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Enhanced biodegradation of phenanthrene in a biphasic culture system

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Abstract

Degradation of phenanthrene by *Pseudomonas aeruginosa* AK1 was examined in (i) an aqueous mineral salts medium to which phenanthrene particles of varying size (i.e. diameter) were added, and (ii) an aqueous/organic biphasic culture system consisting of mineral salts medium supplemented with 2,2,4,4,6,8,8-heptamethylnonane (HMN) as the phenanthrene-carrying organic phase. In both systems, the rate of phenanthrene biodegradation could be significantly enhanced by manipulations leading to improved phenanthrene mass transfer into the aqueous phase. With crystalline phenanthrene, the rate of biodegradation was found to be directly correlated to the particle surface area, whereas in the biphasic system the rate of biodegradation of the dissolved phenanthrene was mainly governed by the HMN/water interface area. In the latter system, exponential growth with a doubling time t_d of 6–8 hours has been achieved under conditions of intensive agitation of the medium indicating that phenanthrene degradation by strain AK1 is limited mainly by physicochemical parameters. Addition of selected surfactants to the culture medium was found to accelerate phenanthrene degradation by strain AK1 only under conditions of low agitation (in the presence of HMN) and after pretreatment of phenanthrene crystals by ultrasonication (in the absence of HMN). Evidence is presented that the stimulating effect of the surfactants was primarily due to improved dispersion of phenanthrene particle agglomerates (in the aqueous mineral salts medium supplemented with phenanthrene crystals) or of the phenanthrene-carrying lipophilic solvent drops (in the aqueous/organic biphasic culture system) whereas the solubilizing activity towards phenanthrene was neglectible. Under conditions of intensive mixing of the culture medium (i.e. if a high particle surface area or HMN/water interface area, respectively, is provided), the addition of surfactants did not enhance phenanthrene biodegradation.

Introduction

Polycyclic aromatic hydrocarbons (PAH) are formed during combustion of organic matter and, therefore, are found throughout the environment in low concentration (Andelman & Snodgrass 1974; Blumer 1976). These airborne PAH may be destroyed by chemical oxidation or photolysis, the major process in nature, however, is microbial degradation together with strong adsorption to humic substances (Sims & Overcash 1983). In addition, considerably higher levels of PAH are found in the ground of PAH-releasing industrial plants like gas works, coking plants and wood-preserving facilities. At these sites PAH-containing coal gasification products like crude benzene or tar oil seeped into the soil over decades. Consequently, PAH are present in the pores of soil and adhesively fixed to soil particles even in great depth remaining unaffected from microbial attack over long time periods. Microbial degradation of PAH with up to 3 rings is well documented and reviewed by Cerniglia (1984) while reports on pathways of bacterial degradation of PAH with more than 3 rings are relatively rare (Heitkamp et al. 1988; Mahaffey et al. 1988; Weissenfels et al. 1991; Kelley at al. 1991; Walter et al. 1991). It is generally accepted that biodegradation of PAH is limited by their low bioavailability resulting from extremely low water solubility and strong adsorption to soil components

(Alexander 1973, 1975; Weissenfels et al. 1992). Water solubility of PAH ranges from 1.29 mg/l (phenanthrene – 3 rings) to 0.14 μg/l (coronene – 7 rings) (Dzombak & Luthy 1984). As Goswami et al. (1983) and Huang & Chou (1990) lined out, three possible modes of uptake of poorly water-soluble substrates by microorganisms are considered: (i) the homogenous mode where substrates are utilized by microorganisms from the dissolved state. This kind of uptake had been reported for naphthalene (Wodzinski & Bertolini 1972) and phenanthrene (Wodzinski & Coyle 1974) and the resulting rates of PAH utilization are restricted due to the extreme low rates of solubilization of these substrates. (ii) by the pseudosolubilization mode. Microbial cells may produce metabolites such as biodetergents which help to dissolve solid substrates in aqueous media. This sort of substrate uptake was reported by Chakravarty et al. (1975) and Cameotra et al. (1983) for the degradation of liquid and solid alkanes. (iii) The interfacial adsorption mode is defined by the cells being attached to substrate particles and may transfer substrate through adsorption-desorption of substrate molecules on the cell envelope having direct contact to the solid material. Zilber et al. (1980) observed adhesion of a *Pseudomonas* sp. on *n*-tetracosane particles and the uptake of sterol by an *Arthrobacter* sp. was reported to be realized by direct surface attachment of the cells to the insoluble substrate particles (Goswami et al. 1983). Preliminary data from our laboratory clearly indicate that *P. aeruginosa* AK1 neither adhere to the phenanthrene particles nor produce any emulsifying agents during growth on phenanthrene as the sole carbon and energy source in mineral salts medium. Obviously phenanthrene is used by this organism from the dissolved state only. This corresponds to observations with another phenanthrene utilizing *Pseudomonas* sp. (Wodzinski & Coyle 1974). In the present work, different approaches have been examined in order to enhance degradation of phenanthrene by *Pseudomonas aerugiosa* AK1 by accelerated mass transfer.

