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Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds

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Abstract

Two-liquid-phase culture systems involve the addition of a water-immiscible, biocompatible and non-biodegradable solvent to enhance a biocatalytic process. Two-liquid-phase bioreactors have been used since the mid-seventies for the microbial and enzymatic bioconversion of hydrophobic/toxic substrates into products of commercial interest. The increasing popularity of bioremediation technologies suggests a new area of application for this type of bioreactor. The toxicity and the limited bioavailability of many pollutants are important obstacles that must first be overcome in order to improve biodegradation processes. Two-liquid-phase bioreactors have the potential to resolve both limitations of biotreatment technologies by the enhancement of the mass-transfer rate of compounds with low bioavailability, and by the controlled delivery of apolar toxic compounds. This technology can also be useful in accelerating the enrichment of microorganisms degrading problematic pollutants. In this paper, we discuss the application of two-liquid-phase bioreactors to enhance the biodegradation of toxic/poorly bioavailable contaminants. Important microbial mechanisms involved in this type of system are described. Uptake of the substrates can be achieved by microorganisms freely dispersed in the aqueous phase and/or bound at the interface between the aqueous and the immiscible phases. Production of surface-active compounds and adhesion abilities are microbial features involved in the process. General guidelines for the design of two-liquid-phase bioreactors for biodegradation purposes are presented. Solvent selection should be established on specific criteria, which depend on the characteristics of target compound(s) and the microorganism(s) implicated in the biodegradation process. The central importance of maximizing the interfacial surface area is highlighted. The potential of this approach as an alternative to current biotreatment technologies is also discussed.

Abbreviations: NAPL, non-aqueous-phase liquid; PAH, polycyclic aromatic hydrocarbon; TLPB, two-liquid-phase bioreactor; TLPS, two-liquid-phase system.

Introduction

Bioremediation, the biodegradative removal or reduction of hazardous pollutants, represents an increasingly popular application and active area of research in biotechnology (Alexander 1994). A wide variety of bioreactor technologies have been developed for the treatment of solids, liquids and gases contaminated with multiple organic compounds (Cookson 1995). The main advantage of bioreactors is that physico-chemical conditions can be controlled and optimized, in order to enhance the activity of microorganisms. They are mainly used for complex contamination cases, but their usefulness is sometimes limited by their associated costs and limited treatment efficiency for some toxic/hydrophobic compounds.

Two-liquid-phase bioreactors have been used since the mid-seventies for the microbial and enzymatic bioconversion of hydrophobic/toxic substrates into products of commercial significance (Schwartz & Mc-

Coy 1977; Furuhashi et al. 1986; Wubbolts et al. 1996). Recently, there has been increasing interest in the application of two-liquid-phase bioreactors to enhance the biodegradation of organic compounds of environmental relevance (Ascon-Cabrera & Lebeault 1993; Vanneck et al. 1995; Jimenez & Bartha 1996).

Usually, two-liquid-phase systems consist of an aqueous phase and a water-immiscible liquid phase present in excess of mutual saturation (special situations such as aqueous/aqueous or organic/organic systems will not be considered in this review). Although they are often referred to as biphasic cultures, at least four phases are actually present in this type of system: hydrophobic phase, aqueous phase, gas phase, and cells (van der Meer et al. 1992a). The expressions "two-liquid-phase" systems (TLPSs) or bioreactors (TLPBs) are, therefore, used in the text.

TLPBs can be divided in two categories (Erickson et al. 1969). The first category encompasses systems where the water-immiscible liquid is the substrate for the microorganisms. This includes: (1) industrial processes for the biotransformation of poorly-soluble substrates into products of interest, e.g. petroleum hydrocarbon fermentations for the production of single-cell proteins (Amin et al. 1973; Shennan 1984); and (2) biosynthesis of value-added chemicals from compounds like *n*-alkanes (Favre-Bulle et al. 1991; Preusting et al. 1993) or other lipophilic substrates (Harbron et al. 1986; Williams et al. 1990).

In the second category of TLPB, the non-aqueous-phase liquid (NAPL) is not the substrate but is added to the bioreactor to enhance a process. The NAPL acts as a non-biodegradable and biocompatible liquid phase reservoir in which apolar organic substrates are dissolved. Such a system has three different areas of application:

(1) The NAPL may be added to the bioreactor to continuously remove the end-products of a biotransformation process. This can alleviate a decrease in productivity due to product-induced feedback inhibition or toxicity, and facilitate downstream product recovery. This process is called extractive fermentation (or biocatalysis) (Freeman et al. 1993; Daugulis 1994);

(2) The NAPL may be introduced in the bioreactor to control the aqueous concentration of a toxic, low polarity substrate. In this situation, the hydrophobic phase acts as a reservoir for controlled delivery of the inhibitory compound to the aqueous phase where biocatalysis occurs (e.g. Collins et al. 1995; Tsai et al. 1996);

(3) Finally, the best known application of TLPBs is the addition of a NAPL to enhance the biotransformation of a water-insoluble substrate by increasing its bioavailability to biocatalysts (Van Sonsbeek et al. 1993; Nikolova & Ward 1993).

The last two applications of TLPBs (partitioning of toxic and water-insoluble substrates by the addition of an inert NAPL) have been used both in biotransformation processes for the synthesis of industrial chemicals and for the complete biodegradation of compounds, e.g. organic pollutants.

