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Development of bioremediation strategies based on the improvement of biomass production from isolated strains in hydrocarbon contaminated soils and their application in bioremediation technologies

Desarrollo de estrategias de biorremediación basadas en la mejora de la producción de biomasa a partir de cepas aisladas en suelos contaminados con hidrocarburos y su aplicación en tecnologías de biorremediación

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RESUMEN

Es frecuente la contaminación ambiental generada por el petróleo o sus derivados, debido al uso generalizado de los mismos a nivel mundial. La descarga y derrame accidental de dichos compuestos en el medio ambiente resultan ser peligrosos para el entorno y los seres vivos. La biorremediación es una estrategia eficiente para limpiar sitios contaminados, siendo no invasiva y rentable. La misma se basa en la biodegradación natural utilizando microorganismos nativos aislados de áreas contaminadas. El centro industrial Zárate-Campana, ubicado en Buenos Aires, representa una de las áreas petroquímicas más importantes de Argentina, con varias empresas que realizan actividades petroquímicas. En este estudio, se seleccionaron 4 cepas previamente aisladas de sitios contaminados e identificadas dentro de los géneros *Pseudomonas sp, Cellulosimicrobium sp y Ochrobactrum sp.* La cepa MT1A3 perteneciente al género *Pseudomonas* fue seleccionada para los ensayos de optimización de la producción de biomasa a partir del uso de diferentes fuentes de carbono que van desde una mezcla de hidrocarburos hasta co-productos agroindustriales y fuente nitrógeno. MT1A3 fue capaz de crecer en una mezcla de hidrocarburos obteniéndose 1,79 g/L de biomasa a 25 ºC a los 7 días. Al comparar el uso de diferentes co-productos agroindustriales de bajo costo como fuente

alternativa de carbono, la producción de biomasa fue significativamente mayor en el aceite de maní crudo en comparación con todos los demás sustratos ($p<0,05$), dando como resultado una biomasa de 7,29 g/L luego de 7 días de cultivo. La fuente de nitrógeno más eficiente para obtener biomasa de MT1A3 fue $NaNO_3$. A partir de estos resultados, en sistemas de microcosmos se evaluó la efectividad de remediación mediante el monitoreo de la degradación de hidrocarburos en los diferentes tratamientos de atenuación natural, bioestimulación y bioaumentación, durante 120 días. El mejor tratamiento, que involucró bioaumentación (MT1A3) y bioestimulación, mostró una degradación del 40,05% del total de hidrocarburos con respecto al tratamiento de atenuación natural utilizado como control.

Palabras claves: biorremediación, suelos contaminados, hidrocarburos, microcosmos

ABSTRACT

Contaminated sites with petroleum compounds are frequently observed, requiring the development of innovative technologies for its remediation. The problem is caused due to the widespread usage of petroleum-based products. Their discharge and accidental spillage in the environment prove to be hazardous both to the surroundings and life forms. Bioremediation is an efficient strategy for cleaning up sites contaminated with organic pollutants. It is a non-invasive and cost-effective technique that relies on natural decontamination using microbes of isolated strains from contaminated areas for the clean-up of these petroleum hydrocarbons. The Zárate-Campana industrial center, located in Buenos Aires, represents one of the most important petrochemical areas in Argentina, with several companies carrying out petrochemical activities. In this study, we have investigated the ability of microorganisms to degrade these hydrocarbons. Samples were collected in the surroundings of the Campana area and screened for hydrocarbon degrading bacteria. 4 of the 13 strains previously isolated from contaminated sites were screened and identified as *Pseudomonas sp*, *Cellulosimicrobium sp* and *Ochrobactrum sp*. A new approach using MT1A3, belonging to *Pseudomonas* genus in petroleum biodegradation from the use of different carbon and nitrogen sources, was proposed to provide maximum biomass production and was evaluated for its degradation characteristics. MT1A3 grew in all carbon sources tested and was able to grow in a hydrocarbon mixture obtaining 1.79 g/L of biomass production at 25 ºC after 7 days. When comparing the use of different low-cost agro-industrial co-products as an alternative carbon source, the biomass production was significantly higher in crude peanut oil in comparison to all other substrates ($p < 0.05$), thus resulting in a biomass of 7.29 g/L. The most efficient nitrogen source for obtaining biomass from MT1A3 was $NaNO_3$. Based on these results, the effectiveness was evaluated by monitoring total hydrocarbons (THs) and n-alkanes degradation as well as changes in bacterial population of natural attenuation, biostimulation and bioaugmentation treatments in microcosm design over a 120-day period. The best treatment, which involved bioaugmentation (MT1A3) and biostimulation strategies, showed a degradation of 40.05 % of total hydrocarbons with respect to the natural attenuation treatment used as control. The highest concentration of THAB and HDB was recorded, reaching a value of $2,17x10^{10}$ CFU and $8,91x10^{6}$ UFC respectively.

Keywords: bioremediation, contaminated soils, hydrocarbons, microscom.

1- INTRODUCTION

Petroleum-based products are the major source of energy for several industries and it is also the raw material for many chemical products such as plastics, paints and cosmetics.

