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Biphenyl degradation kinetics by *Burkholderia xenovorans* LB400 in two-phase partitioning bioreactors

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Abstract

Biphenyl could be successfully degraded by *Burkholderia xenovorans* LB400, initially described as *Pseudomonas* sp. LB400, in two-phase partitioning bioreactors (TPPBs). TPPBs are comprised of an aqueous, cell containing phase, and an immiscible, biocompatible organic phase that partitions toxic and/or poorly soluble substrates (in this case biphenyl) based on maintaining a thermodynamic equilibrium. The critical $\mathrm{Log}\,K_{O/W}$ of the organism was found to be approximately 5.5, indicating that solvents with a $\mathrm{Log}\,K_{O/W}$ larger than 5.5 are suitable as delivery phases for *B. xenovorans* LB400. Two solvents selected for the TPPB system were octadecene and bis(2-ethylhexyl)sebacate (BES). In one experiment a total of 6.6 g biphenyl per l aqueous-phase-equivalent (biphenyl delivered in solvent, at an aqueous phase to solvent ratio of 10) could be degraded in 25 h during batch operation with octadecene. The specific growth rate and the half saturation constant of the Monod model were estimated to be $\mu_{max} = 0.25\ h^{-1}$ and $K_S = 0.0001\ g\ l^{-1}$, and the yield coefficient was $Y_{X/S} = 0.48$ g biomass per g biphenyl. These parameter estimates were used to predict the time course of biphenyl degradation at different initial substrate concentrations and with biphenyl delivered from the two solvents with different partitioning behaviour for biphenyl. The predictions were validated by experimental data, confirming the microbial kinetics as well as the expected partitioning effects.

Keywords: Availability; Insoluble; Monod; Modelling; Intermediates

1. Introduction

Biphenyl is an aromatic hydrocarbon, comprised of two, six-sided aromatic rings connected at one carbon on each ring. It is used as a fungistat in transportation containers of oranges and other citrus fruits (Ambrose et al., 1960). Biphenyl is also used as an intermediate for the production of emulsifiers, optical brighteners, plastics, crop protection products and other organic compounds. It is used as a heat transfer medium, as a dyestuff carrier for textiles and copying paper, as a solvent in pharmaceutical production, and it was the parent compound of polychlorinated biphenyls (PCBs) (Weaver et al., 1979). Biphenyl is considered to be one of the most thermally stable organic compounds (HSDB, 1991). Animal studies have indicated that biphenyl exposure results in morphological and histopathological changes in the urinary system and it is considered to be a possible mutagen based on in-vitro studies (Boehncke et al., 2005).

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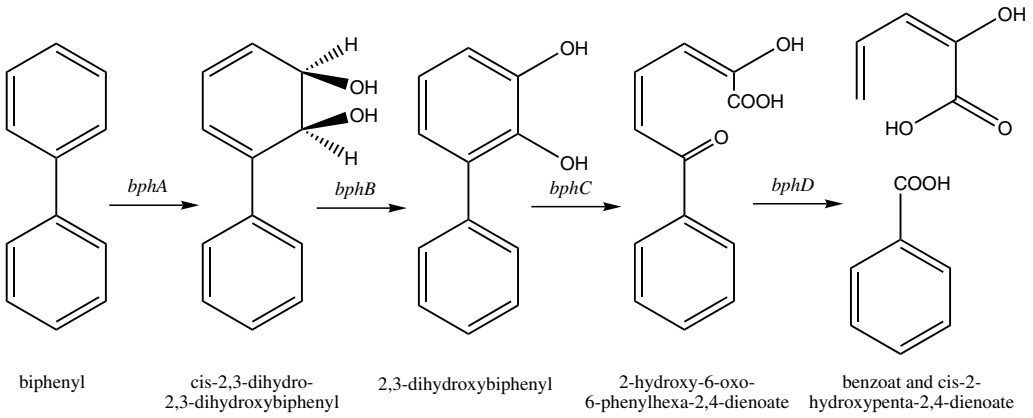

Fig. 1. Dioxygenation of biphenyl and ring fission mediated by the *bhp* genes (Kimura et al., 1997; Mondello et al., 1997).

