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Isolation and identification of Bacteria in Sarir Refinery Wastewater in Libya

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ABSTRACT: Forty isolates of bacteria were isolated from the wastewater system of Sarir Refinery. All isolates were cultivated in liquid media with crude oil as a sole carbon and energy source.

Four bacteria isolates (D2, S3, SI1 and SI2) were identified as *Cellulosimicrobium cellulans*, *Brevibacterium liquefaciens*, *Brevibacterium mcbrellneri* and *Enterococcus saccharolyticus*, respectively.

The results concluded that *C. cellulans* (D2), *E. saccharolyticus* (SI2) and *B. liquefaciens* (S3) have the potential to be used in bioremediation of crude oil because they showed good growth on hydrocarbons.

INTRODUCTION: Crude oil hydrocarbons may be discharged to soil as a result of accidental spillage (e.g., pipeline ruptures, tank failures) or aerial deposition of partially combusted oil particles (Wang and Bartha, 1990). Estimates suggest that there are between 100,000 and 300,000 leaking petroleum and petroleum based product tanks in the USA. It has been estimated that there are 350,000 contaminated sites in Europe, of which the largest proportion are contaminated by petroleum products (Scragg, 2005). Oily wastes may also be deliberately spread on soil under controlled conditions, such as in land farming. Crude oil is physically, chemically and biologically harmful to soil because it contains many toxic compounds in relatively high concentrations (Francoa *et al.*, 2004).

Petroleum refining involves the transformation of crude oil into final useful products such as gasoline, gas oil, kerosene and jet fuel, and petrochemical feed stocks. After initial crude desalting and fractionation, several treatment and conversion processes are employed to reach the final blending stocks. Treatment processes on the other hand include naphtha and gas oil desulfurization, sour water strippers and catalyst regeneration units. Petroleum refining uses relatively large quantities of water, especially for cooling systems, desalting water, stripping steam, and water used for flushing during maintenance and shut down (Dold, 1989). The quantity of wastewater generated and their characteristics depend on the process configuration. As a general rule, approximately 3.5–5m^3 of wastewater are generated per ton of crude oil processed when cooling water is recycled (Dold, 1989).

Almost a century has passed since the first hydrocarbon-degrading bacteria were isolated and described, and the most recent list includes almost 200 bacterial, cyanobacterial, algal

and fungal genera, representing more than 500 species and strains (Prince *et al.*, 2003; Head *et al.*, 2006). In many ecosystems there is already an adequate indigenous microbial community.

None of the microorganisms are able to utilize all hydrocarbons; so each organism can utilize only a certain range of compounds (Alvarez, 2003).

The ability to degrade petroleum hydrocarbons is not restricted to a few microbial genera; a diverse group of bacteria and fungi have been shown to have this ability, and in review noted that more than 100 species representing 30 microbial genera had been shown to be capable of utilizing hydrocarbons (Atlas, 1981).

The most common hydrocarbon-degrading organisms in the marine environment are *Pseudomonas, Acinetobacter, Nocardia, Vibro,* and *Achromobacter.*

Verma *et al.* (2006) reported that there are differences in the relative ability of the *Acinetobacter, Bacillus* and *Pseudomonas* strains to degrade hydrocarbon and the ability of these strains to degrade hydrocarbons in oily sludge suggests that they could be used for the treatment of other oil-wastes.

Bartha and Atlas (1973) reported that a *Flavobacterium* sp. and a *Brevibavterium* sp. were able to mineralize *n*-paraffins which found as the best substrates for both organisms, but *Flavobacterium* sp. exhibited higher rates of mineralization. However, branching prevented utilization by *Flavobacterium* sp. but not by *Brevibavterium* sp.

Many reports mentioned that the bacterial strains belonged to the genera *Gordonia, Brevibacterium, Aeromicrobium, Dietzia, Burkholderia* and *Mycobacterium* are new and not yet described which were isolated from petroleum-polluted soil (Chaillan *et al.*, 2004).

This study was aimed to isolate and identify the hydrocarbon utilizing bacteria from Sarir Refinary wastewater in Libya.

MATERIALS AND METHODS

Sampling locations

Eleven water samples and sludge were collected from the wastewater system of Sarir Refinery Picture (1) with the following characters:

Two samples were collected from the effluents of wastewater and coded as A1 and A1.1, three samples were collected from the drying beds (upper layer of water level) and coded as B1, B1.1 and B1.2, two samples were collected from the bottom of evaporation ponds and coded as C1 and C1.1, and four samples were collected from the bottom of landfill that receives wastewater and coded as D1, D1.3, D1.4 and Soil.

