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Microbial characterization of a facultative residual sludge obtained from a biogas plant with ability to degrade commercial B10 diesel oil

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ABSTRACT

Biodegradation of commercial B10 diesel oil (DB10) by the microbial consortium present in a biogas residual sludge was first investigated. Maximum removal efficiencies of the petrochemical and fatty acids methyl ester (FAME) fractions of DB10 were 55 ± 3% and 94 ± 6%, respectively. Taxonomic profiling of the microbial consortium present in the residual sludge was carried out in order to identify potential hydrocarbon-degrading bacteria. After mild selection pressure using DB10, three *Pseudomonas* species (*P. aeruginosa*, *P. stutzeri* and *P. mendocina*) were mostly found in the treated sample. However, only *P. aeruginosa* and *P. stutzeri* showed a rapid growth and high dehydrogenase activity at the expense of DB10 as the sole carbon source. In addition, both strains removed 42 ± 5% and 53 ± 2% of the petrochemical fraction, and >75% of the FAME fraction, respectively. Similar results were found with a bacterial consortium of the three isolated *Pseudomonas*, indicating no synergistic hydrocarbon degradation by these strains. As expected, biosurfactant production by the pseudomonads was directly associated with the bacterial DB10 biodegradation performance. These results are the first describing petroleum-based hydrocarbon biodegradation ability of a crude facultative residual sludge obtained from a biogas facility; and represent a rational first step in order to understand which bacteria in the sludge may act in petroleum-based fuels degradation.

1. Introduction

Nowadays, despite the great advances made in the study and application of renewable energies, petroleum and its derivatives are still the main sources of energy used by society. Most transport infrastructure, personal mobility, and global trade, besides a significant proportion of heat and electricity requirements, are based on production and consumption of petroleum-derived liquid hydrocarbons (Thornley and Gilbert, 2013).

Diesel oil is one of the most globally commercialized petroleum-derived fuels often used in diesel engines. Regular diesel is a specific fractional distillate of crude oil mainly consisting of iso- and cycloparaffins and, to a lesser extent, olefinic compounds. In addition, it can contain up to 25% aromatic hydrocarbons (including naphthalenes and alkylbenzenes) (Wang and Shao, 2013). Since it is heavier than gasoline (composed mainly of C_5-C_{11} alkanes) the environmental persistence of diesel fuel is considerable. In fact, diesel oil-polluted sites are difficult to remediate because of its low rate of evaporation and fewer biodegradable characteristics compared to gasoline (Silva-Castro et al., 2015; Wang and Shao, 2013). In addition, it has been shown that diesel seriously affects health since many of its components have proven carcinogenic and mutagenic properties (Dai et al., 2016; Pedeli et al., 2011; Xu et al., 2013).

Petroleum-derived liquid hydrocarbons are also a key source of global greenhouse gas emissions. As substitute alternatives, the use of biofuels produced from renewable biomass can help to reduce greenhouse gas emissions in the long term (Thornley and Gilbert, 2013). In the last decades, biodiesel production from crops and other bio-resources has gained considerable environmental and economic impor-

Abbreviations: BE, biodegradation efficiency; BHB, Bushnell-Haas broth; CFU, colony forming unit; CSH, cell surface hydrophobicity; DB10, commercial diesel containing 10% biodiesel, EI: emulsification index; FAME, methyl esters of fatty acids; GC-FID, gas chromatography with flame ionization detector; OTU, operational taxonomic unit; TTC, 2,3,5-triphenyltetrazolium chloride.

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tance. Biodiesel consists of the alkyl esters, usually methyl esters, of the fatty acids (FAME) derived from a variety of fats and oils by a trans-esterification reaction. Advantages of biodiesel include domestic origin, reduced dependency on imported petroleum, high flash point, inherent lubricity, and biodegradability (Demirbaş, 2008). Biodiesel can be used as an alternative renewable fuel for transportation either in the neat form or, more typically, blended with varying proportions of conventional oil-derived diesel (Thomas et al., 2019). In Latin-American countries (Argentina, Brazil, Chile, Colombia, and Peru), Australia and the United States, levels of FAME in diesel fuel range from 5% to 20% considering that the local mandates that govern FAME use vary considerably from one country to another. In Argentina, since 2006, commercial diesel must contain 10% of biodiesel (namely DB10 in the present work) (Resolution N°1283/06 Art. 7°, Secretaría de Energía, Argentina).

Due to the high petroleum and biodiesel exploitation, refinement, use, and transport, every year tons of persistent hydrocarbons enter the environment. This causes a serious scenario of environmental contamination that can cause a substantial decrease in biodiversity, affecting not only regional economies, but especially the health of the population (Thornley and Gilbert, 2013). Conventional remediation processes of sites contaminated with hydrocarbons include *in situ* and *ex situ* technologies, being the *in situ* strategies of choice when intended to be applied in large-scale contaminated sites because of reliability and cost-effectiveness (Azubuike et al., 2016; Megharaj et al., 2011). The efficiency of *in situ* technologies for fuel hydrocarbons biodegradation can be promoted *via* indigenous bacteria stimulation by supplementing with additives such as nutrients or oxygen (*i.e., biostimulation*), and/or amending contaminated sites with a specific hydrocarbon-degrading bacterial consortium (*i.e.*, *bioaugmentation*) (Adams et al., 2015; Jasmine and Mukherji, 2014; Liu, 2014; Ros et al., 2010). In the last decades, efficient *in situ* bioremediation strategies using microbial consortia composed of selected microorganisms able to use hydrocarbons as carbon and energy source have been studied. In general, such consortia are based on gram-negative bacteria belonging to *Bacillus* spp., *Acinetobacter* spp., *Pseudomonas* spp., *Alkanivorax* spp. and related genera, which are well-known for their ability to metabolize petroleum hydrocarbons (Azubuike et al., 2016; Megharaj et al., 2011; Mnif et al., 2011). Application of such consortia has been also recognized as a powerful eco-friendly alternative to conventional methods for resolving environmental problems (Joutey et al., 2013). However, the susceptibility of hydrocarbons to microbial degradation varies according to their chemical structure, solubility, and bioavailability (Ciric et al., 2010). In addition, the ability of microbial consortia to degrade hydrocarbons depends on several factors, including microorganisms adaptation between them and the environment, substrate concentration and toxicity, and the production of bacterial hydrocarbon-degrading enzymes, bioemulsifiers or biosurfactants, among others (Megharaj et al., 2011; Sumarsih et al., 2017). Then, all these characteristics should be considered in order to improve the remediation performance of hydrocarbon-polluted environments by bioaugmentation with a heterogeneous consortium of bacteria.

