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ORIGINAL PAPER

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Cometabolic biodegradation of methyl *t*-butyl ether by *Pseudomonas aeruginosa* grown on pentane

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Abstract A bacterial strain identified as *Pseudomonas aeruginosa* was isolated from a soil consortium able to mineralize pentane. *P. aeruginosa* could metabolize methyl *t*-butyl ether (MTBE) in the presence of pentane as the sole carbon and energy source. The carbon balance for this strain, grown on pentane, was established in order to determine the fate of pentane and the growth yield (0.9 g biomass/g pentane). An inhibition model for *P. aeruginosa* grown on pentane was proposed. Pentane had an inhibitory effect on growth of *P. aeruginosa*, even at a concentration as low as 85 μg/l. This resulted in the calculation of the following kinetic parameters (μ_{max} = 0.19 h^{-1}, K_s = 2.9 μg/l, K_i = 3.5 mg/l). Finally a simple model of MTBE degradation was derived in order to predict the quantity of MTBE able to be degraded in batch culture in the presence of pentane. This model depends only on two parameters: the concentrations of pentane and MTBE.

Introduction

Methyl *t*-butyl ether (MTBE) is one of several fuel oxygenates added to gasoline to improve fuel combustion and reduce the resulting concentrations of carbon monoxide and hydrocarbons. The massive production of MTBE, combined with its mobility, persistence and toxicity, make it an important pollutant. Biological treatment of soil, water or air contaminated with MTBE may offer a simpler, less expensive alternative to chemical and physical processes. In order for such treatment to be possible, specific microorganisms need to be identified and characterized.

It has taken a large effort and many years of research to find aerobic (Cowan and Park 1996; Salanitro et al. 1994) and anaerobic (Suflita and Mormile 1993; Mormile et al. 1994; Yeh and Novak 1994) consortia able to degrade MTBE as the sole source of carbon and energy. A number of degradation rates are quoted in the literature, but they are often difficult to compare because of differences in their methods of determination. Examples are 34 mg h^{-1} g^{-1} dry biomass (Salanitro et al. 1994) or 97.8% 170 mg/l MTBE in 95 h (Cowan and Park 1996). In anaerobic conditions the consortium is totally inefficient in its ability to metabolize MTBE (Mormile et al. 1994; Yeh and Novak 1994) with a degradation, in the best case, of 50% of MTBE over a period of 152 days. The biodegradation of MTBE in presence of alkanes by a consortium was recently illustrated (unpublished results). It was demonstrated that the consortium degraded MTBE in the presence of pentane at a rate of 800 μg mg $biomass^{-1}$ day^{-1}.

Pure microorganisms have also been studied for their ability to degrade MTBE as sole source of carbon and energy. Three strains characterized by Mo and coworkers (1997) were able to degrade 28% of the MTBE present at a concentration of 200 ppm, over a period of 2 weeks. In anaerobic conditions, *Acetobacterium woodi* and *Eubacterium limosum* were unable to degrade any MTBE, but were able to degrade several unbranched ethers (Mormile et al. 1994). Few studies have addressed the cometabolic biodegradability of MTBE by pure cultures. In one recent study (Hardison et al. 1997), MTBE was degraded by a filamentous fungus (*Graphium* sp.) in the presence of butane at rates varying between 1.3 and 10.5 nmol h^{-1} mg dry $weight^{-1}$. Another recent study reported the biodegradation of MTBE, mainly by three propane-oxidizing strains, with rates of 9.2, 4.2 and 0.4 nmol min^{-1} mg cell $protein^{-1}$ (Steffan et al. 1997).

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A demonstration of the ability of a microorganism (Hardison et al. 1997; Steffan et al. 1997) to degrade MTBE in the presence of alkanes could provide important insights into the possible environmental fate for such compounds, which are found in gasoline in high concentration. The objective of this work was to isolate and characterize the organism responsible for the cometabolic degradation of MTBE in the presence of pentane from a consortium that was capable of degrading gasoline to completion.