Sampling

Samples were collected in sterile bottles and kept cool until they were used for the isolation process.

Isolation of hydrocarbon degrading bacteria

Samples collected from different locations (water, soil, and sludge), were used as inoculums in the isolation process.

One gram from collected soil and sludge samples was taken and added to 30 milliliters of sterilized water and shaken well, then a aliquit was taken from each sample for the isolation process.

Two kinds of techniques were used to select the bacterial isolates capable of degrading hydrocarbons (crude oil) and the details are as follows:

Direct Method

Ten ml of the basal medium employed for this study consisted of (g/L); 0.5 K_2HPO_4, 1.0 NH_4Cl, 2.0 Na_2SO_4, 2.0 KNO_3 and 0.2 $MgSO_4.7H_2O$ (Foght *et al.,* 1990), was piptted in Universal bottles of 30 ml volume and then supplemented with 0.01ml of 0.1% (v/v) crude oil as the sole carbon source. Bottles were sterilized by autoclaving at 121°C for 15 minutes. One ml from each sample was taken and added to the bottles, then bottles were incubated at 30°C ± 2°C. The growth of bacteria was monitored daily for 5 days and 0.1 ml of medium was taken from each bottles, that showed good growth and spreaded on nutrient agar plates. Colonies of different appearance were selected, and successive streaked onto nutrient agar plates to obtain pure culture from each strain.

Enrichment method

This method is similar to the direct method but in this method the cultures were incubated at 30°C ± 2°C in shaker at 100 rpm. The procedure was repeated two times to select cultures that have the best growth.

The growth of bacteria was monitored daily for 5 days and one ml of medium was taken from bottles that have a good growth and spreaded on nutrient agar plates.

Colonies of different appearance were selected and successive streaking of these isolates onto nutrient agar plates allowed giving the isolation of pure cultures.

Maintenance of stock cultures

The stock cultures of bacterial isolates (40) were maintained on nutrient agar slants for a short-term period and the other techniques using glycerol and nutrient broth medium for long-term period of maintenance at -20°C.

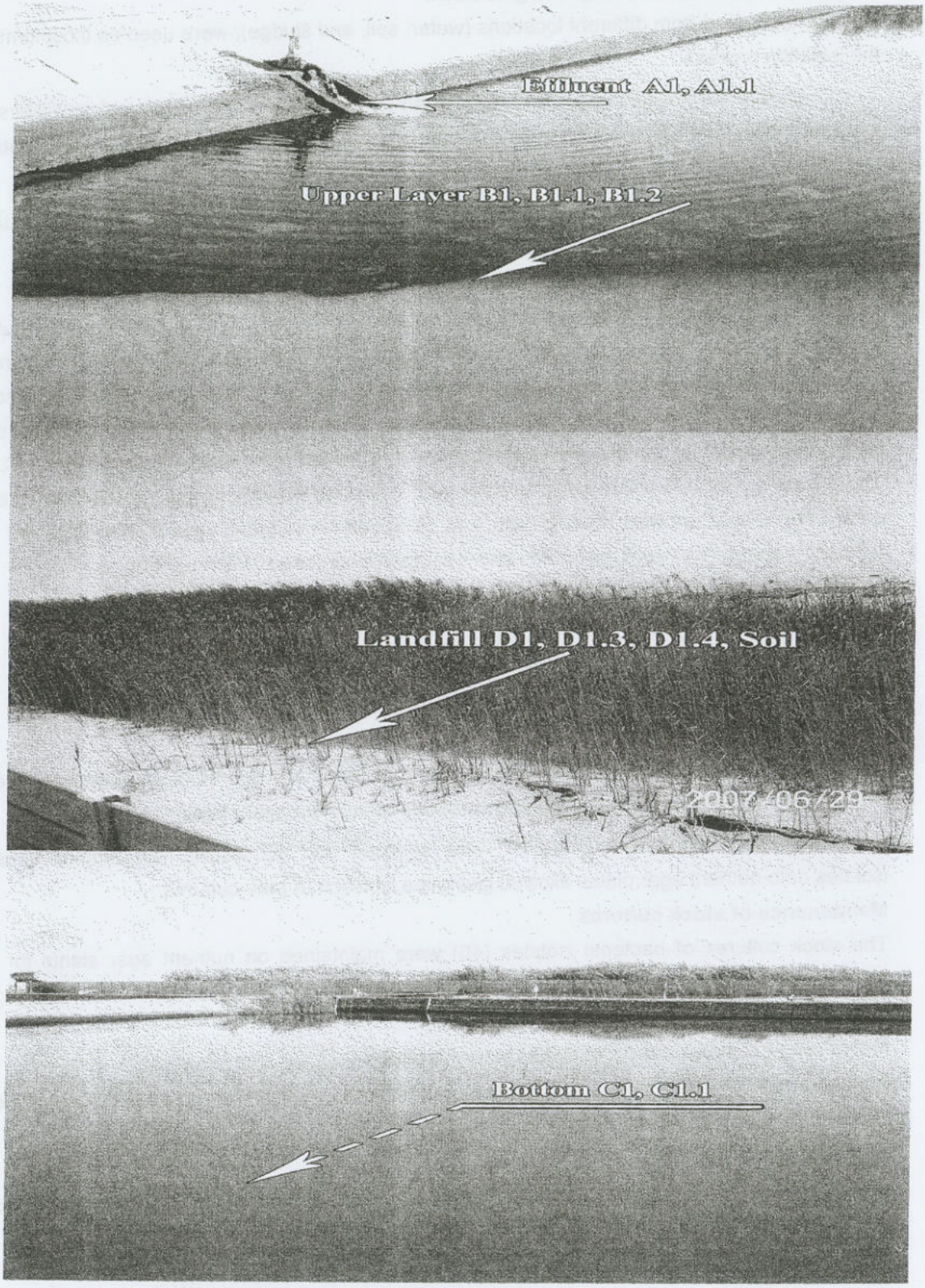
Morphological examination of the bacterial isolates

