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Stenotrophomonas maltophilia isolated from gasoline-contaminated soil is capable of degrading methyl tert-butyl ether

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ABSTRACT

Background: Methyl tert-butyl ether (MTBE) is a pollutant that causes deleterious effects on human and environmental health. Certain microbial cultures have shown the ability to degrade MTBE, suggesting that a novel bacterial species capable of degrading MTBE could be recovered. The goal of this study was to isolate, identify and characterize the members of a bacterial consortium capable of degrading MTBE.
Results: The IPN-120526 bacterial consortium was obtained through batch enrichment using MTBE as the sole carbon and energy source. The cultivable fraction of the consortium was identified; of the isolates, only Stenotrophomonas maltophilia IPN-TD and Sphingopyxis sp. IPN-TE were capable of degrading MTBE. To the best of our knowledge, this report is the first demonstrating that S. maltophilia and Sphingopyxis sp. are capable of degrading MTBE. The degradation kinetics of MTBE demonstrated that S. maltophilia IPN-TD had a significantly higher overall MTBE degradation efficiency and rate (48.39 ± 3.18% and 1.56 ± 0.12 mg L^{-1} h^{-1}, respectively) than the IPN-120526 consortium (38.59 ± 2.17% and 1.25 ± 0.087 mg L^{-1} h^{-1}, respectively). The kinetics of MTBE removal by both cultures fit first-order and pseudo-first-order reaction models.
Conclusions: These findings suggest that S. maltophilia IPN-TD in axenic culture has considerable potential for the detoxification of MTBE-contaminated water.

1. Introduction

Methyl tert-butyl ether (MTBE) is an octane enhancer added to gasoline worldwide in order to reduce emissions of carbon monoxide, ozone, and unburned hydrocarbons [1]. Taking into consideration the high production levels of MTBE, it is not surprising to note that MTBE is often found in the environment. The main source of environmental contamination with MTBE is accidental fuel leakage during storage or transportation. According to its physicochemical properties (water solubility, 49 g L^{-1}; vapor pressure, 250 mm Hg at 25°C; Henry's law constant, 5.87×10^{-4} atm m^3 mol^{-1}), MTBE is the most water-soluble compound of reformulated gasoline [2,3]. It was reported that concentrations of MTBE in groundwater exceeding 20 μg L^{-1} are becoming a factor of huge global concern [4]. The toxic effects of MTBE are varied and range from the induction of neoplasms in both males and females of various animal species [5,6], to DNA damage causing single-strand breaks, double-strand breaks and oxidative base modification of human lymphocytes [7,8]. Furthermore, according to the criteria applied by several international agencies (International Agency for Research on Cancer, National Toxicology Program and US Environmental Protection Agency), there is sufficient scientific evidence to classify MTBE as a potential carcinogenic compound for humans [7].

Due to the deleterious effects of MTBE on human and environmental health, it is essential to remove this pollutant from contaminated surface and ground water streams. Currently, there are several options for remediating MTBE-contaminated waters. Traditional strategies include physical and chemical treatments, such as air stripping, adsorption, and advanced oxidation processes, all which have several drawbacks, primarily high economic and energetic costs [9]. In contrast, biodegradation, the most utilized groundwater remediation process [1],

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is economical and has the potential to achieve total mineralization of contaminants while offering an environmentally friendly technique.

The first work that involved the degradation of MTBE was the isolation of a mixed microbial culture capable of degrading MTBE from a chemical plant's biotreater sludge [10]. Since this work, a number of pure microbial cultures able to use MTBE as a primary carbon source have been isolated, such as *Achromobacter xylosoxidans* [11], *Enterobacter* sp. [12], *Methylibium petroleiphilum* PM1 [13], *Ochrobactrum cytisi* [14], *Mycobacterium austroafricanum* IFP 2012 [15], *Rhodococcus* and *Arthrobacter* [16]. From these microorganisms capable of degrading MTBE various degradation pathways of the compound have been proposed. The most recognized is in which the MTBE is oxidized to tert-butoxy methanol by the MTBE monooxygenase. The resulting tert-butoxy methanol may spontaneously dismutate to tert-butyl alcohol (TBA) and formaldehyde or is oxidized to tert-butyl formate (TBF). The TBF can be hydrolyzed to TBA and formic acid by an esterase enzyme. A second monooxygenase (TBA monoxygenase) catalyzes the hydroxylation of TBA to 2-methyl-1,2-propanediol (2-M1,2-PD). Later, 2-M1,2-PD is oxidized via MpdB enzyme generating the corresponding hydroxyisobutyraldehyde. Subsequently, hydroxyisobutyraldehyde is oxidized by MpdC enzyme to 2-hydroxyisobutyric acid (2-HIBA). The latter is then isomerized to the common metabolite 3-hydroxybutyryl-CoA or decarboxylated to acetone and CO_2 [17].

Despite the isolation and identification of bacterial genera with the potential to degrade MTBE, novel bacterial genera or species capable of removing MTBE with a similar or higher efficiency to the already reported values could be found. In the present work, a bacterial consortium with MTBE biodegradation capacity was obtained from gasoline-contaminated soils of an ex-refinery; its members were isolated and molecularly identified, and their MTBE degradation activity was compared with the bacterial consortium in order to select a bacterial culture suitable for the aerobic bioremediation of MTBE-polluted water.

2. Materials and methods

2.1. Soil samples

Soil samples were collected from an ex-refinery located at 19°29′28.73″N, 99°11′25.04″O in Mexico City, Mexico, which exhibited a concentration of hydrocarbons from the light fraction of 195.50 mg kg^{-1} (dry weight basis) according to the PROY-NMX-AA-105-SCFI-2013 norm [18]. Ten soil samples were taken from the area surrounding gasoline storage tanks at a depth of 30 cm and to a distance of 1 m between each sample. By mixing uniform weight subsamples (10 g) from each soil sample, a composite sample was formed. Subsequently, the composite sample was sieved through a 2-mm mesh opening and maintained at 4°C until use.