The amount of petroleum stored in developed countries is significantly high and its transport across the world is frequent and has generated a great deal of interest in recent decades due to their mutagenic and carcinogenic properties and their recalcitrance into the environment. Petroleum is a complex mixture of different hydrocarbons including aliphatic compounds (linear or branched), cycloalkanes, mono- and polyaromatics, asphaltenes and resins (Philip et al., 2005; Yemashova et al., 2007).Traditionally, the elimination of organic contaminants has been addressed through proven effective technologies that involve physical and / or chemical treatments (Arauna, 2004). In our country, large areas affected by this type of contaminants such as Patagonia, have increased as a consequence of mining, metallurgical activities and atmospheric deposition from fossil fuel power plants, resulting in serious and harmful accumulation of polycyclic aromatic hydrocarbons (PAH). The extent and importance of such pollution depends on several factors: amount of product spilled, characteristics of the soil (composition, texture), water flow and wind direction and intensity. Because of this, remediation techniques were developed to mitigate the impact of such pollution and to aid in the recovery of these sites (Riser-Roberts, 1998). The results derived from this study will permit us to gain new insight into the applicability of biological strategies to deal with the removal of PAH-polluted soil and to consider a new option to clean the environment. This is why environment-friendly bioremediation methods used as an alternative to chemical or physical methods are promising these days (Boer and Wagelmans, 2016). Biological treatments such as bioremediation techniques involve the use of phytoremediation based on plants and their associated microorganisms to remove pollutants from the environment or to make them less harmful (Salt et al., 1998; Arora, 2018). This is why the pressing need for the isolation and identification of efficient degrading microbes and for the design of ways to accelerate the biodegradation rate of oil pollutants components into fatty acids and carbon dioxide or pollutants in much less toxic substances arises. These kind of processes are environmentally friendly. Therefore, a subsequent treatment is rarely required, presenting a situation which position them as a very interesting alternative when compared to other treatments. Crude oil is constituted from thousands of components which are separated into saturates, aromatics, resins and asphaltenes. Hydrocarbons differ in their susceptibility to microbial attack and generally degrade in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low molecular weight aromatics >cyclic alkanes, > polyaromatic hydrocarbons (Leahy and Colwell, 1990; Salleh et. al. 2003), although many of these compounds can be relatively easily degraded under soil and freshwater environments (Van Hamme et. al., 2003) . In order to improve bioremediation performance, two techniques can be applied to clean up contaminated soils: bioaugmentation (Das and Mukherjee 2007; Guarino et al. 2017) and biostimulation (Tyagi et al. 2011). Bioaugmentation is, besides biostimulation, one of the existing possibilities to enhance the soil decontamination process

by increasing the indigenous microbial population of the ecosystem with the external addition of metabolically active microorganisms. It has been reported by Covino et al. 2016 that bioaugmentation, through the use of white-rot fungi, is a profitable approach for cleaning up polluted soils. Usually, bioaugmentation and biostimulation techniques must be used together so that the bioremediation process can be successfully applied (Carberry and Wik 2001). In all cases, the application of precise and reliable methods for evaluation of pollutant biodegradation through bioremediation processes is necessary. A microcosm can be considered as a model at laboratory scale of a natural system that provides, in a short time, information on the biochemical processes involved. Besides the information presented above, application of agro-industrial waste and co-products in bioprocesses provides an alternative way to replace the refined and costly raw materials. In addition, the bulk use of agroindustrial waste residues will help to solve environmental issues, will present an alternative for reuse and will serve as well as energy source for microbial growth. (Pandey et al. 2000; Makkar & Cameotra 2002; Fochesato et. al. 2018). In the present work, Hydrocarbon Degrading Bacterial (HDB) population, identification of the efficient degrader, its molecular characterization and phylogenetic analysis based upon 16s RNA gene sequencing from the samples collected were studied and characterized. We have investigated the ability of microorganisms to degrade these hydrocarbons and we have assessed pollutant bioremediation in microcosm system through the employment of bioaugmentation and biostimulation techniques. For the fulfillment of this objective, this work was carried out on the design of culture media from the use of hydrocarbon mixture and agroindustrial co-products as energy sources.

2 MATERIALS AND METHODS

2.1. BACTERIAL STRAIN ISOLATED

Hydrocarbons Degrading Bacteria (CO1A1, CO1A2, MT1A3, TK1A2) from contaminated soil of petrochemical Zárate-Campana areas in the Province of Buenos Aires, specifically in R.H.A.S.A. Refinery (34°10´ 5.1” S, 58°56´ 23” W) were isolated according to Liporace et al. 2018.

2.1.1. Molecular identification.

Strains were identified by 16s RNA sequencing. Total genomic DNA was extracted using the isolate assaying Wizard ® Genomic DNA Purification Kit (Promega®) and amplified by using the universals primers 27F [5´-AGAGTTTGATCCTGGCTCAG-3´] and 1492R [5´-GGTTACCTTGTTACGCTT-3´]. The PCR conditions were as follows: Cycling began with an initial denaturation at 94 ºC for 2 min, followed by 30 cycles of denaturation at 94 ºC for 1 min, annealing at 55 ºC for 1 min, extension at 72 ºC for 2 min and a final extension step at 72 ºC for 10 min. The PCR amplified DNA was purified using AxyPrep PCR Clean-up Kit (Axygen Bioscience). DNA

sequencing data was performed by INTA Castelar, Argentina. The 16s RNA gene sequences were edited by Bioedit software and compared with the sequences from Alignment Search Tool EzTaxon (Ez BioCloud). The strains showed more than 97% 16s RNA gene sequence similarity were considered to be of the same species.