Materials and methods

Organism

Strain AK1 was isolated from an enrichment culture which has been inoculated with samples of a PAH-contaminated soil from a coking plant. Purity of the bacterial isolate was confirmed by plating on nutrient broth agar medium. Strain AK1 was identified as *Pseudomonas aeruginosa* by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM – German Collection of Microorganisms and Cell Cultures).

Medium

For cultivation of strain AK1, a mineral salts medium was used containing per litre: Na_2HPO4 – 2.225 g, $(NH_4)_2SO_4$ – 1 g; $CaCl_2 \cdot 2\ H_2O$ – 36.6 mg; Fe(III)NH_4-citrate – 10 mg; $MgSo_4 \cdot 7\ H_2O$ – 200 mg; H_3BO_3 – 0.3 mg; $CoCl_2 \cdot 6\ H_2O$ – 0.2 mg; $ZnSO_4 \cdot 7\ H_2O$ – 0.1 mg; $MnCl_2 \cdot 4\ H_2O$ – 0.03 mg; $Na_2MoO_4 \cdot 2\ H_2O$ – 0.03 mg; $NiCl_2 \cdot 6\ H_2O$ – 0.02 mg; $CuCl_2 \cdot 2\ H_2O$ – 0.01 mg. The final pH of the medium was adjusted to 6.8.

Cultivation with crystalline phenanthrene as the substrate

Crystalline phenanthrene was passed through analytic sieves yielding fractions of particles with a maximum diameter of > 400 μm, 250–400 μm and < 250 μm, respectively. Portions (40 mg each) of these phenanthrene particle fractions were transferred to baffled 100-ml-Erlenmeyer flasks. After addition of 20 ml sterile mineral salts medium, the flasks were incubated for 24 h at 30°C on a rotary shaker. After inoculation with phenanthrene-pregrown AK1 cells, the cultures were incubated in the dark at 30° C on a rotary shaker. For further reduction of the particle size some of the flasks containing phenanthrene particles with a diameter < 250 μm were additionally treated in an ultrasonic bath for 75 seconds (35 kHz, 250 W, Sonorex RK 255, Bandelin). Since crystalline phenanthrene could not be homogeneously dispersed in the aqueous medium for determination of the phenanthrene concentration one of the parallel cultures was completely extracted into an equal volume of diethylether. An aliquot of the ether extract was then evaporated under N_2-atmosphere to dryness, redissolved in methanol, diluted and analyzed by HPLC.

Cultivation with phenanthrene dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN)

Experiments with HMN as the organic substrate-carrying phase were carried out in baffled Erlenmeyer flasks of different working volume. HMN containing phenanthrene (8 g/l) was added to mineral salt medium in a 1:4 (v/v) ratio. As inoculum (5% v/v)

phenanthrene-pregrown cells of AK1 were used. The cultures were then incubated at 30° C on a rotary shaker. To record the disappearance of phenanthrene samples were collected from the emulsified culture fluid and centrifuged for 20 minutes at 12 000 rpm to separate the aqueous and organic phases. Portions of 100 μl of the HMN-phase were analysed by HPLC after dilution with methanol, taking into account that HMN can be dissolved in methanol only up to 5%.

The effect of surfactants on phenanthrene biodegradation

Various commercially available surfactants were examined with respect to their phenanthrene-solubilizing potential: Portions (10 mg) of crystalline (unsieved) phenanthrene were mixed with 20 ml each of a 0,01% (w/w) aqueous solution of the particular surfactant in baffled 100 ml Erlenmeyer flasks. After 48 hours of incubation on a rotary shaker at 21°C, aliquots (5 ml) from the various incubation mixtures were passed through cellulose acetate filters (pore size, 0.2 μm). Concentration of dissolved phenanthrene was then determined spectrophotometrically at 254 nm. To test for possible toxic effects of the surfactants, colony forming units were counted in samples taken from AK1 cultures growing with crystalline phenanthrene as the carbon source in the presence of 0.01 – 0.1% (w/w) of the particular surfactant. Surface tension in these test cultures was measured using a tensiometer (Krüss; Hamburg, Germany).

Analytical methods

Phenanthrene was quantified by reversed-phase HPLC (chromatograph with pumps 510, gradient controller 680, and autosampler Wisp 712 (Waters), UV-detector Spectroflow 783 (Kratos), integrator C-R3A (Shimadzu), Grom PAH column 125 by 4 mm or 250 by 4 mm, respectively, (Grom, Herrenberg, Germany), isocratic eluent system, acetonitrile/water (70:30% vol/vol); flow rate, 1 ml/min; injection volume, 10 μl; detection wave length, 254 nm. Biomass concentration in liquid AK1 cultures was determined by measuring the optical density at 578 nm (OD_{578}). To remove crystalline phenanthrene from the aqueous phase, 10% (v/v) HMN was added. Biomass could be determined by measuring OD_{578} because the bacterial cells remained homogenously suspended in the aqueous phase after HMN addition and phase separation.

Chemicals

Phenanthrene and 2,2,4,4,6,8,8-heptamethylnonane (HMN) were purchased from Aldrich (Steinheim, FRG). All chemicals used for HPLC analysis were of LiCrosolve quality and were obtained from Merck (Darmstadt, Germany).

