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ORIGINAL PAPER

Mineralization of methyl *tert*-butyl ether and other gasoline oxygenates by Pseudomonads using short *n*-alkanes as growth source

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Abstract Biodegradation of methyl *tert*-butyl ether (MTBE) by cometabolism has shown to produce recalcitrant metabolic intermediates that often accumulate. In this work, a consortium containing Pseudomonads was studied for its ability to fully degrade oxygenates by cometabolism. This consortium mineralized MTBE and TBA with C3–C7 *n*-alkanes. The highest degradation rates for MTBE (75 ± 5 mg $g_{protein}^{-1}$ h^{-1}) and TBA (86.9 ± 7.3 mg $g_{protein}^{-1}$ h^{-1}) were obtained with *n*-pentane and *n*-propane, respectively. When incubated with radiolabeled MTBE and *n*-pentane, it converted more than 96% of the added MTBE to ^{14}C–CO_2. Furthermore, the consortium degraded *tert*-amyl methyl ether, *tert*-butyl alcohol (TBA), *tert*-amyl alcohol, ethyl *tert*-butyl ether (ETBE) when *n*-pentane was used as growth source. Three Pseudomonads were isolated but only two showed independent MTBE degradation activity. The maximum degradation rates were 101 and 182 mg $g_{protein}^{-1}$ h^{-1} for *Pseudomonas aeruginosa* and *Pseudomonas citronellolis*, respectively. The highest specific affinity ($a°_{MTBE}$) value of 4.39 l $g_{protein}^{-1}$ h^{-1} was obtained for *Pseudomonas aeruginosa* and complete mineralization was attained with a MTBE: *n*-pentane ratio (w/w) of 0.7. This is the first time that Pseudomonads have been reported to fully mineralize MTBE by cometabolic degradation.

Keywords Biodegradation · Cometabolism · Methyl *tert*-butyl ether · Mineralization · Oxygenated additives · Pseudomonads

Abbreviations

ETBE	Ethyl *tert*-butyl ether
MTBE	Methyl *tert*-butyl ether
TAA	*Tert*- amyl alcohol
TAME	*Tert*-amyl methyl ether
TBA	*Tert*-butyl alcohol
CC	Cometabolic Coefficient
V_{max}	Maximum degradation rate
Ks	Half saturation constant

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Introduction

The use of oxygenated fuels has increased since the late eighties as a consequence of regulations established to improve air quality. Compounds used to attain the required oxygen level include ethers such as methyl-*tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), *ter*t-amyl methyl ether (TAME) and alcohols such as *tert*-amyl alcohol (TAA), *tert*-butyl alcohol (TBA) and ethanol. MTBE has been the most frequently used additive due to its low production cost, high oxygen content and ease of blending. MTBE promotes a better fuel combustion reducing the exhaust emissions of carbon monoxide and hydrocarbons. However, its release to the environment, mainly by gasoline storage and distribution systems leakages, has provoked extended groundwater pollution (Shih et al. 2004). The relatively recalcitrance of MTBE to microbial attack is intrinsic to its structure containing both an ether link and a branched moiety. Alkyl ethers are stable molecules (ΔG of the ether bond formation is 360 kJ mol^{-1}) and the high-energy demand for MTBE degradation is reflected by the low biomass yield efficiency (Fortin et al. 2001). Initial studies showed MTBE was highly recalcitrant but recently some authors have demonstrated that certain bacteria can degrade MTBE as their sole carbon and energy source (Hanson et al. 1999; Hatzinger et al. 2001; Ferreira et al. 2006) or in anaerobic conditions (Wilson et al. 2005).

Cometabolism has shown to be a mechanism for removing paraffinic, aromatic and chlorinated compounds in nature (Hovarth 1972) and has been reported to be relevant for MTBE biodegradation (Nava et al. 2007). Cometabolism involves the use of an additional carbon source for growth and some compounds present in gasoline have been reported for their ability to induce enzymes able to degrade MTBE. Cometabolic MTBE degradation has been reported with gaseous alkanes (Hardison et al. 1997; Steffan et al. 1997; Liu et al. 2001), *n*-pentane (Garnier et al. 1999), camphor (Steffan et al. 1997), ethanol (Piveteau et al. 2000) and cyclohexane (Corcho et al. 2000). Bacteria reported to degrade MTBE by cometabolism include: *Nocardia* (Steffan et al. 1997), *Mycobacterium* (Smith et al. 2003a), *Alcaligenes, Rhodococcus* (Hyman et al. 2000; Haase et al. 2006), *Gordonia* (Piveteau et al. 2000) and *Pseudomonas* (Steffan et al. 1997; Garnier et al. 1999; Smith et al. 2003b). However, a major concern in MTBE cometabolism is the accumulation of TBA. Only few studies have reported complete MTBE mineralization under cometabolism with consortia or single strains (Steffan et al. 1997; Morales et al. 2004; Haase et al. 2006). The detection of TBA in groundwater has created concern because it is a suspected human carcinogen (Cirvello et al. 1995). Currently, there are no federal regulatory limits for TBA in drinking water; however, some states in the USA have set their own limits (Martison 2005). Based on the above, it is now recognized that effective bioremediation strategies should promote MTBE mineralization.

