BIOREMEDIATION OF OIL CONTAMINATED SOIL BY AXENIC AND MIXED CULTURES OF BACTERIA AND FUNGI[+]

المعالجة الحيوية للتربة الملوثة بالنفط بواسطة مزارع مفردة ومختلطة من البكتريا والفطريات

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Abstract:

In this study, several species and genera of oil degrading bacteria and fungi were isolated from the soil of Al–Rumella region, Basrah, Iraq. These were *Staphylococcus* sp., *Arthrobacter* sp., *Micrococcus* sp., *Corynebacterium* sp., *Streptomyces* sp., *Bacillus pumilis*, *Bacillus subtilis*, *Bacillus polymyxa*, *Pseudomonas putida*, *Aremonas* sp., *Nocardia* sp. (bacteria), *Candida* sp., *Rhizopus* sp., *Psidxybe cubensis*, *Trichoderma* sp., *Aspergillus niger*, *Pencillum* sp., *Pencillum chrysogenum*, *Cunninghamella* sp. and *Ssccharomyces cerevisiae* (fungi). A mutant of *P. putida* with high ability to degrade crude oil was obtained from subjected this bacteria to UV. radiation.

Axenic cultures of bacteria and fungi, a mixed cultures of bacteria and fungi and a mixed cultures of mutant bacteria and fungus (*P. chrysogenum*) were added to the soil of Al-Rumella region, was contaminated with Basrah regular crude oil and supplemented with nutrients. Favorable conditions for microbial growth were available.

The ability of microorganisms to degrade crude oil was measured directly by determination the residual oil by spectrofluorometer and indirectly by measuring the amount of CO_2 liberated in a controlled system. It was found that a mixed cultures of mutant bacteria and fungus degraded the crude oil more than a mixed cultures of bacteria and fungi. The last cultures can be reduced that the oil pollution better than the axenic cultures of bacteria and fungi. The mixed cultures of mutant bacteria and fungus [*P. putida* (mutant)+*P. chrysogenum*] can be used and applied in bioremediation of polluted soils.

المستخلص:

تضمنت الدراسة الحالية عزل وتشخيص عدة أنواع وأجناس من البكتريا (*Staphylococcus* sp. و*Arthrobacter* sp. و*Micrococcus* sp. و*Corynebacterium* sp. و*Streptomyces* sp. و *Bacillus pumilis* و*Bacillus subtilis* و*Bacillus polymyxa* و*Pseudomonas putida* و*Aremonas* sp.) والفطريات (*Nocardia* sp. و*Candida* sp. و*Rhizopus* sp. و*Psidxybe cubensis* و *Trichoderma* sp. و*Aspergillus niger* و*Pencillum* sp. و*Pencillum chrysogenum* و*Cunninghamella* sp.) المفككة للنفط من تربة منطقة الرميلة الواقعة في محافظة البصرة جنوب العراق. تم الحصول على بكتريا طافرة من النوع البكتيري (*P. putida*) ذات قدرة عالية على تفكيك النفط الخام عند تعريض أنواع من البكتريا المفككة للنفط للأشعة الفوق البنفسجية.

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درس التفكيك الحيوي للنفط مختبريا في تربة منطقة الرميلة حيث لقحت التربة بمزارع مفردة من البكتريا والفطريات ومزارع مختلطة من البكتريا والفطريات ومزارع مختلطة من البكتريا الطافرة والفطر (*P. chrysogenum*) ولوثت بنفط خام البصرة الاعتيادي وجهزت بالمغذيات ووفرت الظروف الملائمة لنمو الأحياء المجهرية.

تم قياس قابلية التكسير الحيوي للنفط الخام بصورة مباشرة عن طريق تقدير النفط المتبقي في التربة بوساطة جهاز الفلورة وبصورة غير مباشرة عن طريق قياس كمية ثاني اوكسيد الكاربون المتحرر خلال عملية التكسير الحيوي في نظام مسيطر عليه. وجد بان المزارع المختلطة من البكتريا الطافرة والفطر تكسر النفط الخام في التربة أفضل من المزارع المختلطة من البكتريا والفطريات. وكانت فعالية المزارع الأخيرة في تفكيك النفط الخام أفضل من المزارع المفردة من البكتريا والفطريات. نستنتج من ذلك انه يمكن استخدام وتطبيق المزارع المختلطة من البكتريا الطافرة والفطر في المعالجة الحيوية للترب الملوثة بالنفط.