Although bioaugmentation is a commonly used method to remediate hydrocarbon-polluted soils, the application of compost and/or residual sludge as sources of organic materials and microorganisms to clean up diesel and lubricant oil has not been thoroughly discussed. Since residual sludge can contain significant amounts of carbon sources and nutrients, its use as additive to enhance contaminant removal efficiency has been investigated. Moreover, residual sludge contains a high and diverse microbial population, so its application on contaminant biodegradation protocols can be expected (Li et al., 2015; Megharaj et al., 2011). In the present study, residual sludge obtained from the biogas facilities of a local company treating non-hazardous organic industrial wastes was assessed in its ability to degrade commercial diesel fuel. In addition, a bioprospecting assay was carried out to investigate the presence of DB10-degrading bacteria in the residual sludge, in order to evaluate the utilization of this by-product for DB10 bioremediation as a cost-effective bioaugmentation strategy.

2. Materials and methods

2.1. Collection of residual sludge samples

Residual sludge from an 8 years-operating biogas plant was kindly provided by a local company (SOLAMB S.R.L, Timbúes, Santa Fe, Argentina). Different batch samples of residual sludge were collected in 1 L glass brown colour bottles and transported under refrigeration to the laboratory within 24 h to determine pH (7.4 ± 0.1), conductivity (13.4 ± 2.2 mS/cm), chemical oxygen demand (COD, 1300 ± 350 mg/L), total solids (29,800 ± 9100 mg/L) and volatile solids (13,100 ± 5200 mg/L), (SM2540B, SM2540E). For microbiological analyses, samples were preserved at 4 °C until use. Heterotrophic microbial load of the residual sludge was estimated by plate-count method on PCA (Britania S.A., Buenos Aires, Argentina) nutritive agar (5.5 ± 0.6 log CFU/mL). Biogas composition (7.1 ± 0.4% O_2, 9.1 ± 0.5% CO_2, 71.1 ± 0.5% CH_4, 162 ± 15 mg/L H_2S) from the sludge samples was measured using an Optima 7 multigas analyzer (MRU Instruments, Germany).

2.2. DNA extraction, sequencing and taxonomic assignment

Genomic microbial DNA was extracted from the residual sludge samples using PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc.), following the manufacturer's instructions. The amount of DNA was quantified using Quant-iT™ PicoGreen® dsDNA assay kit (Invitrogen Corporation, Carlsbad, CA, United States). 16S rRNA gene library was made based on the procedure described in Illumina Guide for Sample Preparation. Then, the library was quantified using Quant-iTTM PicoGreenR dsDNA assay kit (Invitrogen Corporation, Carlsbad, CA, United States) and finally sequenced on Illumina MiSeq platform (Illumina, Inc.). Sequence data pre-processing, classification, and taxonomic assignment was done as described by Piazza et al., 2019). Sequences were clustered to operational taxonomic units (OTUs) at 97% similarity level with an open reference strategy implemented in QIIME. All the analysis was performed at the *Instituto de Agrobiotecnología Rosario* (https://www.indear.com/).

2.3. Isolation and identification of commercial B10 diesel oil-degrading bacteria

Commercial diesel containing 10% biodiesel (DB10) was purchased from a local service station. For sample enrichment in potentially DB10-degrading bacteria, 1 mL of residual sludge was incubated with 30 mL of Bushnell-Haas broth (BHB) amended with 1% (v/v) filter-sterilized DB10 in flask bottles of 125 mL, and incubated with agitation (130 rpm) at 37 °C for 15 days in a SHZ-88 shaking water bath (Arcano, Shanghai, China). Composition of BHB was (g/L): $MgSO_4$, 0.2; $CaCl_2$, 0.02; KH_2PO_4, 1.0; K_2HPO_4, 1.0; NH_4NO_3, 1.0; $FeSO_4$, 0.05; pH 7.0. After incubation, 10 μL of serial dilutions (10^{-1} to 10^{-4}) of the enriched sample were plated on a nutrient agar (PCA, Britania S.A., Buenos Aires, Argentina) and incubated overnight at 37 °C. A pre-selection of the most abundant microorganisms was performed by morphologic and phenotypic analysis of the colonies. Then, the selected bacteria were pure-cultured on Bushnell-Haas agar amended with 1% (v/v) filter-sterilized DB10 as the only carbon source, and plates were incubated for 8 days at 37 °C. Those strains that were able to grow after this last incubation process were used for DB10 biodegradation experiments. All the isolated strains were identified using the RapID NF Plus system (Remel, Lenexa, KS, USA), following the manufacturer's instruc-

tions. Additionally, it was performed a Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) at the *Centro de Especialidades Médicas y Ambulatorias de Rosario* (CEMAR, Rosario, Santa Fe, Argentina) to confirm/resolve bacteria classification when the RapID NF Plus System results were not conclusive (<95% identity). The bacterial mass spectra were analysed using the BioTyper software (version 3.1.66; Bruker, Daltonics, Bremen, Germany) and the MBT DB-5627 database provided by the manufacturer with identification scores to the species level of ≥2.0.

2.4. Microbial growth

To test microbial growth of the potentially DB10-degrading bacteria isolated from the residual sludge, 100 μL of a bacterial suspension adjusted to McFarland 0.5 standard (approx. 1.5×10^8 CFU/mL) in saline solution were inoculated in 125 mL sterilized glass flask containing 50 mL of BHB (pH 7.0) and 1% (v/v) filter-sterilized DB10 as solely carbon source. The flasks were incubated at 37 °C with agitation (130 rpm) for 15 days in a SHZ-88 shaking water bath (Arcano, Shanghai, China). Cell density at 600 nm ($O.D._{600nm}$) was measured periodically using a UV–Vis Lambda 25 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). The experiments were performed in triplicate ($n = 3$).

2.5. Dehydrogenase activity

Conversion of 2,3,5-triphenyltetrazolium chloride (TTC) to red insoluble 1,3,5-triphenylformazan by microbial dehydrogenases was used as a quali-quantitative method for detection of metabolically active cells in broth media (Derrossi Meyer et al., 2014). Briefly, sterilized glass flasks containing 50 mL BHB (pH 7.0) amended with 1% (v/v) filter-sterilized DB10 and 500 μL TTC (50 mg/mL) were separately inoculated with 1 mL of residual sludge (approx. 5.5×10^5 CFU/mL) or 100 μL pre-selected bacteria suspension adjusted to McFarland 0.5 standard in saline solution (approx. 1.5×10^8 CFU/mL), and then incubated for 15 days (37 °C, 130 rpm) in a shaking bath (SHZ-88, Arcano, Shanghai, China). The ability of the microorganisms to grow at the expense of DB10 was determined qualitatively by the observation of a red colour, either in the solution or in the DB10/broth interface, as a surrogate marker of viable diesel-attached bacteria forming a biofilm (Brown et al., 2013). Additionally, at the end of assay (day 15), the product of hydrolytic reaction (*i.e.*, triphenylformazan) was extracted in 5 mL of 80% acetone. After centrifugation (3000 *g*, 10 min), formazan was determined by measuring the absorbance at 485 nm as described by Kumari et al. (2016).