Materials and methods

Microorganism

The enrichment cultures were developed from various soil samples by successive MTBE addition. Those showing growth were transferred to 125-ml bottles containing fresh mineral salt solution (Whittenbury et al. 1970) with 1 g/l KNO_3 as nitrogen source and pentane. After ten consecutive transfers, the cultures were plated (agar plate with salt solution) in a pentane gas atmosphere in a desiccator and incubated at 30 °C for 3 days. Isolated colonies were characterized for their general morphology and microbial properties. The pure culture was identified by Gram staining and by cellular fatty acid (FAME) analyses by Analytical Services Inc. (Williston, Vt., USA). It was deposited at the National Culture Collection of Microorganisms under number CDBB-B-1230 (Colección Nacional de Cultivos Microbianos, CINVESTAV – Departamento de Biotecnología; Apdo. Postal 14-740; México D.F. 07000; Mexico).

For the kinetic and cometabolism studies with *P. aeruginosa*, 0.08 mg (2 ml) dry biomass was added to 125-ml bottles in the presence of minimum mineral salt (20 ml) and the bottles were stoppered with Teflon Mininert valves. A source of carbon and energy (pentane) was added with a syringe at different concentrations with or without MTBE. Bottles were incubated at 30 °C on a rotatory shaker at 250 rpm. The degradation of pentane and MTBE as well as O_2 consumption and CO_2 production were monitored by gas chromatography.

Chemicals

Methyl *t*-butyl ether (98%, ρ = 0.740 g cm^{-3}), Pentane (99%, ρ = 0.626 g cm^{-3}), *t*-butyl alcohol (99%, ρ = 0.77 g cm^{-3}) were from Aldrich Chemicals (Milwaukee, Wis.).

Analyses

The concentrations (from the headspace of each microcosm) of pentane and MTBE were measured by taking a 200-µl gas sample and injecting it into a gas chromatograph (Hewlett Packard (HP), model 5890 series II) connected to a flame ionization detector (FID). An HPI column (methyl-silicone gum) 5 m × 0.53 mm was used. The injector temperature was set at 150 °C, the detector at 210 °C and the column at 60 °C. Nitrogen was used as a carrier gas with a flow rate of 1.7 ml/min.

t-Butyl alcohol concentration was measured from 1 ml sample and passed through a 0.22-µm filter (type GV) to separate microorganisms. Samples of 5 µl were then injected into the FID gas chromatograph with a column temperature of 120 °C.

Oxygen and CO_2 concentrations were measured in a thermal conductivity detection gas chromatograph (Gow Mac series 550) with a concentric CTR-1 column, Alltech, USA) with helium as the carrier gas at a flow rate of 65 ml/min. The operating temperatures were: injector 30 °C, detector 70 °C and column 30 °C.

Determination of kinetic parameters

Equation 1: logistic model

This logistic model, adapted from Wolf and Venus (1992), was used to relate substrate (S) consumption with time. At given conditions the substrate degradation (pentane) is directly proportional to biomass production (low maintenance rate and initial biomass of 0.08 mg/bottle). The equation can therefore be written as:

$$S = \frac{S_0}{1 + [(S_0 - S_i)/S_i]e^{-\mu t}} \tag{1}$$

where S = substrate consumed (mg/bottle), S_i = initial substrate consumed (mg/bottle), S_0 = maximum substrate consumed or initial substrate (mg/bottle), μ = specific growth rate (h^{-1}) and t = time (h).

The specific degradation rate (v) was also determined using this model. It is a symmetrical function: at the inflexion point ($S_0/2$), the degradation rate is equal to $\mu S_0/4$ and the biomass is equal to Y_g S_m)/2 + initial biomass (which is negligible in this case. Y_g represents the growth yield. (v) is therefore equal to $\mu/2Yg$ expressed in milligrams of substrate consumed per h and per gram of dry biomass.

