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Degradation of polycyclic aromatic hydrocarbons and long chain alkanes at 6070 °C by *Thermus* and *Bacillus* spp

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Abstract

Although polycyclic aromatic hydrocarbons (PAH) and alkanes are biodegradable at ambient temperature, in some cases low bioavailabilities are the reason for slow biodegradation. Considerably higher mass transfer rates and PAH solubilities and hence bioavailabilities can be obtained at higher temperatures. Mixed and pure cultures of aerobic, extreme thermophilic microorganisms (*Bacillus* spp., *Thermus* sp.) were used to degrade PAH compounds and PAH/alkane mixtures at 65 °C. The microorganisms used grew on hydrocarbons as sole carbon and energy source. Optimal growth temperatures were in the range of 60–70 °C at pH values of 6–7. The conversion of PAH with 3–5 rings (acenaphthene, fluoranthene, pyrene, benzo[e]pyrene) was demonstrated. Efficient PAH biodegradation required a second, degradable liquid phase. *Thermus brockii* Hamburg metabolized up to 40 mg $(l\ h)^{-1}$ pyrene and 1000 mg $(1\ h)^{-1}$ hexadecane at 70 °C. Specific growth rates of 0.43 h^{-1} were measured for this strain with hexadecane/pyrene mixtures as the sole carbon and energy source in a 2-liter stirred bioreactor. About 0.7 g cell dry weight were formed from 1 g hydrocarbon. The experiments demonstrate the feasibility and efficiency of extreme thermophilic PAH and alkane biodegradation.

Abbreviations: PAH – polycyclic aromatic hydrocarbons; HPLC – high pressure liquid chromatography; IR – infrared (spectroscopy); CDW – cell dry weight; NAPL – nonaqueous phase liquid

Introduction

Many waste materials, contaminated soils as well as industrial wastewaters are polluted with hydrophobic organic compounds which are only sparingly soluble in water. One important example are polycyclic aromatic hydrocarbons (PAH). The biodegradation of these pollutants at ambient temperature in biological treatment plants has become a widely used technology (Samanta et al. 2002). The mesophilic biodegradation of hydrophobic organic pollutants such as aliphatic and aromatic hydrocarbons has been investigated using pure and mixed cultures (Liu 1985; Cuno 1996). However, in some cases the biodegradation is very slow. One limiting factor is the low bioavailability of the hydrophobic pollutants.

Several approaches have been developed to overcome the low bioavailability of hydrophobic pollutants, e.g. the use of surfactants (Tiehm et al. 1997), a chemical pre-treatment, e.g., ozonization (Stehr et al. 2001; Kornmüller & Wiesmann 1999) or simply elevating the temperature during biodegradation. The following list summarizes the advantages of high temperature biodegradation. The order of magnitude of the expected benefits is estimated by citing a typical example:

(1) *Higher solubility of hydrophobic pollutants:* For example, a temperature increase from 20 to 60 °C increases the solubility of anthracene by a factor of 25 (IUPAC solubility data series, 1989).
(2) *Decreased viscosity of the contamination:* For example, a temperature increase from 20 to 60 °C decreases the viscosity of tar oil by a factor of 5 (Bohl 1991).
(3) *The diffusion of pollutants is considerably faster:* For example, the diffusion coefficient for the diffusion of benzene in water is increased by a factor of 3.5 at a temperature increase from 20 to 60 °C (Yaws et al. 1995).
(4) *Many pollutants such as aliphatic hydrocarbons are solid al lower temperatures, but liquid at higher temperatures:* For example, eicosane is solid at 20 °C, but liquid at 60 °C (Griesbaum 1989).

Concluding the benefits of higher temperatures, considerably higher mass transfer rates are feasible at high temperatures and hence problems related to the limited bioavailability of hydrocarbons will be attenuated. Although increasing the hydrocarbon solubility is not the only factor influencing the bioavailability of a hydrophobic pollutant, increasing the solubility is an efficient way to increase the bioavailability of pollutants, e.g., in contaminated soils. The higher mass transfer rates at higher temperatures also contribute to this increased bioavailability.

Industrial effluents are often discharged at 50–130 °C, with temperatures frequently exceeding 80 °C (Hamer et al. 1989). Treatment of such effluents at elevated temperatures would be inexpensive. Additionally, treatment at high temperatures is a process alternative for the processing of e.g., industrial waste or excavated contaminated soil in slurry reactors due to the possible shortening of treatment time by increasing the bioavailability of the pollutant.

