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MICROBIAL BIODEGRADATION POTENTIAL OF PETROLEUM-AND NATURE OILS BY INDIGENOUS HYDROCARBON DEGRADING BACTERIA ISOLATED FROM PETRO-CONTAMINATED SITES

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ABSTRACT: The study was carried out as an effort at developing active natural petroleum hydrocarbon degraders that could be of relevance of bioremoval of petro-and non-petro oil pollutants from contaminated sites, a collection of 10 bacterial isolates as well as 3 yeast strains and *Enterobacter clocace* spp. *disssolvens* strain as efficient petro-hydrocarbon degraders were employed in this study to reach to this target. These bacterial isolates were identified to genus and species level using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALD-TOF-MS) with high degree of precision (2.111-3.361) as an efficient approach for unambiguous identification process in real sample within minutes. Two experimental techniques, namely respirometric method (CO_2 production) and the redox indicator 2,6-dichlorophenol indophenol (DCPIP) test were carried out to evaluate the capacity of the tested organisms to utilize monoaromatic hydrocacbons (BTEX-mixture) and/or *n*-hexadecane as an excellent substrates in the study of hydrocarbon biodegradation in the tested media, individual and/or mixed bacterial and yeast consortium. The results indicated that the tested organisms showed different rate and extent of growth as well as mixed bacterial–or yeast consortium showed more growth and degradation. Also, some growth condition factors that affect the success of biodegradation were determined, and obtained results revealed that pH (7.0), temperature (30-35˚C), salinity (0.0% and 0.5% NaCl) and incubation period (11 days) were suitable for the most tested bacteria. The results showed also that only five bacterial strains out of 10 strains namely: *Sphingobacterium thalpophilum* QBII-6, *Pseudomonas nitroreducens* RdI-14, *Bacillus subtilis* ssp *subtilis* GH-5, *B. atrophaeus* GH-6 and *B. licheniformis* RdI-17 as well as *Ent. clocace* spp. *disssolvens* exhibited the highest capability to metabolize a diverse range petroleum oils (crude oil, diesel oil, engine oil, used engine oil) and natural oils (corn oil, used corn oil, sunflower oil and used sunflower oil), and these five bacterial types could be applicable in bioremediation process of pollutants after carrying out future studies in field condition as a final goal.

Key words: Petroleum hydrocarbon, CO_2, DCPIP, natural oils, *n*-hexadecane, BTEX.

INTRODUCTION

Petroleum hydrocarbons are the most common environmental pollutants and oil spills pose a great hazard to terrestrial and marine ecosystems. Oil pollution may arise either accidently or operationally whenever oil is produced, transported, stored, processed or used at sea or on land. Fortunately, the degradation of these oils in the environment is possible through several techniques: physical (Costes and Druelle, 1997), chemical (Chu and Kwan, 2003) or biological (Mittal and Singh, 2009). The technology commonly used for the soil remediation includes: mechanical, burying, evaporation, dispersion and washing (Sawadogo *et al*., 2014). However, these methods are expensive and can lead to incomplete depollution (Sonawdekar, 2012; Das and Chandran, 2011). In view of this situation, bioremediation gives a better solution, owing to, it provides efficacy, safety on the long term use,

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cost and simplicity of administration with promising opportunity for better environment (Bento *et al.,* 2003, Williams *et al.,* 2006; Mandri and Lin, 2007).

Recently, biodegradation of pollutants by microbes has been received significant interest as mankind attempts to reduce contamination and construct a pollution free environment (Das and Chandran, 2011; Dindar *et al.,* 2013; Koshlaf and Ball, 2017). Biodegradation by natural population of microorganisms is the most basic and the most reliable mechanisms by which thousands of pollutants including crude oil and its product (Cappello *et al.,* 2007; Haasanshahian *et al.,* 2012) as well as natural oils and their products (Mahalingam and Sampath, 2014). Biodegradation, is a mineralization of organic chemicals, which ultimately leading to the formation of CO_2, H_2O and biomass (Benedek *et al*., 2010; Mrozik and Piotrowska-Seget, 2010).

However, a number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons along with temperature, salinity, oxygen concentration, oil concentration, nutrients and hydrocarbon chemical composition (Leahy and Colwell, 1990; Walworth *et al.,* 2001 ; Das and Chandran, 2011).

In the light of these information, the present study was carried out to utilize already existing indigenous bacteria, which were isolated from petro-contaminated Egyptian sites and primary screened for their abilities to utilize monoaromatic hydrocarbon(benzene, toluene, etthylbenzene, and xylene-BTEX-mixture), as well as reference strains from bacteria and yeasts to degrade petroleum and non-petroleum oils through lab-scale experiments, as well as the growth conditions for optimizing their abilities to utilize of these organic contaminants were monitored.

MATERIALS AND METHODS

Microbial Used

After screening 170 bacterial isolates for their efficiencies to biodegrade different hydrocarbons, the highest 10 active bacterial isolates namely :RdI-13, QBII-6, RDI-14, GH- 5, RdI-8, QRII-7, GH-6, RDI -3, RdI-17 and RdI -1 were selected and used in this study. Also, a bacterial strain namely *Enterobacter clocace* spp. *disssolvens*, was kindly provided by Dept. Agric. Micro., Fac. Agric., Zagazig Univ., Egypt and it is known as an efficient in hydrocarbon degradation. In addition three yeast strains, namely *Candida parapsilosis Complex* 9163, *Rhodotorula mucilaginosa Harrison* 8802, and *Pichia anomala Hansen*10186, were purchased from Mycology Centre at Asiut University, Egypt. They characterized by the centre as active hydrocarbon degraders.

Identification of Bacteria

One large colony of bacterial isolate (enough to fill about one half of a 10-µl inoculating loop) was suspended in 70% ethanol in a 1.5 ml microcertifuge tube. Protein mass identification of bacteria using a Bruker MALD-TOF. Micro Flex mass spectrometer were performed at Academic Park, Faculty of Medicine, Alexandria, University, Egypt (Sauer and Kliem, 2010; Biswas and Rolain, 2013).

Efficiency of Biodegradation

Two techniques were used to confirm and evaluate the capability of the tested organisms to biodegrade the tested hydrocarbon used in this study (BTEX blend and/or n-hexadecane at 2.0%), as an excellent substrates in biodegradation studies.

Microbial CO_2 Production

Respirometric biodegradation experiment was conducted in a special unit consists of one-liter wide mouth jar, Pyrex, with special lid as recommended by Isermeyer (1952) and Mariano *et al.* (2009), using 100 g/d. wt. soil, sandy loam, treated with 2.0 ml BTEX-blend, 2.0 ml of Tween 80, 2.0 ml of the tested organism (10^7 CFU/ml), and the water content was brought to 55% of water holding capacity (WHC). The prepared soil jars were incubated at 28˚C and the titration process was carried out after 0, 3, 7, 14 and 28 days of incubation. The output of CO_2 was trapped in 100 ml KOH and measured by titrating the residual KOH with standard solution of HCl (0.1N), (1 ml of 0.1N HCl equivalent 2.20 mg CO_2), and the obtained results were expressed as mg CO_2/100 g. d. wt. soil.