In this work, we studied the cometabolic degradation of MTBE with C3–C7 *n*-alkanes by a consortium containing Pseudomonads and the kinetic characterization of two strains that were isolated from it. This consortium was previously reported for its ability to grow on gasoline and *n*-pentane (Morales et al. 2004) and developed an extended capability to mineralize MTBE and TBA using C3–C7 *n*-alkanes and to degrade structurally similar oxygenates with *n*-pentane as a results of continuous exposure to MTBE.

Materials and methods

Microorganisms

A consortium was obtained from samples of gasoline polluted soils and adapted to *n*-pentane and MTBE. Pure strains were isolated from the consortium on nutrient agar and re-adapted to grow on pentane in liquid medium during 3 months. Previous analysis by partial sequencing of 16S-rDNA (Morales et al. 2004) showed that three Pseudomonads, a *Pseudomonas aeruginosa*, a *Pseudomonas citronellolis* and one *Pseudomonas* sp. were present. Two strains (*Pseudonomas aeruginosa* BM-B-450 and *Pseudomonas citronellolis* BM-B-447) were deposited in the Industrial Culture Collection of the Instituto de Investigaciones Biomédicas (Universidad Nacional Autónoma de México) IIBM-UNAM WDCM48 (http://wdcm.nig.ac.jp/CCINFO/CCINFO.xml?48). Cell propagation was done in a 1-l cell culture spin flask (29300-02 Cole Parmer, USA) by replenishing every week half of the liquid volume with the mineral

medium and adding 35 mg MTBE. Additions of 75 mg of pentane were made twice a week.

Kinetics

Degradation experiments were carried out in microcosms under aerobic conditions using 125 ml serum bottles. The initial biomass was 20 $mg_{protein}$ l^{-1} in 20 ml of mineral medium, its composition in g l^{-1} was: K_2HPO_4, 2; KH_2PO_4, 1; NH_4Cl, 0.75; $MgSO_4$, 0.5; $CaCl_2$, 0.018; and 1 ml l^{-1} of trace element solution (Morales et al. 2004). In consortium degradation experiments, 1.5 mg of MTBE or TBA and variable amounts (0.5–5 mg_{carbon}) of *n*-alkanes (C3–C7) were added to microcosms. Bottles supplemented with *n*-pentane, *n*-hexane and *n*-heptane were sealed with mininert valves (614250 VICI, USA) and gaseous *n*-alkanes (propane and butane) were added directly to the serum bottles sealed with viton rubber stoppers. Experiments to compare protein and CO_2 production were also conducted adding only *n*-alkanes and no oxygenates. Additionally, the consortium was tested for the biodegradation of 1.5 mg of TAME, ETBE, TBA and TAA with variable amounts of *n*-pentane (0.5–5 mg_{carbon}).

The adaptation of isolated strains was done during 6 months, adding 1.5 mg of MTBE and 2.5 mg of *n*-pentane once a week. Kinetic parameters of isolated strains were obtained experimentally using 2.5 mg of *n*-pentane and MTBE in the range 0.7–7 mg of MTBE. Incubation of the microcosms was at 30°C on a rotatory shaker at 100 rpm. The experiments were carried out by triplicate.

Degradation rates were calculated using the Gompertz model (Morales et al. 2004). The cometabolic coefficient (CC) is the ratio of degraded oxygenated compound to the consumed *n*-alkane. CC_{100} is referred to the maximum CC obtained with 100% removal of the oxygenated compound. Mineralization was calculated from the carbon balance data as the ratio of experimentally measured CO_2 and the theoretical CO_2 from the mineralization of both the alkane and the oxygenated compound.