Purified bacterial isolates were examined morphologically by using a spread-plate technique, Gram stain and oxidase test (Harley and Prescott, 1993).

Bacterial identification

Among the 40 isolates of bacteria only fourteen had specified growth on crude oil. Four of them were chosen for further study and identification.

The identification was done in the Agricultural Research Center, Cairo, Egypt.



Picture 1: The locations of collected samples.

The BioLog3 microbial identification system was applied to identify the bacteria strains which included incubator, analyzer, computer, printer, and software to read the Biolog Micro Plates.

Growth of the isolated bacterial strains on Bushnell-Hass agar containing 1% crude oil

To investigate the capability of bacterial isolates to degrade crude oil, they were cultured on Bushnell-Hass medium (HIMEDIA) (Okoh *et al.*, 2001) which is used as a selective medium for hydrocarbons degrading bacteria.

The forty bacterial isolates were streaked onto nutrient agar plates and incubated at 30°C ±1°C for 18-24h, then they were streaked onto Bushnell-Hass agar medium supplemented with 1 % (v/v) crude oil and incubated at 30°C for three days. Visual monitoring of growth of bacteria was daily done.

RESULTS

Isolation of crude oil degrading bacteria

Direct method was applied to select the bacteria, which have the ability to degrade crude oil. Bacteria isolates were isolated from eleven samples presented in Table (1). Four samples showed very good growth and one sample shown good growth and four samples were found to have a weak growth and the rest (A1 and A1.1) gave negative results.

Enrichment method

Enrichment method was applied to select the efficient bacteria able to grow on and degrade crude oil which were carried out three times. The final results of enrichment method presented in Tables (1) and showed that four samples have very good growth and two samples showed good growth and three samples showed weak growth. Samples A1 and A1.1 did not show any significant growth.

Purification of isolated bacteria

After the direct and enrichment methods, suspensions of samples were used to inoculate duplicate on nutrient agar plates. Separated single colony were obtained to be used for maintenance of cultures.

Forty colonies with different microbial morphology were isolated from different locations of Sarir Refinery wastewater as shown in Table (1) using direct and enrichment method. Isolates were stored in slant agar and 30% of glycerol and refrigerated at -20°C.

Designation of isolates

Bacteria isolates were designated based on the locations of samples; twelve bacteria were isolated from the upper level of an drying bed (designated as B1 to B12), eight bacteria were isolated from the sludge in the drying bed (designated as C1 to C8), ten bacteria were isolated from the bottom of landfill (designated as D1A to D9) and ten bacteria were isolated from the soil of landfill (six designated as S1 to S6 and four designated as E1, E2, SI1 and SI2).