2.2. GROWTH AND HYDROCARBON BIODEGRADATION

2.2.1. Bacterial Media and culture conditions: Shake flask experiments

2.2.1.1. Carbon Sources

In order to evaluate the growth conditions, the most efficient hydrocarbon degraders were chosen to be tested in biomass production using different carbon sources. Commercial hydrocarbons (from YPF: **Yacimientos Petrolíferos Fiscales S. A)** as carbon sources were assayed in a mix of these three sources (4,5 % v/v): gasoline (RON95), kerosene and diesel. They were cultivated in 125 ml Erlenmeyer flasks containing a minimal saline medium (MSM) (Liporace, 2012) and incubated at 25 ºC on the rotary shaker at 135 rpm for 5 days. The HC mixture used was sterilized by filtering through Millipore filter of 0.45µ diameter and stored in sterile bottles.

Considering this result, the ability to grow under different concentrations of hydrocarbon mixtures was evaluated under different concentrations of the mixture: 2, 4.5, 20, 50 and 60 % v/v), and incubated at two temperatures (20 and 25 ºC). Cultures were kept at 135 rpm between 7 and 14 days, and the isolated growth efficiency using sole carbon for each one: gasoline (1,5 % v/v), kerosene (1,5 % v/v), diesel (1,5 % v/v) and HC mixture (4,5 % v/v) used as a control sample. The cultures were incubated at 25 ºC, at 135 rpm for 10 days. The pH of the medium was adjusted to 7.0.

The viability of a biotechnological process includes the strategic optimization of a culture medium using agro-industrial waste and co-products. Different substrates were assayed for biomass production adding easily available carbon sources like glucose, and biodegradable wastes as low cost alternatives: glycerol, bovine milk serum, ovine milk serum, refined sunflower oil, oleic high sunflower oil, raw peanut oil, fried peanut oil, camelina oil and milled pressed peanut (2% v/v) from different industries in the surrounding areas.

2.2.1.2. Nitrogen Sources

On the other hand, the effects of three different nitrogen sources in culture media were tested to improve the biomass production: $NaNO_3$ (4,00 g/L), NH_4Cl (2,50 g/L) and urea (1,39 g/L) (Santa Ana et. al. 2002; Abouseoud et. al. 2008; Liporace, 2018) using raw peanut oil (2% v/v) as carbon source in each assay and incubated at 25 ºC, at 135 rpm for 5 days.

All media were homogenized and autoclaved at 121 C for 15 min.

2.2.3. Biomass and pH measurements

Bacterial growth was estimated by cell dry weight method. For this, an aliquot 18 mL of culture broth was collected every 24 h. Each sample was centrifuged at 4ºC and 13500 rpm for 15 minutes. The pellet was dried at 100ºC in a hot air oven until constant weight. The pH of the culture broth was measured with digital pH-meter (Adwa).

2.3. MICROCOSM DESIGN

The hydrocarbon contaminated soils used in the microcosm design were sampled from RHASA refinery areas (Liporace et. al., 2012). This area contained contaminated soils with hydrocarbons produced by spills during the routine operations of the plant (Fig. 1). The collected samples were pooled and transferred to pre-sterilized, labeled, self-sealed plastic bags and transported to the laboratory and preserved at 4°C during experiments. The homogenized and sieved soil sample was dispensed into cylindrical glass flasks (60 mm diameter and 360 ml of volume capacity), each containing 200 g of soil. Different treatments were carried out for each system. Natural attenuation as a control assayed and biostimulation/bioaugmentation strategies were applied according to Table 1. 5000 mg/kg of soil ammonium nitrate (NH_4NO_3, Biopack, analytical grade) was used as the N source, whereas a 1000 mg/ kg of soil sodium dihydrogen phosphate ($NaH_2PO_4.12H_2O$) solution (Biopack analytical grade) was used as the P source (Márquez-Rocha et. al. 2001; Ruberto et al . 2003; Ruberto el al. 2009). The microorganisms used in the bioaugmentation process were grown in MSM and raw peanut oil (2% $_{v/v}$) as a carbon source culture media was incubated for 5 days at 135 rpm and at 25 ºC. Flasks were kept at room temperature (between 20-25°C according to the environmental conditions) during the experimental period (120 days). The samples were taken once every 30 days. The content of each flask was aseptically mixed three times a week to ensure a homogeneous distribution of nutrients and contaminants, as well as appropriate aeration of the soil, and the process was controlled by water addition when necessary.

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Figure 1: Hydrocarbon contaminated soil collection pools for final disposal

2.4. BIOLOGICAL ACTIVITY

Spilled petroleum hydrocarbons in soils represent a direct resource for hydrocarbon degrading bacteria (HDB). The determination of microbial activity and its comparison among treatments is a useful tool to follow the biological processes occurring in these types of areas. 1g of the sample was aseptically transferred into 100ml of NaCl 0.9%.and transferred in a series of eight 10 fold serial dilutions. HDB and population was enumerated by inoculating 0.1 ml aliquot from these dilutions onto sterile agar plates containing MSM medium spread with 100µl of a HC mixture in duplicates (Dias et. al 2012; Jiang et. al 2016) .