DCPIP Redox Indicator Technique

2,6-dichlorophenol indophenol indicator was used to confirm the ability of the tested organisms to utilize BTEX blend and/or n-hexadecane as carbon source in Bushnell Haas mineral medium in the test tubes as recommended by Hanson *et al.* (1993) and Mariano *et al*. (2009). In this technique, time taken for decolourization of the blue (oxidized) DCPIP to colorless (reduced) form was recorded for each isolate per hour.

Optimizing the Growth Conditions

Optimizing the bacterial growth conditions (temperature, pH, salinity, and incubation periods) for the 10 selected active strains, were determined using trypticase soy broth medium supplemented with *n*-hexadecane (2.0%) as a carbon source as follows:

Effect of Temperature on Bacterial Growth

The influence of temperature (25, 30, 35 and 40˚C) on the growth of the tested bacteria in 250 ml Erlenmeyer flasks containing 100 ml of trypticase soy broth supplemented with 2.0 ml n-hexadecane (after sterilization). The flasks were inoculated with 1 ml of overnight cultures at the log phase of growth was studied. The experiment was conducted at pH 7 and incubated for 11 days for all levels of temperature using rotary shaker at 150 rpm. The growth was monitored through culture densities measuring light absorption spectrophotometrically (Jenway 6405 UV-VIS spectrophotometer, UK) at 600 nm. (Mahalingam and Sampath, 2014).

Effect of pH on Bacterial Growth

The effect of the initial pH values of 6.5, 7.0, 7.5 and 8.0 on the growth of the 10 bacterial strains were estimated by growing them as mentioned above, and incubated at 30˚C ± 0.5˚C for 11 days using rotary shaker at 150 rpm. The bacterial growth of the tested cultures was determined spectrophotometrically at 600 nm. (Mahalingam and Sampath, 2014).

Effect of Salinity on Bacterial Growth

The influence of sodium chloride at different concentrations in trypticase soy broth media (0, 0.5, 2.5 and 5.0%) supplemented with hydrocarbon n–hexadecane on the growth of the tested bacteria was measured for all concentrations. The experiment was conducted at pH 7.0 and 30˚C for 11 days of incubation using rotary shaker at 150 rpm. The growth was monitored through culture densities measuring light absorption spectrophotometrically at 600 nm. (Margesin and Schinner, 2001; Qin *et al.,* 2012).

Effect of the Incubation Periods on Bacterial Growth

In this experiment, the effect of incubation periods at 3, 7, 11 and 15 days on the growth of the tested strains was studied using tripticase soy broth media (100 ml) supplemented with 2.0 ml of *n*-hexadecane at pH 7.0, temperature 30˚C and NaCl 0.0% using rotary shaker at 150 rpm. The growth of the tested cultures (at 600 nm) was determined in the desired time interval to record the best incubation period for these strains (Mishra and Singh, 2012).

Evaluation the Growth and Ability of the Tested Bacteria for Using Different Hydrocarbon and Organic Compounds

In this experiment, the 10 hydrocarbon degraders bacteria, were tested to study their ability to grow on some other petroleum hydrocarbons (diesel oil - used engine oil-engine oil and crude oil) as well as some organic compounds (corn oil, used corn oil, sunflower oil and used sunflower oil) as a carbon and energy sources (Malatova, 2005). This experiment was conducted in duplicates using Bushnell-Haas broth medium in 250 ml Erlenmeyer flasks containing 100 ml supplemented with 2.0 ml of the tested compounds. The experimental conditions were: pH 7.0, incubation temperature 30˚C, salinity NaCl 0.5 g and incubation period 11 days and shaking at 150 rpm. The bacterial growth (at 600nm) of the cultures was estimated at the final phase of the experiment.

Statistical Analyses

Each treatment was run in three replicates and the data were statistically analyzed by CoStat version 6.311 Copyright(c) 1998-2005 CoHort Softwar, http://www.cohort.com.

RESULTS AND DISCUSSION

Direct Identification of the Tested Bacteria Using MALDI-TOF-MS

The 10 active hydrocarbon bacterial isolates codes: RdI-13, QBII-6, RDI-14, GH- 5, RdI-8, QRII-7, GH-6, RDI-3, RdI-17 and RdI-1 were

identified at Academic Park Fac. Medicine Alex. Univ., Egypt, using MALDI-TOF- MS. (matrix-assisted layser desorption inonization-time of flight mass spectrometry). This strategy generates a spectrum based on protein detected directly from intact microorganisms, allowing a rapid identification, and it took the leading position in just a few years. This success in based on MALD's short analysis, high sensitivity and intact cell measurement, as well as the possibility of automation, therefore it has become the method of choice throughout the third millennium, (Krasny *et al.*, 2013). This strategy was the good argument to have accurate and direct identification of the tested bacteria. In this advanced method, the identification process was confirmed and the prospective strains with their numbers as conserved in the International Cultural Centers for Microorganisms were registered. The obtained results of score value for each bacterial isolates are shown in Table 1.

Of the 10 isolates with a score value between 2.111 to 2.361 (100%) organisms were correctly identified by MALDI- TOF- MS to genus and species levels. All the tested bacterial strains were type strains that are included in the Bruker Database, and all spectrum scores were greater than 2.0. Thus, all of the tested bacterial hydrocarbon degraders were correctly identified to genus and species levels by the Micro Flex LT mass spectrophotometer with biotype software score values greater than 2.0, and all of them had high degree of precision. On the other hand, two isolates only were identified to genus, species and subspecies levels as recorded in isolate No. 2 and isolate No. 6 (Table 1). Thus, they were completely identified as they were as follows *Bacillus subtilis* ssp.*subtilis* DSM 10 T DSM and *B. subtilis* ssp. *spizizenii* DSM 618 DSM and their similarity scores were 2.332 {C1(+++) (A)} and 2.323 {C6(+++) (A)}, respectively. In this respect, Bille *et al.* (2012) pointed out that this method allowed a proper identification of bacteria grown on solid media to reach 99.20% cases (2609 out of 2630 organisms) Also, it is well known that in most cases, the 16S rRNA sequencing results agreed with MALDI- TOF- MS identification (Bizzini *et al.,* 2010 ; Wang *et al.,* 2013) presumably owing to co-evolution of ribosomal proteins and ribosomal nucleic acids (Sauer and Kliem, 2010).

Microbial Biodegradation Potential of Petroleum Hydrocarbon

The time required to decolourize redox indicator DCPIP

By incorporating an electron acceptor such as DCPIP to the culture medium, it is possible to ascertain the ability of organism to utilize the substrate by observing the colour change of DCPIP from Blue (oxidized) to colorless (reduced), (Hanson *et al.,* 1993 ; Mariano *et al*., 2009), and the obtained results are giving in Table 2.