2.2. Obtaining a bacterial consortium through enrichment culture

The composite soil sample (1 g) was introduced in a flask containing 100 mL minimal medium (MM) with the following composition per liter: 1 g of $KH_2PO_4 \times 3H_2O$, 1 g of Na_2HPO_4, 0.1 g of $MgSO_4 \times 7H_2O$, 1 g of NH_4NO_3, 0.001 g of $CaCl_2 \times 2H_2O$, and 0.4 mg of $FeSO_4 \times 7H_2O$. MTBE (99.9% purity, Sigma-Aldrich, St. Louis, MO, USA) was added to the MM as the sole carbon and energy source to achieve a final concentration of 1% (v/v).

The flask was incubated at 30°C and 100 rpm until the culture reached an optical density at 590 nm ranging from 0.2 to 0.4. Enrichment subcultures were performed as follows: 1 mL of the culture was transferred to 100 mL of fresh MM supplemented with 1% (v/v) MTBE and incubated under the conditions described above. This procedure was performed nine times successively.

2.3. Metagenomic DNA extraction and evaluation of the consortium stabilization using PCR-DGGE

Metagenomic DNA from enrichment subcultures was extracted using a modified cetyltrimethylammonium bromide extraction protocol described by Murray and Thompson [19]. Briefly, 5 mL of subcultures were incubated at 37°C for 1 h with 6.25 μL of lysozyme (20 mg mL^{-1}), 2.5 μL of RNase (10 mg mL^{-1}), 1.24 μL of proteinase K (20 mg mL^{-1}) and 20 μL of 20% SDS. The DNA was obtained following a sequential extraction procedure with phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and precipitated with ethanol at 70% (v/v). DNA quality was estimated via electrophoresis in 1% (w/v) agarose gel in 1× TAE buffer (40 mmol L^{-1} tris, pH 8.3; 20 mmol L^{-1} acetic acid; 1 mmol L^{-1} ethylenediaminetetraacetic acid) at 80 V. The DNA was stained with a 0.5 μg mL^{-1} ethidium bromide solution and observed under ultraviolet light.

Next, DNA extracted from enrichment subcultures was used as a template to amplify the V6–V8 hyper variable regions of the bacterial 16S rRNA. The PCR reactions were carried out using the forward primer U968-GC [57-(GC clamp) CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-39] and reverse primer L1401 (59-CGG TGT GTA CAA GAC CC-39) in the following reaction mixture: 50 ng template DNA, 2.5 μL of 10× reaction buffer, 50 mM $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer, 1.5 U of Taq DNA polymerase (Invitrogen, Waltham, MA, USA), and adjusted to 25 μL total with water for injection. The PCR conditions were 1 cycle at 95°C for 10 min, followed by 25 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and finally, 1 cycle at 72°C for 7 min. The PCR products were analyzed using a DCode mutation detection system, following the procedure described by the manufacturer (Bio-Rad Laboratories, Hercules, CA, USA). Products of PCR reaction were applied to 8% (w/v) polyacrylamide gels prepared in 1× TAE buffer. The denaturing gradients contained 35–65% denaturant [100% denaturant corresponds to 7 M urea and 40% (w/v) formamide]. Electrophoresis was performed at 85 V and 60°C for 16 h. DGGE gel was fixed and DNA bands were visualized by silver staining with procedures previously reported [20]. A 100% match of DGGE band patterns between enrichment subcultures was used as the criterion to indicate that the consortium had reached a stable state.

2.4. Isolation and identification of the cultivable fraction

Successive decimal dilutions of the stabilized consortium were carried out until 10^{-8}, and 100 μL of each dilution was spread onto plates containing MM. The plates were incubated under MTBE atmosphere at 28°C for 24 h. The isolates were grouped according to their colonial morphology and their microscopic morphology.

2.5. Molecular identification of the cultivable fraction

Each morphotype was grown on nutrient broth overnight. Then, genomic DNA was extracted according to the protocol described in the Section 2.3.

The 16S rRNA gene was amplified by PCR with primers 8 (5′-CCG CGG GCG CCG CTG GAT AGT TTG CAG ATC CTG GCT CAG-3′) and 1492 (5′-GGC TCG AGC GGC CGC CCG GGT TAC CTT GTT ACG ACT T-3′) [21]. The reaction mixture and the PCR conditions were similar to those described above, except that the number of amplification cycles was 35 and the amplification stage was 2 min. The amplified fragments were purified using the Zymoclean™ Gel DNA recovery kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions, and the integrity was confirmed by electrophoresis in 1% (w/v) agarose gel in 1× TAE buffer. The sequencing of the amplified DNA fragments was performed on an ABI PRISM 310 Genetic Analyzer PE (Applied Biosystems, Foster City, CA, USA).

A collection of taxonomically related sequences obtained from EzTaxon database [22] using Blast [23] was included in the multiple alignment analyses with CLUSTAL X [24] and was manually edited using SEAVIEW software [25]. Similitude analysis was estimated using nucleotide sequences with MEGA [26]. Phylogenetic affiliations were based on the limits proposed by Rosselló-Mora and Amann [27], where <95, 95–97.5 and >97.5% define the taxonomic levels of family, genus and species, respectively. The sequences of the 16S rRNA genes reported in this paper have been deposited in the GenBank database under accession numbers from KP165412 to KP165416.