Equation 2

Equation 2 is the integrated form of the first-order growth equation coupled with Monod kinetics as presented by Pirt (1975). This model was used to determine the apparent saturation constant when parameters Y_g, X_0, S_0 and μ_{max} had been determined. The growth yield was determined from experimental data and the maximum growth rate was calculated from Eq. 1.

$$t = \frac{P\ \mathrm{In}\{[Y_g(S_0 - S) + X_0]/X_0\} - Q\ \mathrm{In}(S/S_0)}{\mu_{max}} \tag{2}$$

where $P = (K_s'Y_g + S_0Y_g + X_0)/(Y_gS_0 + X_0)$, $Q = (K_s'Y_g)/(Y_g\ S_0 + X_0)$, K_s' = apparent saturation constant (mg/bottle), Y_g = growth yield (g/g), X_0 = initial biomass (0.08 mg/bottle) and μ_{max} = maximum growth rate (h^{-1}).

Equation 3

Equation 3 is derived from the inhibition model also described by Pirt (1975) and allows real saturation and inhibitory constants to be evaluated under inhibitory conditions:

$$\mu = \frac{\mu_{max}SK_i}{(SK_i) + (K_sK_i) + S^2} \tag{3}$$

where S = initial substrate (mg/bottle), K_s = real saturation constant (mg/bottle) and K_i = inhibitory constant (mg/bottle).

At the inflexion point of the curve predicted by equation 3, the substrate concentration is determined as: $S_{crit} = (K_i\ K_s)^{1/2}$. At S_{crit} the growth rate is maximum (μ_{max}).

Results

Identification and characterization of the isolate

A pentane-degrading microorganism was isolated from soil samples obtained from gasoline-polluted sites and identified as *P. aeruginosa* from cellular fatty acid analysis. The isolated strain of *P. aeruginosa* was able to degrade MTBE when cometabolized in the presence of pentane (Fig. 1). No MTBE degradation was observed in the absence of pentane.

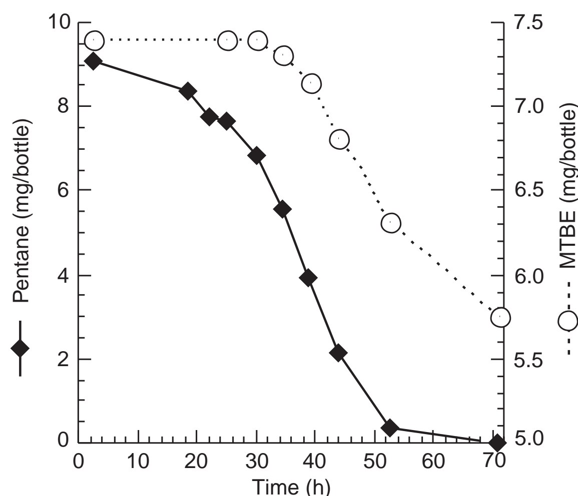
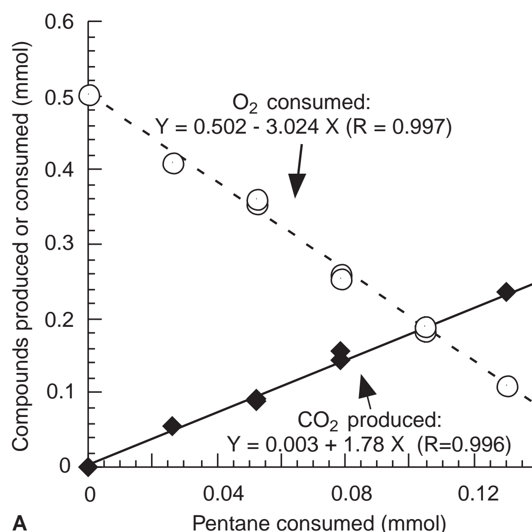


Fig. 1 Cometabolic degradation of methyl *t*-butyl ether (MTBE) by *Pseudomonas aeruginosa* grown on pentane