Results presented in this Table show that there were great variations in the time (in hours) to decolourize the DCPIP indicator by the tested bacteria as well as yeast strains and consortium from yeasts or bacterial strains. This time was ranged from 22 to 49 hr., in the case of n-hexadecane and 39 to 86 hr., in the case of BTEX- blend.

As for n-hexadecane, as an example of alkanes group, two strains, namely *Bacillus licheniformis* RdI-17 and *Pseudomonas nitroreducens* RdI-14 caused a discolourization of DCPIP indicators after 29 and 30 hr, respectively. These strains showed a maximum utilization of the tested hydrocarbon *n*-hexadecane in shortest time and can be considered as the best alkanes biodegraders among the tested bacteria. On the other hand, the longest time was recorded in the case of strain *Bacillus mojavensis* QRII 7 and *Bacillus pumilus* RdI-1 being just in 40 hr., and 49 hr., respectively.

Regarding the BTEX blend, as an example of monoaromatic hydrocarbon compounds used in this experiment, only two active strains, *Bacillus licheniformis* RdI-17 and *Pseudomonas nitroreducens* RdI-14 which were recorded in the case of n-hexadecane, and showed also the shortest time in the case of BTEX-blend being 51 and 52hr., respectively, among the tested bacteria for the decolourization of the blue BCPIP to the colorless form. These results are in harmony with those obtained by Pirôllo *et al.* (2008) who mentioned that the *Pesudomonas aeruginosa* LBI strain caused a discolourization the DCPIP redox indicator in the presence of diesel oil and crude oil after 23 hr., in the presence

Table 1. Classification results rate by Bruker Daltonik MALDI Biotyper

Isolate number	Isolate code	Analyte name	Organism (best match)	Score value
1	**RdI-13**	C5(++) (B)	*Bacillus licheniformis 992000432 LBK*	**2.111**
2	**GH-5**	C1(+++) (A)	*Bacillus subtilis* ssp. *subtilis DSM 10T DSM*	**2.332**
3	**GH-6**	C2(++) (B)	*Bacillus atrophaeus DSM 5551 DSM*	**2.121**
4	**RdI-14**	C3(++) (B)	*Pseudomonas nitroreducens LMG 20221T HAM*	**2.129**
5	**RdI-8**	C4(++) (B)	*Bacillus subtilis 107_W_7_QSA IBS*	**2.120**
6	**RdI-3**	C6(+++) (A)	*Bacillus subtilis* ssp. *spizizenii DSM 618 DSM*	**2.323**
7	**RdI-17**	C7(++) (A)	*Bacillus licheniformis CS 54_1 BRB*	**2.361**
8	**QRII-7**	C8(++) (B)	*Bacillus mojavensis* DSM 9205T DSM	**2.130**
9	**QBII-6**	C9(++) (B)	*Sphingobacterium thalpophilum DSM 11723T HAM*	**2.119**
10	**RdI-1**	C10(++) (B)	*Bacillus pumilus DSM 1794 DSM*	**2.151**

- Category A= species consistency (2.300-3.000) , Category B = genus consistency (2.000-2.299)

Table 2. Changes in the time (in hours) for decolorization of DCPIP indicator at 0.1 % in Bushnell-Haas broth by the tested bacterial and yeast strains, alone and with a consortium

	Organism	Time of decolorization of DCPIP during the experiment (96 hr.)	
		***n* –hexadecane**	**BTEX-blend**
	Control	No decolorization	No decolorization
Bacteria	***Bacillus licheniformis* RdI-13**	37 g	65 h
	***Sphingobacterium thalpophilum* QBII-6**	33 e	56 f
	***Pseudomonas nitroreducens* RdI-14**	30 c	52 d
	***Bacillus subtilis* ssp *subtilis* GH-5**	31 d	54 e
	***Bacillus subtilis* RdI- 8**	37 g	75 i
	***Bacillus mojavensis* QRII -7**	40 h	77 j
	***Bacillus atrophaeus* GH- 6**	31 d	55 d
	***Bacillus subtilis* ssp *spizizenii* RDI- 3**	33 e	64 g
	***Bacillus licheniformis* RdI-17**	29 b	51 c
	***Bacillus pumilus* RdI-1**	49 h	82 k
Yeasts	***Candida parapsilosis Complex 9163***	41 i	82 k
	Rhodotorula mucilaginosa Harrison 8802	42 j	85 l
	Pichia anomala Hansen10186	41 i	86 m
Mixed	**Mixed 10 bacteria strains**	22 a	39 a
	Mixed 3 yeasts strains	34 f	50 b
	LSD 0.05	0.0962	0.0962

of kerosene after 68 hr., and partial discolourization in oil sludge after 72 hr. Smilar results were also reported by Bidoia *et al.* (2010) who found that the tested cultures of bacteria can completely reduce DCPIP in 75, 87, 125 and 138 hr., for mineral oil, used oil, semi-synthetic and synthetic oil, respectively.

As seen for yeast strains, results in Table 2 reveal that they showed shortest time to decolorize of the DCPIP indicator within 41-42 hr., in the case of *n*-hexadecane and within 82 - 86 hr., of incubation in the case of BTEX- blend was shown. It seems that the tested yeast strains had the lower ability than the tested bacterial strains to biodegrade the tested hydrocarbon compounds under this study. In general, it is well known that fungi withstand harsher environmental conditions than bacteria and could play an important role in the degradation of petroleum hydrocarbons in the soil (Bossert and Bartha, 1984). Also, the obtained results in Table 2 clearly reveal that the mixed 10 bacterial strains (as consortium no.1) showed maximum *n*-hexadecane and BTEX-blend biodegradation as indicated by the shortest time of DCPIP discolouration being 22 and 39 hr., respectively as compared to the tested individual bacteria. The same trend was also recorded in the case of the addition of yeast strains, as consortium No. 2, it enhanced the total discolouration of DCPIP in the presence of n-hexadecane and BTEX-blend after 34 and 50 hr., respectively as compared to single yeast strains tested. In general, these results are in agreement with those reported by Rahman *et al.* (2002) who stated that individual bacterial cultures, *Micrococcus* sp. GS2-22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, *Bacillus* sp. DS 6-86 and *Pseudomonas* sp. DS 10-129, showed less growth and degradation of Bombay high crude oil than did the mixed bacterial consortium prepared using the above strains.

Changes in the Amounts of CO_2 Evolution

Mineralization studies involving measurement of total CO_2 production can provide excellent information on the biodegradability potential of hydrocarbon (Balba *et al.,* 1998). This process rely on the natural ability of microorganisms to carry out the mineralization of organic chemicals leading ultimately to the formation of CO_2, H_2O and biomass. Monitoring of CO_2 produced during 28-day assays by the tested microorganisms is shown in Figs. 1, 2 and 3.