Model for the kinetics of phenanthrene dissolution

A simplified mathematical model was developed of to describe the decrease of crystalline phenanthrene particles caused by bacterial degradation. The model is based on the assumption that:

- phenanthrene crystals or their aggregates are of a spherical form and unique size,
- the aqueous concentration of phenanthrene is constant during growth of the organism as long as insoluble phenanthrene remains in the medium,
- the boundary transition layer between the surface of the phenanthrene crystal and the aqueous solution is constant, and
- the concentration gradient $\triangle c$ within the aforementioned boundary is constant.

Given spherical symmetry as indicated by the first assumption, diffusive mass transfer m_D* inside the boundary layer at radius r is expressed by the first Fick's law:

$$m_{D}* = -DA(r)\frac{dc}{dr} \qquad (1)$$

where D is the diffusion coefficient ($m^2 \cdot s^{-1}$), A(r) = $4\pi r^2$ is the surface area of the sphere of radius r, and $\frac{dc}{dr}$ $kg \cdot m^{-4}$) is the boundary layer, which will be called Z. To conserve mass balance, the rate of addition of mass to the aqueous phase must equal the rate of loss from the solid phase $4\pi p r^2 \frac{dr}{dt}$. Substitution and integration leads to

$$r = r_0 - \frac{k}{\rho}t \qquad (2)$$

where k = DZ (in $kg \cdot h^{-1} m^{-2}$). The undissolved mass of phenanthrene can be determined from the original mass M_O and radius r

$$M = M_0 \left(\frac{r}{r_0}\right)^3 \qquad (3)$$

Substitution from equation (2) gives:

$$M = M_0 \left(1 - \frac{kt}{r_s(0)\rho}\right)^3 \qquad (4)$$

Results

Utilization of crystalline phenanthrene

Evidence has been presented by Thomas et al. (1986) and Stucki & Alexander (1987) that the rate of biodegradation of sparingly water-soluble solid chemicals mainly depends on their dissolution rate. Particularly, Thomas et al. (1986) reported that rates of naphthalene disappearance in mixed bacterial culture were inversely related to the size of particles of solid naphthalene.

According to these observations, experiments have now been performed with sieved phenanthrene particles of varying diameter. The results obtained (Fig. 1) with strain AK1 are indicative of a significant correlation between the rate of phenanthrene biodegradation and growth of AK1 cells, on one hand, and the average size of phenanthrene particles added to the medium, on the other hand. Exponential cell growth, however, did not occur with any of the particle fractions tested.

Based on the relation between the size and surface area of a particle, a simplified mathematical model has been developed to describe phenanthrene degradation by strain AK1. Equation (4) was fitted with respect to the phenanthrene concentration values observed in the experiments. The radius (r_o) of spherical phenanthrene particles at the beginning of the experiment and the dissolution rate (k) were varied according to the pore size of the analytical sieves used. The r_o-average values were calculated as being 160, 130, 113 and 100 μm. These values reflect the fact that the phenanthrene particle fractions were inhomogeneous due to imperfect sieving yielding smaller phenanthrene particles and subsequent aggregation of these smaller crystals.

The value which fitted best for k was 4.5×10^{-4} kg h^{-1} m^{-2}. The resulting theoretical curves are shown as dotted lines in Fig. 1. Assuming a diffusion coefficient (D) estimated by the Method of Wilke and Chang (1955) as being about 8×10^{-10} $m^2 s^{-1}$ the boundary layer has been calculated to be < 10 μm. This very small boundary layer indicates that under the experimental conditions used turbulent flow took place in the culture medium rather than laminar flow, for which a boundary layer of ≥ 100 μm is characteristic (R. Lacmann, Institut für Physikalische und Theoretische Chemie, Technische Universität Braunschweig, Germany, personal communication).

Table 1. Solubilization of crystalline (unsieved) phenanthrene in 0.01% (w/w) aqueous solutions of representative[1] nonionic[2] surfactants at 21°C.

Surfactant (tradename)	solubilized phenanthrene [mg/l]
(Aqua dest.)	0.86
Biosoft HR40[3]	9.55
Emulan A[4]	9.06
Biosoft HR33[3]	6.45
Biosoft HR18[3]	6.31
Emulan EL[4]	5.60
Syperonic A7[5]	4.64
Dobanol 45-7[6]	4.47
Genapol O-120[7]	4.19
Plurafac LF 600[4]	3.60
Lutensol AO 5[4]	3.14
Arkopal N[7]	3.12
Marlipal 1618/25[8]	2.92
Dehydrol 100[9]	2.85
Sapogenat T-100[7]	1.92
Tween 20[5]	1.54
Fluowet OT[7]	1.22
FSN-100[10]	1.19
Invadin JFC[11]	1.12

[1] Only nonionic surfactants solubilizing more than 1 mg/l phenanthrene are listed. From families of surfactants with nearly identical chemical structure or composition only the best phenanthrene solubilizing ones are considered in this table. Exceptions are surfactants solubilizing more than 5 mg/ml phenanthrene.

[2] Further were tested: anionic surfactant: a biosurfactant (rhamnolipid) from *Pseudomonas aeruginosa* DSM 2659, gift from Th. Gruber (Fh-IGB; Stuttgart, Germany); cationic: surfactants: Abil B 8842, Abil B 9905; ampheroteric surfactants: Abil B 9905; (all obtained from Goldschmit: Essen, Germany), Zonyl FSK (obtained from [10]).

