



## Isolation and characterization of crude oil degrading bacteria from soil of Ouargla (Algeria)

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**Key words:** Hydrocarbonoclast bacteria , Bioremédiation, Hydrocarbons, Soil, Conditions

<http://dx.doi.org/10.12692/ijb/10.6.13-19>

Article published on June 16, 2017

### Abstract

The application of hydrocarbonoclast bacteria in bioremediation depends on their degradation capabilities and their environmental resistance to the distended site of treatment, understanding their adaptive physiology can avoid problems during application. The bacterial hydrocarbonoclast strains isolated from the unpolluted and polluted Saharan soil in the Hassi Messoud and Haoud Berkaoui oil fields of Ouargla and identified by sequencing 16S rDNA show a degrading degradation of oil and a fraction diesel, benzen, xylene, toluene at pH = 7, almost all strains degrade the fraction tested, but the addition of NaCl has no effect on the degradation of the oil, Fraction, their percentage decreased in proportion to the fraction used.

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## Introduction

Petroleum hydrocarbons are the most common environmental pollutants and oil spills pose a great hazard to terrestrial and marine ecosystem. Oil pollution may arise either accidentally or operationally whenever oil is produced, transported, stored, processed or used at sea or on land. Oil spills are a major threat to the environment as they severely damage the surrounding ecosystem; (Head *et al.*, 2006). Various groups of bacteria, fungi, cyanobacteria and algae are involved in hydrocarbon degradation, (Okerentugba and Ezeronye, 2003; Matsui *et al.*, 2013). They are widely distributed in marine, freshwater and soil habitats (Geetha *et al.*, 2013). The importance of the growth rate of bacteria in the presence of crude oil can be explained by the diversity and combination of fractions easily assimilated by these organisms (Nayerh and Mulligan, 2015). Biodegradation of hydrocarbons is one of the mechanisms leading to the first transformation of the pollutants into less toxic products. The ecology of hydrocarbon degradation by microbial populations in the natural environment is reviewed, emphasizing the physical, chemical, and biological factors that contribute to the biodegradation of petroleum and individual hydrocarbons. Rate of biodegradation depends greatly on the composition, state, and concentration of the oil or hydrocarbons. Temperature and oxygen and nutrient concentrations are important variables in both types of environments. Salinity and pressure may also affect biodegradation rates in environments, and moisture and pH may limit biodegradation in soils (Grajat *et al.*, 2013; Criste *et al.*, 2016).

The natural biodegradation process can be enhanced by addition of nutrients and optimizing the growth parameters. In order to remediate the crude oil pollution, crude oil biodegradation is necessary to isolate and characterize unique microbial species for evolution of their efficacy in utilization of crude oil before application of the contaminated sites. Therefore an attempt has made to examine the oil contaminated soil as a source of carbon utilizing bacteria to clean environmental contamination.

## Material and method

### *Preparation of samples*

Analyzed soil samples were collected with a sterile spatula on March, 2011 and January 2012 from the quagmires at the Hassi Messoud and Haoud Barkaoui oil fields and other unpolluted samples. The samples were then entered into a 50 mL polypropylene conical tube and placed in sterile polyethylene bags containing ice to be brought to the laboratory.

### *Isolement and purification of aerobic and oil degrading bacteria*

From a soil sample collected from different fields from Hassi Messoud sloughs, fields of Berkaoui  $\hat{a}$  amendil at Ouargla Algeria, 5g were taken and mixed with 30 ml of liquid mineral medium M63 0M (100 mM/LK<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>, 15 mM/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 4 mM/lMgSO<sub>4</sub> 7H<sub>2</sub>O and 9m MFeSO<sub>4</sub> 7H<sub>2</sub>O). The medium is supplemented with 2 ml of crude oil. The mixture is then incubated at 30 and 40 ° C with stirring at a rate of 150 rpm. after a week of incubation, isolation and purification of cultures at solid midium M63 0M of NaCl at 30 °C and 40°C enriched with 60µl oil as the sole carbon and energy sources; us to obtain pure cultures from different isolated colonies. The selection is based on colony macroscopic aspect, the color, the shape, diameter and opacity. A sample of each type of colony was removed and then purified by sub-culturing method streaks.