Various aerobic bacteria are capable of degrading biphenyl via the *bhp* encoded pathway (Catelani et al., 1971; Haddock et al., 1993), which is also capable of degrading low chlorinated PCBs. *Burkholderia xenovorans* LB400 is the most studied aerobic biphenyl and PCB degrader (Bopp, 1986; Haddock et al., 1993; Billingsley et al., 1997; Seeger et al., 1999; Fain and Haddock, 2001). A yellow metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (Bruhlmann and Chen, 1999), (Fig. 1) is often used as an indicator for biphenyl degradation. However, biphenyl, PCBs and high molecular weight polycyclic aromatic hydrocarbons (PAHs) share many physical properties which limit engineered bioremediation processes, and one of these properties is their low solubility and therefore low availability to the degrading microorganisms. Two-phase partitioning bioreactors (TPPBs) have been used in various situations where aqueous solubility is a limiting degradation factor (Kohler et al., 1994; Daugulis, 2001; Janikowski et al., 2002). TPPBs are typically stirred-tank bioreactors consisting of an aqueous phase which contains the required nutrients to support microbial growth and an immiscible organic solvent which can either function as a delivery phase for hydrophobic compounds or can remove toxic products from the aqueous phase (Daugulis, 1997). The solvent used in a TPPB should be immiscible with water, non-toxic and non-biodegradable by the employed organism(s), as well as being cheap, non-toxic to humans, and possessing a high boiling point and a low volatility. Another feature of interest in the selection of a suitable TPPB solvent is the partitioning of the compound to be degraded, in this case biphenyl, between the two phases. High affinity of the solvent for the degradable substance is generally an advantage, as it allows high initial substrate loadings. A detailed description of solvent selection for TPPBs can be found in the literature (Collins and Daugulis, 1999).

This work examined the degradation kinetics of biphenyl by *B. xenovorans* LB400 in a TPPB using two delivery solvents. The use of a TPPB allows for continued provision of substrate for microbial degradation over a long period of time and facilitates the study of microbial degradation kinetics of highly hydrophobic substances. Kinetic studies are otherwise limited by the aqueous solubility of the substrate, which results in very short degradation times at very low substrate concentrations close to the analytical detection limit, and therefore poor estimates of the intrinsic kinetic parameters. A good understanding of the microbial degradation kinetics of hydrophobic compounds such as biphenyl may provide valuable information for engineered bioremediation processes of other poorly soluble contaminants such as PAHs and PCBs.

It was the objective of this work to develop a methodology to estimate kinetic parameters of the biodegradation of poorly water soluble substrates and to use this system to kinetically characterize biphenyl degradation by *B. xenovorans* LB400.

2. Materials and methods

2.1. Chemicals

All nutrients used in the fermentation media, and solvents, were obtained from either Sigma Aldrich (Canada) or Fisher Scientific (Canada). Biphenyl, 99% (assay) was obtained from Alfa Aesar (USA).

2.2. Bacterial strain

Pseudomonas strain LB400 (strain NRRLB-18064), isolated by researchers at General Electric (Schenectady, NY), was obtained from the Northern Regional

Research Laboratory (Peoria, IL). The strain has since been reclassified as *B. xenovorans* sp. nov. (Goris et al., 2004). Cultures were maintained on solid mineral salts medium (Bedard et al., 1986), pH 7, with biphenyl as the sole carbon source supplied as vapours from crystals in the lid of the Petri dish. The Petri dishes were incubated at 30 °C for 48 h and then stored at 4 °C. For long-term storage of cultures, 12% dimethyl-sulfoxide (DMSO) was added to the liquid cell culture in standard growth medium and 1 ml aliquods of the mixture were stored at −70 °C in 1.5 ml vials.