Radiolabeled ^{14}C-MTBE experiments

Uniformly labeled ^{14}C-MTBE in ethanol with a specific activity of 5.8 mCurie $mmol^{-1}$ was used in consortium mineralization tests. The experiment was performed in 160 ml serum flasks sealed with viton rubber stoppers with 20 ml of medium containing 20 $mg_{protein}$ l^{-1} of pre-grown cells. The initial radioactivity was 1.66×10^{-4} mCurie. Incubation was performed under the conditions described previously. The released ^{14}C–CO_2 was trapped in a glass tube with 3 mL of 4 M NaOH. Aliquots (1 ml) from the culture and from the NaOH solution were sampled and replenished with an equivalent volume of fresh NaOH 8 M at regular intervals. ^{14}C–CO_2 was quantified after fourfold dilution in ScintiVerse II liquid using a scintillation counter (Triathler multilabel tester, Hidex, Finland). The apparatus counting efficiency coefficient was 89%. A simultaneous experiment was similarly performed with unlabeled MTBE (1.5 mg), *n*-pentane (3 mg) and ethanol (1.6 mg) for chromatographic analysis. The mineralization experiments were carried out by triplicate.

Analysis

Chromatographic analysis

Ethers and *n*-alkanes were quantified by headspace analysis using gas chromatography (model 6890, Agilent Technologies, USA) with a flame ionization detector. The column used was a 30 m dimethylpolysiloxane × 250 µm (HP-1, Agilent Technologies, USA) with helium at 1.5 ml min^{-1}; hydrogen and air flow rates at 30 and 300 ml min^{-1}, respectively. Oven temperature was 40°C. All oxygenated compounds were analyzed using the same equipment and detector coupled to an autosampler for liquid analysis and a 30 m length × 320 µm OD × 0.25 µm film thickness acid modified polyethyleneglycol column (AT-1000, Alltech, USA). Helium flow was 1 ml min^{-1} and the oven temperature was 110°C.

Headspace analysis for CO_2 and O_2 were also performed by gas chromatography (model 580, Gow Mac, USA) equipped with a thermal conductivity detector and a concentric column (CTRI, Alltech, USA) at room temperature and a helium flow of 65 ml min^{-1}.

Protein quantification

The cell suspension was centrifuged and washed twice with mineral medium. It was then hydrolyzed with 0.1 M NaOH (1:1 volume) in boiling water

for 15 min. The Coomassie Brilliant Blue method (Sedmak and Grossberg 1997) was used with bovine serum albumin as standard.

Chemicals

MTBE (98% purity), ETBE (99% purity), TAME (97% purity), TBA (99% purity) and TAA (99% purity) were from Sigma Aldrich Chemicals Co (Milwaukee, Wis., USA). *n*-Pentane (99% purity), *n*-hexane (99% purity) and *n*-heptane (99% purity) were from J. T. Baker (Phillispurg, NJ, USA). *n*-Propane (99%), *n*-butane (99%) were from Matheson Tri-gas Alltech Associates (Twinsburg, OH, USA). ^{14}C-MTBE in ethanol with a specific activity of 5.8 mCurie mmol^{-1} was from Perkin Elmer Life Sciences Inc. (Boston, MA, USA) lot. 3498-63 and the scintillation liquid ScintiVerse II from Fisher Scientific (Fair Lawn, New Jersey, USA). Serum albumin bovine (96% purity) was from Sigma Aldrich Chemicals Co (Milwaukee, Wis., USA).