2.6. Search for putative MTBE degradation genes in the Stenotrophomonas maltophilia genome

BLASTP searches were performed with the protein sequences of the MTBE degradation pathway from *M. austroafricanum* IFP2012 using default parameters. The reference for the *Stenotrophomonas* genome was *S. maltophilia* K279a (GenBank accession NC_010943.1). Based on the putative genes, the following primers were designed: alkane 1-monooxygenase A, with the primer AMA-Fw (5′-TGG AGG ACT ATC TGT CCG GC-3′) and AMA-Rv (5′-TGG AGG ACT ATC TGT CCG GC-3′); alkane 1-monooxygenase B with AMB-Fw (5′-GAA GGC CCA CTC AAC ATC CA-3′) and AMB-Rv (5′-GAT CTT CAC GTC ATT GCC GC-3′); carboxylesterase with CE-Fw (5′-AAC GGT GGA ACA GGA AAC CG-3′) and CE-Rv (5′-CAT CGG ATA GCT GTG CCA CT-3′); propanediol-utilization with PU-Fw (5′-TCA TTC CCG AGT TCG ACC G-3′) and PU-Rv (5′-GATGCTCGATATCCAGGCCG-3′); and NADP-betaine with NB-Fw (5′-CAT GAG CAT CCT CGC CTA CG-3′) and NB-Rv (5′-ATT CCT TGT AGC CAC CGA CC-3′). Obtaining DNA from the isolates and the consortium was carried out according to the protocol previously described by Murray and Thompson [19] and reaction mixture and the PCR conditions were similar to those described in Section 2.3.

2.7. MTBE degradation assays

Bacterial cultures were propagated overnight in nutrient broth and the biomass was aseptically separated by centrifugation at 10,000 rpm for 15 min. Next, the cell pellets were washed three times with 0.85% saline solution. Finally, the cell suspensions were adjusted to the 0.5 nephelometric McFarland standard with 0.85% saline solution, and 3 mL of the resulting cell suspensions were inoculated into serological vials containing 27 mL MM supplemented with MTBE at 110 mg L^{-1}. Vials were sealed with PTFE/silicone septa and agitated at a constant shaking speed of 100 rpm.

For the selection of microorganisms with a high potential to degrade MTBE, a 2-d degradation assay was performed. In this test, the biomass and MTBE concentration were quantified at the beginning and end of the incubation period. For the degradation kinetics experiments, the biomass and MTBE concentration were monitored periodically every 6 h for 36 h. Additionally, experiments without biomass and experiments with heat-inactivated biomass were used as abiotic controls to evaluate MTBE losses by photolysis and/or adsorption to the cells or glass. All experiments were performed in triplicate, and mean values are reported.

2.8. Cell growth and MTBE degradation parameters

The maximum specific growth rate (μ_{max}, h^{-1}), the efficiency (E, %), and overall rate (V_g, mg L^{-1} h^{-1}) of MTBE degradation by the microbial cultures were estimated from the experimental data using [Equation 1], [Equation 2] and [Equation 3], respectively:

$$\mu_{max} = \frac{\ln\left(X_f - X_i\right)}{t_f - t_i} \qquad \text{[Equation 1]}$$

$$E = \left(\frac{C_f - C_i}{C_i}\right)(100\%) \qquad \text{[Equation 2]}$$

$$V_g = \frac{C_i - C_f}{T_f - T_i} \qquad \text{[Equation 3]}$$

where:

X_i = biomass concentration at the beginning of the exponential phase; X_f = biomass concentration at the end of the exponential phase; C_i = initial MTBE concentration; C_f = final MTBE concentration; T_i = initial time of incubation; T_f = final time of incubation; t_i = time at the beginning of the exponential phase; t_f = time at the end of the exponential phase.

2.9. MTBE degradation kinetics modeling

The experimental data of the degradation kinetics of MTBE were analyzed using zero-order [Equation 4], first-order [Equation 5] and pseudo-first-order [Equation 6] models, which have been widely used to understand degradation kinetics of xenobiotics. The kinetic models are expressed as follows:

$$C_t = C_0 - K_0\, t; \ T_{1/2}\ \frac{C_0}{2K_0} \qquad \text{[Equation 4]}$$

$$InC_t = InC_0 - K_1\, t; \ T_{1/2}\ \frac{In2}{K_1} \qquad \text{[Equation 5]}$$

$$C_t = C_0 - e^{-K_1' t}; \quad T_{1/2} = \frac{\ln 0.5}{-K_1'} \qquad \text{[Equation 6]}$$

where:

C_t = MTBE concentration at time $t = t$; K_0, K_1, K_1' = apparent rate constants; t = time; C_o = initial concentration of MTBE; $T_{1/2}$ = half-life period.

2.10. Statistical analysis

Statistical analyses of the experimental data were performed using ANOVA, and significant differences ($p < 0.05$) were determined using a Bonferroni test. All statistical analyses were conducted using GraphPad Prism® software version 5.03.

2.11. Analytical techniques

2.11.1. Cell concentration
Biomass was quantified by dry cell weight by filtering 10 mL of the culture samples through previously weighed 0.7 μm filters (Whatman GF/F) and subsequently drying the samples at 105°C to a constant weight. The cell concentration was obtained by calculating the amount of weight lost during the drying procedure. The filtrates were used to determine the residual MTBE concentration.

2.11.2. MTBE quantification
MTBE was recovered from each culture sample according to an organic extraction method described by Karimi et al. [28]. Briefly, 0.3 mL of a solution containing methanol, 42 μL trichloroethylene and 1 mg L^{-1} of n-hexane was injected rapidly into the sample solution using a 1 mL syringe and the mixture was gently shaken. The mixture was then centrifuged for 3 min at 4500 rpm. The dispersed fine particles of extraction phase were sedimented in the bottom of the test tube. 0.5 μL of the sedimented organic phase was removed and quantified. An MTBE standard (99.9% purity, Sigma-Aldrich) was used to construct a calibration curve. MTBE quantification was carried out using a mass spectrophotometer (Agilent Series 5975C) coupled

to a gas chromatograph (Agilent 6850 Series II Network System). The samples were injected into the capillary column (Agilent 19091S-433E; 30 m × 0.25 mm × 0.25 μm) of the gas chromatograph using an automated injector. The split ratio was 100:1. The initial oven temperature was 40°C, increased to 50°C at a rate of 3°C min^{-1}, and then increased to 58°C at a rate of 10°C min^{-1}. The temperature of the injector and detector was set at 250°C. Helium was used as the carrier gas at a constant flow of 0.7 mL min^{-1}.

