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МОЛЕКУЛЯРНЫЕ ХАРАКТЕРИСТИКИ БАКТЕРИЙ, ВЫДЕЛЕННЫХ ИЗ ОТРАБОТАННОГО ЭЛЕКТРОТРАНСФОРМАТОРНОГО МАСЛА

А. де Систо, Э. Фуселла, Г. Урбина, В. Леон, Л. Наранхо

(Университет Биотехнологии Нефтепродуктов, Центр биотехнологии; Фундаментальный Институт Передовых Исследований; C/ Hoyo de la Puerta- Baruta, Sartenejas, Caracas 1080, Venezuela. http://www.idea.gob.ve)

Отработанное электротрансформаторное масло (ЭТМ) включает такие опасные соединения, как полициклические углеводороды и иногда полихлорированные бифенилы в качестве диэлектрических жидкостей. Они очень ядовиты и устойчивы к разложению. Из ЭТМ были выделены три штамма бактерий, принадлежащих к *Acinetobacter lwoffii, Bacillus amyloliquefasciens* и *Bacillus pumilus*. Фенотип и молекулярные исследования показали высокий потенциал *A. lwoffii* к усвоению фенантрена. Впервые выделены, идентифицированы и охарактеризованы бактериальных сообщества, скрытые в ЭТМ.

Electrical transformer oil (ETO) is a highly-refined mineral oil that is stable at high temperatures and has excellent electrical insulating properties. Polychlorinated Biphenyls (PCBs), synthetic chlorinated organic chemicals resistant to the heat and chemical stable, were widely used as dielectric fluids and heat transfer agents in ETO. Nevertheless, the PCBs are highly mutagenic and carcinogens compounds belonging to the larger group of persistent organic pollutants (POPs) [1, 2]. Besides being lipophilic (bioaccumulative) and hardly degraded by microorganisms, the PCBs incineration produce compounds like dioxins and dibenzofurans causing toxic effects on living beings and environment [3, 4]. In Venezuela currently exists about 2600 ton of PCBs corresponding to the electric and basic national industries. Due to the incorporation of Venezuela in the Stockholm agreements, is necessary to safeguard the final disposition, management and degradation of the POPs as the PCBs [5, 6]. Bacteria have been shown to degrade PCBs compounds directly and cometabolically under anaerobic and aerobic conditions [7]. The aim of this work is to isolate, identify, and characterize autochthonous cultivable bacterial strains from an ETO mixture wasted; in order to use them as biocatalyst in novel green processes for ETO-biodegradation.

MATERIALS AND METHODS

Isolation of bacteria from ETO wasted and culture conditions

ETO sample wasted was proceeding from Electricity of Caracas Company (Venezuela). Standard enrichment technique was used to isolate cultivable bacteria from ETO wasted. 500 mL Erlenmeyer flasks containing a mixture of 100 mL Luria-Bertani (LB) medium [8] and 25 mL of ETO were incubated at 30 єC in a rotary shaker (200 rpm). Following five cycles of enrichment, 1 mL of the culture was diluted and plated on basal salt medium (BSM) agar plates [9] supplemented with 25% (v/v) of ETO as sole carbon source and energy.

ETO chemical analysis

To determine saturated and aromatics fraction in ETO samples the method that cover separation of defined fractions from petroleum products and lubricants was used [10]. The PCBs concentration was analyzed through analytical methods for Aroclors included in the Environmental Protection Agency (EPA) approved Method 8082; using capillary column with GC/ECD [11–13].

Molecular identification of bacterial strains from ETO wasted

Genomic DNA from each bacteria strain was extracted as described previously [14]. Two different DNA fingerprinting techniques were performed using the ERIC-PCR [15, 16] and GIRRN-LIRRN [17] set of primers. PCR amplifications of 16S rRNA gene were performed as described previously [18, 19]; purified and sequenced using an ABI Prism^{TM} 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). DNA Sequence analysis was performed using the Lasergene software package

DNASTAR Programs (DNASTAR, Inc., UK), BLASTN [20] and FASTA [21]. All other nucleic acid manipulations were carried out by standard methods [22].