There are numerous publications concerning research work on TLPBs in the context of biotechnological production processes, and this topic has been extensively reviewed (Daugulis 1997; Van Sonsbeek et al. 1993; Nikolova & Ward 1993; Brink et al. 1988; Laane et al. 1987; Lilly et al. 1987; Brink & Tramper 1985). The purpose of this paper is consequently not to address this area of application but rather to present a review of the literature in which this technique has been used in biodegradation/biotreatment processes. We discuss the following points: (1) the specific microbial mechanisms involved in TLPSs, (2) the use of TLPBs to enhance the biodegradation of toxic/poorly bioavailable compounds, (3) the parameters that should be considered in the design and optimization of TLPBs in biotreatment situations, and (4) the potential of this approach as an alternative to current biotreatment technologies.

Microbial mechanisms involved in TLPBs

Erickson et al. (1969) were the first to describe growth models for TLPSs where the growth substrate is dissolved in an inert dispersed phase. They, and others (e.g. Woodley et al. 1991), have indicated that in this type of system the reaction can take place: (1) uniformly throughout the aqueous phase after diffusion or solubilization of the substrate, and (2) at the liquid-liquid interface by direct attachment to the immiscible solvent phase (Figure 1). Therefore, understanding and predicting microbial growth behavior on water-insoluble (and toxic) chemicals in TLPBs depends on the location of the reaction. Surface-attached microbial cells may utilize the substrate directly from the water-immiscible phase, while cells that grow only in the aqueous phase take up dissolved or pseudosolubilized substrate from the aqueous medium (Nakahara et al. 1977; Miura et al. 1977). As discussed by Westgate et al. (1995), these two mechanisms are difficult

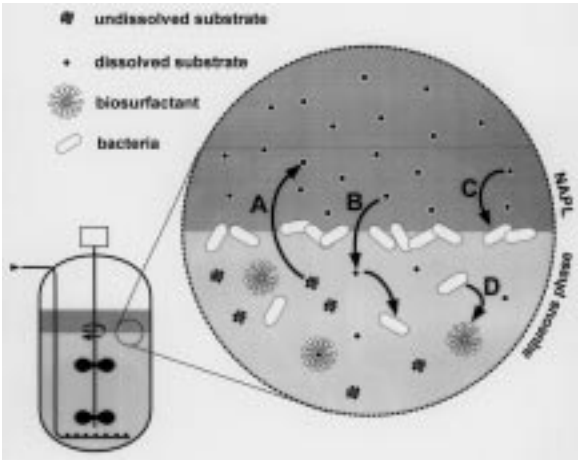

Figure 1. Physico-chemical and biological mechanisms implicated in TLPBs. (A) Dissolution of hydrophobic/toxic compounds in the NAPL. (B) Partitioning of the molecules in the aqueous phase and uptake by microorganisms in suspension. (C) Interfacial uptake of the substrate by adhering microorganisms. (D) Production of surface-active and solubilizing substances.

to distinguish experimentally because an increase in interfacial area will increase not only the surface for cell contact but also for diffusional mass-transfer rate.

Three modes of uptake of poorly water-soluble substrates by microorganisms are generally considered (Nakahara et al. 1977; Huang & Chou 1990; Goswami & Singh 1991). In the first mode, only the substrate dissolved in the aqueous phase is bioavailable and the degradation rate depends on the mass-transfer rate from the nonaqueous to the aqueous phase. This is the model generally accepted for solid compounds such as low molecular weight polyaromatic hydrocarbons (Wodzinski & Coyle 1974; Volkering et al. 1992) but considered not to contribute significantly to growth on insoluble substrates, such as long chain ($\geq C_{12}$) alkanes (Erickson & Nakahara 1975; Singer & Finnerty 1984). In TLPBs, biodegradation rates of compounds dissolved in NAPL are often higher than partitioning rates from nonaqueous to aqueous phases (Efroymson & Alexander 1991, 1994; Labare & Alexander 1995; Ortega-Calvo et al. 1995; Osswald et al. 1996; Bouchez et al. 1997b). This indicates that the maximum biodegradation rate of compounds present in NAPLs cannot be predicted from their dissolution rate. Consequently, growth in TLPSs will be best described by the next two uptake mechanisms where microbial growth and activity increase the bioavailability of the NAPL-dissolved substrate.

In the second uptake mode, cells come in direct contact with the NAPL. A biofilm may develop at the interface (Rosenberg 1989; Marin et al. 1996), and microorganisms acquire the substrate by transfer near the point of contact through their membrane (interfacial uptake). The way by which attached cells acquire their substrate from the NAPL is still obscure. Nevertheless, it may simply be that substrate concentration is superior in the aqueous phase near the interface. Cell adhesion onto surfaces is known to depend on the culture medium, culture conditions and the physiological activity of the cells (Rosenberg 1991). For some microorganisms, cell attachment is of great significance and even a prerequisite for substrate uptake. Nonadhesive mutants of alkane-degrading bacteria could not grow on hexadecane (Rosenberg & Rosenberg 1981). Initial adhesion is due to nonspecific hydrophobic interactions (Singer & Finnerty 1984). Cell surface hydrophobicity correlates well with the ability to adhere to surfaces (van Loosdrecht et al. 1987; Vanhaecke et al. 1990). Bell (1973) found that when long chain fatty acids dissolved in pristane were used as carbon source, the growth of *Candida tropicalis* having hydrophobic surface was improved, but that of *Saccharomyces cerevisiae* having hydrophilic surface was unaffected. It has been demonstrated that cells cultured on hydrocarbon or oil surfaces become more hydrophobic than cells growing on soluble substrates (Gutierrez & Erickson 1977; Neufeld et al. 1980; Ng & Hu 1989; Ascon-Cabrera & Lebeault 1995a). Likewise, many studies showed a decrease in the number of cells in the aqueous phase and an accumulation of cells at the interface (Efroymson & Alexander 1991; Vanneck et al. 1995; Jimenez & Bartha, 1996). In a TLPB where paraffin oil was added to enhance the biodegradation of pyrene, bacteria collected at the interface mineralized pyrene 8.5 times faster than free cells from the aqueous phase (Jimenez & Bartha 1996). Under certain circumstances, preventing the adhesion of microorganisms to liquid hydrocarbon substrates by a surfactant suppresses their activity. For example, the nontoxic surfactant Triton X-100 suppressed the adhesion of an *Arthrobacter* strain to heptamethylnonane containing the growth substrate hexadecane, preventing its biodegradation (Efroymson & Alexander 1991).

