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Optimization and Statistical Evaluation of Medium Components Affecting Crude Oil Biodegradation by Some Locally Isolated Bacteria

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TOWARDS an efficient crude oil bioremediation, optimization and statistical evaluation of medium components for four local bacterial isolates were investigated. Morphological and biochemical analysis identified the most active species as *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus sphaericus* and *Bacillus brevis*. 16S rRNA sequencing analysis identified the most potent isolate as *Pseudomonas aeruginosa* KH6 and was submitted to GenBank under the accession number (KM194714). Preliminary experiments of crude oil degradation revealed that the four bacterial species, gave the highest total petroleum hydrocarbon (TPH) removal efficiency after 15 days incubation in the presence of crude oil as a carbon source at 35°C, and pH 7.5. Statistical medium optimization on the biodegradation efficiency using Plackett-Burman design showed that decreasing in crude oil concentration was significant for maximum biodegradation efficiency for the isolates. While, the increase in Fe_2SO_4 and $MgSO_4.7H_2O$ factors resulted in an elevation in the TPH removal efficiency for *Bacillus sphaericus* and *Bacillus brevis*, respectively. The optimized media achieved by statistical design raised the removal efficiency for the four isolates, reaching 1.13 times higher than that of the control medium for *Pseudomonas aeruginosa* KH6.

Keywords: Crude oil biodegradation, Optimization, Plackett-Burman design, *Bacillus*, *Pseudomonas*.

The discharge of oil and grease containing wastewater to the environment increases every year due to rapid urbanization and industrial development (Affandi *et al*., 2014). Crude oil is the original mixture of a variety of petroleum hydrocarbons. One of the most important means by which oil is removed from the marine environment is natural biodegradation, especially the nonvolatile components of crude or refined petroleum. Man-made bioremediation technologies are intended to improve the effectiveness of natural biodegradation (Von Storch *et al*., 2008). Bioremediation is suggested for treating contaminated soil sites because of its low cost and ability to convert contaminants to harmless end products. Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders of spilled oil. Recently, growing interest in the use of several Pseudomonades during degradation of crude oil have been reported (Berekaa, 2013).

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The process of natural bioremediation is usually slow. It was shown that factors such as temperature, pH and salinity played an important role in the bioremediation process. Therefore, adding allogenetic microorganisms and changing degradation conditions (temperature, pH and salinity) are effective methods that can improve the rate of biodegradation. Different microorganisms have different optimum growth conditions and oil degrading efficiencies, and so does the same microorganism in various environments (Bao *et al*., 2013). The rates of uptake and mineralization of many organic compounds by a microbial population depend on the concentration of the compound (Olivera *et al*., 1997). High concentrations of hydrocarbons associated with heavy, undispersed oil slicks in water can cause inhibition of biodegradation by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons. The temperature also influences petroleum biodegradation by its effect on the physico-chemical properties of the oil, rate of hydrocarbon metabolism by microorganisms and composition of the microbial community (Atlas, 1981).

Experimental design techniques present a more balanced alternative to one-variable-at-a-time approach (OVAT) in which single factor is varied, while others are kept fixed. However, Plackett and Burman design comprises one type of two-level screening and can be constructed on the basis of fractional replication of a full factorial design (Plackett and Burman, 1946). This design is appropriate to face the large number of cultivation conditions under investigation and allow obtaining unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations (Akhnazarova and Kafarov, 1982).

The main aim of this work was to investigate the possible improvement of crude oil degradation by different oil degrading bacteria and to evaluate the influence of different cultivation condition on efficiency of crude oil degradation.

Materials and Methods

Bacterial isolates were isolated from wastewater samples collected in sterile glass containers from oil-polluted wastewater of the API (American Petroleum Institute) separators (1, 2 and 4) of Alexandria Petroleum Company (APC), Alexandria, Egypt. A suitable dilution of water sample was used to inoculate mineral salt medium (MSM) of the following composition (g/L): $(NH_4)_2SO_4$, 1g; K_2HPO_4, 0.3g; $CaCl_2$, 0.01g; $MgSO_4.7H_2O$, 0.2g; $FeSO_4$, 0.01g; Yeast extract, 0.1g; Agar, 20g. After 48 hr, the produced colonies were subsequently plated out into the same medium for further purification. Screening of the purified colonies for crude oil utilization was carried out by cultivation on nutrient agar medium supplemented with crude oil as carbon source for 15 days. Colonies were chosen and maintained on nutrient agar slants. Subcultures of the isolates were cultivated in 4 ml nutrient broth medium at 35ºC for activation of the organisms for crude oil biodegradation. Different isolates from overnight cultures at the log phase of growth were transferred to 250 ml conical flasks, each containing 50 ml of sterile defined mineral salts medium (MSM), supplemented with 1% crude oil.