Data in Figs. 1 and 2 revealed that the evolution of CO_2, in the case of the tested bacteria, reached its peak after 3 days of incubation, as indicated by the rate of CO_2 output during the time course of the experiment, in the case of *Pseudomonas nitroreducens* RdI-14, *B. subtilis* RdI-8 and *B. mojavensis* QRII-7 and after 7 days in the remainder bacterial strains. Also, the same trend was recorded in the case of yeast strains used, since the peak was reached after 3 days in the case of *Candida parapsilosis Complex* 9163 and *Pichia anomala Hansen*10186*,* and 7 days in the case of *Rhodotorula mucilaginosa Harrison* 8802. The same trends of daily CO_2 production during incubation period were also reported by Mariano *et al.* (2007), who found that respirometric data indicated that a maximum hydrocarbon mineralization of 19%, obtained through the combination of the three agents, addition of nitrogen and phosphorus solutions or Tween 80 surfactant, with a total petroleum hydrocarbon removal of 45.5% in 55 days of treatment.

Also, it is obvious from Data in Figs. 1 and 2 that as the time of the experiment proceeded thereafter, the amount of CO_2 evolved were gradually decreased up to the end of the experiment in both bacteria and yeast strains. This decline in CO_2 production might be attributed to the decline in density of microbial population, which was deduced to the exhaustion of the available degradable organic fraction. In this connection, Sabate *et al.* (2004) mentioned that, this decline could be attributed to the absence of assimilable sources of carbon and energy or to the presence of toxic compounds in soil.

The rate and extent of biodegradtion might be attributed to the strain ability as well as the number of aromatic ring present and the nature of the linkage between these rings in the tested monoaromatic hydrocarbon, BTEX-mixture. However, Mariano *et al*. (2007) mentioned that, this variation may be caused by a transition of carbon sources. As the labile hydrocarbon

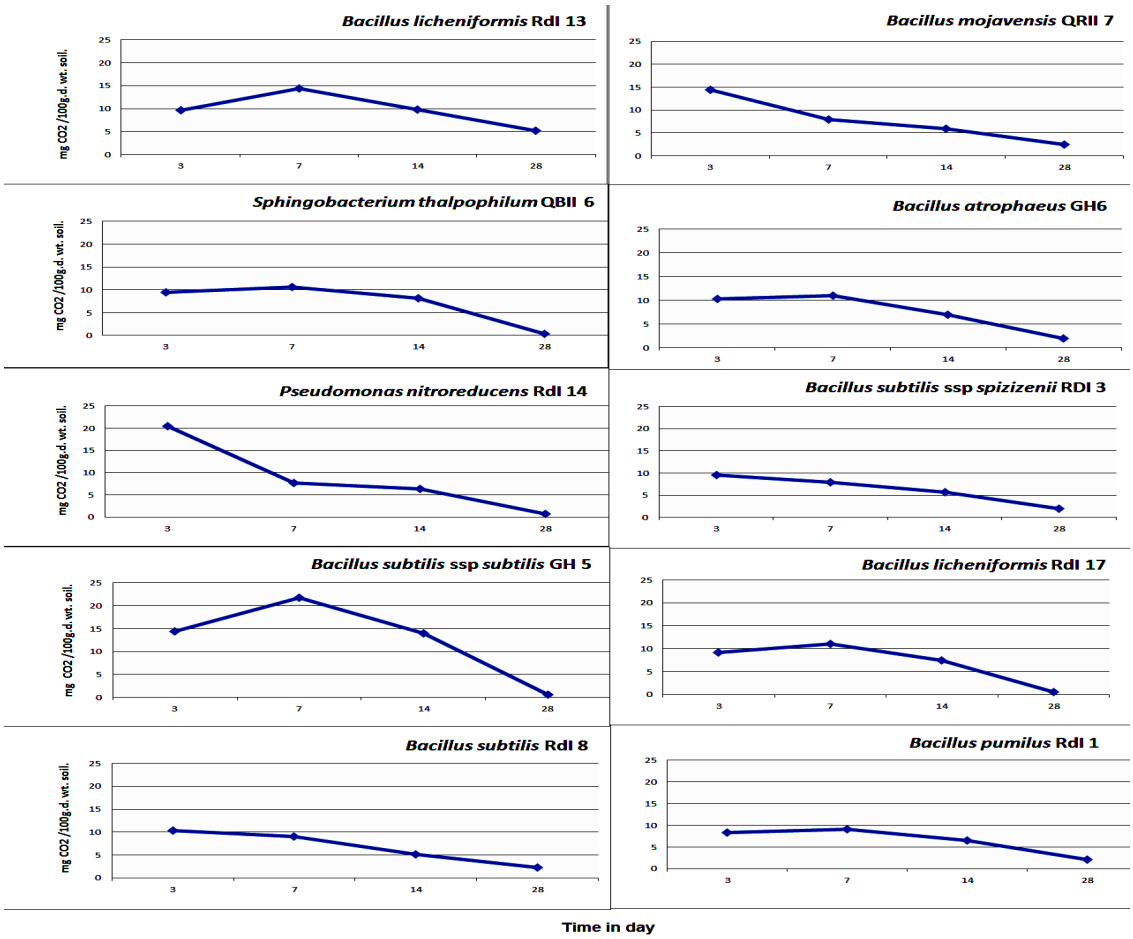

Fig. 1. Changes in the amounts of CO_2 from sandy loam soil output during the biodegradation of BTEX blend at 2% by the best bacterial strains during incubation at 28˚C for 28 days

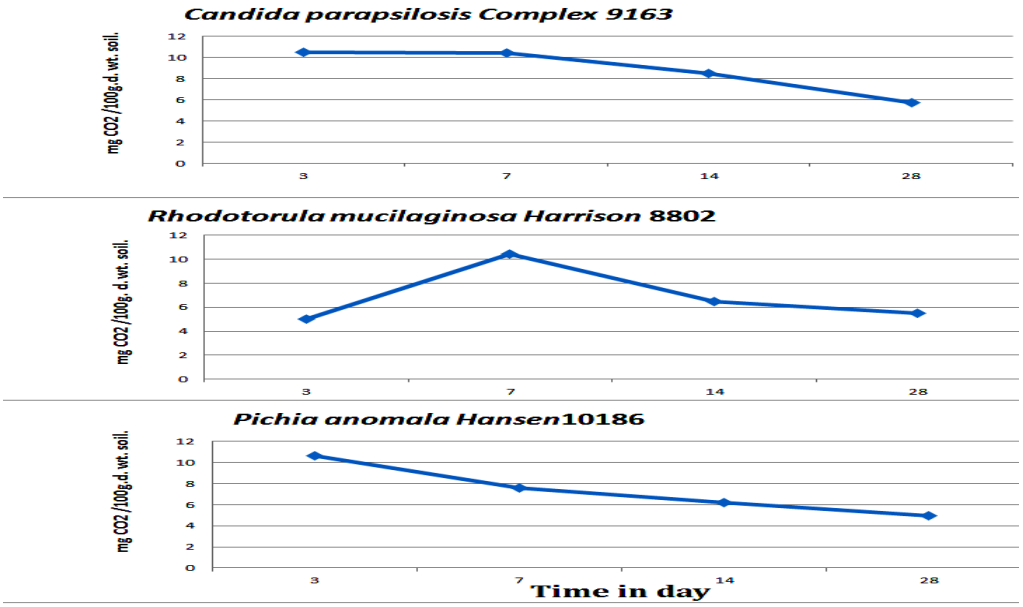

Fig. 2.Changes in the amouts of CO_2 output from sandy loam soil during the biodegradation of BTEX blend at 2% by three standard yeasts strains during incubation at 28˚C for 28 days