Introduction:

The pollution of petroleum hydrocarbons caused a major changes in the physical and chemical properties of the soil. It is an environmental concern, because contaminated soils may be unsuitable for agricultural, industrial, or recreational use and also potential sources for surface and ground water contamination [1]. [2] showed that the petroleum did not persist for long periods in the most soils even when relatively large quantities of petroleum have spilled. This is probably due to large part to the initial degradation by the action of sunlight followed by microbial attack when the oil sinks. The individual aliphatic, olefinic and naphthenic compounds are mostly susceptible to attack by microorganisms [3]. Such microorganisms are widely and abundantly distributed in nature and include a large number of genera. Such decomposition of hydrocarbons takes place in very simple salt media with ammonia as nitrogen supplement, in both liquid and solid states and over a wide range of environmental conditions [2].

Despite the many species of microbes which may be involved in the attack on petroleum, the degradation of individual components generally follows similar catabolic pathways [3] (Figure 1).

1- Alkanes: Oxidation of the terminal (C_1) methyl group by molecular oxygen gives an alcohol, aldehyde and subsequently a carboxylic acid. To lesser extent the C_2 carbon may be oxidized to give a methyl ketone or the oxidative attack may take place at both ends to give an α dicarboxylic acid. The mechanism of the oxidation is not finally agreed upon but it seems most likely to be catalysed by a mono oxygenase. Subsequent breakdown of the carboxylic acids is by β oxidation and gives rise to acetyl coenzyme A as final product [4].

2- Cyclo alkanes: Fewer studies have been made on compounds such a un-substituted cyclohexanes. In alkane substituted cyclo alkanes the initial attack is generally on the substituted group and attack on the ring probably involves a hydroxylation by an oxygenase [5].

3- Aromatic compound: A wide range of substituted benzene derivatives as well as benzene itself are readily degraded by many species of microbes. Two main pathways are involved after an initial hydroxylation by deoxygenases to form phenols.

a- Via cis-cis muconic acid which subsequently gives rise to succinate and acetyl coenzyme A.

b- α hydroxymuconic aldehyde which will also give a carbonyl compound, pyruvate and carboxylic acid depending on the substituents of the aromatic ring.

These pathways are also involved in the degradation of polycyclic aromatic rings such as the naphthalenes [6].

4- Heterocyclic compounds: Substituted pyridines, e.g. nicotinic acid and its analogues are degraded by hydroxylation adjacent to the nitrogen atom which dose not involve molecular oxygen but water (hydration and dehydrogenation). Consequently by using a suitable hydrogen acceptor these compounds may be assimilated anaerobically as succinate, acetate and formate by the usual pathways. The work on substituted thiophenes has shown that the initial ring-opening step releases H_2S but the subsequent steps by which carbon is assimilated are not yet clear. Substituted phenanthrene derivatives have been much worked on in connection with microbial steroid transformation: it appears likely that the compounds are assimilated as carboxylic acids and acetyl CoA and that the initial attack is by hydroxylation reactions most of which involve oxygenases and molecular oxygen [2].

The assimilation of these constituents of petroleum by microbes may be summed up by pointing out that the initial attack in almost every case depended on reactions involving molecular oxygen. Subsequent degradation to low molecular weight carboxylic acids involve steps such as β oxidation, in which no molecular oxygen is required. However, breakdown of the compound completely to CO_2 and H_2O will need further oxygen which is used as the terminal electron acceptor. In the initial step no energy in the form of adenosine triphosphate (ATP) is made available to the organisms and bond breakage is often accompanied by the formation of heat. Subsequent metabolism of carboxylic acids by the Krebs cycle or fermentation is the main source of ATP. The oxidation of alkanes, where acetyl CoA is the main or only product, demands that an anaplerotic reaction such as glyoxalate by-pass function and that alkane metabolism is similar to acetate metabolism [2, 7].

Microbial degradation of petroleum hydrocarbons in the ecosystems is influenced strongly by a wide variety of abiotic and biotic factors which include; temperature, pH, aeration, nutrients, water availability, microbial adaptations, previous chemical exposure concentration of hydrocarbons, bioavailability, diffusion, soil type, organic matter, toxicity, depth, pressure, salinity, physico–chemical properties of the hydrocarbons, seasonal factors and presence of hydrocarbons degrading microorganisms [8]. Many of these are reviewed extensively by Farid [4], with special emphasis on the soil environment.

The use of biodegradation to degrade hazardous pollutants is a practical alternative to traditional remediation treatment technologies [9]. The two approaches taken for the bioremediation of petroleum pollutants are the addition of microorganisms (seeding) that able to degrade hydrocarbons and the modification of environment, for example, by adding fertilizers or by aerating the contaminated site [10].

The objective of the present study was to use axenic and mixed cultures of indigenously species of bacteria and fungi (with capabilities to degrade petroleum hydrocarbons) in addition mutant bacteria for development inoculum to increase the usefulness of biodegradation process as an effective remedial tool of polluted soils with oil.