3. Results and discussion

Previous studies have shown that an adequate adaptation and stabilization process of a microbial community is essential for the biodegradation of any recalcitrant organic compound [29]. Therefore, nine enrichment subcultures using MTBE as the sole carbon and energy source were analyzed by PCR-DGGE profiles in order to determine the moment when the stabilization of the bacterial consortium was reached. Based on the PCR-DGGE profiles of the V6–V8 region from the 16S rRNA genes, it was evident that stabilization was reached in the 8th transfer because most of the bands were conserved in the last two subcultures and six to eight discrete bands and other diffuse bands were observed (Fig. 1). In addition, a less complex bacterial community was observed from the first to the last subculture. Some bands were present in all subcultures (bands A and B), while other bands disappeared in the last subcultures (band D); this indicates that one or some bacteria are the main members of the consortium that adapted to subculture conditions. Conversely, bands appearing from the 7th subculture (bands C and E) suggest that the selection or enrichment of some members of the consortium occurred, but due to their low abundance, they were not displayed in the first subcultures. In a study similar to the one presented here, the PCR-DGGE profiles were used to determine the stability of subcultures [30], indicating that this molecular technique is efficient for determining the stability of subcultures. In this study, the stabilization of the bacterial consortium was obtained in the 9th subculture and it was maintained using MM supplemented with 100 mg MTBE L^{-1}. The obtained bacterial consortium was defined as the IPN-120526 consortium.

In order to know which members integrate the IPN-120526 consortium and to determine which ones have the ability to degrade MTBE, the isolation and identification of the cultivable fraction was performed. Five gram-negative bacterial strains were isolated from the IPN-120526 consortium and identified as *Pseudomonas delhiensis*, *Ochrobactrum* sp., *Aminobacter aminovorans*, *S. maltophilia* and *Sphingopyxis* sp. by 16S rRNA gene sequencing (Table 1). Assays for MTBE degradation by five isolates at an initial concentration of 110 mg L^{-1} and 2 d of incubation showed that only *S. maltophilia* IPN-TD and *Sphingopyxis* sp. IPN-TE exhibited the highest MTBE degradation efficiency, with values of 51.27 ± 3.74% and 33.60 ± 4.5%, respectively (Fig. 2). These efficiencies were significantly different ($p < 0.05$) compared to the values obtained for the heat-inactivated biomass and biomass-free controls (with values less than 1%). *P. delhiensis* IPN-TA, *Ochrobactrum* sp. IPN-TB and *A. aminovorans* IPN-TC were not able to degrade MTBE, as there was no difference between the MTBE degradation efficiency of these strains with respect to their abiotic controls ($p > 0.05$). The inability of these isolates to use MTBE as the sole carbon and energy source suggests that they have a symbiotic relationship with *S. maltophilia* IPN-TD and *Sphingopyxis* sp. IPN-TE, as they are not viable individually under the culture conditions for a long period of time. Although the genera *Pseudomonas* [31,32] and *Ochrobactrum* have been previously reported as degraders of MTBE [14], in this work *P. delhiensis* IPN-TA and *Ochrobactrum* sp. IPN-TB did not exhibit MTBE degradation ability. Furthermore, Sun. et al. [33] detected the presence of *Sphingopyxis* sp. as a member of a microbial community capable of removing MTBE under anaerobic conditions. However, the study did not demonstrate the ability of *Sphingopyxis* sp. to remove MTBE under aerobic conditions. Therefore, this is the first time that a member of the *Sphingopyxis* genus has been linked to aerobic MTBE degradation.

In regard to *S. maltophilia* IPN-TD, the results suggest that it is the main member of the bacterial IPN-120526 consortium involved in metabolizing MTBE. However, a cooperative association between *S. maltophilia* IPN-TD and the other members of the IPN-120526 consortium could occur for MTBE degradation. For example, *S. maltophilia* IPN-TD could initiate MTBE degradation, generating such intermediaries as TBF and TBA, which are later used by the other members of the consortium during MTBE degradation. However, the exact roles of each bacterial member of the consortium need to be further elucidated. In an attempt to prove the participation of *S. maltophilia* IPN-TD in the degradation of MTBE, a bioinformatics search for the putative genes involved in MTBE degradation was carried out. The selected genome was *S. maltophilia* K279a and the organism of reference was *M. austroafricanum* IFP2012 because this organism possesses the complete pathway of MTBE degradation [17]. The first and third step in the MTBE degradation pathway is performed by MTBE monooxygenase and TBA monooxygenase, respectively. The *S. maltophilia* K279a genome contains two related contiguous genes homologous to the organism of reference named as alkane 1-monooxygenase A and B (*alkBA*) (genes Smlt2102 and Smlt2103), with 22 and 19% homology, respectively. Similarly, a carboxylesterase gene (gene Smlt4132) was found, which is involved in the second step of the degradation pathway. Regarding the *mpdB* gene it was detected one gene (gene Smlt3611) related to *M. austroafricanum* IFP2012 and named as propanediol-utilization. Whereas *mpC* gene, four dehydrogenases genes were detected and one of them, the NADP-betaine (Smlt2238), had 39% homology to *M. austroafricanum* IFP2012. The detection of genes by PCR, previously identified in silico, only managed to amplify the alkane 1-monooxygenase A in *S. maltophilia* IPN-TD and the IPN-120526 consortium (Fig. 3). These preliminary results suggest that *S. maltophilia* IPN-TD initiates MTBE degradation, however, more molecular studies are needed to confirm the presence of genes involved in the degradation of MTBE in *S. maltophilia* IPN-TD.

