ISOLATION AND CHARACTERIZATION OF CRUDE OIL-DEGRADING BACTERIA FROM PETROLEUM OIL CONTAMINATED EGYPTIAN SOILS

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ABSTRACT

Eleven bacterial strains were isolated from two different oil contaminated Egyptian soils. They were characterized according to their phenotypic examination as species of the genus *Bacillus.* These bacterial populations showed counts ranging between 8 and 10 log CFU/g soil on the agar plates. An experimental study was undertaken to assess the efficiency of these bacterial isolates in degradation of the petroleum oil in their culture medium amended with 1% v/v petroleum oil. Results revealed that both CS1.1 and CS1.7 are the most effective isolates to degrade two crude oils from Suez and Agiba Company for the first time by growing the individual isolates separately on oil agar media. In addition, CS1.1and CS1.7 exhibited 55.6% and 53.5%, respectively emulsification activity after 48 hrs. incubation time. Also, isolates CS1.1 and CS1.7 could reduce the surface tension to 36.5 ± 0.25mN/m and 37.5 ± 0.45mN/m, respectively in less than 12 hrs. in glucose based media. The gravimetric analysis revealed that both CS1.1 and CS1.7 isolates were able to degrade the petroleum oil and utilize it as a sole carbon source for growth, energy and reproduction. These isolates, CS1.1 and CS1.7 degraded 65% and 64 % of added petroleum oil respectively, over 15 days incubation time. Also, the change in absorbance values of the tested samples from 0.936 O.D. to 1.832 O.D. compared with the control sample 0.815 O.D. indicates the breakdown of the molecular structure of oil and confirms the degradation of crude oil by bacterial isolates. Physiological and biochemical parameters for those the most potent degrading isolates were determined. They were identified as *Bacillus subtilis* and *Bacillus licheniformis.*

Keywords: Bioremediation, crude oil, degrading bacteria, emulsification activity.

INTRODUCTION

Petroleum based products are the major of energy for industry and daily life, leaks and accidental spills occur regularly during the exploration, production, refining, transport and storage of petroleum and petroleum products. The amounts of natural crude oil seepage were estimated to be 600000 metric ton per year **(Kvenvolden and Cooper, 2003).**

Oil spillage in water and soil environments have been a major threat to the ecosystem and human being through the transfer of toxic organic materials such as polycyclic aromatic hydrocarbons (PAHs) into the food chain **(Sei and Fathepure, 2009).** Due to its complicated composition, petroleum has the potential to elicit multiple types of toxic effects. It can cause acute lethal toxicity, sub-lethal chronic toxicity, or both depending on the exposure, dosage, and the organism exposed. Physical and chemical methods like photooxidation, volatilization, chemical oxidation, and bioaccumulation are rarely successful in rapid removal and cleaning up PAHs **(Prince, 1997 and Zhao *et al*., 2008)**. These methods are not safe and cost effective when compared to microbial degradation. Bacteria have long been considered as one of the predominant hydrocarbon degrading agents found in the environment, which are free living and ubiquitous **(Dasgupta *et al*., 2013)**.

Bioremediation is an ecologically sound and state-of-the-art technique that employs natural biological processes to completely eliminate toxic contaminants through biochemical transformation or mineralization. Biodegradation by natural populations of microorganisms like bacteria, fungi or their enzymes represents one of the primary mechanisms by which petroleum pollutants can be return the natural environment altered to its original condition **(Chakraborty *et al*., 2012; Mani and Kumar, 2014)**. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and environmental soundness **(Kumar *et al*., 2011)**.

Among the microorganisms able to grow on hydrocarbons, bacteria remain qualitatively and quantitatively the most active agents **(Bertrand and Mille, 1989; Brooijmans *et al*., 2009; Das and Chandran, 2011)**. Based on the frequency of isolation, the predominant bacterial genera found on this issue are *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Vibrio*, *Flavobacterium*, *Achromobacter*, *Micrococcus*, *Nocardia*, *Bacillus* and *Corynebacteria* **(Leahy and Colwell, 1990; Floodgate, 1995; Adebusoye *et al*., 2007).** Also, **Jalilzadeh *et al*., (2014)** studied the bioremediation ability of *Bacillus subtilis* and *Bacillus cereus* on MTBE, which is one of the oil derivations using environmental-friendly methods in order to remove or reduce oil pollutants and their derivations found in the environment.

The purpose of the present study was to isolate, identify and characterize crude oil utilizing bacterial strains, from oil contaminated soil samples to estimate the biodegradation potential of the most promising strains.

MATERIALS AND METHODS

1. Source of isolation

The soil samples were collected from two different locations contaminated with crude oil spills (each of 100 g). One obtained from local mechanic workshop of Egyptian Petroleum Research Institute (EPRI), Nasr City, Cairo, Egypt, while the other from Suez Oil Processing Company (SOPC), Suez, Egypt. Samples were taken down to 15 cm depth, after discarding the upper 3 cm of the soil surface, sealed separately in a sterile polyethylene bag and transferred immediately to the laboratory. The sieved soils were kept at 4°C until used for isolation of oil-degrading microorganisms (**Saadoun, 2002).**

2. Petroleum crude oil

Two different crude oil samples were used in this study; one oil sludge sample was collected from oil reservoir tanks in Agiba Petroleum Company and the Arabian crude oil sample was obtained from Suez Oil Processing Company. The oil sample (250 ml) was collected in sterile screw cap brown glass bottle, transferred to the laboratory and stored in away from direct sun light after sterilization until use.