2.3. Standard growth medium

Phosphate buffered mineral salt medium supplemented with yeast extract was used as the standard growth medium (Bedard et al., 1986). The medium composition per litre was as follows: 75.5 ml stock solution (56.77 g l^{-1} K_2HPO_4, 21.94 g l^{-1} KH_2PO_4 and 27.61 g l^{-1} NH_4Cl), 10 ml trace element solution (19.5 mg l^{-1} $MgSO_4$, 5 g l^{-1} $MnSO_4 \cdot H_2O$, 7 g l^{-1} $FeSO_4 \cdot 7H_2O$ and 0.2 g l^{-1} $CaCl_2 \cdot 2H_2O$) and 50 mg yeast extract. The trace element solution was added to the autoclaved medium. Biphenyl was either dissolved in the chosen delivery phase or in acetone, filter sterilized using a 0.22 μm nylon syringe filter (Fisher Scientific (Canada)) and added to the autoclaved medium. Acetone was allowed to evaporate, leaving biphenyl crystals suspended in the medium.

2.4. Inoculum preparation

Cells from a Petri dish were inoculated into 50 ml growth medium with biphenyl as the sole carbon source in a 125 ml Erlenmeyer flask and incubated for 22–24 h at 30 °C on an orbital shaker set at 180 rpm. The cell suspension was filtered through sterile glass wool to remove excess biphenyl crystals, centrifuged at 3400 rpm for 20 min, washed twice with 0.05 M sodium phosphate buffer, pH 7.5, and resuspended in biphenyl-free medium to an OD of 2.0 at 600 nm (OD_{600}), using a 1-cm light path.

2.5. Biphenyl degradation in TPPBs

Experiments were undertaken in 5-l New Brunswick BioFlo® III bioreactors, agitated with two Rushton turbines at 800 rpm (to avoid mass-transfer limitation) and aerated (sterile air) at 3 l min^{-1}. The aqueous-phase volume was 2800 ml in all experiments. The solvent in the first experiment was 280 ml octadecene with an initial biphenyl concentration in the solvent of 66 g l^{-1}, corresponding to 6.6 g biphenyl per litre aqueous phase. The solvents in the second experiment were 175 ml and 1750 ml bis(2-ethylhexyl)sebacate (BES), with initial biphenyl concentrations in the solvents of 80 g l^{-1} and 8 g l^{-1}, respectively, corresponding to 5 g biphenyl per litre aqueous phase. The reactors were inoculated with 50 ml inoculum. Conditions were automatically maintained at 30 °C, and at pH 6.9 by adding 3 M KOH. Dissolved oxygen levels were measured with a polarographic-membrane electrode (Broadley and James Corp., USA). All experiments were done in duplicate and replicates were not done simultaneously.

2.6. Solvent selection

Bioavailability of selected solvents was assessed by inoculating 50 ml standard growth medium and 5 ml solvent in the absence of any other carbon source with *B. xenovorans* LB400. The OD_{600} of the aqueous phase was measured after 72 h. Biocompatibility was tested by inoculating 50 ml standard growth medium and 5 ml solvent containing biphenyl (50 g l^{-1}), and 50 ml tryptic soy broth (TSB) and 5 ml solvent, with *B. xenovorans* LB400. The OD_{600} of the aqueous phase was measured after 72 h.

2.7. Biphenyl analysis

The biphenyl concentration in solvent samples was analyzed via a Varian 3400 Gas Chromatograph (GC) (Varian, Inc., CA, USA), equipped with a flame ionization detector (FID) and a 30 m DB5.625 0.25 micron column (J&W Scientific/Agilent Technologies Canada Inc., Mississauga, ON). The following temperature program was used: Initial column temperature 90 °C holding time 2 min, 10 °C min^{-1} to 180 °C, 3 °C min^{-1} to 250 °C followed by a holding time of 60 min. The injector temperature was 150 °C and the detector temperature was 300 °C. Data analysis and peak integration was performed by the software package Millenium[32] (Workstation Version 3.05.01 Waters Corp., USA). The detection limit in the organic phase was 10 μg l^{-1}.

2.8. Biomass analysis

Aqueous phase samples were centrifuged at 3400 rpm for 20 min. The pellet was washed twice with buffer, resuspended to the original volume and diluted to an OD_{600} between 0.1 and 0.6 to ensure that samples were measured in the linear range of the instrument. The OD_{600} was converted to dry cell weight (DCW) using the factor 0.3648 g l^{-1} OD_{600}^{-1} which was determined by filtering and drying cell suspensions and correlating the dry weight to the previously recorded OD_{600} readings.