2.6. Indirect evaluation of bacterial biosurfactant production

One hundred microliters (100 μL) of a bacterial suspension adjusted to McFarland 0.5 standard in saline solution (approx. 1.5×10^8 CFU/mL) for each isolated bacterium were inoculated in 50 mL BHB (pH 7.0) amended with 1% (v/v) filter sterilized DB10 as solely carbon source. Then, the flasks (125 mL) were put on a shaker incubator (SHZ-88, Arcano, Shanghai, China) for 15 days (37 °C, 130 rpm). After incubation, samples were centrifuged at 7000 *g* for 20 min and separated into cells and cells-free supernatant (CFS). Finally, bacteria capacity to produce biosurfactant was studied based on two methods: emulsification index (EI) and cell surface hydrophobicity (CSH).

2.6.1. Emulsification index (EI)

The EI was assayed by adding red kerosene to the CFS in 1:1 ratio (v/v). The solution was vortexed at high speed for 3 min and incubated for 24 h (EI_{24}%) at room temperature. After incubation, EI_{24}% (Eq. (1)) was calculated through the height of the emulsion layer according to (Shahaliyan et al., 2015):

$$EI_{24}\% = 100 * \left(\frac{\text{total height of the emulsified layer}}{\text{total height of the liquid layer}} \right) \quad (1)$$

All experiments were performed in triplicate ($n = 3$).

2.6.2. Cell surface hydrophobicity (CSH)

Bacterial adherence to DB10 was used to quantify CSH. Harvested washed cells were suspended in 3 mL of sterilized saline solution and adjusted to an initial optical density of 0.5 at 600 nm ($O.D._{600nm}$). Cell suspension and filter-sterilized DB10 (400 μL) were mixed in a screw-top test tube and vortexed at high speed for 3 min. After mixing, the DB10 and aqueous phases were left to separate at room temperature. The aqueous phase was carefully removed and the $O.D._{600nm}$ was measured again. Cellular hydrophobicity in terms of bacterial adherence was quantified using the following equation:

$$\text{Bacteria adherence } (\%) = 100 * \left(1 - \frac{\text{O.D.of vortexed suspension}}{\text{initial O.D.of cell suspension (without shaking)}} \right) \quad (2)$$

Bacterial adherence values over 75% were considered as significant biosurfactant production by bacteria. The experiments were performed in triplicate ($n = 3$).

2.7. Biodegradation experiments

DB10 biodegradation experiments were carried out in 125 mL glass bottles (bioreactors) containing 50 mL of BHB (pH 7.0) amended with 1% (v/v) filter sterilized DB10 as the sole carbon source. One millilitre (1 mL) of the residual sludge (approx. 10^5 CFU/mL) or 1 mL of a suspension (approx. 10^8 CFU/mL) of potentially DB10-degrading bacteria (either each bacterium alone or forming a consortium) were used as test inoculum in independent experiments. The flasks were then incubated for 15 days at 37 °C at 130 rpm in a shaking water bath (SHZ-88, Arcano, Shanghai, China). After incubation, 5 mL of *n*-hexane were added to each bioreactor in order to extract the remaining DB10, agitated vigorously for 1 min, and centrifuged for 10 min (Tyfon II, Zelian Instruments, Buenos Aires, Argentina). Then, the supernatant was collected and filtered through a PTFE filter (pore size = 0.45 μm) to analyse the residual DB10 by gas chromatography. Un-inoculated reactors containing DB10 were used as control to monitor abiotic losses of DB10. The experiments were performed in triplicate ($n = 3$).

2.8. Gas chromatography analysis of commercial diesel (DB10)

Gas chromatography analyses were carried out on a SCION 436 GC equipped with a flame ionization detector (GC-FID) and a 30-m fused silica capillary column (Rtx-5, Restek Co., Bellefonte, PA, USA). The injector and detector temperatures were set at 275 °C. The column temperature was initially set at 90 °C, then raised to 300 °C at 7 °C/min, and finally set at 300 °C for 1 min. Samples were injected (1 μL) in the split mode (split ratio, 1:20). Nitrogen (N_2) was used as carrier gas at 1 mL/min. The biodegradation efficiencies (BE) of the petrochemical and the FAME fractions of DB10 were calculated using the expression (Eq. (3)) described by Michaud et al. (2004):

$$\text{BE } (\%) = 100 - \frac{A_s * 100}{A_{ac}} \quad (3)$$

where A_s is the total area of the selected peaks (*i.e.*, C_{11}-C_{22} or FAME compounds) in each sample after the incubation and A_{ac} is the total

area of the selected peaks in the appropriate abiotic control after the same incubation time.

2.9. Statistical analysis

All experiments were conducted using three independent replicates ($n = 3$). Statistical analyses were performed using the SigmaStat 3.5 software (Systat Software Inc., San Jose, CA, USA) *via* analysis of variance (ANOVA). The difference of means between groups was resolved using the Tukey's *post hoc* test at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Commercial B10 diesel (DB10) degradation by the residual sludge

Fig. 1A shows a GC-FID chromatogram of a control system consisting of DB10-contaminated (1% v/v) minimum salt medium (BHB) after 15 days of incubation (37 °C, 130 rpm), without any treatment. As expected, a characteristic chromatogram of diesel oil can be seen, consisting principally of n-alkanes (C_{11}-C_{22}) as major constituents. In particular, four distinctive peaks can also be observed (namely J, L, M and N) that correspond to the biodiesel fraction of the commercial B10 diesel fuel utilised in the present work (Fig. 1C). This fraction consists of FAME compounds, mostly produced from soy bean oil (Prince et al., 2008). Table 1 shows the identification and quantification of some of the major peaks that appear in Fig. 1A, assigned by comparing the chromatograms obtained for pure alkanes standard solutions.

On the other hand, Fig. 1B shows a chromatogram obtained from a DB10-contaminated (1% v/v) mineral salt medium (BHB) inoculated with the residual sludge obtained from a biogas facility, after 15 days of incubation (37 °C, 130 rpm) under the same conditions as the control system (Fig. 1A). As it can be seen, the presence of the residual sludge caused a significant decrease in the areas of all the principal components (hydrocarbons and FAME compounds) identified in the DB10 (Fig. 1B). Maximum removal efficiencies calculated for C_{11}-C_{22} hydrocarbons and FAME compounds were 55 ± 3% and 94 ± 6%, respectively. These results suggest that the microbial consortium present in the sludge sample could be involved in DB10 fuel degradation. Many microorganisms are able to degrade hydrocarbons under oxic (Das and Chandran, 2011) or anoxic conditions (Rabus et al., 2016). Under anoxic conditions, microbes respire electron acceptors other than molecular oxygen, including nitrate, iron, and sulphate, or thrive in syntrophic methanogenic consortia. In the latter case, hydrocarbons are degraded by fermenting bacteria to intermediate products (*e.g.*, hydrogen, acetate), which are subsequently converted to methane by methanogens (Gieg et al., 2014). The dynamics of aerobic and anaerobic hydrocarbon degraders have been studied mainly in short-term oil contamination of marine open waters, sediments or coastal environments (Michas et al., 2017). However, knowledge about metabolization processes undergone by DB10 fuel during exposure to a biogas residual sludge microbiome is very scarce.