Results

Biodegradation of MTBE and TBA with different *n*-alkanes

The consortium was initially grown only on *n*-pentane and adapted for MTBE degradation during approximately 6 months. *n*-Propane, *n*-butane, *n*-hexane and *n*-heptane were tested alternatively at different alkane concentrations (between 0.5 and 5 mg C added to the 125 ml microcosms) to degrade 1.5 mg of MTBE or TBA. Table 1 summarizes the main kinetic results. The consortium grew in all the tested alkanes with higher cell yield obtained with shorter alkanes. The highest value was 0.47 $mg_{protein}$ mg_{alkane}^{-1} for *n*-pentane and the lowest 0.17 $mg_{protein}$ mg_{alkane}^{-1} for *n*-heptane. A comparison in protein production from experiments amended with *n*-alkanes and MTBE or TBA and only with *n*-alkanes was conducted (results not shown) and no differences were found between both experiments, confirming that MTBE and TBA were not used as growth source by the consortium. Apparent Ks values of the consortium (referred to the gas phase concentration) showed a better affinity to shorter *n*-alkanes but higher V_{max} rates with longer *n*-alkanes. MTBE and TBA degradation was carried out by the consortium with all tested *n*-alkanes obtaining values between 22 and 75 mg $g_{protein}^{-1}$ h^{-1} for MTBE and 29.1–86.9 mg $g_{protein}^{-1}$ h^{-1} for TBA. The highest MTBE degradation rates were observed with *n*-pentane and *n*-hexane while the maximum values for TBA were with *n*-propane and *n*-butane. The highest MTBE CC_{100} values were obtained for *n*-pentane (1.16) and *n*-hexane (1.09). In all cases, MTBE and TBA mineralization was above 90%. MTBE and TBA degradation was not observed in controls without *n*-alkanes. Due to the higher yield, CC and MTBE degradation rate, *n*-pentane was used in all the following experiments.

MTBE and other oxygenated compounds biodegradation with *n*-pentane

Figure 1 shows the kinetic behavior by the consortium with 1.5 mg of MTBE and 2.8 mg of *n*-pentane in a 125 mL microcosm. *n*-Pentane was totally

Table 1 Kinetic parameters for *n*-alkanes biodegradation and MTBE or TBA degradation by the consortium

	n-Propane	*n*-Butane	*n*-Pentane	*n*-Hexane	*n*-Heptane
Apparent Ks mg_{alkane} l^{-1}	20	57	67	37	73
V_{max} mg_{alkane} $g_{protein}^{-1}$ h^{-1}	323	270	769	1250	2000
Cell yield $mg_{protein}$ mg_{alkane}^{-1}	0.37	0.45	0.47	0.26	0.17
MTBE deg. rate mg $g_{protein}^{-1}$ h^{-1}	51.2 ± 2.1	63.3 ± 2.5	75 ± 5	67.7 ± 4.7	22 ± 0.9
TBA deg. Rate mg $g_{protein}^{-1}$ h^{-1}	86.9 ± 7.3	83.4 ± 6.2	57.1 ± 4.3	29.7 ± 3.2	29.1 ± 1.8
CC_{100} mg_{MTBE} mg_{alkane}^{-1}	0.38	0.4	1.16	1.09	0.48
% MTBE mineralization	95	93	96	98	92
% TBA mineralization	95	96	100	92	95

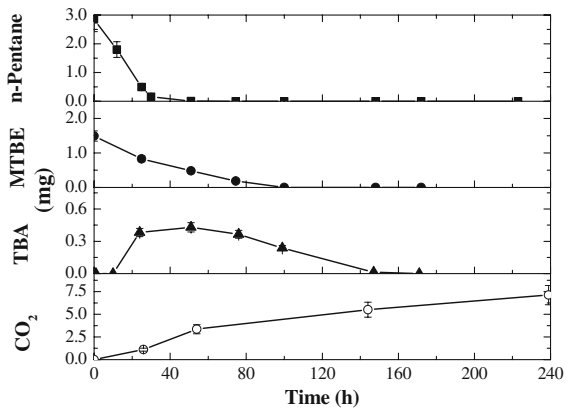

Fig. 1 Time course of *n*-pentane, MTBE, TBA and CO_2 evolution by the consortium in experiments amended with 2.8 mg of *n*-pentane and 1.5 mg of MTBE in 125 ml serum bottles. Error bars indicate standard deviation

consumed in approximately 30 h, at this time MTBE conversion was around 50%. Degradation continued and MTBE was not further detected at 100 h, corresponding to a degradation rate of 45 mg $g_{protein}^{-1}$ h^{-1}. The intermediate TBA was detected with a maximum value at around 50 h and total consumption at 150 h. Associated to the hydrocarbon degradation, maximum CO_2 production was after 240 h. Figure 2 shows the MTBE and *n*-pentane degradation rates for experiments with the consortium amended with different initial *n*-pentane concentrations and MTBE. The *n*-pentane degradation rate was a linear function of *n*-pentane added and a value of 625 ± 25 mg $g_{protein}^{-1}$ h^{-1} was observed with 5.3 mg of *n*-pentane. The maximum MTBE degradation rate was 75 ± 5 mg $g_{protein}^{-1}$ h^{-1} and was reached when more than 4 mg of *n*-pentane were added to the microcosms.