Finally, in the third uptake mechanism, microorganisms produce surface-active compounds causing the formation of small (less than 1 μm) droplets (emulsification) or micelles (pseudosolubilisation) that are directly assimilated. The production of surfactants and emulsifiers by microorganisms growing on liquid hydrocarbons is well documented (Desai

& Banat 1997; Hommel 1990). However, the exact mechanism by which the producing microorganisms take advantage of this ability is poorly understood. Emulsification of the NAPL is sometimes encountered in TLPBs, suggesting that microorganisms are producing surface-active agents. The presence of adherent microbial cells at the surface of oil droplets also stabilises emulsions by preventing coalescence (Erickson & Nakahara 1975; Rosenberg 1989). Ascon-Cabrera & Lebeault (1995a) attributed the increase in the interfacial area between the two liquid phases during a culture, caused by the formation of an emulsion, to a reduction in the interfacial tension. Osswald et al. (1996) observed that bacterial activity increased the rate of styrene partitioning in a TLPB. From the biodegradation rate that was higher than the partitioning rate and by a similar behavior when a synthetic surfactant was added, they deduced that the bacteria were releasing solubilizing agents. In addition, surface-active compounds retained on the cell surface could be involved in the regulation of microbial surface hydrophobicity, further linking the second and third uptake modes (Neu 1996).

All three uptake mechanisms may happen consecutively or simultaneously in a culture system, depending on the properties of microorganisms, the type of substrate, and growth conditions. A growth model can be described by the following sequence of events (Ascon-Cabrera & Lebeault 1995a; Ortega-Calvo & Alexander 1994; Prokop et al. 1972): (1) at low cell concentration, growth occurs on the dissolved substrate portion; (2) while biomass and substrate requirements are increasing, cells become more hydrophobic and adherent and/or the more adherent cells are located near the interface where the substrate is more abundant; (3) cells grow as a biofilm covering the available surface on large NAPL droplets, consuming the substrate from the inert reservoir solvent; (4) growth in the continuous phase slows down because of the decreasing mass-transfer rate resulting from the depletion of the content of droplets; (5) the increasing number of cells at the drop surface and the production of surface-active substances give rise to a reduction in interfacial tension, which leads to the emulsification of the NAPL and an increase in the interfacial area for growth and mass-transfer. In some cases, the emulsification and solubilization of the NAPL result in the formation of submicron droplets or pseudosolubilisation into micelles (Goma et al. 1974; Velankar et al. 1975; Chakravarty et al. 1975) that are rapidly consumed; and finally (6) cells come off the depleted NAPL droplets and colonize fresh solvent droplets (Rosenberg 1989).

A special situation is encountered with toxic apolar substrates, since both emulsification and interfacial contact may have inhibitory effects on microbial activity. Nevertheless, as discussed in the section Design of TLPBs for biodegradation purposes, toxicity problems can be avoided with careful selection of the solvent and parameters of bioreactor operations.

In summary, microbial growth and activity in TLPBs increase the interfacial area between the two liquid phases, and this in turn promotes the mass-transfer rate of hydrophobic substrates from the NAPL to the aqueous phase, increases the surface available for attachment and ultimately improves biodegradation rates.

Use of TLPBs to enhance biodegradation of toxic/poorly bioavailable compounds

Rationale

Biological treatment of hazardous wastes and polluted effluents is an increasingly popular alternative to traditional physico-chemical and thermal technologies (Head 1998). Most organic contaminants can be detoxified, and often mineralized, by microorganisms (Allard & Neilson 1997). A variety of bioremediation processes have been designed for several pollutant classes and sources (Cookson 1995). However, even if microorganisms with the required degradative capacity are present and adequate environmental conditions for growth and degradation are found, the applicability of bioremediation is sometimes restricted. Two important obstacles, limited bioavailability and toxicity, are emphasized in this section.

Firstly, the pollutant may be insufficiently available to the microflora (Harms & Bosma 1997; Mihelcic et al. 1993; Providenti et al. 1993). For example, some organic contaminants have very low aqueous solubilities (e.g. polyaromatic hydrocarbons), however only the water-dissolved fraction of chemicals is usually assumed to be available to microorganisms (Wodzindski & Coyle 1974; Thomas et al. 1986; Stucki & Alexander 1987). Secondly, some pollutants (e.g. styrene, phenol) are toxic to microorganisms (Sikkeman et al. 1995), and growth inhibition at high substrate concentration prevents the effective biodegradation of these contaminants.

TLPBs have the potential to resolve these two limitations of biotreatment technologies by controlling

the mass-transfer rate and aqueous phase concentration of hydrophobic/toxic compounds. This is especially interesting when it is recognized that both problems usually require opposite solutions. Moreover, TLPBs can be used to select and isolate microorganisms that can degrade these types of molecules. Key information from studies that used TLPBs to evaluate the biodegradation of environmentally relevant compounds is presented in Table 1.