In the last years new sources for extreme thermophilic microorganisms have been exploited, e.g., deep sea hydrothermal vents (Gonzales et al. 1996). In contrast, Wilhelms et al. (2001) presented evidences that oilfields were virtually sterilized at temperatures of 80–90 °C. Therefore, many of the microorganisms isolated from oilfields might have been only recently injected to the oilfield with production water and originate from other sources such as hydrothermal vents (Wilhelms et al. 2001). Chen & Taylor (1997) isolated consortia of anaerobic, BTEX degrading bacteria at 60 °C from such produced water of an oilfield. Compost was the source of several thermophilic microorganisms isolated which degrade aromatic hydrocarbons. Hebenbrock (1997) isolated a *Bacillus thermoleovorans* sp. that degrades naphthalene as sole carbon and energy source from contaminated compost piles. Another thermophilic, naphthalene degrading *Bacillus* sp. (Shimura et al. 1999) was capable of degrading polychlorinated biphenyls at 60 °C. Nearly no information is available concerning the thermophilic degradation of higher PAH compounds.

Thermophilic alkane degrading microorganisms have already been isolated in 1967 (Mateles et al. 1967). A few more thermophilic microorganisms have been isolated and characterized during the following years. The obligate alkane degrading *Thermoleophilum album* (Zarilla & Perry 1984) and *T. minutum* (Perry 1985), the facultative n-alkane degrading *Thermomicrobium fosteri* (Phillips & Perry 1976), *Bacillus stearothermophilus* (Sorkoh et al. 1993) and *B. thermoleovorans* (Zarilla & Perry 1987). Kato et al. (2001) isolated and characterized long-chain alkane degrading *Bacillus thermoleovorans* species from deep subterranean petroleum reservoirs. Five hydrocarbon-oxidizing strains were isolated by Nazina et al. (2001) from the formation water of oilfields.

Emulsified hydrocarbons and hydrocarbons dissolved in a second nonaqueous phase liquid (NAPL) were used as tools to investigate the fate of xenobiotics as well as a technology to increase the bioavailability of this compounds. The use of surfactants for an enhanced bioavailability has been study as a promising method to increase biodegradation rates (Tiehm et al. 1997). Benzo[a]pyrene dissolved in different nonaqueous phases was used by Kanaly et al. (2001) to prove the biodegradation of this substance and to investigate the parameters influencing its degradation. Birmann & Alexander (1996) assessed the interactions of several determinants in NAPL governing the biodegradation of phenentrene.

In this paper PAH and alkane biodegradation is examined using a mixed thermophilic culture. Uptake mechanisms are investigated using PAH mixtures dissolved in degradable and non- degradable alkanes. In a further step, a simplified model system is used to identify kinetic parameters and estimate obtainable degradation rates at 70 °C. The substrate in this model system was a hexadecane/pyrene mixture; and the process was inoculated by a *Thermus* sp.

Materials and methods

Microbial cultures

Several aerobic microorganisms capable of degrading aromatic and aliphatic hydrocarbons were isolated from hot springs, compost piles and industrial wastewater (Feitkenhauer 1998). Seven of the eight isolated microorganisms were identified to be *Bacillus* spp., while one isolate was identified to be a *Thermus* sp. All isolates possessed optimal growth temperatures of 60–70°C. The pH-values of optimal growth were determined to be between pH 6 and pH 7. All microorganisms isolated grew on hydrocarbons as sole carbon and energy source (Feitkenhauer 1998). A mixed culture of these strains plus 2 thermophilic Bacillus strains degrading lower molecular weight aromatic hydrocarbons (Hebenbrock 1997) and a phenol degrading microorganisms (*Bacillus thermoleovorans* A2; Mutzel et al. 1996) was used in the presented mixed culture experiments. This mixed culture was cultivated in a 1-l reactor on tar oil (Feitkenhauer 1998) before the presented experiment.