Designation of isolates

Bacteria isolates were designated based on the locations of samples; twelve bacteria were isolated from the upper level of an drying bed (designated as B1 to B12), eight bacteria were isolated from the sludge in the drying bed (designated as C1 to C8), ten bacteria were isolated

from the bottom of landfill (designated as D1A to D9) and ten bacteria were isolated from the soil of landfill (six designated as S1 to S6 and four designated as E1, E2, SI1 and SI2).

Table 1: Bacteria isolated from direct and enrichment methods.

Samples	Purified isolated bacteria from direct method		Purified isolated bacteria from enrichment method	
	Number	Designated	Number	Designated
A1	0	Nil	o	Nil
A1.1	0	Nil	0	Nil
B1	2	B1& B2	2	B8 & B9
B1.1	3	B3, B4 & B5	2	B10 & B11
B1.2	2	B6 & B7	1	B12
C1	2	C1 & C2	2	C5 & C6
C1.1	2	C3 & C4	2	C7 & C8
D1	2	D1A & D1B	1	D6
D1.3	1	D2	1	D7
D1.4	3	D3, D4 & D5	2	D8 & D9
Soil	7	S1, S2, S3, E1, E2, SI1 & SI2	3	S4, S5 & S6
Total	24	Total	16	

Characteristics of bacterial colonies

Gram stain was used as a differential procedure to distinguish between Gram-positive and Gram-negative bacteria. Table (2) presented the results of Gram stain and oxidase tests. 90% and 10% of isolates were classified as Gram - positive and Gram- negative, respectively. On the other hand, 47.5% and 52.5% were oxidase positive and negative, respectively.

Morphology of fortybacterial colonies

Bacterial isolates were morphologically analysed after performing the Gram- stain test. The shape, form and elevation of bacteria were observed and the results are demonstrated in Table (2).

Growth of forty bacterial isolates on Basal agar medium

The bacteria isolates were streaked onto basal agar medium plates which were supplemented with crude oil 1% (v/v) as a sole carbon source, to evaluate its ability to grow in the presence of crude oil.

Growth was monitored for three days (Table 4) and the results show that out of forty bacteria isolates, thirty-four isolates were able to grow whereas six isolates namely, B2, B4, B8, C6, D1B and S2 failed to grow a period of 72h of incubation.

Growth of forty isolates on Bushnell-Hass agar medium

To select the isolates, which have the ability to grow in the presence of crude oil, all isolates, were streaked onto Bushnell-Hass agar medium plates supplemented

with 1% (v/v) as a sole carbon source. The plates were incubated at 30°C for three days and the growth was monitored visually.

Results in Table (3) indicate that thirty two bacteria isolates were able to grow on Bushnell-Hass agar medium and the rest showed no growth.

Selection of the best bacteria isolates

Fourteen bacteria isolates were chosen for further study with different hydrocarbon compounds as a sole carbon and energy source, and based on their ability to grow in different concentrations of crude oil.

After the evaluation of the results four bacteria isolates, (D2, S3, SI1 and SI2) were selected to be identified and used for further studies.

Identification of the four bacterial isolates

The selected bacteria isolates were identified by using BioLog3 microbial identification system by Agricultural Research Center in Cairo, Egypt and Table (4).

Table 2: Morphological and Bacterial characteristics of forty isolates

Bacterial isolates	Bacterial morphology			Bacterial Characteristics	
	Shape	Form	Elevation	Gram stain	Oxidase
B1	Rod	Circular	Convex	Negative	Negative
B2	Coccus	Irregular	Convex	Positive	Negative
B3	Rod	Circular	Convex	Positive	Positive
B4	Rod	Irregular	Convex	Negative	Positive
B5	Rod	Irregular	Umbonate	Negative	Negative
B6	Coccus	Irregular	Pulvinate	Positive	Negative
B7	Coccus	Irregular	Convex	Negative	Positive
B8	Rod	Circular	Convex	Positive	Negative
B9	Coccus	Irregular	Convex	Positive	Positive
B10	Coccus	Irregular	Pulvinate	Positive	Negative
B11	Coccus	Irregular	Umbonate	Positive	Negative
B12	Coccus	Irregular	Convex	Positive	Positive
C1	Coccus	Irregular	Convex	Positive	Positive
C2	Coccus	Irregular	Pulvinate	Positive	Negative
C3	Coccus	Irregular	Umbonate	Positive	Positive
C4	Coccus	Irregular	Umbonate	Positive	Positive
C5	Coccus	Irregular	Undulate	Positive	Positive
C6	Coccus	Circular	Pulvinate	Positive	Negative
C7	Coccus	Irregular	Convex	Positive	Positive
C8	Coccus	Irregular	Umbonate	Positive	Positive
D1A	Coccus	Circular	Pulvinate	Positive	Positive
D1B	Coccus	Circular	Pulvinate	Positive	Positive
D2	Rod	Circular	Convex	Positive	Positive
D3	Coccus	Circular	Pulvinate	Positive	Negative
D4	Coccus	Circular	Convex	Positive	Positive
D5	Coccus	Circular	Convex	Positive	Negative
D6	Coccus	Irregular	Umbonate	Positive	Positive
D7	Coccus	Circular	Convex	Positive	Negative
D8	Coccus	Irregular	Convex	Positive	Negative
D9	Rod	Irregular	Umbonate	Positive	Positive
S1	Coccus	Irregular	Umbonate	Positive	Negative
S2	Coccus	Irregular	Pulvinate	Positive	Negative
S3	Rod	Circular	Pulvinate	Positive	Negative
S4	Rod	Irregular	Convex	Positive	Positive
S5	Coccus	Circular	Umbonate	Positive	Positive
S6	Coccus	Irregular	Umbonate	Positive	Negative
E1	Coccus	Circular	Convex	Positive	Negative
E2	Coccus	Irregular	Pulvinate	Positive	Negative
SI1	Rod	Irregular	Umbonate	Positive	Negative
SI2	Coccus	Circular	Pulvinate	Positive	Negative