3.2. Identification of potentially DB10-degrading bacteria from residual sludge

In order to evaluate the presence of potentially DB10-degrading bacteria in the crude sludge sample, a characterization of the microbial community was performed by the 16S rRNA gene-based amplicon sequencing and analysis. Sequence analysis of OTUs indicated that the dominant phyla in the residual sludge was *Firmicutes*, followed by *Synergistetes* and *Proteobacteria* (with a majority of *Gammaproteobacteria*, *Deltaproteobacteria,* and *Epsilonproteobacteria* subclasses) as the second most abundant phyla (Fig. 2A). Consistent with these results, an important role of *Gammaproteobacteria* for hydrocarbons removal had been demonstrated (Kostka et al., 2011; Liu and Liu, 2013). Also, *Bacteroidetes*, *Chloroflexi*, *Spirochaetes*, *Tenericutes,* and *Thermotogae* were found (Fig. 2A). Interestingly, 4.5% of the OTUs were assigned to a bacterial group called WWE1 (Wastewater of Evry). This particular uncultured group of bacteria was discovered by molecular inventories of the anaerobic mesophilic (35 ± 0.5 °C) digester of Evry (Chouari et al., 2005) and it was often observed in anaerobic ecosystems (Limam et al., 2014; Sun et al., 2015). However, the *in situ* ecophysiology of the WWE1 group in complex microbial ecosystems is poorly documented.

Fig. 2B shows the relative abundance (%) of the most representative orders present in the sludge sample. *Deltaproteobacteria* (*e.g.*, *Desulfobacterales*, *Desulfovibrionales,* and *Desulfomonadales*) were less abundant, while *Clostridiales*, *Bacteroidales*, and *Synergistales* showed a higher one. As expected, due to its origin (a biogas facility), all archaeal reads

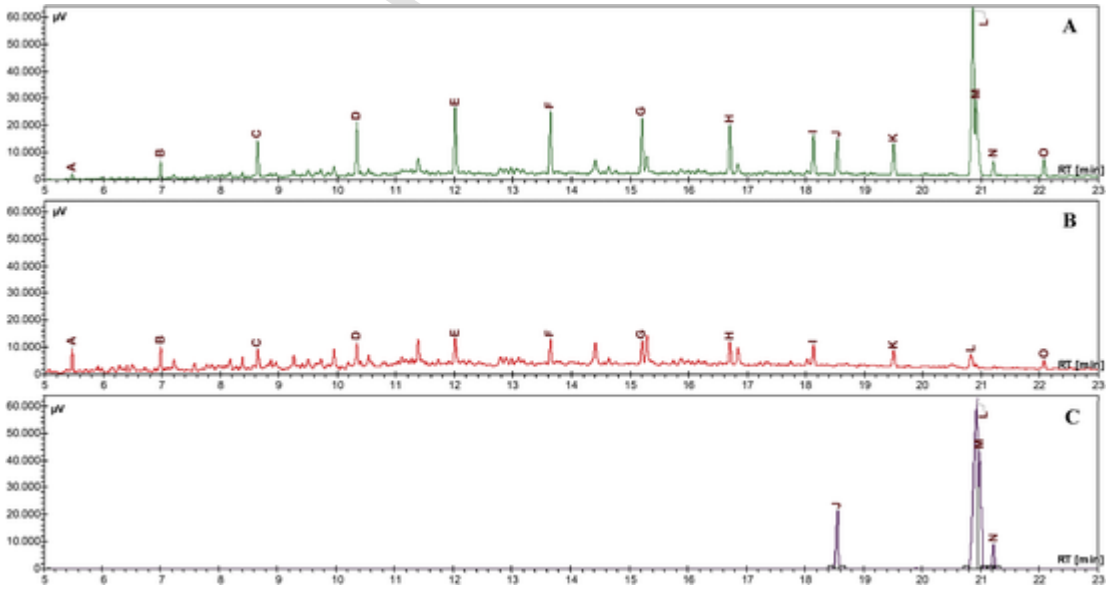


Fig. 1. GC-FID chromatograms of residual diesel hydrocarbons extracted with hexane from Bushnell-Haas broth (BHB) with 1% (*v*/v) B10 diesel after 15 days of incubation (37 °C, 130 rpm). **(A)** Abiotic B10 diesel degradation control (*i.e.*, without bacterial inoculation) and, **(B)** inoculated with biogas residual sludge. Fifteen major components named A-N were observed in the commercial diesel used at the present study. **(C)** GC-FID profile of 1% (v/v) pure biodiesel in hexane.

Table 1
Identification and quantification of diesel oil hydrocarbons and FAME compounds in the commercial fuel used at the present study.

Commercial B10 diesel major components	
Petrochemical fraction	
A	*n*-undecane (3.2)
B	*n*-dodecane (3.5)
C	*n*-tridecane (3.6)
D	*n*-tetradecane (4.4)
E	*n*-pentadecane (11.2)
F	*n*-hexadecane (4.3)
G	*n*-heptadecane (4.9)
H	*n*-octadecane (3.4)
I	*n*-nonadecane (2.8)
K	*not identified*
O	*not identified*
Biodiesel fraction	
J	Methyl palmitate (3.1)
L	*not identified*
M	Methyl oleate (5.2)
N	*not identified*

Note: concentration (% v/v) of major identified components in DB10 are informed between parentheses.

obtained from the residual sludge were assigned to the phylum *Euryarchaeota* (mainly *Methanomicrobiales* and *Methanosarcinales*), which includes methanogens (Fig. 2). Noticeably, our findings revealed some similarities between the microbial communities found in other ecosystems with a long history of oil contamination. For example, the orders *Thermotogales*, *Clostridiales*, *Desulfobacterales*, *Desulfuromonadales*, and *Syntrophobacterales* were identified in sediment microbiomes with the potential for syntrophic degradation of hydrocarbons linked to methanogenesis (Michas et al., 2017). In addition, 3.5% of the OTUs were assigned to the order *Pseudomonadales*. This finding is of particular interest for the aim of the present work since it is well-known that some facultative bacteria clustered into this order, such as *Acinetobacter* spp. and *Pseudomonas* spp., are very versatile in the biodegradation of several pollutants including petroleum hydrocarbons (Jasmine and Mukherji, 2014; Liu, 2014; Mnif et al., 2011; Parthipan et al., 2017).