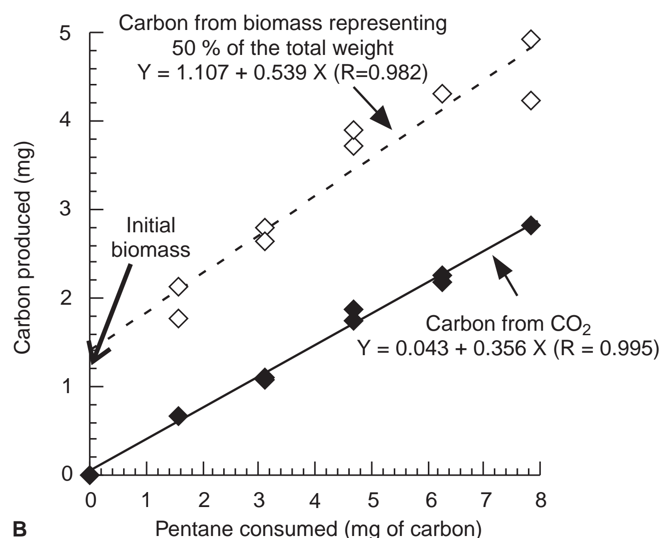


Fig. 2 **A** Consumption of O_2 and production of CO_2 by *P. aeruginosa* grown on pentane. **B** Carbon balance of pentane degradation by *P. aeruginosa*

Determination of the carbon balance of *P. aeruginosa* grown on pentane

From respirometry studies (Fig. 2A), oxygen consumption and CO_2 production were determined. Three moles of O_2 were consumed and 1.75 mol CO_2 was produced per mole of pentane consumed giving a CO_2/O_2 ratio of 0.6. The carbon balance was established to determine the fate of pentane by measuring biomass and CO_2 production (Fig. 2B). Ninety percent of the total initial carbon was accounted for (the remaining 10% could be in the carbonate form or present in intermediary compounds); 54% of the carbon was transformed into biomass (crude formula: $CH_{1.8}O_{0.5}N_{0.2}$) and 36% appeared as CO_2. A Y_g value of 0.9 g of biomass/g pentane was therefore obtained, assuming a biomass carbon content of 50% (Pirt 1975).

Determination of kinetic parameters of *P. aeruginosa* grown on pentane

The results shown in Fig. 2B suggest a negligible maintenance rate (m_0) as the biomass produced was proportional to the amount of pentane consumed even at low initial pentane concentrations in the bottle and up to the maximum value of 15 µl pentane/bottle. The specific growth rate (μ) and the apparent saturation constant (K_s') were calculated on the basis of the initial pentane. The specific growth rate was obtained by fitting from Eq. 1 [conditions: negligible m_0 and low initial biomass (0.08 mg/bottle)] for each experiment. Then, using Eq. 2 and fixing parameters Y_g as 0.9, X_0 as 0.08 mg/bottle and S_0 as 6.6 mg/bottle (determined by Eq. 1), the respective K_s' values were calculated. As seen in Fig. 3 the maximum growth rate (μ_{max}) was around 0.19 h^{-1} and the specific degradation rate of pentane (V_{max}) was 105 mg h^{-1} g dry biomass^{-1} (see calculations following Eq. 1 in Materials and methods). As shown in Fig. 3, the apparent saturation constant K_s' increases while the growth rate μ decreases, when the initial pentane introduced into each bottle was augmented. This indicated a probable substrate inhibition of enzyme activity, such as that reported by Pirt (1975) with the same substrate.