3. Physicochemical characteristics of the collected samples:

3.1. Oil contaminated soil samples

The chemical and physical analysis of both soil samples were carried out in the laboratories of Soils, Water and Environment Research Institute (SWERI), Agriculture Research Center (ARC), Giza, pH of the soil samples was determined with a pH meter (3505 Jenway, UK). The other physiochemical testes were determined according to (**Obayori *et al.*, 2008).**

3.2. Petroleum crude oil samples

The general physicochemical characteristics such as, the kinematic viscosity, the sulfur content, molecular weight and wax content of the tested crude oil samples have been determined according to the ASTM, IP and UOP standard methods **(El-Sheshtawy *et al.*, 2017).**

4. Isolation and purification of crude oil degrading bacteria from oil contaminated soil samples:

Isolation of bacteria was done according to the method of **Richard and Vogel (1999)**; **Shahian *et al*. (2012)**. 10gm of each soil was suspended in 90 ml sterile distilled water and incubated for 2hr. Five ml of the supernatant from each sample were added to 250 ml Erlenmeyer flask containing 100 ml of a synthetic Bushnell Hass Mineral Salts medium (BHMS) according to **(Shahian *et al.*, 2012)**. The pH was adjusted 7 and the BHMS medium was supplemented with 1% (v/v) crude oil as the sole source of carbon and energy. The flasks were incubated for 7 days at 30^{o}C on a rotary shaker operating at 150 rpm. Then, 5 ml aliquots from all were transferred to fresh BHMS medium and incubated again for 14days and then for 21 days at the same

conditions. After a series of three subcultures, 0.1ml from each flask was streaked on Tryptone Glucose Yeast Extract (TGY) Agar plates for 24hrs. Phenotypically different colonies were purified on BHMS agar medium for 3-5 days. The procedure was repeated and only isolates that exhibited pronounced growth on crude oil were preserved and kept in the refrigerator at 4 ºC for further characterization (**Maneerat and Phetrong, 2007).**

5. Enumeration of total and hydrocarbon utilizing bacteria in the collected soil samples:

After different time intervals, the enumeration of the total and hydrocarbon bacterial cells according to **Benson (1995) and Khalida *et al.*, (2006)** was implemented by pour plate count technique using TGY agar medium. The cultures were then incubated at 30^{o}C for 24 h. Thereafter, plate count in the range of 30 and 300 colonies were recorded. The bacterial counts were described as log CFU/ml and the CFU (colony forming unit) was calculated from the following equation:

$$\mathbf{CFU/ml} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Volum of plated inoculum}}$$

Where, the dilution factor is defined as the inverse of the dilution bacterial count.

6. Screening of crude oil degradation by bacterial isolates:

6.1. Growth on oil agar medium

Each bacterial isolate was streaked on a plate of oil agar medium (BHMS) containing 1% Suez or Agiba crude oil as a sole source of carbon. The plates were incubated at 35^{o}C. The bacterial growth was determined by visual observation within 3-7 days at. High growth of bacteria was denoted by (+++) indicating high degrading ability; (++) moderate degrading ability; (+) low degrading ability **(Abdel Rahman, 2011).**

6.2. Biosurfactant production ability

Synthesis of biosurfactant by bacterial isolates was determined using Mineral Salt Medium (MSM) with the following composition (g/l): KH_2PO_4 2.0, Na_2HPO_4 2.0, $NaNO_3$ 2.5, NaCl 0.8, KCl, 0.8, $CaCl_2$, 0.2$MgSO_4.7H_2O$ 0.01, $FeSO_4.7H_2O$, 0.001 and 5 ml of a trace element solution. The carbon source (glucose) was added to make a final concentration of 2% (v/v). Glucose is widely used for biosurfactant production by many bacteria **(Haghighat *et al.*, 2008).**The cultivation condition was carried out in 250 ml flasks containing 100 ml medium at 30^{o}C, 150 rpm, for different time intervals (24, 48, 72 and 96 hours). All measurements were made on cell-free broth after centrifugation for 20 min and analyzed at room temperatures.

6.2.1. Oil displacement method

Oil displacement experiment was performed as described by **Morikawa and Hirata (2000)**. To a Petri- dish containing 30 ml of distilled water, a 100 µl of Suez crude petroleum oil was added, forming a thin film on the water surface. Then, 20 µl of cell-free supernatant was gently pipetted into the center of the oil film. After one minute if biosurfactant is present (the sample was +ve), the oil will be displaced with an oil free clearing zone. The diameter of this clearing zone indicates the oil displacement activity of the biosurfactant. Distilled water was employed as the negative control; in which no oil displacement or clear zone was observed, and Triton X-100 was used as the positive control.

6.2.2. Emulsification index (E_{24})

The emulsification activity of the produced biosurfactant in the cell- free supernatant was estimated according to **Cooper and Goldenberg (1987).** This was performed by adding 4 ml of kerosene to 4 ml of cell-free supernatant, vortex at high speed for 5 min, and the mixture was incubated at ambient temperature for 24 hrs. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

6.2.3. Surface tension measurement

The surface tension (ST) of the cell-free culture broth was measured by a digital surface tensiometer (Krüss K6 model). All measurements were performed after dipping the platinum ring in the surfactant solution for a while, in order to attain the equilibrium state. The bacterial supernatant solution was tested at 25^{o}C to evaluate the surface tension of surfactant **(Nitschke and Pastore, 2006)**. The value of surface tension was expressed as mN/m. The validity of estimated ST readings was checked with distilled water before each reading. Fresh un-inoculated MSM was used as a control.

7. Experimental confirmation for determination of the most potent degrading bacterial isolates:

- Preparation of inoculum and biodegradation ability of crude oil by bacterial isolates

Five ml of 24 hrs. nutrient broth culture was inoculated into 50 ml MSM with 2% (v/v) crude oil in 100ml conical flasks set up in duplicates. The control flasks contained the same amount of MSM and crude oil but without organism. The flasks were incubated with shaking (150rpm) at 30°C for 15 days and pH 7.5 **(Obuekwe and Al-Zarban, 1998)**.Total viable count (TVC) of cells was determined by agar plate technique, modified method **(Bao *et al.*, 2012)**. The residual crude oil samples were extracted from different microcosm and determined spectrophotometrically and gravimetrically.

