Journal of Genetic and Environmental Resources Conservation, 2014, 2(3):283-289.
www.jgerc.com

Bioremediation of contaminated soils by hydrocarbons

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Abstract

Several researchers in many parts of world studied the degradation of gas oil in soil, at the same time, many studies do report the incomplete degradation. In chronically contaminated environments, the selective pressure could enrich the soils of hydrocarbon degrading microorganisms. This work were staged evidence of biodegradation of the hydrocarbons and then isolated bacterial strains considered most interesting from the standpoint of biodegradation. The presence of an indigenous microflora, potentially capable of degrading the hydrocarbon mixture contaminant. Under aerobic conditions and in the presence of nutrients (Nitrogen N and Phosphorus P) the community autochthonous showed the ability to degrade some of the main components of the contaminant. The degradative capacity of the isolate MS2 evaluated by using the mixture of contaminant and a gas oil traction as a carbon source. The isolated MS2 result is able to degrade the major components of the mixture and the percentage contaminant general degradation was 23% after 14 days of incubation. The ratio of degradation In the presence of gas oil as a carbon source was 49%.

Keywords: Bioremediation, Contaminated, Hydrocarbons

Introduction

Petroleum hydrocarbons, due to geological events, through a slow infiltration may fall into the biosphere where they used by microorganisms that have evolved over time the metabolic pathways that allow degradation.

However, the huge amounts of hydrocarbons introduced into the ecosystem by human activities exceeds the self-purifying capacity of the environment contamination with hydrocarbons is most often due environmental disasters. Therefore, a huge amount of contaminated oil, which every day released into the environment, is causing pollution by hydrocarbons (Atlas and Bartha, 1997). Soils inhabited by indigenous (native) of bacteria, fungi, algae and protozoa. Are Also phages or viruses that are able to infect each of these classes of organisms, but information about it are still limited (Maier and Pepper, 1995). In addition to the populations, we can find indigenous micro-organisms introduced by human activity (control agents biological agents or biodegradation) or animal (droppings). The microorganism may release enzymes into the soil. The enzymes have ability to catalyze the oxidation reactions of a variety of different hydrocarbons, and some of them characterized by a broad substrate specificity (Gibson and Yeh, 1973). The activities of enzymes in the soil are the sum of the activity of all enzymes together. The activity of the native enzyme is result of many processes that lead to partial combination of the enzymes produced locally in the soil. In other words, these enzymes immobilized on the surface of soil particles (McLaren, 1975). The bioremediation is a process based on the activity of aerobic and anaerobic organisms heterotrophic (Bodour *et al.*, 2003), which is based on stimulation of their catabolic capacity, for remove the contaminants from the soil. The microorganisms are adsorbed onto soil particles through mechanisms of ion-exchange: In general, the soil particles have a charge negative, and soil bacteria can bind through ionic bonds involving the cautions polyvalent (Killharn, 1994). They can destroy the contaminant present in the soil through microbial metabolism, which is the vital process of microbial cells in which they carried out the activities of a nutritional and functional body (Pelczar *et al.*, 1986). Generally, soil microorganisms perform two functions: draw a source of carbon from an organic contaminant and use the electrons supplied by the same compound obtain energy. Schematically the phenomena of biodegradation of the organic molecules can be represented by redox reactions, catalyzed by the enzymes produced by microorganisms, ie; a process of transferring one or more electrons from

compounds highly energy, electron donors (oxidized), to compounds with lower energy, electron acceptors (reduced), with final storage of energy in ATP molecules (Ewis, 1998). The responsible microorganisms of biodegradation processes compete with each other for sources organic carbon available. The stoichiometry of the processes for the conversion of the substance organic synthetically represented by CH_2O, in different redox environments, it may be schematically represented by the general reactions and the corresponding values of the free energy 34 Gibbs, at pH = 7 (Schaeffer *et al.*, 1979).