A more detailed study was therefore undertaken to determine the degradation of pentane by *P. aeruginosa* and the influence of MTBE. The K_s (0.016 mg/bottle) and the K_i (13.69 mg/bottle) of *P. aeruginosa* in the presence of pentane were calculated by Eq. 3 with a regression coefficient of 0.995 (Fig. 4). Considering the gas/liquid equilibrium for pentane, as expressed by Henry's constant (128×10^3 Pa m^3 mol^{-1} at 25 °C), saturation and inhibitory constants were calculated in the liquid as K_s equal to 2.9 µg/l and K_i = 3.5 mg/l. The growth rate increased up to a critical value of S

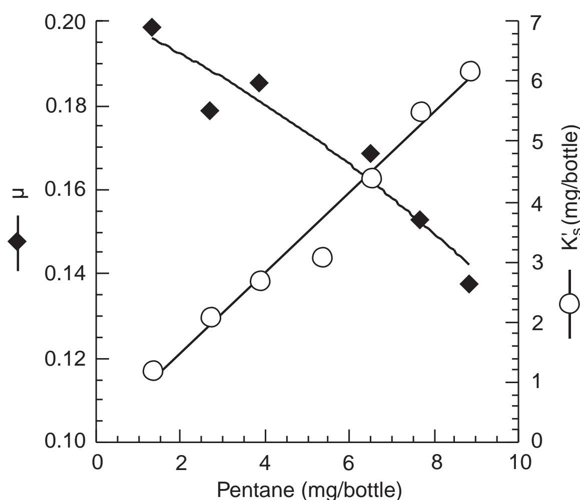


Fig. 3 Calculated μ and K_s' values as a function of initial pentane added per bottle

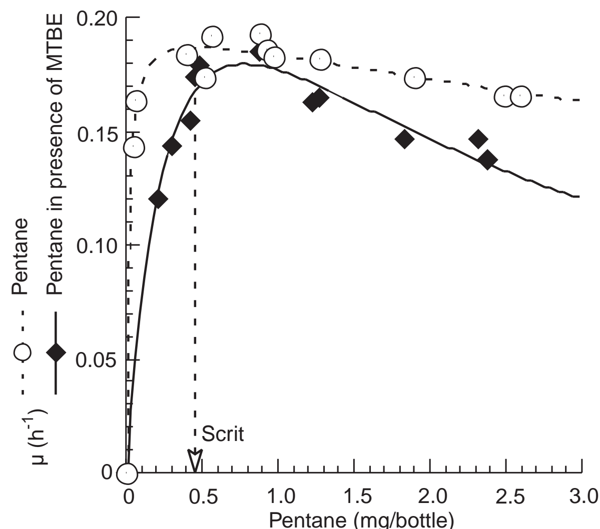


Fig. 4 Calculated μ values with and without MTBE (10 µl per bottle) when *P. aeruginosa* was grown on pentane

(S_{crit}) equal to 0.47 mg/bottle or 85 µg/l as shown in Fig. 4.

The same experiment was done in the presence of a fixed initial MTBE concentration (10 µl/bottle) to determine its influence on pentane degradation by *P. aeruginosa*. As seen in Fig. 4 for pentane concentrations lower than 0.5 mg/bottle, the calculated μ is lower with MTBE. After S_{crit}, a constant maximum growth rate (μ_{max} around 0.19 h^{-1}) between 0.5 mg and 1 mg pentane per bottle was detected. This result indicates a competitive inhibition between pentane and MTBE. At higher concentrations of pentane, the growth rate is lower with MTBE, indicating an inhibition by MTBE of pentane degradation. For MTBE the Henry constant at 25 °C is 55 Pa m^3 mol^{-1}, about 2000 times lower than for pentane. The K_s of MTBE, as determined by kinetic modeling (Materials and methods, Eq. 2), was about 4.5 mg/bottle or 185 mg/l.

Determination of the degradation rate of MTBE and *t*-butyl alcohol

Figure 1 shows the degradation curve for pentane and MTBE over time with a bottle containing 10 µl MTBE (300 mg/l in the liquid phase) and 14 µl pentane (1.6 mg/l in the liquid phase), which was incubated with *P. aeruginosa*. During the early stages (0–30 h), pentane was degraded, producing biomass, but there was no degradation of MTBE. Between 30 h and 50 h, the degradation rate of MTBE was maximum (3.9 nmol min^{-1} mg cell $protein^{-1}$). After 60 h, pentane was completely consumed and the degradation rate of MTBE slowed down.