2.9. Biphenyl solubility tests

Biphenyl solubility in various solvents was tested by adding 5 g biphenyl to 5 ml solvent in 15 ml glass vials

closed with foil-lined cap. The vials were placed on a rotary shaker at 25 °C for 48 h. Un-dissolved biphenyl crystals were removed by filtering through glass wool. The filtrate was subsequently analyzed via GC-FID.

3. Results and discussion

3.1. Solvent selection

A wide range of possible TPPB solvents were initially considered based on their costs, availability and desirable physical properties (low vapour pressure, high boiling point, etc.), resulting in a short list of solvents to be examined experimentally. The biocompatibility of these selected solvents with *B. xenovorans* LB400 was then tested by comparing microbial growth on biphenyl and TSB in the presence and absence of solvents. A plot of the relative cell growth as a function of the octanol water partitioning coefficient of the solvents ($\mathrm{Log}\,K_{O/W}$) is shown in Fig. 2. All solvents shown in this figure were shown not to support cell growth of *B. xenovorans* LB400 within 72 h if provided as the sole carbon source (data not shown) and are therefore considered to be not bioavailable (i.e. not used as a substrate). From Fig. 2 the critical $\mathrm{Log}\,K_{O/W}$ of *B. xenovorans* LB400 is estimated to be between 5.5 and 6. Solvents with a $\mathrm{Log}\,K_{O/W}$ higher than 6 can therefore be considered as possible delivery phases during biphenyl degradation using *B. xenovorans* LB400 in a TPPB. This critical

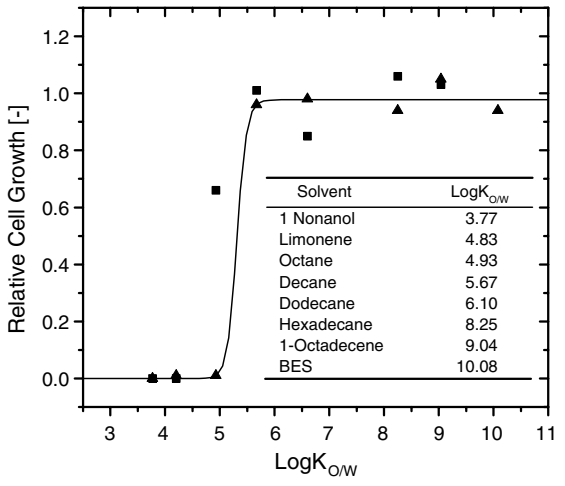

Fig. 2. Ability of *B. xenovorans* LB400 to grow in the presence of various solvents with different $\mathrm{Log}\,K_{O/W}$. The relative cell growth is the optical density of the cell suspension in the presence of solvent divided by the optical density in the absence of solvent after incubation of 72 h. The squares represent incubation in TSB medium and triangles show growth on biphenyl as a carbon source in mineral salt medium.

$\mathrm{Log}\,K_{O/W}$ is within the reported ranges found for bacteria; typical values for gram negative bacteria are between 3 and 5 and for gram positive bacteria between 5 and 7 (Inoue and Horikoshi, 1989, 1991; Collins and Daugulis, 1999). The only significant difference in the ability of the organism to grow in the presence of solvents on the two carbon sources employed (biphenyl and TSB) was in the case of octane. Octane is the only solvent that promoted growth on TSB and not on biphenyl. The $\mathrm{Log}\,K_{O/W}$ of octane is 4.9, relatively close to the critical $\mathrm{Log}\,K_{O/W}$ of *B. xenovorans* LB400, and it is speculated that the organism may able to tolerate a larger stress imposed by the presence of a solvent when growing on an easily accessible carbon source (TSB). Two solvents, BES and octadecene were chosen for further consideration as delivery phases for biphenyl degradation due to their high $\mathrm{Log}\,K_{O/W}$ values.