PAH and alkane biodegradation monitoring

For the measurement of the cell dry weight, the shake flask was opened and 1 ml samples were removed. The samples were centrifuged (11,000 g, 15 min) and the resulting pellets were treated with 1 N NaOH for 15 minutes at 95 °C. In the supernatant after centrifugation (5,000 g, 15 min) cell proteins were measured by the method of Lowry as described by Süßmuth et al. (1987). For the calculation of the cell dry weight (CDW) a protein content of the microorganisms of 50% was assumed (Bailey & Ollis 1986).

For PAH extraction, the solvent (1,1,2-trichlorotrifluoroethane, spectroscopy grade, Merck, Darmstadt, Germany) was added to the entire shake flasks (0.2 ml solvent per ml medium). The flask was vortexed and subsequently placed in an ultrasonication bath for 15 min (Sonorex RK 1028, Bandelin, Berlin, Germany). The organic phase was separated and stored in 1.5 ml autosampler Teflon® sealed vials at −18 °C.

The amount of hydrocarbons in the extract was measured by IR-spectroscopy in an 'oil in water' analyzer (Model OCMA, Horriba, Japan) as described by Wilichowski (1994). The individual PAH-compounds in the extract were determined with a HPLC method. The set up was: pump: L-6000a, Hitachi, Tokyo, Japan; auto sampler: Perkin-Elmer, Überlingen, Germany; column: RP 18, 125 mm, Merck, Darmstadt, Germany; UV/VIS detector: Spectro Monitor 3100, LDC Analytical, Gelnhausen, Germany; data collection and integration: Axxiom Chromatography system 727, Axxiom Chromatography, Calabasas, CA, USA. Injected sample volume: 20 μl. Flow rate: 1 ml/min; Column temperature: 25 °C. Solvent: Methanol/water 80/20 (v/v).

Bioreaction systems and growth medium

Baffled shake flasks sealed with a Teflon® lined screw cap were closed air tight to avoid an abiotic PAH loss via the gas-phase. The flasks were incubated in a rotary shaker (160 rpm) at 65 °C and an initial pH of 6.5. The amount of oxygen inside the flasks was sufficient for a complete mineralization of the investigated compounds. A 2-l bioreactor (Bioengineering, Wald, Switzerland) equipped with pH-control and dissolved oxygen indicator was used for the determination of kinetic parameters as described before (Feitkenhauer et al. 2001). A standard mineral salt medium (Feitkenhauer et al. 2001) was used and the hydrocarbon substrates are specified in the figures.

Results and discussion

Biodegradation of PAH-mixtures using a mixed culture

The degradation of 3–5 ring PAH at 65 °C was investigated in the presence of a degradable aliphatic hydrocarbon (emulsified hexadecane) and a non-degradable aliphatic hydrocarbon (heptamethylnonane) in shake flasks. The flasks were inoculated with the mixed culture of extreme thermophilic microorganisms described above. In case of hexadecane after 3 days the lower molecular weight PAH acenaphthene and fluoranthene were nearly completely metabolized (58 of the initial 60 mg/l). About half of the benzo[e]pyrene (12 of 30 mg/l) and pyrene (17 of 30 mg/l) was degraded in the same period (Figure 1). Microbial growth started after a lag-phase of 18 hours. Within 55 hours the cell dry weight increased from below 10 to about 825 mg/l and more than 90% of the total hydrocarbon was degraded.

Lower conversions of the PAH were measured in shake flasks containing 750 mg/l heptamethylnonane and the same PAH components than in flasks with the

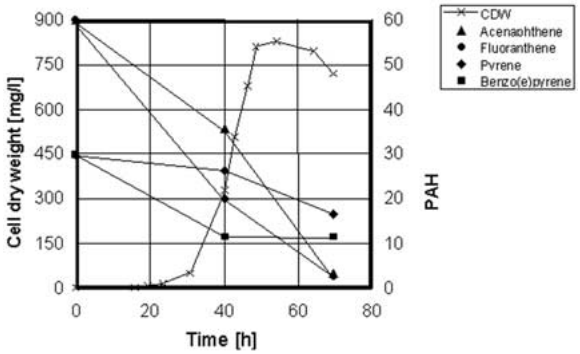

Figure 1. Degradation of 4 PAH compounds (acenaphthene and fluoranthene 60 mg/l, pyrene and benzo[e]pyrene 30 mg/l) dissolved in 750 mg hexadecane as the main carbon and energy source and 25 mg/l of the emulsifier ET 5. The shake flask was incubated at 65 °C, pH 6.5 and a shaker speed of 160 rpm with the mixed culture described in materials and methods. The cell dry weight increase was measured (CDW) using a modified Lowry protein test.