Abilities of bacterial strains to use Polyaromatics Hydrocarbons (PAHs) as sole carbon source and energy

The bacterial strains were tested for their abilities to grow on BSM agar (2% w/v) plates supplemented with 5% (w/v) of naphthalene or phenanthrene as sole carbon source and energy and incubated at 30єC.

Molecular detection of genes involved in the PAHs degradation

The *nah*Ac gene encoding the large subunit of the naphthalene dioxygenase was amplified by PCR using following set of primers: FAc (5′-CCCYGGCGACTATGT-3r) and RAc (5′-CCTCRGGCATGTCTTTTTC-3‘) [23]. An 840 bp DNA fragment internal to the *nah*Ac gene from *Pseudomonas putida* R1 was used as probe in the hybridization experiments.

Abilities of the bacterial strains to use ETO wasted as sole carbon source

To study the skill to use ETO as sole carbon source and energy, the bacterial strains were grew in different carbon sources: i) BSM supplemented with 1% of Yeast Peptone Glucose medium (YPG); ii) BSM supplemented with 1% of YPG and 1% of ETO; iii) BSM supplemented with 1% of ETO as sole carbon source and; iv) BSM without carbon source (as control). The Erlenmeyer flasks were inoculated by duplicate with 2% (v/v) of inoculum of each bacterium (1×10^8 CFU/ml) and were incubated in a rotatory shaker (200 rpm) at 30°C for four days. Three samples of 0.5 mL was collected each 3 h and the microbial growth was monitored at OD_{610} using UVwinlab version 2.85.04 2000 software for PerkinElmer Instruments Lambda 35 UV/VIS spectrophotometer.

RESULTS AND DISCUSSION

Bacterial strains isolated from ETO wasted only included species of the *Bacillus* and *Acinetobacter* genera

There are some bacteria involved in BPCs-biodegradation [24–26] but it is unknown about bacterial diversity present in ETO wasted and their potential in ETO-biodegradation. In order to determinate the genetic diversity present in ETO, bacterial strains were isolated by mean of standard enrichment technique and identified through several molecular methods. Eleven autochthonous bacterial strains were isolated and axenically cultivated. DNA fingerprinting using the ERIC-PCR [15] or GIRRN-LIRRN [17] primers was performed for identifying and differentiating among individual bacteria in the population. The results (Fig. 1) revealed the presence of three different groups using GIRRN-LIRRN, which were confirmed using the ERIC-PCR [17] (data not shown). Three defined groups of strains were confirmed by 16S rRNA gene sequencing, belonging to three different species: *Acinetobacter lwoffii* (one strain); *Bacillus amyloliquefasciens* (eight strains); and *Bacillus pumilus* (two strains), suggesting a low genetic diversity present in ETO wasted. The results indicated that both DNA fingerprinting methods used are useful to discriminate bacteria at specific levels. This work reports for the first time the isolation, identification and the bacterial diversity present in an ETO wasted.

A. lwoffii is able to use phenanthrene as sole carbon source via Phthalate pathway

In order to study the capacity of bacterial strains to catabolize PAHs, their abilities to grow using naphthalene or phenanthrene as sole carbon source and energy were tested. Phenotype assays (Fig. 2) showed that *Bacillus* and *Acinetobacter* species were incapable to grow using naphthalene or phenanthrene, while *A. lwoffii* was able to grow using phenanthrene. These results are according to the implication of *Acinetobacter* species in the biodegradation of diverse pollutants such as aromatic compounds and chlorinated biphenyl's [27–29]. To detect the presence of genes involved in the degradation of PAHs

