Isolation and characterization of bacteria with potential naphthalene-degradation from “la Escondida” lagoon, reynosa, Mexico

Gilberto Pinto-Liñan[a], Alma D. Paz-González[a], Valeria González-Muñoz[a], Isidro Palos[b], Maribel Mireles-Martínez[a], María A. Cruz-Hernández[a], Lenci K. Vázquez-Jiménez[a], Gildardo Rivera[a]*

[a]Laboratorio de Biotecnología Farmacéutica, Centro de Biotecnología Genómica, Instituto Politécnico Nacional, 88710 Reynosa, México.

[b] Unidad Académica Multidisciplinaria Reynosa-Rodhe, Universidad Autónoma de Tamaulipas, 88779 Reynosa, México.

Aislamiento y caracterización de bacterias con potencial de degradación de naftaleno de la laguna"La Escondida", Reynosa, México

Aïllament i caracterització de bacteris amb potencial degradació del naftalè de la llacuna “La Escondida”, Reynosa, Mèxict

RECEIVED: 30 AUGUST 2021; REVISED: 23 MAY 2022; ACCEPTED: 26 MAY 2022

SUMMARY

Bioremediation, is currently one of the most studied techniques for the elimination of polycyclic aromatic hydrocarbons in soils. However, in order to have a more efficient degradation process, the use of autochthonous bacteria from the contaminated region is recommended because these bacteria are adapted to the climatic and environmental conditions of the site to be remedied. The aim of this work was to isolate and characterize bacteria strains from the city of Reynosa, Mexico, with the potential to degrade naphthalene. Strain isolation was carried out whit soil samples from the shore of “La Escondida” lagoon, a former landfill of pollutants of the petrochemical industry. The isolates were subjected to the emulsion index test as a selective factor to later evaluate their effect in the drop collapse, drop displacement and naphthalene tolerance tests. Finally, sixty-two strains were identified by amplification of the 16S rRNA gene. Ten strains showed the best values in the drop collapse, oil displacement and naphthalene tolerance tests. Four strains had the best naphthalene degradation potential; *Pseudomonas aeruginosa* (1P2 and 5P2), *Bacillus cereus* (5S1) and *Bacillus subtillis* (P52). A degradation of naphthalene was observed in the IR spectrum and UPLC chromatogram after 12 days by 1P2 strain.

Keywords: bioremediation, soils, polycyclic aromatic hydrocarbon, *Pseudomonas*.

RESUMEN

La biorremediación, es actualmente una de las técnicas más estudiadas para la eliminación de hidrocarburos aromáticos policíclicos en suelos. Sin embargo, para tener un proceso de degradación más eficiente, se recomienda el uso de bacterias autóctonas de la región contaminada debido a que estas bacterias están adaptadas a las condiciones climáticas y ambientales del sitio a remediar. El objetivo de este trabajo fue aislar y caracterizar cepas de bacterias de la ciudad de Reynosa, México, con potencial para degradar naftaleno. El aislamiento de la cepa se realizó con muestras de suelo de la orilla de la laguna "La Escondida", antiguo vertedero de contaminantes de la industria petroquímica. Los aislados se sometieron a la prueba del índice de emulsión como factor selectivo para luego evaluar su efecto en las pruebas de colapso de gota, desplazamiento de gota y tolerancia a naftaleno. Finalmente, se identificaron sesenta y dos cepas mediante amplificación del gen 16S rRNA. Diez cepas mostraron los mejores valores en las pruebas de colapso de gota, desplazamiento de aceite y tolerancia a naftaleno. Cuatro cepas tenían el mejor potencial de degradación de naftaleno; *Pseudomonas aeruginosa* (1P2 y 5P2), *Bacillus cereus* (5S1) y *Bacillus subtillis* (P52). Se observó una degradación de naftaleno en el espectro IR y cromatograma UPLC después de 12 días por la cepa 1P2.

***Corresponding author: gildardors@hotmail.com**

Palabras clave: biorremediación, suelos, hidrocarburo aromático policíclico, *pseudomonas*

RESUM:

La bioremediació, és actualment una de les tècniques més estudiades per a l'eliminació d'hidrocarburs aromàtics policíclics en sòls. Tanmateix, per tal de tenir un procés de degradació més eficient, es recomana l'ús de bacteris autòctons de la regió contaminada perquè aquests bacteris s'adapten a les condicions climàtiques i ambientals del lloc a esmenar. L'objectiu d'aquest treball era aïllar i caracteritzar soques de bacteris de la ciutat de Reynosa, Mèxic, amb el potencial de degradar naftalè. L'aïllament de soques es va dur a terme amb mostres de sòl de la riba de la llacuna "La Escondida", un antic abocador de contaminants de la indústria petroquímica. Els aïllats es van sotmetre a la prova d'índex d'emulsió com a factor selectiu per avaluar posteriorment el seu efecte en les proves de col·lapse de gota, desplaçament de gota i tolerància a la naftale Finalment, es van identificar seixanta-dues soques mitjançant l'amplificació del gen de l'ARNr 16S. Deu soques van mostrar els millors valors en les proves de col·lapse de gota, desplaçament d'oli i tolerància a la naftale. Quatre soques tenien el millor potencial de degradació del naftalè; *Pseudomonas aeruginosa* (1P2 i 5P2), *Bacillus cereus* (5S1) i *Bacillus subtillis* (P52). Es va observar una degradació de naftalè a l'espectre IR i al cromatograma UPLC després de 12 dies per la soca 1P2.

Paraules clau: bioremediació, sòls, hidrocarburs aromàtics policíclics, *pseudomonas*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds derived from petroleum, from incomplete combustion, from industrial processes and from insecticide residues. These hydrocarbons have been considered highly carcinogenic and mutagenic compounds for humans. Due to their abundance in the environment and their carcinogenic characteristics, they have been considered one of the most abundant pollutants in ecosystems. Several techniques have been developed to solve pollution problems caused by hydrocarbons. Chemical oxidation using oxidants such as ozone, hydrogen peroxide, permanganate and persulfate, seems to be a promising technique[1, 2, 3]. However, chemical oxidation causes negative effects in the environment and in humans. In recent decades, an environmentally friendly and interesting strategy to solve this problem is the remediation of soils using microorganisms[4, 5] that have the ability to use hydrocarbons as an energy source in contaminated environments[6]. Microbial degradation helps eliminate hydrocarbons that are spilled in the environment through various physical and chemical methods[7]. This is possible because microorganisms have enzymes that degrade and use hydrocarbons as a source of carbon and energy[8, 9].