7.1. Bacterial count (Log CFU/ ml) method

The total bacterial counts (TBC) in liquid culture were determined by the plate count method according to **Khalida *et al*. (2006).** The bacterial counts were described as log CFU/ml. The CFU was calculated as mentioned before in **(5).**

7.2. -Loss of degraded oil using UV- Visible spectrophotometer:

The loss of crude oil due to biodegradation by isolated crude-oil utilizing bacteria was measured spectrophotometrically according to the method described by **Odu (1972)**. One ml of 48 hrs. grown culture was inoculated into 50 ml of MSM **(Zajic and Supplisson, 1972)** plus 0.5 ml of used crude oil in 250 ml conical flasks. Control flasks containing the same amount of MSM and crude oil but without bacterial culture were prepared. The flasks were incubated with shaking at 150 rpm for 15 days at 30°C. The residual crude oil was extracted from different microcosm by adding 100 ml of toluene to MSM-oil mixture and reading the optical density of the oil extract at different wavelength until λ_{max} for control sample was determined, using CARY 100 Conc. UV- visible spectrophotometer, varian E103077203.

7.3. Determination of biodegradation percentages for residual crude oil by Gravimetric analysis

At the end of incubation period (15 days), the whole content of each flask (50 ml) was taken to assess the residual hydrocarbons of crude oil. The extraction was carried out using 3ml sample: 1ml chloroform. Samples with chloroform were placed in a separating funnel with continuous shaking, after which the contents were allowed to settle; two layers were formed: watery layer and chloroform layer containing the residual hydrocarbons. The organic layer was dried over anhydrous sodium sulphate and the solvent was removed using rotary evaporator until a constant weight. After chloroform evaporation, the residual oil was quantified gravimetrically. The consumed oil was calculated by subtracting the residual hydrocarbons from the original weight of hydrocarbons **(Panda *et al.*, 2013)**.

8. Identification based on Morphological and biochemical characterization of the most potent bacterial strains

The most potent bacterial isolates which showed higher biodegradation and growth rates on BHMS and MSM media with crude oil as carbon source were cultivated separately on TGY agar plates and incubated for 24 hours at 30°C. Morphological characterization as a preliminary identification was confirmed according to **Romero *et al*. (1988) and Benson (1994)**. They were characterized on the basis of different morphological features as color, form and cell shape **(Holt *et al*., 1994).**Gram stain test was performed for each isolate.

While, the biochemical identification was confirmed using Biomeriux VITEK-2 **(Pincus, 2006).** This was done at Animal Health Research Institute (AHRI), Bacteriology lab., Ministry of Agriculture, Dokki, Giza. The VITEK 2 is an automated microbiology system utilizing growth-based technology. It is a rapid identification method using Gram positive card based on developed biochemical methods and newly developed substrates that measure carbon source which utilize

enzymatic activities and resistance. There was one negative control well and 46 biochemical tests giving final results in approximately 10 hours or more **(Chatzigeorgiou *et al.*, 2011).**

RESULTS AND DISCUSSION

1. Physicochemical characteristics of the collected samples

1.1. Oil contaminated soil samples

The physicochemical properties of the soil samples were performed to determine the physical factors, limiting nutrients, and pollutants that could indicate the types of microorganisms recovered from the soils. The pH of the two soil samples was weakly acidic (5.6 and 6.2). The presence of various heavy metals, such as cadmium, lead and nickel, in high concentration emphasized the pollution of the soil samples. Results of these soil analyses are presented in **Table 1.**

Table (1): Physicochemical characteristics of soil samples.

Parameters	Sample No.	
	Soil (1)	**Soil (2)**
pH	5.6	6.2
Moisture (%)	8.46	8.22
Total Organic Carbon (%)	5.2	3.8
Micro Elements (mg/kg)		
Fe	964	656
Cu	23	17
Zn	27.3	174.3
Mn	22	26
Heavy Metals (mg/kg)		
Cd	15	24
Pb	192	162
Ni	30.9	48.1

1.2. Physicochemical properties and composition of crude oil sample

The different physicochemical parameters of the crude oil sample were analyzed. Density and specific gravity were measured according to **American Society for Testing and Materials (ASTMD-1298) (2001a)** and its API gravity was calculated. Pour point and viscosity in accordance with **ASTM D-445 (2001d)** were recorded. Sulfur content was measured according to **ASTM D-4294 (2001e)**. The crude oil sample obtained from (SOPC) has a Kinematic viscosity of 66.89 cst at 50^{o}C, high salinity 3000 mg/l and PH= 6.5. The crude oil composed of low wax content 4.6% and high asphaltene 8.9% as shown in Table 2. Also, it has a high specific gravity of 0.9249, low API gravity of 21.49 at 60^{o}F and a pour point was 50^{o}F.

Table (2): Physicochemical characteristics of Suez crude oil.