The nonionic surfactants were obtained from:

[3] Stepan; Voreppe, France; [4] BASF; Ludwigshafen, Germany; [5] ICI; Frankfurt, Germany; [6] Shell; Frankfurt, Germany; c[7] Hoechst; Frankfurt, Germany; [8] Hüls; Marl, Germany; [9] Henkel; Düsseldorf, Germany; [10] DuPont; Basel, Switzerland; [11] Ciba Geigy; Basel, Switzerland.

Influence of surfactants and ultrasonication

Various authors have considered the possibility of accelerating the process of solubilization and subsequent biodegradation of poorly water-soluble growth substrates by adding appropriate surfactants. The stim-

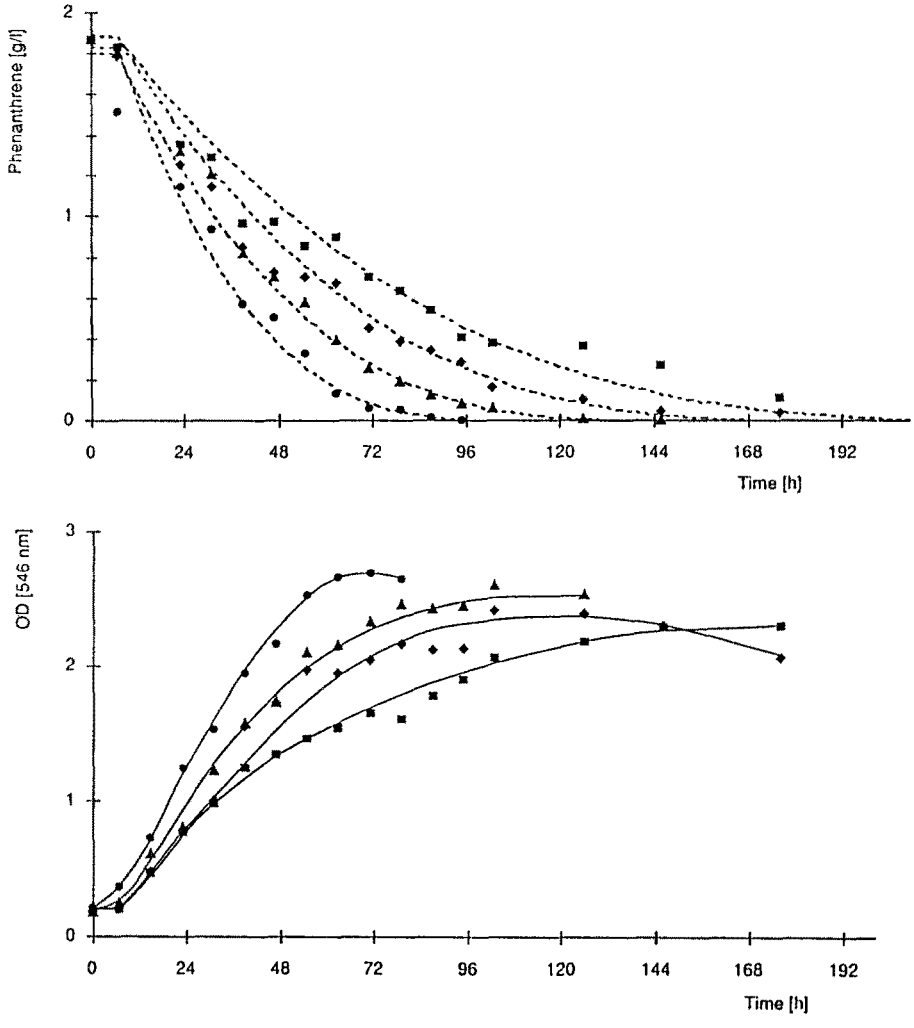

Fig. 1. Influence of the particle size on (A) Disappearance of solid phenanthrene and (B) growth of strain AK1 in mineral salts medium. Each experimental point represents the average phenanthrene content in three parallel cultures which were completely extracted and individually analyzed. The corresponding theoretical curves are shown as dotted lines. Symbols represent phenanthrene of different particle size: —■— 400 – 1000 μm; —◆— 250–400 μm; —▲— < 250 μm; —●— < 250 μm, medium treated by ultrasonication.

ulating effect of surfactants has been postulated to be based on their ability to solubilize the solid substrates (Foght et al. 1989; Oberbremer et al. 1990; Rosenberg 1986; Hommel 1990). In the present work, a systematic study has been undertaken in order to elucidate the mechanism of action of surfactants onto biodegradation of PAH using phenanthrene and strain AK1 as a model system. Thus a total of 38 non-ionic, 2 cationic, 2 amphotheric and 1 anionic surfactants from 12 suppliers were examined with respect to their phenanthrene-solubilizing capacity. All surfactants were used at a concentration of 0.01% (w/w) which was below the concentration causing cytotoxic effects, but above their critical micelle-forming concentration (cmc). The non-ionic surfactants (Table 1) proved to be most efficient in solubilizing phenanthrene. By increasing the surfactant concentration in the medium considerably more phenanthrene was solubilized. However, some of the tested surfactants inhibited growth of strain AK1 at concentrations above 0.05% (data not shown). Surfactants solubilizing more than 6 mg/l phenanthrene and one biosurfactant were used for further studies.