The solubility of biphenyl in the delivery solvent is an additional factor to consider in solvent selection. High biphenyl solubility in the solvent allows high initial substrate loadings, which permits the use of smaller amounts of solvents, thus reducing cost. Although $\mathrm{Log}\,K_{O/W}$ can be used to asses the hydrophobicity and the biocompatibility of a solvent it cannot be used as an indicator of the solubility of biphenyl in these solvents. Solvents with high $\mathrm{Log}\,K_{O/W}$ value do not necessarily have a stronger affinity for biphenyl than less hydrophobic solvents, as shown in Table 1. This table includes not only the tested solvents (upper section), but several additional common solvents for comparison purposes (lower section). The $\mathrm{Log}\,K_{O/W}$ for *n*-alkanes increases from 3.8 for hexane to 8.15 for hexadecane, as the length of the alkane chain increases. The molar solubility (mol mol^{-1}) of biphenyl in these alkanes increases with the chain length, whereas the mass solubility (g l^{-1}) decreases slightly due to the increased molecular weight of longer alkanes. The solvent/water partitioning coefficient (Eq. (1)) of biphenyl, estimated as the solubility of biphenyl in a given solvent divided by its solubility in water, therefore decreases with the length of the alkanes as well.

$$K_{S/W} = \frac{S_{sol.}}{S_{aq.}} = \frac{S^*_{sol.}}{S^*_{aq.}} \quad (1)$$

where $K_{S/W}$ is the equilibrium partitioning coefficient of biphenyl between a given solvent and water $S_{sol.}$, and $S_{aq.}$ are the concentrations of biphenyl in the given solvent and water, and $S^*_{sol.}$ and $S^*_{aq.}$ are the solubilities in the solvent and in water, respectively.

The highest solubility for biphenyl can be found in substances with aromatic structures, such as benzene and toluene. These solvents, however, do not fulfil the $\mathrm{Log}\,K_{O/W}$ biocompatibility requirements of the selected organism. The selected solvents, BES and octadecene, show high solubility of biphenyl and were therefore both chosen as possible delivery phases for biodegradation of biphenyl.

Table 1
Properties of various solvents and the solubility of biphenyl in these solvents at 25 °C

Solvent	Boiling point [°C]	Molar weight [g mol^{-1}]	Density [g cm^3]	Log $K_{O/W}$ of solvent	$S^*_{sol.}$ [mol mol^{-1}]	$S^*_{sol.}$ [g l^{-1}]	Log $K_{S/W}$ of biphenyl
1-Nonanol	215	144.50	0.827	3.77	0.13	117.15	4.23
d-Limonene	176	136.26	0.839	4.20	0.50	471.24	4.84
n-Octane[a]	125	114.23	0.703	4.76	0.15	139.51	4.31
n-Decane[b]	174	142.29	0.730	5.67	0.16	129.44	4.28
n-Dodecane	216	170.34	0.750	6.60	0.18	125.43	4.26
n-Hexadecene[b]	287	226.45	0.773	8.15	0.22	113.23	4.22
n-Octadecene	315	252.48	0.790	9.04	0.21	101.26	4.17
BES	>300	426.70	0.914	10.08	0.50	165.94	4.39
n-Hexane[a]	69	86.18	0.660	3.84	0.12	146.45	4.33
n-Heptane[a]	98	100.20	0.684	4.31	0.13	139.90	4.31
n-Nonane[b]	150	128.26	0.718	5.23	0.16	133.90	4.29
Benzene[a]	80	78.11	0.870	2.04	0.38	654.38	4.98
Toluene[a]	111	92.14	0.865	2.66	0.38	545.79	4.90
Methanol[b]	65	32.04	0.780	−0.66	0.02	69.49	4.01
1-Octanol[b]	196	130.23	0.827	–	0.11	107.43	4.20

[a] Data from Chang (1969).
[b] Data from De Fina et al. (1999).

