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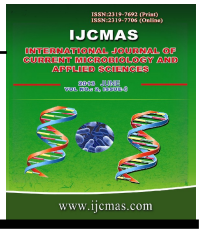

Original Research Article

Environmental studies on the microbial degradation of oil hydrocarbons and its application in Lebanese oil polluted coastal and marine ecosystem

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A B S T R A C T

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry, therefore, accidental releases of petroleum products are of particular concern in the environment, which could lead to consequences for the biotic and abiotic components of the ecosystem if not restored. Remediation of hydrocarbon-contaminated system could be achieved either by physiochemical or biological method. Present study aims to use biological methods for the remediation of the contaminated sites. Screening study for the isolation of the promising hydrocarbon degraders and optimization experiments to evaluate the best environmental and physiological factors that lead to maximum degradation of hydrocarbons revealed that, *Bacillus cereus* A , *Bacillus cereus* B and *Bacillus* sp. ZD the most promising hydrocarbon degraders were isolated from Lebanese marine ecosystem and identified using 16S rRNA. *Bacillus cereus* A showed maximum diesel oil degradation (82.41% and 81.56% of aliphatic and aromatic hydrocarbons) after 2 days incubation under shaken condition, at pH 7, 100 ml culture volume and 2% inoculum size. Seven nutritional factors were examined for their significance on hydrocarbon degradation using a statistical design known as Plackette-Burman. Maximum diesel oil degradation produced by *Bacillus cereus* A was revealed by the statistical design. Immobilization technique showing that *Bacillus cereus* A can be used for diesel oil degradation on large environmental scale, solving by this one of the problems of oil spill management that results from the petrochemical industry and saving the environment from additional water and soil pollution.

Keywords

Biostimulation stratagem; promising hydrocarbon degraders; degradation of hydrocarbon; *Bacillus cereus*; Plackett-Burman;

Introduction

Petroleum which is the major source of energy for industry and daily life is an extremely complex mixture of hydrocarbons. From the hundreds of individual components, four classes, based on related chemical structures can be recognized, and named as : aromatic, saturate or aliphatic, asphaltic and resins (Gopinathan *et al*., 2012). However, after the sinking of the super tanker *Torney Canyon* in 1967 the attention of the scientific community was drawn towards

the problems of oil pollution. These pollution problems often result in huge disturbances of both the biotic and abiotic components of the ecosystems (Al-Jumaily and Al- wahab, 2012), furthermore some hydrocarbon components have been known to belong to a family of carcinogenic and neurotoxic organopollutants (Tevvors and Saier, 2010). Thus, release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution (Kumar *et al*., 2008).

Several physical and physiochemical techniques have been used to clean up the oil residues but, compared to physiochemical methods; biological method such as biodegradation offers to be non-invasive, simple to maintain, applicable over large areas and relatively cost effective method for the treatment of oil contamination because : first, the majority of molecules in the fuel oil and refined products are biodegradable (Prince, 2002), and oil-degrading microorganism are ubiquitous (Chaîneau *et al*., 2000 and Joo *et al*., 2008).

Although many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon and energy for metabolic activities, but the microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants and biodegradability of the these compounds generally decreases in the following order: n-alkanes, branched-chain alkanes, branched alkenes, low molecular- weight n-alkyl aromatics monoaromatics, cyclic alkanes polycyclic aromatic hydrocarbons asphaltenes (Van Hamme *et al*., 2003).

Biodegradation, is a mineralization of organic chemicals, which ultimately leading to the formation of CO2, H2O and biomass (Hamdi *et al*.,2007). Biodegradation being an economical and eco-friendly approach, has emerged as the most advantageous soil and water clean-up technique for contaminated sites containing oil spills, is applied with different strategies, but well accomplished with a process called biostimulation, that is, the addition of several nutrients and fertilizers to a contaminated matrix (Tyagi *et al*., 2011).

However, a number of limiting factors have been recognized to affect the biodegradation of petroleum hydrocarbons. The success of bioremediation is dependent upon physical and chemical conditions such as nutritional requirements (carbon, nitrogen and phosphorous), oxygen, pH and biosurfactant.

The present study aimed for isolation and selection of hydrocarbon degrading bacteria from oil polluted seawater and studies the environmental and physiological factors of the most promising and selected isolates lead to maximum hydrocarbon degradation and its application for the biodegradation as a biostimulation strategy for oil bioremediation in polluted seawater.

Materials and Methods

Water sample

Oil contaminated seawater sample were collected under aseptic conditions from Saida port in south Lebanon at 50 cm depth from seawater surface in sterile containers.

Oil samples

Diesel oil and fuel oil Samples were obtained from the Jieh power plant in south Lebanon.

Microorganisms

The microorganisms used throughout the present investigation were isolated from seawater at Saida port in south Lebanon and identified genotypically using 16S rRNA as three different Bacillus sp.

Media

Unless otherwise indicated, all media were prepared with distilled water, adjusted to initial pH 7 and sterilized by autoclaving for 20 min at pressure of 15 lb/inch^2 to raise the temperature to 121°C.

Nutrient agar medium (NA): was used for the bacterial cultures and had the following composition (g/l); peptone,5; yeast extract,2; sodium chloride, 5 and bacteriological agar, 15 (Goldman and Green, 2009).

Fermentation medium: used for the growth of seawater autochthonous oil degraders and contained (g/l): NH_4Cl, 2.5; KH_2PO_4, 0.3; Na_2HPO_4, 0.7; $MgSO_4.7H_2O$, 0.2; Yeast extract, 0.1; Thiamine HCl, 0.001(Dutta and Harayama 2000).

Isolation and purification of oil degrading bacteria

Serial dilutions-agar plating technique of the seawater sample ($1/10$,$1/10^2$,$1/10^3$,$1/10^4$ and $1/10^5$) were prepared in sterile distilled water and plated on nutrient agar media plates and incubated at 30°C for 48 hrs. The bacterial colonies obtained were further purified on seawater nutrient agar media plates containing either diesel or fuel oil as sole carbon and as energy for metabolic activities and incubated at 30°C for 48 hrs (Sathishkumar *et al*., 2008; Jyothi *et al*., 2012).