Tests	Results
Density at 15°C g/cm^3	0.9244
Specific gravity@ 60°F	0.9249
API gravity	21.49
PH value at 25°C	6.5
Salinity mg/l	3000
Total sulfur content WT.%	3.00
Nitrogen content WT.%	0.42
Ash content WT.%	0.034
Salt content	13
Water content Vol.%	0.15
Carbon residue, WT%	11.8
Sediment content, WT.%	0.014
Viscosity @ 100°F, sec.	489
Kinematic viscosity @ 37.5°C, cst.	121
Kinematic viscosity @ 50°C, cst.	66.89
Pour point °F	50
Asphaltene content WT.%	8.9
Wax content WT. %	4.6
Mercaptan sulphur ppm	36
hydrogen sulfide ppm	Nil
Vanadium content ppm	102
Nickel content ppm	75
Total acidity mg KOH/g	0.13
Inorganic acidity mg KOH/g	Nil
ASTM Distillation (ASTM D):	
Recovered at 100°C	3
Recovered at 150°C	9
Recovered at 200°C	15
Recovered at 250°C	21
Recovered at 300°C	31
Light Hydrocarbons:	
Total C4 and lighter WT%	0.78
Ethane WT%	0.00
Propane WT%	0.13
l-Butane WT%	0.24
n-Butane WT%	0.41

2. Isolation, purification and enumeration of total, crude oil degrading bacterial isolates

The oil degrading organisms which can utilize hydrocarbons as sole sources of energy and carbon are extremely diverse and widely distributed in nature. They can adapt to survive in unsuitable environments **(ZoBell, 1946; Sohal and Srivastava, 1994)**. Using crude oil as a sole source of carbon, eleven bacterial strains were isolated from the soil samples collected from crude oil contaminated sites. The isolates from the different sites were given code numbers. Bacterial strains isolated from SOPC given code CS1. While, those isolated from EPRI were given code CS2. A total of eleven crude oil degrading bacterial strains isolated from the soil samples are; seven from site CS1 and four from site CS2. The total and hydrocarbon degrading bacterial counts of the soil samples were determined gravimetrically as shown in **Table (3)**.These isolates were purified and stored for further screening and selection.

Table (3): Total and hydrocarbon degrading bacterial count (Log CFU/ml) of oil contaminated soil samples.

Soil Samples	Viable Bacterial Count (Log CFU/ 1 gm soil)			
	Total Bacterial Count (TBC)	Count of Hydrocarbon Degrading Bacteria (HDB)		
		After 7 days	After 14 days	After 21 days
CS1	10.82	8.32	4.77	3.90
CS2	8.00	7.60	4.34	2.47

In agreement with the present work **Afifi *et al.*, (2015)** isolated native bacteria from oil sludge produced in Abadan refinery, Iran using a mineral base medium. Native bacteria were screened based on their potential in reduction of total petroleum hydrocarbons.

3. Evaluation of growth and bio-degradation ability of the bacterial isolates

Qualitative estimations of biodegradation potential were carried out on each of the bacterial isolates. Detection of bacterial growth is taken as an indication of ability to degrade crude oil. The ability of all isolates to utilize crude oil was initially evaluated visually by growing on oil agar media and by biosurfactant production before being confirmed quantitatively.

3.1. Growth on oil agar media

The ability of the bacterial isolates to grow on two crude oils from Suez and Agiba Companies was tested visually by growing the individual isolates separately on oil agar media. This measurement is rapid and simple being used by several workers **(Ijah, 1998; Sepahi *et al*., 2008)**. It gives a preliminary screening of the degradation ability of all isolates within a short time. Results in **Table (4)** show the ability of all bacterial isolates to utilize crude oil as a sole source of carbon in different levels of growth as high, moderate and low.

Table (4): Qualitative data of Egyptian crude-oil biodegradation by growth of bacterial isolates on oil agar media.

Bacterial Isolates code	Growth on oil agar	
	Suez crude oil	Agiba crude oil
CS1.1	+++	+++
CS1.2	+++	+++
CS1.3	+++	+++
CS1.4	++	+
CS1.5	++	+
CS1.6	++	++
CS1.7	+++	+++
CS2.1	+	+
CS2.2	+	+
CS2.3	+++	++
CS2.4	+++	++

(+) Low growth, **(++)** Moderate growth, **(+++)** High growth.

The results indicated that six isolates showed high growth, three showed moderate growth and two showed low growth on Suez crude oil, while on Agiba company crude oil four isolates gave high growth, three gave moderate growth and four gave low growth. Only four of all bacterial isolates showed the highest rates of

growth on both crude oils. Similar results were recorded by **Hamzah *et al*. (2010)** who isolated four species of bacteria, *Acinetobacter lwoffii*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *P. putida*, from soil contaminated with hydrocarbons. The isolates were testes for their ability to degrade petroleum hydrocarbon by growing in MSM supplemented with two types of crude oil, either Sumandak or South Angsi at 1% (v/v) concentration.

3.2. Biosurfactant analysis

3.2.1. Oil displacement activity of the bacterial isolates

The oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms; a larger diameter represents a higher activity of the testing solution **(Huy *et al.*, 1999 and Rodrigues *et al.*, 2006)**. Many methods were used for screening of biosurfactant production, some are qualitative and the others are quantitative methods. In the present investigation, isolates CS1.1 and CS1.7 gave the highest biosurfactant productivity with 6.35, 4.25 cm, respectively as compared with the positive control, while CS2.1 gave the lowest biosurfactant productivity of 0.85cm **(Table 5)**. In contrast, isolates CS2.2 and CS2.3 showed no displaced circle as well as negative control **(Figure 1)**.

Table (5): Screening the biosurfactant productivity of the bacterial isolates using oil spreading assay.

No.	Bacterial isolates code	MDCZ (cm)	No.	Bacterial isolates code	MDCZ (cm)
1	CS1.1	6.35	**8**	CS2.1	0.85
2	CS1.2	3.00	**9**	CS2.2	0.00
3	CS1.3	3.25	**10**	CS2.3	0.00
4	CS1.4	1.85	**11**	CS2.4	1.80
5	CS1.5	1.25	**12**	Negative control	0.00
6	CS1.6	1.55	**13**	Positive control	9.50
7	CS1.7	4.25			

MDCZ: Mean Diameter of Clearing Zone, **Negative control:** oil with dist. H_2O, **Positive control:** oil with surfactant.