Furthermore, THAB (Total Heterotrophic Aerobic Bacteria) population was enumerated using 1ml of the aliquot from each of the dilutions and was inoculated by pour plate method onto Nutrient agar (NA) in duplicates. The plates were incubated at 25° C for 7 days. The total number of colonies is referred to as the Total Viable Count (TVC) and was performed according to Brock & Madigan 2015.

Table 1. Conditions applied to the different microcosms.

System	Condition
C	Natural attenuation control
B	Biostimulation with N and P
MT1A3	Biostimulation with MT1A3
Mix	Biostimulation with strains mixture*
MT1A3 + B	Bioenrichment with MT1A3 + Biostimulation with N and P
Mix + B	Biostimulation with strains mixture* + Biostimulation with N and P

* Strains mixture MT1A3, CO1A1, CO1A2, TK1A2.

2.5. HYDROCARBON EXTRACTION AND QUANTITATION

For hydrocarbon quantification, soil samples (5 g) were extracted with 10 mL of dichloromethane in 20 ml glass tubes. Samples were vortexed for 5 minutes and shaken (135 rpm) overnight at 25°C. Afterwards, the organic phase fraction was recovered.

Chromatographic analysis was carried out in a Shimadzu GC-2010 Plus Gas Chromatograph (Shimadzu, Corp., Japan) equipped with a flame ionization detector (FID) and a 100-m-long 0.25 mm i.d. (0.5 μm film thickness) fused-silica capillary column (Petrocol DH Sepulco). Oven temperature was kept at 45°C, then ramped to 8 °C/min to 150°C, 4 °C/ min at 250 °C and 8°C/min at 300 and kept at this temperature during 48 min. Injector temperature was 300°C and detector temperature was 320°C. Carrier gas (H2) flow was 2 ml/min. Data were collected using the GC-Solution software (Universidad Tecnológica Nacional-Argentina). The total area of all peaks ranging from C8 to C40 in samples was compared to those from suitable standards 50.16.512 gravimetric standard DHA classic (PAC) and alkanes mix C8-C40 (Accustandard) (Ramirez et al. 2012; Jiang et al. 2016). For quantification purposes, initial calibration curves were carried out using a non-contaminated soil with different concentrations of a HC mixture in a range of 1000 to 20000 ppm. This procedure allowed to trace a straight line correlation between the peak area and the total HC concentration used. Hydrocarbons removal was then calculated using the following equation:

$$\text{CTH: total hydrocarbons concentrations (ppm)} = \frac{\text{A (The peak area obtained) - b (slope)}}{\text{m (cut of the straight line obtained)}}$$

3- RESULTS AND DISCUSSION

We chose to study 4 of the 13 strains previously isolated from RHASA refinery which is described by Liporace, 2018. Strains were chosen based on their profiles of degradation of petroleum derivatives, (Table 2) their biomass production and their maintenance under laboratory conditions. The genetic identification of the bacterial strains was done in Instituto de Nanobiotecnología Conicet-Universidad de Buenos Aires by a comparative analysis of the sequence of the 16S rRNA gene. The genetic sequences obtained were processed with the BioEdit® software and compared with those existing in the databases: EZ Taxon, RDP and SILVA. The partial sequencing of 16srDNA of the strains revealed the isolates to be (Table 2) *Pseudomonas (*MT1A3 and TK1A2), *Cellulosimicrobium (*CO1A1) and *Ochrobactrum* (CO1A2).

Table 2. Genetic identification of strains by its 16S rRNA gene partial sequence.

	Sequence length (nt)	Related to	Identity (%)
CO1A1	1373	*Cellulosimicrobium cellulans* LMG 16121 (T)	99.9
CO1A2	1338	*Ochrobactrum anthropi* ATCC49188 (T)	100
MT1A3	1402	*Pseudomonas kunmingensis* HL22-2 (T) JQ246444	99.4
TK1A2	1380	*Pseudomonas panipatensis* Esp-1 (T) EF424401	100

After isolation and identification, the hydrocarbon degrading capacity of the selected bacterial isolates was studied. The bacteria were able to use a 4.5 % v/v mix of hydrocarbons as a sole carbon source. All strains tested showed good biomass production (Table 3 and figure 2) resulting in values ranging from 0.11 to 1.86 g/L respectively. In this case, the fastest-growing strain was MT1A3, and after 5 days of incubation, the biomass obtained was significantly higher than the rest of the strains up to 93.01 % (1, 86 g/L). Regarding pH values, MT1A3 registered the highest pH and this correlates with the fact that it presented the highest biomass development as well. Probably, this effect is due to a greater metabolic activity, since it is known that the pH of the microbial culture medium is modified by the terminal products of metabolism.

Table 3. Biomass and pH of cultures obtained in MSM + HC (4.5%), at 5 days of incubation.