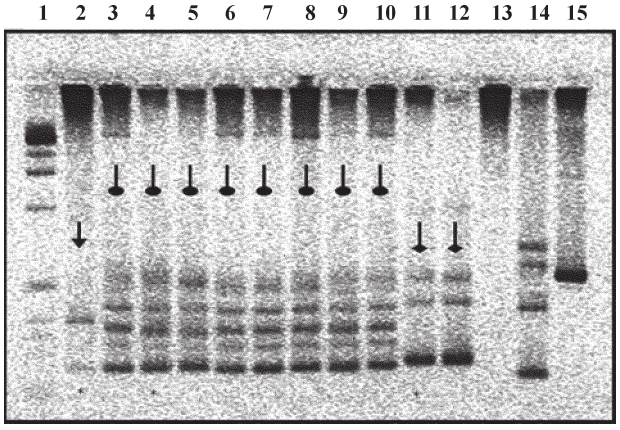

Fig. 1. DNA Fingerprinting of the eleven bacterial strains isolated from ETO wasted using GIRR-LIRRN primers pairs. Note that the presences of three different groups of strains are indicated with different symbols. The 16S rRNA gene sequencing confirmed that the three definite groups belong to: *Acinetobacter lwoffii* (1 strain); *Bacillus amyloliquefasciens* (8 strains) and *Bacillus pumilus* (2 strains). 1. 1 Kb DNA ladder; 2. BPC1; 3. BPC2; 4. BPC3; 5. BPC4; 6. BPC5; 7. BPC6; 8. BPC7; 9. BPC8; 10. BPC9; 11. BPC10; 12. BPC11; 13. PCR reaction control; 14 and 15. *P. putida* 9816 and *Pseudomonas* sp. R1 used as controls respectively

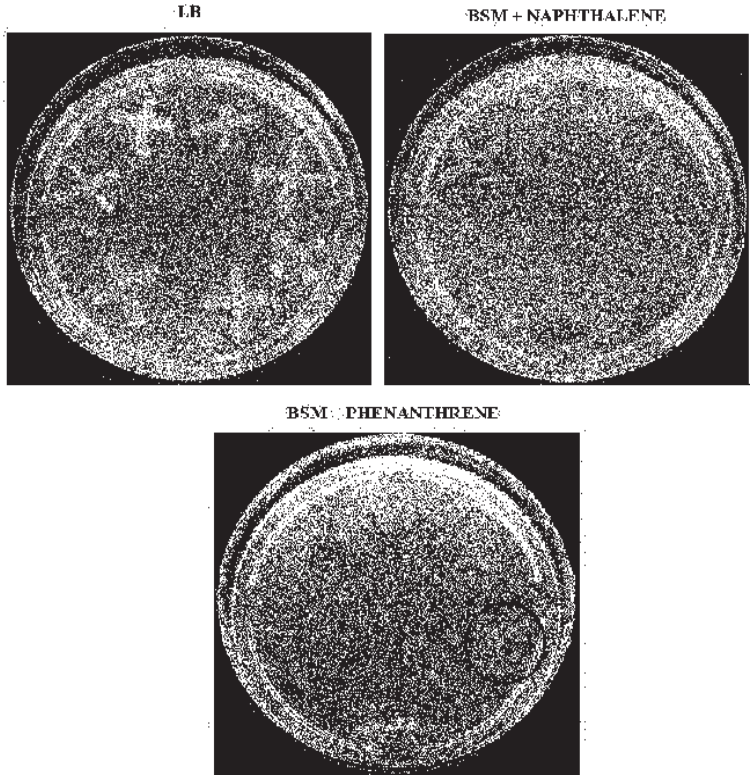

Fig. 2. Phenotypic characterization of bacterial strains according to their abilities to grow up using naphthalene or phenanthrene as sole carbon source and energy. Note that all bacteria were incapable to grow up using naphthalene or phenanthrene, excepting *A. lwoffi* that was able to grow up using phenanthrene. BPC2.2: *A. lwoffii*; BPC2.1 y BPC11 *B. pumilus*; BPC4 and BPC7: *B. amyloliquefasciens*; R1 and DH5a: *Pseudomonas* sp. R1 and *E. coli* DH5± used as positive and negative control respectively

in the bacterial strains, the *nah*Ac gene encoding the large subunit of the naphthalene dioxygenase from naphthalene upper catabolic pathway described in *Pseudomonas* strains 30] was amplified by PCR (Fig. 3A). A DNA fragment of 840 bp in *P. putida* R1 (positive control) was expected. The results showed that *nah*Ac gene was not present in all bacteria studied, excepting for a DNA band of minor size (about 800 bp) obtained in *A. lwoffii* (Fig. 3B). To determine if this band was corresponding to the *nah*Ac gene, a hybridization analysis was performed. The results (Fig. 3C) showed an 840 bp hybridization band in *P. putida* R1 (positive control) as expected. However, this band was not present in all bacteria studied, discarding the presence of the *nah*Ac gene, characteristic from the naphthalene catabolic pathway. Apparently, *A. lwoffii* has different genes for phenanthrene degradation from those reported in *Pseudomonas* strains. *Nocardioides* sp. KP7 (*phd* cluster genes) or *Alcaligenes faecalis* AFK2 (*phn* cluster genes) using Phthalate pathway for phenanthrene degradation are unable to degrade naphthalene [30]. Both molecular and phenotypic results suggest that *A. lwoffii* is able to catabolize phenanthrene via Phthalate pathway.