Maintenance of the Microorganisms

All the bacterial strains used throughout the present investigation were maintained on nutrient agar slants and stored at 4°C with regular transfer at monthly intervals.

Preparation of seed culture

Transfer from single slant cultures (48 hour old) were taken into 50 ml aliquots of the seed medium containing (g/l): Beef extract, 1; Yeast extract, 2; Pepton, 5 and 1L of distilled water. Dispensed in 250 ml of Erlenmeyer flask to initiate the growth (OD≤1). Standard inocula 2% (v/v) were taken from the latter liquid culture after growth for 18 hours at 30 °C on a reciprocal shaker to start growth in the fermentation flask which is equivalent to 1.5 x 10^8 colony forming unit/ml (CFU/ml) according to McFarland scale 0.5.

Screening for hydrocarbon degrading activities of the isolates under investigation

The cultivation of the isolated and purified bacterial strains was achieved in replicate in 250 ml Erlenmeyer flasks each containing 50 ml of fermentation medium .The media were sterilized by autoclaving for 15 min at 121°C then 2ml of the diesel and fuel oil was added one at a time with a chemical intermediate tween 20, which is act as emulsifying agent and incubated with 2 % (v/v) inoculum level unless otherwise stated and then incubated at

30°C for different time interval 12, 24, 36, 48 and 72 hours respectively under shaken conditions using rotary shaker(160 rpm.) and static conditions.

Residual hydrocarbon analysis

Analytical determination of oil hydrocarbons

Cell free extract preparation

At the end of incubation period the culture was sampled and the cells were removed by centrifugation at 6000 r.p.m for 15 min. The supernatant (cell free extract) was used for analysis of residual oil fractions immediately or stored at 4°C for further analysis.

Quantification and Extraction of aliphatic and aromatic fractions in both diesel and fuel oil

The residual aliphatic and aromatic fractions in both diesel and fuel oil from culture flasks (supernatent) were extracted twice in separatory funnel, one time with one volume of n- hexane (40 ml) for the extraction of aliphatic hydrocarbon fractions and shaken vigorously for 3 min and allowed to settle for 5 min. The solvent layer (liquid phase) was separated by allowing the (diesel oil or fuel oil "aliphatic fractions" – n-hexane) to pass gradually through a funnel fitted with filter paper. Anhydrous sodium sulphate, spread on the filter paper was employed to remove any moisture in the mixture. The liquid phase was collected in a 50-ml flask .The flask containing the extract (aliphatic fractions"- n-hexane) was placed in an hot-plate and the extractant solvent (n-hexane), allowed to evaporate at 90 °C. The residual diesel/fuel oils were poured in pre-weighted vials and weighed to determine the quantity of residual diesel oil/fuel oil by difference. The second time, extraction done in same manner but with dichloromethane (40 ml) for the extraction of aromatic hydrocarbon fractions.

The percentage of aliphatic or aromatic fraction in diese/fuel oils degraded at different time course was determined from the equation: % degraded of aliphatic or aromatic fraction = (weight of aliphatic or aromatic fractions degraded / original weight of diesel oil or fuel oil introduced) × 100. Where the weight of aliphatic or aromatic fraction degraded was determined as original weight of diesel oil/fuel oil minus weight of residual aliphatic or aromatic fraction obtained after evaporating the extractant (Nwaogu *et al*., 2008; Kebria *et al*., 2009).

Morphological characterization

Growth characterization of the cultures was determined on the basis of color appearance, size and shape of colonies developed, and the reaction towards the stain.

Genotypic characterization

The genomic DNA of isolate (A,B,C) were isolated according to Sambrook *et al*., (1989). The 16S rRNA was amplified by polymerase chain reaction (PCR) using universal eubacteria specific primers, designated to amplify 1500bp fragment of the 16S rRNA regions. The forward primer was: 27F (5'AGAGTTTGATCMTGGCTCAG 3') and the reverse primer was: 1492R (5'TACGGYTACCTTGTTACGACTT3') , which yielded a product of approximately 1500 bp.Successful amplification was confirmed by agarose gel electrophoresis and the remnant mixture was purified

using QIA quick PCR purification reagents (Qiagen). DNA sequence were obtained using an ABI PRIMSE 377 DNA Sequencer and ABI PRIMSE BigDye Terminator Cycle Sequencing (Perkin Elmer). The PCR product was sequenced using the same PCR primers. Blast program was used to assess the DNA similarities and multiple sequence alignment and molecular Phylogeny were performed using BioEdit software (Hall, 1999). The Phylogenetic tree was displayed using TREEVIEW program (Page. 1996).

Environmental and Physiological factors affecting diesel oil degradation

Different environmental factors such as culture volume, inoculum size, pH and carbon source were tested and screened for maximum diesel oil degradation.

Optimization of the nutritional factors affecting biodegradation using multifactorial statistical design (Plackett-Burman design)

Plackett-Burman design (Placket and Burman,1946), well established statistical technique for medium component optimization (Xiong *et al*.,2005) was applied to screen the medium components critically affecting degradation of diesel oil by the selected bacterium. Seven independent variables were screened in eight combinations organized according to the Plackett-Burman design matrix described in the result section. For each variable, a high (+) and low(-) was tested. All trials were performed in duplicates and the percentage of hydrocarbon consumptions was treated as the response for each trial. The main effect of each variable was determined with the following equation:

$$E_{xi}=(\sum M_i^{+} - \sum M_i^{-})/N$$

Where E_{xi} is the variable main effect, M_i^{+} and M_i^{-} are percentage of consumed oil in trials where the independent variable (x_i) was present in high and low levels, respectively, and N is the number of trials divided by 2.

The standard error (S.E) of the variables was the square root of variance and the significance level (Pvalue) of each variables calculated by using the *t*- Test:

$$t = E_{xi}/ S.E$$

where E_{xi} is the effect of the tested variable. The variable with higher confidance levels were considered to influence the response or output variable.