	Biomass (g/L)	pH
CO1A1	0,13 ± 0,01	7,55 ± 0,07
CO1A2	0,66 ± 0,06	7,63 ± 0,09
MT1A3	1,86 ± 0,07	8,34 ± 0,15
TK1A2	0,47 ± 0,01	7,77 ± 0,10

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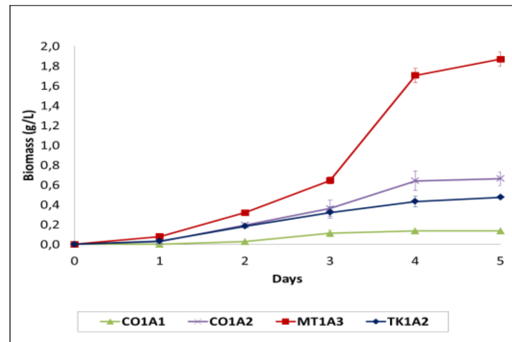

Figure 2. Cell growth of strains in MSM + HC (4.5%), after 5 days of incubation.

In terms of MT1A3 biomass tolerance and production capacity, it was observed that MT1A3 had the ability to grow at concentrations of up to 50% (v/v) in HC (0.90 g/L at 20 ºC and 1.17 g/L at 25 ºC) after 14 days (figure 2). This represents a high tolerance to tested hydrocarbons as correlated with studies where microorganisms with high tolerance to compounds such as toluene, xylene, styrene and phenol have been reported (Heipieper et. al. 1994; Rabodonirina et. al. 2018). There was no growth at 60 % (v/v) of HC. This presents favorable conditions for bioremediation strategies of hydrocarbon contaminated sites. Strain MT1A3 presented the highest biomass production profile at 4.5 % (v/v) HC, obtaining 1.36 g/L at 20 ºC and 1.79 g/L at 25 ºC at 7 days, as discussed above (Table 4 and 5).

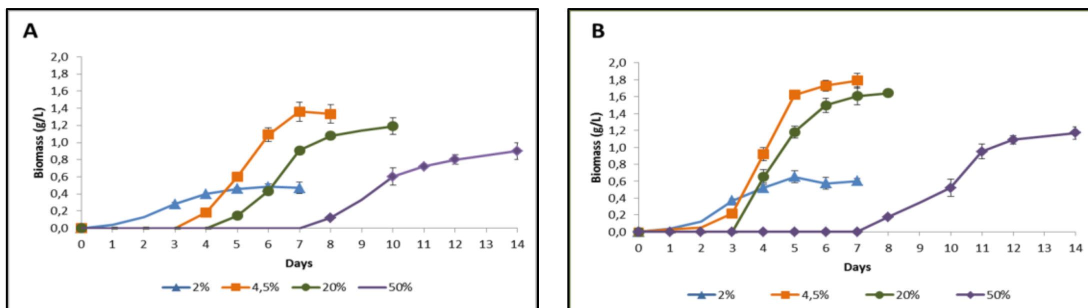

Figure 2. Cell growth of MT1A3 cultivated in medium with 2, 4.5, 20 and 50 % HC, incubated at 20 ºC (A) and 25 ºC (B).

Table 4. Biomass, pH and time to reach stationary phase of MT1A3 cultures in medium with 2, 4.5, 20 and 50 % HC, incubated at 20 ºC.

HC	Biomass (g/L)	pH	Time (days)
2 %	0.47 ± 0.07	7.46 ± 0.06	7
4.5 %	1.32 ± 0.11	8.02 ± 0.09	8

20 %	1.19 ± 0.10	7.86 ± 0.10	10
50 %	0.90 ± 0.07	7.66 ± 0.12	14

Table 5. Biomass, pH and time to reach stationary phase of MT1A3 cultures in medium with 2, 4.5, 20 and 50 % HC, incubated at 25 ºC.

HC	**Biomass (g/L)**	**pH**	**Time (days)**
2 %	0.60 ± 0.03	7.75 ± 0.10	7
4.5 %	1.79 ± 0.09	8.13 ± 0.06	7
20 %	1.64 ± 0.01	8.05 ± 0.08	8
50 %	1.17 ± 0.07	7.94 ± 0.11	14

When MT1A3 was cultivated in the three hydrocarbon fractions separately, a differential biomass growth was obtained, being 1.49 g/L in diesel culture medium, 0.69 g/L in gasoline and 0.15 g/L in kerosene after 10 days of incubation in comparison to HC mixture culture (1,76 g/L) . These studies indicate that MT1A3 has a greater capacity to use diesel as first substrate, then gasoline and kerosene.

By gas chromatography technique, the amount of total hydrocarbons (HT) present in all the assays was determined at 0, 5 and 10 days of incubation. HT was defined as the total hydrocarbon concentration expressed in ppm. In the case of MT1A3, culture grown in HC showed a HT degradation of 43.53 % obtained after 5 days; where the concentration decreased from 31023 ppm (HC control, 5 days) to 17928 ppm (MT1A3 in HC, 5 days) (Table 6). Likewise, the amount of HT degraded by MT1A3 action was maintained, between 5 and 10 days ($p > 0.05$).

Table 6. Concentration (ppm) of total hydrocarbons (TH) in controls and MT1A3 cultures, in medium with HC, diesel, kerosene or gasoline; at 0, 5 and 10 days of incubation.