3.3. Isolation of potentially DB10-degrading strains from residual sludge

Sample enrichment in DB10-tolerant bacteria was performed by the inoculation of the residual sludge in a minimal salt medium (BHB) with 1% (v/v) DB10 as the only added carbon source. After 15 days of incubation, a total of 15 colonies with different morphologies and colours were chosen as potential DB10-degrading microorganisms. Further, the isolated bacteria were identified using biochemical tests (RapID NF Plus System) and MALDI-TOF MS. Using these approaches, seven strains were positive identified as *Pseudomonas stutzeri*, two as *Pseudomonas aeruginosa*, and one as *Pseudomonas mendocina*. On the other hand, identification of the 5 remaining isolated strains by both methods was ambiguous or not strongly conclusive (identity $< 90\%$).

Bacteria belonging to the *Pseudomonas* genera are facultative anaerobes, and it has been reported that these microorganisms are involved in the hydrolysis and degradation of organic material in anaerobic digesters (Gerardi, 2003). In addition, *Pseudomonas* have been also found in anaerobic sediments (Sánchez-Andrea et al., 2011), activated sludges (Jilani and Khan, 2006), and anoxic zones of anaerobic-anoxic-aerobic reactors (Atkinson et al., 2001). Moreover, *Pseudomonas* is highly recognized as an environment-pollutant degrader, being largely employed in bioaugmentation protocols for hydrocarbons biodegradation both alone or as part of mixed consortia (Das and Chandran, 2011; Mnif et al., 2011; Obayori et al., 2009; Wasi et al., 2013).

3.4. Screening of selected bacteria for DB10 utilization

In order to advance in the knowledge about the possible microorganisms involved in DB10 biodegradation by the residual sludge, the three isolated *Pseudomonas* species were evaluated in their individual capacities to grow at the expense of DB10 as the sole carbon and energy source. To this aim, kinetics of growth of *P. stutzeri*, *P. aeruginosa,* and *P. mendocina* on mineral salt medium BHB with DB10 (1% v/v) as carbon source was followed by measuring the evolution of the optical density at 600 nm ($O.D._{600nm}$) for 15 days at 37 °C. Microbial growth at the expense of DB10 was verified for the three *Pseudomonas* by demonstrating an increase in the $O.D._{600nm}$ during the tested period, suggesting that these microorganisms are potential DB10 biodegraders (Fig. 3). Noticeably, the growth profile for *P. aeruginosa* and *P. stutzeri* did not display a substantial lag phase. Also, both strains showed a similar growth pattern in which the stationary phase was rapidly reached at the second incubation day. This behaviour could be owed to a better assimilation of DB10 as a unique source of carbon and energy (Mnif et al., 2015).

On the other hand, the growth kinetics for *P. mendocina* showed a lag phase of 4 days and, from there, a slight progressive growth until the end of the assay. However, until day 15, the stationary phase was not reached, suggesting that nutrient deprivation or detrimental environmental changes that can affect their survival (such as the build-up of toxic wastes) did not occur.

Degradative enzyme activity and microbial count are common indices to describe the microbial activities during pollutants bioremediation (Kumari et al., 2016). The reduction of tetrazolium salts, such as triphenyltetrazolium chloride (TTC), to red-coloured formazan is a widely accepted method to assess microbial growth as an indicator for dehydrogenase activity (Alef, 1995; Mosher et al., 2003). So, quali-quantitative determination of dehydrogenase activity as a surrogate marker of bacterial hydrocarbon respiration was assessed since bacterial dehydrogenase may be involved in hydrocarbon degradation processes (Borowik et al., 2017; Kaczyńska et al., 2015; Lipińska et al., 2014; Zhang et al., 2010). In Fig. 4, it can be seen a considerable dehydrogenase activity for *P. aeruginosa* (Fig. 4B) and *P. stutzeri* (Fig. 4C) after 15 days of incubation in BHB with 1% (v/v) DB10 as solely carbon source. Noticeably, for both strains, a distinctive number of red metabolically active cells adhered to the superficial organic phase could be appreciated. A similar observation was perceived for the residual sludge sample (Fig. 4E). Conversely, no red-coloured cells were observed for *P. mendocina* (Fig. 4D). However, a significant turbidity could be appreciated compared to the control system (Fig. 4A) after 15 days of incubation, consistent with the increment in the $O.D._{600nm}$ values showed in Fig. 3 for this strain. The lack of red-coloured cells could be due to inability of *P. mendocina* to use TTC as electron acceptor since microorganisms have distinct dehydrogenase systems with different capacity to reduce TTC (Praveen-Kumar and Tarafdar, 2003). Moreover, degradative enzyme induction during the degradation of fuels depends on the fuel oil composition and their metabolic products (Kumari et al., 2016). In addition, as TTC is a metabolic dye, growth conditions should be optimal when using the TTC stain. In such sense, suboptimal growth or stressful conditions, such as nutrient limitation, could led to inefficient TTC reduction by *P. mendocina* (Brown et al., 2013; Ghaly and Mahmoud, 2006). Taking together, the different growth profiles and degradative enzyme activity observed for the analysed microorganisms demonstrate different ability of *Pseudomonas* strains to adapt and use DB10 as the solely carbon and energy source.