Table 1. Recovery rates of mixtures of 4 PAH (acenaphthene and fluoranthene 60 mg/l, pyrene and benzo[e]pyrene; 30 mg/l) in the presence of heptamethylnonane (HMN, 750 mg/l) from sterile flasks, and from flasks inoculated with the mixed culture described in materials and methods grown on tar oil. The sealed shake flasks were incubated at 65 °C, pH 6.5 and a shaker speed of 160 rpm. Within 5 days the protein content in the shake flask with bacteria increased from 61 to 74 mg/l

% Recovery	Sterile 5 days	With bacteria 5 days
Acenaphthene	94	47
Fluoranthene	103	35
Pyrene	107	77
Benzo[e]pyrene	108	72

substrate hexadecane. However, more than half of the amount of the 3 ring PAH and a quarter of the pyrene and benzo[e]pyrene was degraded within 5 days. From sterile control flask the PAH contamination was completely recovered (Table 1).

These data confirm the bioconversion of 3–5 ring PAH compounds. However, an efficient PAH biodegradation was only achieved in the presence of a degradable liquid phase in the simulated contamination. For applications, e.g., in the treatment of hot wastewater this is not a major drawback as long as such a degradable phase is present and not only very high PAH concentrations alone. The favorable physico-chemical conditions for degradation of dissolved PAH compounds at 65 °C such as the higher solubility were not fully exploited by the mixed culture, as the biodegradation was neither complete nor very fast without a degradable co-substrate. These results demonstrate that an adsorption/desorption kind of uptake mechanisms as well as transport of dissolved pollutants through the liquid phase is not the preferred PAH uptake mechanism of the microorganisms of the mixed culture used in this experiment. At least this substrate uptake method is not as efficient as the uptake of PAH dissolved in a second degradable substrate. The emulsifying agent (surfactant) used also contributed to an increased degradation, as previously described in literature (Tiehm et al. 1997). One strategy to overcome this problem is to continue the search for efficient, extreme thermophilic PAH degrading microorganisms which do not require a second liquid, degradable phase.

Kinetic parameters of hexadecane/pyrene biodegradation at 70 °C

The optimal growth temperature of *Thermus brockii* sp. Hamburg used in this experiment was determined to be 70 °C. Slow growth was still observed at a temperature of 80 °C. It was observed in fed-batch fermentations with hexadecane/pyrene mixtures as substrates that the lag-phase was longer in cases where higher stirrer speeds were employed. The lag phase lasted about 4 h at 400 rpm, but was longer than 40 h at 3000 rpm (Feitkenhauer 1998). At low stirrer speeds, however, the reactor was insufficiently mixed and the oxygen transfer rate was too low to support high cell densities. To combine short lag-phases with high growth rates, a two phase approach was developed: the fermentation was started at a stirrer speed of 800 rpm and switched to 2000 rpm in the exponential growth phase (Figure 2). Higher salt concentrations have been shown to decrease the growth rate of this strain significantly (Feitkenhauer 1998) and therefore not the whole amount of mineral salts was added at the beginning of the fermentation. However, in order to avoid a situation where a nutrient from the mineral salt solution becomes limiting, four times the initial amount of mineral salts was added in 8 steps during the fermentation. To make sure that always sufficient substrate was available in the fermentation broth hydrocarbons were added to the reactor at 4 times during the experiment (Figure 2). Within 36 hours about 16.5 g hexadecane and 0.7 g pyrene were converted into 11.5 g biomass (CDW). Growth was almost linear and up to 1.05 g $(l\ h)^{-1}$ hexadecane and 40 mg $(l\ h)^{-1}$ pyrene were metabolized in the