The medium was adjusted to pH 7 and flasks were incubated at 30ºC and 120 rpm in shaker incubator for 15 days. Flasks were grown in replica to measure the physiochemical effects on growth and total petroleum hydrocarbon (TPH) removal efficiency.

The isolates were identified and characterized to the species level according to Bergey's manual of systematic bacteriology (Holt *et al*., 1994) by the help of the Fermentation Biotechnology and Applied Microbiology Center (Ferm-BAM) at El Azhar University, Nasr city, Egypt Furthermore, the 16S rRNA gene of the most potent species was amplified by PCR using the primers 16F27 and 16R1492 from the genomic DNA and sequenced as described by Sambrook *et al*. **(**1989**)**. DNA sequencing was performed by Macrogen Inc., Korea. Blast Program (www.ncbi.nlm.nih.gov/blast) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999).

To study the effect of time on growth and biodegradation, 50 ml of MSM medium supplemented with 1% crude oil in 250 ml Erlenmeyer flasks were incubated at 30ºC, and 120 rpm in shaking incubator for 20 days. Sampling was performed at 5 days interval for 20 days.

The effect of temperature was studied in the temperature range of 15 ºC to 45ºC. Different pHs from 6.5 to 9.5 were examined by using sodium hydroxide solution 1 N (NaOH) and 1 N (HCl).

Plackett-Burman design

The Plackett-Burman design, which is a fraction of a two level fraction design, allows the investigation of n-1 variables in at least n experiments (Plackett and Burman, 1946). In this experiment, 7 variables which included nutrients were screened in eight combinations organized according to the Plackett-Burman design matrix. For each variable, a high (+) and low (-) level was tested (Tables 1 and 2). All trials were performed in duplicates and the average results were treated as the responses.

The main effect of each variable was determined with the following equation: $E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / n$. Where E_{xi} is the variable main effect, M_{i+} and M_{i-} are the TPH removal efficiency in trials where the independent variable (xi) was present in high and low concentration respectively, and n is the number of trails divided by 2. A main effect with a positive sign indicates that the high concentration of this variable is near to the optimum and a negative sign indicates that the low concentration of this variable is near to the optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significances.

Optical density determination

The culture broths were taken for growth determination photometrically at 600 nm using spectrophotometer (Pharmacia Biotech, Novaspec II).

TABLE 1. Different levels of the seven independent variables used in the plackett-burman design.

Factors (g/L)	Low level (-)	Level (0)	High Level (+)
$(NH_4)_2SO_4$	0.5	1	1.5
$CaCl_2$	0.005	0.01	0.015
K_2HPO_4	0.15	0.3	0.45
$MgSO_4.7H_2O$	0.1	0.2	0.3
Fe_2SO_4	0.005	0.01	0.015
Yeast extract	0.05	0.1	0.15
Hydrocarbon	5	10	15

TABLE 2. Plackett-burman design for seven variables.

Trials	Independent variables						
	$(NH_4)_2SO_4$	$CaCl_2$	K_2HPO_4	$MgSO_4.7H_2O$	Fe_2SO_4	Yeast extract	Hydrocarbon
1	+	+	+	-	+	-	-
2	+	+	-	+	-	-	+
3	+	-	+	-	-	+	+
4	-	+	-	-	+	+	+
5	+	-	-	+	+	+	-
6	-	-	+	+	+	-	+
7	-	+	+	+	-	+	-
8	-	-	-	-	-	-	-
9	0	0	0	0	0	0	0

Determination of total petroleum hydrocarbons (TPH)

The total petroleum hydrocarbon in the investigated samples was determined spectrophotometrically, at wavelength 420 nm (Odu *et al*., 1985), at the Central Laboratory of the Faculty of Science, Alexandria University, Egypt (PERKIN-ELMER, Lambda 4B, UV/VIS Spectrophotometer). A standard curve was prepared using known concentrations of crude oil which was used to estimate the amount of hydrocarbons in the samples. The removal efficiency (RE %) of the crude oil was calculated according to the following equation :

Removal efficiency (RE %) = (TPH Control – TPH Treated) / TPH Control) X 100

In this equation, TPH Control is the oil concentration of the control, and TPH Treated is the oil concentration of the biodegraded sample. All the biodegradation experiments were carried out in duplicate with the live bacterial cultures and the controls. The oil removal efficiency was acquired as the average value.