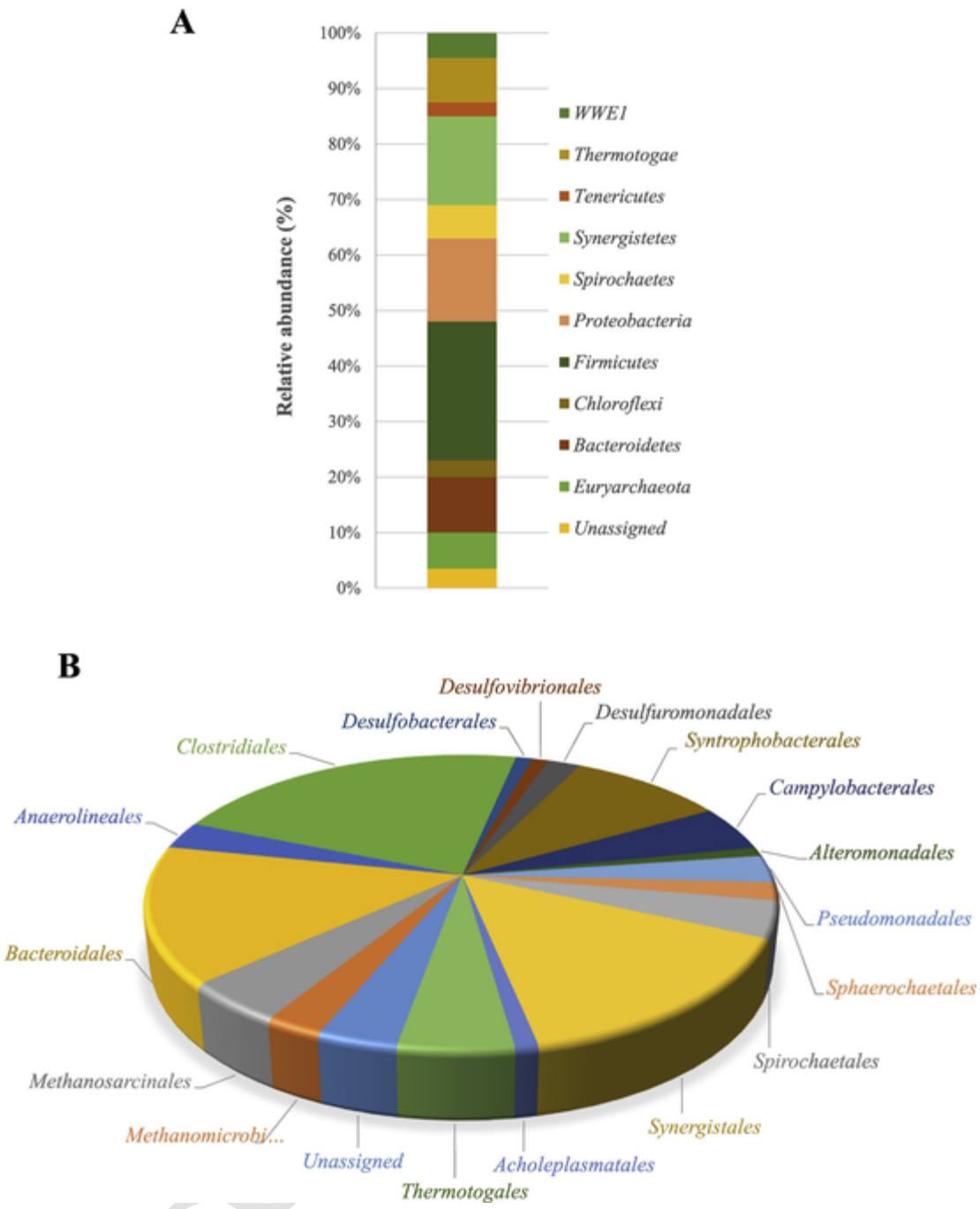


Fig. 2. Taxonomic composition of bacteria from residual sludge sample. Relative taxa abundance (%) is represented with different colours at the **(A)** phyla and **(B)** order level.

Noticeably, the highest dehydrogenase activity after 15 days of incubation was quantified for the residual sludge (Fig. 4F). This result supports the view that consortia with broad enzymatic capacities are more effective in the degradation of contaminants in any environment (Kumari et al., 2016). At this point, it is important to mention that dehydrogenase activity was only detected when the residual sludge was inoculated with the commercial diesel. Moreover, control systems without additional added organic material or prepared with the inoculation of heat-inactivated sludge sample did not showed measurable dehydrogenase activity (data not shown).

3.5. Screening of selected bacteria for biosurfactant production

Growth of certain microorganisms on hydrophobic substrates can be limited by numerous factors, most vital of which are substrate chemical structure, environmental conditions, and low solubility of organic compounds (Mnif et al., 2015). It is well known that fuel hydrocarbons are slightly soluble in aqueous systems. Therefore, for bacteria to potentially use them as carbon source, hydrocarbons must be bioavailable in the growing media. Certain hydrocarbon-degrading bacteria can synthesize natural emulsifier agents called biosurfactants. These compounds are totally or partially extracellular amphipathic molecules which facilitate hydrocarbons uptake by bacteria (Preethy and Das,

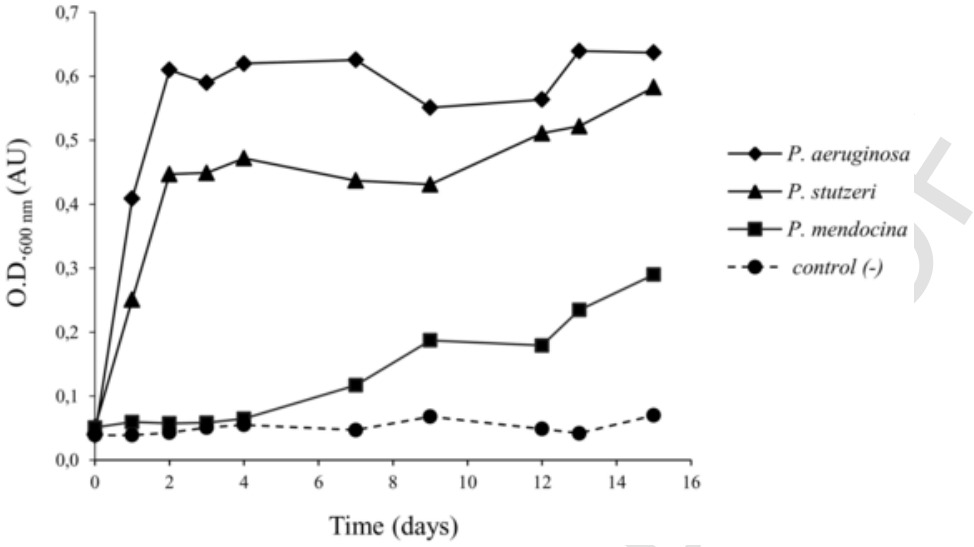


Fig. 3. Growth profiles of the selected bacteria in Bushnell-Haas broth (BHB) with 1% (v/v) B10 diesel as the only carbon source. *P. aeruginosa* (♦); *P. stutzeri* (▲); *P. mendocina* (■); *negative control* (●).

2010). In such sense, the emulsification index (EI_{24}%) is considered a reliable method to assay the capability of microorganisms to produce biosurfactants (Mendes Lopes et al., 2014; Shahaliyan et al., 2015).

In our study, among the tested strains, maximum EI_{24}% (48.8 ± 1.9) was obtained for *P. stutzeri,* while the lowest EI_{24}% (15.0 ± 3.5) was found for *P. mendocina* (Table 2). In addition, the hydrophobic nature of bacterial surface plays a significant role in the attachment of microorganisms to hydrophobic substrates, being a growth limiting factor (Zhang and Miller, 1994; Zhao et al., 2011). Between the isolated pseudomonads, only *P. aeruginosa* and *P. stutzeri* showed a significant cellular surface hydrophobicity (CSH > 80%) (Table 2). These results could also explain the observed adherence of red-coloured active cells to the DB10 phase, notably perceived for *P. aeruginosa* and *P. stutzeri* in Fig. 4 (B and C, respectively). Moreover, the presence of biosurfactants can also enhance the cellular hydrophobicity due to modification of the cell surface of *gram*-negative bacteria by the removal of lipopolysaccharides and the exposure of hydrophobic tail of biosurfactant to hydrophobic compounds, such as fuel hydrocarbons (Ali Khan et al., 2017).