The mineralization rate was then determined (Fig. 5) by studying production of CO_2 resulting from pentane degradation with or without MTBE, after 3 days of culture. CO_2 from pentane degradation represented 0.317 mg expressed in milligrams of carbon. CO_2 from the degradation of pentane in the presence of MTBE (10 µl/bottle) represented 0.351 mg expressed as milligrams of carbon. The difference between the two values of CO_2 comes from the cometabolic biodegradation of MTBE, which was equal to 0.034 mg carbon. The total MTBE degraded was equal to 0.173 mg, expressed in milligrams of carbon. Mineralization of MTBE was calculated by taking into account the following ratio: mass of CO_2 (from MTBE degradation) over total mass

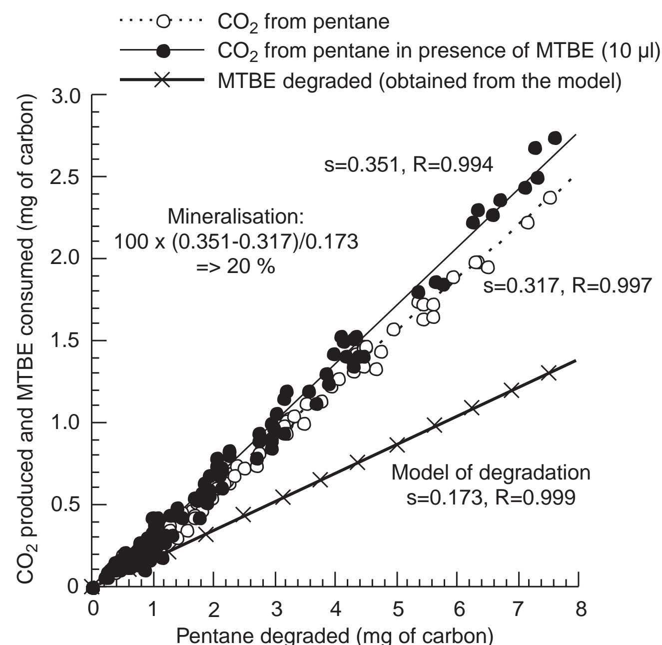


Fig. 5 Determination of the mineralization rate of MTBE after 3 days in batch culture (complete degradation of pentane and end of degradation of MTBE)

of MTBE degraded. Each value is expressed in mg carbon. Under these conditions, mineralization of MTBE was 20%. It is important to note that, after 3 days of incubation, MTBE degradation stops a few hours after complete degradation of pentane (*t*-butyl alcohol was not measured in these experiments).

In order to measure the ability of *P. aeruginosa* to degrade, *t*-butyl alcohol, its degradation was measured over a 4-day period in the presence of pentane. The initial concentration in the liquid phase was 100 mg/l with an absorbance of 1.4 at 540 nm. The degradation rate was calculated as 0.95 ± 0.05 nmol min^{-1} mg cell $protein^{-1}$ at 30 °C. Another experiment consisted in leaving *P. aeruginosa* without pentane over a period of 2 days. The resulting bacterial cells were unable to degrade any *t*-butyl alcohol, demonstrating that pentane was required for their cometabolic activity.

Degradation rate of MTBE by *P. aeruginosa* grown on pentane in batch culture

The aim of the following experiment was to establish a model for MTBE degradation by *P. aeruginosa*, in batch culture and in cometabolism with pentane. The degradation of MTBE was analyzed after 3 days relative to the concentration of MTBE (mg/bottle) and the concentration of pentane (mg/bottle), which determines the quantity of final biomass and the time necessary for complete degradation (a maximum of 3 days).