Experimental Results

Identification

The bacterial isolates were identified as *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus sphaericus* and *Bacillus brevis*. *Pseudomonas aeruginosa* was the most potent isolate, it was further identified by 16S rRNA gene sequencing analysis, ten closest bacteria to the isolate were depicted and listed in Table 3 from the BLAST program using the multiple alignment method with the 1187 b obtained from the previous sequence query.

TABLE 3. The most similar bacteria to *Pseudomonas aeruginosa* based on reverse sequence analysis of 16S rRNA gene.

Description	Similarity	Accession
Pseudomonas aeruginosa RP73, complete genome	97%	NC_021577.1
Pseudomonas aeruginosa B136-33, complete genome	97%	NC_020912.1
Pseudomonas aeruginosa DK2 chromosome, complete genome	97%	NC_018080.1
Pseudomonas aeruginosa NCGM2.S1 chromosome 1, complete genome	97%	NC_017549.1
Pseudomonas aeruginosa M18 chromosome, complete genome	97%	NC_017548.1
Pseudomonas aeruginosa LESB58 chromosome, complete genome	97%	NC_011770.1
Pseudomonas aeruginosa PA7 chromosome, complete genome	97%	NC_009656.1
Pseudomonas aeruginosa UCBPP-PA14 chromosome, complete genome	97%	NC_008463.1
Pseudomonas aeruginosa PAO1 chromosome, complete genome	97%	NC_002516.2
Pseudomonas aeruginosa PA1R, complete genome	97%	CP004055.1

From the previous data in Table 3 and Fig. 1, the isolate is 97% similar to *Pseudomonas aeruginosa* RP73. Therefore, it can be identified as *Pseudomonas aeruginosa* KH6 and was submitted to GenBank under the accession numbers (KM194714).

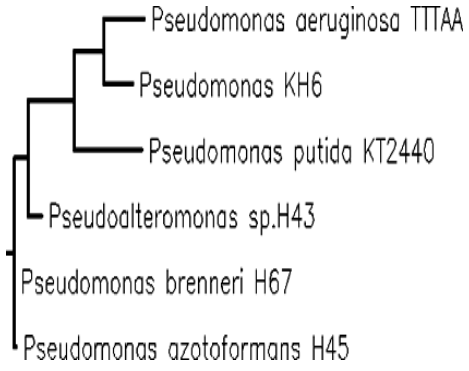

Fig. 1. Phylogenetic tree of isolated *Pseudomonas aeruginosa* KH6 (accession number KM194714), showing its phylogenetic position among related bacterial species. The tree was constructed by BioEdit.

Effect of incubation period on growth and TPH removal efficiency

Crude oil biodegradation and growth with reference to time (0-25 days) were shown in Fig. 2 and 3. The growth was increased with increasing the incubation time until 15 days for all species, it was for *Pseudomonas aeruginosa* KH6 the highest (0.678) (Fig. 2). The highest TPH removal efficiency was obtained after 15 days of incubation for the four species (Fig. 3). *Pseudomonas aeruginosa* KH6 gave the best removal efficiency (75.04 %).

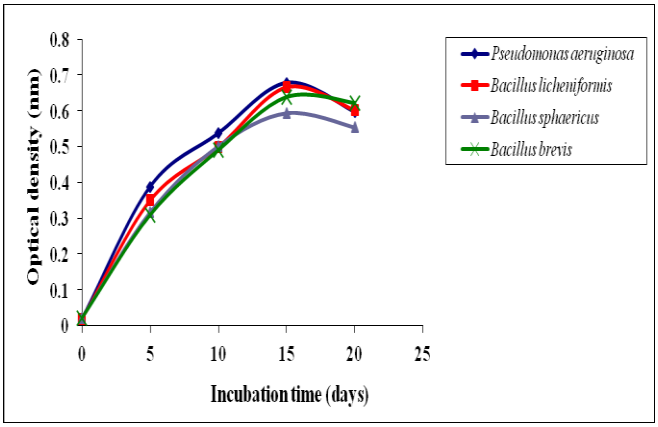

Fig. 2. Effect of incubation period on growth of different bacterial species.