### *Identification of strains*

The oil-degrading isolates were identified by Gram stain, biochemical test and confirmed by 16S rRNA sequencing. The isolates were further confirmed using polymerase chain reaction (PCR) assay targeting 16S rDNA gene. The bacterial DNA was extracted DNA Mini Kit according to the manufacturer's instruction. The PCR reactions were performed in a Biometra T-Gradient thermocycler . Amplification was performed in a 50-µl reaction mixture using 16S rDNAs (universal F: 5-AGAGTTTGATCCTGGCTCAG-3, universal R: 3-GGTTACCTGTTACGACTT-) (Weisburg *et al.*, 1991) and 1 µ Taq polymerase.

Clones were sequenced sequencer and compared against the National Center for Biotechnology Information (NCBI) ([www.blast.ncbi.nlm.nih.gov](http://www.blast.ncbi.nlm.nih.gov)) using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997). For long term preservation, the bacterial isolates were stored in 20% glycerol at -80°C.

#### *Growth of the isolates at different salinities and temperatures and on different strations*

To achieve our experiment consists in the evaluation of the effects of pH, and salinity on the growth of the hydrocarbon degrading bacterial strains, the cultures were performed on solid M63 medium added the 60

µl of oil, gasoil, benzen or xylene or toluen ) in different Conditions of PH( 5.5,7,8.5) and temperature (30C°,40C°) for 24 -48h (Table 1) .

#### *Statistical analyses*

All analyses were conducted in triplicate and the results were expressed as means ± standard deviation (SD). It is performed using the statistical XLSTAT software.

#### **Results**

Hieghteen alkane-degrading bacterial strains were isolated from enrichment cultures that established at 30°C for one week.

**Table 1.** Characteristics of culture media used.

Conditions	Solide medium of M63 + 60µl of fraction ( Oil, Diesel, Benzen, Xylene, Toluen)					
Salinity (NaCl)	0 mM			400mM		
pH	5.50	7.00	8.50	5.50	7.00	8.50
Temperature of incubation	C°/40C°30					

Thirteen strains were identified by classical biochemical tests. Molecular identification of the isolated bacteria were perform by amplification and sequencing the 16S rDNA gene sequencing and comparing them to the database of known 16S rDNA sequences (Table 2).

Table records the effects of pH, temperature and salinity on the growth of isolated in the presence of different strains of hydrocarbon fractions. The effects of each parameter will be represented in the following figures.

**Table 2.** The results of identification of strains.

N° strain	Gender or species determined by sequencing the gene RNA16S
S1	<i>Klebsiella</i> sp
S2	<i>Klebsiella pneumoniae</i>
S3	<i>Staphylococcus haemolyticus</i>
S4	<i>Acinetobacter calcoaceticus</i>
S5	<i>Pseudomonas putida</i>
S6	<i>Acinetobacter radioresistans</i>
S7	<i>Stenotrophomonas maltophilia</i>
S8	<i>Rhodococcus equi</i>
S9	<i>Pseudomonas parfulva</i>
S10	<i>Acinetobacter radioresistans</i>
S11	<i>Enterobacter hormaechi</i>
S12	<i>Bacillus licheniformis</i>
S13	<i>Ochrobactrum cicerti</i>

Effect of pH on bacterial growth: The change in pH has no apparent effect on the growth of bacterial strains in the presence hydrocarbonoclastes 2% Crude Oil. Indeed, we are witnessing a growth rate of 100% of seeded bacteria after 48 hours of incubation. In the presence of 2% of Diesel, the effects of pH vary. At pH 5.5, only 33% of the incubated strains were able to grow, whereas at pH 7, there is a growth rate of 66%. The pH 8.5 only allows the growth of 16.66% of the bacteria tested (Fig. 4).