Immobilization of *Bacillus cereus* A by adsorption on sponge and luffa

Sponge and luffa of a known mass were cut into small cubes were added individually into a 250 ml Erlenmeyer flask containing 100 ml fermentation media inoculated with the selected bacterium and allowed to grow for 48 hrs at 30°C under shaken condition (160 rpm).

Application in a bioreactor

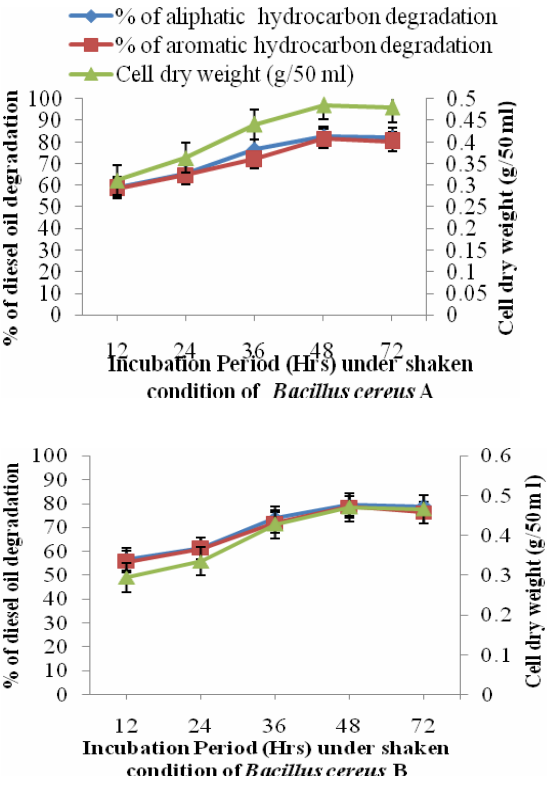
A glass column was sterilized with its caps. A known mass of sponge and luffa cubes loaded with the selected bacterium cells were transferred under aseptic conditions to the columns. Fifty ml of distilled water fortified with 1 ml diesel oil were added to the bioreactors. After oil degradation was observed, the medium was drained. The process was repeated for several times until degradation of oil was decreased. In every run medium taken was centrifuged, and the supernatant was used to measure the degradation of oil.

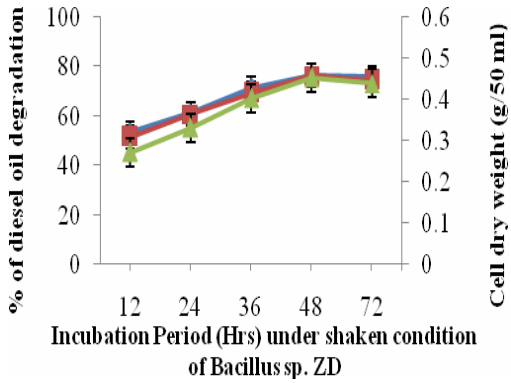
Results and Discussion

Screening for hydrocarbon degrading activities of the isolates under investigation

Data revealed that the highest oil degradation (82.41% and 81.56% of aliphatic and aromatic diesel oil hydrocarbons respectively), was achieved with isolates A when grown on fermentation medium fortified with diesel oil for 2 days under shaken conditions, followed by isolate B(79.51% and 78.64% of aliphatic and aromatic diesel oil hydrocarbons respectively) than by isolate C(76.44% and 76.25% of aliphatic and aromatic diesel oil hydrocarbons respectively when grown under the same conditions.(Figure 1).

Figure.1 Diesel oil degrading capacity of the bacterial isolates under test using shaken conditions





Phenotypic characterization

The cultural and colonial characteristics showed that the colonies were circular, whitish, medium in size with serrated edge, raised elevation and smooth surfaces. Morphological characterization showed gram positive, long rod shape, spore-forming isolates were identified as *Bacillus* sp.

Genotypic characterization and phylogeny

The most promising bacterial isolates A,B,C were identified by sequencing PCR amplified 16S rRNA. The obtained sequences submitted to FASTA3 data base in order to find homologies with other 16S rRNA. Tables (1,2,3)shows the similarities percentages and accession numbers obtained after comparing the sequences of the tested strains(isolate A, isolate B and isolate C) to the submitted sequences in gene bank respectively. The tested strains were affiliated to the genus *Bacillus* with 99%, 99% and 93% similarity to *Bacillus cereus* respectively. The phylogeny of the tested strains and closely related species were analyzed using multi sequence alignment program (TREEVIEW program) and the results are presented in phylogenetic cladogram (Figure 2,3, 4 respectively). Isolate A showed 99% identity to different strains of *Bacillus cereus* so it is named *Bacillus cereus* A.

Table.1 *Bacillus* strains showing 99 % identity to *Bacillus cereus* A

Accession number	Description	Identity (%)
FJ686822.1	*Bacillus cereus* strain LB5	99
EU272862.1	*Bacillus cereus* strain MK30	99
JN206612.1	*Bacillus* sp. SR2	99
HQ844479.1	*Bacillus cereus* strain JSYM28	99
JN182694.1	*Bacillus* sp. ANIOT10	99

Table.2 *Bacillus* strains showing 99 % identity to *Bacillus cereus* B

Accession number	Description	Identity (%)
GU471204.1	*Bacillus* sp. Q1BY4	99
EU162024.1	*Bacillus cereus* isolate PGBw4	99
EF503534.1	*Bacillus cereus* strain HPC1414	99
N206612.1	*Bacillus* sp. SR2	99
HQ844479.1	*Bacillus cereus* strain JSYM28	99
JF728871.1	*Bacillus cereus* strain Tcb1	99
JF736840.1	*Bacillus cereus* strain Tcb2	99
JF895490.1	*Bacillus cereus* strain Cr-50	99
JF939041.1	*Bacillus cereus* strain AIMST Nse4	99

Figure.2 Phylogenetic relationships among representative experimental isolate A and the most related bacteria based on 16S rRNA sequences.