	TH (ppm)				
	0	**5**		**10**	
	Control	**Control**	**MT1A3**	**Control**	**MT1A3**
HC	36582 ± 409	31023 ± 251	17928 ± 309	26051 ± 305	14702 ± 210

Diesel	12881 ± 223	10081 ± 154	7510 ± 103	8780 ± 301	4724 ± 166
Kerosene	11664 ± 352	10382 ± 232	9943 ± 214	9555 ± 195	6194 ± 233
Gasoline	11520 ± 275	6020 ± 201	4962 ± 239	5422 ± 122	3412 ± 169

When the level of degradation of each HC mixture component was analyzed, it was observed that the highest value was the one corresponding to the diesel culture, being 46.24% (Table 6). This result correlates with the values previously obtained in terms of biomass production (Figure 2). In this way, MT1A3 proved to be an indigenous strain with a high potential to be used in bioremediation processes in hydrocarbons contaminated sites. In accordance with the development of sustainable technologies, the growth of MT1A3 using different alternative carbon sources including low-cost agro-industrial co-products was studied. Carbon substrates such as HC mixture, glycerol and glucose (as easily assimilable sources of carbon), bovine milk whey, sheep milk whey, refined sunflower oil, high oleic sunflower oil, crude peanut oil, fried peanut oil, camelina oil, pressed peanut as low-cost agro-industrial co-products were used. MT1A3 grew in all carbon sources tested, but growth was significantly higher in crude peanut oil compared to all other substrates ($p < 0.05$), resulting in a biomass of 7.29 g/L (Table 7).

Table 7. Biomass and pH of MT1A3 cultures, at different carbon sources.

Carbon source	Biomass (g/L)	pH
HC (4.5%)	1.74 ± 0.11	8.12 ± 0.09
Glucose (2%)	4.01 ± 0.14	7.15 ± 0.05
Glycerol (2%)	2.69 ± 0.13	8.25 ± 0.12
Bovine milk serum (2%)	2.21 ± 0.09	6.86 ± 0.08
Ovine milk serum (2%)	1.94 ± 0.06	6.67 ± 0.09
Refined sunflower oil (2%)	3.98 ± 0.12	7.35 ± 0.13
High oleic sunflower oil (2%)	2.78 ± 0.15	7.49 ± 0.10
Crude peanut oil (2%)	7.29 ± 0.49	7.61 ± 0.11

Fried peanut oil (2%)	2.08 ± 0.11	7.27 ± 0.07
Camelina oil (2%)	1.23 ± 0.19	7.02 ± 0.05
Milled pressed peanut (2%)	4.42 ± 0.25	7.25 ± 0.13

Based on the biomass results obtained from the use of several carbon sources (Table 7), nitrogen sources were tested using crude peanut oil as the carbon substrate. The sources for nitrogen were: $NaNO_3$, NH_4Cl and urea. The most efficient nitrogen source for obtaining biomass from MT1A3 was $NaNO_3$, with a value of 7.14 g/L. The values for NH_4Cl and urea were significantly lower, being 1.71 and 0.62 g/L, respectively (Table 8). It is important to note that in the case of NH_4Cl, the culture had a drop in pH to 5.35, and it is likely that acidification of the medium produces inhibition of the growth of the strain, since ammonium chloride augments acidity by increasing the concentration of free H+. Therefore, the low biomass registered for NH_4Cl is probably influenced by changes in the chemical composition of the medium.

Table 8. Effect of nitrogen source on biomass and final pH in MT1A3 cultures at 5 days.

Nitrogen source	**Biomass (g/L)**	**pH**
$NaNO_3$	7.14 ± 0.35	8.15 ± 0.10
NH_4Cl	0.62 ± 0.12	5.36 ± 0.11
Urea	1.71 ± 0.18	7.08 ± 0.05

Five different bioremediation treatments in microcosms were carried out in order to evaluate the efficiency of hydrocarbon degradation in contaminated soils. We assessed the following treatments: (a) monitored natural attenuation (C); (b) biostimulation (B); (c) bioaugmentation (MT1A3); (d) bioaugmentation (Mix); (e) bioaugmentation + biostimulation (MT1A3 + B); (f) bioaugmentation + biostimulation (Mix + B) (Table 1). The average humidity on all trials was recorded at 31.67 ± 4.45. The values of pH presented a slight increase of the order from pH 7 reached up to 8, except treatment C that lowered up to pH 6, 88 during the 120 days. The effect of the different treatments on total heterotrophic aerobic bacterial counts (THAB) is shown in Figure 3. All treatments evidenced an early stimulation of the counts during the first 30 d. Likewise, in the MT1A3 + B system, the highest concentration of THAB was recorded, reaching the value of $2.17x10^{10}$ CFU at 120 days. After that, all systems tended to stabilize the THAB levels until the end of the study. The number of

hydrocarbon degrading bacteria (HDB) demonstrated a similar behavior as that of THAB at the end of the test (120 d). In comparison to treatment C, the highest HDB values were observed in MT1A3 + B, showing 8,91x10^{6} UFC at the end of the essay. The rest of the values in all treated systems showed no significant differences when compared to control system, revealing that they were not influenced by the bioremediation treatments.