3.2. Biphenyl degradation

B. xenovorans LB400 is the most studied aerobic biphenyl and PCB degrader (Bopp, 1986; Haddock et al., 1993; Billingsley et al., 1997; Seeger et al., 1999; Fain and Haddock, 2001). The degradation pathway has been studied extensively on a molecular and genetic level and a draft of the entire genome is available through the Joint Genome Institute (JGI; http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html). However, no extensive kinetic studies on biphenyl degradation in a controlled bioreactor system can be found in the literature, possibly because of operational difficulties with low substrate solubility in conventional (single aqueous phase) bioreactor systems. Degradation of biphenyl by *B. xenovorans* LB400 has been assumed to follow the Monod model (Monod, 1949), which is the most frequently used model to describe microbial growth in substrate-limited systems. The Monod model in its simplest form describes the microbial growth rate as a function of the concentration of one limiting substrate, in this case biphenyl. In the given system, only biphenyl dissolved in the aqueous phase is considered available to the microorganisms. Biphenyl functions as the sole carbon and energy source and all other nutrients, including oxygen, are in excess. Eqs. (2) and (3) describe the biomass and total substrate concentration as a function of time. The total substrate concentration is defined as the total amount of substrate present in both aqueous phase and solvent, normalized by the volume of the aqueous phase (Eq. (4)). Eq. (1), defined earlier, describes the partitioning of biphenyl between the two phases.

$$\frac{dX}{dt} = X \cdot \frac{\mu_{max} \cdot S_{aq.}}{K_S + S_{aq.}} \quad (2)$$

$$\frac{dS_{tot.}}{dt} = \frac{X}{Y_{X/S}} \frac{\mu_{max} \cdot S_{aq.}}{K_S + S_{aq.}} \quad (3)$$

$$S_{tot.} = \frac{S_{aq.} \cdot V_{aq} + S_{sol} \cdot V_{sol.}}{V_{aq.}} \quad (4)$$

where X is the biomass concentration in the aqueous phase [g l^{-1}], μ_{max} is the maximum specific growth rate [h^{-1}], K_S is the half saturation concentration [g l^{-1}], $Y_{X/S}$ is the yield coefficient [g biomass per g substrate], $S_{aq.}$ and $S_{sol.}$ are the substrate (biphenyl) concentrations in aqueous phase and the solvent [g l^{-1}], respectively. $S_{tot.}$ is the total substrate available in the system normalized to the aqueous phase volume [g l^{-1}] and $V_{aq.}$ and $V_{sol.}$ are the volume of the aqueous phase and the solvent [l], respectively.

This set (Eqs. (1)–(4)) of coupled non-linear ordinary differential equations (ODEs) can be solved in a number of different ways (Mohamed and Hatfield, 2005). The most common numerical method to solve stiff systems of ODEs is Gear's method (Gear, 1971); however the finite difference method, which is simpler and faster to compute has been shown to be equally accurate on similar sets of ODEs (Mohamed and Hatfield, 2005) and was therefore used here.

Fig. 3a shows biomass formation and biphenyl degradation in a TPPB by *B. xenovorans* LB400. The delivery phase was octadecene with a Log $K_{S/W} = 4.17$. Biphenyl degradation was completed after approximately 25 h, and at the same time, no further biomass formation was observed. The solid lines represent the solutions to the previously defined set of equations,