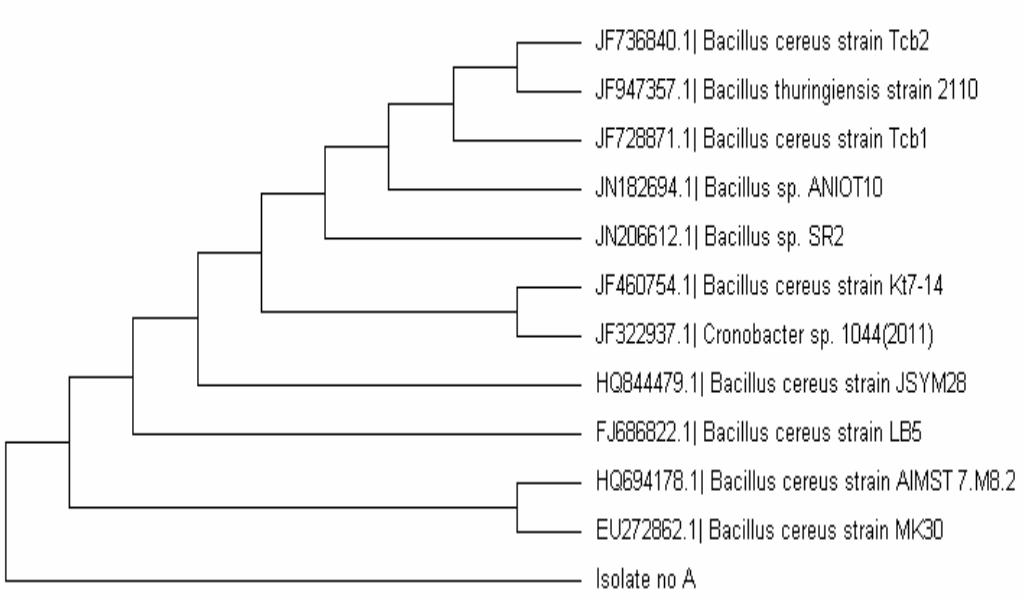


Figure.3 Phylogenetic relationships among representative experimental isolate B and the most related bacteria based on 16S rRNA sequences

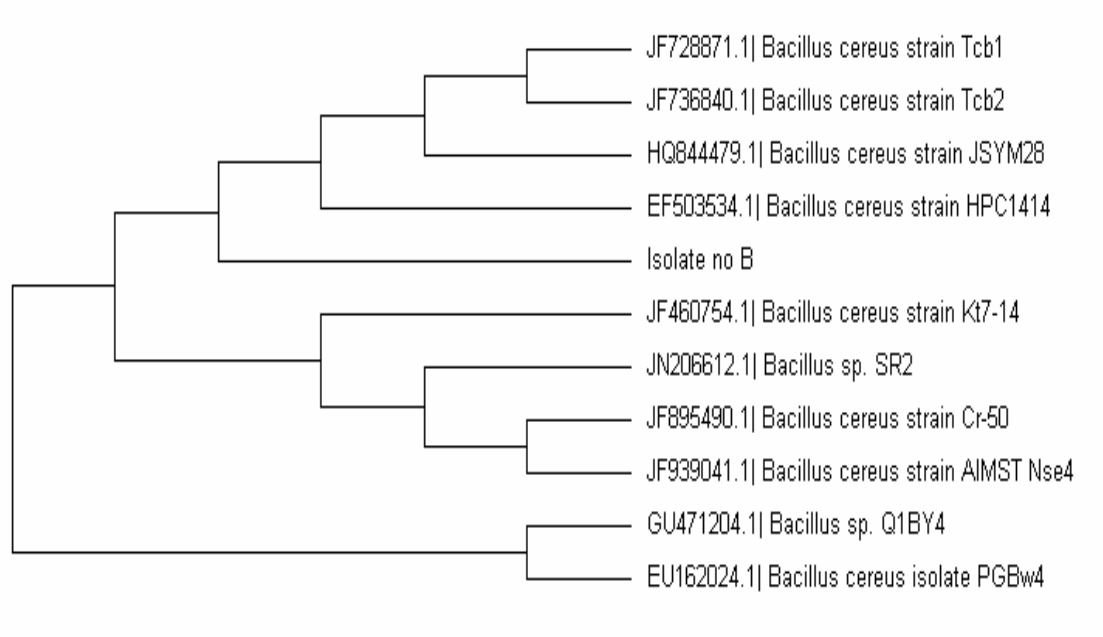
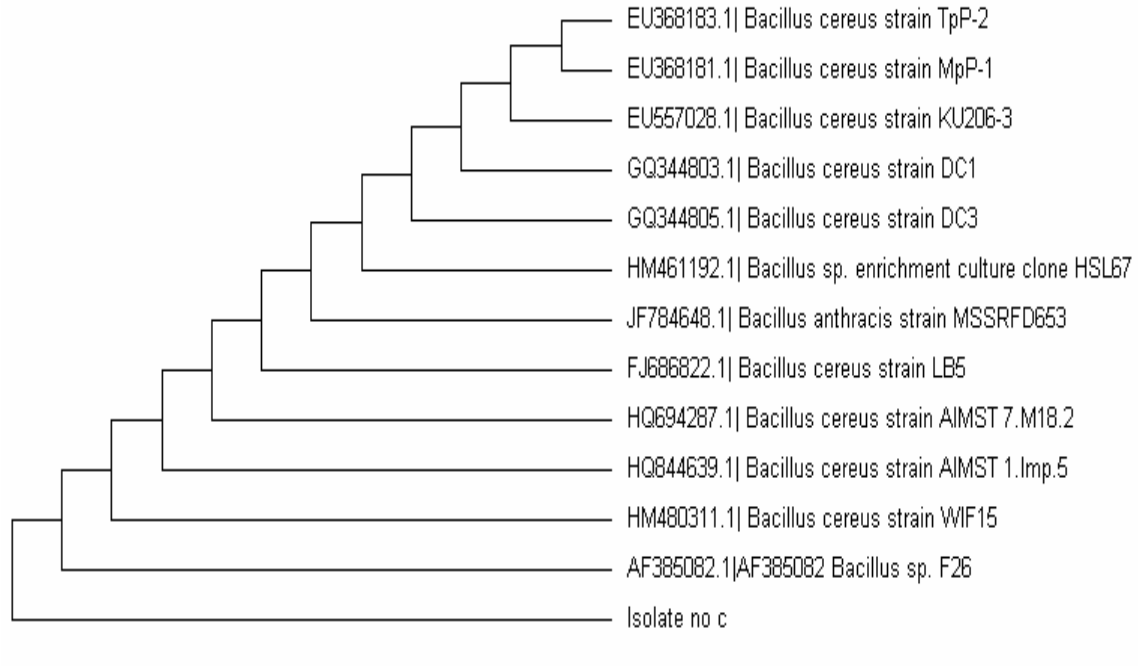


