuwait where crude oil constitutes a major pollutant and the environment is hostile for microbial activity rendering bioremediation to be a trivial option. Thus, in the present study, the leaves of *Conocarpus* and *Tamarix* were used to enrich the low organic content soils that showed the potential of soil supplementation with organic matter to enhance the growth and activity of soil indigenous microbiota. The amendments of soils with 20 mg g^{-1} soil significantly increased the counts of crude oil-degrading bacteria in crude oil-contaminated and uncontaminated soils to 999.4 x 10^{-3} CFU g^{-1} and 358.8 x 10^{-3} CFU g^{-1}soil, respectively. The identification of isolated bacteria revealed the dominance of the genus *Microbacterium* (39.6%), *Sphingopyxis soli* (19.3%), and *Bordetella petrii* (19.6%) in unamended, *Conocarpus*-amended and *Tamarix*-amended contaminated soils, respectively. The 16S rRNA analyses showed the high diversity of isolated bacteria. Also, the diversity of the majority of isolated bacteria decreased after soil amendments with plant-derived material. Therefore, the recycling of plant-derived materials could be an excellent option under conditions tested and confirm the commandments of sustainable planning.
Keywords: bioremediation, Conocarpus, Tamarix, crude oil-degrading bacteria, Microbacterium, Sphingopyxis, 16S rDNA, amendments.

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1 Introduction

Pollution is not just the addition of substances that damage or kill organisms; it is any man-made impact that increases the risk of damage to a natural system (Moss [10]). Pollutants and contaminants are produced yearly through natural and anthropogenic activities such as industrial activities, agricultural practices, and waste disposal systems. High level, medium-level, and low-level wastes in solid, liquid, or gaseous forms are released into the environment at separate intervals or on a continuous basis (Balonov [4]). Several proposals were made by companies for remediation of heavily polluted soils after the oil lakes are drained of liquid crude oil. These included conversion of oily soil to road base material or a topping layer for car parks and roads after mixing with aggregate or consolidation agents. Other methods include containment in large burial sites, incineration, biological methods, absorption methods, soil washing methods, air stripping, thermal treatment (Lefebvre and Moletta [9]) and vacuum extraction and separation by centrifugation. However, physical and chemical approaches are expensive and byproducts may cause secondary contamination of soil and water resulting in the need for additional post-treatments. As such, there is a widespread interest in bioremediation for the complete mineralization of hydrocarbons to carbon dioxide and water. Bioremediation is a cost effective method and provides *in situ* remediation without disturbing native ecosystems (Bragg *et al*. [6]). Generally speaking, several bioremedial approaches have been applied to contaminated soils to reduce impacts of crude oil pollution. These include stimulation of indigenous oil-degrading microbiota by the addition of fertilizers high in nitrogen and phosphorus and/or oxygen; a technology known as biostimulation; seeding of the oil-fouled areas with hydrocarbon-degrading microorganisms; and application of surfactants to the oil-contaminated zone (Edwards *et al*. [8]). In strictly aerobic conditions, adding nutrient at an adequate rate is sufficient for the biodegradation of saturated hydrocarbons. It was suggested that excess of amendment reduces the assimilation of hydrocarbons. Also, the extent of assimilation of aromatics and hexane insoluble molecules are not increased with high inputs of nutrient as compared to natural attenuation. There are three strategies of nutrient application that are generally used for bioremediation purposes. The first strategy encompasses the addition of soluble mineral nutrients to soil. The second strategy involves the addition of organic nutrient formulations. The third strategy comprises the addition of slow-release inorganic fertilizers (SRIFs) to soil or sediments (Xu *et al*. [12]). In addition, during the past years, composting strategies have been introduced in the bioremediation of contaminated soils by adding high amounts of agro-industrial wastes (pine sawdust, sugarcane bagasse, wheat straw, compost, etc.) for the removal of organic pollutants (Civilini *et al*. [7]). However, the cost of applying the aforementioned bioremediation technologies created a need for cheaper methods. In order to overcome the problem of high cost of added nutrients to soil, the addition of plant leaves (litter) especially of local dominant plants in Kuwait such as *Tamarix aphylla* and *Conocarpus spp*. to soil was proposed. Therefore, the aim of the present investigation was to investigate the potential for

enhancing the rates of crude oil bioremediation in crude oil-contaminated soil via the addition of organic materials (as nutrient sources) derived from two local plants *Conocarpus* and *Tamarix spp*.

2 Materials and methods

2.1 Collection, preparation and characterization of plant-derived material

Leaves of *Tamarix aphylla* and *Conocarpus* were collected, washed thoroughly three times with sterile water, air dried, and ground then stored at -20ºC.