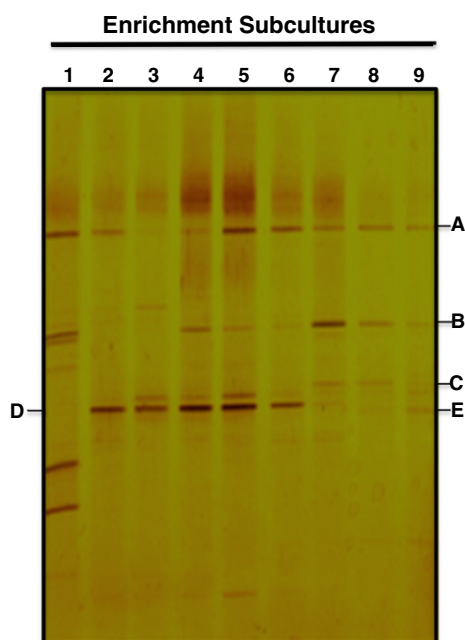### Enrichment Subcultures

Fig. 1. Stabilization of the enrichment subcultures by DGGE profiles of the 16S rDNA gene. 1–9 indicates the numbers of the enrichment subcultures. The stabilization of the bacterial consortium was reached in the ninth subculture. A–E shows representative bands.

Table 1
Phylogenetic affiliation of bacteria isolated from the IPN-120526 consortium.

Isolate name	Best math database[a] (GenBank Accession number)	Similarity (%)[b]	Microbial affiliation group
IPN-TA	*Pseudomonas delhiensis* RLD-1 (DQ339153)	98.26	*P. delhiensis*
IPN-TB	*Ochrobactrum triticy* SCII24 (AJ242528)	95.47	*Ochrobactrum* sp.
IPN-TC	*Aminobacter aminovorans* DMS7048 (AJ011759)	98.17	*A. aminovorans*
IPN-TD	*Stenotrophomonas maltophilia* ATCC 19861 (AB021406)	98.99	*S. maltophilia*
IPN-TE	*Sphingopyxis ginsengisoli* Gsoil 250 (AB245343)	97.23	*Sphingopyxis* sp.

[a] The best match was identified by using the EzTaxon server on the basis of 16S rRNA sequence data [22].
[b] Similarity percentage was estimated by considering the number of nucleotide substitutions between a pair of sequences divided by the total number of compared bases × 100. Taxonomic limits to define family, genera and species: x < 95, 95 < x < 97.5 and x > 97, respectively [27].

To our knowledge, this is the first work demonstrating that *S. maltophilia* IPN-TD has the capability to use MTBE as the sole carbon and energy sources.

To determine whether the bacterial consortium is a better MTBE degrader than the pure culture of *S. maltophilia* IPN-TD, a comparative kinetic study of cell growth and MTBE degradation was performed in the present work. Fig. 4a shows that as incubation time proceeded, the concentration of residual MTBE progressively diminished in both cultures. However, the most MTBE degradation carried out by *S. maltophilia* IPN-TD occurred during the first 18 h of incubation, and no significant difference was observed between the evaluated points after this period of time ($p > 0.05$). After 36 h of incubation, the levels of residual MTBE obtained in the *S. maltophilia* IPN-TD culture was lower than that obtained in the IPN-120526 consortium culture. Similarly, a higher efficiency of MTBE degradation by *S. maltophilia* IPN-TD was observed than was observed in the IPN-120526 consortium. During the MTBE degradation kinetics experiment, the cell growth of *S. maltophilia* IPN-TD tended to increase until 18 h of incubation, after which it remained almost constant until 36 h, whereas the cell growth of the IPN-120526 consortium increased until 24 h, after which no additional growth was observed (Fig. 4b). The kinetic parameters of cell growth and MTBE degradation were evaluated. There was no significant difference between the maximum specific growth rate (μ_{max}) achieved by *S. maltophilia* IPN-TD (0.070 ± 0.01 h^{-1}) and that reached by the IPN-120526 consortium (0.071 ± 0.022 h^{-1}). Comparing the μ_{max} of the cultures with those reported in others studies, both cultures obtained higher μ_{max} than that reported for pure (0.0083 h^{-1}) and mixed bacterial cultures (0.0083 h^{-1}) grown aerobically in culture media containing MTBE as the carbon and energy source [34].

Likewise, there was significant difference for the values of overall degradation efficiency and rate reached by *S. maltophilia* IPN-TD with respect to those obtained for the IPN-120526 consortium (Table 2). Both bacterial cultures were capable of degrading MTBE regardless of their growth phase (Fig. 4). These data suggest that *S. maltophilia* IPN-TD is the main bacteria responsible for degrading MTBE in the consortium; as above, it is supported by the amplification of the alkane 1-mooxygenase A (the first gene involved in the MTBE degradation pathway). However, we cannot discard the possibility that the other members of the consortium could contribute to the cleavage of metabolic intermediates produced by *S. maltophilia* IPN-TD. Because only residual MTBE was measured during the kinetics of degradation and not the intermediate metabolites, the IPN-120526 consortium may be required to carry out complete MTBE mineralization. Additionally, this work was focused on the cultivable fraction, and it is likely the non-cultivable fraction contributes to MTBE degradation.