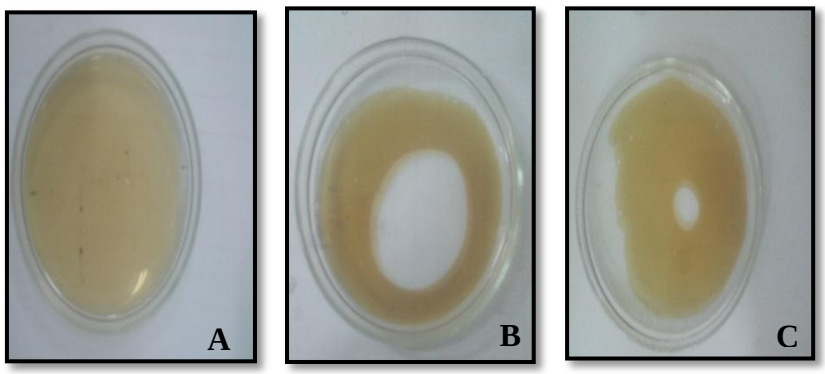

Fig. (1): A: Negative control, **B, C:** Oil spreading assay technique showing the highest and lowest displacement of the oil present in the systems as compared to the negative control.

3.2.2. Emulsification index and Surface tension measurements

Data represented in **Table (6)** and **Figure (2)** show that the emulsification index ranged from 6.6 % to 55.6 %. The optimum time for maximum biosurfactant production varied from organism to another. Their production is increased by time until it reached its maximum productivity then it decreased until losing its production ability with time and it reached zero in some cases. After the optimum incubation time (48hrs.), the bacterial isolates CS1.1, CS1.7and CS1.3 produced the highest emulsification index of 55.6%, 53.5% and 46.6% respectively with reduction in surface tention range from 36.5 to 39 mN/m while, the bacterial isolates CS2.1, CS1.5 and CS2.3 recoded the lowest emulsification index (20 %, 14 % and 6.6% respectively) with surface tension reduction of 47, 46.8 and 48.9 mN/m respectively. The bacterial isolates CS2.2 didn't have the ability to produce any emulsification index such as a control sample **(Figure 2, E)**.

Table (6): Screening the biosurfactant production by the bacterial isolates using emulsification index (E_{24}) and surface tension.

Bacterial isolates code	Surface tension (mN/m)	Emulsification index (E_{24}) %					
		Time (hours)					
		0	12	24	48	72	96
CS1.1	36.5	0.0	37.1	55.6	55.6	55.6	55.6
CS1.2	39.8	0.0	0.0	20	40	40	35
CS1.3	39	0.0	17.7	20	46.6	46.6	46.6
CS1.4	40.7	0.0	0.0	33.3	33.3	15.5	8.8
CS1.5	46.8	0.0	0.0	14	14	14	10
CS1.6	44	0.0	26.6	26.6	26.6	26.6	26.6
CS1.7	37.5	0.0	35.5	35.5	53.5	53.5	51.7
CS2.1	47	0.0	0.0	13.3	20	20	20
CS2.2	59.0	0.0	0.0	0.0	0.0	0.0	0.0
CS2.3	48.9	0.0	0.0	6.6	6.6	6.6	0.0
CS2.4	45.4	0.0	0.0	25.3	25.3	25.3	25.3
Control (Medium)	59.30 ± 0.70	0.0	0.0	0.0	0.0	0.0	0.0
Dist.H_2O	72.0 ± 0.68	0.0	0.0	0.0	0.0	0.0	0.0

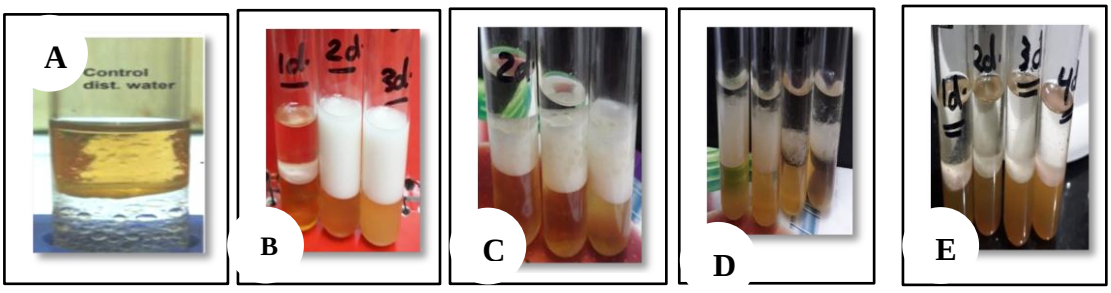

Fig. (2): A: The control without emulsion, **B-E:** Emulsification assay technique of oil-contaminated soil bacterial isolates showing changes compared to the control sample.

The results of the present work are in agreement with those recorded by **Abdurrahim *et al.*, (2009)** who showed that the extracellular surface-active agent produced by the indigenous strain of *P. aeruginosa* was identified as rhamnolipid, which is one of the most commonly biosurfactant use to reduce surface tension of water. Additionally, there were direct relationships between both the emulsification activity (E_{24}) and the decrease in surface tension with increasing growth rate on hydrocarbons **(Shahian *et al.*, 2012 and Ismail *et al.*, 2013a, b)**.

While, **Ibrahim (2016)** reported that the two bacterial strains namely, *Ochrobactrum anthropi* HM-1 and *Citrobacter freundii* HM-2, previously isolated from used engine oil contaminated soil are capable of producing biosurfactants using their cell-free culture broth. The highest E_{24} value recorded by *O. anthropic* biosurfactant was 90% compared with 89% for *C. freundii* and they reduced the surface tension of growth medium (70 ± 0.9) to 30.8 ± 0.6 and 32.5 ± 1.3 mN/m, respectively.