In the absence of surfactants, phenanthrene was usually present in the medium forming large particle agglomerates which were associated with the surface or

the glass wall. Treatment of uninoculated mineral salts medium by ultrasonication dispersed the phenanthrene agglomerates into very small particles which, however, subsequently reagglomerated. Addition of surfactants to the otherwise untreated aqueous medium resulted in dispersion of the phenanthrene crystals in the aqueous phase without reagglomeration. If surfactant addition was combined with ultrasonication, the phenanthrene agglomerates were dispersed and formed stable suspensions containing very small particles.

Model experiments have now been performed to study the influence of surfactants on degradation of crystalline phenanthrene by strain AK1. For this purpose, the anionic surfactant Biosoft HR33 has been chosen among the different surfactants examined because it proved to be the most appropriate one: it caused a 6–7 fold increase of the phenanthrene concentration in the aqueous phase, did not inhibit growth of AK1 cells at concentrations $\preceq$ 0.01% and was not degraded by strain AK1. As shown in Fig. 2, degradation of crystalline phenanthrene by AK1 cells was considerably enhanced by combined treatment of the medium by ultrasonication and surfactant addition. No significant effect was observed after either ultrasonication or surfactant addition alone. In all these cultures linear growth was observed.

Use of 2,2,4,4,6,8,8-heptamethylnonane (HMN)

In the context of biodegradation testing, Boethling (1984) suggested to dissolve poorly water-soluble solid organic chemicals in a water-immiscible non-biodegradable organic solvent (e.g. HMN) in order to allow microorganisms to utilize the respective growth substrate directly at the water-solvent interface. Based on this idea, model experiments were conducted in the present work using phenanthrene-pregrown AK1 cells and HMN as the organic phenanthrene-carrying phase. The intention was to study the influence of (i) varying interface area, and/or (ii) decreased interface tension on biodegradation of PAH in such aqueous/organic biphasic culture systems. From microscopic studies it was evident (not shown), that AK1 cells did not attach to the interface during growth in the biphasic system. This indicated, that phenanthrene utilization by this organism took place only in the aqueous phase. Taking into account that the partition coefficient of phenanthrene in the HMN/water system is rather high (13 100), a relatively high phenanthrene concentration (8 g/l; corresponding to about 50% of saturation) in the organic solvent has been used to achieve a sufficiently high substrate concentration in the aqueous phase. Under conditions of a constant volume ratio (1:4) of HMN and the aqueous phase, the interfacial area in the aqueous/organic biphasic system has been varied by agitation using different frequencies of the rotary shaker and/or flasks of different size. As seen from Fig. 3 and Table 2, improved mixing of the biphasic incubation medium clearly resulted in accelerated phenanthrene degradation and growth of AK1 cells. This indicated, that under the given experimental conditions substrate transfer was growth limiting and could be accelerated by intensified mixing of the two phases. Under conditions of intensive agitation (i.e. shaking of 250-ml-cultures in baffled 1-liter-Erlenmeyer flasks at 110 rpm) of the biphasic medium, the bacterial cultures even exhibited a distinct exponential growth phase characterized by a doubling time t_d of 6–8 hours. Obviously bacterial growth was not limited by either oxygen, nutrient salts or trace elements because doubling time of strain AK1 growing under the same conditions with glucose as the sole source of carbon and energy was approximately 2 hours.

To study the influence of surfactants on phenanthrene degradation and cell growth in the aqueous/organic biphasic system 250-ml-cultures of strain AK1 were incubated in baffled 1000-ml-Erlenmeyer flasks under conditions of low agitation (i.e. shaking at 70 or 90 rpm), and intensive agitation (i.e. shaking at 110 rpm). The following surfactants were compared: Biosoft HR18, Biosoft HR33, Biosoft HR40 (each 0.01%), and the Rhamnolipid (0.005%). All of these substances proved to be non-toxic to and non-degradable by cultures of strain AK1 and (with the exception of the Rhamnolipid) were found to have a high phenanthrene-solubilizing capacity (see Table 1). As shown for Biosoft HR33 in Fig. 4, addition of the surfactants considerably stimulated phenanthrene degradation by AK1 cells under conditions of low agitation (i.e. shaking at 70 rpm) as compared to unamended control cultures. Similar results were obtained with the other nonionic surfactants and even with the poorly phenanthrene-solubilizing rhamnolipid (Table 3). In contrast, there was no significant effect of surfactant addition on phenanthrene biodegradation and on growth if the cultures were incubated under conditions of intensive agitation (data not shown).