MTBE degradation was linear as a function of the ratio of MTBE to pentane, as seen in Fig. 6 for all five experiments (3, 5, 9, 12 and 15 µl initial pentane/bottle). Slopes (percentage of MTBE degraded) were calculated from Fig. 6 and compared with the initial concentration of pentane. When the concentration of pentane increased (regression coefficient = 0.99), the fraction of MTBE degraded increased as well (slope = 0.0235).

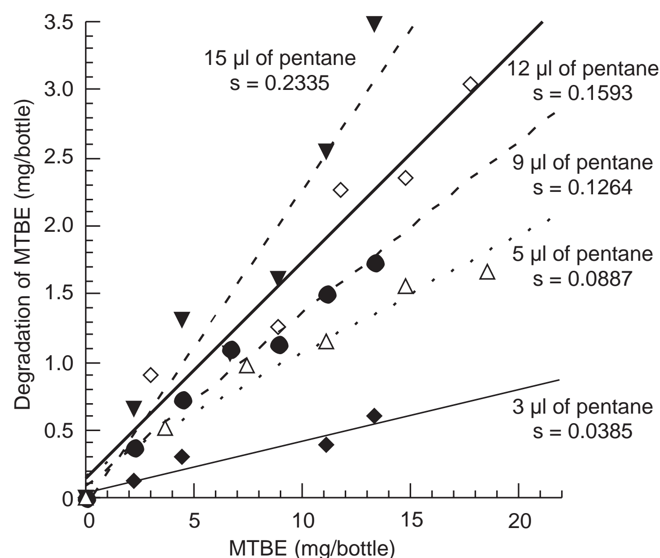


Fig. 6 Degradation of MTBE as a function of the concentration of pentane per bottle and the ratio of pentane in each bottle

To correlate the data a multiple linear model yielded:

$$Q_{\mathrm{MTBE}} = 0.0235 \times c_{\mathrm{pentane}} \times c_{\mathrm{MTBE}}$$

where Q_{MTBE} is the mass of MTBE degraded per bottle after 3 days of culture (at which time the degradation of pentane was complete), c_{MTBE} is the initial concentration of MTBE (mg/bottle) and c_{pentane} is the initial concentration of pentane (mg/bottle).

Discussion

P. aeruginosa isolated from gasoline-contaminated soil degraded pentane as sole source of carbon and energy and was able to degrade MTBE when cometabolized with pentane as seen in Fig. 1. A maximum growth rate on pentane of 0.19 h^{-1} was calculated, which is equivalent to a biomass doubling time, t_d, of 3.65 h. Real saturation (K_s = 2.9 µg/l) and inhibitory constants (K_i = 3.5 mg/l) for pentane were obtained by incorporating the equilibrium between the gas and liquid phases.

The ratio between the K_s of MTBE (185 mg/l) and the K_s of pentane (2.9 µg/l) was calculated as 64 000. The enzymes involved in the degradation process therefore have a much higher affinity for pentane than for MTBE. The K_s for microorganisms able to degrade MTBE as the sole source of carbon and energy has been reported (Cowan and Park 1996) to be 12.9 mg/l, which is 14 times lower than that calculated with *P. aeruginosa* during degradation of MTBE by cometabolism. The value of K_s reported for MTBE is much higher than those usually reported for microbial substrates (Pirt 1975).

A kinetic study in the presence of MTBE indicated, in the presence of low amounts of pentane (less than 0.5 mg/bottle), a lower growth rate for the same quantity of pentane per bottle, but with approximately the same maximum growth rate (about 0.19 h^{-1}). Figure 5 illustrates this competitive inhibition between MTBE and pentane, thus suggesting a single enzymatic site for the two compounds as reported by Steffan et al. (1997) and Hardison et al. (1997) on alkane-oxidizing microorganisms.