Some authors have shown that a useful strategy is to combine a chemical remediation method with a bacterial consortium to exploit the complementarity of the two approaches; thus, the organic solvents are used to solubilize the total petroleum hydrocarbons (TPH), and the PAHs increase contact with the degradation bacteria[10, 11]. In addition, studies show that the use of a consortium of native bacterial isolates directly from contaminated soil increases efficiency in remediation[12]. The use of the native microbiota for bioremediation of contaminated soils is of great interest, since it is expected that these microorganisms are much more adapted to the particular environment of the soil microbial inoculants than the non-native commercial ones. Although, many times the natural microbiota does not show the capacity to efficiently degrade polluting compounds, the use of these native microorganisms in inoculants with improved activity (native bioaugmentation) is being sought[13, 14] as a possible means to improve the success of the process. These findings encourage the isolation of native bacterial strains from contaminated soils. Based on the aforementioned, the objective of this work was to isolate and characterize native bacteria from Reynosa, Mexico with a potential for PAHs degradation, in particular naphthalene.

MATERIALS AND METHODS:

Soil Samples

Samples of contaminated soil were collected in the "La Escondida" lagoon, a site recognized as a landfill of the "PEMEX" refinery in Reynosa, Mexico. In this place (26.047692, -98.270419), two samples from each of the five points (1 kg) were collected: one at 10 cm (surface sample "S") and another at 20 cm (depth sample "P"). The soil samples were oven dried at a range of 35 to 40 °C for 3 to 5 days. Then, the samples were crushed and sieved (No. 20, 850 μM) and weighed at 1 g to process them later.

Isolation of Microorganisms

Initially, 1 g of the homogenized soil sample was inoculated in 100 mL of minimal saline medium (MSM: NaCl 24 g, KCl 0.7 g, KH_2PO_4 2 g, 1 g $MgSO_4.7H_2O$, Na_2HPO_4 3 g, 0.2 g CaCl, $2H_2O$; pH 7.0, 1 L sterile distilled water); pH 7.0 was adjusted using NaOH, in a 250 mL Erlenmeyer flask. The flasks were incubated at 30 °C and 37 °C at 180 rpm for 24 to 72 h. Subsequently, serial dilutions were made at 1:1000 and 100 μL of the medium was inoculated into Petri dishes with Nutrient agar (NA) (Bioxon) and Pseudomonas agar (PA) (Bioxon). The inoculated plates were incubated 24 to 72 h at the set temperatures. Each colony with a different macroscopic morphology was selected and inoculated with a platinum loop in 50 mL falcon tubes with 10 mL of nutrient broth. These were then incubated at the set temperatures, at 180 rpm during, until obtain an absorbance of 1 to 600 nm.

Macroscopic and Microscopic Identification of the Strains

Identification of the strains was carried out considering the criteria established[15, 16] according to the characteristics of culture and morphology of the colonies obtained. In addition, Gram staining and the catalase test were carried out.

Emulsion Index

Initially, the emulsion index (EI) at different times was determined as a first condition (EI> 30 %) for the selection of isolates with polycyclic aromatic hydrocarbon degradation potential. Subsequently, the selected isolates were tested for drop collapse, oil displacement and naphthalene tolerance.

The EI was determined in all isolates according to the procedure reported[17] modifying the growth conditions. The EI was determined in two suspensions: cells and free cells at 2 min, 2, 24 and 48 h. The calculation of the EI was made using the formula and categorization established[18]. Milli-Q® ultrapure water was used as a negative control and 10 % Tween 80 (non-ionic surfactant) and 10 % sodium dodecyl sulfate (SDS) (anionic surfactant) as positive controls. The data of the EI obtained were subjected to an analysis of variance and the Tukey test with a significance of p= 0.05.

To carry out the drop collapse, oil displacement and tolerance to naphthalene tests, the isolates from the tubes with inclined NA and PA were grown in 50 mL conical tubes with 10 mL of nutrient broth (NB) until optical density $(OD)_{600}$ = 1 nm. Once the isolates were grown, each of the tests was continued following the methodology described below.

Drop Collapse Test

The drop collapse test was used to determine in the isolates, the production of biosurfactants with solubilizing activity. The test was carried out according to the method described[19] modifying the growth conditions of the isolates to be evaluated. The hydrocarbon used was SAE 40 motor oil. Milli-Q® ultrapure water was used a negative control and 10 % Tween 80 and 10 % SDS as positive controls.

Oil Displacement Test

This test was performed based on the method described[20], modifying the growth conditions and the volumes reported. PBS 1X (phosphate buffered saline) was used as a negative control and 10 % Tween 80 and 10 % SDS as positive controls.

Naphthalene Tolerance Test

This test was carried out according to the method[21], modifying the growth conditions, the contaminant and the volumes reported. Naphthalene was used at concentrations of 1 %, 5 % and 10 %. As a control, plates with NA without naphthalene were used. The evaluation of tolerance to the different concentrations of naphthalene was done based on the number of colony-forming units (CFU) present in each isolate. All the experiments were performed in triplicate.

Molecular Identification Using 16S rRNA

To perform molecular identification as a first step, genomic DNA extraction (gDNA) was carried out following the gDNA Isolation protocol of Gram-positive and Gram-negative bacteria from the Wizard® Genomic DNA Purification Kit (Promega). Subsequently, it was done by amplifying the 16S rRNA region with the polymerase chain reaction (PCR) using universal oligonucleotides for bacteria, 27F (5'GAAGTTTGATCMTGGCTCAG 3') and 1492R (5'GCGCTACCTTGTTACGACTT 3')[22]. As amplification conditions, 94 °C was used for 1 min; 30 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min; 72 °C for 3 min with a SimpliAmp thermocycler (Applied Biosystems). The reaction was prepared using 1 X Taq DNA polymerase buffer, 2.5 mM $MgCl_2$, 0.2 µM foward primer, 0.2 µM reverse primer, 0.2 mM dNTPs, 1.25 U of Taq DNA polymerase, 50 ng of DNA, adjusted to a volume of 25 µL with sterile Milli-Q® ultrapure water. The PCR products were purified with EXO-SAP and sequenced in Eurofins GTM Operon LLC (Louisville KY, USA). The sequences were analyzed and assembled in LASERGENE subsequently aligned in NCBI.