Table 3: Growth of the forty isolates in Basal agar medium and Bushnell-Hass agar medium with **1%** (v/v) crude oil at 30 °C after 72h.

Isolates	Basal agar medium	Bushnell-Hass agar medium	Isolates	Basal agar medium	Bushnell-Hass agar medium
B1	G	N	D1A	G	G
B2	N	N	D1B	N	N
B3	G	G	D2	G	G
B4	N	N	D3	G	G
B5	G	G	D4	G	G
B6	G	G	D5	G	G
B7	G	G	D6	G	G
B8	N	N	D7	G	G
B9	G	G	D8	G	G
B10	G	N	D9	G	N
B11	G	G	S1	G	G
B12	G	G	S2	N	N
C1	G	N	S3	G	G
C2	G	G	S4	G	G
C3	G	N	S5	G	G
C4	G	G	S6	G	G
C5	G	G	E1	G	G
C6	N	N	E2	G	N
C7	G	G	SI1	G	G
C8	G	N	SI2	G	G

N - No growth, G - Growth

Table 4: Identification of selected bacteria

Isolate Designated	Identified Bacteria Isolates
D2	*Cellulosimicrobium cellulans*
S3	*Brevibacterium liquefaciens*
SI1	*Brevibacterium mcbrellneri*
SI2	*Enterococcus saccharolyticus*

DISCUSSION

The fate of petroleum hydrocarbons in the environment is largely controlled by a biotic factors which influence the rates of microbial growth and enzymatic activities that determine the petroleum hydrocarbon utilization. The ability to isolate high numbers of certain oil-degrading microorganisms from oil-polluted environment is commonly taken as evidence that these microorganisms are the active degraders of that environment (Ojo, 2006). Discharging of wastewaters without treatment into a natural water body is undesirable, since it can deteriorate the water quality of the receiving water body (Pereira *et al.,* 1998; Carmen *et al.*, 1990). Isolation, identification, and genetic manipulation of a vast number of indigenous bacterial species for bioremediation of pollutants have been the focus of many investigations worldwide (Van Hamme *et al.*, 2003; Ghazali *et al.*, 2004).

The present work deals with the isolation bacterial isolates from the wastewater of Sarir Refinery which contains a small amount of crude oil discharged to the soil. Forty bacteria were isolated. Different experiments were planned to reach the best available bacteria isolates which could help in the bioremediation of oil.

In the initial screening, the number of bacteria isolated from the enrichment method is less than that from the direct procedure and these results confirmed the fact that repeated exposure to petroleum products at a site will usually increase the adaptive capabilities of the microorganisms, though it increases the rate of degradation with a new exposure to a compound. This results are agree with Miyagi *et al.* (2001) they reported that 67 strains were isolated. The strains of 38 (56.7%) were isolated only by direct plating, and 25 (37.3%) strains were isolated by both direct plating and after enrichment. Four strains (6·0%) were isolated after enrichment but not by direct plating.

The morphology and type of bacterial colonies were also investigated in cell counts experiments. One of the objectives of this study was to isolate as many colourable strains as possible in order to determine their hydrocarbon biodegradation potential in standardized culture conditions. For this reason, a first screening of isolates was done after Gram staining and microscopic examination for bacteria to eliminate apparently similar isolates.