Materials and Methods:

The present study comprised collection of soil samples from the field and crude oil samples, isolation and characterization of oil degrading microorganisms from soil samples, mutation of oil degrading bacteria species, seeding the soil with oil degrading microorganisms, addition of crude oil and nutrients into soil, monitoring the residual oil and amount of CO_2 released and comparatively the microbial growth in soil samples.

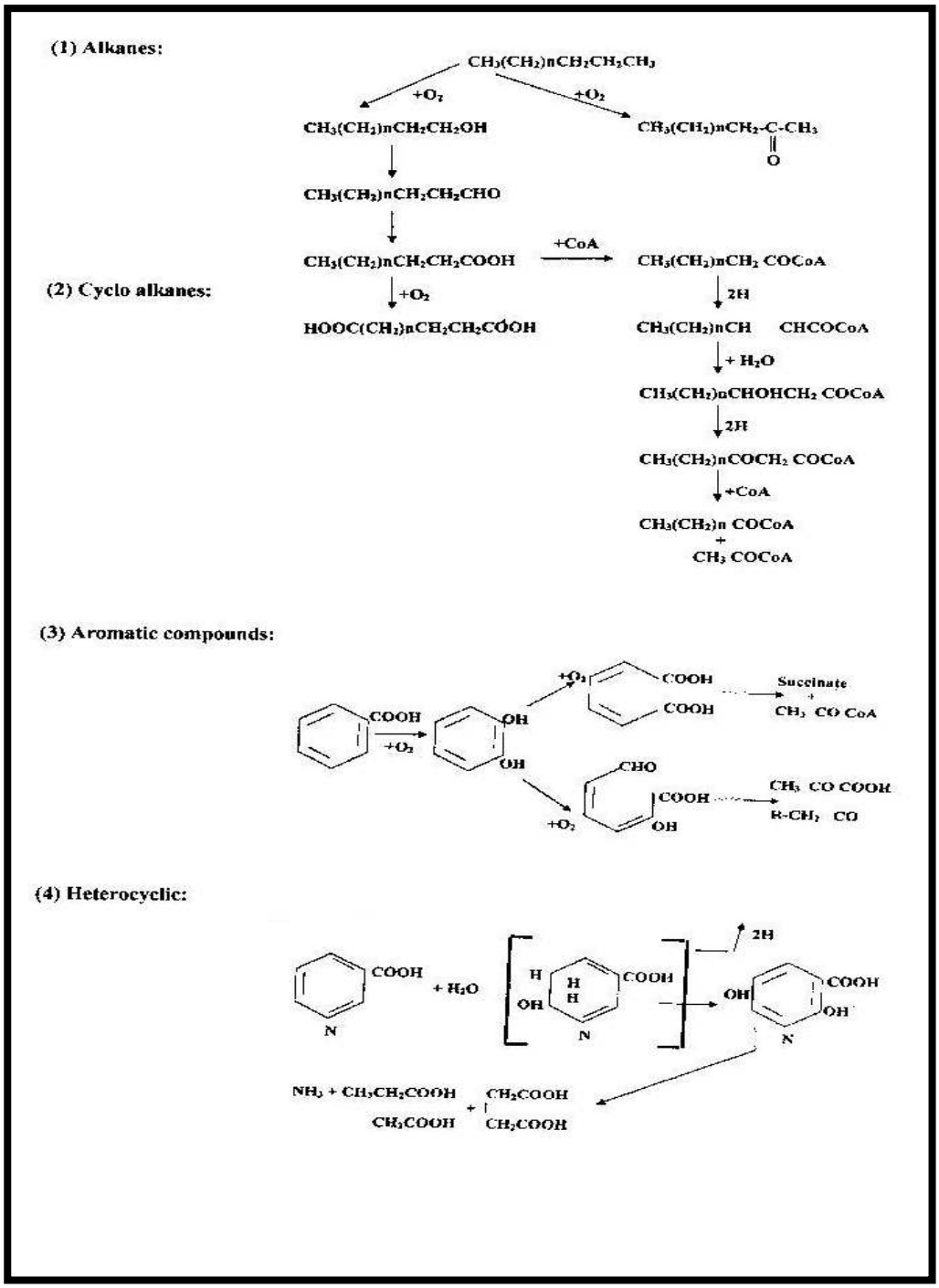

Figure (1): The catabolic pathways of individual components of petroleum .

All the analytical methods used in the present investigation were standard analytical methods such as the microbial isolation and characterization methods, CO_2 production analysis method and spectrofluorometeric analysis technique of extracted oil. The microbial count was done using the viable counting technique.

Sample of crude oil

Crude oil (Basrah regular-medium-API gravity between 28–34) was supplied by Iraqi South Oil Company. Basrah, Iraq. It was transferred to laboratory in dark glass bottle closed tightly and kept in a cold and dark place prior to use.