2.2 Collection and characterization of soil samples

The effects of nutrients addition on the rate of crude oil degradation were studied using crude oil contaminated and uncontaminated soils collected from Al-Sabreia oil field, north of Kuwait. Soil samples were collected in sterile plastic containers, kept at 4˚C and transported immediately to the laboratory for treatment. Soil samples were sieved (2mm) and stored for two weeks at 4˚C for stabilization. The total carbon, hydrogen, nitrogen and sulfur in soil samples were determined using standard methods (Alef [1]).

2.3 Amendments of soil with plant-derived materials

Prior to the onset of the biodegradation/mineralization experiments, soil samples were amended with varying amounts of prepared organic supplements (20 mg, 60 mg and 100 mg g^{-1} soil) to provide known amounts of nutrients. Then, soil samples (50g) were transferred to sterile reaction glass bottles (250 ml). The water content was adjusted to 30%, the contents of the reaction bottles were mixed gently for 10 min and incubated at 25˚C. The bottles were used for the mineralization and biodegradation experiments.

2.4 Determination of biodegradation potentials of soil microbiota

The rate of crude oil biodegradation was determined using gas chromatography (GC). Soil samples (50g) were transferred to screw-capped bottles (Duran) followed by the addition of organic supplement (20mg, 60mg and 100mg g^{-1} soil). Water content was adjusted to 30% and bottle contents were mixed for 10 min and incubated at 25˚C. After one month of incubation, residual crude oil in three flasks was extracted three times with equal volumes of pentane at 5˚C. All pentane extracts were pooled for crude oil analysis.

2.5 Enumeration, isolation and identification of bacteria in soil

The culturable total heterotrophic (THB) and crude oil-degrading bacteria in contaminated and uncontaminated soils were counted before and after the amendments experiments in order to study the effects of nutrients amendments on the THB and crude oil-degrading bacterial populations. Bacteria in soil were counted using standard plate dilution method. Three portions from soil samples

were pooled, thoroughly mixed, and passed through a sterile sieve (mesh size, 2mm). Ten grams (wet weight) of soil was suspended in 40 ml of 50 mM phosphate buffer (pH 7.2) and vortexed for 1 minute at low speed. Aliquots (100 µl) in direct and 10^{-1}-10^{-6} dilutions were spread on Hutner's minimal agar plates and incubated under crude oil vapor in a desiccator at 25˚C for up to 21 days. Grown colonies were counted and the numbers of crude oil-degrading bacteria g^{-1} soil were calculated and expressed as Colony Forming Unit (CFU) g^{-1} soil.

2.6 Identification and fingerprinting of isolated crude oil-degrading bacteria by 16S-RFLP

The isolated bacteria were identified by sequencing of the 16S rDNA and fingerprinted using 16S-RFLP following previous methods (Al-Saleh and Obuekwe [3]).

3 Results

The total amounts of carbon, hydrogen, nitrogen and sulfur of soil samples were determined. As can be seen in Table 1, the total amounts of hydrogen, nitrogen, sulfur and carbon were significantly higher ($P<0.05$) in the contaminated soil (2.027%, 0.050%, 0.419% and 14.32%, respectively) compared to those in the uncontaminated soil samples (0.245%, 0.020%, 0.034% and 0.040%, respectively).

Table 1: Amount of total carbon, hydrogen, nitrogen and sulfur in soil samples.

Elements	Uncontaminated soil (%)	Contaminated soil (%)
Hydrogen	0.245±0.02	2.027±0.21
Nitrogen	0.020±0.001	0.050±0.001
Sulfur	0.034±0.001	0.419±0.03
Carbon:	1.830±0.15	14.32±0.32
Inorganic carbon	0.040±0.001	0.220±0.02
Organic carbon	1.790±0.14	14.10±0.33

The mineralization studies to assess the effects of the addition of plant-derived material on the mineralization of crude oil in soil (Tables 2 and 3) were conducted and the results showed that the addition of 20, 60 and 100 mg *Conocarpus*-derived material significantly ($P<0.05$) increased the amounts of carbon dioxide evolution by the microbiota of the uncontaminated soil (61992, 21060 and 6669 µg carbon dioxide, respectively). Furthermore, the addition of crude oil (4.0 µl crude oil g^{-1} soil) in presence of *Conocarpus*-derived material (20, 60 and 100 mg g^{-1} soil) resulted in additional increase in the amounts of

carbon dioxide evolution in the uncontaminated (68900, 22572 and 6850 μg carbon dioxide, respectively) soil samples (Table 2). Moreover, the addition of *Tamarix*-derived materials to soil samples resulted in similar observations (Table 3). However, the addition of *Conocarpus*-derived material to soils in presence/absence of crude oil resulted in significantly higher ($P<0.05$) amounts of carbon dioxide evolution than that determined when *Tamarix*-derived materials were added to soil.