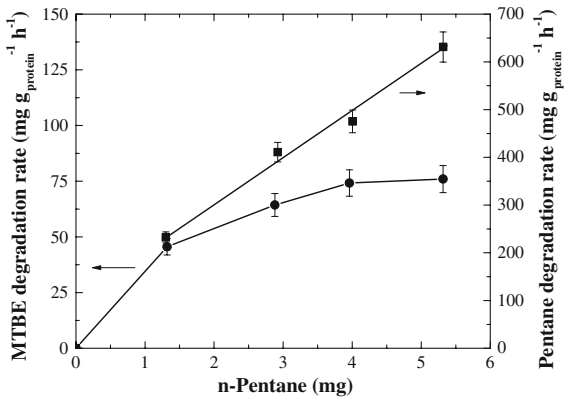

Fig. 2 MTBE (●) and *n*-pentane (■) degradation rates with different amounts of *n*-pentane added to the microcosms using the consortium containing Pseudomonads. Initial MTBE added was 1.5 mg in 125 ml bottles. Error bars indicate standard deviation

Table 2 summarizes the results for the degradation of different oxygenates with *n*-pentane. Degradation rates were obtained varying the amount of *n*-pentane between 0.5 and 5 mg of carbon in the 125 mL serum bottles. The highest degradation rates obtained for MTBE, TAME and ETBE were 75, 71.5 and 78.8 mg $g_{protein}^{-1}$ h^{-1}, respectively. The CC_{100} values obtained for MTBE and TAME with *n*-pentane were 1.16 and 1.31 $mg_{oxygenates}$ $mg_{n\text{-pentane}}^{-1}$, respectively; the lower CC_{100} value obtained for ETBE (0.53) indicated that more *n*-pentane was necessary to fully degrade it. Degradation rates for alcohols were 57.1 ± 2.3 and 105 ± 4.2 mg $g_{protein}^{-1}$ h^{-1} for TBA and TAA, respectively. Transient accumulation of TBA in the medium was observed in those MTBE degradation experiments when TBA degradation rate was lower than MTBE. On the other hand, TAA had a higher degradation rate than TAME and TAA accumulation was not observed.

In all cases, mineralization was >90% of the stoichiometric value expected from the degradation of the oxygenates. This result, coupled with the complete TBA and TAA disappearance, suggests complete MTBE and TAME mineralization.

Radiolabeled MTBE experiment

Experiments with radiolabeled MTBE were carried out to confirm its mineralization by the consortium. A triplicate unlabeled assay was conducted for chromatographic analysis in parallel with the radiolabeled MTBE experiment. Figure 3 shows the evolution of *n*-pentane, MTBE and the recovery of produced ^{14}C–CO_2. *n*-Pentane was degraded in approximately 85 h and MTBE in 250 h. Due to the ethanol presence in the assay, there was a degradation delay of around 150 h for MTBE and 50 h for *n*-pentane when compared to the results without ethanol (Fig. 1). Analysis of ^{14}C–CO_2 indicated a recovery of approximately 30% when *n*-pentane was totally consumed at 85 h, afterwards this value increased to 80% when MTBE was completely depleted and the maximum recovery was 96%.

Table 2 Summary of results by the consortium for different oxygenated compounds (1.5 mg) with *n*-pentane in 125 ml serum bottles. Maximum degradation rates were observed when 4 mg *n*-pentane were added

	Maximum degradation rate ($mg_{oxygenate}\ g_{protein}^{-1}\ h^{-1}$)	CC_{100} ($mg_{oxygenate}\ mg_{alkane}^{-1}$)	Mineralization (%)
MTBE	75 ± 5	1.16	96
TAME	71.5 ± 2.9	1.31	92
TBA	57.1 ± 2.3	1.22	100
TAA	105 ± 4.2	0.92	100
ETBE	78.8 ± 3.1	0.53	92

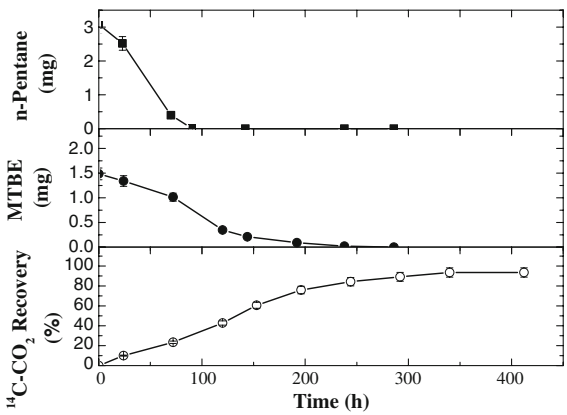

Fig. 3 Time course of *n*-pentane, MTBE mineralization and ^{14}C–CO_2 recover in microcosms experiments with the consortium containing Pseudomonads. Profiles of *n*-pentane and MTBE were obtained from unlabeled MTBE experiments whereas ^{14}C–CO_2 profile was obtained with radiolabeled MTBE. Error bars indicate standard deviation