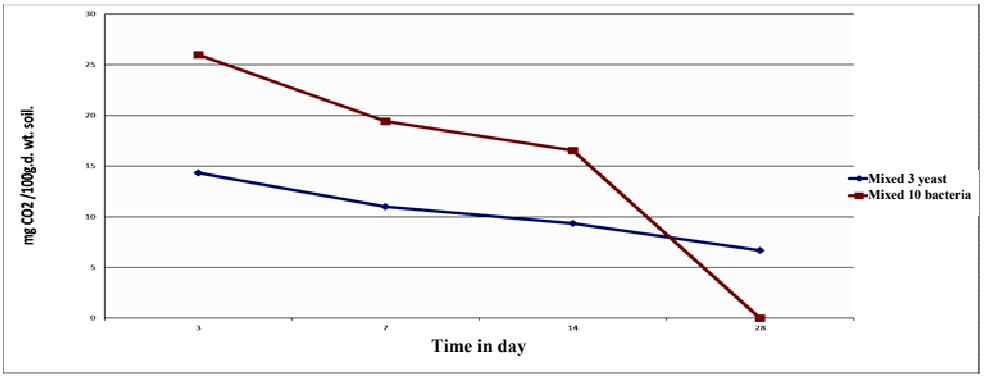

Fig. 3. Changes in the amouts of CO_2 output from sandy loam soil during the biodegradation of BTEX blend at 2% by either mixed bacterial strains or mixed yeasts strains during incubation at 28˚C for 28 days

sources (probably linear and open-chain hydrocarbons) are consumed, their bioavilability decreases. The shortage of these sources forces the microorganisms to adapt to more recalcitrant sources (probably aromatic hydrocarbon with higher molecular weight), resulting in decrease of the CO_2 production.

When each of the active hydrocarbon bacterial or yeast degraders were mixed then added to the soil as consortium (No.1 and No.2), considerable amounts of CO_2 uptake were recorded during the time course of the experiment. Also, the rate of CO_2 production reached its peak after 3 days of incubation, being 25,978 and 14.345 mg CO_2/day/100 g.d.wt. soil, as well as the average daily rate of CO_2 production were 2.477 and 1.502 mg CO_2/ day in the case of consortium No. 1 and consortium No. 2, respectively. The mixed bacterial strains and yeast strains as a specific consortium showed more growth and degradation of BTEX-blend used in this study as indicated by the values of CO_2 production and the average daily rate of CO_2 production. Also, it is interesting to notice from Figs. 1, 2 and 3 that the amount of CO_2 evolved at 28 days, in the case of consortium No.1, reached to zero value. This means that the used 10 bacterial strains, in association form, exhausted or mineralized completely the available degradable hydrocarbon, BTEX-components. Thus, using this bacterial consortium as inoculum provides certain advantages over biostimulation of indigenous microorganisms cases where there is pollutant toxicity or lack of appropriate microorganisms (quantity or quality). In this respect, Leahy and Colwell (1990) reported that mixed population with overall broad enzymatic capacities are required to degrade complex mixture of hydrocarbons such as crude oil and diesel fuel. Such mixed cultures display metabolic superiority to pure cultures (Van Hamme *et al.,* 2000).

In general, microbial consortium can degrade hydrocarbon from oil-contaminated sites more efficiently than pure isolated microorganisms mainly due to synergetic effects. Recently, El-Naas *et al.* (2014) explained this enhancement to either the presence of different microbial species with a number of metabolic pathways or to interspecies interactions. Therefore, mixed cultures may be more effective than single cultures in biotreatment systems for the complete biodegradation of multicomponent hydrocarbons.

Factors Affecting Biodegradation of Petroleum Hydrocarbons

Effect of temperature

It is well known that temperature affects rate of hydrocarbon degradation and the physicochemical compositions of oil, results in enhanced hydrocarbon bioaviability as well as the composition and metabolic activity of the microbial communities (Okoh, 2006; Perfumo *et al.,* 2007; Koshlaf and Ball, 2017). The changes in the bacterial growth of the tested 10 bacterial strains in trypticase soy broth supplemented with *n*-hexadecane at 2.0% as affected by

different incubation temperatures are given in Table 3. It is obvious from the results that the tested bacterial strains were able to grow at different temperature degrees *viz,* 25, 30, 35 and 40˚C. Moreover, there were, in general, from two to three- fold increase in the bacterial growth (O.D 600 nm) when the incubation temperature raised from 25 to 30˚C. Also, the results revealed that the highest bacterial biomass of the tested bacterial strains were observed at 35˚C and showed a positive correlation between the temperature degrees and the bacterial biomass production except in the case of *Pseudomonas nitroreducens* RDI-14*,* which showed its maximal growth at 30˚C. At 40˚C, there were noticeable declines in the bacterial growth of all tested bacterial strains. These results confirmed those obtained by Sathishkumar *et al.* (2008), who reported that temperature of 35˚C was found to be an optimum for maximum biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium (*Bacillus* sp.IO1.7, *Corynebacterium* sp. Bp2-6, *Pseudomonas* sp. HPS2.5, and *Pseudomonas* sp. BPS1.8). Also, Rahman *et al.* (2002) reported that temperature of 30 ˚C was found to be optima for maximum biodegradation of crude oil (from 1 to 10%) by *Micrococcus* sp. GS2.22, *Corynebacterium* sp. GS5-66, *Flavobacterium* sp. DS5-73, and *Pseudomonas* sp. DS10.129 isolates.

In fact, temperature is responsible or controlling the nature and extent of microbial metabolism in hydrocarbons as well as diffusion rates, biovailability and solubility (El-Naas *et al.,* 2014).

Effect of pH

The bacterial growth in the synthetic media are sensitive to the alternative of pH. Thus, it was necessary to determine the optimum pH value suitable for petroleum degradation by the selected bacteria. Results in Table 4 show that there were a progressive increase in the bacterial growth (O.D 600 nm) for all the tested strains when pH value of the tested media increased from 6.5 to 7.0. This alteration in pH value caused considerable bacterial growth rates being seven- and nine-fold increase in the case of *Bacillus subtilis* RdI-8 and *B. pumilus* RdI-1, respectively, while the rest of the bacterial strains showed two to four -fold increase in the growth rate.

Results also revealed that only 6 out of 10 bacterial strains, namely *Sphingobacterium thalpophilum* QBII-6, *Bacillus subtilis* ssp *subtilis* GH-5, *B. mojavensis* QRII-7, *B. atrophaeus* GH-6, *B. licheniformis* RdI-17 and *Bacillus pumilus* RdI-1, showed the highest bacterial growth rates at pH 7.0, while the rest of bacterial strains showed the highest bacterial growth rates at pH 7.5. Such variation could be attributed to bacterial type *per se*, microbial behaviour and interaction to utilize *n*-hexadecane as carbon and energy source.