A. lwoffii is capable to use efficiently ETO wasted as sole carbon source

ETO included hazardous compounds such as PAHs and sometimes PCBs, which are highly toxic and difficult to degrade. Chemical analysis revealed that ETO used in this work include, among other fractions, 65.7 and 6.6 % of saturates and aromatics respectively; with <0.1 ppm of Aroclors 1242, 1254, and 1260. To determine the potential of the bacterial strains in ETO-biodegradation, their abilities to grow using different carbon sources were tested. The main results showed that *B. amyloliquefasciens* was incapable to use ETO as sole carbon source (compared with control) even there was difference in absorbance at 3 h ($p<0.01$) after inoculation, suggesting its low potential to be used as biocatalyst in ETO-biodegradation (Fig. 4A). *B. pumilus* and *A. lwoffii* showed significant difference at 18 to 27 h ($p<0.05$) and at 9 to 40 h ($p < 0.01$) respectively, using ETO as sole carbon source compared with the control

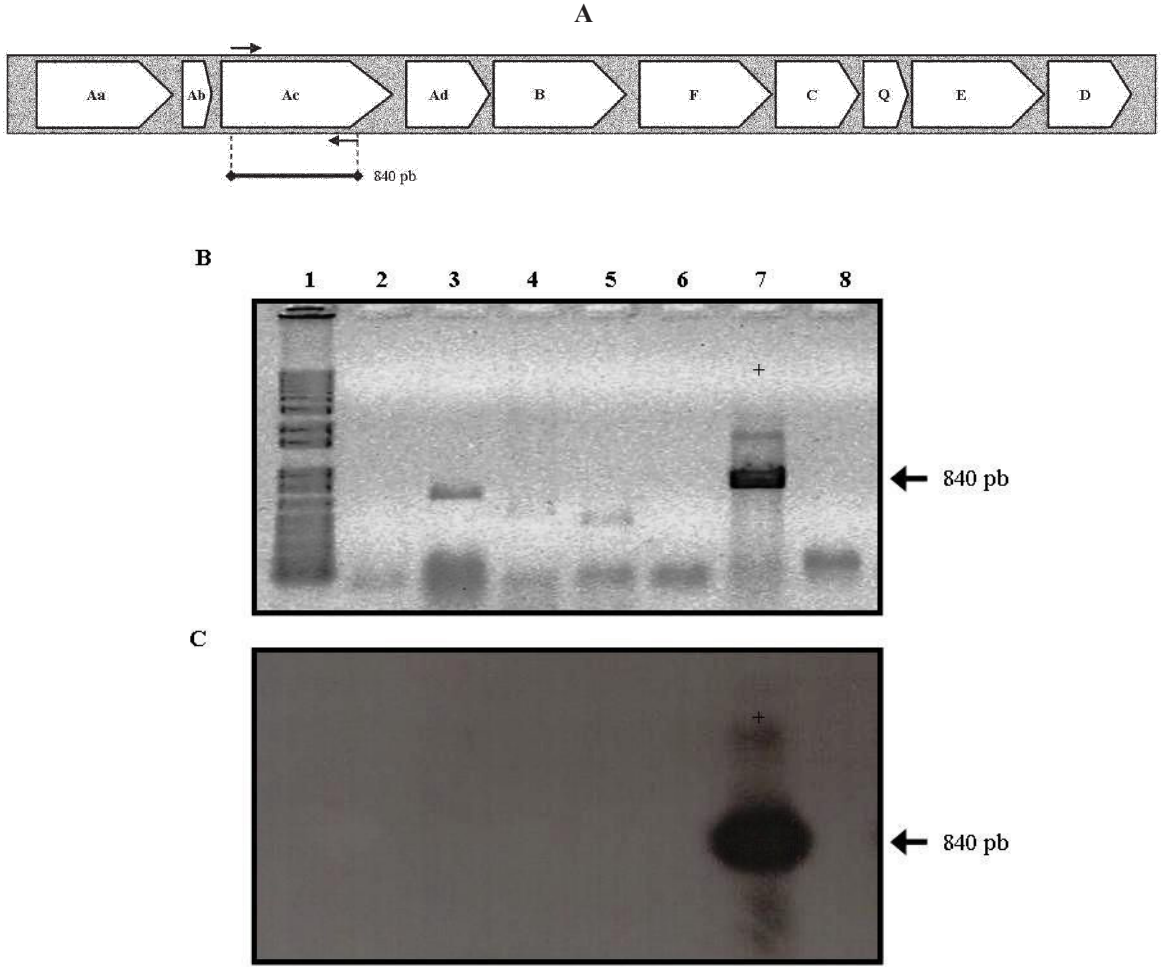

Fig. 3. Naphthalene upper catabolic pathway genes of Pseudomonas strains named *na*h operon (A). PCR amplification of the *nah*Ac gene encoding the naphthalene dioxygenase from operon *na*h using genomic DNA from bacterial strains isolated from ETO mixture disused and from *P. putida* R1 used as positive control (B). Southern hybridisation using an 840 bp DNA fragment internal to the *nah*Ac gene from *P. putida* R1 as probe (C). Note that the presence of the *nah*Ac gene was discarded in all bacteria studied. 