MTBE degradation by pure strains

The three strains were adapted separately to *n*-pentane and MTBE in liquid media, after they were isolated from the consortium. Kinetic experiments, with 1.5 mg of MTBE and 2.8 mg of *n*-pentane, were periodically done and purity of the strains was verified. Only degradation activity in the *Pseudomonas aeruginosa* and *Pseudomonas citronellolis* strains was observed, whereas MTBE biodegradation was not significant (data not shown) by *Pseudomonas* sp. For both strains, MTBE degradation activity slowly increased along the 6 month adaptation period, during this period *Pseudomonas aeruginosa* showed a degradation rate of 73 ± 6 mg $g_{protein}^{-1}\ h^{-1}$ and cellular yield based on *n*-pentane consumption of 0.19 $mg_{protein}\ mg_{n\text{-pentane}}^{-1}$; for *Pseudomonas citronellolis,* the average degradation rate was 21 ± 3.2 mg $g_{protein}^{-1}\ h^{-1}$ with a cellular yield of 0.42 $mg_{protein}\ mg_{n\text{-pentane}}^{-1}$. In both cases TBA accumulation was not detected.

Table 3 shows the results from the experiments with different initial MTBE concentrations. Ks values and maximum degradation rates were 23 mg l^{-1} and 101 mg $g_{protein}^{-1}\ h^{-1}$ for *Pseudomonas aeruginosa* and 546 mg l^{-1} and 182 mg $g_{protein}^{-1}\ h^{-1}$ for *P. citronellolis*. Specific affinity ($a°_{MTBE} = V_{max}/Ks$) is generally used as an index reflecting substrate specificity. The value for *P. aeruginosa* ($a°_{MTBE}$ = 4.39 l $g_{prot}^{-1}\ h^{-1}$) was one order of magnitude higher than the value for *P. citronellolis* ($a°_{MTBE}$ = 0.33 l $g_{prot}^{-1}\ h^{-1}$) mainly due to the large difference in Ks values.

According to Table 3, TBA accumulation was related to MTBE: *n*-pentane ratio. While *Pseudomonas aeruginosa* showed no TBA accumulation for a ratio below 0.7, it was over 50% for *Pseudomonas citronellolis*. Table 3 also shows that higher MTBE: *n*-pentane ratios affect the *n*-pentane degradation in both strains indicating possible substrate competition.

Discussion

The results of this study provide evidence of complete MTBE mineralization by cometabolism with the consortium containing Pseudomonads and by two isolated strains. Our consortium previously showed the capacity to degrade MTBE in cometabolism with *n*-pentane (Morales et al. 2004) with a degradation rate of around 40 mg $g_{protein}^{-1}\ h^{-1}$. In this work, all the *n*-alkanes tested were able to induce MTBE degradation (Table 1) although, the highest degradation rate, 75 mg $g_{protein}^{-1}\ h^{-1}$, was still obtained with *n*-pentane. By comparison, MTBE degradation rates using short chain *n*-alkanes, such as *n*-propane, *n*-

Table 3 Kinetic parameters for MTBE cometabolic biodegradation with *Pseudomonas aeruginosa* BM-B-450 and *Pseudomonas citronellolis* BM-B-447