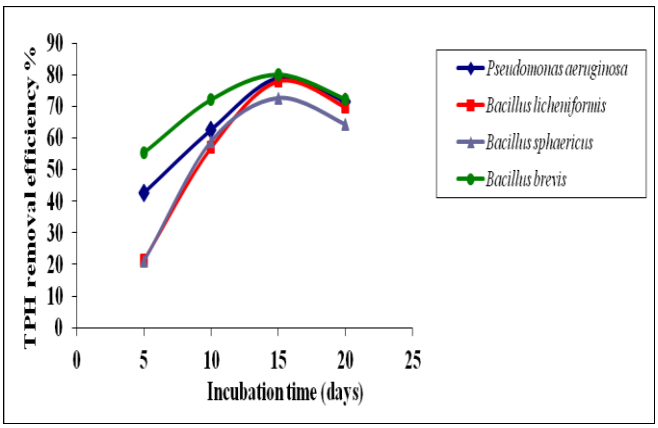

Fig. 3. Effect of incubation period on TPH removal efficiency of different bacterial species.

Effect of temperature on growth and TPH removal efficiency

Growth and crude oil degradation were determined at different temperatures ranging from 15ºC to 45ºC. Flasks were incubated at 120 rpm in a shaking incubator for 15 days. The results in Fig. 4 indicated that the best crude oil removal efficiencies were recovered at temperature 35 ºC, and the best TPH

removal efficiency for *Pseudomonas aeruginosa* KH6 was 76.01 % at 35ºC followed by *Bacillus brevis* (75.0 %), *Bacillus sphaericus* (68.55 %), while *Bacillus licheniformis* gave the lowest crude oil degradation (67.33%). Also, the growth was increased with increasing the temperature up to 35ºC, and then decreased afterwards (data not shown).

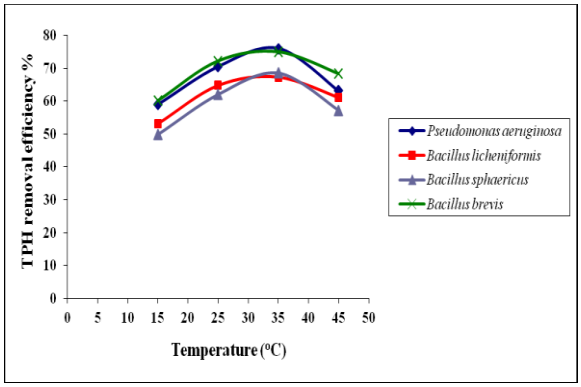

Fig. 4. Effect of incubation temperature on TPH removal efficiency ofdifferent bacterial species.

Effect of pH on growth and TPH removal efficiency

Different pHs were also observed between 6.5 to 9.5. Flasks were incubated at 35ºC and 120 rpm in a shaker incubator for 15 days. The results in Fig. 5 revealed that pH 7.5 was favorable for all the bacterial isolates and the highest percentage of TPH removal efficiency was achieved at pH 7.5 by *Pseudomonas aeruginosa* KH6 followed by *Bacillus brevis, Bacillus sphaericus, and Bacillus licheniformis* with 77.76 %, 76.18 %, 68.77 % and 67.36 % respectively. Also, the growth was increased with increasing pH up to 7.5 and decreased with increasing the pH from 7.5 to 9.5. Therefore, pH 7.5 was selected for further experiments (data not shown).

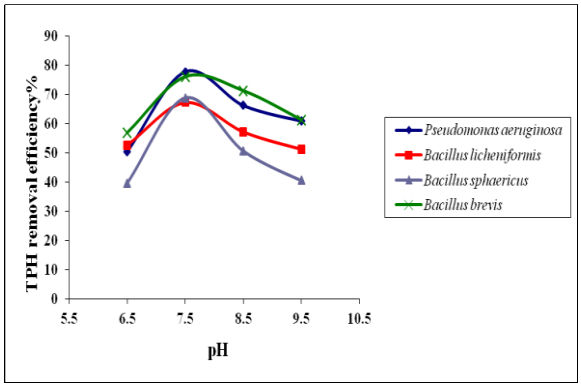

Fig. 5. Effect of pH on TPH removal efficiency of different bacterial species.