At pH 8.0, there were obvious reduction in the bacterial growth rates for all tested bacterial strains used in this study. Similar results were also obtained by Salmon *et al.* (1998) and Rahman *et al.* (1999). Meredith *et al.* (2000) found that a neutral pH of 7.0 has been reported to be optimal for petroleum biodegradation and extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons. Also, the optimal growth of many tested other diesel degrading bacteria were reported at neutral or near pH, (Ueno *et al.,* 2007 ; Kwapisz *et al.,* 2008). However, El-Naas *et al.* (2014) stated that most bacteria are neutrophils, so the optimum pH at which the highest degree of BTEX biodegradation is achieved at pH 7.5, and bacteria and actinomycetes was optimal in a range typically between 7 and 8.

Effect of salinity

In this experiment, the ability of the selected 10 active bacterial strains to grow at different NaCl concentrations *viz*, 0, 0.5, 2.5 and 5.0% were determined at 30˚C, pH 7.0 and incubation for 11 days.

The obtained results in this respect are shown in Table 5. It was found that all bacterial strains were very sensitive to NaCl concentrations used in this study, as indicated by the obvious declines in the bacterial growth rates. On the other hand, these tested bacterial strains were mainly isolated from soil contaminated with hydrocarbons or petroleum sludge samples not from marine environments.

Table 3. Bacterial growth (O.D 600 nm) of the tested 10 bacterial strains in trypticase soy broth supplemented with *n*–hexadecane at 2.0% as affected by different incubation temperatures

Temperature degree / Bacterial strain	Bacterial growth (O.D 600 nm)			
	25 ˚C	30 ˚C	35 ˚C	40 ˚C
***Bacillus licheniformis* Rd-I13**	0.331	1.111	1.328	0.500
***Sphingobacterium thalpophilum* QBII- 6**	0.561	1.342	1.480	0.806
***Pseudomonas nitroreducens* RDI- 14**	0.500	1.471	1.330	0.880
***Bacillus subtilis* ssp. *subtilis* GH -5**	0.610	1.169	1.401	0.776
***Bacillus subtilis* RdI- 8**	0.444	1.111	1.216	0.593
***Bacillus mojavensis* QRII -7**	0.500	1.091	1.309	0.716
***Bacillus atrophaeus* GH -6**	0.499	1.126	1.421	0.730
***Bacillus subtilis* ssp. *spizizenii* RDI- 3**	0.385	1.041	1.221	0.513
***Bacillus licheniformis* RdI- 17**	0.600	1.141	1.340	0.832
***Bacillus pumilus* RdI-1**	0.501	1.000	1.136	0.725
LSD 0.05	0.0012	0.0033	0.0011	0.0033

Table 4. Bacterial growth (O.D 600 nm) of the tested 10 bacterial strains in trypticase soy broth supplemented with *n*–hexadecane at 2.0% as affected by different starting pH levels

pH value / Bacterial strain	Bacterial growth (O.D 600nm)			
	6.5	7.0	7.5	8.0
***Bacillus licheniformis* RdI- 13**	0.277	1.032	1.121	0.482
***Sphingobacterium thalpophilum* QBII -6**	0.482	1.391	1.321	0.832
***Pseudomonas nitroreducens* RdI -14**	0.464	1.361	1.421	0.944
***Bacillus subtilis* ssp. *subtilis* GH -5**	0.365	1.436	1.312	0.998
***Bacillus subtilis* RdI -8**	0.164	1.323	0.885	0.711
***Bacillus mojavensis* QRII -7**	0.053	1.190	1.130	0.666
***Bacillus atrophaeus* GH -6**	0.316	1.451	1.174	0.831
***Bacillus subtilis* ssp. *spizizenii* RdI -3**	0.464	1.131	1.160	0.83
***Bacillus licheniformis* RdI -17**	0.492	1.490	1.441	0.885
***Bacillus pumilus* RdI -1**	0.125	1.195	1.130	0.500
LSD 0.05	0.0011	0.0670	0.0033	0.0033

Results in Table 5 show also that, the control treatment was the most suitable for most tested bacterial strains, with some exceptions in the case of *Bacillus subtilis* RdI-8 and *B. licheniformis* RdI-17, which showed the highest bacterial biomass formation at 0.5% NaCl.

The obtained results are in line with those reported by Minani-Tehrani *et al.* (2006) who studied the effect of different NaCl concentrations (0.0% -5.0%) on polycyclic aromatic hydrocarbons (PAHs) reduction from the heavy crude oil contaminated soil. They pointed out that the biodegradation of total crude oil was higher at 0.0% NaCl (41%), while a higher total PAHs reduction was observed at 1.0% NaCl (35%). The lower reduction in total crude oil and PAHs reduction were observed at 5.0% NaCl giving 12% and 8%, respectively. Also, de Carvalho and de Fonseca (2005) observed that degradation of alcohol and hydrocarbons at different concentrations of NaCl ranged from 0.13% to 2.0% and found a better growth rate at 0.5%. Thus, biodegradation of oil by microorganisms in the presence of high salinity was slow, this was because of high NaCl in medium might be disrupt cell membrane, denature some proteins, such as enzymes, or change the osmatic force, which any of these factors could be lethal for microorganisms (Kargi and Dincer, 2000 ; Minani- Tehrani *et al.,* 2006).

Effect of Incubation Periods

The influence of incubation periods at 3, 7, 11 and 15 days on the growth of bacterial strains tested and their abilities to degrade *n*-hexadecane at 2.0% at different time intervals was studied using trypticase soy broth, at pH 7.0 and 30˚C (Table 6).

The results demonstrated that as the time of incubation proceeded, there were an obvious increases in the bacterial growth (biomass) of the most bacterial strains. The highest bacterial biomass formation was observed at 11 days of incubation, this means that the tested bacterial strains had the abilities to utilize the tested substrate at this time. At 15 days of incubation, the end of the experiment, there was a progressive reduction in the bacterial biomass formation, these results were true with all the tested bacteria. This reduction could be attributed to the absence of assailable sources of carbon and energy in the food medium and/or to the presence of toxic metabolite compounds in the food medium. The obtained results are in the same line with those found by Mishra and Singh (2012), since they studied *n*-hexadecane degradation in mineral salt medium (MSM) as mediated by degrative enzymes using three bacteria identified as *Pesudomonas aeruginosa,* PSA5, *Rhodococcus* sp. NJ2 and *Ochrobacterum intermedium* P2 isolated from petroleum sludge. They reported also that *n*-hexadecane was degraded to 99%, 95% and 92% during 10 days of incubation, respectively. On the other hand, Yasin *et al.* (2014) found that, the percentage of crude oil consumption by *Pesudomonas aeruginosa* after 7 days of incubation was 70.7%, while it was 71.5%, 74.1% and 78.11% for incubation period of 14, 20 and 28 days, respectively. Also, Mahalingam and Sampath (2014) pointed that the better degradation of diesel oil was recorded by isolates of *Pesudomonas* spp. and *Bacillus* spp. with 1% diesel oil at 37˚C on the 7th day.