Analysis of Degradation of Naphthalene by IR and UPLC-MS

The selected strain was inoculated in 50 mL falcon tube with 10 mL of NA and incubated for 24 h at 37 °C at 180 rpm until obtaining an absorbance of 1 at 600 nm. The analysis of the degradation was evaluated using a co-substrate (yeast extract), in an Erlenmeyer flask (250 mL) 100 mL of Bushnell Haas liquid medium (BH), 0.5 g of yeast extract and 500 mg/L of naphthalene were added. Subsequently, the flask was inoculated with 1 mL of the previously grown selected isolate. The growth of the isolate was monitored by taking 1 mL aliquots at different times (1, 2, 3, 4, 5, 6, 7 and 8 days), the aliquots were read at 600 nm. Once growth was observed, the selected isolate was reintroduced in fresh medium BH with 0.5 % yeast extract for 24 h at 37 °C at 180 rpm, until obtaining an absorbance of 1 at 600 nm. Subsequently, from the growth described above, 1 mL samples were taken and inoculated in flask containing 100 mL of BH medium with 0.5 g of yeast extract and 500 mg/L of naphthalene.

To determinate the degradation of naphthalene, the strain was incubated in triplicate for 12 days at 37 °C at 180 rpm. After the incubation time, the extraction of the naphthalene residue was carried out, adding 50 mL of ethyl acetate to the samples and two washes were carried out with 25 mL of the aforementioned solvent. Subsequently, the separation of the solvent was carried out with a Buchi rotavapor (at 240 mbar, at 50 °C for 30 min) and the extracted product was analyzed by FTIR ("BRUKER" ALPHA's Platinum ATR). Also, the extracted product was dissolved with HPLC-grade dichloromethane and transferred to Eppendorf tubes of 1.5 mL, the dichloromethane was allowed to evaporate for 24 h and 100 µL of dimethylsulfoxide (DMSO) and 900 µL of HPLC-grade methanol were added. The Eppendorf tubes were vortexed for 1 min and 100 µL was taken to transfer them to another Eppendorf tube with 900 µL of methanol. The samples were filtered

with 22 µm filters and transferred to vials for analysis in Ultra-Performance Liquid Chromatographic (UPL). The analysis was performed with an ACQUITY QDA® mass detector from Waters (Milford, MA, USA), using an ACQUITY UPLC®BEH C18 1.7 µm 2.1 x 100 mm column and the mobile phases "A" (formic acid at 1 % in water), "B" (methanol) and "C" (acetonitrile). The execution time was 0.5 to 5 minutes, with a flow rate of 0.3 mL/min of the phases (27 % A: 25 % B, 48 % C), an injection volume of 3.0 µL and a column temperature of 50 °C.

RESULTS:

Isolation of Microorganisms

Of the five sampling points, both superficial and deep, 62 isolates of the "La Escondida" lagoon were obtained in two different solid mediums (NA and PA) at two different temperatures. Twenty isolates grew at 30 °C and 42 were isolated at 37 °C (Appendix A), according to the origin, 28 isolates were from the surface and 34 were from depth.

Macroscopic and Microscopic Identification of the Strains

Macroscopic and microscopic identification (Appendix B) as well as Gram staining and the catalase test were performed on the isolates obtained. In Annex A, 62 colonies obtained are shown; 42 grew in NA and 20 grew in PA, observing a different macroscopic and microscopic morphology in the strains identified. According to the macroscopic identification, 31 % isolated showed a white color, 72 % presented a circular shape, 62 % maintained a pulvinated elevation, 66 % an entire margin and 45 %, a creamy consistency. According to the microscopic identification 78 % were bacilli, with 23 % of small size, 64 % are gram-negative and 55 % presented a positive catalase test.

Emulsion Index Test

The EI was determined for all isolates; 10 isolates (4S3, MP31, 1P2, 5S1, 5P1, 5P2, S53, P52, P32 and P41) presented the best EI (Fig. 1); therefore, theses isolates were selected for subsequent tests.

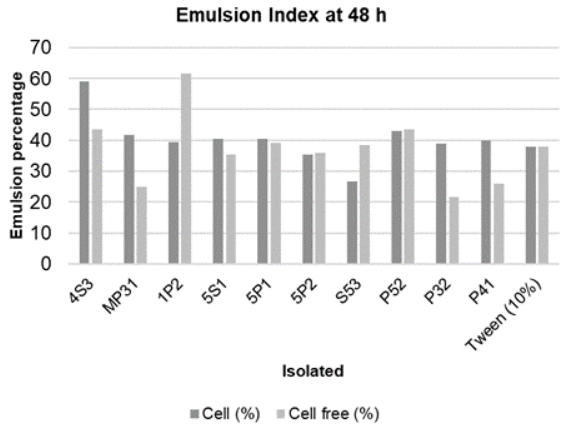


Fig. 1. *Isolates (top ten) with the best emulsion index (value> 30%).*

Drop Collapse, Oil Displacement and Naphthalene Tolerance Test

The results obtained from the drop collapse, oil displacement and naphthalene tolerance tests of the 10 selected isolates are shown in (Appendix C). The purpose of this classification was to differentiate and detect the bacteria that had the best potential for naphthalene degradation. The isolates that showed the best degradation profile (3 positive tests) were 1P2, P52, 5S1 and 5P.