Plackett – Burman Design

Plackett – Burman design was applied to reflect the relative importance of various medium factors involved in the utilization of crude oil by the bacterial isolates. The medium nutrient components were screened by applying the Plackett-Burman matrix. The principle statistical analysis of this experiment for each isolate was shown in Table 4, 5, 6, and 7.

The tested degrees of significance for *Bacillus licheniformi*s and *Pseudomonas aeruginosa* KH6, *Bacillus sphaericus*, and *Bacillus brevis* of hydrocarbon factor were 99, and 95%. At 95% tested degree of significance, the hydrocarbon was significant, in which the decrease in hydrocarbons concentration resulted in an increase in TPH removal efficiencies of both species (Table 4, 5, 6, and 7). It was deduced from Fig. 6, 7, 8, and 9 that hydrocarbon was the most significant variable for removal efficiency.

The tested degrees of significance for *Bacillus sphaericus* of Fe_2SO_4 factor were 99, 95, and 90 %. At 90% tested degree of significance, the Fe_2SO_4 was significant, in which the increase in Fe_2SO_4 factor resulted in an evaluation in the TPH removal efficiency. It was deduced from Table 6 and Fig. 8 that Fe_2SO_4 and hydrocarbons were the most significant variables for removal efficiency of the tested bacterium.

The tested degrees of significance for *Bacillus brevis* of $MgSO_4.7H_2O$ factor were 99, 95, and 90%. At 90% tested degree of significance, $MgSO_4.7H_2O$ was significant, in which the increase in $MgSO_4.7H_2O$ factor resulted in an evaluation in the TPH removal efficiency. It was deduced from Table 7 and Fig. 9 that $MgSO_4.7H_2O$ and hydrocarbons were the most significant variables for removal efficiency of the species.

TABLE 4. Degree of positive and negative effects of independent variables on the TPH removal efficiency % by *Pseudomonas aeruginosa* KH6 according to levels in the Plackett–Burman experiment.

Variable	TPH removal efficiency (%)					Mean	Main effect	T-value	Degree of significance (%)
$(NH_4)_2SO_4$	+	76.44	45.35	56.39	77.75	63.982	-7.328	-0.68301	95
	-	68.35	52.32	79.02	85.55	71.310			
$CaCl_2$	+	76.44	45.35	68.35	79.02	67.290	-0.710	-0.06399	95
	-	56.39	77.75	52.32	85.55	68.002			
K_2HPO_4	+	76.44	56.39	52.32	79.02	66.042	-3.210	-0.29000	95
	-	45.35	68.35	77.75	85.55	69.250			
$MgSO_4.7H_2O$	+	45.35	77.75	52.32	79.02	63.610	-8.070	-0.75878	95
	-	76.44	56.39	68.35	85.55	71.682			
Fe_2SO_4	+	76.44	68.35	77.75	52.32	68.715	2.140	0.19251	95
	-	45.35	56.39	79.02	85.55	66.577			
Yeast extract	+	56.39	68.35	77.75	79.02	70.377	5.460	0.50059	95
	-	76.44	45.35	52.32	85.55	64.915			
Hydrocarbon	+	45.35	56.39	68.35	52.32	55.602	-24.090	-4.60643	95
	-	76.44	77.75	79.02	85.55	79.690			

Degree of sign. : Degree of significance, $t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439

TABLE 5. Degree of positive and negative effects of independent variables on the TPH emoval efficiency % by *Bacillus licheniformis* according to levels in the Plackett–Burman experiment.

Variable		TPH removal efficiency (%)				Mean	Main effect	T-value	Degree of significance (%)
$(NH_4)_2SO_4$	+	77.80	64.44	55.34	61.50	64.770	2.858	0.36250	95
	-	47.28	56.39	75.76	68.22	61.912			
$CaCl_2$	+	77.80	64.44	47.28	75.76	66.320	5.958	0.78508	95
	-	55.34	61.50	56.39	68.22	60.362			
K_2HPO_4	+	77.80	55.34	56.39	75.76	66.322	5.963	0.78581	95
	-	64.44	47.28	61.50	68.22	60.360			
$MgSO_4.7H_2O$	+	64.44	61.50	56.39	75.76	64.522	2.363	0.29867	95
	-	77.80	55.34	47.28	68.22	62.160			
Fe_2SO_4	+	77.80	47.28	61.50	56.39	60.742	-5.198	-0.67668	95
	-	64.44	55.34	75.76	68.22	65.940			
Yeast extract	+	55.34	47.28	61.50	75.76	59.970	-6.743	-0.90163	95
	-	77.80	64.44	56.39	68.22	66.712			
Hydrocarbon	+	64.44	55.34	47.28	56.39	55.862	-14.958	-2.92158	95
	-	77.80	61.50	75.76	68.22	70.820			

Degree of sign. : Degree of significance, $t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439

TABLE 6. Degree of positive and negative effects of independent variables on the TPH removal efficiency % by *Bacillus sphaericus* according to levels in the Plackett–Burman experiment.