Biodegradation of Petroleum and Non-Petroleum Oils

The registered bacterial growth (at 600 nm) of the tested 10 bacterial strains as well as one bacterial *Enterobacter clocace* spp. *disssolvens* strain, used in this study as efficient bacteria in petroleum hydrocarbon degradation as affected by different carbon sources are shown in Table 7.

As for the first group of carbon sources, crude oil, and their products, (diesel oil, engine oil and used engine oil), the tested bacteria had the ability to grow and utilize them as indicated by the values of their optical densities which were ranged as follows: 0.500 - 1.200, 0.555 - 1.216, 0.604 - 1.321 and 0.583 - 1.272, respectively. In fact, it is well known that petroleum products are considered to be recalcitrant to microbial degradation and persist in ecosystems because of their hydrophobic nature and low avlability and they pose a significant threat to the environment (Abed *et al.,* 2002; Sawadogo *et al.,* 2014). Also, diesel oil and lubricants are hydrocarbon commonly used in Egypt and over the world. Soils and surface water contamination by these forms is common occurrence in most developing countries.

Table 5. Bacterial growth (O.D 600 nm) of the tested 10 bacterial strains in trypticase soy broth supplemented with *n*–hexadecane at 2.0% as affected by different salinity levels

Salinity level (%) NaCl / Bacterial strain	Bacterial growth (O.D 600 nm)			
	0.0%	0.5%	2.5%	5.0%
***Bacillus licheniformis* RdI -13**	0.858	0.659	0.529	0.025
***Sphingobacterium thalpophilum* QBII -6**	1.480	1.355	1.197	0.482
***Pseudomonas nitroreducens* RdI -14**	1.380	1.060	0.845	0.465
***Bacillus subtilis* ssp. *subtilis* GH -5**	1.330	1.165	0.919	0.365
***Bacillus subtilis* RdI -8**	0.605	0.629	0.342	0.164
***Bacillus mojavensis* QRII -7**	1.236	1.011	0.538	0.277
***Bacillus atrophaeus* GH -6**	1.332	1.220	0.724	0.316
***Bacillus subtilis* ssp. *spizizenii* RdI -3**	0.980	0.742	0.672	0.053
***Bacillus licheniformis* RdI -17**	1.450	1.482	1.355	0.487
***Bacillus pumilus* RdI -1**	0.816	0.585	0.538	0.465
LSD 0.05	0.0033	0.0033	0.0016	0.00116

Table 6. Bacterial growth (O.D 600 nm) of the tested 10 bacterial strains in trypticase soy broth supplemented with *n*–hexadecane as affected by different incubation periods

Incubation period / Bacterial strain	Bacterial growth (O.D 600 nm)			
	3 days	7 days	11 days	15 days
***Bacillus licheniformis* RdI -13**	0.563	0.580	1.052	0.838
***Sphingobacterium thalpophilum* QBII -6**	0.640	1.181	1.203	1.00
***Pseudomonas nitroreducens* RdI- 14**	0.662	1.095	1.377	1.211
***Bacillus subtilis* ssp. *subtilis* GH -5**	0.861	0.986	1.278	1.110
***Bacillus subtilis* RdI- 8**	0.095	0.561	0.769	0.6605
***Bacillus mojavensis* QRII -7**	0.201	0.657	1.077	0.948
***Bacillus atrophaeus* GH- 6**	0.600	0.741	1.228	1.000
***Bacillus subtilis* ssp. *spizizenii* RdI -3**	0.059	0.700	1.180	0.999
***Bacillus licheniformis* RdI -17**	0.905	1.128	1.277	1.156
***Bacillus pumilus* RdI -1**	0.340	0.611	1.062	0.893
LSD 0.05	0.0033	0.0033	0.0033	0.0033

This has been shown to have harmful effects on the environment and human being (Abioy *et al.,* 2012).

Results presented in Table 7 show also, that only 5 bacterial strains, namely: *Sphingobacterium thalpophilum* QBII-6*, Pseudomonas nitroreducens* RdI-14, *Bacillus subtilis* ssp. *subtilis* GH-5*, Bacillus atrophaeus* GH-6 and *Bacillus licheniformis* RdI-17 as well as *Enterobacter clocace* spp. *disssolvens* strain showed the highest capacities to utilize these carbon sources used in this study. On the other hand, all the tested bacteria showed also considerable reduction in their growth rates on crude oil as carbon source in the growth medium. This could be considered a logic, since it is well known that petroleum contains numerous compounds of varying structural complexities. It is composed mainly of 95-99.5% hydrocarbons, as well as some bitumen and other materials. Hydrocarbons involved with alkanes, aromatic hydrocarbons, cyclic alkanes, fused ring compounds, and heterocyclics (Leahy and Colwell, 1990). Also, many microorganisms possess the capability to degrade petroleum, but in different degradation abilities for different components of petroleum (Li *et al.,* 2002; Akbari and Ghoshal*,* 2014).

As to the presence of engine oil and used engine oil as a carbon source in the growth medium, it is clear from the previous results that all the tested organisms exhibited the ability to utilize them but in different degrees as indicated by the values of their optical densities at the end of the experiment. These values were lower with all tested organisms in the case of used engine oil. Recently, Sawadogo *et al.* (2014) studied the ability of two isolated strains of *Acinetobacter* (S2) and *Pseudomonas* (S7) to use the following hydrocarbons: Diesel, Total Quartz 9000 motor oil, SAE 40 used oil and gasoline as a sole carbon and energy source at a concentration of 3% (*V/V*). They found that no significant difference was recorded among hydrocarbons for strains S2 and S7 as well (P=0.235).

As for the second group of carbon sources, natural oils, the active 10 bacterial strains as well as *Enterobacter clocace* spp. *disssolvens* strains had the ability to utilize some vegetable oils namely corn oil and sunflower oil as well as their spent forms produced after cooking and repeated uses, and to find out its carbon source depletion could further oil consumption by the tested organisms.

The bacterial growth (at 600 nm) of the tested organisms, used in this study as affected by the type of organic materials (corn oil- used corn oil-sunflower-used sunflower oil) as carbon source in Bushnell-Haas medium are shown in Table 7. Results showed that all the tested bacteria had the ability to metabolize and utilize all the tested fresh and used vegetable oils but in different degradations as indicated by the values of their optical densities owing to their growth. These values were ranging from 0.627 to 1.459 and from 0.551 to 1.400 in the case of corn oil, and used corn oil, respectively, and from 0.624 to 1.431 and from 0.584 to 1.205 in the case of sunflower, and used sunflower oils, respectively. Similar results on the efficient degradation of spent vegetable oils by the tested bacteria were ascertained. In line with that, Al-Darbi *et al.* (2005) studied the influence of nutrient and microbial environment on changes in bacterial numbers and exent and rates of degradation for various test oil (olive, mustard, canola and olive oils). They found that different oils respond in different ratios and extent to biodegradation depending on their stability, viscosity and composition. Also, in the same year Malatova (2005) studied the ability of seven bacterial strains to utilize larger and more complex structures of hydrocarbons, and other organic compounds (olive oil- peanut oil- corn oil-canola oil), and the author pointed out that the highest bacterial growth on olive oil and canola oil was elucidated by their composition, with high percentage of monounsaturated fatty acid (62-72%) and lower percentage of polyunsaturated (9-32%) make them an easy target for microbial utilization. Recently, Mahalingam and Sampath (2014) used different carbon sources such as diesel oil, corn oil and sunflower oil to study the bacterial growth of three efficient diesel oil degrading bacteria. The higher percentage of microbial biomass was recorded in Bushnell-Haas medium supplemented with sunflower oil as a carbon source at 1% in the presence of ammonium sulfate as a nitrogen source.