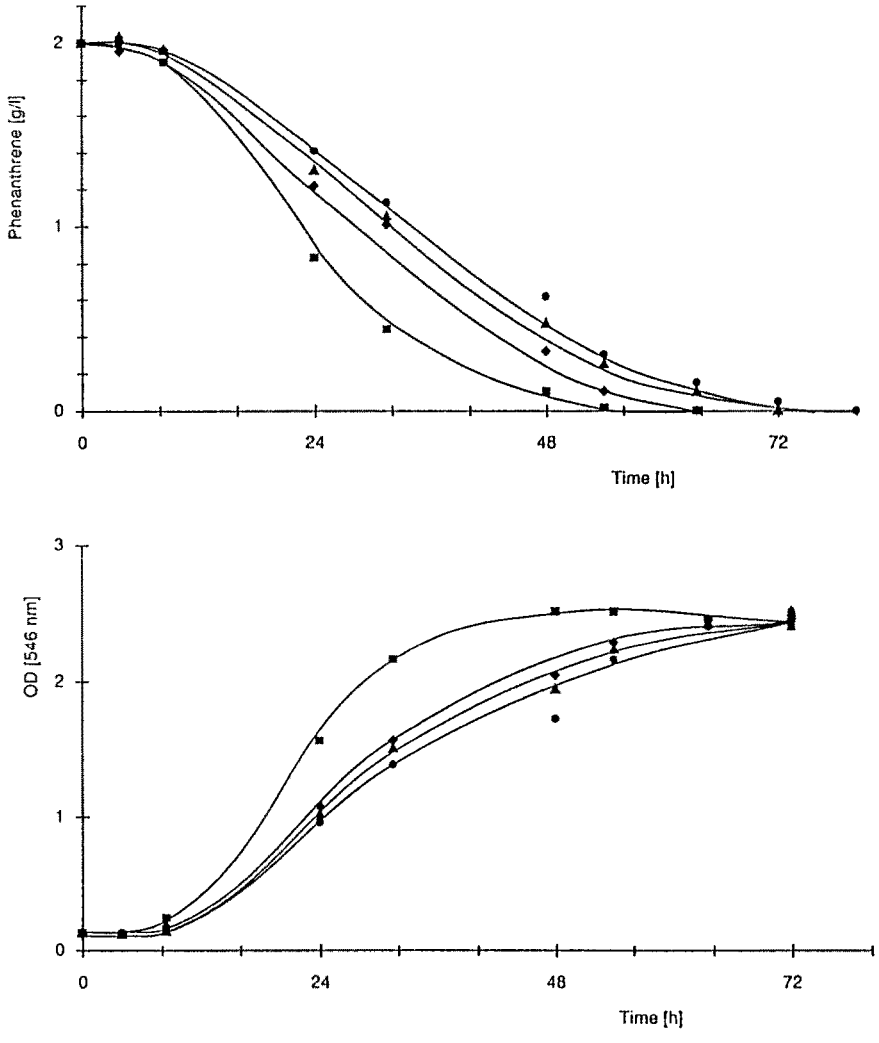

Fig. 2. (A) Disappearance of crystalline phenanthrene (particle fraction < 250 μm) and (B) growth of strain AK1 in mineral salts medium under various medium conditions: —●— without pretreatment of ultrasonication, no surfactant added; —▲— medium pretreated by ultrasonication, no surfactant added; —◆— no ultrasonication, Biosoft HR33 (0.01%) added; —■— medium with phenanthrene pretreated by ultrasonication, Biosoft HR33 (0.01%) added. Each experimental point represents the average phenanthrene concentration of three parallel cultures which were completely extracted and individually analyzed.

Discussion

Alexander (1975) postulated ten commandments concerning mechanisms of recalcitrance of organic compounds. In one of them he predicted the degradation of compounds, which are present in aqueous solution in exceedingly low concentrations as generally difficult.

As shown in the present work, biodegradation of PAH primarily is limited by its bioavailability. Different kinetic models have been proposed to describe substrate utilization and biomass formation during growth on poorly water soluble carbon substrates. Chakravarty et al. (1975) described bacterial growth on poorly water soluble n-alkanes, Huang and Chou (1990) the degradation of (solid) stearic acid by anaerobic microorganisms. In the latter model particular attention is paid to the form of the particles. It is based on the assumption that the microorganisms are attached to the solid surface and assimilate the substrate at the point of contact. Therefore it cannot directly be compared with the model proposed in the present work. In addition, the model of Huang and Chou relates to particle sizes (of ≤ 2 μm) in the same order of magnitude as the size of bacterial cells.