Collection of soil sample

Soil samples were collected from petroleum contaminated region of Al–Rumella in Basrah governorate, Iraq, during 2008 (Figure 2). All samples were taken randomly from upper surface of soil at depth 5–10 cm. The samples were then transferred to laboratory in sterile nylon sacs.

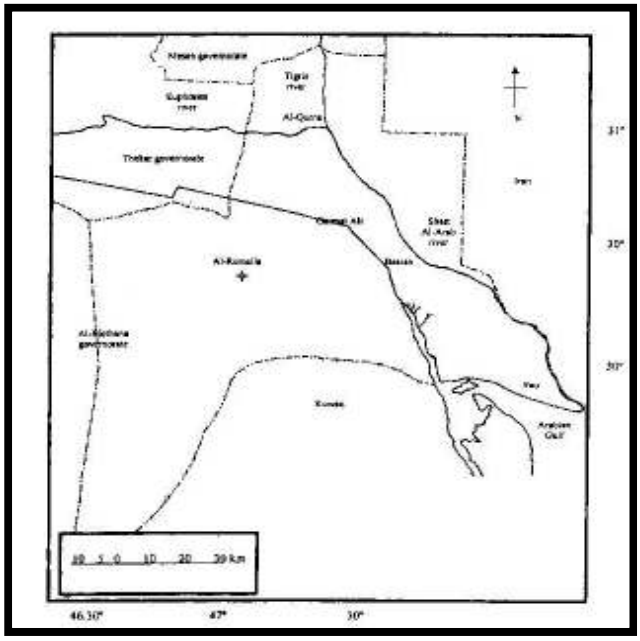

Figure (2): Map of sampling location.

Isolation and characterization of oil degrading microorganisms:

Isolation of oil degrading bacteria and fungi were carried out on liquid culture media of Cerniglia [6] and Al–Dossari [11] respectively (Table 1). Both media were overlaid with 0.1ml of Basrah regular crude oil (as the sole source of carbon and energy)/100ml media in 250ml flasks. The liquid media then inoculated with one gram soil sample. The control flasks were not inoculated with soil sample. All flasks were incubated at 37°C in an orbital shaker set at 100rpm. for 7–28 days.

The growth of bacteria and fungi were weekly monitored at 7–28 days intervals by culturing 0.1ml of appropriate dilution on nutrient agar media (bacteria) and potato carrot agar media (fungi). The pH and turbidity of the liquid media were also monitored. The colonies of bacteria and fungi were purified, enumerated and examined microscopically. When the cultures were considered to be pure, they were further inoculated on slants (contained nutrient agar overlaid with Basrah regular crude oil for growing of bacteria) and Petri dishes (contained potato carrot agar overlaid with Basrah regular crude oil for fungi) as stock cultures. The stock cultures were maintained at 4°C and subculture every 6–8 weeks.

Identification of bacteria and fungi were carried out according to their morphological characteristics (i.e. rate of growth, motility, spores formation, form of growth, elevation, margin, pigmentation and structural changes in the fruiting bodies of fungi). Gram stain and Albert's stain techniques were applied for bacteria. Whereas lactophenol technique was applied for fungi.

Biochemical tests involved sugar utilization (fermentation test of glucose, lactose and maltose), gas production, hydrogen peroxide formation, hydrolysis of starch, carbohydrate metabolism, nitrate reduction, arginine dihydrolase, gelatin hydrolysis, and enzymes production (test of oxidase and catalase) were carried out for bacteria characterization [11, 12, 13, 14, 15].
The microbial isolates (bacteria and fungi) were all examined for their ability to degrade crude oil.

Mutation of bacteria:

Four bacterial isolates were subcultured on to nutrient agar plates, incubated for 6h, suspended in the 20ml of phosphate buffer pH7 (Na_2PO_4, 6.0g; Na_2PO_4, 9.52g; 1LDW) and diluted to 10^{-4}. The suspensions were then subjected to UV radiation for different periods (5min and 30min). Another replicates of cultures were incubated for 12h, suspended in phosphate buffer pH7, diluted to 10^{-4} and then subjected to UV radiation for 60min period. Different rates and stages of irradiation were designed to cover the different phases of exponential growth of different bacterial cells in order to effectively induce mutation [16].
The UV light source was a germicidal lamp (254 nm). The immediately output of the lamp was 10erg/mm/sec. The irradiation was only applied when the fluency of lamp was in a stable maximum.
After irradiation, the bacteria in each cultured on nutrient agar media were observed morphologically for phenotypic expression. The cultures were then examined for their ability to degrade of crude oil.

Table (1): Composition of liquid media of Cerniglia [6] and Al–Dossari [11] for isolation of oil degrading bacteria and fungi.