Also, **Diallo *et al.*, (2020)** selected four bacterial strains belonging to genera *Acinetobacter* and *Pseudomonas* after constitute three different consortia of them based on their initial concentration. The highest degradation rate (78%) after 4 weeks of incubation was recorded when the concentration of biosurfactant (BS) producing isolate was high in the presence of 1% (v/v) crude oil.

4. Quantitative measurement (Growth in oil broth medium):

4.1. Crude oil utilization studies using UV-visible spectrophotometer

Results in **Table (7)** show the UV-visible studies of the residual oil samples after 15 days of incubation time at λ_{max} 260 nm. The UV spectral studies of the tested crude oil were recorded and compared with the control sample.

Table (7): The spectrophotometric analytical data of the crude oil residual compounds after extraction by chloroform at 260 nm.

Bacterial isolates code	Optical density (O.D.)
CS1.1	1.832
CS1.2	1.470
CS1.3	1.223
CS1.4	1.119
CS1.5	1.217
CS1.6	1.326
CS1.7	1.703
CS2.1	0.955
CS2.2	0.936
CS2.3	1.032
CS2.4	1.126

The UV-visible studies of the crude oil were recorded for the tested samples and it shows change in absorbance value range from 0.936 O.D. to 1.832 O.D. This change indicates the breakdown of the molecular structure of the oil and this confirms the degradation of crude oil by bacterial isolates due to the breaking of conjugates in the molecular structure of the crude oil. The increase in population densities with simultaneous increase in degradation rate correlates with the UV spectrophotometry results. Similarly, **Evdokimov and Losev (2007)** suggested that, as the long chain hydrocarbons break, the C-C linkage at which the breaking occur results in the shift of

the atom's energy state and hence the peak symbolizes the highest absorption for that atom.

4.2. Gravimetric analysis and bacterial count (Log CFU/ml) determination

The isolated bacteria were tested for their growth ability to grow on MSM supplemented with 1% petroleum oil as a carbon source. Emulsification of crude oil and change in oil state during the incubation period was shown in **Figure (3)** and the extraction of residual petroleum crude oil from microbial cultures is shown in **Figure (4)**. The efficiency of crude oil degradation by the individual bacterial cultures was determined quantitively by estimating the consumed hydrocarbons after the biodegradation process. The percentage of biodegradation of crude oil by the bacterial isolates was estimated gravimetrically as shown in **Figure 5** and **Table 8**. The growth of the bacterial isolates was determined as a Log of bacterial count after 15 days incubation.

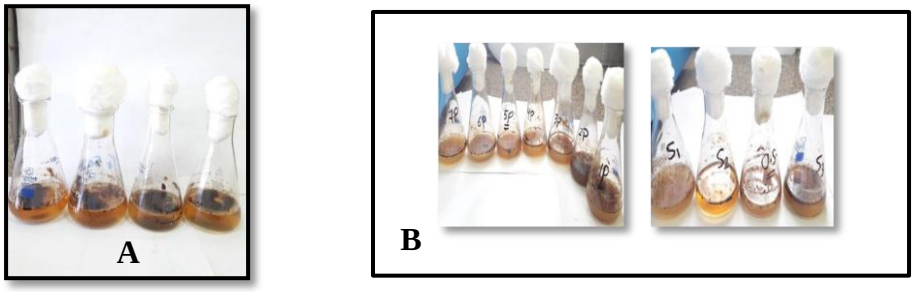

Fig. 3: A: Test flasks at zero time, **B:** Visible observations of crude oil degradation by bacterial isolates after 15 days of incubation on MSM broth.

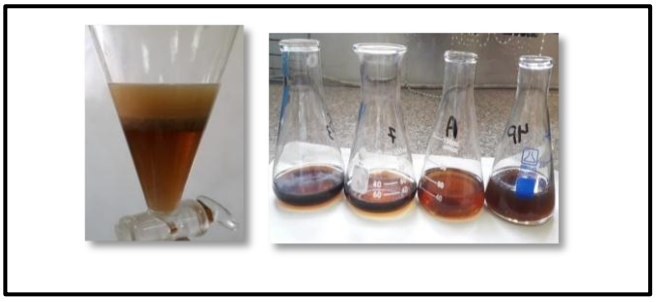

Fig. 4: Residual crude oil extraction using chloroform solvent for gravimetric analysis.

Table (8): Relation between bacterial count (Log CFU/ml) of bacterial isolates and biodegradation Percentage of crude oil after 15 days of incubation.

Bacterial isolates	Bacterial count (Log CFU/ml)	Weight of residual crude oil (gm/100ml)	Weight of consumed crude oil (gm/100ml)	Biodegradation percentage (%)
CS1.1	14.36	0.70	1.32	65
CS1.2	12.87	1.10	0.90	45
CS1.3	13.10	0.80	1.20	60
CS1.4	12.55	1.30	0.70	35
CS1.5	12.62	1.09	0.91	45.5
CS1.6	11.70	1.05	0.95	47.5
CS1.7	13.21	0.72	1.28	64
CS2.1	11.19	1.30	0.70	35
CS2.2	9.94	1.50	0.50	25
CS2.3	12.98	1.10	0.90	45
CS2.4	11.52	0.78	1.22	61

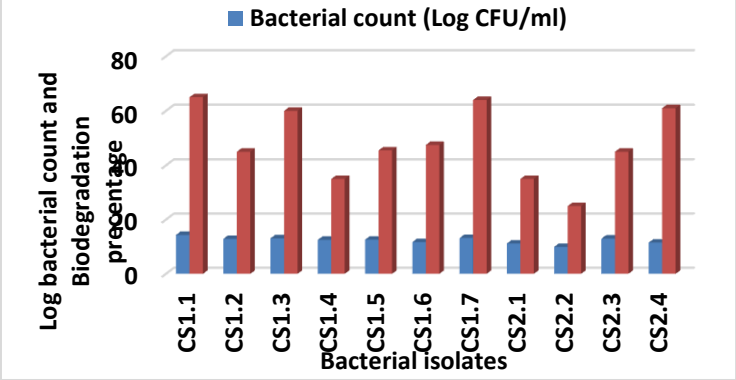

Fig. (5): Relation between bacterial count and biodegradation percentage of crude oil by bacterial isolates after 15 days incubation period.