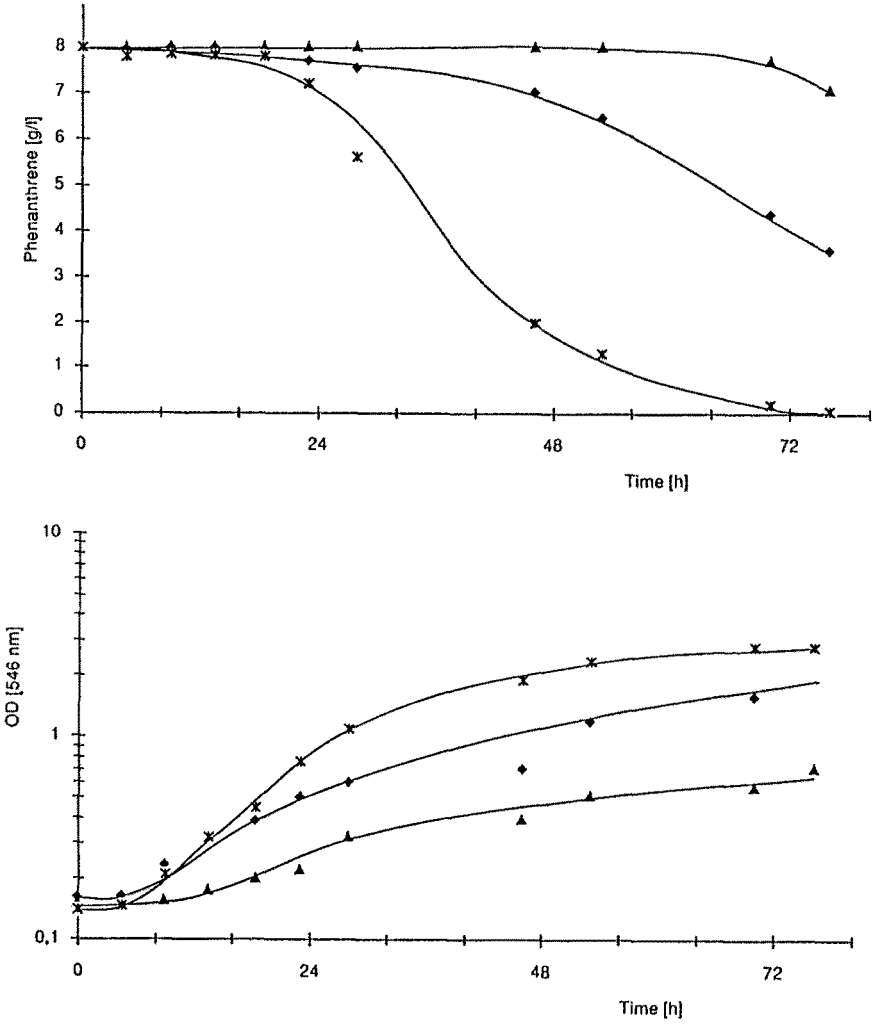

Fig. 3. Disappearance of phenanthrene (data given as phenanthrene concentrations in the HMN-phase; average values obtained from three parallel cultures) during incubation in baffled Erlenmeyer flasks of *Pseudomonas aeruginosa* AK1 in the aqueous/HMN biphasic medium under varying conditions of agitation: —◆— 250-ml-Erlenmeyer flask/culture medium, 62.5 ml/shaking at 90 rpm; —▲— 250-ml-Erlenmeyer flask/culture medium, 62.5 ml/shaking at 70 rpm; —*— 250-ml-Erlenmeyer flask/culture medium, 62.5 ml/shaking at 110 rpm.

Volkering et al. (1992) presented a mathematical approach to describe growth of bacteria utilizing PAH from the dissolved state only. In that model bacterial growth has been related to the mass transfer which limits growth at the expense of poorly water-soluble substrates.

In the present work a mathematical model has been proposed which describes the decrease in surface and volume of spherical substrate particles. The model is based on the assumptions that (i) bacterial growth on PAH like phenanthrene primarily depends on mass transfer into the aqueous phase, and (ii) the solid substrate is present in the form of particles of unique size. Assuming laminar flow in the agitated aqueous cultures, application of that model to degradation of crystalline phenanthrene by strain AK1 in mineral salts medium yielded theoretical curves of substrate disappearance which fitted the experimental curves of phenanthrene biodegradation quite well. As shown in the experiments and confirmed by the proposed mathematical model, rates of degradation of crystalline phenanthrene by strain AK1 depend exclusively on the actual particle surface and rates of mass transfer into the aqueous phase.

The present results demonstrate that under laboratory conditions biodegradation of crystalline phenan-

Table 2. Rates of degradation of phenanthrene by strain AK1 in the aqueous/HMN biphasic culture system under different conditions of agitation in the absence or presence of surfactant.

Agitation (rpm)/flask size – culture volume	Maximum degradation rate[1] [g phenanthrene/l·d]	
	surfactant Biosoft HR33 added (0.01%)	in the absence of surfactant
70 rpm		
100 ml – 25 ml	0.3 (46–76)	n.r.[2]
250 ml – 62.5 ml	0.5 (53–76)	n.r.[2]
1000 ml – 250 ml	0.8 (13–28)	0.7 (53–76)
90 rpm		
100 ml – 25 ml	0.9 (18–52)	0.7 (46–76)
250 ml – 62.5 ml	1.2 (23–46)	0.8 (46–70)
1000 ml – 250 ml	1.5 (18–23)	1.4 (18–23)
110 rpm		
100 ml – 25 ml	1.5 (23–46)	1.1 (23–52)
250 ml – 62.5 ml	1.6 (23–46)	1.4 (23–46)
1000 ml – 250 ml	2.0 (18–23)	2.0 (18–28)

[1] values in parentheses indicate the particular time interval of incubation (in hours) during which biodegradation occurred with maximum rate

[2] no substantial removal of phenanthrene during 76 hours of incubation.