Liquid medium	Composition
Cerniglia (bacteria)	NaCl, 0.3g; $(NH_4)_2\ SO_4$, 0.6g; KNO_3, 0.6g; KH_2PO_4, 0.25g; $K2HPO_2$, 0.75g; $MgSO_4.7H_2O$, 0.15g; LiCl, 20µg; $CuSO_4.5H_2O$, 80µg; $ZnSO_4.H_2O$, 100µg; $Al_2\ (SO_4)_3.16H_2O$, 100µg; $NiCl.6H_2O$, 100µg; $CoSO_4.7H_2O$, 100µg; KBr, 30µg; KI, 30µg; $MnCl_2.4H_2O$, 600µg; $SnCl_2.2H_2O$, 40µg; $FeSO_4.7H_2O$, 300µg; and distilled H_2O, 1000ml. PH=7.5
Al–Dossari (fungi)	neopepton, 10g; dextrose, 10g; and distilled H_2O, 1000ml. PH=7.5

Determination of biodegradation potential bacteria and fungi:

To determine the biodegradation potential of oil-degrading isolate bacteria and fungi. The liquid culture media of Cerniglia [6] and Al–Dossari [11] were overlaid with 0.1ml of Basrah regular crude oil/100ml media in 250 ml flasks. The liquid media were then inoculated with the pure isolate bacteria or fungi ($2.9x10^3$cell/100ml). Control flasks were left without microbes. All flasks were incubated at 37°C in an orbital shaker set at 100rpm for 28 days.
The growth of bacteria and fungi were monitored. The pH and turbidity of the liquid media were also monitored.
After incubation period was over, the oil hydrocarbons were extracted from the liquid cultures with 50ml n–hexane three times. The extracts were then filtered through sterile 0.45µm Millipore filter to remove the culture materials. To these extracts 10g of anhydrous sodium sulfate was added to remove excess water. The hexane extracts were reduced in

volume to less than 5ml by using a rotary evaporator. Further concentrate was made by stream of pure nitrogen for measurement the residual petroleum by spectroflurometer [6].

Study of biodegradation in soil:

Fifteen g of soil samples were sieved (through a 2mm sieve) and air–dried . The soils was then placed in plastic jar (10x7x1.5cm in size). 0.4g of nutrients [$(NH_4)_2SO_4$+K_2HPO_4] was added to the jars. The jars were then overlaid with axenic (pure) or mixed cultures (seeding) ($6.6x10^2$cell/ml) (Tables 3, 4 and 9). To these jars one ml of sterile water were added. The jars were supplemented with 10ml of Basrah regular crude oil (as a source of carbon and energy).
Control jars were left without crude oil. All seeded jars were incubated at 37°C for 7–28 days. One g of soil was sampled and serially diluted in sterile water to 10^{-4} and 10^{-6} every 7 days intervals for microbial viable count.
Duplicate soil samples were also taken every 7 days intervals for measurement the residual petroleum following the procedure of [17]. The soil were placed in a per–extracted cellulose thimble and soxhlet extracted with 150ml methanol:benzene (1:1ratio) for 24h. At the end of this period the extract was transferred to a storage flask and the sample was further extracted with fresh solvent. The combined extracts were reduced in volume to Ca 10ml in a rotary vacuum evaporator and was then saponified for 2h with a solution of 4N KOH in 1:1 methanol:benzene. After extraction the unsaponified matter with hexane, the extract was dried over anhydrous Na_2SO_4 and concentrated by stream of N_2 for analysis by spectrofluorometer.

Measurement of CO_2:

In one liter covered jars, 90 ml. of Matale's enrichment media, 5ml of Basrah regular crude oil and 5ml of cultured inoculum were added. The jars were also supplied with 20ml vial containing 1g of BaO_2 and 1ml of sterile water to absorb the CO_2 formed during the biodegradation. The vials were withdrawn every 7 days intervals for analysis the quantity of CO_2 liberated. Tow controls were done in this section. The first one did not contain the cultured medium but the volume was made up with sterile water. Another control was inoculated with cultured inoculum but without crude oil [18].

Spectrofluorometer:

The Shimadzu RF–540 spectrofluorometer equipped with a DR–3 data recorder was used to determine the concentration of residual petroleum hydrocarbons. The basis quantitative measurements were made by measuring the emission intensity at 360nm with excitation set at 310nm and monochrometer slits of 10nm. The reference oil used for calibration was Basrah regular crude oil.

Statistical analysis:

The data of the present study was analyzed by Randomized Complete Block Design (RCBD). Significant results were then analyzed by RLSD test [19].

Results and Discussion:

The Al–Rumella region in Basrah governorate was chosen for this study. (Figure 1). The choice of this region was governed by the presence of possible sources of oil contamination (gas product station and oil field and wells).