TLPBs for the enhancement of mass-transfer of compounds with limited bioavailability

The bioavailability of a molecule may be characterized by its mass-transfer rate relative to the rate of its uptake, and degradation, by the microorganisms (Bosma et al. 1997). Microbial degradation of sparingly soluble compounds, such as polycyclic aromatic hydrocarbons (PAHs), is restricted by their solubilization rate (Volkering et al. 1992). Consequently, methods to improve the bioavailability and the biodegradation of hydrophobic compounds are required. The principal approach to increase the mass-transfer to the aqueous phase is by enhancing the solubilization or dissolution rates. This can be achieved by increasing the total surface area between the substrate and the aqueous phase. It is often accomplished by homogenization (e.g. mechanical agitation), and/or by the addition of surface-active agents, i.e. surfactants (Edwards et al. 1994). Surfactants are amphipathic molecules with both hydrophobic and hydrophilic moieties that partition preferentially at the interface between fluid phases with different polarities, such as oil-water or air-water interfaces (West & Harwell, 1992). They can reduce surface and interfacial tension and promote the solubilization, emulsification and dispersion of poorly soluble compounds (Rouse et al. 1994). Synthetic surfactants have been extensively used to solubilize, and occasionally increase the biodegradation of hydrophobic contaminants under both laboratory and field conditions (Vigon & Rubin, 1989; Aronstein & Alexander 1992; Tiehm 1994). However, results concerning their effectiveness have been highly contradictory (Rouse et al. 1994; Liu et al. 1995). Many difficulties related to the utilization of synthetic surfactants are recognized. Indeed, they can prevent the biodegradation of contaminants due to their toxicity to microorganisms (Tiehm 1994) or sequestration of compounds within surfactant micelles (Laha & Luthy 1991; Guha & Jaffé 1996). Moreover, they are often costly, poorly specific toward contaminants, difficult to biodegrade and may adsorb onto soil particles.

Water-miscible solvents can also be used to enhance the solubility and dispersion of hydrophobic compounds; however they are usually toxic towards microorganisms (Inoue & Horikoshi 1991; Vermuë et al. 1993), thus, preventing their effective utilization to increase the bioavailability of non-polar compounds.

In 1966, Miller & Johnson observed that the usefulness of surfactants to disperse and enhance growth of a mixed yeast culture on solid *n*-alkanes was limited. They successfully circumvented this problem by dissolving these insoluble hydrocarbons in pristane [2,6,10,14-tetramethylpentadecane], an immiscible liquid not attacked by the microorganisms. A prolonged exponential growth of a *Pseudomonas aeruginosa* strain was achieved with phenanthrene as the substrate, when dissolved in the solvent 2,2,4,4,6,8,8-heptamethylnonane (Köhler et al. 1994). Jimenez & Bartha (1996) tested several NAPLs to enhance pyrene mineralization by a *Mycobacterium* sp. strain. With the ones mentioned in Table 1, they reported initial rates of mineralization of pyrene between 116 and 182 μg/mg of protein/h compared to 81 μg/mg/h in the culture without NAPL. Under anaerobic conditions, reductive dechlorination of the extremely insoluble compound octachloro-dibenzo-*p*-dioxin (octaCDD) by a mixed microflora was increased by the addition of decane (Barkovskii & Adriaens 1995). Higher transfer rates provided by the NAPL/water interfacial area help to overcome bioavailability limitations.

TLPBs for the controlled delivery of apolar toxic compounds

Several poorly water-soluble organic compounds are biodegradable but toxic to microorganisms when supplied above a particular inhibitory concentration. The low concentrations tolerated before inhibition of microbial activity limit the efficiency and the performance of biotreatment systems. Various methods have been used to protect cells from toxic substrates, such as membrane reactors, biofilm systems, immobilization, and encapsulation. TLPSs represent an effective and flexible alternative. By careful selection of the NAPL and the operating conditions, the immiscible phase of a TLPB can act as a ‘reservoir’ providing inhibitory substrates continuously via diffusion into the aqueous phase. The concentration within the aqueous phase is maintained at a non-toxic level, based on the

Table 1. TLPBs in which the biodegradation of environmentally relevant compounds has been observed

NAPL	Substrates	Microorganisms	References
Pristane	Eicosane, docosane, octacosane	*Candida intermedia*, *C. lipolytica*	Miller & Johnson 1966
Pristane	Liquid fraction of *n*-alkanes	*Torulopsis* sp.	McLee & Davies 1972
HMN	Naphthalene	*Pseudomonas* sp.	Wodzinski & Larocca 1977
Dibutyl phtalate	Benzene	*Pseudomonas* sp. 50	Rezessy-Szabó et al. 1987
Dibutyl phtalate	Styrene	*P. putida*	Bestetti et al. 1989
HMN	Naphthalene, hexadecane	*Arthrobacter* sp.	Efroymson & Alexander 1991
Hexadecane	1,2,3-trichlorobenzene (reductive dechlorination)	Anaerobic mixed culture	Holliger et al. 1992
Silicone oil	1,2-dichlorobenzene, 1,2,3- and 1,2,4-trichlorobenzene, ethyl butyrate, 2-ethylbutyraldehyde	Mixed culture	Ascon-Cabrera & Lebeault 1993
Silicone oil	Styrene	Mixed culture	El Aalam et al. 1993
HMN	Phenanthrene	*P. aeruginosa* AK1	Köhler et al. 1994
Decane, dodecane, hexadecane	Naphthalene	*Corynebacterium* sp.	Gamerdinger et al. 1995
Silicone oil	Naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]pyrene	Mixed culture	Vanneck et al. 1995
Silicone oil	Ethyl butyrate	*Candida* sp. CF3	Ascon-Cabrera & Lebeault 1995a
Silicone oil	2,4,6-trichlorophenol	*Pseudomonas* sp. SP1 and SP2, *Arthrobacter* sp. AR2, *Alcaligenes* sp. AL2	Ascon-Cabrera & Lebeault 1995b
Decane, octadecane, isooctane, hexane, cyclohexane, toluene, benzene	Naphthalene, biphenyl	*Pseudomonas* sp. DS-1051, *Pseudomonas* sp. DS-313, *Bacillus* sp. DS-1051	Abe et al. 1995
Decane	Polychlorinated dibenzo-*p*-dioxins (reductive dechlorination)	Anaerobic mixed culture	Barkovskii & Adriaens 1995
Silicone oil	Styrene	Mixed culture	Osswald et al. 1996
Diethyl sebacate	Pentachlorophenol	*Arthrobacter* sp. ATCC 33790	Munro & Daugulis 1996
Paraffin oil, tridecylclohexane, *cis*-9-tricosene, squalane, squalene	Pyrene	*Mycobacterium* sp.	Jimenez & Bartha 1996
2-Undecanone	Phenol	*P. putida* ATCC 11172	Collins & Daugulis 1996, 1997a,b
Silicone oil, HMN	Phenanthrene	*Pseudomonas* sp. SPheNa1	Bouchez et al. 1995
HMN	Phenanthrene, fluoranthene, pyrene	*Pseudomonas* sp. SPheNa1, *Rhodococcus* sp. SFltNa1 and SPyrNa1	Bouchez et al. 1997b