Molecular Identification Using 16S rRNA

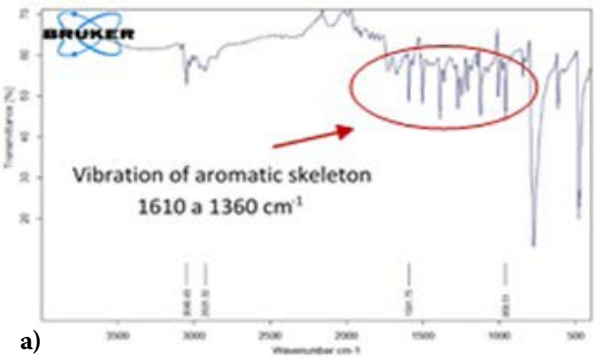
Once the isolates with the greatest degradation potential were detailed, they were identified by amplification of the 16S rRNA gene. The results obtained from the bacterial identification by homology analysis with the 16S rRNA gene are shown in table I. The 1P2 and 5P2 bacteria were identified as *Pseudomonas aeruginosa*, obtaining 99-100 % identity. The bacterium P52 was identified as *Bacillus subtilis* with 98 % identity, and the bacterium 5S1 as *Bacillus cereus* with 99 % identity.

Table 1. *Molecular identification of the isolates obtained..*

Isolated	Description of the organism	Identity	Access number
4S3	*Pseudomonas sp.* SWI36	92 %	CP026675.1
MP31	*Bacillus cereus* strain ATCC 14579	99 %	NR_074540.1
1P2	*Pseudomonas aeruginosa* DMW	100 %	MH760805.1
5S1	*Bacillus cereus* DFT-6	99 %	KY750690.1
5P1	*Brachybacterium paraconglomeratum* strain LMG 19861	97 %	NR_025502.1
5P2	*Pseudomonas aeruginosa* VRF_227	99 %	JX970975.1
S53	*Bacillus thuringiensis* strain ATCC 10792	98 %	NR_114581.1
P52	*Bacillus subtilis* 168	99 %	NR_102783.2
P32	*Lysinibacillus boronitolerans* strain NBRC 103108	97 %	NR_114207.1
P41	*Lysinibacillus boronitolerans* strain NBRC 103108	96 %	NR_114207.1

Degradation of Naphthalene

The 1P2 strain (*Pseudomonas aeruginosa*) was selected for the degradation of naphthalene. This experiment was analyzed by IR and UPLC in two times: 0 and 12 days after incubation. Naphthalene at 0 h shown two representative peaks at 773 cm^{-1} (C-H aromatic) and 1360-1610 (C-H, vibration of aromatic skeleton) (**Fig. 2a**). After the incubation by 12 days with the 1P2 strain, the sample obtained showed new peaks at 3200-3400 (vibration peak of hydroxyl group) and 1724 (carboxyl group) (**Fig. 2b**).



a)

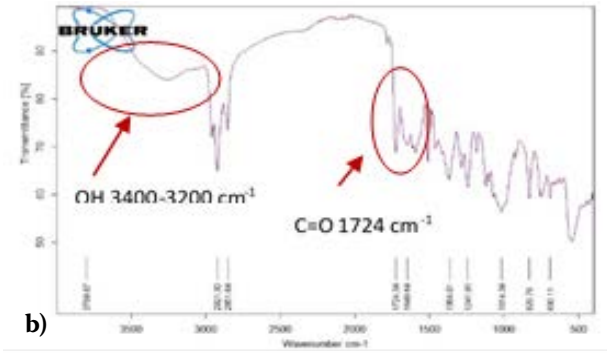


Fig. 2. *Infrared spectrums of naphthalene in two times: 0 h (a) and 12 days (b) after incubation with the 1P2 strain.*

The UPLC chromatogram of the samples after 12 days of incubation shown three peaks of metabolites of degradation (**Fig. 3**): Salicylaldehyde (rt= 0.62 min; 122.12 m/z), 1,2-dihydroxynaphthalene (rt= 0.8 min; 160.17 m/z) and 3-fumaril pyruvate (rt= 0.82, 186.12 m/z).

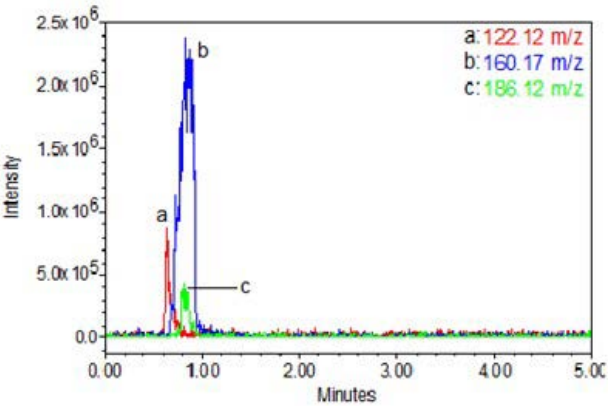


Figure 3. *Chromatogram of naphthalene sample incubated with 1P2 strain after 12 days.*

DISCUSSION

Regarding the isolation of microorganisms, 62 isolates were obtained. The greatest number of colonies were obtained from the depth (34 isolates); this can be attributed to the greater number of nutrients in the depth and to more stable environmental conditions according to[23]. The results of the isolation are similar to those presented[24], who reported 66 bacteria isolated from solid residues of crude oil. Also, in 2017[25] reported 45 isolates from samples from a refinery of the Indian Oils Corporation, where 15 isolates were grown in enriched media and 30 isolates were grown in non-media enriched. The macroscopic and microscopic identification of the isolates showed differences between strains from NA and PA medium. This could be attributed to the fact that NA is a basic medium that facilitates the culture of various bacteria, while PA is a selective medium for the genus of Pseudomonas. Results showed few isolates of this genus.

The emulsion test (cell and cell-free) has been used for the detection of biosurfactants with emulsifying activity by various authors and could be used as an inclusion criterion to select isolates with a potential degradation of PAHs[26, 27, 28]. This test was carried out at different times to measure (EI). From the 60 isolates tested, 10 strains showed a better EI percentage (>30 %) than the positive control (10 % Tween 80) after 48 h in cell or free-cell. Only 4 strains (4S3, 1P2, 5P1 and PS2), showed better eI percentage in both samples than the positive control. However, we decided to test the 10 strains in the next experiments.