The objectives of the research were to monitoring of the microbial community and characterization of the indigenous community degrading

Materials and Methods

Culture media:

Minimal medium culture Bushnell-Haas (BH; Difco cod. 0578-17): Minimal medium BH used to assess the activity of degrading hydrocarbons by of microorganisms. There were no sources of carbon in soils, therefore could provide hydrocarbons of which one wants to search for degradation. The formula (per liter) of the soil Bushnell-Hass is the following: Magnesium sulfate: 0.2g, Calcium chloride: 0.02g, Potassium dihydrogen phosphate: 1g, Ammonium phosphate, dibasic: 1 g Potassium nitrate: 1 g, Ferric chloride: 0.05 g, Final pH: 7.0+ 0.2 at 25 °C. To prepare 1 liter of soil, needed 3:27g of BH; then sterilize by autoclaving (121°C for 15 minutes).

Determination of the Mmost probable number (MPN): In each multi-well plate are added 20µl of each of dilutions serial supernatant. The first vertical column of each multiwell inoculated with 20µl of serial dilution 10^{-1}. The plates sealed with parafilm and then incubated for 15 days at 32°C in the plastic bags to prevent evaporation of soil.

The multiwell are prepared in this way:

- At first were added to each well, 180µl of medium BH; then were added 5µl of hexadecane.
- At second added to each well, 180µl of medium BH; then were added 5µl of sterile mixture.
- At third added to each well, 180 µl of medium TSB.

The title calculated on five horizontal lines (BF) of the multiwell (MPN 5-tube). In the case of the plates containing BH and hydrocarbons, were added to each well of 50 wells, 1µl of iodionitro tetrazolium (INT, 3g/l) and "pipetting" for 4 times, to homogenize the sample. The plates then incubated 24 hrs. at 32°C and positive wells identified by the appearance of the purple color. The title of vital calculated on five horizontal lines (BF) of the multiwell (MPN 5-tube).

Chemical analytical procedures for determining degradation of strains isolated from: Gas chromatography combined with Mass Spectrometry used to study the degradation of products in the sample. This technique is essential to perform a unique identification of different each components of the complex mixture of hydrocarbons. For mass analysis, gas chromatograph interfaced Thermo Quest TOP 8000 has been used with a quadrupole mass spectrometer MD 800, chromatographic column VARIAN CPSIL 8, 30m, 0.25, uM 0:25 movie ,Carrier : Helium at 120 kPa,Injector: split with split ratio 140:1, Ramp: C/2min 40° C , 10°C /min up to 310 °C for 2 min, MS : full scan range 50-450 a.m.n. ; detector 550.

Amounts of 1µl for all samples injected with AS 800 autosampler Thermo Quest. The samples analyzed under different experimental analytical conditions, under particular modulating the temperature ramp and the split ratio, in order to achieve the best experimental conditions and to obtain a statistically significant. Ultimately, the final figure showed the result of 12 different determinations.

Results and Discussion

Preliminary characterization of the degradative potential of the microbial community: For the microbiological characterization, MPN (Most probable number) was used .This method is very rapid and lends itself to the analysis on the field (Alexander, 1982). This method widely used for monitoring microbiological remediation of soils contaminated by petroleum hydrocarbons, similar to the present, and is considered the method of reference for microbiological analysis (Wrenn and Venosa , 1996; Bachoon *et al.* , 2001; Eriksson *et al.* , 2001). In literature, the microorganisms enumerated by the MPN technique, in the presence of hydrocarbons as the sole source of carbon, considered "degrading". The organisms listed by MPN in BH soil spiked with the contaminant as the soil carbon source would therefore be able to degrade at least one of the components of the hydrocarbon mixture and therefore, between the metabolic groups listed in this work, would be the most important for the reclamation (Wrenn and Venosa, 1996; Bachoon *et al.*, 2001; Eriksson *et al.,* 2001). However, we consider, that the exclusive use of MPN method, to obtain an enrichment of degrading microorganisms, does not allow drawing definitive conclusions on the Figure (1).