Again the only 5 bacterial strains namely: *Sphingobacterium thalpophilum* QBII-6*, Pseudomonas nitroreducens* RdI-14, *Bacillus subtilis* ssp *subtilis* GH-5*, Bacillus atrophaeus*

Table 7. Growth and ability of the tested bacteria, to use different petroleum hydrocarbon and natural oils as carbon source as grown in Bushnell-Haas broth for 11 days of incubation

Organic compound / Bacterial strain	Bacterial growth (O.D 600 nm)							
	Petroleum oils				Non-petroleum oils			
	Diesel oil	Used engine oil	Engine oil	Crude oil	Corn oil	Used corn oil	Sunflower oil	Used sunflower oil
***Bacillus licheniformis* RdI -13**	0.666	0.684	1.00	0.500	1.051	1.00	1.051	1.001
***Sphingobacterium thalpophilum* QBI -6**	1.1065	1.133	1.156	1.055	1.444	1.333	1.344	1.139
***Pseudomonas nitroreducens* RdI 14**	1.159	1.183	1.200	1.060	1.456	1.367	1.431	1.191
***Bacillus subtilis* ssp. *subtilis* GH- 5**	1.1665	1.250	1.311	1.050	1.450	1.343	1.394	1.078
***Bacillus subtilis* RdI -8**	0.555	0.606	0.650	0.500	0.777	0.655	0.774	0.584
***Bacillus mojavensis* QRII -7**	0.599	0.583	0.615	0.583	0.700	0.555	0.806	0.606
***Bacillus atrophaeus* GH- 6**	1.216	1.260	1.311	1.100	1.459	1.400	1.429	1.079
***Bacillus subtilis* ssp. *spizizenii* RdI- 3**	0.630	0.646	0.649	0.610	0.677	0.616	0.685	0.585
***Bacillus licheniformis* RdI -17**	1.255	1.322	1.383	1.200	1.479	1.433	1.494	1.139
***Bacillus pumilus* RdI- 1**	0.580	0.601	0.604	0.565	0.627	0.551	0.624	0.611
Enterobacter clocace* spp. *disssolvens	1.205	1.272	1.321	1.200	1.416	1.315	1.415	1.205
LSD 0.05	0.0097	0.0097	0.0097	0.0097	0.0096	0.0097	0.0092	0.0097

GH-6 and *Bacillus licheniformis* RdI-17 as well as *Enterobacter cloacae* strain showed the highest capability to metabolize and utilize these vegetables oils in fresh and spent forms. On the basis of these results, it could be concluded that these five bacterial strains as well as *Enterobacter cloacae* strain have high ability to utilize a diverse range of organic compounds, vegetable oil, and petroleum hydrocarbon products.

Finally, in order to increase the feasibility of these bacteria as the potential commercial strains, future studies need to clarify the factors affecting the capability and efficiency of hydrocarbons and crude oil degradation such as nutrients, oxygen content and physical state of oil in the field under realistic conditions.

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إمكانية التحلل الحيوى الميكروبى لزيوت البترول والزيوت الطبيعية بواسطة البكتريا الطبيعية المحللة للهيدروكربون والمعزولة من مواقع ملوثة بالبترول

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أُجريت هذه الدراسة كمحاولة للوصول إلى بكتريا نشطة في تحلل الهيدروكربونات البترولية والتى تكون ذات قدرة عالية على الإزالة الحيوية للملوثات من الزيوت البترولية وغير البترولية ولتحقيق هذا الهدف فقد تم استخدام ١٠ عزلات بكتيرية و٣ سلالات من الخمائر وسلالة قياسية لبكتريا الانتيروباكتر كلواسى ذات الأنشطة الفعالة في تحلل المواد البترولية، وقد تم تعريف العزلات البكتيرية العشرة إلى مستوى الجنس والنوع باستخدام تقنية ال MALDI-TOF-Ms بدرجة دقة عالية ٢,١١١-٣,٣٦١ وهى استراتيجية حديثة في تعريف الميكروبات في العينة مباشرة وفي دقائق معدودة، وقد تم استخدام اثنين من التقنيات هما طريقة قياس التنفس (قياس CO_2 الناتج من التربة) وطريقة اختبار دليل الأكسدة والاختزال DCPIP بهدف تقييم قدرة الميكروبات المستخدمة علي الاستفادة من مخلوط BTEX ومركب الهكساديكان (كأدلة نموذجية تستخدم في دراسة التحلل للمواد البترولية ميكروبياً) وذلك بصورة منفردة أو من خلال لقاح بكتيرى خليط أو لقاح خميره خليط وقد أشارت النتائج إلى وجود تباين في قدرات الميكروبات تحت الدراسة على تحليل المواد المستخدمة وأن اللقاحات المختلطة كانت أكثر كفاءة على النمو وتحليل المواد المستخدمة في الدراسة، وقد تم تقدير بعض عوامل النمو الهامة المسئولة عن نجاح التحلل الحيوى للمواد تحت الدراسة وأظهرت النتائج أن درجة ال pH (٧) والحرارة (٣٠-٣٥°م) والملوحة (صفر إلى ٠,٥% كلوريد صوديوم) وفترة تحضين (١١يوم) كانت الأكثر مناسبة لمعظم البكتريا المستخدمة تحت الدراسة، ولقد أشارت النتائج أيضاً أمتلاك خمسة أنواع من البكتريا العشرة المستخدمة فى الدراسة وهى: *Sphingobacterium thalpophilum* QBII-6, *Pseudomonas nitroreducens* RdI-14, *Bacillus subtilis* ssp *subtilis* GH-5, *B. atrophaeus* GH-6 and *B. licheniformis* RdI-17 as well as *Ent. clocace* spp. *disssolvens*، قدرة عالية على تمثيل مدى واسع من المواد البترولية (زيت البترول - زيت الديزل- زيت المحرك- زيت المحرك المستعمل) والزيوت الطبيعية (زيت ذرة- زيت ذره مستعمل- زيت عباد الشمس- زيت عباد شمس مستعمل) وتلك الانواع الخمسة والسلالة المرجعية يمكن تطبيقها بعد عمل دراسات مستقبلية فى ظروف الحقل في عمليات الإزالة الحيوية للملوثات كهدف نهائي.

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