Drop collapse and oil displacement are two tests used for the detection of biosurfactants with emulsifying, solubilizing, and/or dispersing characteristics. These molecules could help better degrade PHAs; however, the results only showed that P52 had a similar biological behaviour to positive controls. The isolates, 1P2, 5S1, and 5P2, were positive in both tests but with an effect less than the positive control. P32 and P41 only showed positive oil displacement, which suggests that these isolates only produce dispersing molecules.

Additionally, tolerance to naphthalene has been related to the ability to degrade PHAs; therefore, the isolates 4S3, 1P2 and 5P2, could have the best degradation potential. When analyzing all the tests, only four strains (1P2, P52, 5S1 and 5P2) that presented positive tests were identified as *P. aeruginosa*, *B. subtilis* and *B. cereus*. Also, 4S3 isolated with high tolerance to naphthalene was identified as *P. aureginosa*. The strain *P. aeruginosa* in several reports has been identified as a naphthalene degrader[29]. Similarly, strains of *B. subtilis* (M16K and M19F) have been reported as petroleum oil and phenanthrene degraders[30].

Based on the results of the selective tests and the background of bacteria reported from the same genus, it is possible that the 10 isolates evaluated and identified have potential for the degradation of PHAs. Therefore, 1P2 strain was selected to analyze degradation of naphthalene. Results of the IR and UPLC analysis shown that the naphthalene after 12 days of incubation produce metabolites as salicylaldehyde, 1,2-dihydroxynaphthalene and 3-fumaril pyruvate. These metabolites suggest a degradation of naphthalene by the 1P2 strain.

CONCLUSIONS

Based on the results of the selective tests and the background of bacteria reported from the same genus, it is possible that the 10 isolates evaluated and identified have potential for the degradation of PAHs. Therefore, 1P2 strain was selected to analyze degradation of naphthalene. Results of the IR and UPLC analysis shown that the naphthalene after 12 days of incubation produce metabolites as salicylaldehyde, 1,2-dihydroxynaphthalene and 3-fumaril pyruvate. These metabolites suggest a degradation of naphthalene by the 1P2 strain.

ACKNOWLEDGMENTS

We are grateful for the financial support granted to the Secretaria de Investigacion y Posgrado del Instituto Politecnico Nacional for the implementation of this project, through the projects with registration number SIP-20201007 and SIP-20210433.

REFERENCES

1. Otaraku J., Uchechukwu A. and Nwambo Yirakpoa P. (2013). *In situ*, chemical remediation of crude oil-polluted soil using hydrogen peroxide and its modelling. Int. J. Innov. Eng. Res. Manag. 2 (8), 344-347.
2. Andrade V., de Souza C., Godinho S.C., Lourenço R. and Vidal D. (2012). Remediation of oil-contaminated soil by chemical oxidation. Rev. Bras. Ciênc. Solo. 36 (5), 1656-1660. DOI: 10.1590/S0100-06832012000500030
3. Goi A., Trapido M. and Kulik N. (2009). Contaminated Soil Remediation with Hydrogen Peroxide Oxidation. Int. J. Chem. Biomol. Eng. 3 (4), 209-213.
4. Smułek W., Zdarta A., Guzik U., Dudzińska-Bajorek B. and Kaczorek E. (2015). *Rahnella* sp. strain EK12: Cell surface properties and diesel oil biodegradation after long-term contact with natural surfactants and diesel oil. Microbiol. Res. 176, 38-47. DOI: 10.1016/j.micres.2015.04.008
5. Jørgensen K.S., Puustinen J. and Suortti A.M. (2000). Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ. Pollut. 107 (2), 245-254. DOI: 10.1016/s0269-7491(99)00144-x
6. Jurtshuk P. Jr. (1996). Bacterial Metabolism. En: Medical Microbiology. (S. Baron, Ed). Galveston, Texas, U.S.
7. Ljah U.J.J. and Okang C.N. (1993). Petroleum degrading capabilities of bacteria isolated from soil, W.A.J. Boil. Appl. Chem. 38: 9-15.
8. Antai S.P. and Mgbomo E. (1993). Pattern of degradation of bonny light crude oil by *Bacillus spp.* and *Pseudomonas spp.* isolated from oil spilled site. W.A.J. Boil. Appl. Chem. 38, 16-20.
9. Ljah U. J. J. and Antai S. P. (1998). Degradation and Mineralization of crude oil by bacteria, Niger. Biotechnol. J. 5: 79-86.
10. Gong Z., Alef K., Wilke B. M. and Li P. (2005). Dissolution and removal of PAHs from a contaminated soil using sunflower oil. Chemosphere. 58(3), 291-298. DOI: 10.1016/j.chemosphere.2004.07.035
11. Taylor L. T. and Jones D.M. (2001). Bioremediation of coal tar PAH in soils using biodiesel. Chemosphere. 44 (5), 1131-1136. DOI: 10.1016/S0045-6535(00)00344-1
12. Gan S., Lau E.V. and Ng H.K. (2009). Remediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs). J. Hazard. Mater. 172 (2-3), 532-549 DOI: 10.1016/j.jhazmat.2009.07.118
13. Szulc A., Ambrożewicz D., Sydow M., Ławniczak Ł., Piotrowska-Cyplik A., Marecik R. and Chrzanowski Ł. (2014). The influence of bioaugmentation and biosurfactant addition on bioremediation efficiency of diesel-oil contaminated soil: feasibility during field studies. J. Environ. Manage. 132: 121-8. DOI: 10.1016/j.jenvman.2013.11.006
14. Gentry T.J., Rensing C. and Pepper I.L. (2004). New approaches for bioaugmentation as a remediation technology. Crit. Rev. Env. Sci. Tec. 34 (5), 447–494. DOI: 10.1080/10643380490452362
15. Harrigan W.F. and McCance M.E. (1966a). Biochemical Tests for Bacteria. Laboratory Methods in Microbiology. ACADEMIC PRESS INC. (LONDON) LTD, New York, U. S. 362 pp.
16. Harrigan W.F. and McCance M.E. (1966b). The Description of the Morphological and Cultural Characteristics of an Organism. Laboratory Methods in Microbiology. Elsevier, 374 pp. DOI: 10.1016/C2013-0-12452-X
17. Menezes B.F., de Oliveira F.A., Okeke B.C. and Frankenberger W.T. (2005). Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiol. Res. 160 (3), 249–55. DOI: 10.1016/j.micres.2004.08.005
18. Cooper H.A., Bennett W.P., Kreger A., Lyerly D. and Wagner R.H. (1981). The effect of extracellular proteases from gran-negative bacteria on the interaction of von Willebrand factor with human platelets. J. Lab. Clin. Med. 97 (3), 379-389.
19. Tugrul T. and Cansunar E. (2005). Detecting surfactant-producing microorganisms by the drop-collapse test. World J. Microbiol. Biotechnol. 21 (6-7), 851–853. DOI: 10.1007/s11274-004-5958-y
20. Youssef N.H., Duncan K.E., Nagle D.P., Savage K.N., Knapp R.M., McInerney M.J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods. 56 (3), 339–347. DOI: 10.1016/j.mimet.2003.11.001
21. Zhang G.Y., Ling J.Y., Sun H.B., LuoJ., Fan Y.Y. and Cui Z.J. (2009). Isolation and characterization of a newly isolated polycyclic aromatic hydrocarbons-degrading *Janibacter anophelis* strain JY11. J. Hazard. Mater. 172 (2-3), 580–586. DOI: 10.1016/j.jhazmat.2009.07.037
22. Weisburg W.G., Barns S.M., Pelletier D.A. and Lane D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173 (2): 697–703. DOI: 10.1128/jb.173.2.697-703.1991
23. Birkeland P.W. (1984). Soils and geomorphology. Oxford University Press. Oxford, UK. Pp 372.
24. Toledo F.L., Calvo C., Rodelas B. and González-López J. (2006). Selection and identification of bacteria isolated from waste crude oil with polycyclic aromatic hydrocarbons removal capacities. Syst. Appl. Microbiol. 29 (3), 244–252. DOI: 10.1016/j.syapm.2005.09.003
25. Sarkar P., Roy A., Pal S., Mohapatra B., Kazy S.K., Maiti M.K. and Sar P. (2017). Enrichment and characterization of hydrocarbon-degrading bacteria from petroleum refinery waste as potent bioaugmentation agent for *in situ* bioremediation. Bioresour. Technol. 242, 15–27. DOI: 10.1016/j.biortech.2017.05.010
26. Smułek W., Zdarta A., Guzik U., Dudzińska-Bajorek B. and Kaczorek E. (2015). *Rahnella* sp. strain EK12: Cell surface properties and diesel oil biodegradation after long-term contact with natural surfactants and diesel oil. Microbiol. Res. 176, 38-47. DOI: 10.1016/j.micres.2015.04.008