Table 2: Effects of the addition of *Conocarpus*-derived material and crude oil on the evolution of carbon dioxide by soil microbiota.

Amount of *Conocarpus*-derived material (mg/g soil)	Carbon dioxide evolution (μg)	
	Conocarpus	*Conocarpus* and crude oil
Non	2700	2832
20	61992	68900
60	21060	22572
100	6669	6850

Equal amounts of soil (50 g), water (0.4 ml g^{-1} soil) and crude oil (4.0 μl g^{-1} soil) were added to reaction flasks.

Table 3: Effects of the addition of Tamarix-derived material and crude oil on the evolution of carbon dioxide by uncontaminated soil microbiota.

Amount of Tamarix-derived material (mg/g soil)	Carbon dioxide evolution (μg)	
	Tamarix	Tamarix and crude oil
Non	2700	2832
20	27675	29977
60	7209	7529
100	6939	7126

Equal amounts of soil (50 g) and water (0.4 ml g^{-1} soil) and crude oil (4.0 μl g^{-1} soil) were added to reaction flasks.

The potential encouragement of crude oil utilization by soil microbiota due to the addition of plant-derived materials was assessed by using gas chromatography method to determine the residual amounts of crude oil in soil. As presented in Table 4, the addition of 20 and 60 mg g^{-1} soil of *Conocarpus*-derived material to soil in the presence of crude oil (4.0 μl g^{-1} soil) significantly

($P<0.05$) increased the amounts of crude oil biodegradation by the microbiota of uncontaminated (75.2% and 66.8%, respectively) soil. In addition, , the addition of 20 and 60 mg g^{-1} soil of *Tamarix*-derived material to soil in the presence of crude oil (4.0 µl g^{-1} soil) resulted in analogous observations (Table 4). Nonetheless, the addition of 100 mg g^{-1} soil of *Conocarpus*- and *Tamarix*-derived material to soil in the presence of crude oil (4.0 µl g^{-1} soil) decreased the potentials of crude oil biodegradation by soil microbiota.

Table 4: Biodegradation of crude oil in presence of plant-derived materials in uncontaminated soil.

Amount of plant-derived material (mg/g soil)	Biodegradation of crude oil (%)	
	Conocarpus	Tamarix
Non	42.5	42.5
20	75.2	60.1
60	66.8	49.4
100	43.7	40.2

Equal amounts of soil (50 g), water (0.4 ml g^{-1} soil) and crude oil (4.0 µl g^{-1} soil) were added to reaction flasks. The amounts of crude oil were determined by gas chromatography.

The occurrences of the total heterotrophic and crude oil-degrading bacteria in the crude oil-contaminated and uncontaminated soil samples were determined using plate-dilution method and the results are presented in Table 5. The counts of the total heterotrophic bacteria in contaminated soil samples (13.15×10^3 to 29.85×10^5 CFU g^{-1} soil) before and after the addition of plant-derived materials were significantly higher ($P<0.05$) than that determined in uncontaminated soil samples (10.12×10^3 to 22.96×10^5 CFU g^{-1} soil). The counts of crude oil-degrading bacteria in unamended contaminated (9.75 x 103 CFU g^{-1} soil) and uncontaminated (3.51×10^3 CFU g^{-1} soil) soil samples were lower than the counts of crude oil-degrading bacteria in amended contaminated (2.123×10^5 to 22.38×10^5 CFU g^{-1} soil) and uncontaminated (61.25×10^3 to 80.58×10^4 CFU g^{-1} soil) soil samples. Additionally, the increases in the counts of the crude oil-degrading bacteria in soils amended with *Conocarpus*-derived material were significantly higher ($P<0.05$) than those in soils amended with *Tamarix*-derived material (Table 5). Furthermore, the molecular identification of crude oil-degrading bacteria in the contaminated soil revealed the dominance of the genus *Microbacterium* (39.6%), *Sphingopyxis soli* (19.3%), and *Bordetella petrii* (19.6%) in unamended, *Conocarpus*-amended and *Tamarix*-amended contaminated soils, respectively. The 16S rRNA analyses of isolated crude oil-degrading bacteria showed the high diversity of isolated bacteria from the contaminated soil. In addition, the diversity of the majority of isolated bacteria decreased after soil amendments with plant-derived material (Table 6).

Table 5: Counts of total heterotrophic and crude oil-degrading bacteria in soil.