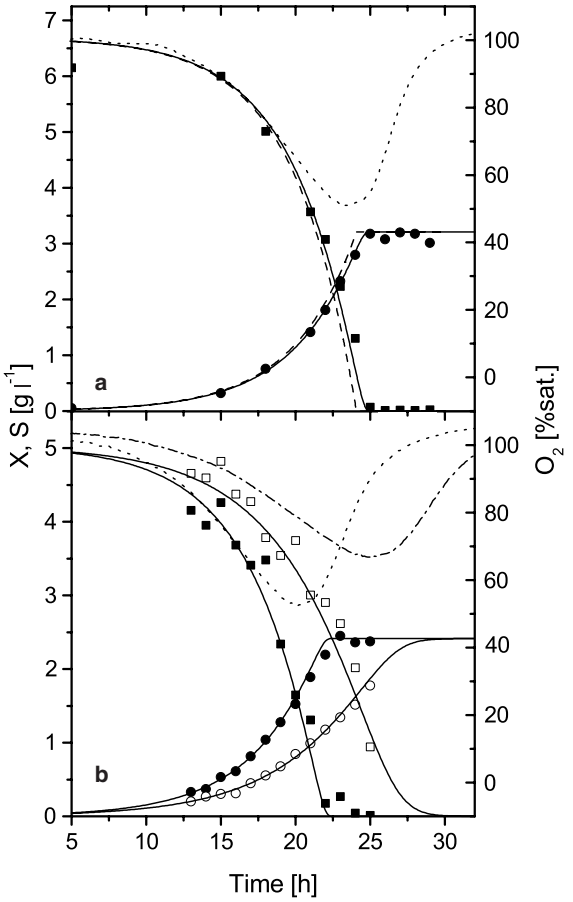

Fig. 3. Biomass formation (circles) and biphenyl degradation (squares) in TPPB by *B. xenovorans* LB400. (a) The initial amount of biphenyl was 6.6 g l^{-1} based on the aqueous-phase volume. Biphenyl was delivered from octadecene at an initial concentration of 66 g l^{-1}. The solid lines show the Monod simulation and the dashed lines a Monod simulation using the same parameter estimates and assuming a single liquid phase reactor in which biphenyl is provided at an equivalent aqueous-phase biphenyl concentration as solid crystals. The dotted line shows the dissolved oxygen concentration. (b) The initial amount of biphenyl was 5 g l^{-1} based on the aqueous-phase volume. Biphenyl was delivered from BES at initial concentrations of 8 g l^{-1} (open symbols) and 80 g l^{-1} (closed symbols). The solid lines show the Monod simulations. The dash-dot line shows the dissolved oxygen concentration in reactor 1 (open symbols) and the dotted line shows the dissolved oxygen concentration in reactor 2 (closed symbols).

and the parameter estimates are $\mu_{max} = 0.25\ h^{-1}$, $K_S = 0.1$ mg l^{-1} and $Y_{X/S} = 0.48$ g biomass per g biphenyl. These estimated parameters are in the typical range for bacteria (Bailey and Ollis, 1986), although the half saturation concentration K_S is at the low end of the spectrum, which can be expected of a hydrophobic substrate. Reichardt et al. (1981), one of the very few studies on biphenyl degradation kinetics, reported $\mu_{max} = 0.067\ h^{-1}$ and $K_S = 0.00023$ mg l^{-1} for a microbial consortium growing aerobically on biphenyl. Although the maximum specific growth rate of *B. xenovorans* LB400 is significantly higher than that of the consortium, the half saturation constant of the consortium is substantially lower, suggesting that the members of the consortium may have been highly adapted to extremely low substrate concentrations. The half saturation constant found for *B. xenovorans* LB400 indicates that it might show poor degradation performance at low substrate concentrations, compared to the microbial consortium. The initial aqueous phase substrate concentration in the above-discussed experiment was still approximately 50 times higher than the half saturation concentration, but the substrate concentration in the aqueous phase might become the limiting factor in applications with more hydrophobic substrates.

TPPBs will always provide aqueous-phase substrate concentration below the saturation concentration of the specific compound in the aqueous phase, due to thermodynamic equilibrium of the activities of the target compound in both phases. This feature has been proven highly beneficial in cases where the substrate is toxic in high concentration as in the case of phenol (Prpich and Daugulis, 2004), however, it might result in substrate-limited systems in the case of batch degradation of highly hydrophobic substances with no or low toxicity to the degrading organisms, as in the case of biphenyl, or high molecular PAHs and PCBs. The dashed lines in Fig. 3a show simulated biphenyl and biomass concentrations (assuming the same kinetic parameters) for a hypothetical single-phase case in which biphenyl is provided at an equivalent aqueous phase biphenyl concentration with the biphenyl provided as solid crystals. In this case, assuming instantaneous dissolution of the solid crystals, the aqueous phase would be saturated with biphenyl over essentially the entire time of the fermentation. The hypothetical saturated aqueous-phase reactor would perform faster than the TPPB only at the very end of the fermentation. These results confirm that TPPBs can provide a valuable technology for the degradation of highly hydrophobic compounds, as discussed earlier (Daugulis, 2001). Large quantities of biphenyl were completely dissolved in the solvent, delivered to the degrading organisms and degraded to completion. The above-mentioned single-phase system would be difficult to examine experimentally, mainly because of difficulties in the analysis of the total substrate concentration.