Figure.4 Phylogenetic relationships among representative experimental isolate C and the most related bacteria based on 16S rRNA sequences



However Isolate B also showed 99% identity to different strains of *Bacillus cereus* so it was named *Bacillus cereus* B, while Isolate C showed 93% identity to *Bacillus cereus* so it was named *Bacillus* sp ZD.

Environmental and Physiological factors affecting hydrocarbons degradation by *Bacillus cereus* A:

The aim of the present study is to evaluate the optimum physiological and environmental factors for maximum hydrocarbon degradation by *Bacillus cereus* A.

Effect of culture level

Bacillus cereus A has attained maximum diesel oil degradation (85.89% and 83.33% of aliphatic and aromatic hydrocarbon respectively with 100 ml culture volume (Figure 5) that was utilized as the optimum culture volume in the next experiments. The finite volume of the culture medium means the limitation of the nutrients for the microorganism. The consumption of the nutrients is largely dependent on the bacterial population. To ensure high diesel oil degradation in limited culture volume, the inoculum size should therefore be controlled (Abusham *et al*., 2009).

Effect of Inoculum Size

Results revealed that maximum diesel oil degradation by *Bacillus cereus* A (85.89 % and 83.33 % of aliphatic and aromatic hydrocarbon) was achieved with 2ml inoculum level/flask; however, minimal diesel oil degradation(50.1 % aliphatic and 48.51 % aromatic hydrocarbon) was achieved with 4ml inoculum level/flask (Figure 6). Accordingly, 2ml/100ml inoculum level was selected to carry out the next part of the research.

Low inoculum size required longer time for cells to multiply and produce the desired product (Jiff *et al*., 1998). A small amount of inoculum can lead to insufficient number of microbial cells and a reduced amount of the secreted enzymes while a much higher inoculum could lead to or cause a lack of oxygen and depletion of nutrients in the culture media (Abusham *et al*., 2009).

Effect of pH

Maximum diesel oil degradation by *Bacillus cereus* A (85.99% and 83.84% of aliphatic and aromatic hydrocarbon) was at pH 7 (Figure 7). Therefore pH 7 was selected for further experimentation. Higher or lower pH values showed inferior results; metabolic processes are highly susceptible to even slight changes in pH (Wang *et al*., 2012).

Meredith *et al*., (2000) and Rahman *et al*., (2002) were reported that any extremes in pH were shown to have a negative influence on the ability of microbial populations to degrade hydrocarbons. A change in pH has an effect into the biodegradative activity of microbial populations, as well as on the solubilization and absorption/desorption of ions and pollutants (San Martín, 2011).

Effect of carbon source

The maximum hydrocarbon degradation by *Bacillus cereus* A was achieved in the presence of arabinose as a carbon source (88.03 % aliphatic and 84.28 % aromatic hydrocarbons), while lactose and mannose were not favorable for bacterial growth as compared to the other tested carbon sources(Figure 8).

Table.3 *Bacillus* strains showing 93 % identity to *Bacillus* sp ZD

Accession number	Description	Identity (%)
HQ844639.1	*Bacillus cereus* strain AIMST 1.Imp.5	93
HQ694287.1	*Bacillus cereus* strain AIMST 7.M18.2	93
HM480311.1	*Bacillus cereus* strain WIF15	93
FJ686822.1	*Bacillus cereus* strain LB5	93
AF385082.1	*Bacillus* sp. F26	93
HM461192.1	*Bacillus* sp. enrichment culture clone HSL67	93
GQ344805.1	*Bacillus cereus* strain DC3	93
GQ344803.1	*Bacillus cereus* strain DC1	93
EU557028.1	*Bacillus cereus* strain KU206-3	93
EU368183.1	*Bacillus cereus* strain TpP-2	93
EU368181.1	*Bacillus cereus* strain MpP-1	93

Table.4 The experimental design using Plackett-Burmans method for screening of medium components affecting diesel oil degradation by *Bacillus cereus* A

Variables / Run	NH_4CL	KH_2PO_4	Na_2HPO_4	$MgSO_4.7H_2O$	Yeast Extract	Thiamine HCL	Oil	% of oil degredation	
								aromatic	aliphatic
1	-	+	+	-	-	+	+	82.26	83.3
2	+	-	+	+	-	-	+	96.54	96.90
3	+	+	-	+	+	-	-	74.45	75.67
4	-	+	+	-	+	+	-	98.30	98.78
5	-	-	+	+	-	+	+	70.44	71.86
6	+	-	-	+	+	-	+	62.50	63.64
7	+	+	-	-	+	+	-	82.37	83.47
8	-	-	-	-	-	-	-	57.02	58.82

Figure.5 Effect of the culture volume (ml) on diesel oil degradation by *Bacillus cereus* A.

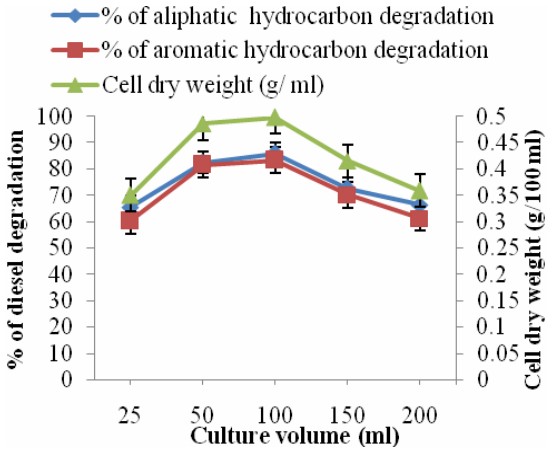


Figure.6 Effect of the inoculum size on the diesel oil degradation by *Bacillus cereus* A.