27. Panjiar N., Sachan S.G. and Sachan A. (2015). Screening of bioemulsifier-producing micro-organisms isolated from oil-contaminated sites. Ann. Microbiol. 65 (2), 753–764. DOI: 10.1007/s13213-014-0915-y
28. Bezza F.A. and Chirwa E.M.N. (2015). Production and applications of lipopeptide biosurfactant for bioremediation and oil recovery by *Bacillus subtilis* CN2. Biochem. Eng. J. 101, 168–178. DOI: 10.1016/j.bej.2015.05.007.
29. Qi J., Wang B., Li J., Ning H., Wang Y., Kong W. and Shen L. (2015). Genetic determinants involved in the biodegradation of naphthalene and phenanthrene in *Pseudomonas aeruginosa* PAO1. Environ. Sci. Pollut. Res. Int. 22 (9): 6743–6755. DOI: 10.1007/s11356-014-3833-4
30. Oladunjoye G., Chienb M., Ikeda-Ohtsubo W., Suzuki H., Sunday O., Sunday Adekunle S., Olusoji M., Oladipo O. and Endo G. (2017). Biodegradation of crude oil and phenanthrene by heavy metal resistant *Bacillus subtilis* isolated from a multi-polluted industrial wastewater creek. Int. Biodeter. Biodegr. 120, 143–151. DOI: 10.1016/j.ibiod.2017.02.021

APPENDIX

Appendix A
Isolates from the "La Escondida" lagoon

Sampling points		Liquid Medium	Solid Medium			
			Nutrient Agar		Pseudomonas Agar	
			30°C	37°C	30°C	37°C
1	S	Minimum Saline Medium 30 and 37°C Dilutions 1:10, 1:100	1S-1 (MS1-1)	1S-1	1S-1	1S-1
			1S-2 (MS1-2)	1S-2	1S-2	1S-2
			1S-3 (MS1-3)	-	1S-3	1S-3
	D		1P-1 (MP1-1)	1P-1	1P-1	1P-1
			1P-2 (MP1-2)	1P-2	-	1P-2
			1P-3	1P-3	-	-
2	S		2S-1 (MS2-1)	2S-1	2S-1	2S-1
			2S-2 (MS2-2)	2S-2	2S-2	2S-2
			2S-3	2S-3	2S-3	-
			2S-4	2S-4	-	-
			2S-5	-	-	-
	D		2P-1 (MP2-1)	2P-1	2P-1	2P-1
			2P-2 (MP2-2)	2P-3	2P-2	2P-2
			2P-3 (MP2-3)	-	2P-3	2P-3
			2P-4 (MP2-4)	-	-	-
3	S		3S-1 (MS3-1)	3S-1	3S-1	3S-1
			-	3S-2	3S-2	3S-2
			-	3S-3	3S-3	3S-3
			-	-	-	3S-4
	D		3P-1 (MP3-1)	3P-1	3P-1	3P-1
			3P-2	3P-2	3P-2	3P-2
			3P-3 (MP3-3)	3P-3	3P-3	3P-3
			3P-4 (MP3-4)	3P-4	-	3P-4
			-	3P-5	-	-
4	S		4s-1 (MS4-1)	4S-1	4S-1	4S-1
			4S-2	4S-2	4S-2	4S-2
			4s-3 (MS4-3)	4S-3	4S-3	4S-3
			4s-4	4S-4	4S-4	4S-4
			-	4S-5	4S-5	-
	D		4P-1 (MP4-1)	4P-1	4P-1	4P-1
			4P-2 (MP4-2)	4P-2	4P-2	4P-2
			4P-3 (MP4-3)	4P-3	4P-3	4P-3
			4P-4	4P-4	4P-4	4P-4
			4P-5 (MP4-5)	4P-5	4P-5	-
5	S		5s-1 (MS5-1)	5S-1	5S-1	5S-1
			5S-2 (MS5-2)	5S-3	5S-2	5S-2
			-	5S-4	5S-3	5S-3
			-	-	-	5S-4
	D		5P-1 (MP5-1)	5P-1	5P-1	5P-1
			5P-2 (MP5-2)	5P-2	5P-2	5P-2
			5P-3 (MP5-3)	5P-3	5P-3	-
			-	-	5P-4	-
			-	-	5P-5	-

Appendix B

Morphological identification of bacterial isolates.