Variable		TPH removal efficiency (%)				Mean	Main effect	T-value	Degree of significance (%)
$(NH_4)_2SO_4$	+	79.51	48.91	64.07	80.32	68.20	4.795	0.60444	95
	-	56.37	68.35	67.20	61.71	63.40			
$CaCl_2$	+	79.51	48.91	56.37	67.20	62.99	-5.615	-0.71594	95
	-	64.07	80.32	68.35	61.71	68.61			
K_2HPO_4	+	79.51	64.07	68.35	67.20	69.78	7.955	1.06097	95
	-	48.91	56.37	80.32	61.71	61.82			
$MgSO_4.7H_2O$	+	48.91	80.32	68.35	67.20	66.19	0.780	0.09553	95
	-	79.51	64.07	56.37	61.71	65.41			
Fe_2SO_4	+	79.51	56.37	80.32	68.35	71.13	10.665	1.54246	90
	-	48.91	64.07	67.20	61.71	60.47			
Yeast extract	+	64.07	56.37	80.32	67.20	66.99	2.370	0.29211	95
	-	79.51	48.91	68.35	61.71	64.62			
Hydrocarbon	+	48.91	64.07	56.37	68.35	59.42	-12.760	-2.02697	95
	-	79.51	80.32	67.20	61.71	72.18			

Degree of sign. : Degree of significance, $t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439

TABLE 7. Degree of positive and negative effects of independent variables on the TPH removal efficiency % by *Bacillus brevis* according to levels in the Plackett–Burman experiment.

Variable	TPH removal efficiency (%)					Mean	Main effect	T-value	Degree of significance (%)
$(NH_4)_2SO_4$	+	58.65	60.77	50.30	79.18	62.225	-5.060	-0.71309	95
	-	56.66	68.45	71.45	72.58	67.285			
$CaCl_2$	+	58.65	60.77	56.66	71.45	61.882	-5.745	-0.81974	95
	-	50.30	79.18	68.45	72.58	67.627			
K_2HPO_4	+	58.65	50.30	68.45	71.45	62.212	-5.085	-0.71692	95
	-	60.77	56.66	79.18	72.58	67.297			
$MgSO_4.7H_2O$	+	60.77	79.18	68.45	71.45	69.962	10.415	1.72298	90
	-	58.65	50.30	56.66	72.58	59.547			
Fe_2SO_4	+	58.65	56.66	79.18	68.45	65.735	1.960	0.26678	95
	-	60.77	50.30	71.45	72.58	63.775			
Yeast extract	+	50.30	56.66	79.18	71.45	64.397	-0.715	-0.09682	95
	-	58.65	60.77	68.45	72.58	65.112			
Hydrocarbon	+	60.77	50.30	56.66	68.45	59.045	-11.420	-1.99154	95
	-	58.65	79.18	71.45	72.58	70.465			

Degree of sign. : Degree of significance, $t_{\alpha 95}$= 1.943 $t_{\alpha 90}$= 1.439

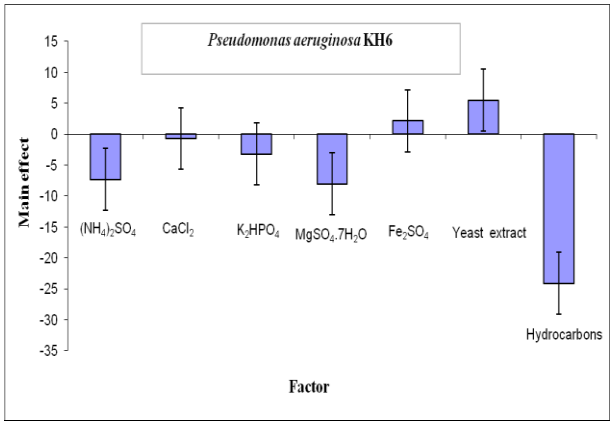

Fig. 6. The main effect of each variable upon TPH removal efficiency by *Pseudomonas aeruginosa* KH6.