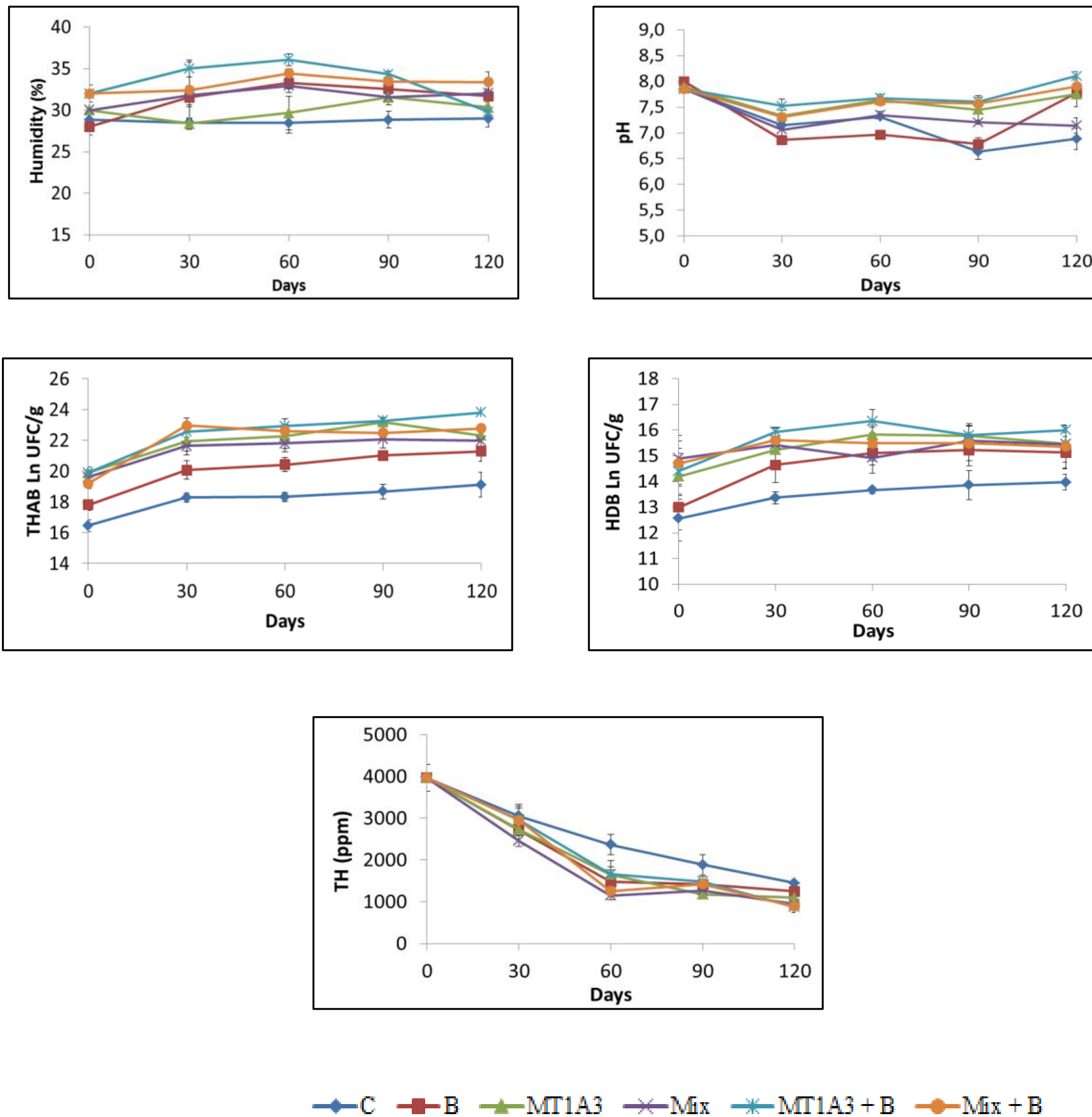

Figure 3: Influence of the different treatments on humidity, pH, the number of heterotrophic aerobic bacteria (THAB) and hydrocarbon-degrading bacteria (HDB), and hydrocarbon concentration during microcosms assay.

At the end of the test at 120 days, hydrocarbon degradation in assayed bioaugmented treatments showed a removal significantly higher than the C (natural attenuation) rate between 25.12 and

40.05%. Meanwhile, in treatment B (biostimulation), the potential for pollutant reduction was not influenced by stimulation with N and P sources added in the above mentioned concentrations with respect to C system. One possible reason for our results is the great energy requirement of the specific microflora, responsible for hydrocarbon degradation and also, if N and P are less available for microbial growth, it could be necessary to add higher amounts of these nutrients than the levels used. Despite of this, it may also occur that such results are not related with the activities of aerobic hydrocarbon utilizing microorganisms such as immobilization onto soil matrices, leaching or nitrification. Initially, a concentration of HT of 3960.24 ppm was registered in the soil. The percentages of hydrocarbon removal obtained in treatments C, B, MT1A3, Mix, MT1A3 + B and Mix + B, were 63.64; 68.52; 72.47; 76.35; 77.95; 77.53 %. Natural attenuation of soil allows the biodegradation of recalcitrant compounds by autochthonous microbial communities, which is commonly considered to be the primary mechanism for the natural removal of contaminants (Declercq et al., 2012). Considering the initial level of hydrocarbon measured in contaminated soil in this study, C system (natural attenuation) exhibited a reduction of 63.64 %, probably as a result of abiotic processes and as the result of microbiota biological activity of the soil already adapted. Taking into account that the studied soil contained a high initial level of contaminants and that it was chronically contaminated, a removed fraction, that is 77.95% of the initial level using bioaugmentation and biostimulation strategies, can be considered as promissory technology (Figure 4). Nevertheless, further research will be necessary to keep studying the adequate bioremediation process required by optimum strategies of biodegradation in hydrocarbon-contaminated soils.