Xylene 2% led to the growth of all the strains when the pH is adjusted to 5.5 or 8.5. These same pH, have allowed the growth of 33.33% incubated with 2% Toluene strains. At pH 7, only 77% of the strains are able to grow in the presence of benzene and toluene and in the presence of 67% xylene. The strains studied saw their slow at pH 5.5 and 8.5 in the presence of benzene growth. Their respective growth rates are 33.33% and 11.11% (Fig. 1).

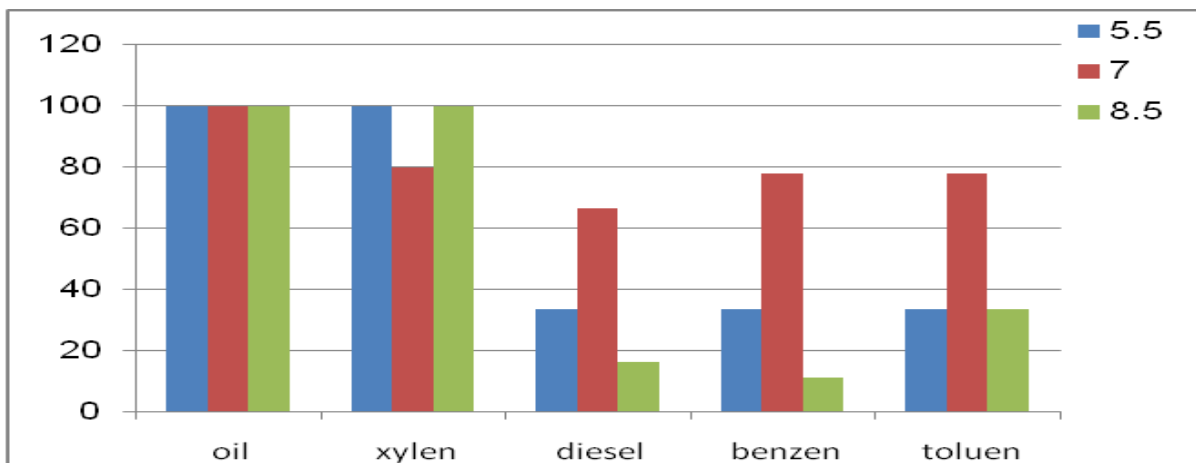


Fig. 1. Effects of pH on bacterial growth

Effect of Salinity on bacterial growth: Fig.2 reveals that in the presence of crude oil in the culture medium, the addition of 0.4 M NaCl did not affect the growth of the bacteria studied. We are witnessing a growth rate of 100% under these experimental conditions. In contrast, the NaCl seems negatively affect bacterial growth when added in the presence of other fractions (Fig. 2). The incubation of the tested at 30 °C and pH 7 in the presence of xylene or Diesel

strains led to the growth of 66% of these strains. In the presence of toluene or benzene is the highest growth rate (77%) (Fig. 2). The addition of NaCl (0.4 M) to the culture medium containing allowed Gasoil 55.55 growth of the strains tested. Xylene, toluene and benzene hardly allow better growth of these bacteria. Rates are respectively 33.33%; 22.22% and 16.66% (Fig. 2).