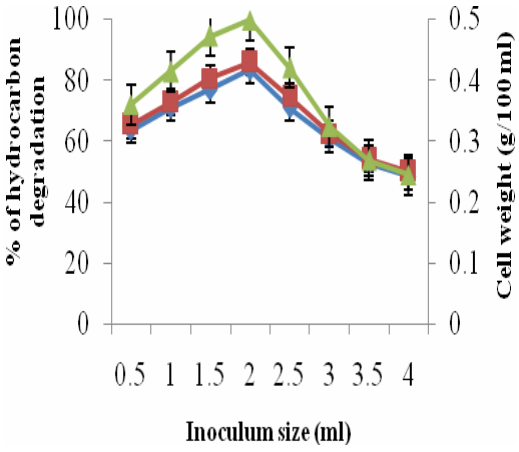


Figure.7 The effect of pH on diesel oil degradation by *Bacillus cereus* A

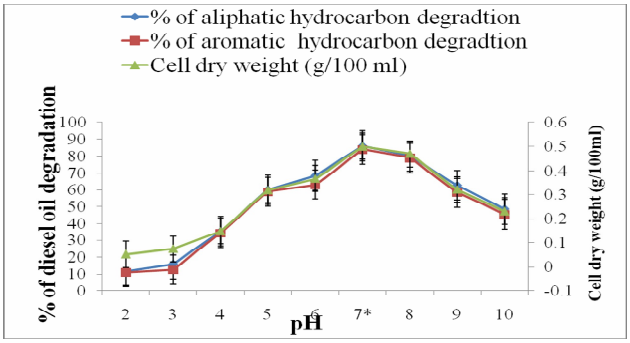


Figure.8 The effect of different carbon sources on diesel oil degradation by *Bacillus cereus* A

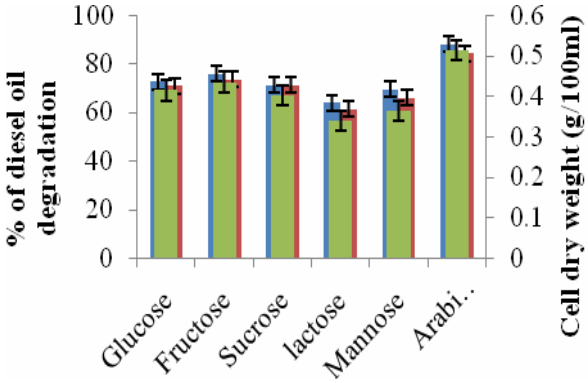


Figure.9 Main effect of the medium components factors on diesel oil degradation by *Bacillus cereus* A based on Plackett Burman design's result

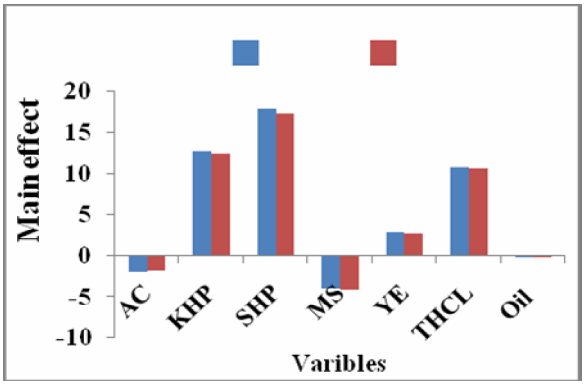
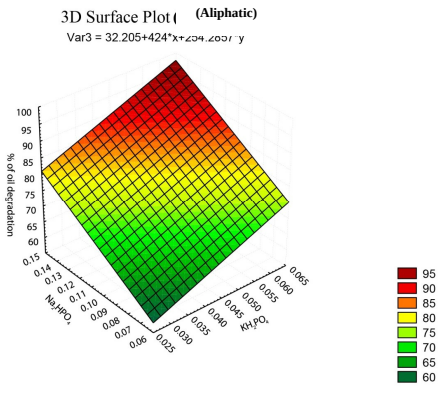


Figure.10 Effect of interaction between % of aliphatic diesel oil degradation,Na_2HPO_4 and KH_2PO_4 by *Bacillus cereus* A.



Bayoumi *et al.*, 2011, reported that maximum yield of biosurfactant and degradation of crude oil is occurred in the presence of sucrose. Batista *et al*., (2006) reported that, glucose is a better carbon source than fructose and sucrose for biosurfactant production and biodegradation of crude oil by Gram-positive and Gram-negative bacteria.

Optimization of the Best Nutritional Factors Affecting Hydrocarbon Biodegradation Using Multifactorial Statistical Design

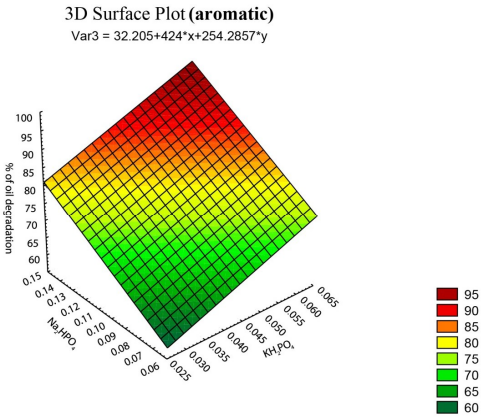
Sequential optimization approaches were applied in the present part of the study. The approach was to optimize the nutritional factors that control hydrocarbon degradation process. The best culture conditions such as, incubation time for 2 days; initial pH at 7; 2 ml inoculum level; 100ml culture volume and 30ºC incubation temperature were used for the optimization of the nutritional factors using the Plackett-Burman statistical design.