Pristane: 2,6,10,14-tetramethylpentadecane, HMN: 2,2,4,4,6,8,8-heptamethylnonane.

equilibrium partitioning coefficient of the compound, which partitions preferentially into the hydrophobic phase.

As demonstrated by Collins & Daugulis (1996, 1997a,b), one of the benefits of TLPBs is the self-regulating nature of the process, since the delivery rate of toxic substrates is determined by the metabolic requirements of the microorganisms (uptake rate). Using a strain of *Pseudomonas putida* and 2-undecanone as the NAPL in a TLPB operated in a fed-batch mode, a volumetric consumption rate of 175 mg/l · h of phenol was reached without problems of substrate toxicity (Collins & Daugulis 1997a). It was favorably compared to the maximum volumetric phenol consumption rate of 37.5 mg/l · h previously reported for a conventional bioreactor (Fugita et al. 1993). In 1987, Rezessy-Szabó et al. cultivated a *Pseudomonas* strain on high concentrations of benzene, circumventing the substrate toxicity by the addition of dibutyl phtalate. The same NAPL was use by Bestetti et al. (1989) to grow a *P. putida* strain with styrene as substrate. With a chemostat containing 20% v/v of silicone oil, El Aalam et al. (1993) cultivated a *P. aeruginosa* strain on styrene (lethal aqueous concentration, 70 mg/l) up to a very high specific activity of 293 mg · g^{-1} dry cells · h^{-1}. The toxic wood-preservative compound pentachlorophenol (PCP), a solid, xenobiotic chemical, is inhibitory at a low concentration (McAllister et al. 1996). *Arthrobacter* sp. ATCC 33790 is able to utilise PCP as sole substrate, but inhibition occurs above a concentration of 130 mg/l in batch cultures (Stanlake & Finn 1982). Using this strain, Munro & Daugulis (1997) designed a TLPS capable of degrading 7.5 g of PCP in 40 h (187.5 mg/l · h), a 2 l fermenter containing 1 l aqueous medium, 10 g of PCP and 500 ml of diethyl sebacate as the specifically selected NAPL.

TLPBs for the enrichment of xenobiotic-degrading microorganisms

The selection and the isolation of microorganisms able to degrade compounds that are poorly water-soluble and/or toxic is often one of the first difficulties encountered in biodegradation processes. Addition of a NAPL has the potential to shorten the acclimation period required for the isolation of adapted microorganisms. Only low concentrations of toxic substrates can be added in classical aqueous enrichment cultures because of substrate inhibition problems. However, these could well be below the threshold concentration required for the creation of a selective advantage (Boethling & Alexander 1979; Spain & van Veld 1983).

A TLPB with silicone oil as the NAPL has been used for the rapid selection of microorganisms able to degrade two mixtures of chlorinated and nonchlorinated hydrophobic/toxic compounds (see Table 1; Ascon-Cabrera & Lebeault 1993). The authors obtained a stable consortium on chlorobenzenes in a relatively short acclimation period (30 days). This was attributed to the high concentrations of toxic substrates that they could add to the enrichment culture via the water-immiscible phase. Similarly, Holliger et al. (1992) used hexadecane as a NAPL for the enrichment and the maintenance of an anaerobic mixed culture that reductively dechlorinates 1,2,3-trichlorobenzene (1,2,3-TCB) to 1,3-dichlorobenzene. Since TLPBs offer the possibility to circumvent the limitations usually encountered when studying hydrophobic or toxic compounds, they could represent potentially useful research tools for studies on biodegradability testing, catabolic pathways, kinetics of biodegradation, etc. (Prokop et al. 1971; Boethling 1984; Bouchez et al. 1995, 1997a,b).

Design of TLPBs for biodegradation purposes

Selection of an appropriate NAPL

A variety of water-immiscible organic solvents have been used to facilitate the biodegradation of hydrophobic/toxic pollutants (Table 1). As stated by Munro & Daugulis (1997), the criteria to consider for the selection of an appropriate NAPL for biotreatment processes are essentially the same as for extractive fermentation, except that the compound of interest is the substrate rather than the product. Based on a strategy established for the choice of a solvent in extractive biocatalysis (Bruce & Daugulis 1991), they proposed a systematic approach to simplify the selection of a NAPL for improved biodegradation. The properties that should be considered include: biocompatibility, high solubilization capacity for the substrate, immiscibility, and non-biodegradability (Daugulis 1997).