Results in **Table (8)** indicate that, after 15 days of treatment, all the bacterial isolates exert a percentage of biodegradation compared to the control (0.0%). The consumed hydrocarbons after crude oil biodegradation varied greatly among the bacterial isolates from 0.50 to 1.32 gm/100ml. The lowest biodegradable strain was CS2.2 which resulted in biodegradation percentage of 25%. Meanwhile, strains number CS1.1 and CS1.7 showed appreciable biodegradation percentage of 65% and 64%, respectively. So, the results revealed that both CS1.1 and CS1.7 isolates were able to degrade the petroleum oil and utilize it as a sole carbon source. Finally, the previous results indicated that there is relation between bacterial count and biodegradation percentage of crude oil by all bacterial cultures.

This result is in agreement with **El-Sheshtawy *et al.* (2014)** who illustrated that biodegradation percentage of the crude oil by the isolated bacterial strains *Pseudomonas xanthomarina* KMM1447 and *P. stutzeri* ATCC 17588 were from 30 to 50% after 7 days incubation. Also, **Al-Wasify and Hamed (2014)** reported that the efficiency of crude oil degradation by the 3 bacterial strains *Pseudomonas aeruginosa, Bacillus subtilis, and Acinetobacter lwoffi*, was determined qualitatively by estemating the consumed hydrocarbons at the end of the four incubation periods

(7, 14, 21, and 28 days). The consumed hydrocarbons after crude oil biodegradation were in the range from1.18 to1.91gm/l.

5. Biochemical identification of the most potent hydrocarbon degrading bacterial isolates:

Biochemical tests were carried out by using a turbidity meter VITEK® 2 DensiCHEK^{TM}. The isolated strains were identified on the basis of morphological features. Eleven bacterial strains were isolated and identified as different species of the genus *Bacillus*. The BCL card is used for the automated identification of these bacterial strains as the most significant aerobic endospore-forming species of the family *Bacillaceae.* The results included in **Table (9)** present a summary of morphological and biochemical identification of the most potent bacterial isolates (CS1.7andCS1.1).

Both CS1.7 and CS1.1 bacterial strains were identified as *Bacillus subtilis*, and *B. licheniformis.* Gram staining and endospore staining according to **Cheesbrough (1991) and Yan *et al.* (2013)** revealed that the two isolates are Gram positive and spore forming bacteria. They mostly live as saprophytes in water, soil, dust, and vegetation **(Cheesbrough, 1991)**, and diesel oil contaminated sites **(Rahman *et al.*, 2002b).** The microscopic examinations of the most potent bacterial isolates are shown in **Figure (6)**.

Table (9): Biochemical and Morphological features of the most potent hydrocarbon degrading bacterial isolates.

S.No.	Identification tests	Bacterial isolates	
		Bacillus subtillis **(CS1.7)**	***Bacillus licheniformis*** **(CS1.1)**
Analysis time (Hours)		14.25	14.25
Probability		89%	89%
A) Preliminary tests			
1	Colony color	Gray-white	Creamy
2	Colony Form	Circular	Circular
3	Cell shape	Rod	Rod
4	Gram staining	+ve	+ve
5	Spore staining	+ve	+ve
6	Motility	+ve	+ve
7	KOH (3%) reaction	-ve	-ve
B) Carbohydrate fermentation tests			
1	D-glucose	+ve	+ve
2	D-galactose	-ve	-ve
3	D-ribose	+ve	+ve
4	D-mannitol	+ve	+ve
5	D-mannoes	+ve	+ve
6	D-melezitose	-ve	-ve
7	D-tagatose	-ve	-ve
8	D-trehalose	+ve	+ve
9	Myo-inositol	-ve	-ve
10	Palatinose	+ve	+ve
11	L-rhamnose	+ve	+ve
12	Maltotriose	+ve	+ve
C) Other test substrates on BCL Card			

S.No.	Identification tests	Bacterial isolates	
		Bacillus subtillis **(CS1.7)**	***Bacillus licheniformis*** **(CS1.1)**
1	Beta-Xylosidase	+ve	+ve
2	L-Lysine-Arylamidase	-ve	-ve
3	L-Aspartate-Arylamidase	-ve	-ve
4	Leucine-Arylamidase	-ve	+ve
5	Phenylalanine- Arylamidase	+ve	+ve
6	L-Proline Arylamidase	-ve	-ve
7	Beta-Galactosidase	-ve	-ve
8	L-Pyrrolydonyl- Arylamidase	+ve	+ve
9	Alpha-Galactosidase	-ve	+ve
10	Alanine Arylamidase	-ve	+ve
11	Tyrosine Arylamidase	+ve	+ve
12	Beta-N-Acetyl-Glucosaminidase	-ve	+ve
13	Ala-Phe-Pro- Arylamidase	-ve	-ve
14	Glycogen	+ve	-ve
15	Methyl-A-D Glucopyranoside Acidification	-ve	-ve
16	Ellman	-ve	-ve
17	Methyl-D-Xyloside	-ve	-ve
18	alpha-Mannosidase	-ve	-ve
19	glycine Arylamidase	-ve	-ve
20	N-Acetyl-D-Glucosamine	+ve	+ve
21	Beta-Glucosidase	+ve	-ve
22	Beta-Mannosidase	-ve	-ve
23	Phosphoryl Cholin	-ve	-ve
24	Pyruvate	-ve	-ve
25	Alpha-Glucosidase	-ve	-ve
26	Inulin	-ve	-ve
27	Putrescine Assimilation	-ve	-ve
28	Growth in 6.5%NaCl	+ve	+ve
29	Kanamycin Resistance	+ve	+ve
30	Oleandomycin Resistance	+ve	+ve
31	Esculin Hydrolysis	+ve	+ve
32	Tetrazolium Red	+ve	+ve
33	Polymixin-B Resistance	+ve	+ve