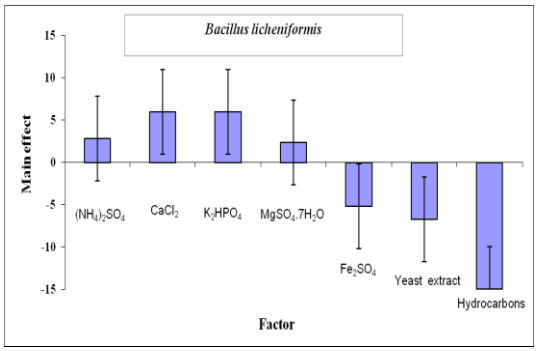

Fig. 7. The main effect of each variable upon TPH removal efficiency by *Bacillus licheniformis*.

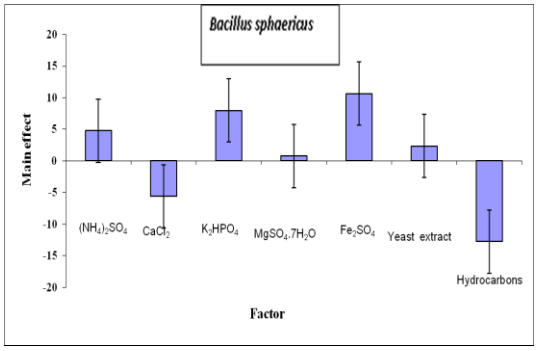

Fig. 8. The main effect of each variable upon TPH removal efficiency by *Bacillus sphaericus*.

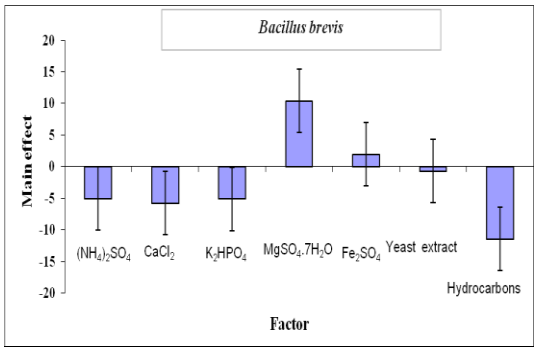

Fig. 9. The main effect of each variable upon TPH removal efficiency by *Bacillus brevis.*

A Verification experiment was carried out in triplicate. The predicted optimum levels of independent variables were examined and compared to the basal condition setting. The TPH removal efficiencies are shown in Table 7. For all isolates, the results indicated that the TPH removal efficiencies from optimized culture gave the highest value and it was 1.13 times higher than that of the control medium for *Pseudomonas aeruginosa* KH6.

TABLE 7. The results of optimized media, antioptimized media and the control media for the TPH removal efficiency % of crude oil by the bacterial species.

Media	TPH removal efficiency (%)			
	Bacillus brevis	***Bacillus licheniformis***	***Bacillus sphaericus***	***Pseudomonas aeruginosa* KH6**
Optimized media	82.57	79.43	78.88	87.90
Control media	77.92	69.63	70.51	78.00
Antioptimized media	58.87	55.61	56.76	59.20

Discussion

The study of the effect of incubation time on the biodegradation of crude oil revealed that a remarkable increase in biomass was observed in the first 10 days of incubation. After 5 days, cell count increased very slowly. These results were supported by another study, which recorded that a large increase in biomass was observed in the first 7 days of the study of the biodegradation of petroleum refinery wastewater by *Alcaligenes odorans*, *Bacillus subtilis*, *Corynebacterium propinquum* and *Pseudomonas aeruginosa*. After 7 days, count increased very slowly (Bujang *et al*., 2013).

All the isolates in the present study were mesophilic in nature; they all exhibited optimum activity at 35ºC (Banat, 1995; Shahriari *et al*., 2014 and Kavynifard *et al*., 2015). The highest and lowest growth values for *Bacillus salmalaya* 139SI remediating water polluted with crude oil waste were recorded at 40°C and 25°C, respectively (Ismail and Dadrasnia, 2015). Some studies showed that a temperature drop from 25 to 5ºC caused a tenfold decrease in response. Petroleum also becomes more viscous at low temperature. Hence, less spreading occurs and less surface area is available for colonization by microorganisms (So *et al*., 2003).