The slow growth rates of *S. maltophilia* IPN-TD exhibited during MTBE degradation could be attributed to the effect of the MTBE on the bacterial metabolism. It has been reported that MTBE acts as an electron transport inhibitor or an uncoupler of ATP synthesis, and can result in the formation or accumulation of metabolic intermediates that inhibit cellular growth [35]. Furthermore, it has been suggested that the small amount of biomass produced from MTBE utilization may cause low MTBE degradation rates [36]. To evaluate the potential of *S. maltophilia* IPN-TD and the IPN-120526 consortium to degrade MTBE in aqueous solutions, the results obtained in this work were compared with other studies. Table 3 compares the overall degradation efficiencies and rates of MTBE degradation achieved by different microbial cultures. The degradation efficiency of MTBE at 110 mg L^{-1} by *S. maltophilia* IPN-TD ($48.39 \pm 3.18\%$) and the IPN-120526 consortium ($38.59 \pm 2.17\%$) was higher than those reported for pure cultures, such as *Rhodococcus* sp. (28%), *Sphingomonas* sp. (20%), and *Streptomyces* sp. (30%) [37], but not
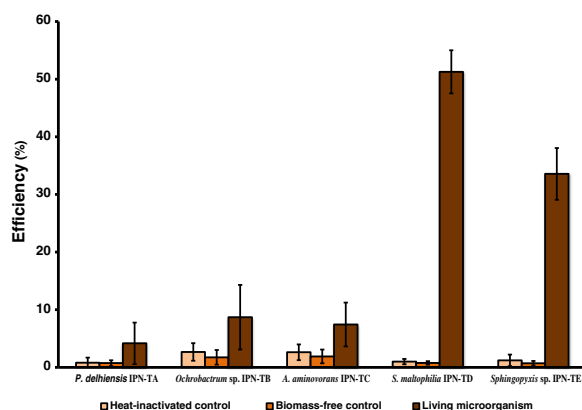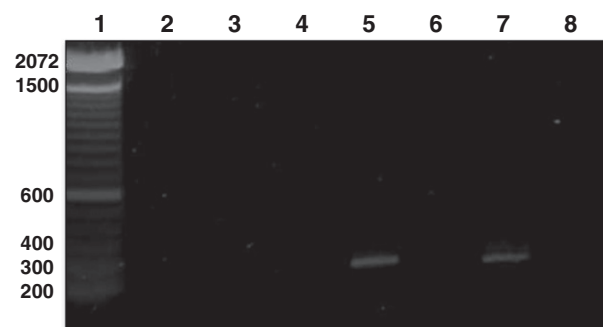

Fig. 2. MTBE degradation capacity by the cultivable fraction of the IPN-120526 consortium. The assay was performed at an initial concentration of MTBE of 110 mg L^{-1} and for 2 d at 28°C and 100 rpm. MTBE degradation efficiency was calculated according to [Equation 2]. Statistical analyses (ANOVA and Bonferroni test) were performed with residual MTBE concentrations at the end of the kinetic study. The comparison was made between each isolate with its respective abiotic controls. Each point on the graph represents the mean and standard error of 3 independent assays.

Fig. 3. Detection of alkane 1-monooxygenase A gene in the cultivable fraction and IPN-120526 consortium. Amplification by PCR was realized with signed primers described in Materials and methods section and the fragment was according with the expected size of 295 bp. Lane 1, molecular weight marker; lanes 2–6 represent *P. delhiensis* IPN-TA, *Ochrobactrum* sp. IPN-TB, *A. aminovorans* IPN-TC, *S. maltophilia* IPN-TD, *Sphingopyxis* sp. IPN-TE; lane 7, IPN-120526 consortium and lane 8, negative control.
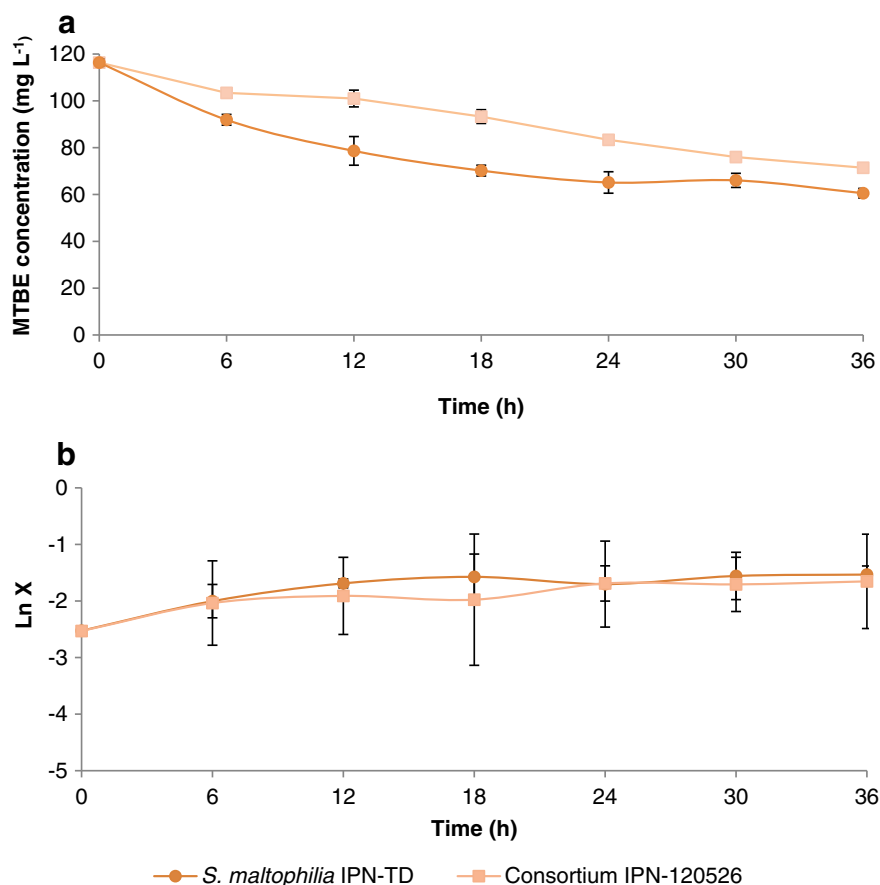
Fig. 4. Comparison of the kinetics of MTBE degradation between *S. maltophilia* IPN-TD and IPN-120526 consortium. a) Determination of the kinetics of MTBE degradation by the IPN-120526 consortium and *S. maltophilia* IPN-TD was carried out at an initial concentration of 110 mg L^{-1} and evaluated every 6 h until 36 h at 30°C and 100 rpm. b) Growth of the IPN-120526 consortium and *S. maltophilia* IPN-TD, X represents biomass concentration (dry weight). Each point on the graph represents the mean and standard error of 3 independent assays.