A B

Figure (1): A: Multiwell with TSB, B: hexadecane

Isolation of microorganisms enriched in the MPN: This strategy allows the isolation of microorganisms numerically representative able to employ the mixture hydrocarbon contaminant as a substrate for growth. Strains were isolated in three steps in order to obtain pure cultures (Table 1).

Table (1): Strains isolated by enrichment MPN samples of soil in mineral soil with contaminant BH.

Isolate	Morphology
MS1	Orange rounded opaque
MS2	Orange glossy rounded
MS3	Orange wrinkled
MS4	Orange wrinkled
MS5	Orange glossy rounded
MS6	Orange jagged rough

Determination of the best hydrocarbon-degrading strains isolated from contaminated site: During the remediation of hydrocarbon, microflora monitored as a microbiological parameter, the title of microorganisms capable of growing in the presence of a contaminant as a carbon source (determined by the MPN method). As already mentioned, we believe that the MPN enumeration, to obtain an enrichment of degrading microorganisms, does not allow draw definitive conclusions on the degradative activity of the isolated microorganisms. In the soil, may be mineral used for enrichment but in fact, growing microorganisms oligotrophic, chemiolitotrofi and autotrophic. Among isolated bacteria from the contaminated site and selected due to the ability for employing the mixture of hydrocarbon contaminant as substrate, in general the best growth capacity: isolated MS2 showed,

- Grows faster, compared to all other blocks, in an agar medium with the mixture hydrocarbon contaminant as a source of carbon and energy, forming colonies visible in a week of growth at 32 °C.
- Among all isolates is the only one able to break the layer of NAPL in culture BH2 with liquid contaminant (2% w/v) after 7 days of growth at 32 °C under stirring.

Chemical characterization of the contaminant:
Total petroleum hydrocarbons (TPH); The residual hydrocarbon concentration measured in duplicate on both the inoculated culture that in the abiotic control (uninoculated), the time kinetic reported in Table (2).

Table (2): Degradation of diesel fuel made from the strain MS2 (TPH)

Time	medium diesel fuel degraded (mg / L)	ST.DEV degraded	medium diesel fuel Abiotic control (mg / L)	ST.DEV abiotic
0	932	49	940	42
1	589	20	956	20
2	568	22	916	19
3	466	36	925	12
7	479	21	945	4
14	458	40	899	25

After 14 days of incubation at 37 °C in a soil BH2 containing 1g/l of diesel oil, the limit general degradation was 51%.

Analysis for classes of contaminants: The analysis of degradation for classes of contaminants was carried Processing chromatograms obtained using the mass spectrometer as a detector, by selecting the same ions used for the analysis for the contaminant mixture. Figure (2) shows the profile chromatography GC / MS of cultures inoculated at time 0, 1 and 7 days, the tests in batch. One can easily notice the disappearance of some peaks, representing some of the components hydrocarbon diesel.

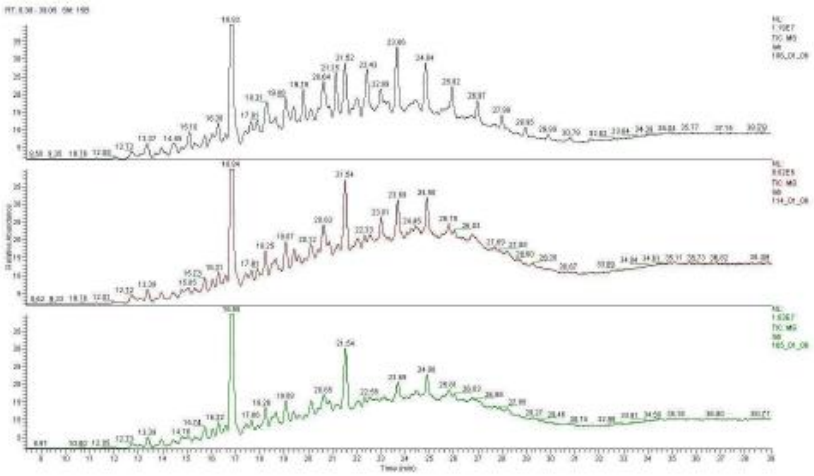

Figure (2): GC / MS analysis of a diesel traction extracted from cultures inoculated with isolate MS2 at time 0, 1 and 7 days at 32 °C.