The maximum population and degradation rates were observed at initial pH 7.5. This was agreed with previous studies, which have shown that degradation

of oil increased with increasing pH, and that optimum degradation occurred under slightly alkaline conditions (Venosa and Zhu, 2003). The optical density for *Bacillus salmalaya* 139SI remediating water polluted with crude oil waste, was the highest, at a pH 7, followed by pH 8, pH 6 and pH 5 (Ismail and Dadrasnia, 2015). This finding is supported by the idea that pH affects the activity of the enzyme, if too extreme then the enzymes will be denatured. This decrease in pH affects the growth of bacteria because bacteria have an optimum pH range of about pH 6.5-7.5. Below pH 5 and above 8.5, the bacteria cannot grow well unless the bacteria resistant to acid and alkaline (Alami *et al*., 2014). However, pH does not fluctuate much in the oceans, it remains between 7.6 and 8. l and does not appear to have an important effect on biodegradation rates in most marine environments. In salt marshes, however, the pH maybe as low as 5.0, and thus may slow the rate of biodegradation in these habitats (Li *et al*., 2007).

Some studies used Plackett–Burman design; three important trace elements (Mg^{2+}, Zn^{2+} and Fe^{2+}) were identified that have significant positive effects on the THF (tetrahydrofuran) degradation by some bacterial species (Yao *et al*., 2009). Also, Shahriari *et al*. (2014) used the statistical methodology, combination of the Plackett-Burman and Taguchi designs, to be effective in selecting statistically significant factors and finding the optimal concentration of factors for crude oil biodegradation.

Consequently, the present study revealed the possibility of utilizing four crude oil-degrading bacteria isolated from industrial oil polluted wastewater from API separators of the Alexandria Petroleum Company, Alexandria, Egypt. Physiological factors affecting biodegradation could be optimized for maximum removal efficiency. This potential of the species towards high crude oil utilization could be directed for industrial effluent treatment and decontamination of natural polluted areas and will be reported in future correspondence.

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تحسين عملية التكسير الحيوى للهيدروكربونات البترولية بواسطة بعض البكتيريا المعزولة محليا باستخدام النظام الاحصائى

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تختص هذه الدراسة بتحسين بعض العوامل الفيزيائية و إستخدام طرق التحليل الاحصائي لأربع عزلات معزولة محليا للحصول على أفضل ظروف وأفضل التركيزات للوصول الى أعلى كفاءة فى التكسيرالحيوى للبترول الخام. تم تعريف السلالات البكتيرية الأربعة التى أظهرت كفاءة عالية من خلال التعريف الظاهرى و الكيمياء الحيوى به الى بسودومونس اريجنوزا، باسيلاس ليكينوفورمس، باسيلاس سفيريكس، باسيلاس بريفس. تم تعريف الكائن الأعلى كفاءة وهو بسودومونس اريجنوزا من خلال التعريف الجزيئى .KM194714 رقم وقد أعطى 16S rRNA

أظهرت التجارب الأولية لعملية تكسير المواد البترولية الخام أن أفضل فترة للسلالات الأربعة كانت بعد ١٥ يوم من التحضين فى وجود البترول الخام كمصدر للكربون عند درجة حرارة ٣٥ درجة مئوية وأس هيدروجينى ٧،٥. وقد أظهرت دراسة النظام الاحصائى أن البترول الخام يعتبر عنصر مؤثر حيث أن إنخفاض التركيز منه يؤدى الى زيادة فى الكفاءة لعملية إزالة الهيدروكربونات البترولية للسلالات الأربع و أن زيادة التركيز من مركب كبريتات الحديدوز فى الوسط الغذائى لسلالة باسيلاس سفريكس يؤدى إلى زيادة فى الكفاءة لعملية الازالة ، بينما زيادة التركيز من مركب كبريتات الماغنيزيم فى الوسط الغذائى لسلالة باسيلاس بريفس يؤدى إلى زيادة فى الكفاءة. و أدى الوسط المحسن بالنظام الاحصائى الى زيادة فى كفاءة عملية التكسير الحيوى للسلالات الاربع و وصل إلى زيادة فى الكفاءة بحوالى ١،١٣ ضعف لسلالة بسودومونس اريجنوزا.