The biocompatibility and potential toxicity of solvents to microorganisms have been extensively discussed in the literature (Bruce & Daugulis 1991; Nikolova & Ward 1993). It is known that many organic solvents suppress microbial proliferation and metabolism (Lilly et al. 1987). The ability of a microorganism to grow in the presence of an organic solvent is related

to the solvent polarity, where higher activity retention correlates with lower polarity (Brink & Tramper 1985). Many solvent parameters have been studied in order to correlate biocatalytic activity and solvent hydrophobicity. The logarithm of the octanol-water partition coefficient ($\log P_{ow}$) is currently accepted as the best measure of a solvent's biocompatibility (Laane et al. 1985, 1987; Inoue & Horikoshi 1991). As a general trend, solvents with high values of $\log P_{ow}$ (4.0 or higher) are not toxic to biocatalysts (Laane et al. 1987; Vermuë et al. 1993). However, tolerance to solvents is highly variable between different bacterial species, Gram-negative bacteria being usually more tolerant than Gram-positive ones (Harrop et al. 1989; Inoue & Horikoshi 1991; Vermuë et al. 1993). For example, bacterial strains able to tolerate and even degrade solvents with $\log P_{ow}$ as low as 2.3 have been isolated (Cruden et al. 1992; Weber et al. 1993), whereas some bacteria are inhibited at a $\log P_{ow}$ below 7 (Inoue & Horikoshi 1991). Biocompatibility experiments with a range of solvents with different $\log P_{ow}$ should therefore be conducted in order to determine the critical $\log P_{ow}$ for the microorganism(s) to be used (Collins & Daugulis 1997a). The $\log P_{ow}$ of any solvent can be obtained experimentally, but it can also be calculated accurately from hydrophobic fragmental constants by the procedure described by Rekker (1977).

A high solubility of substrate in the NAPL is beneficial since it will insure a good capacity for the substrate and allow the use of lower volumes of NAPLs. For toxic substrates, the mass partition coefficient, defined as the ratio of the substrate concentration in the NAPL to the substrate concentration in the aqueous phase (Bruce & Daugulis 1991), should be as high as required for the aqueous phase concentration to remain below the inhibitory level (Collins & Daugulis 1997a; Munro & Daugulis 1997), especially at a high substrate concentration in the system. On the contrary, this coefficient should not be restrictive for hydrophobic substrates (Prichanont et al. 1998); if the microbial activity is exclusively located in the aqueous phase, the mass partition coefficient should allow the aqueous concentration of hydrophobic/non-toxic compounds to approach their saturation concentration (as determined under abiotic conditions).

The aqueous solubility of the NAPL will influence the solubility of the substrate in the aqueous phase (co-solvent effect) and the equilibrium concentration. It should also be low enough to minimize solvent loss. Furthermore, the interfacial tension between the NAPL and the aqueous phase will affect the formation of an emulsion. The increased surface area resulting from emulsion formation is beneficial for biodegradation activities (Ascon-Cabrera & Lebeault 1995a; as discussed in the Microbial mechanisms section), but if the emulsion is too stable, this may represent a major problem in sampling of the two phases for analysis purposes (Munro & Daugulis 1997).

Biodegradability of the added NAPL is rarely a concern when using a single microorganism as biocatalyst since the limits of the carbon source spectrum can be rather easily determined, first by a survey of the literature, then by few cultivation tests (Munro & Daugulis 1997; Collins & Daugulis 1996, 1997a). However, in the case of mixed microbial cultures, the choice of solvents is greatly reduced because, after an adaptation period, microorganisms will acquire the ability to attack almost any organic molecules (van der Meer et al. 1992b). If the NAPL is eventually used as a growth substrate, then competition with the target compounds to be degraded could arise (Morrison & Alexander 1997) and, even, an enrichment for solvent-degrading microorganisms is likely.

Higher-molecular-weight compounds, especially those with multiple condensed ring structures and with highly branched or substituted compounds, are relatively resistant to microbial attack (Atlas 1988). Branched alkanes that are liquid at room temperature are therefore suitable choices. In one of the first investigations conducted to study the bioavailability and degradation of a poorly water-soluble compound (naphthalene) dissolved in a NAPL, 2,2,4,4,6,8,8-heptamethylnonane (HMN) was chosen because it was not biodegraded by the *Pseudomonas* sp. strain used (Wodzinski & Larocca 1977). The same NAPL was later selected by others (Table 1) and it resisted degradation by mixed microbial cultures (Ghoshal et al. 1996; Morrison & Alexander 1997). Apparently, HMN can withstand biodegradation because of its terminal methyl groups (Schaeffer et al. 1979).

Ascon-Cabrera & Lebeault (1993, 1995a,b), and subsequently others (Table 1), have used silicone oils (polydimethylsiloxanes) because of their hydrophobicity, chemical stability and resistance to hydrolytic and oxidative breakdown. Although this NAPL was apparently not attacked by the microorganisms in several cases (Ascon-Cabrera & Lebeault 1993; Osswald et al. 1996), some studies suggest that it may in some conditions be degradable via abiotic and biotic reactions (Carpenter et al. 1995; Stevens 1998).

As mentioned previously, hexadecane was chosen to control the delivery of a toxic substrate (1,2,3-TCB) and decane was used to enhance the bioavailability of an hydrophobic one (octaCDD) to anaerobic mixed cultures (Holliger et al. 1992; Barkovskii & Adriaens 1995). Although they are easily attacked under aerobic conditions, saturated alkanes were selected because they are generally considered non-biodegradable under highly reduced, methanogenic conditions (Zehnder & Svensson 1986).