MTBE: *n*-pentane (w/w)	*n*-Pentane degradation rate ($mg_{n\text{-pentane}}\ g_{protein}^{-1}\ h^{-1}$)	Consumed MTBE (%)	Accumulated TBA (%)	CC ($mg_{degraded\ MTBE}\ mg_{degraded\ n\text{-pentane}}^{-1}$)	Ks (mg l^{-1}) V_{max} ($mg_{MTBE}\ g_{protein}^{-1}\ h^{-1}$)
Pseudomonas aeruginosa					
0	489	0	0	0	Ks = 23
0.2	ND	100.0	0.0	0.2	V_{max} = 101
0.5	404	94.3	2.8	0.4	
0.7	ND	96.0	2.8	0.7	
1.0	320	97.4	16.4	1.0	
1.3	280	97.2	29.9	1.2	
1.6	250	81.9	99.6	1.3	
2.0	ND	72.5	100.0	1.4	
Pseudomonas citronellolis					
0	393	0	0	0	Ks = 546
0.2	381	100.0	15.0	0.2	V_{max} = 182
0.5	ND	93.6	19.1	0.5	
0.7	ND	83.4	52.2	0.6	
1.0	308	69.8	58.1	0.7	
1.6	283	65.4	56.9	1.0	
2.2	ND	61.8	58.8	1.4	
2.5	255	54.8	65.0	1.4	

MTBE: *n*-pentane ratio was modified varying the amount of MTBE with a fixed initial amount (2.5 mg) of *n*-pentane in 125 ml serum bottles

ND—not determined

butane and *n*-pentane, have been reported to be in the range of 0.88 to 186 mg $g_{protein}^{-1}$ h^{-1} (Hardison et al. 1997; Smith et al. 2003b; Haase et al. 2006).

No substrate inhibition (Fig. 2) was present in the *n*-pentane concentration range tested. A different behavior was observed for *Pseudomonas aeruginosa* (Garnier et al. 1999) where inhibition was present even at low *n*-pentane amounts as 0.5 mg.

On the other hand, the MTBE: *n*-pentane ratio is relevant to attain the balance between the *n*-pentane necessary for growth and the induction of the MTBE-oxidizing activity. The CC_{100} for *n*-pentane was 1.16 and lower values (Table 1) were obtained for the other *n*-alkanes. The possible reasons for the different *n*-alkane requirement to achieve MTBE complete degradation could be: (a) different permeability of the cell to the tested *n*-alkanes, (b) the relative activities of the different consortium members to the different *n*-alkanes, (c) the presence of isoenzymes (d) the solubility of the *n*-alkane (C3 > C4 > C5 > C6 > C7). Until now, the highest CC has been obtained in our work by consortium containing Pseudomonads followed by a *Pseudomonas aeruginosa* with a CC of 0.7 (Garnier et al. 1999) with *n*-pentane and finally the fungus *Graphium* sp. with a CC of 0.04 (Hardison et al. 1997) with *n*-propane. An interesting characteristic is the ability of our consortium to use gaseous alkanes (*n*-propane and *n*-butane) as carbon growth source. *Pseudomonas mendocina* KR-1 grew well in C5–C8 *n*-alkanes but not on gaseous *n*-alkanes (C1–C4) (Smith et al. 2003b). *Pseudomonas aeruginosa* has been reported to grow on C5–C8 *n*-alkanes and also to oxidize *n*-butane, even though this compound does not support cell growth (van Eyk and Bartles 1968). There is only one report about a Pseudomonad that was able to grow on gaseous *n*-alkanes (Johnson and Hyman 2006). *Pseudomonas butanovora* utilizes *n*-alkanes ranging from C2 to C9 (Hamamura et al. 1999) but MTBE degradation was not studied. As far as we know our study is one of the few reports for *Pseudomonas* growth on gaseous alkanes.

A major concern in MTBE cometabolism is that TBA accumulation may result in an incomplete site cleanup. So if bioaugmentation is used, the microorganisms used should promote complete MTBE transformation. ^{14}C-MTBE assays by our consortium confirmed the mineralization (Fig. 3) previously suggested by carbon balances and high TBA degradation rates (Table 1). The cometabolic MTBE degradation by Pseudomonads has been previously reported associated, in most of the cases, to the alkane hydroxylase activity. The best-characterized alkane degradation is that by *Pseudomonas putida* GPol where the initial oxidation step (MTBE oxidation to TBA) is performed through an alkane hydroxylase system composed of a membrane bound non-heme iron monooxygenase. This alkane hydroxylase is known to dealkylate a variety of methoxylated alkane derivatives (Katapodis et al. 1988). The ability of this microorganism to degrade MTBE was initially tested with *n*-octane-grown cells with negative results (Steffan et al. 1997) and just recently the transformation of MTBE into TBA was positive (Smith and Hyman 2004). Other microorganism from the same genus, a *Pseudomonas aeruginosa* strain, was reported to degrade MTBE at a rate of 20 mg $g_{protein}^{-1}$ h^{-1}. However, MTBE mineralization was only of 20% corresponding to the methoxy group oxidation (Garnier et al. 1999). MTBE degradation was also reported for *Pseudomonas mendocina* KR-1 which was grown on 2-methylpentane (Hyman et al. 2000) and was able to transform MTBE to TBA when it was pre-grown on C5–C8 *n*-alkanes (Smith et al. 2003b). The MTBE degradation rate by the strain pre-grown in *n*-pentane was 186 mg $g_{protein}^{-1}$ h^{-1} and an average value of 322.6 mg $g_{protein}^{-1}$ h^{-1} was obtained when the growth was in *n*-hexane, *n*-heptane or *n*-octane; MTBE degradation rate was two-fold lower with *n*-butane and *n*-propane and no TBA degradation was observed (Smith et al. 2003b). In our work, high TBA degradation rates (Table 1) by the consortium containing Pseudomonads in cometabolisn were observed.