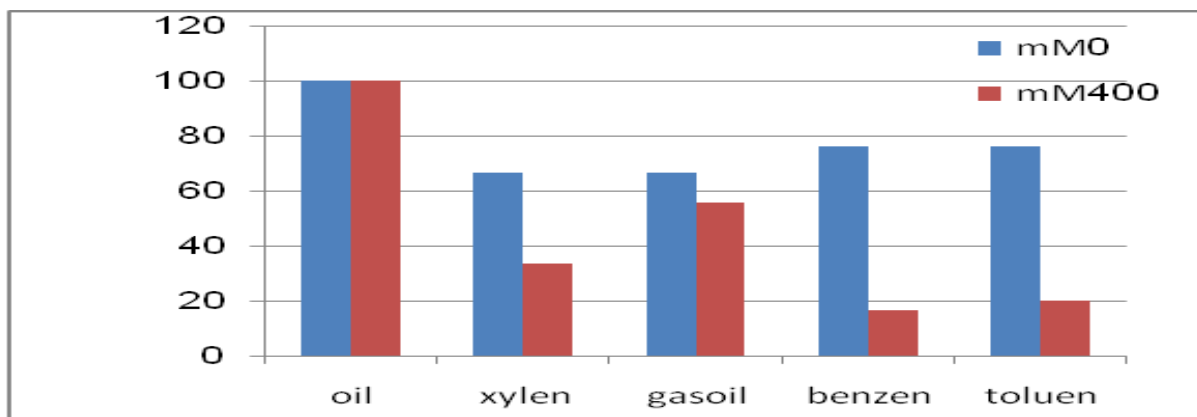
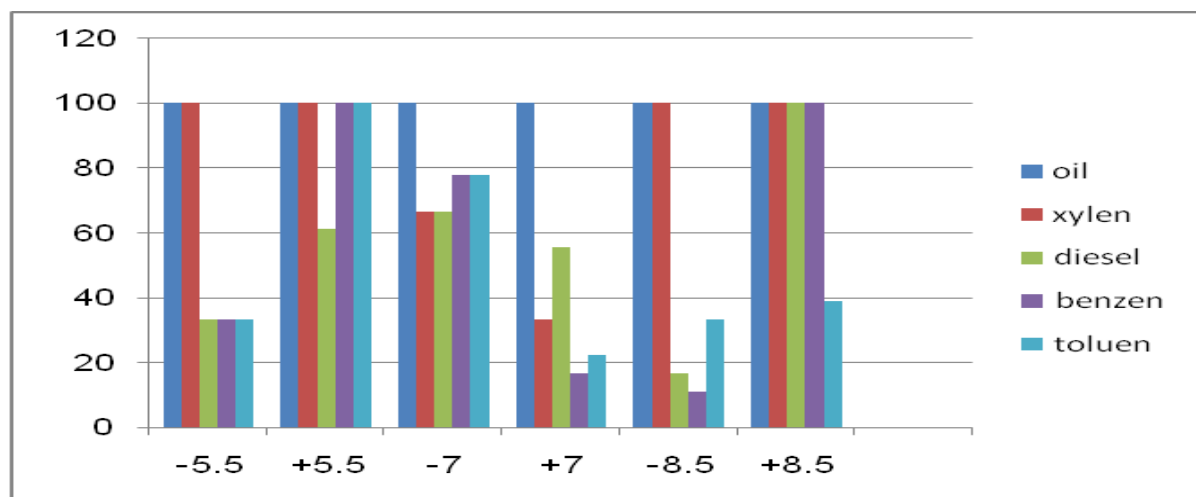


Fig. 2. Effect of Salinity on bacterial growth

Effects of pH and salinity on bacterial growth: Fig 3 shows the results of the action of pH and salinity on the isolated strains at 30 °C in the presence of 2% of various hydrocarbon fractions. The variation of pH in the presence of 0.4 M NaCl seems to have no effect on the growth of bacterial strains in the presence hydrocarbonoclastes 2% Crude oil. Indeed, we are witnessing a growth rate of 100% of seeded bacteria after 48 hours of incubation.

In the presence of 2% of Diesel, the effects of pH and salinity are variable. Indeed, in a saline medium, pH 5.5 resulted in the growth of the incubated strains 61.11%, while at pH 7, there has been a growth rate no more than 55.55%.

The positive effect of pH and salinity arises at pH 8.5 where the growth rate of the strains tested reached 100% (Fig. 3).



**Fig. 3.** Effect of pH and salinity on the growth of strains isolated at 30 °C.

Adding 2% of xylene, toluene or benzene, and 400 mM NaCl in the culture medium and adjusting the pH to 5.5, there is the growth of all strains incubated. At pH 7, only 33% of the strains are able to grow in the presence of xylene, 22.22% in the presence of toluene and 16.66% in the presence of benzene. Increasing the pH to 8.5 in the medium supplemented with NaCl led to improved growth rate hydrocarbonoclastes bacteria reaches 100% in the presence of benzene and xylene. In the presence of toluene, only 38.88% of the sown strains come to grow.