A variety species and genera of indigenous oil degrading microorganisms (bacteria and fungi) were isolated from the soil of Al-Rumella region. These were *Staphylococcus* sp., *Arthrobacter* sp., *Micrococcus* sp., *Corynebacterium* sp., *Streptomyces* sp., *B. pumilis*, *B. subtilis*, *B. polymyxa*, *P. putida*, *Aremonas* sp., *Nocardia* sp. (bacteria), *Candida* sp., *Rhizopus* sp., *P. cubensis*, *Trichoderma* sp., *A.. niger*, *Pencillum* sp., *P. chrysogenum*, *Cunninghamella* sp. and *S. cerevisiae* (fungi) (Table 2).

[7] reported that in the most environment systems which may become contaminated with petroleum hydrocarbons. There were indigenous oil degrading microorganisms capable of seeding the oil spilled and initiated microbial attack. [20] in their study about the effect of oil spill on the composition of microbes in a soil, They found that the soil was dominated by a diversity of oil degrading microorganisms including *Aeromonas* sp., *Flavobacterium* sp., *Pseudomonas* sp., *Micrococcus* sp., *Bacillus* sp., Actinomyces, *Aspergillus* sp., *Penicillum* sp., *Rhizopus* sp., *Thamnidium* sp., *Cunninghamella* sp. and *Candida* sp.. When petroleum hydrocarbons polluted various habitats the indigenous microflora was two fold. The hydrocarbons may inhibited or caused by a death of certain microorganisms. On the other hand, there will also be increasing in numbers of certain microorganisms especially those capable of degrading the hydrocarbons [21].

In the present study, the isolated species of oil degrading bacteria were subjected to different periods of UV light. The UV radiation was commonly used to generate mutant strains of microorganisms. It was less energetic than X–and gamma rays (ionizing radiation), but its wavelengths were preferentially absorbed by nucleotides of DNA and by aromatic amino acids of proteins, so it has important biological and genetic effects [22].

Four species of oil degrading bacteria (*B. pumilis*, *B. subtilis*, *B. polymyxa* and *P. putida*) were subjected to UV radiation. The first two species were only mutated and one species (*P. putida*) was exhibited a high ability to degrade crude oil.

[23] reported that UV light damaged the cells DNA, while DNA repair mechanisms corrected this damage. Mutations occurred. UV light stimulated adjacent pyrimidine bases, usually thymines, in DNA to react with one another, becoming linked to form a thymine dimmer (a dimmer is a chemical compound composed of two identical parts). This damage activates several repair systems, one of which allowed DNA replication across the damaged region at the cost of accuracy, the resulting errors were mutations.

The microbial biodegradation in this investigation was studied in a small scale experimental jars contained the soils from Al–Rumella region. Basrah regular crude oil was subjected into soils contained jars to degradation by indigenous microorganisms and exogenous microorganisms (i.e. axenic cultures of bacteria and fungi, a mixed cultures of bacteria and fungi and a mixed cultures of mutant bacteria and fungi) were applied. The optimum conditions of temperature, nutrients and adequate aeration were available. The conclusions for the present study were based on the oil degrading microorganisms density, the percentage loss of crude oil concentration, and CO_2 released during the biodegradation.

It was observed that the density of crude oil had increased in all soil experimental jars with time. This increasing in density of crude oil was suggestive of decreasing in the low molecular weight hydrocarbons and to increasing of asphaltenes mainly due to evaporation, chemical oxidation , adsorption , leaching and microbial degradation. The same conclusion was arrived by [24].

In the present study, a significant differences in the numbers of oil degrading bacteria and fungi were evident among the time of biodegradation (P>0.05) (Table 5 and 7). [24] had shown that a change in the number of microbes during biodegradation was the simplest way to measure their activity. A higher numbers of bacteria and fungi were observed in the soil after 28 days of incubation (Table 6 and 8). This indicated that these microorganisms had adapted to degrade the petroleum hydrocarbons. Such conclusion had also been previously reported by [25].

Bacteria utilized the crude oil as a sole source of carbon and energy. In contrast, fungi did not utilize the petroleum hydrocarbons as their sole source of carbon and energy but transformed them co–metabolically to detoxified chemical products [7]. In spite the fact that the microorganisms counts were useful index to microbial activity, they suffered from some difficulties. Since the growth conditions available in the present study were different from those present in the natural environment. Therefore, it was difficult to interpret the counts in the terms of natural situation. However, the microbial counts were a direct indicator of petroleum biodegradation activity [26]. It had been shown that the numbers of oil degrading fungi were more than the numbers of oil degrading bacteria in the soil of present study (Table 3 and 4) . This may indicate that fungi are more active than bacteria in the biodegradation of petroleum hydrocarbons. The same result was obtained by [27] in their study on the changes in the flora of soil fungi following oil waste application, and study of [28] on the biodegradation potential of hydrocarbon–assimilating tropical fungi. They had been shown that fungi were as active or more active than bacteria in the biodegradation of hydrocarbons. Whereas, [29] found that the individual bacteria strains were the most active than fungi in the biodegradation of fuel oil in a temperate agricultural soil in France.