Linear and Branched aliphatic: Figure (3) shows the normalized values of the areas of individual peaks at various times kinetic analysis. The value of the degradation, calculated for the individual peaks and for the totality of compounds studied, refers to the time 7 days.

In contrast to the contaminant mixture, commercial gas oil has a high percentage of linear alkanes. These compounds were rapidly and completely degraded, while the degradation of those branched chain was minor overall degradation of aliphatic hydrocarbons was 88%. Figure (4) shows the chromatogram of alkanes: is even more evident, than observed in the degradation of the contaminant mixture, the decrease of the peak areas.

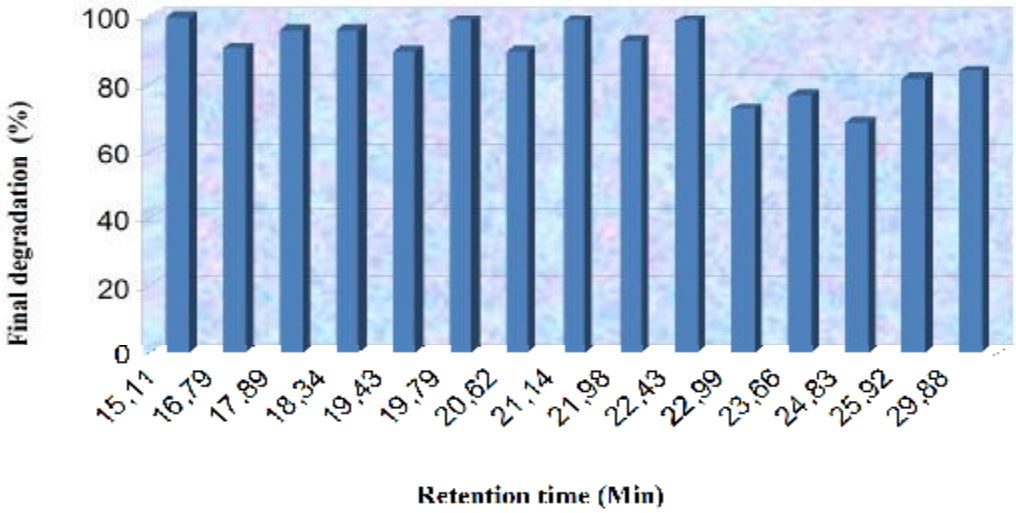

Figure (3): Diesel: linear and branched aliphatic

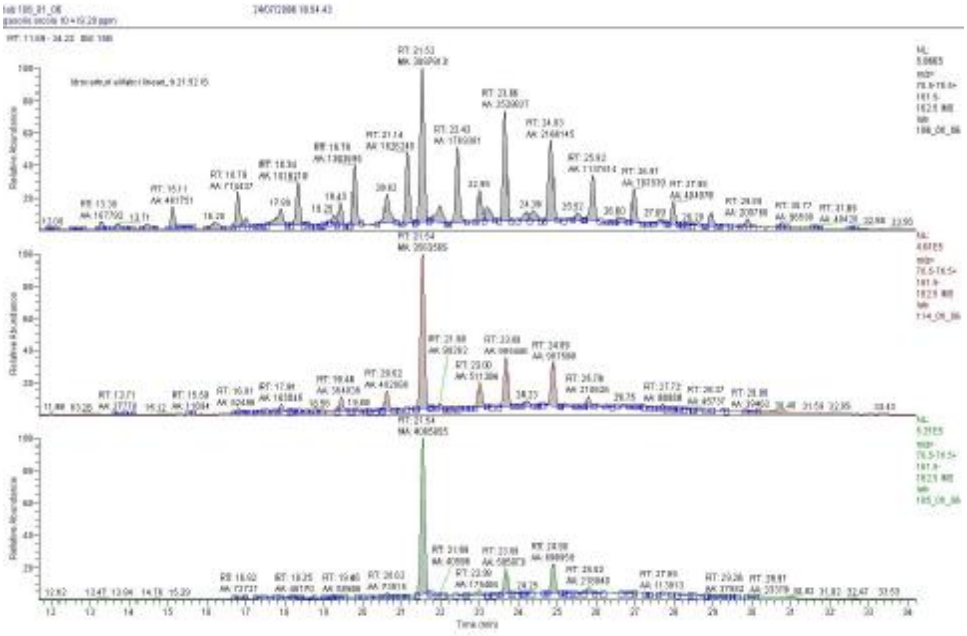

Figure (4): GS / MS of diesel: linear and branched aliphatic

Monoaromatic Alkyl-Replaced, Naphthaline Alkyl-Replaced, Naphthenes Alkyl-Replaced: Figure (5), Figure (6), and Figure (7) shows the areas of individual peaks at various times kinetic analyzed, respectively, for the mono-alkyl-substituted, the alkyl-substituted naphthalenes and alkyl-substituted naphthenes. The value of the degradation, calculated for individual peaks and for all the compounds studied, refers to the time 7 days. The mono-alkyl-substituted, alkyl-substituted naphthalenes and alkyl-substituted naphthenes were partially degraded, 33%, 35% and 23% respectively. For what concerns the degradation of the oil, then we can observe how the isolate MS2, despite primarily degraded classes aliphatic, is capable of also degrade aromatic fraction of diesel.