		IDENTIFICATION									
		Isolated	Macroscopic					Microscopic		Gram stain	Catalase test
			Color	Form	Elevation	Margin	Consistency	Form	Description		
Nutrient Agar	30 °C	1S-1 (MS1-1)	-	Rhizoid	Crateriform	Dispersed	-	Bacilli	Small	-	+
		1S-2 (MS1-2)	-	Circular	Pulvinated	Entire	-	Cocci	Staphylococci	+	-
		1S-3 (MS1-3)	-	Circular	Pulvinated	Entire	-	Bacilli	Short	+	-
		1P-1 (MP1-1)	White	Circular	Pulvinated	Wavy	Filamentous	Bacilli	Big- sporulated	+	+
		1P-2 (MP1-2)	White	Circular	Pulvinated	Entire	Arenosa	Bacilli	Thin	-	-
		2S-1 (MS2-1)	White	Irregular	Convex	Filiform	Creamy	Bacilli	Small	-	+
		2S-2 (MS2-2)	Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small	-	-
		2P-1 (MP2-1)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	medium -thin	-	-
		2P-2 (MP2-2)	Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small	-	+
		2P-3 (MP2-3)	White	Circular	Convex	Entire	Creamy	Cocci	Streptococci	+	-
		2P-4 (MP2-4)	Translucent	Circular	Convex	Entire	-	Bacilli	Short	+	-
		3S-1 (MS3-1)	Translucent	-	Convex	Wavy	-	Bacilli	Small	-	+
		3P-1 (MP3-1)	White	Circular	Convex	Entire	Creamy	Bacilli	Short	+	-
		3P-3 (MP3-3)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	-	-
		4s-1 (MS4-1)	Translucent	Circular	Convex	Entire	-	Bacilli	Small	-	-
		4s-3 (MS4-3)	Yellow	Circular	Pulvinated	Entire	Creamy	Diplo-coccus	-	-	+
		4P-1 (MP4-1)	White Translucent	Irregular	High	Wavy	-	Bacilli	Small	-	-
		4P-2 (MP4-2)	Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small	-	-
		4P-3 (MP4-3)	Beige Translucent	Circular	Pulvinated	Entire	-	Diplo-coccus	-	-	+
		4P-5 (MP4-5)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Inclined -me-dium	-	-
		5s-1 (MS5-1)	White	Circular	Convex	Entire	Creamy	Bacilli	Medium	+	+
		5S-2 (MS5-2)	Beige	Irregular	Pulvinated	Wavy	Creamy	Bacilli	Inclined -me-dium	+	+
		5P-1 (MP5-1)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Big- chain	+	-
		5P-2 (MP5-2)	White Translucent	Circular	Pulvinated	Entire	-	Bacilli	Big- chain	-	+
		5P-3 (MP5-3)	White	-	Flat	Wavy	Creamy	Bacilli	Big- spores	+	+
	37 °C	1S-1 (S1-1)	Yellow	Punctiform	High	Wavy	Creamy	Bacilli	Long- thin	-	+
		1S-2	Translucent	Circular	Convex	Entire	-	Bacilli	Short-wide	-	-
		1P-1 (P1-1)	Cream	Rhizoid	Pulvinated	-	Filamentous	Bacilli	Thin-big	-	-
		1P-2 (P1-2)	Pink	Circular	Flat	Wavy	-	coco-bacilos	Small	-	+
		1P-3	-	Irregular	Flat	Entire	Creamy	Bacilli	Short	-	-
		2S-1 (S2-1)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	-	+
		2S-3 (S2-3)	-	Irregular	Pulvinated	Wavy	-	Bacilli	Short-wide	+	-
		2P-1	Translucent	Circular	Convex	Entire	-	Bacilli	Short-Thin	-	-
		2P-3 (P2-3)	Cream	Circular	Pulvinated	Wavy	-	Cocco-bacilli	Short-Thin	-	+
		3S-1 (S3-1)	-	Rhizoid	Convex	-	Filamentous	Bacilli	Short	+	-
		3S-2 (S3-2)	White	Irregular	Flat	Filiform	Creamy	Bacilli	Short-Thin	-	+
		3S-3 (S3-3)	White	Circular	Pulvinado	Entire	Creamy	Bacilli	Small	-	+
		3P-1 (P3-1)	Yellow	Circular	Pulvinado	Entire	-	Cocco-bacilli	Small	+	-
		3P-2 (P3-2)	Cream	Rhizoid	Convex	-	Filamentous	Bacilli	Short	-	-
		3P-3 (P3-3)	Cream	Irregular	Pulvinated	Wavy	-	Bacilli	Thin	-	+
		3P-4 (P3-4)	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Medium	-	-
		4S-1		Circular	Convex	Entire	Creamy	Bacilli	Short	-	-
		4S-2 (S4-2)	Cream	Irregular	Convex	Wavy	-	Bacilli	Short-small	-	-
		4S-3	-	Irregular	Convex	Wavy	-	Cocci	Small	-	-
		4S-4	-	Irregular	Flat	Wavy	-	Bacilli	Short-wide	-	-
		4P-1 (P4-1)	White	Circular	Pulvinated	Entire	Creamy	Cocco-bacilli	Small	-	+