The residual crude oil resulting from biodegradation was measured by fluorescence spectrophotometric technique. Fluorescence was a simple, rapid and sensitive technique that had been widely used to determine the rates of microbial degradations of oils by [4, 12, 30]. Chemical compounds present in oil can absorb radiant energy at certain wavelength and remitted energy at anther wavelength. The absorbed energy was of a shorter wavelength and therefore a higher energy than the emitted fluorescence energy. Because of the large variety of compounds present in oil, a unique fluorescent emission finger–prints can be obtained when an oil was exposed to radiant energy [31].

The results showed that the axenic cultures of fungi degraded the crude oil in soil better than the axenic cultures of bacteria. The highest percentage loss of crude oil concentration by the axenic cultures of fungi was 76% (by the fungus *P. chrysogenum*) after a month period of biodegradation Table (3 and 4). Whereas was 65% for the axenic cultures of bacteria (by the species *P. putida*) in the same period (Table 3 and 4). The mixed cultures of bacteria and fungi found to degrade the crude oil in similar soil more than the axenic cultures of bacteria and fungi. The highest percentage loss of crude oil concentration by the mixed cultures of bacteria and fungi was 87% (by the species *P. putida*+*P. chrysogenum*) after a mouth period of biodegradation (Table 9). The mixed cultures of mutant bacteria and fungus [*P. putida* (mutant)+*P. chrysogenum*] was identified to be capable of rehabilitating the oil polluted soil better than another cultures. The ability of the cultures to degrade crude oil within reasonably desired period (30 days) was observed 97% (Table 9).

The CO_2 released is proportional with increasing in microbial numbers as well as the degrading capacity of the microorganisms (Table 3, 4 and 9). This confirmed the utilization of crude oil by microorganisms. The same conclusion was arrived by [3] and [32].

Conclusions:

1- The oil degrading microbes isolated from the soil of the present study were not restricted to one or few species and genera. They included *Staphylococcus* sp., *Arthrobacter* sp., *Micrococcus* sp., *Corynebacterium* sp., *Streptomyces* sp., *Bacillus pumilis*, *B. subtilis*, *B. polymyxa*, *Pseudomonas putida*, *Aremonas* sp., *Nocardia* sp. (bacteria), *Candida* sp., *Rhizopus* sp., *Psidxybe cubensis*, *Trichoderma* sp., *Aspergillus niger*, *Pencillum* sp., *P. chrysogenum*, *Cunninghamella* sp. and *Ssccharomyces cerevisiae* (fungi). The density of oil degrading fungi were more than the oil degrading bacteria. The microbes were well adapted to degrade and utilize the crude oil.
2- The mount of CO_2 produced was relatively proportion with increasing of density and biodegradation ability of oil degrading microorganisms.
3- The axenic cultures of fungi degraded the crude oil better than the axenic cultures of bacteria. Whereas, their mixed together in cultures to accelerate the biodegradation of crude oil. The mixed cultures of mutant strain of bacteria and fungus [*P. putida* (mutant)+*P. chrysogenum*] given the greatest capability to degrade the crude oil.
4- Rehabilitation of oil contaminated soil by the culture of mutant bacteria and fungus [*P. putida* (mutant)+*P. chrysogenum*] was promising as it can reduce the oil pollution to acceptable levels (97%) for reuse of land within a short period.

Table (2): Oil degrading bacteria and fungi isolated from Al–Rumella region.

Bacteria	Fungi
Staphylococcus sp. *Arthrobacter* sp. *Micrococcus* sp. *Corynebacterium* sp. *Streptomyces* sp. *Bacillus pumilis* *B. subtilis* *B. polymyxa* *P. putida* *Aremonas* sp. *Nocardia* sp.	*Candida* sp. *Rhizopus* sp. *P. cubensis* *Trichoderma* sp. *A. niger* *Penicillum* sp. *P. chrysogenum* *Cunninghamella* sp. *S. cerevisiae*

Table (3): The percentage loss of crude oil, the amount of CO_2 released and the number of oil degrading bacteria (cfu/10g) and control during four weeks of incubation.