Evaluation of the Factors Affecting diesel oil degradation

In screening and optimizing the factors affecting diesel oil degradation, it is very important to test as much factors as possible and to identify the significance of each of them. Plackett-Burman design offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component (Srinivas *et al*., 1994). The design is recommended when more than five factors are under investigation (Abdel-Fattah *et al*., 2005).

The influences of seven factors on diesel oil degradation were tested (Table 4). It was shown that potassium dihydrogen phosphate and disodium hydrogen phosphate had a significant effect on diesel oil degradation (Figure 10, 11), whereas the other factors affected slightly the hydrocarbon degradation process.

Figure.11 Effect of interaction between % of aromatic diesel hydrocarbon degradation,Na_2HPO_4 and KH_2PO_4 by *Bacillus cereus* A**.**



The main effect that was estimated as a difference between both average of measurements made at the high level (+1) and at the low level (-1) of the factor of the examined factors affecting diesel oil degradation was calculated and presented graphically (Figure 9).

On the analysis of the regression coefficients of the seven variables, ammonium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, yeast extract and thiamine HCl had shown a positive effect, whereas magnesium sulfate and oil had shown a negative effect.

In the present study, nitrogen and phosphorus supply promoted the biodegradation of oil hydrocarbons with lower level for nitrogen and higher level for phosphorous. These results are in

accordance with that obtained by Dongfeng *et al*., (2011), Onuoha *et al*., (2011) and Al-Jumaily and Al- wahab (2012) which explained that excess amount of nitrogen fertilizer could suppress the microbial growth, leading to a reduced ability to degrade petroleum hydrocarbons by decreasing its ability for production of metabolic enzymes. However, the data of the present study indicated that KH_2PO_4 and Na_2HPO_4 showed a significant effect on diesel oil biodegradation which is in agreement with that obtained by Haines *et al*., (2003) and Farag and Soliman (2011) who stated that the presence of phosphate plays a critical role and its inadequate supply may result in slowing the rate of biodegradation and phosphorus can be used as source of storage energy in the form of ATP. On contrary Wenxiang *et al*., (2012) reported that the presence of an excess amount of phosphorus decreased diesel oil removal efficiency through inhibition of bacterial growth. Furthermore, it would increase the cost of bioremediation and may cause eutrophication in sea ecosystems. The phosphorus addition can stimulate the biodegradation of petroleum hydrocarbons, however some sources (phosphate and ortho- phosphate) can have diverse effect on the biodegradation, depending on its toxicity and solubility (Changyi *et al*., 2009).

Magnesium (principal inorganic cation in cells and constitutes approximately 1 % of the dry weight of the microbial cell) plays a role as a cofactor for many enzymatic reactions or cell wall components; it stimulate enzyme reactions associated with a synthesis of cell materials (Cameotra et al ., 2008). In the present investigation , magnesium in its low level negatively affected the biodegradation which is in agreement with Abdel-Fattah and Hussein (2002) who reported that metal ion (Mg^{+2}) may favor fungal and bacterial enzymatic activity and the net result of the effect created by the metals (Mg^{+2}) used was favoring petroleum bioremediation by the *Penicillium* strain. On the other hand Dongfeng *et al*., (2011) which showed that an excessively high concentration of metal ions (Mg^{+2}) could have an intoxicating effect on growth of the strain KL2-13 to reduce the strain's ability to utilize petroleum hydrocarbons.

The influence of some additives on hydrocarbon biodegradation was tested; present investigation revealed that the addition of yeast extract in its higher level promoted the biodegradation of oil hydrocarbons and this is in agreement with Abdel-Fattah and Hussein (2002) who reported that the addition of yeast extract to the medium components was also induced the bioremediation process since the microorganisms require a primary growth substrate to cooxidize hydrocarbon compounds. Nicolson & Fathepure (2004) reported that the addition of yeast extract to halophilic and halotolerant bacteria enhanced the biodegradation of benzene, toluene, ethylbenzene and xylene (BTEX) compounds. In addition to that Arulazhagan *et al*., (2010) studied the influence of yeast extract as an additional substrate by the bacterial consortium and they reported that yeast extract is the water soluble portion of autolyzed yeast containing vitamins, nitrogen, amino acids and carbon for bacterial growth which promote the degradation of PAHs. On the other hand , Okeke and Frankenberger (2003) demonstrated that good growth and degradation rate of crude oil in presence of 1% yeast extract as nitrogen source. On the contrary Khleifat (2006) reported that the nitrogen sources supply, except yeast extract and casein, led to the enhancement of the phenol biodegradation of *Ewingellaamerica.*

The present study also revealed that the

addition of Thiamine HCl in its higher level favor the biodegradation of oil hydrocarbons .On other hand Todar (2008) reported that some bacteria do not require any growth factors; they can synthesis all essential vitamins, starting with carbon source, as a part of their own intermediary metabolism.

Diesel oil used as a carbon and energy sources was negatively significant factor once it present in its lower level (2ml/ 100ml). Atlas and Hazen (2011) microorganisms are able to consume petroleum hydrocarbons as the sole source carbon and energy for metabolism, although inhibition to microorganisms by toxins is also possible(Wenxiang, 2012). When crude oil in the environment reached a definite concentration it would have toxic effect on microorganisms, because the strain could not tolerate the high concentration of crude oil, which would inhibit the normal microbial growth to dramatically reduce the hydrocarbon degradation rate (Changyi *et al*., 2009). Ferreira *et al*., (2012) reported that some compounds in petroleum can be toxic to *Y. lipolytica* IMUFRJ 50682 above a certain concentration, inhibiting the metabolism of the microorganism. An increase in diesel degradation corresponded to an increase in cell number during the degradation processes demonstrating the microbial ability of utilizing diesel as the energy source (Zhu *et al*., 2001). Thus reinforcing the micobial ability to utilize both aliphatics and aromatics as sole source of carbon and energy. Biodegradation of crude oil by microorganisms appears to be the natural process by which the bulk of the polluting oil is used as an organic carbon source, causing the breakdown of petroleum components to lower molecularcompounds or transformed into the other organic compounds such as biosurfactants (Zhang *et al*., 2005).