No specificity for the methoxy group in MTBE and TAME was observed in our consortium and both the ethoxy group in the ETBE and the tertiary methyl group in TBA were also degraded (Tables 1, 2). This differs to the observation by Smith and Hyman (2004) where these two compounds were not degraded. The degradation values (29–86.9 mg $g_{protein}^{-1}$ h^{-1}) observed with C3–C7 *n*-alkanes were slightly higher than the maximum value (27 mg $g_{protein}^{-1}$ h^{-1}) reported for *Mycobacterium* IFP 2015 pre-grown on *n*-hexane (Ferreira et al. 2007).

Mineralization by microbial consortia has been observed by joint cometabolism and commensalism among different members with specific roles in the

degradation (Beam and Perry 1974). In our case, both *Pseudomonas aeruginosa* and *P. citronellolis* showed MTBE degradation capacity (Table 3) and their different Ks and V_{max} values suggest cooperative degradation in the consortium. The high Ks value by *P. citronellolis* indicated its low affinity and it was lower than the value obtained (Ks = 1763 mg l^{-1}) by *Pseudomonas putida* GPol (Smith and Hyman 2004). *P. aeruginosa* was found to have one of the highest MTBE affinity but lower than an *Arthrobacter* strain (Liu et al. 2001) (Ks = 2.1 mg l^{-1}) although the maximum velocity reported in that case was lower (35.8 mg $g_{protein}^{-1}$ h^{-1}). Higher V_{max} were reported with *Xanthobacter* sp. (369.4 mg $g_{protein}^{-1}$ h^{-1}) (Hyman et al. 1998) and *P. mendocina* (322.6 mg $g_{protein}^{-1}$ h^{-1}) (Smith et al. 2003b), but in both cases TBA accumulation was observed.

Similar $a°_{MTBE}$ values were obtained with *Xanthobacter* sp. (1.27 l g_{prot}^{-1} h^{-1}) (Smith et al. 2003b), *M. vaccae* JOB5 (1.08 l g_{prot}^{-1} h^{-1}) (Smith et al. 2003a) and with *P. mendocina* (0.28 l g_{prot}^{-1} h^{-1}) (Smith et al. 2003b), although in those reports the authors observed TBA accumulation. In spite that a higher $a°_{MTBE}$ (16.95 l $g_{protein}^{-1}$ h^{-1}) was calculated for *Arthrobacter* (Liu et al. 2001), the authors observed low degradation rates and incomplete MTBE mineralization.

Possible hypotheses to explain the MTBE mineralization by individual Pseudomonads are based on the acquired ability to degrade TBA and include the evolution in the specificity of the alkane monooxygenase and the expression of isoenzymes. Further studies on genetic analysis and protein expression should generate evidence to evaluate the above hypotheses to explain bacterial adaptation.

Conclusions

The results of this study have shown special features of the cometabolic activities by the consortium containing Pseudomonads. One of them was the ability to fully mineralize MTBE supporting a high TBA degradation rate when *n*-alkanes (C3–C7) were used as carbon and energy sources. Pure strains isolated from the consortium, *Pseudomonas aeruginosa* and *Pseudomonas citronellolis*, degraded and mineralized MTBE. Cometabolic biodegradation constitutes an alternative for MTBE removal in aquifers. The use of an alternative carbon source provides a solution to overcome some specific problems of the bacteria that use MTBE as carbon source such as low cell yield and slow growth. The results of this work showed the potential for cleanup of MTBE polluted sites through the stimulation of microbial population by adding degradable non toxic and easily available *n*-alkanes.

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