Additional characteristics to consider include low viscosity, low volatility, high melting/boiling points, low flammability, availability, and cost (Bruce & Daugulis 1991; Nikolova & Ward 1993). We can conclude from these numerous criteria that the selection process for a non-biodegradable NAPL should be systematic and specific to each situation. A lot of information about the microorganisms implicated in the biodegradation and the targeted substrates are required before a sensible choice can be made.

Determination of optimal operation parameters

In a TLPB, the most critical factor to optimize is the interfacial area between the two liquid phases (Nakahara et al. 1977; Woodley et al. 1991). Microbial activity, both at the interface and in the aqueous phase, relies upon the mass-transfer rate of substrate molecules from the NAPL to the aqueous phase, which is mainly governed by the size of the interfacial area (Dunn 1968; Wodzinski & Larocca 1977; Ascon-Cabrera & Lebeault 1993, 1995a). Since higher growth rates are observed at maximal interfacial area, it is essential to maintain the interfacial area high enough not to restrict microbial activity.

The volumetric liquid-liquid interfacial area, a ($m^2 \cdot m^{-3}$), is calculated following (Bailey & Ollis 1986):

$$a = \frac{6 \cdot \phi}{d_{sm}}$$

where d_{sm} ($m^3 \cdot m^{-2}$) is the Sauter mean (surface averaged) droplet diameter and ϕ corresponds to the dispersed-phase volume fraction, which is the ratio of the NAPL volume to the total liquid volume (phase ratio). It can be seen that the interfacial area increases with a decrease in the mean drop size. The Sauter mean diameter can be estimated directly from droplet size distribution measurements (Prokop et al. 1972; Gutierrez & Erickson 1977) and correlated to Calderbank's expression, which takes into account the phase ratio (solvent phase holdup), the turbulence intensity (agitation rate), and the interfacial tension (Chatzi et al. 1989). Consequently, the interfacial area is principally influenced by these three parameters, plus microbial growth (Ascon-Cabrera & Lebeault 1995a; Nakahara et al. 1977; Wang & Ochoa 1972).

The optimal phase ratio which will generate the highest interfacial area is a parameter influenced by many determinants, such as the physical conformation of the bioreactor, the properties of the NAPL (density, viscosity, etc.) and the mixing rate. The equation shows that the interfacial area increases with an increased phase ratio. However, it is also known that NAPL drop diameters have a tendency to increase with an increase in the phase ratio (Prokop et al. 1972). Ascon-Cabrera & Lebeault (1993, 1995a) have studied the effect of variations of a NAPL phase fraction (8.3 to 83% v/v silicone oil) on the interfacial area and observed maximal values between 20 and 40%. A wide range of phase ratios have been used but few studies have been carried out in order to find the ideal proportions. Other factors influence the choice of a proper phase ratio. For example, if the apolar substrate is toxic, the concentration of the compound in the aqueous phase must be kept under the inhibitory level; this is achieved by knowing the partition coefficient and then adjusting the phase ratio accordingly (Collins et al. 1995).

A direct relationship between intensity of agitation and specific growth rate in TLPB has been frequently reported (e.g. Wang & Ochoa 1972; Velankar et al. 1975; Ascon-Cabrera & Lebeault 1993). Although the formation and the stabilization of an emulsion is facilitated by a decrease in interfacial tension, a mechanical input is required for the initial formation of NAPL droplets. As the agitation intensity is increased, the Sauter mean diameter is decreased and the number of droplets increased (Erickson & Nakahara 1975). The stable-state distribution of drop sizes, as well as mean diameter of droplets formed by turbulent mixing, are a result of the equilibrium between two simultaneous processes – break-up and coalescence of drops: high mixing rate will produce smaller NAPL drops, e.g. via interaction with the impeller, but also multiply the probability of drop contact and coalescence. Also, elevated agitation energy might prevent contact of microorganisms with substrate-containing NAPL droplets and shear stress could eventually be detrimental to the microbial cells (McLee & Davies 1972). With cultures of a *Candida* sp. on 2-ethylbutyraldehyde incubated in a baffled re-

actor at different agitation speeds (between 200 and 800 rpm) with 20% silicone oil, Ascon-Cabrera & Lebeault (1993) obtained an optimal growth rate at mixing speeds between 500 and 600 rpm.

The Sauter mean diameter is strongly dependant on interfacial tension (Erickson & Nakahara 1975). Addition of surfactants increases the interfacial area (Wang & Ochoa 1972) and prevents coalescence of NAPL droplets. In TLPBs, the interfacial tension between NAPL and aqueous phase decreases as cultivation proceeds (Prokop et al. 1971, 1972; Velankar et al. 1975; Nakahara et al. 1977). Microorganisms using liquid hydrocarbons as substrate secrete emulsifying and tensio-active substances such as glycolipids and lipopeptides to facilitate uptake (Haferburg et al. 1986; Georgiou et al. 1992). The cells themselves also can decrease the interfacial tension in TLPSs (Bajpai et al. 1975; Nakahara et al. 1977). Biosurfactant or bioemulsifier production is generally considered beneficial for growth on long-chain liquid aliphatic hydrocarbons (Hommel 1990). However, it was recently reported that excessive biosurfactants concentrations decreased the growth rate of *P. oleovorans* on decane (Schmid et al. 1998). This was attributed to reduced mass-transfer rate of the insoluble substrate to the aqueous phase caused by the strong stabilizing film surrounding the NAPL droplets.

In general, microorganisms growing on a NAPL (as reservoir or substrate) have a strong affinity for the immiscible liquid and colonize the surface of droplets (Miura et al. 1977; Rosenberg 1991). When investigating growth of a *Candida lipolytica* strain on hexadecane dissolved in inert gas oil, Prokop et al. (1971, 1972) observed a tendency for the interfacial area to increase with increases in cell mass. They suggested that cells with hydrophobic surfaces may tend to stabilize emulsions when growing at the drop surface by preventing coalescence. However, not all drops were covered with cells and this could be explained by the exhaustion of substrate contained in the small reservoir droplets (Prokop et al. 1971; Gutierrez & Erickson 1977).