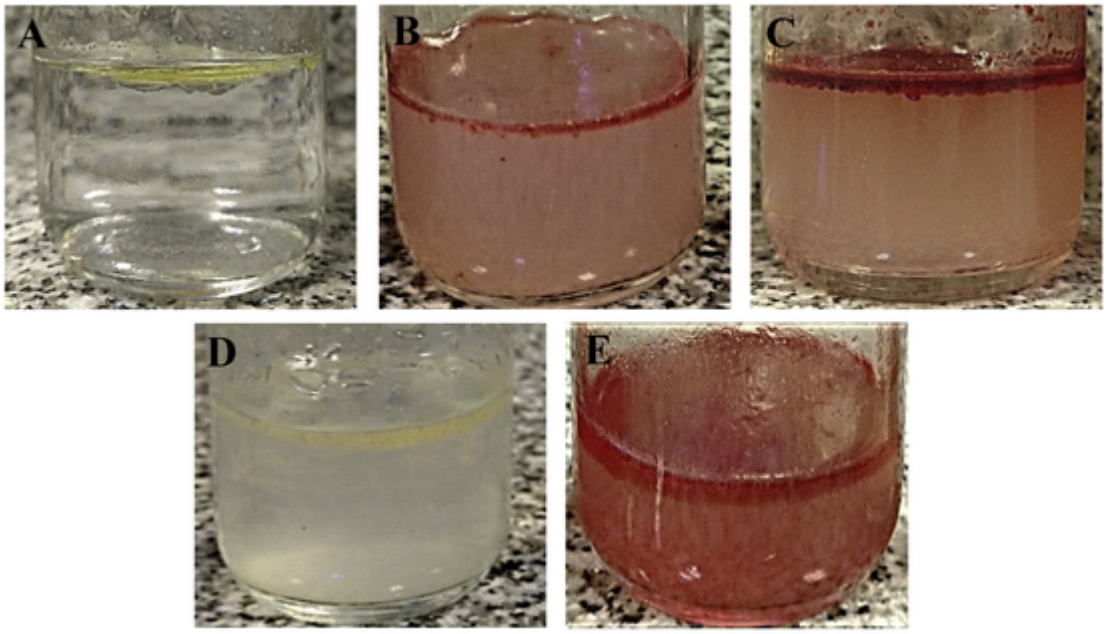
On the other hand, *P. mendocina* presented a minor ability to produce biosurfactant, and this fact correlates with its lowest capacity to emulsify DB10 (Table 2). Therefore, these results are in agreement with the lag phase of 4 days observed for *P. mendocina* (see Fig. 3), as well as with the unnoticed dehydrogenase activity (Fig. 4D and 4F).

Many authors have reported extracellular excretion of biosurfactant (mainly rhamnolipid in nature) by bacteria belonging to the *Pseudomonas* genera (Mnif et al., 2015). In such sense, the use of microbial consortia containing bacteria with the ability to produce biosurfactants would allow the development of improved bioremediation *in situ* strategies for the recovery of polluted environments (Mnif et al., 2011; Reddy et al., 2010). In addition, biosurfactants have advantages over chemical surfactants such as high biodegradability, low toxicity, ecological acceptability, selectivity and specific activity at extreme temperatures, pH and salinity (Preethy and Das, 2010). Thus, understanding the microbial processes involved in hydrophobic pollutants uptake and metabolization is crucial when intended to apply biotechnological techniques for petroleum-based fuel bioremediation.

3.6. Biodegradation experiments

In order to confirm the biodegradation of DB10 by the selected *Pseudomonas* isolated from the biogas residual sludge, a GC-FID analysis was performed. As we have previously described (see Fig. 1), the commercial B10 diesel used in the present work contains a biodiesel fraction composed of FAME that may be easier to degrade by the selected bacteria than the petrochemical fraction (C_{11}-C_{22} hydrocarbons). Therefore, the biodegradation efficiency (BE) for the isolated strains (alone or in a consortium), as well as the analysed residual sludge, were separately calculated for the petrochemical and FAME fractions (Fig. 5). As stated previously (Section 3.1), the presence of the residual sludge obtained from a biogas facility showed a significant decrease in the areas of all the principal components identified in the DB10 (Fig. 1B). This is consistent with the BE (%) for both fractions of DB10 shown in Fig. 5 for the residual sludge. Regarding this, the two most efficient strains for DB10 degradation were *P. stutzeri* and *P. aeruginosa*, which showed a BE of 53 ± 2% and 42 ± 5% for the petrochemical fraction, and >75% for the FAME compounds respectively. The significant BE (%) of C_{11}-C_{22} hydrocarbons in the DB10 reached by *P. stutzeri* and *P. aeruginosa* could be related with the fact that both strains showed a significant biosurfactant-producing ability under the tested conditions (Table 2) that enhanced hydrocarbons degradation. Conversely, *P. mendocina* showed <10% biodegradation performance for both fractions (Fig. 5). These results indicate that *P. mendocina* may be less efficient than *P. aeruginosa* and *P. stutzeri* in DB10 degradation under the assayed conditions. Since bacterial degradation of petroleum-based fuels is limited by the low solubility and bioavailability of the target compounds, the above-mentioned results are consistent with the lowest EI_{24}% and CSH found for *P. mendocina* (Table 2). In addition, the obtained results are in agreement with the low growth rate and dehydrogenase activity determined for *P. mendocina* (Fig. 3 and Fig. 4, respectively).

It has been reported that microbial consortia offer a combination of individual microorganisms metabolic capabilities which may promote the biodegradation of complex hydrocarbon mixtures. This enhancement could be achieved by several ways such as, different biodegradation pathways of each individual bacterium, metabolic intermediates of one bacterium that may act as the starting material of another, different genetic makeup, or by synthesising different variants of the same catalytic enzymes (Obayori et al., 2009; Kumari et al., 2018). Re-