Bacteria	Uncontaminated soil (CFU g^{-1} soil)*	Contaminated soil (CFU g^{-1} soil)*
Total heterotrophic bacteria		
Before the commencement of all soil experiments	10.12±0.12	13.15±0.11
After the end of control experiments (no plant material was added)	450.6±2.33	585.2±2.39
After the end of *Tamarix* experiments (20 mg g^{-1} soil)	1025±4.55	1332±5.11
(60 mg g^{-1} soil)	267.7±3.36	347.1±3.12
(100 mg g^{-1} soil)	175.5±1.52	283.7±3.33
After the end of *Conocarpus* experiments (20 mg g^{-1} soil)	2296±6.11	2985±2.95
(60 mg g^{-1} soil)	780.4±3.14	1014±4.35
(100 mg g^{-1} soil)	585.4±2.46	870.6±4.13
Crude oil-degrading bacteria		
Before the commencement of all soil experiments	3.510±0.09	9.750±0.11
After the end of control experiments (no plant material was added)	157.5±1.61	438.8±2.14
After the end of *Tamarix* experiments (20 mg g^{-1} soil)	358.8±3.24	999.4±4.21
(60 mg g^{-1} soil)	93.45±1.12	260.3±2.96
(100 mg g^{-1} soil)	61.25±0.93	212.3±2.23
After the end of *Conocarpus* experiments (20 mg g^{-1} soil)	805.8±3.78	2238±6.52
(60 mg g^{-1} soil)	273.7±2.88	745.5±3.35
(100 mg g^{-1} soil)	204.8±2.36	652.5±2.67

*Bacterial counts determined (CFU g^{-1} soil x1000).

Table 6: Identity and number of some isolates of crude oil-degrading bacteria isolated from un-amended and amended contaminated soil with *Conocarpus*- and *Tamarix*-derived material.

Identity	Number of isolated bacteria		
	Un-amended soil	*Conocarpus*-amended soil	*Tamarix*-amended soil
Microbacterium spp.	21	8	11
Xanthomonas spp.	9	1	4
Brevundimonas spp.	8	9	4
Stenotrophomonas spp.	5	2	5
Achromobacter spp.	2	0	0
Bacillus spp.	1	0	0

4 Discussion

The significantly ($P<0.05$) higher values of total inorganic and organic carbon determined in the crude oil-contaminated soils (0.220% and 14.10%) compared to those in the uncontaminated soils (0.040% and 1.790%) were most probably due to crude oil contamination. Since the occurrence of total heterotrophic bacteria (THB) in soil can be used as an indicator for the extent of soil support to bacterial growth (Black *et al*. [5]), the THB were enumerated in soil samples before and after the addition of plant-derived materials. The counts of total heterotrophic bacteria (THB) and crude oil-degrading bacteria in soil samples after the addition of plant-derived materials increased significantly ($P<0.05$) where the counts of TBH in *Conocarpus*-amended soil ($585.4 \times 10^3 – 2985 \times 10^3$ CFU g^{-1} soil) were significantly ($P<0.05$) higher than those in the *Tamarix*-amended soil ($175.5 \times 10^3 – 1332 \times 10^3$ CFU g^{-1} soil). This difference in THB counts indicated the higher supportive nature of *Conocarpus*-derived material to the growth of THB compared to that of *Tamarix*-derived material. Also, it was noticed that the addition of 20 mg g^{-1} soil of plant-derived materials to soil resulted in the significantly ($P<0.05$) higher increase in THB and crude oil-degraders counts in *Conocarpus*- and *Tamarix*-amended soils compared to that when 60 and 100 mg g^{-1} soil of plant-derived materials were added to soil. These findings were in agreement with other studies (Al-Saleh and Obuekwe [2]; Obayori *et al*. [11]). The identification of the crude oil-degrading bacteria revealed the predominance of Gram-negative crude oil-degrading bacteria in the unamended and amended contaminated soil samples which might be due to the higher ability of Gram-negative bacteria to adapt to crude oil pollution. The identification of bacteria in the contaminated soil revealed the dominance of the genus *Microbacterium* (39.6%), *Sphingopyxis soli* (19.3%), and *Bordetella petrii* (19.6%) in unamended, *Conocarpus*-amended and *Tamarix*-amended

contaminated soils, respectively. The 16S rRNA analyses of isolated crude oil-degrading bacteria showed high diversity of isolated bacteria from the contaminated soil. Analysis of the distribution of band patterns of 16S-RFLP demonstrated the high diversity of isolated bacteria from the contaminated soil where the band-based similarity calculations revealed wide range of sequence differences among members of the same species. Therefore, the recycling of plant-derived materials could be an excellent option under conditions tested and confirm the commandments of sustainable planning.

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