1. 1 Kb DNA ladder; 2. *B. pumilus* 11; 3. *A. lwoffii*; 4. *B. amyloliquefasciens* 4; 5. *B. amyloliquefasciens* 7; 6. *B. pumilus* 10; 7. *Pseudomonas* sp. R1 (positive control); 8. PCR reaction control

(Fig. 4B y 4C). However, the growth in *A. lwoffii* was highly significant at 15, 24 and 36 h ($p < 0.01$) compared with *B. pumilus*. In order to confirm the potential of *A. lwoffii* in ETO-biodegradation, a great scale assay (100 mL) using ETO as sole carbon source was carried out as described in material and methods. Interestingly, chemical analysis of biotreated ETO at 15 d after inoculation revealed a decrease in 63.1 and 1.6% of saturates and aromatics fractions respectively. Compared with non-biotreated ETO values, a degradation of 4 and 80% of saturated and aromatics fractions were obtained respectively. These results suggest the ability of *A. lwoffii* to use initially low and then high molecular weight fractions presented in ETO. *Acinetobacter* species could use various complex mixtures of alkanes and aromatic compounds and chlorinated byphenils as carbon source, are considered as efficient oil degraders [27]; and important biosurfactants producers, which enhance biodegradation of saturated and aromatic compounds [31, 32]. The results discussed in this article support the high potential of *A. lwoffii* in environmental and biotechnological applications such as ETO-biodegradation.