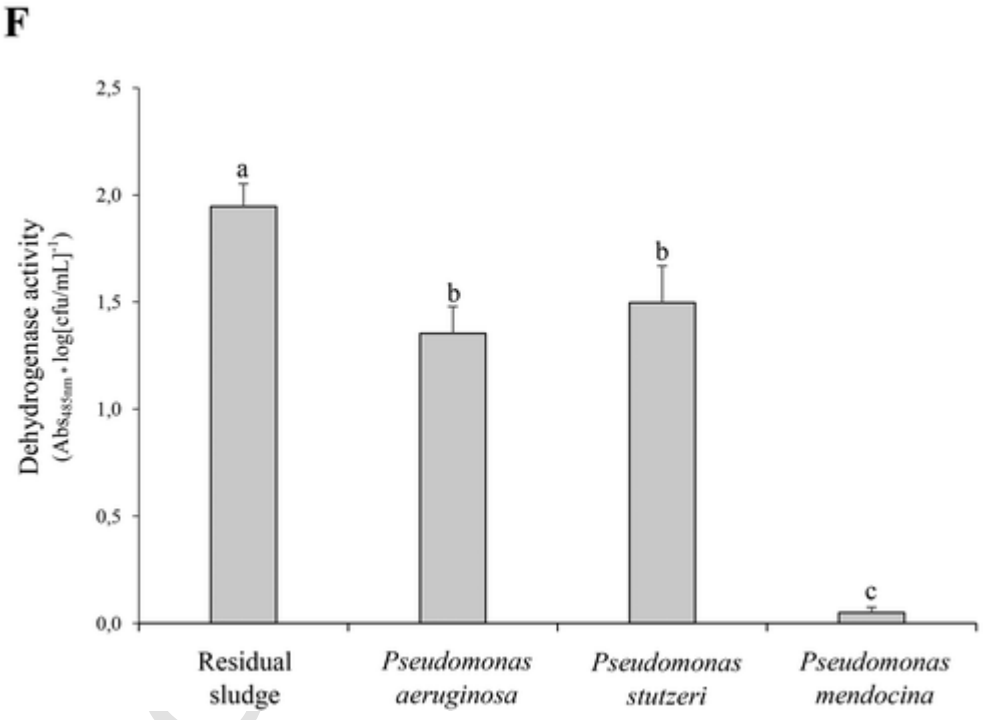


Fig. 4. Dehydrogenase activity of the residual sludge and the isolated *Pseudomonas* spp. after 15 days of incubation (37 °C, 130 rpm) in Bushnell-Haas broth (BHB) with 1% (v/v) B10 diesel as the only carbon source. Development of red formazan colour indicates high bacterial cell activity as a surrogate marker of B10 diesel degradation. **(A)** un-inoculated negative control; **(B)** *P. aeruginosa*; **(C)** *P. stutzeri*; **(D)** *P. mendocina*; **(E)** residual sludge sample. **(F)** Quantification of the dehydrogenase activity expressed during DB10 degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cently, Dutta et al. (2019) have reported that a microbial consortium of *Pseudomonas* sp. may be superior to the free *Pseudomonas* strains in polycyclic aromatic hydrocarbon biodegradation since the epigenetic impacts on the metabolic enzymes. Moreover, microenvironment modification seems to trigger evolutionary divergences for a same enzyme between related bacterial species (Su et al., 2016). However, our results showed that the efficiency of DB10 biodegradation with a bacterial consortium of the three isolated *Pseudomonas* was similar to those obtained for *P. aeruginosa* and *P. stutzeri* alone (Fig. 5), suggesting no synergetic effects between these microbial species during DB10 degradation.

4. Conclusions

In the present study, we have demonstrated for the very first time the potential of a facultative residual sludge generated in an active-operating biogas plant to degrade commercial B10 diesel (petroleum-

Table 2
Bacterial screening for biosurfactant production after 15 days of incubation (37 °C, 130 rpm) in Bushnell-Haas broth (BHB) with 1% (v/v) B10 diesel as the only carbon source.

	EI_{24} (%)	CSH (%)
P. aeruginosa	39.5 ± 2.5 [b]	81.3 ± 2.6 [b]
P. mendocina	15.0 ± 3.5 [a]	33.8 ± 1.2 [a]
P. stutzeri	48.8 ± 1.9 [c]	82.9 ± 0.7 [b]

Data corresponds to mean values ± standard deviations of three independent experiments ($n = 3$).
Values with different letters in each column are significantly different ($p < .0.05$).

based diesel amended with 10% biodiesel). In addition, three potentially DB10-degrading *Pseudomonas* species (*P. stutzeri*, *P. aeruginosa,* and *P. mendocina*) were isolated from the sludge sample under *mild selection pressure*. *P. aeruginosa* and *P. stutzeri* showed a significant degradation rate for C_{11}-C_{22} hydrocarbons and FAME compounds of DB10, demonstrating significant differences in the metabolic response between related pseudomonads. However, a mixed consortium of the three isolated *Pseudomonas* did not improve fuel degradation. In addition, biodegradation efficiency of DB10 by the *Pseudomonas* spp. was signally related to the induction of degradative enzymes (dehydrogenase activity) and biosurfactant production.

In conclusion, the presented information is a rational first step in order to reveal the complex bacterial community present in a residual sludge with the ability to degrade petroleum-based fuels. However, further studies are needed in order to understand how syntrophic consortia may act in hydrocarbons degradation to develop harmless, cost-effective, and efficient biotechnological approaches towards the elimination of persistent pollutants from the environment.

Uncited references

Adams et al., 2015

Declaration of Competing Interest

The authors have no conflicts of interest.

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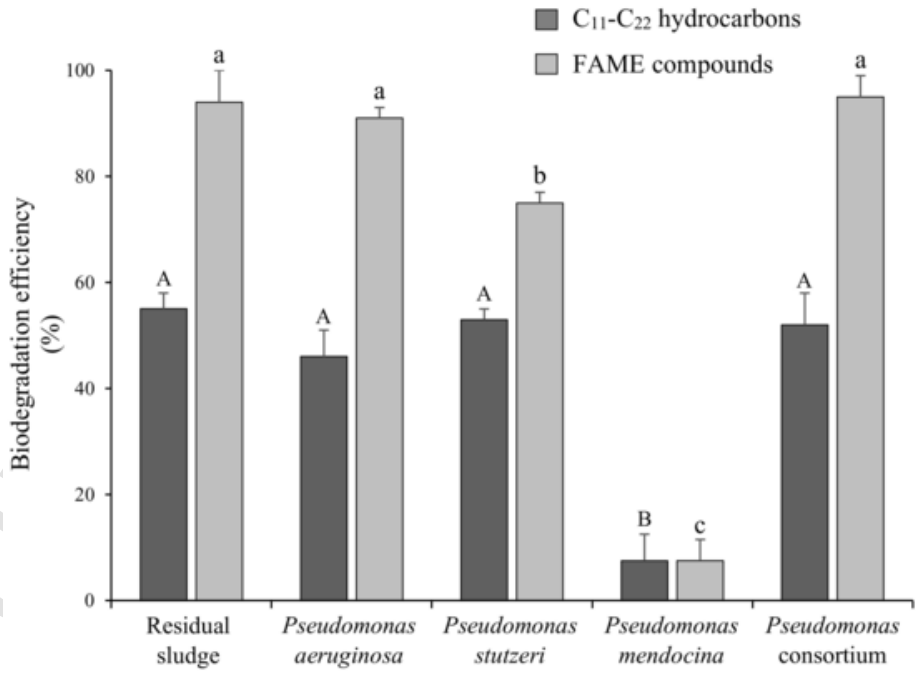

Fig. 5. Biodegradation efficiency (%) of petrochemical (C_{11}-C_{22} hydrocarbons) and FAME fraction of commercial diesel oil by the residual sludge and the isolated *Pseudomonas* spp. (alone and in a consortium) in Bushnell-Haas broth (BHB) with 1% (v/v) B10 diesel as the only carbon source. Different lowercase letters show significant differences between FAME fraction degradation and different uppercase letters show significant differences between middle-chain *n*-alkanes degradation ($p < 0.05$).

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