		IDENTIFICATION									
		Isolated	Macroscopic					Microscopic		Gram stain	Catalase test
			Color	Form	Elevation	Margin	Consistency	Form	Description		
		4P-3	Translucent	Circular	Convex	Wavy	-	Coccobacilli	-	+	-
		4P-4 (P4-4)	White	Circular	Pulvinated	Entire	-	Cocci	Staphylococci	-	-
		5S-1 (S5-1)	Translucent	Irregular	Pulvinated	Wavy	Creamy	Cocci	Staphylococci	+	-
		5S-3 (S5-3)	Cream	Circular	Pulvinated	Wavy	-	Bacilli	Small-thin	-	-
		5P-1	-	Circular	Pulvinated	Entire	Creamy	Cocci	-	-	-
		5P-2 (P5-2)	Brown/Pink	Circular	Pulvinated	Entire	-	Bacilli	Short	+	+
		5P-3 (P5-3)	-	Irregular	High	-	-	Bacilli	Short	-	+
Pseudomonas Agar	30 °C	1S-1	White	Circular	Pulvinated	Wavy	Creamy	Bacilli	Short	-	+
		1S-2	Yellow	Circular	Pulvinated	Entire	Creamy	Bacilli	Short	-	+
		1P-1	Yellow	Circular	Pulvinated	Entire	-	Bacilli	Short	-	+
		2S-3	Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small	+	+
		2P-1	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Short	-	+
		2P-2	Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small	+	+
		2P-3	Yellow	Circular	Pulvinated	Entire	-	Bacilli	Short	+	+
		3S-1	-	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	+	-
		3S-2	-	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	-	+
		3P-1	Translucent	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	+	+
		3P-3	Translucent	Circular	Pulvinated	Entire	Creamy	Bacilli	Small-thin	+	+
		4S-3	-	Circular	Pulvinated	Entire	Creamy	Bacilli	Small-inclined	+	+
		4S-4	Yellow Translucent	Circular	Pulvinated	Entire	-	Bacilli	Short	+	+
		4S-5	White Translucent	-	Crateriform	Entire	Creamy	Bacilli	Short	-	+
		4P-1	White	Circular	Pulvinated	Entire	Creamy	Coccobacilli	Small-inclined	+	+
		4P-2	White	Circular	Pulvinated	Entire	-	Bacilli	Short	-	+
		4P-3	Translucent	Irregular	Umbloid	Wavy	-	Coccobacilli	Small	-	+
		5S-1	Translucent	Circular	Pulvinated	Entire	Creamy	Bacilli	Short	+	+
		5S-2	Ligth Yellow	Circular	Pulvinated	Entire	-	Bacilli	Long-thin	-	+
		5P-2	Translucent	Circular	Pulvinated	Entire	-	Cocci	Small	+	+
		5P-3	Translucent	-	High	Entire	-	Bacilli	Small-short	-	+
		5P-4	White Translucent	Circular	Convex	Entire	Creamy	Bacilli	Short	+	+
		5P-5	Yellow Translucent	Circular	-	Entire	-	Bacilli	Short	+	+
	37 °C	1S-1	Yellow	Circular	Pulvinated	Entire	-	Bacilli	Long-thin	-	+
		1S-3	Translucent	Circular	-	Wavy	Creamy	Bacilli	Small	-	+
		1P-1	Translucent	Irregular	Flat	-	Creamy	Bacilli	Big-long	-	-
		1P-2	Translucent	Circular	-	-	Creamy	Coccobacilli	-	-	+
		2S-1	Translucent	Circular	Pulvinated	Entire	Creamy	Bacilli	Small	-	+
		2S-2	Translucent	Circular	Pulvinated	Entire		Bacilli	Short	-	-
		2P-1	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Short	-	+
		2P-2	Yellow	Circular	Pulvinated	Entire	Creamy	Bacilli	Long	-	-
		3S-2	White	Irregular	-	Filiform	-	Bacilli	Short-thin	-	+
		3S-3	Translucent	Circular	Pulvinado	Entire	-	Bacilli	Small	-	+
		3S-4	White, Translucent	Irregular	High	Filiforme	-	Bacilli	Small-thin	+	+
		3P-1	White	Circular	Pulvinated	Entire	-	Coccobacilli	-	+	-
		3P-2	Translucent	Circular	Pulvinated	Entire	Creamy	Bacilli	Short	-	-
		3P-4	White	Circular	Pulvinated	Entire	Creamy	Bacilli	Medium	-	-
		4S-1	White	Circular	Pulvinated	Wavy	Creamy	Bacilli	Short	+	+
		4S-3	Translucent	Circular	Convex	Wavy	-	Bacilli	Short	-	-
		4S-4	White Translucent	Circular	Pulvinated	Entire	-	Bacilli	Small -short	+	-
		4P-1	White	Circular	Pulvinated	Entire	Creamy	Coccobacilli	-	+	+
		4P-3	-	-	-	-	-	-	-	-	-
		4P-4	White	Circular	Pulvinated	Entire	-	Cocci	Staphylococci	-	-
		5S-1	Translucent	Irregular		Wavy	Creamy	Cocci	Staphylococci	+	-
		5S-4	White	Circular	Pulvinated	Entire	-	Coccobacilli	Long- thin	-	-
		5P-1	White	Circular	Pulvinated	Entire	Creamy	Coccobacilli	Short	-	+
		5P-2	Green Translucent	Circular	Pulvinated	Entire	-	Coccobacilli	Short	-	+

Appendix C.

Results of the drop collapse, oil displacement and naphthalene tolerance.

Isolated	Drop collapse	Oil displacement	Naphthalene tolerance				
			Control	0 %	1 %	5 %	10 %
		30 °C					
4S3	-	-	++++	++++	++++	++++	+
MP31	-	-	+	+	+	-	-
		37 °C					
1P2	++	+++	++++	++++	++++	++++	+
5S1	++	+	+++	+++	+++	+	+
5P1	-	-	++++	++++	++++	-	-
5P2	+	+	++++	++++	++++	++++	-
S53	-	-	++++	++++	+	+	+
P52	+++	++++	++	++++	++	+	+
P32	-	+	++	++	-	-	-
P41	-	+	-	-	-	-	-
Water ultrapure	-	-	-	-	-	-	-
Tween (10 %)	++++	++++	-	-	-	-	-
SDS (10 %)	++++	++++	-	-	-	-	-