### Discussion

In the present study, crude oil utilizing bacteria were isolated from different level of crude oil contaminated soil for evaluating their efficacy in reclamation of crude oil contaminated soil. Higher microbial population in soil may be due to utilization of crude oil as carbon source by native bacteria. (Head *et al.*, 2006; Hassanshahian *et al.*, 2010). In the present study, altogether 32 morphologically distinct bacteria

were isolated from crude oil contaminated soil and 18 different hydrocarbon-degrading bacteria were selected. Though there are reports on screening of large number hydrocarbon degrading bacterial strain from different ecological niches but reports are scanty on screening of hydrocarbon degrading bacteria from varied environmental conditions. Finally 13 isolates were short listed and selected for further study. Sequencing and subsequent phylogenetic analysis of the 16S rDNA gene identified the isolates as *Bacillus*, *Rhodococcus*, *Stenotrophomonas*, *Enterobacter*, *Achromobacter*, *Klebsiella*, *Staphylococcus* and *Pseudomonas* strains (Table 2). The occurrence of these bacteria in oil polluted soil were earlier reported by several workers (Wang *et al.*, 2010; Das *et al.*, 2011; Manif *et al.*, 2011). For first time, the abundance of hydrocarbon utilizing bacteria in different crude oil contaminated soil. (Xue1 *et al.* (2015) described petroleum-degrading bacteria fast part of their evaluation of the distribution of the naturally occurring petroleum-degrading aerobic bacteria.

They isolated some crude-oil-degrading bacteria that belong to the genre a *Pseudomonas*, *Acinetobacter*, *Micrococcus* and *Nocardia*. Bacterial diversity of soils studied suggests a strong hydrocarbonoclaste activity when favorable conditions are put (Pedregosa *et al.*, 1996; Kumar and Khanna, 2010; Nalinee, 2013). The optimum growth of our strains was detected at salinities 0.4mM and temperatures between 30 and 40 °C, suggesting that they are halotolerant and mesophilic.

The salinity and temperature tolerance of the strains is in accordance with the prevailing conditions in the field where the original soil were collected.. The change biodegradability of this hydrocarbon may be due to its nature which is composed of several types of hydrocarbons consisting of different families hardly assimilated by microorganisms (Terry *et al.*, 2012; Mesbaiah and Badis, 2013). The improvement in the growth rate of cultured in the presence of cyclic moieties by increasing the pH (8.5) and bacteria of adding NaCl (0.4 M) suggests the importance of soil salinity and pH in the biodegradation of hydrocarbons. Indeed, the importance of control of the NaCl in the ion permeability of the cell membrane and the regulation of the osmotic pressure inside the cell has often been mentioned (Patil *et al.*, 2012; Marti and Balcázar, 2014). Moreover, the acidification of the soil occurring after partial degradation of hydrocarbons proves detrimental to the microorganisms which degrade; hence the advantage of a slightly basic pH (8.5) was reported to have a positive effect in the degradation of hydrocarbons (Philippe, 2014). The temperature is a parameter that can influence the biodegradation of oil by changing its physical condition, its chemical composition, the physiological activity of microorganisms and consequently the rate of degradation of the latter as well as the nature and concentration of microbial species (Varjani *et al.*, 2015). The maximum metabolic activity of the microorganisms is generally observed at a temperature between 30 and 40 °C. In addition, there has been an increase in the toxicity of oil and decreased metabolic activity (Mishamandani *et al.*, 2015).

## Conclusion

Thirteen crude oil-degrading bacteria were isolated from industrial oil-polluted soil and not polluted soil from Ouargla. The most potent strain was identified using a method based on 16S rDNA gene sequencing. Most of the strains having the capacity to degrade petroleum hydrocarbons under different conditions of temperature and salinity and pH. From the results obtained the observed resistance of these isolates can be interpreted by their adaptation under Saharan conditions, which leaves us to reflect to integrate them and the applications in the treatment of the soils polluted by petroleum hydrocarbons.

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