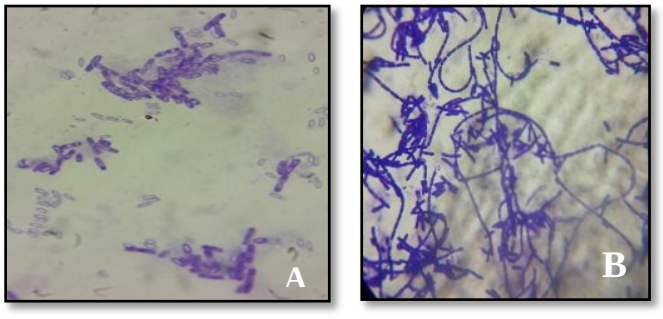

Figure (6): Gram positive vegetative cells of most potent oil degrading bacterial isolates. **A:** A phase contrast photograph of *Bacillus subtillis*, **B:** A phase contrast photograph of *B. licheniformis.*

These results are in accordance with those of **Survey *et al.*, (2004)** who isolated different *Bacillus* spp from soil near petrol pumps in Pakistan. Similarly,

Sorkhoh *et al*. (1993) isolated 368 isolates belonging to genus *Bacillus* from desert soil samples. These *Bacillus* spp. are more tolerant to unsuitable conditions of high temperatures and high levels of hydrocarbons in soil. This may be attributed to the ability of members of the genus to survive periods of unfavorable conditions by producing endospores which rapidly reproduce upon the return of favorable conditions. The isolation of *Bacillus* spp. (as the most potent crude oil degrading bacterial isolates) from petroleum hydrocarbon contaminated sites in this study corroborates literature data about the widespread distribution of *Bacillus* sp. in petroleum associated environments **(Roy *et al*., 2002; Von der Weid *et al*., 2008 and de Vasconcellos *et al,* 2009).** Also, **Malatova (2005)** isolated 20 hydrocarbon degrading bacteria from a tropical soil. Ten isolates were identified phenotypically as species of *Pseudomonas* and eight as those of *Bacillus*.

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عزل وفحص وتوصيف العزلات البكتيرية التي تعمل على تحلل الزيت الخام من أنواع مختلفة من التربة المصرية الملوثة بزيت البترول الخام

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الملخص العربى:

تم عزل ١١ سلالة بكتيرية من عينتين مختلفتين من التربة الملوثة بالزيت من بيئات محلية مختلفة في مصر. وتم تمييزهم وفقًا لفحص النمط الظاهري لديهم أعلى أنهم ينتمون إلى جنس *Bacillus* . أظهرت هذه العزلات البكتيرية أعدادًا تتراوح بين 8,10 log CFU/g لكل جرام من التربة لذلك تشير هذه النتائج إلى أن التلوث الأطول عمرًا أظهر عددًا أكبر من الكائنات الحية الدقيقة في هذه الأماكن.

وقد أجريت دراسة تجريبية لتقييم كفاءة هذه السلالات المعزولة في تحلل (المعالجة الحيوية) لزيت البترول نوعيًا بزراعتها في وسط غذائي صلب يحتوى على الأملاح الأساسية وعلى خام البترول بنسبة 1% v/v.

فأظهرت النتائج أن كلا من CS1.1 و CS1.7 هما أكثر العزلات فاعلية في تحلل نوعين من الزيت الخام من شركتي السويس وعجيبة لتكرير البترول وذلك بعد تحضين كل سلالة في كل خام على حده. بالإضافة إلى ذلك، أظهر CS1.1 و CS1.7 نشاط استحلابى بنسبة ٥٥.٦٪ و٥١.٧٪ على التوالي بعد ٤٨ ساعة من فترة الحضانة. أيضًا، يمكن أن تقلل العزلات CS1.1 و CS1.7 من التوتر السطحي إلى 36.5 ± 0.25 mN/m و 37.5 ± 0.75 mN/m، على التوالي في أقل من ١٢ ساعة في الوسط المزود بالجلوكوز. بينما أظهرالتحليل الجرافيميترى أن كلا من العزلات CS1.1 و CS1.7 كانت قادرة على تحلل زيت البترول واستخدامه كمصدرأساسي للنمو والطاقة والتكاثربنسبة ٦٥٪ و ٦٤٪ من زيت البترول المضاف، على التوالي، خلال ١٥ يومًا.

أيضًا لوحظ ان التغير في قيم الامتصاص للعينات المختبرة من ٠.٩٣٦ .O.D إلى ١.٨٣٢ .O.D بالمقارنة مع العينة الاصلية ٠.٨١٥ .O.D يشير إلى انهيار التركيب الجزيئي في الزيت ويؤكد تحلل الزيت الخام بواسطة العزلات البكتيرية. واخيرا تم تعريف هاتين العزلتين الأكثر فاعلية طبقا للمتغيرات الفسيولوجية والكيميائية على أنها *Bacillus subtilis* و*Bacillus icheniformis*.

الكلمات المفتاحية: المعالجة الحيوية ، الزيت الخام ، البكتيريا المحللة ، نشاط الاستحلاب.