for *A. xylosoxidans* [11] and *Enterobacter* sp. [12]. Furthermore, the overall degradation rate reached by *S. maltophilia* IPN-TD (1.56 ± 0.12 mg L^{-1} h^{-1}) and the bacterial IPN-120526 consortium (1.25 ± 0.087 mg L^{-1} h^{-1}) was higher than that reported for mixed cultures [9,38,39], as well as for diverse pure cultures, such as *A. xylosoxidans* [11], *Enterobacter* sp. [12], *Rhodococcus* sp. [37], *Sphingomonas* sp. [37] and *Streptomyces* sp. [37]. Likewise, the overall degradation rate exhibited by *S. maltophilia* IPN-TD and the IPN-120526 consortium was higher and similar, respectively, than that reported for *M. petroleiphilum* PM1 (1.29 mg L^{-1} h^{-1}), which has been widely used as an indicator microorganism because of its outstanding ability to degrade MTBE [36]. Notably, *S. maltophilia* IPN-TD achieved higher overall MTBE degradation efficiency and rate compared to the IPN-120526 consortium.

Furthermore, the mathematical behavior that fit the MTBE degradation of our cultures was determined. The three mathematical kinetic models are shown in Fig. 5a–c. The apparent kinetic rate constants (K$_0$, K$_1$, K$_1'$), half-lives (T$_{1/2}$) and the regression equations (R^2) under given conditions for each reaction model are

presented in Table 4. Although the determination coefficient (R^2) of the three kinetic models is similar, the RMSE value is lower in both the first-order and pseudo-first-order model, indicating that the degradation kinetics of MTBE by the IPN-120526 consortium and *S. maltophilia* IPN-TD are described better by first-order and pseudo-first-order reaction models. This implies that the biodegradation of MTBE by the IPN-120526 consortium and *S. maltophilia* IPN-TD is a time-dependent process. Additionally, the half-life of the first-order and pseudo-first-order degradation reaction is independent of the initial MTBE concentration. The calculated degradation rate constant is 0.0134 h^{-1} and 0.0126 h^{-1} for the IPN-120526 consortium and *S. maltophilia*

Table 2
Growth and MTBE degradation parameters of the IPN-120526 consortium and *S. maltophilia* IPN-TD.

Culture	μ_{max}	E	Vg
Consortium IPN-120526	0.071 ± 0.022	38.59 ± 2.17[a]	1.25 ± 0.087[a]
S. maltophilia IPN-TD	0.070 ± 0.01	48.39 ± 3.18	1.56 ± 0.12

μ_{max}: maximum specific growth rate (h^{-1}); E: degradation efficiency (%); Vg: overall degradation rate (mg L^{-1} h^{-1}).
[a] Shows statistical difference between parameters $p < 0.05$.

Table 3
MTBE degradation parameters reported for various microbial cultures.