The growth ability of forty bacteria isolates on selective mediums was performed using basal medium agar and Bushnell-Hass agar which were supplemented with1% (v/v) crude oil as a sole carbon source and energy and the results showed that 85% and 80% of bacteria isolates were grown on Basal medium and Bushnell-Hass medium, which are the selected media for hydrocarbon degrading bacteria (Okoh *et al.,* 2001), respectively.

Bacterial isolate D2, S3, SI1 and SI2 were identified as *Cellulosimicrobium cellulans*, *Brevibacterium liquefaciens, Brevibacterium mcbrellneri* and *Enterococcus saccharolyticus* respectively.

Four bacteria strain were screened with a selective medium of PAHs from oily wastewater and were identified and found mostly similar to that strains of *Microbacterium esteraromaticum*, *Cellulosimicrobium cellulans*, *Chelatococcus asacharoeorans* and *Sphingopyxis tarra* (Wang *et al.*, 2007).

Bioremediation is the use of biological systems to destroy or reduce the concentrations of hazardous wastes from contaminated sites. Such systems have the potential site applications include ground water, soils, lagoons, sludge and process waste-streams. Many studies articles have documented the potentials of microorganisms to degrade oil in both the laboratory and field trials. A number of scientific papers including several review articles covered aspects of the biodegradation process as well as results from controlled field experiments designed to evaluate degradation rates in various environments (Gunkel and Gassmann, 1980). Crude oil is a complex but biodegradable mixture of hydrocarbons, and the observation that hydrocarbon degrades can be enriched in many, if not most, types of environments have contributed to the development of oil bioremediation techniques (Margesin and Schinner, 1997).

These experiments showed promising hydrocarbon degrading ability in bacteria isolates. Because our ultimate goal was to select isolates with the ability to utilize hydrocarbons, eventually many complex substrates such as crude oil and industrial organic waste, we chose the four most successful isolates for further investigation. The four isolates are isolates *C. cellulans*, *B. liquefaciens, B. mcbrellneri* and *E. saccharolyticus* were selected due to their increased ability to grow on hydrocarbons in batch experiments.

CONCLUSIONS

In this study, bacteria that are able to growth on crude oil as a carbon and energy source were isolated from the evaporation ponds and landfill of wastewater system of Sarir Refinery. The selective direct and enrichment techniques were used for selecting hydrocarbon degrading bacteria. Growth of the isolated bacteria on crude oil in basal medium liquid culture demonstrated the hydrocarbon and oil-degrading activities of isolated bacterial strains.

This system will allow performing degradation of complex mixture of petroleum hydrocarbons, industrial waste and other contaminates by bacterial blend of *C. cellulans* (D2), *B. liquefaciens* (S3) and *E. saccharolyticus* (SI2) with different levels of process control and optimization.

Further research should focus on the factors affecting the ability and efficiency of hydrocarbon and crude oil degradation, such as nutrient concentration, optimum temperature range, oxygen content, salinity and physical state of the oil and to investigation of the biodegradation capability of crude oil hydrocarbons and industrial waste, by using bacterial inocula containing combinations of more than one isolate.

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عزل وتعريف بعض العزلات البكتيرية من المياه العادمة بمنطقة سرير – ليبيا

1- فرج محمد شعيب و 2- عبد الحميد حسن الغزاوني

1-قسم النبات- كلية العلوم- جامعة عمر المختار – البيضاء و(2) حقل بترول سرير- شركة الحليج العربي للبترول- بنغازي- ليبيا

تم عزل 40 عزلة من البكتريا المحللة للهيدروكربونات وذلك من المياه العادمة بمنقطة السرير- ليبيا وأظهرت النتائج أن جميع العزلات البكترية لها القدرة على النمو في البيئات السائلة التي تحتوي على النفط الخام كمصدر وحيد للكربون والطاقة . كم تما تعريف عدد 4 عزلات بكترية (S12, S11, S3, D2) وذلك على الترتيب :-

Cellulosimicrobium cellulans, Brevibacterium liquefaciens, Brevibacterium mcbrellneri , Enterococcus saccharolyticus,

ومن النتائج المتحصل عليها يمكن استنتاج أن العزلات البكترية :-

Cellulosimicrobium cellulans, Brevibacterium mcbrellneri , Enterococcus saccharolyticus,

ويمكن استخدامهم في مجال التحلل الحيوي للنفط الخام.