By the end of the present study, the conditions achieved nearer to optimum one for diesel oil biodegradation by *Bacillus* cereus A were (g/l): NH_4CL, 2.5; KH_2PO_4, 0.6; Na2HPO4, 1.4 ; $MgSO_4.7H_2O$, 0.2 ; Yeast Extract, 0.2; Thiamine HCL, 0.002 and diesel oil, 2ml (with few drops of tween 20). Flasks with 100 ml culture volume were inoculated with 2 % (v/v) of the bacterial suspension equivalent to 0.5 Macfarland and incubated at 30°C for 48 hrs under shaken condition(160r.p.m.).

Biodegradation of diesel oil using immobilized *Bacillus cereus* A

It was aimed in the present part to test the potentiality of immobilized cells in diesel oil degradation. Different immobilizing agents; sponge and luffa were used as natural support to immobilize the living cells of *Bacillus cereus* A as previously described. To study the adsorption of diesel oil on immobilization matrix, an experiment was carried out with cells free sponge and luffa. The sponge was transferred to glucose containing medium and kept for 48 hrs. The same process was done for other immobilization matrix (luffa).

Use of immobilized *Bacillus cereus* A by adsorption on luffa and sponge

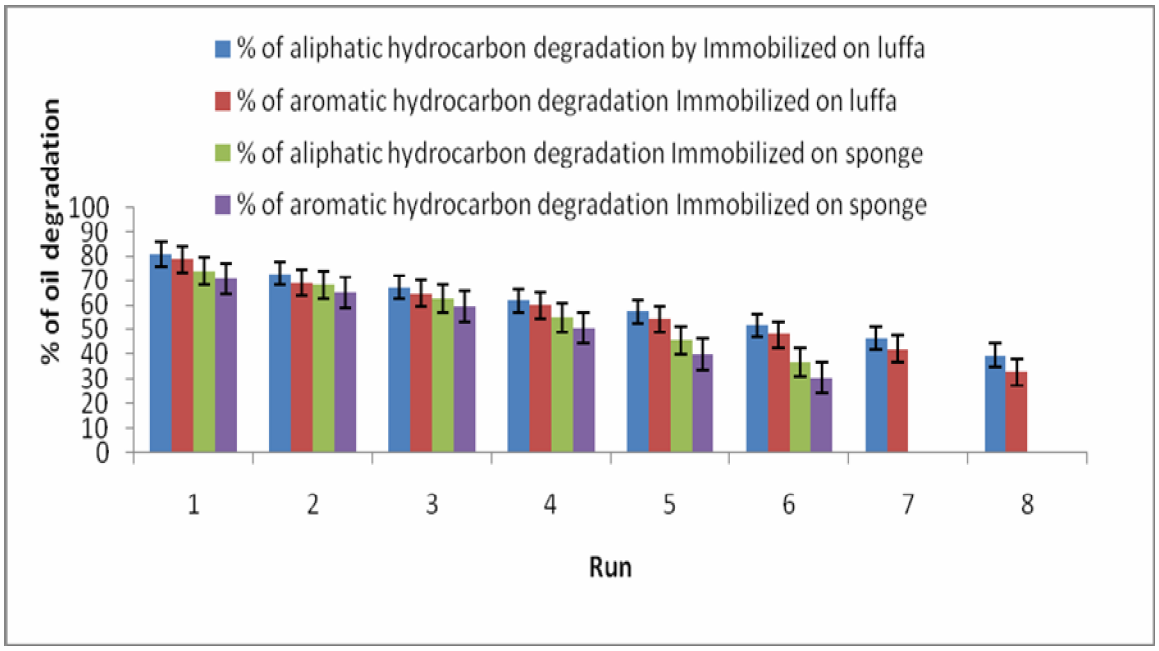
Immobilized *Bacillus cereus* A on luffa and sponge were added individually to bioreactor containing distilled water amended with diesel oil (1ml/50ml) and oil degradation was determined after 48 hours. Maximum percentage of oil degradation (80.67% and 78.83% of aliphatic and aromatic hydrocarbons respectively) was recorded with *Bacillus cereus* A immobilized on luffa. Eight successive runs were overloaded with the oil and the results showing decrease in oil degradation until it reach (39.58 % and 32.76% of aliphatic and aromatic hydrocarbons respectively).

Whereas maximum percentage of oil degradation was recorded with *Bacillus cereus* A immobilized on sponge (71.97% and 68.63% of aliphatic and aromatic hydrocarbons respectively). Six successive runs were overloaded with the oil and the results showing decrease in oil degradation until it reach 36.95 % and 30.43 % of aliphatic and aromatic hydrocarbon) (Figure 12). The major advantages of an adsorption system for water pollution control are less investment in terms of initial cost, and easy operation (Namasivayam and Yamouna, 1992). Tao *et al*., (2010) found that immobilization of strain GY2B with rice straw possesses a good application potential in the treatment of wastewater and bioremediation of estuary and offshore environment contaminated by phenanthrene, since the adsorption of phenanthrene onto the carrier promoting the degradation efficiency of the strain immobilized on it.

Studies showed that natural support such as pumice, modified rice straw and luffa are a good biosorbent for some pollutants in water, such as oil-spills (Sun *et al*., 2002), dyes (Gong *et al*., 2008) and heavy metal ions (Rocha *et al*., 2009). In a trial to apply fed-batch cultures, a bioreactor was used. Immobilized (75.67% and 73.83% of aliphatic and aromatic hydrocarbons respectively) cells on luffa were repeatedly used to degrade diesel oil.

At the end of this study, it was concluded that Saida port seawater autochthonous bacteria had high potential to degrade petroleum hydrocarbons and the present study provided an evidence to recommend that biostimulation of autochthonous microorganisms of the oil contaminated water is a good strategy to bioremediation oil polluted seawater using the free and immobilized bacterial cells on natural support as sponge and luffa.

Figure.12 Oil degradation by immobilized *Bacillus cereus* A as affected by using different immobilizing agents through several cycles.



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