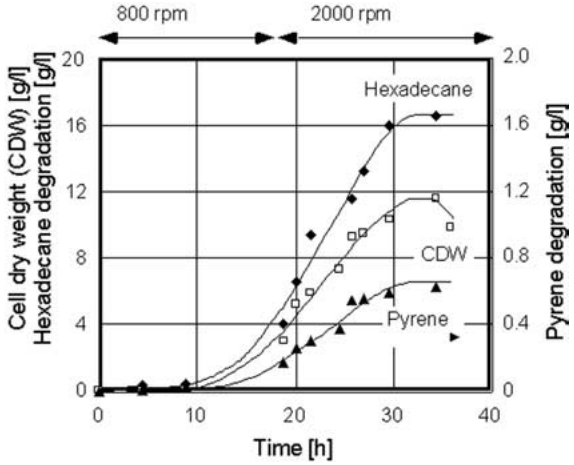

Figure 2. Fed-batch fermentation of *T. brockii* Hamburg at 70 °C and pH 6.25 using hexadecane/ pyrene as the substrates. The process was started at a stirrer speed of 800 rpm and a concentration of 800 mg/l hexadecane and 40 mg/l pyrene. The fermentation conditions were changed as follows: After 8.75 hours, additional 4800 mg/l hexadecane and 200 mg pyrene were added to the reactor, after 20 h 15400 mg/l hexadecane and 440 mg/l pyrene and finally after 27 h 2000 mg/l hexadecane and 200 mg/l pyrene were added. The residual concentrations at the end of the experiment were 6400 mg/l hexadecane and 240 mg/l pyrene. Between the 9 and 30 hours of the experiment, four times the initial amount of mineral salts was supplied to the reactor in 8 steps, except ammonium, which was supplied by using 12.5% NH_3 solution for pH correction. The airflow was increased stepwise from 0.05 vvm to 0.5 vvm during the course of the fermentation to keep the oxygen level above 10% air saturation.

growth phase. In this fed-batch fermentation a specific growth rates of up to 0.43 h^{-1} was measured with hexadecane/pyrene mixtures as the sole carbon and energy source (Figure 2). After 36 hours the experiment was stopped because of intensive foaming.

Compared to mesophilic alkane degrading microorganisms, the measured growth rates of the isolated thermophilic microorganisms were in the same range. However, long-chain alkanes are solid at lower temperatures (Griesbaum 1989) and hence better accessible if they are molten at high temperatures. *Thermus brockii* Hamburg metabolized aliphatic hydrocarbons at an optimal growth temperature of 70 °C (Feitkenhauer 1998), at least 5–10 °C higher than previously described thermophilic alkane degrading microorganisms. The temperature of highest growth rates (70 °C) was in good agreement with other members of the *Thermus* genus (65–75 °C, Beffa et al. 1996). α, ω-chloroalkanes were not degraded by the isolated thermophilic microorganism. The metabolization of polycyclic aromatic hydrocarbons was shown here for the model substance pyrene. However, pyrene was degraded efficiently only in the presence of hexadecane or another degradable long-chain alkane. Several microorganisms are known to degrade PAH compounds at lower temperatures (Cerniglia 1992), but at elevated temperatures so far only *Bacillus stearothermophilus* (Sorkoh et al. 1993) and *Bacillus thermoleovorans* spp. degrading naphthalene (Shimura et al. 1999; Hebenbrock 1997) as well as the *Bacillus* spp. species used in this study were described to possess PAH degrading capabilities. These organisms all grow at temperatures considerably lower than 70 °C, the temperature of optimal growth of the *Thermus* sp. used in this study. The obtained degradation rates were also considerably higher compared to previous studies using phenanthrene as model compound in a NAPL system under mesophilic conditions (Birmann & Alexander 1996). The favorable kinetic parameters of this strain as well as the high pyrene and hexadecane degradation rates obtained in this experiment demonstrate that very efficient thermophilic hydrocarbon degradation is feasible.

Conclusion

High degradation rates of aliphatic and polyaromatic hydrocarbons were measured at 60 and 70 °C using extreme thermophilic hydrocarbon degrading microorganisms originating from various industrial and natural sources. Efficient PAH uptake and degradation required a second degradable liquid phase. High growth rate of a *Thermus* sp. at 70 °C (0.43 h-1) and also high degradation rates of up to 40 mg (1 h^{-1}) pyrene and 1000 mg (1 h)$^{-1}$ hexadecane demonstrate that high temperature hydrocarbon biodegradation is not only feasible, but also very effective. It was also shown that higher PAH compounds up to benzo[e]pyrene can be degraded by extreme thermophilic microorganisms. Based on these encouraging results, industrial applications of this promising biodegradation technique are very likely in the near future.

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