The parameter estimates were further verified by conducting two experiments in parallel with the same initial amount of biphenyl present in the system based on the volume of the aqueous phase. One reactor contained ten-fold amount of solvent compared to the other reactor. Based on the assumption of a constant partitioning coefficient, an initial ten-fold lower biphenyl concentration

in the aqueous phase is expected, which would result in lower specific growth rates and consequently lower degradation rates. The delivery phase was also changed from octadecene ($\text{Log}\,K_{S/W} = 4.17$) to BES ($\text{Log}\,K_{S/W} = 4.39$). Fig. 3b shows the experimental data as well as the predicted time courses. The experimental data follow the model prediction very well, which supports the estimates for the microbial parameters as well as the measured partitioning coefficients ($\text{Log}\,K_{S/W}$ for octadecene and BES). The results show that low concentrations of a target molecule can still be degraded successfully in TPPBs. It also shows that low initial substrate loadings in the organic phase of a TPPB will result in lower microbial growth/degradation rates. For non-toxic substrates, it can be concluded that the most economical way to operate a TPPB is at initial substrate loading close to the substrate solubility in the solvent. This mode of operation will minimize the amount of solvent needed in the reactor and also maximize the degradation rate. It is however necessary that the system be well-mixed. The solvent/aqueous phase ratio will become significant if mass transfer from the solvent into the aqueous phase becomes the rate-limiting step.

A yellow colorization could be observed in the reactor containing the smaller solvent fraction. This was initially interpreted as a sign of oxygen limitation; however the dissolved oxygen trace, shown as the dotted line in Fig. 3b, indicates that the oxygen concentration in the aqueous phase never dropped below 50% of the saturation concentration. The yellow color was observed between 12 h and 22 h of operation, and this is a common phenomenon during biphenyl and PCB degradation (Wesche et al., 2005). The yellow metabolite has been identified by Bruhlmann and Chen (1999) as 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, and is the fourth intermediate during biphenyl degradation, as shown in Fig. 1. Based on the fact that this metabolite only accumulated in the reactor having faster microbial biphenyl degradation, it can be concluded that the breakdown of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, catalyzed by the *bphD* gene product, can become the rate-limiting step in aerobic biphenyl degradation at high substrate concentrations. Kinetic studies of this enzyme can be found in the literature (Speare et al., 2002), but cannot easily be compared to current findings, as the actual concentration of the intermediate as well as its partitioning behaviour has not been studied, and is beyond the scope of this paper. Moody et al. (2002) studied the intermediates formed during biphenyl degradation by *Mycobacterium* sp. PYR-1 and could not detect any earlier metabolites other than 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate in the growth medium. The temporary accumulation of this intermediate can easily be avoided by reducing the aqueous-phase substrate concentration by increasing the solvent to solute ratio and keeping the total mass of substrate constant. TPPBs can therefore be used to control the metabolic activity of microorganisms, and the degradation rate, and in some cases, the accumulation of metabolites, can be controlled. This can help reduce accumulation of possibly toxic metabolites, which will, depending on their hydrophobicity, partition into the organic phase, and reduce the toxicity even further.

4. Conclusions

Biphenyl could successfully be degraded in TPPBs using two different solvents and different initial substrate concentrations. Microbial growth and biphenyl degradation could be modelled using Monod kinetics and assuming equilibrium partitioning. To the best of our knowledge this was the first attempt to model biphenyl degradation by *B. xenovorans* LB400 using the Monod model.

A method to study microbial kinetics for the degradation of poorly soluble substrate has been developed via the use of a two-phase bioreactor system. The system readily allows the kinetic characterization of microbial biphenyl degradation and is expected to be able to characterize microbial degradation of other hydrophobic compounds. These kinetic constants can be used in more complex models for environmental applications.

Acknowledgements

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