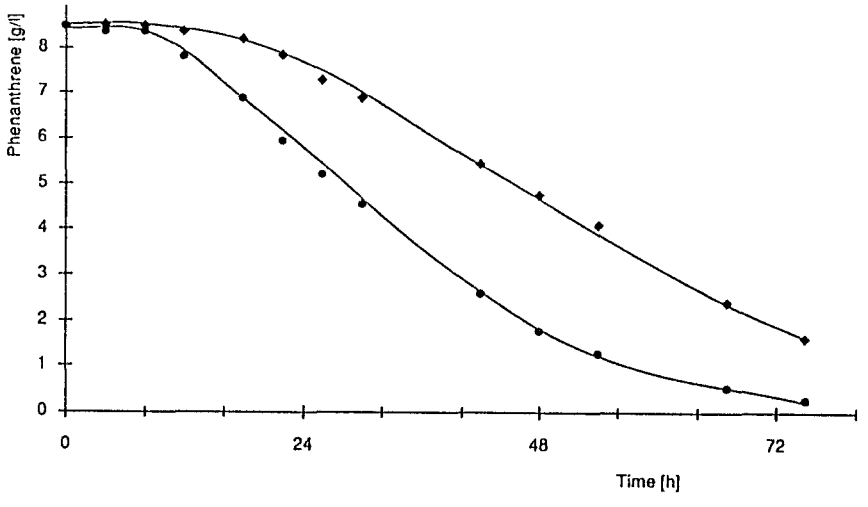

Fig. 4. Disappearance of phenanthrene (data given as phenanthrene concentrations in the HMN-phase; average values obtained from three parallel cultures) during incubation of *Pseudomonas aeruginosa* AK1 in the aqueous/HMN biphasic medium under conditions of low agitation (1000 ml-Erlenmeyer flasks containing 250 ml medium, shaken at 70 rpm) in the absence (—♦—) or presence (—•—) of the nonionic surfactant Biosoft HR33 (0.01%). The initial biomass in the aqueous phase corresponded to an optical density OD_{546} 0.07–0.08.

threne in mineral salts medium can markedly be enhanced by ultrasonication of the aqueous medium and subsequent addition of surfactants. Notably, growth of AK1 cells in such pretreated medium was linear.

As shown in model experiments using phenanthrene-pregrown AK1 cells and HMN as the phenanthrene-

Table 3. Rates of degradation of phenanthrene by strain AK1 in the aqueous/HMN biphasic culture system under conditions of low agitation[1] in the presence of different surfactants.

Surfactant added	Maximum degradation rate[2] [g phenanthrene/l · d]
without surfactant	0.7 (30–54)
Biosoft HR18 (0.01%)	0.8 (30–54)
Biosoft HR33 (0.01%)	1.2 (18–30)
Biosoft HR40 (0.01%)	0.9 (18–30)
Emulan A (0.01%)	0.9 (18–30)
Rhamnolipid (0.005%)	1.3 (18–30)

[1] 1000-ml-Erlenmeyer flask, 250 ml culture medium, shaking at 70 rpm

[2] values in parentheses indicate the particular time interval of incubation (in hours) during which biodegradation occurred with maximum rate.

carrying organic phase, the rate of phenanthrene biodegradation in aqueous/organic biphasic culture media is mainly governed by the size of the interface between the two liquid phases. Under conditions of intensive agitation and sufficiently high substrate concentration in the organic phase, exponential growth (with doubling times of 6–8 hours) of strain AK1 on phenanthrene as the sole carbon and energy source could be achieved in the aqueous/HMN biphasic culture system. This confirms biodegradation of phenanthrene (and possibly of other PAH) to be limited first of all by mass transfer.

Some earlier reports describe the influence of HMN on biodegradation of naphthalene by *Pseudomonas* sp. (Wodzinski and Larocca, 1977) and an *Arthrobacter* sp. (Efroymson and Alexander 1991). In these experiments exponential growth was not achieved, probably because significantly lower substrate concentrations and very low amounts of HMN ($\leq$ 2.4%) had been used compared to the present experiments (25% v/v).

In contrast to the naphthalene degrading bacterial strains mentioned above, *Pseudomonas aeruginosa* AK1 grows exclusively in the aqueous phase and does not attach to the lipophilic surface. So the PAH uptake mechanism of the strains and also the effect of HMN appear to be different. Surface-attached microorganisms may utilize the substrate directly from the HMN phase, while strain AK1 most likely takes up dissolved phenanthrene from the aqueous phase. In the latter case HMN serves only as a reservoir to provide the substrate continuously via diffusion into the aqueous phase.

Addition of surfactants to the culture medium was found to accelerate phenanthrene degradation by strain AK1 under certain experimental conditions. Evidence has been obtained that the stimulating effect was primarily due to improved dispersion of the phenanthrene particles (in mineral salts medium supplemented with phenanthrene crystals) or of the phenanthrene-carrying lipophilic solvent in the aqueous phase. Moreover, the addition of surfactants obviously prevented reaggregation of phenanthrene crystals and coalescence of HMN droplets. There was no correlation between the phenanthrene-solubilizing capacity of surfactants and enhancement of phenanthrene degradation. Thus, under conditions of intensive mixing of the aqueous or biphasic culture media, the addition of surfactants did not enhance phenanthrene degradation by strain AK1. These findings indicate that the PAH-solubilizing effect of surfactants is negligible under the given experimental conditions compared to mechanical effects.

As shown in this work for phenanthrene, use of water immiscible solvents like HMN as lipophilic mediators for facilitated mass transfer of PAH into the aqueous phase leads to several-fold enhanced rates of PAH biodegradation. Therefore it represents a valuable tool for both fundamental research and biotechnology concerning biodegradation of non-polar compounds.

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