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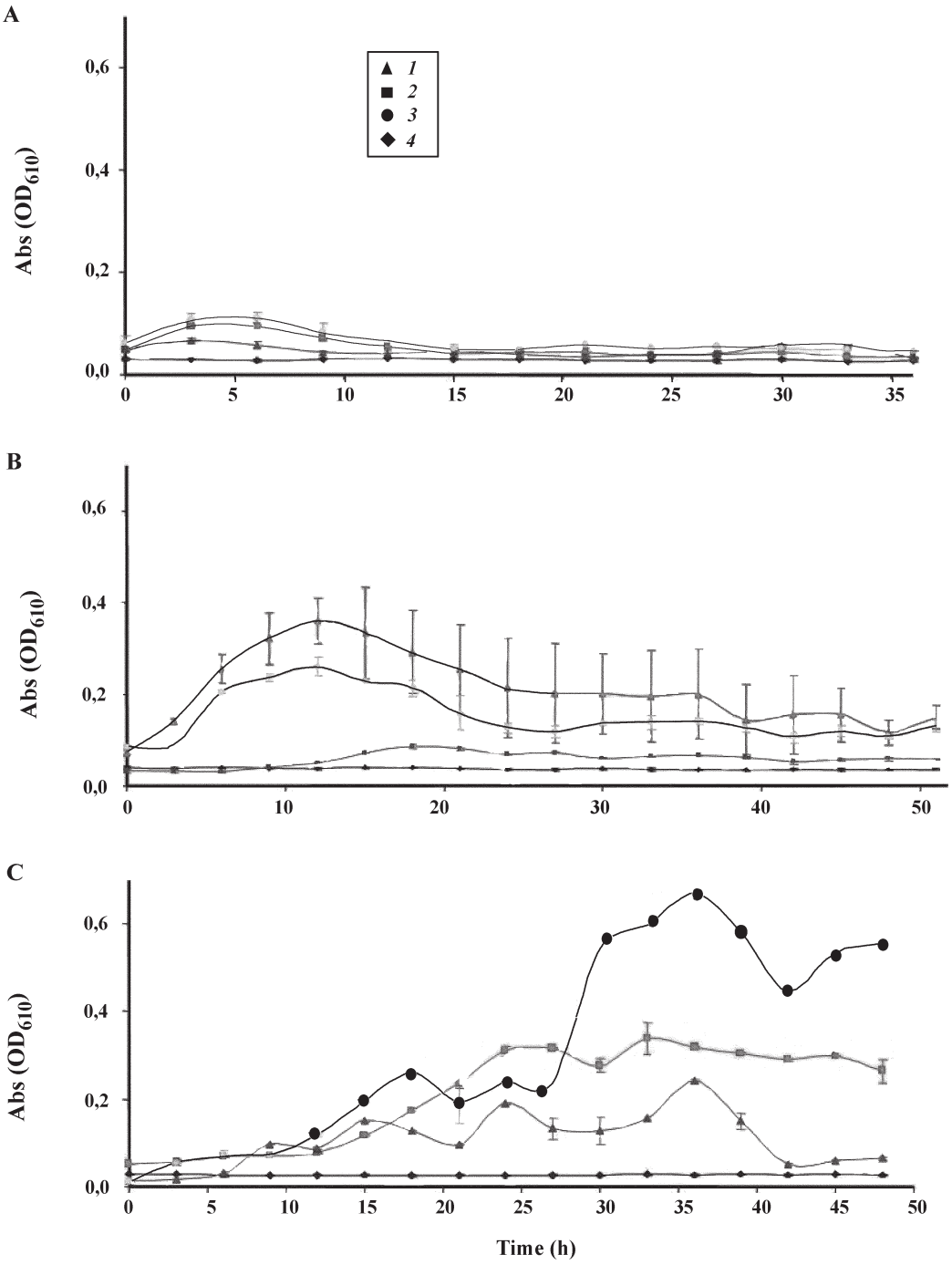

Fig. 4. Bacterial growth using different carbon sources: BSM without carbon source (negative control) (*1*); BSM + ETO 1% (*2*); BSM + YPG 1% (*3*); BSM + YPG 1% + ETO 1% (*4*). Bacterial growth of *B. amyloliquefasciens* (A); *B. pumilus* (B); and *A. lwoffii* (C). Note that the growth of *A. lwoffii* using ETO wasted as sole carbon source was highly significant ($p < 0.01$) compared with both Bacillus strains; indicating its high potential to be used as biocatalyst in ETO-biodegradation

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MOLECULAR CHARACTERIZATION OF BACTERIA ISOLATED FROM ELECTRICAL TRANSFORMER OIL WASTED

A. de Sisto, E. Fusella, H. Urbina, V. Leyn, L. Naranjo

(Unidad de Biotecnologна del Petryleo, Centro de Biotecnologна, Fundaciyn Instituto de Estudios Avanzados (IDEA). C/ Hoyo de la Puerta- Baruta, Sartenejas, Caracas 1080, Venezuela)

Electrical transformer oil (ETO) includes hazardous compounds such as PAHs and sometimes PCBs as dielectric fluids, which are highly toxic and persistent to degrade. Three species of bacteria belonging to: *Acinetobacter lwoffii, Bacillus amyloliquefasciens*, and *Bacillus pumilus* were isolated from an ETO wasted. Phenotype and molecular assays revealed the high potential of *A. lwoffii* to catabolize phenanthrene and ETO as sole carbon source and energy. This article reports for the first time the isolation, identification and the characterization of bacterial diversity hidden in ETO.