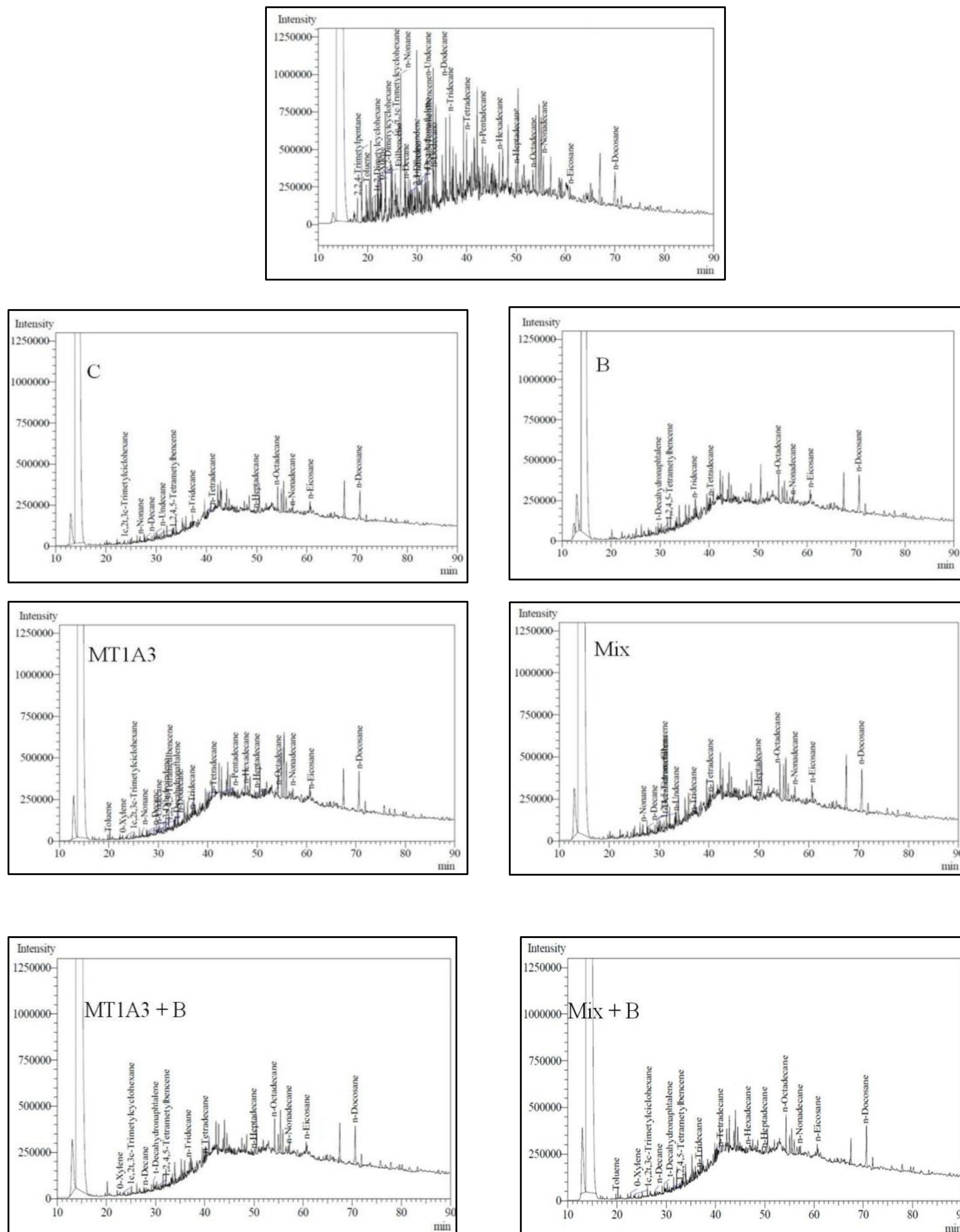

Figure 4. Chromatograms of microcosms: time zero and treatments after 120 days for control of natural attenuation (C), biostimulación (B), bioaugmentación with MT1A3 (MT1A3), bioaugmentation with mix (Mix), bioaugmentation with MT1A3 + biostimulation (MT1A3 + B), bioaugmentation with Mix + biostimulation (Mix + B).

4. CONCLUSION

The strains under study were identified phylogenetically in the genera *Pseudomonas, Cellulosimicrobium and Ochrobactrum*, whose genera present antecedents that relate them with the

metabolism of hydrocarbons, thus positioning them as potential microorganisms to be used in bioremediation strategies. The microcosm-based study presented in this paper has revealed a number of relevant facts. One of these facts is represented by the magnitude detected in bioaugmentation/biostimulation strategies applied when using the treatment MT1A3 + B, which reduced 77.95 % of the initial hydrocarbon concentration. This resulted in the application of autochthonous microorganisms used in this work. These microorganisms are promising to address as bioremediation strategies.

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