| Culture	Initial MTBE concentration (mg L^{-1})	Degradation efficiency (%)	Estimated overall degradation rate (mg L^{-1} h^{-1})	Reference
Mixed culture	10	100	0.06	[38]
Mixed culture	10	100	0.01	[39]
Mixed culture	146	85	0.43	[9]
A. xylosoxidans	100	78	0.65	[11]
Enterobacter sp.	100	60	0.36	[12]
Rhodococcus sp.	200	28	0.17	[37]
Sphingomonas sp.	200	20	0.12	[37]
Streptomyces sp.	200	30	0.18	[37]
M. petroleiphilum PM1	50	100	1.29	[36]
S. maltophilia IPN-TD	110	48.39 ± 3.18	1.56 ± 0.12	This work
Consortium IPN-120625	110	38.59 ± 2.17	1.25 ± 0.087	This work
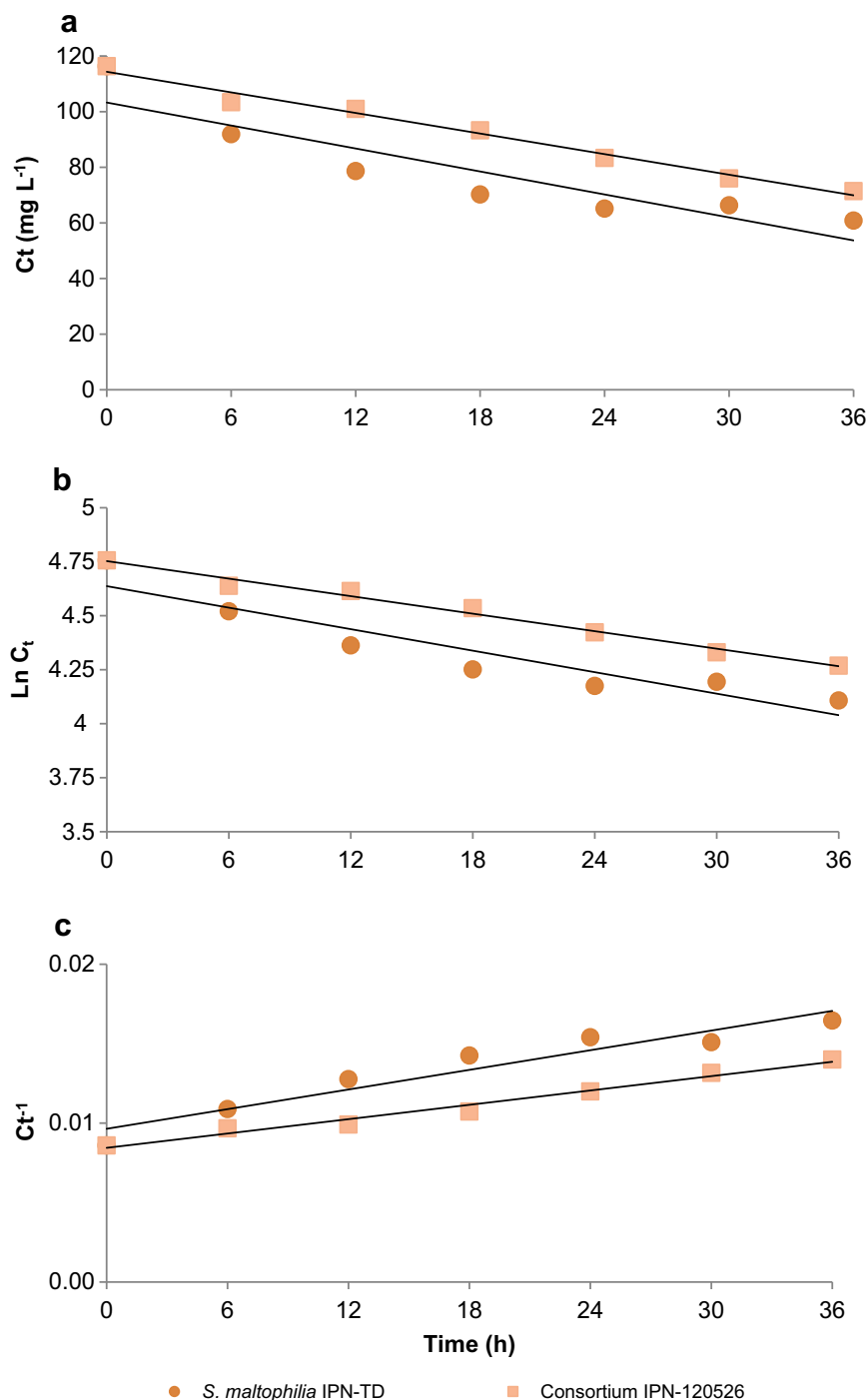
Fig. 5. Mathematical kinetic models of MTBE degradation by *S. maltophilia* IPN-TD and the IPN-120526 consortium. a) Zero-order, b) First-order, c) Pseudo-first order.

IPN-TD, respectively. It was also observed that the time needed to degrade half of the MTBE is almost similar for both microbial cultures (51.7 and 55.0 h, respectively).

The above results suggest that *S. maltophilia* IPN-TD and the IPN-120526 consortium have the ability to reduce high MTBE concentrations at a high rate, which makes these microbial cultures could be attractive for the bioremediation of MTBE-polluted wastewater. In addition, because *S. maltophilia* IPN-TD was the only member of the consortium that presented the alkane 1-monooxygenase A gene and the kinetic parameters of MTBE degradation by *S. maltophilia* IPN-TD are similar to those of IPN-120526 consortium, it is suggested that *S. maltophilia* IPN-TD is the bacteria which initiates the MTBE degradation producing

secondary metabolites that could be utilized by the other consortium members to achieve the mineralization of MTBE.

4. Conclusions

In the present study, a bacterial consortium able to degrade MTBE was isolated. Within the consortium's cultivable fraction, it was found that *S. maltophilia* IPN-TD exhibited the highest MTBE degradation efficiency. *S. maltophilia* IPN-TD degraded $48.39 \pm 3.18\%$ of 110 mg MTBE L^{-1} with an overall degradation rate of 1.56 ± 0.12 mg L^{-1} h^{-1}, which was significantly better than the IPN-120526 consortium. The kinetic behavior of MTBE removal by *S. maltophilia* IPN-TD and the IPN-120526 consortium was best fit

Table 4
Kinetic modeling of MTBE degradation by cultures IPN-120526 and *S. maltophilia* IPN-TD.

Parameters	Consortium IPN-120526	*S. maltophilia* IPN-TD
Zero-order regression equation	$C_t = 112.5 - 1.165\ t$	$C_t = 91.9 - 0.9396\ t$
K_0 (mg L^{-1} h^{-1})	1.165 ± 0.08497	0.9396 ± 0.1897
$T_{1/2}$ (h)	65.53	43.17
R^2	0.9706	0.8598
RMSE	2.13	4.76
First-order regression equation	$\ln C_t = 4.32 - 0.0134\ t$	$\ln C_t = 4.37 - 0.0126\ t$
K_1 (h^{-1})	0.01340 ± 0.001024	0.01260 ± 0.002254
$T_{1/2}$ (h)	51.72	55.01
R^2	0.9772	0.8866
RMSE	0.025	0.05656
Pseudo-first order regression equation	$C_t = 74.65 \cdot e^{-0.0134t}$	$C_t = 79.35 \cdot e^{-0.126t}$
K_1' (h^{-1})	0.01340 ± 0.001024	0.01260 ± 0.002254
$T_{1/2}$ (h)	51.72	55.01
R^2	0.9772	0.8866
RMSE	0.025	0.05656

to first-order and pseudo-first-order reaction models. These results suggest that *S. maltophilia* IPN-TD has potential for the detoxification of MTBE-contaminated water.

Conflict of interest

The authors declare that there are no conflicts of interest.

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