In a study presented by Steffan and coworkers (1997, the best microorganisms ENV421 and ENV425 were able to degrade respectively 9.2 and 4.6 nmol min^{-1} mg cell $protein^{-1}$ at 28 °C with 100 mg/l MTBE with an A_{550} of 4. It has also been reported that a *Pseudomonas putida* strain can degrade 0.4 nmol min^{-1} mg cell $protein^{-1}$. Hardison et al. (1997) obtained, with *Graphium* sp. grown on butane, a maximum rate of 10.5 nmol h^{-1} mg dry $weight^{-1}$ (MTBE concentration = 200 µmol/l), which (considering that proteins from the cell biomass account for 50% of the total weight) is 0.35 nmol min^{-1} mg of cell $protein^{-1}$. With *P. aeruginosa* a degradation rate of 3.9 nmol min^{-1} mg cell $protein^{-1}$ (Fig. 1, with a ratio MTBE/pentane of 0.7) was determined. Steffan et al. (1997) reported a MTBE mineral-

ization of 25% (at the end of the degradation of MTBE and before the degradation of *t*-butyl alcohol) presumably resulting from the complete oxidation of the methoxy methyl group of MTBE for the three microorganisms. The results obtained in the present study are similar, with a MTBE mineralization of 20% (1/5 of the carbon).

The consortium studied by Salanitro et al. (1994) was able to degrade MTBE as the sole source of carbon and energy at a rate of 13 nmol min^{-1} mg cell $protein^{-1}$ (34 mg h^{-1} g dried $biomass^{-1}$). Pure alkane-oxidizing microorganisms (present study; Hardisson et al. 1997; and Steffan et al. 1997) were able to degrade MTBE at rates varying between 0.35 and 9.2 nmol min^{-1} mg cell $protein^{-1}$. For *P. aeruginosa* there were no significant differences in the rates obtained as compared with the consortium from where it was originally isolated (unpublished results)

The results obtained with *P. aeruginosa* can be compared to those of Steffan et al. (1997). The *t*-butyl alcohol degradation rate was 0.95 ± 0.05 nmol min^{-1} mg cell $protein^{-1}$ with *P. aeruginosa* (a *t*-butyl alcohol concentration of 160 mg/l and an A_{540} of 1.4). The best microorganisms in Steffan's study, ENV421 and ENV425 were able to degrade respectively 2.4 and 1.6 nmol min^{-1} mg cell $protein^{-1}$ at 28 °C with 100 mg *t*-butyl alcohol/l and an A_{550} of 4. Hardison et al. (1997), obtained a production rate of *t*-butyl alcohol of 0.47 nmol h^{-1} mg dry $weight^{-1}$, which is equal to 0.016 nmol min^{-1} mg cell $protein^{-1}$ (considering that proteins from the cell biomass account for 50% of the total weight). The ratio of the degradation rate of MTBE to the degradation rate of *t*-butyl alcohol varies between 2.8 (ENV425) and 22 (*Graphium* sp.). *t*-Butyl alcohol in batch culture is degraded and its accumulation is unlikely as long as there is pentane to cometabolize it. However in a system able to degrade MTBE continuously, the difference in degradation rate between MTBE and *t*-butyl alcohol could result in the accumulation of the alcohol.

Finally, MTBE degradation by cometabolism in the presence of pentane by *P. aeruginosa* in batch culture was predicted with a simple model that depends only on the two initial concentrations of MTBE and pentane. No significant inhibitory effects on *P. aeruginosa* were observed with a MTBE concentration of up to 900 mg/l (20 mg/bottle). Moller and Arvin (1990) reported that MTBE concentrations of 200 mg/l showed a weak inhibitory effect on the biodegradation of aromatic hydrocarbons (3.5 mg benzene, toluene, ethyl benzene, and xylenes per liter).

With ever increasing concern about air and water contamination, a search for bioremediation alternatives is underway. Various laboratory-scale models are presently under study to attempt to resolve specific problems such as MTBE-contaminated ground water (Mosteller et al. 1997) and air (Eweis et al. 1997). Similar studies are also underway to determine, in a biofilter, the ability of *P. aeruginosa* to degrade MTBE in the presence of pentane. The use of the controlled addition of a metabolite to foster MTBE degradation remains a very interesting alternative.

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