Culture	First week			Second week			Third week			Fourth week		
	No. of ODB	%loss of crude oil	CO_2 mg	No. of ODB	%loss of crude oil	CO_2 mg	No. of ODB	%loss of crude oil	CO_2 mg	No. of ODB	%loss of crude oil	CO_2 mg
***Arthrobacter* sp.**	$2.2x10^3$	25	19.0	$2.31x10^4$	36	27.7	$1.7x10^5$	53	35.4	$1.2x10^6$	58	47.7
***Streptomyces* sp.**	$9.0x10^3$	27	23.3	$9.2x10^4$	38	33.3	$3.1x10^5$	56	41.3	$9.3x10^6$	60	48.2
B. pumilis	$5.1x10^4$	30	25.2	$6.2x10^5$	40	38.3	$5.3x10^6$	58	45.2	$0.3x10^7$	63	50.4
P. putida	$9.3x10^4$	33	30.1	$8.1x10^5$	42	40.1	$9.2x10^6$	60	49.3	$8.1x10^7$	65	52.2
Control-*P. putida* (without oil)	$3.2x10^2$	---	1.1	$2.2x10^2$	---	2.3	$2.0x10^2$	---	3.5	$1.0x10^2$	---	3.6
Control-crude oil (without culture)	---	4	1.2	---	5	2.5	---	7	3.7	---	9	5.2

ODB=Oil degrading bacteria

Table (4): The percentage loss of crude oil, the amount of CO_2 released and the number of oil degrading fungi (cfu/10g) and control during four weeks of incubation.

Culture	First week			Second week			Third week			Fourth week		
	No. of ODF	%loss of crude oil	CO_2 mg	No. of ODF	%loss of crude oil	CO_2 mg	No. of ODF	%loss of crude oil	CO_2 mg	No. of ODF	%loss of crude oil	CO_2 mg
***Rhizopus* sp.**	$7.2x10^3$	38	31.0	$3.2x10^4$	49	39.1	$1.2x10^5$	58	44.0	$1.2x10^6$	69	50.9
P. cubensis	$3.3x10^4$	41	33.2	$4.7x10^5$	55	41.7	$8.3x10^6$	63	48.2	$9.2x10^7$	71	53.1
A. niger	$9.7x10^4$	45	34.0	$9.2x10^5$	58	44.9	$5.7x10^6$	68	49.0	$8.3x10^7$	74	57.2
P. chrysogenum	$1.1x10^5$	50	37.0	$13.7x10^6$	61	47.6	$6.9x10^7$	72	53.7	$2.1x10^8$	76	60.8
Control-*P. chrysogenum* (without oil)	$3.5x10^2$	---	1.9	$2.5x10^2$	---	2.7	$2.3x10^2$	---	4.0	$1.2x10^2$	---	4.2
Control-crude oil (without culture)	---	5	1.7	---	7	3.1	---	11	3.8	---	12	4.9

ODF=Oil degrading fungi

Table (5): The one layout analysis variance of number of oil degrading bacteria.

Source of variance	Degree of freedom	Sum of square	Mean of square	F
Among time of incubation	3	4.98	1.66	13.83*
Error	9	1.10	0.12	
Total	12			

Table (6): The RLSD test for detecting differences in means of number of oil degrading bacteria.

Time of incubation (week)	Mean
First	4.2212×10^4 D
Second	2.8032×10^5 C
Third	8.8442×10^5 B
Fourth	4.7480×10^6 A

Table (7): The one layout analysis variance of number of oil degrading fungi.

Source of variance	Degree of freedom	Sum of square	Mean of square	F
Among time of incubation	3	7.26	2.42	11.00*
Error	9	2.02	0.22	
Total	12			

Table (8): The RLSD test for detecting differences in means of number of oil degrading fungi.

Time of incubation (week)	Mean
First	4.7726×10^4 D
Second	8.2400×10^5 C
Third	2.9880×10^6 B
Fourth	1.8242×10^7 A

Table (9): The percentage loss of crude oil and the amount of CO_2 released during four weeks of incubation of mutant bacteria, a mixed cultures of bacteria and fungi, a mixed cultures of mutant bacteria and fungus and control.

Culture	First week		Second week		Third week		Fourth week	
	%loss of crude oil	CO_2 mg	%loss of crude oil	CO_2 mg	%loss of crude oil	CO_2 mg	%loss of crude oil	CO_2 mg
P. putida **(mutant)**	50	92.2	68	130.3	70	241.9	79	310.0
Arthrobacter **sp.** + ***Rhizopus*** **sp.**	56	143.3	68	262.0	72	355.2	80	411.5
Streptomyces **sp.** + ***P. cubensis***	60	144.0	71	271.1	76	330.7	82	414.3
B. pumilis + ***A. niger***	62	150.0	73	274.7	80	395.3	85	418.2
P. putida + ***P. chrysogenum***	66	155.1	75	347.2	82	400.8	87	450.9
P. putida **(mutant)** + ***P. chrysogenum***	72	176.3	80	370.9	91	444.9	97	522.8
Control-*P. putida* (mutant)+ *P. chrysogenum* (without oil)	0	1.2	0	2.5	0	4.6	0	5.2
Control-crude oil (without culture)	5	1.0	8	2.1	9	4.5	11	6.3

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