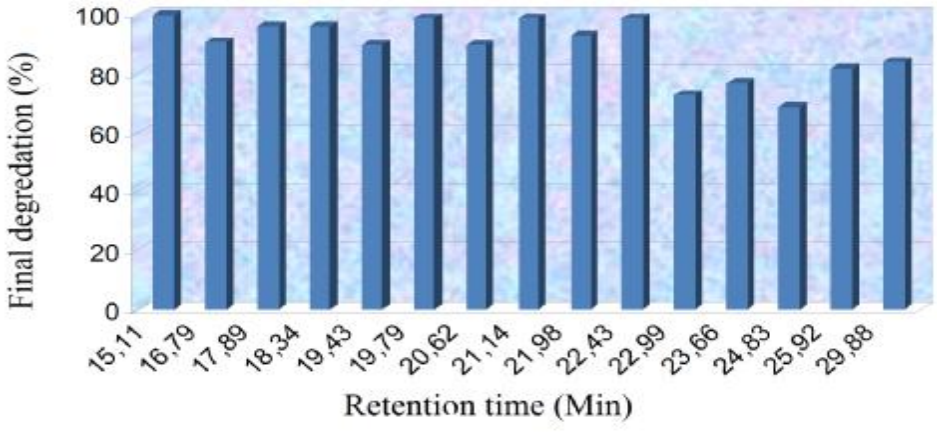

Figure (5): Oil mono-substituted compounds

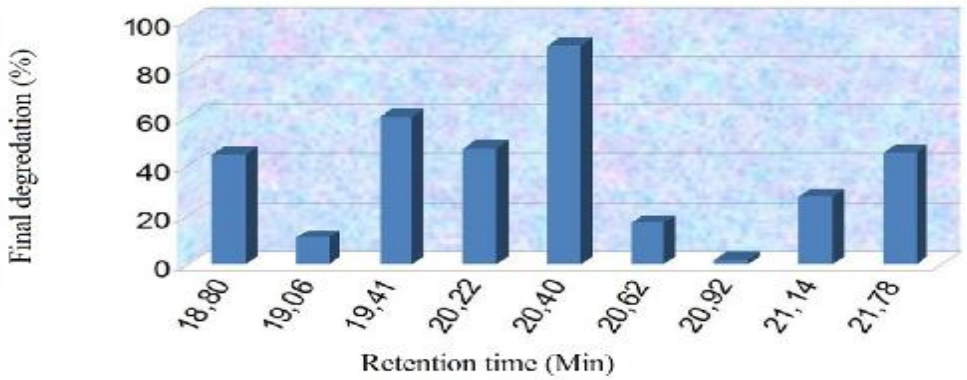

Figure (6): Oil substituted Naphthalenes

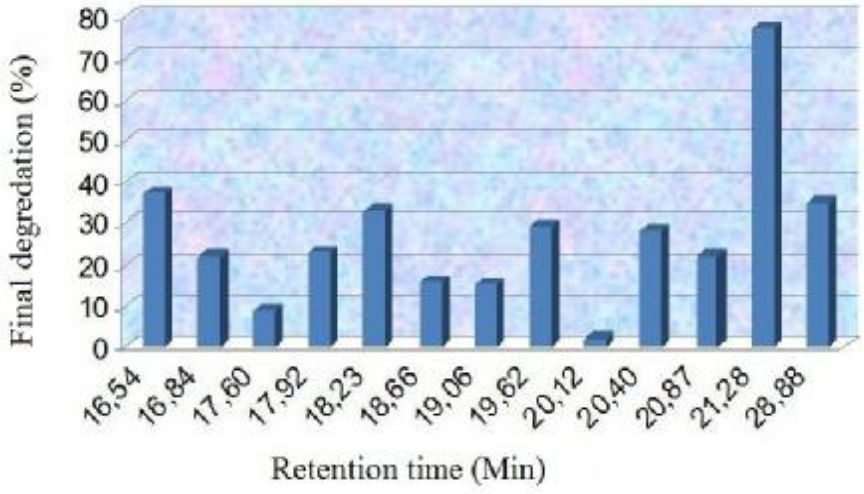

Figure (7): Oil replaced Naphthenes

Conclusions

Among the isolated strains, which isolated from the contaminated site, the isolated strain MS2 showed in general best growth capacity, when using hydrocarbons as a carbon source, the strain MS2 was the fastest among the isolated strains to grow significantly with the mixture as a contaminant substrate. In addition, we have been able to observe that the strain was the only one able to break the layer of NAPL in the cultures in liquid state, after a week of incubation, therefore we have undertaken a characterization of the catabolic properties of this organism also in view of its use in biotechnological applications. Were then staged degradation tests at liquid state, in duplicate, the using of isolate MS2 as inoculating and the contaminant mixture as a carbon source, in one case, and a Gas Oil traction. On the other hand, the characterization of the catabolic properties of the isolated MS2 made mainly in relation of MS2 isolate using as an additive to be used to improve the process of bioremediation of contaminated soils by petroleum products.

After 7 days of incubation at 32 °C in medium containing 0.1% of BH mixture of hydrocarbon contaminants (old diesel), the general limit of degradation was 23%. After 14 days not observed a further degradation. In particular we have have observed degradation of the aliphatic fraction , mono- compounds , and the nafatleni of naphthenes replaced , respectively, equal to 45%, 34%, 23% and 26%. Under the same experimental conditions, general limit of degradation of oil, after 14 days of incubation, was the 51%. The isolated strain of MS2, in the absence of limiting factors, the strain of MS2 is capable of degrade a fair percentage of the contaminant mixture recovered from the contaminated site, and a good percentage of the oil in question. As expected, the percentage of degradation Total (TPH) was significantly higher

in the gas oil that contaminant in the mixture, mainly due to the presence of the fraction aliphatic linear, lower in the mixture due to degradative processes occurred over the years in the field. This fraction in gas oil fuel was in fact be readily degradable. Regarding the mono- compounds observed that degradation for gas oil and for the contaminant mixture does not differ significantly. For naphthenes, you can instead assume that there has been in the mix, albeit modest, greater degradation. The reason for this increased degradation can searched in most presence in the fuel of more easily degradable compounds. It is possible to assume, therefore, that in gas oil at the end of the trial, there is still the presence of naphthenes degradable. However, concerns naphthalenes, the degradation was higher in gas oil: the difference of degradation is attributable to the presence in the fuel of a component of the fraction of naphthalenes easily degradable and no longer present in the mixture contaminant.

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