Most of the design parameters previously discussed apply to TLPSs in which the NAPL is well-mixed with the aqueous phase and for substrates whose biodegradation is principally limited by their bioavailability. However, special attention must be given to substrates exhibiting also a problem of toxicity. Even if the phase ratio has been adjusted to keep the initial concentration of an inhibitory substrate in the aqueous phase under the toxic level, the maximal interfacial area beneficial for optimal mass-transfer rate for hydrophobic substrates may not be ideal for toxic substrates. A distinction between molecular toxicity (caused by dissolved molecules) and phase toxicity (occurring at the liquid-liquid interface) has been proposed (Bar 1987). It is possible that microorganisms coming into direct contact with the interface may be exposed to elevated, inhibitory, levels of toxic compounds (Hocknull & Lilly 1988; Lilly et al. 1987) and consequently emulsified systems with high interfacial area may be detrimental to microbial activity (Prichanont et al. 1998). For example, Collins et al. (1995) observed reduced dioxygenase activity of *P. putida* UV4 converting toluene to toluene *cis*-glycol in a TLPB with tetradecane as the NAPL. The phase ratio (30% v/v) and the toluene concentration in the NAPL (20% v/v) had been calculated based on the partition coefficient in order to have a sub-inhibitory level of toluene in the aqueous phase. Nevertheless, loss of microbial activity was observed, which was attributed to interfacial toxicity because of the formation of an emulsion. Moreover, when the substrate concentration was reduced to 10% the activity was maintained for a longer period. They suggested that operational limits of specific TLPSs could be represented in the form of "windows of operation" where the organic phase substrate concentration is shown as a function of phase ratio. Hence, if the toxic substrate ratio in the NAPL is too low, mass-transfer rate becomes limiting and if it is too high, phase toxicity occurs. Increasing the phase ratio by operating the TLPB with the solvent phase continuous (two phases not intermixed) was proposed to prevent formation of an emulsion (Collins et al. 1995). Daugulis (1997) suggested that it may be preferable to select a NAPL and culture conditions not favoring formation of an emulsion, to prevent phase toxicity. However, most publications about biodegradation of toxic compounds in completely mixed TLPSs have not reported problems of inhibition of microbial activity (El Aalam et al. 1993; Osswald et al. 1996). Collins & Daugulis (1996, 1997a,b) investigated the biodegradation of phenol in a TLPS containing 1 l of medium and 500 ml of 2-undecanone as NAPL. This system was operated without intermixing the two liquid phases. They did not observe inhibitory effects but, unlike others who are using completely dispersed TLPSs (e.g. Ascon-Cabrera & Lebeault 1993, 1995a), microbial activity was oxygen-limited in these conditions of elevated phase ratio. Nevertheless, sufficient oxygen supply should generally not be a problem in TLPBs. Oxygen solubilities in hydrocarbons have

been shown to be much higher than in pure water (Ju & Ho 1989). Ho et al. (1990) even added *n*-hexadecane to enhance the oxygen supply to oxygen-limited cultures (solubility of oxygen in *n*-hexadecane is more than 8 times higher than that in pure water). Dispersion of the NAPL in a TLPB enhanced the gas/liquid mass-transfer coefficient of oxygen during growth of *P. oleovorans* on octane (van der Meer et al. 1992a).

To summarize, the main objective to keep in mind when setting up a TLPS for enhanced biodegradation of apolar compounds is to achieve the highest mass-transfer rate, while preventing microbial inhibition when the substrate is also toxic.

Application of TLPBs as a biotreatment process

Many environmental pollutants are sparingly soluble in water and toxic towards microorganisms. In bioremediation research, much work has focused on the optimisation of microbial activity by improving environmental conditions, and occasionally by bioaugmentation. Increased microbial degrading capacities, however, will not lead to higher biotransformation rates if the mass-transfer rate is limiting or the target substrate inhibits the activity and survival of the microorganisms.

New technologies are needed for lowering the costs and time requirements of biotreatment processes. Since TLPBs are effective at controlling the delivery of apolar/inhibitory organic compounds to biocatalysts, they could represent a valuable tool for both fundamental research and applied processes concerning the biodegradation of environmentally problematic compounds. Some authors have proposed that TLPSs could have a great potential for treating water and effluents contaminated with toxic compounds such as styrene (El Aalam et al. 1993), phenol (Collins & Daugulis 1996, 1997a,b), and pentachlorophenol (Munro & Daugulis 1997), by allowing increased toxic charge load of effluents to be treated. Decontamination of styrene-containing waste gas was also suggested (El Aalam et al. 1993). Likewise, it was proposed that this approach could be applied to effluents polluted by poorly water-soluble compounds, such as PAH-containing water (Vanneck et al. 1995) and refinery sludges (Jimenez & Bartha 1996).

In biotreatment processes using TLPBs, the hydrophobic/toxic substrate would first dissolve into the NAPL, then diffuse to the microorganisms at the interface and in the aqueous phase (Figure 1). In all likelihood, the biotreatment of contaminated soils and sediments in slurry-phase bioreactors might also be improved by the addition of an inert NAPL. The bioavailability of molecules is controlled by a number of physical-chemical properties such as aqueous solubility and tendency to sorb to the soil and sediment matrix (Harms and Bosma 1997; Luthy et al. 1997). Desorption and dissolution rates are the critical limiting steps in the biodegradation of hydrophobic molecules. Since the partitioning of hydrophobic compounds into a NAPL is in many ways comparable to sorption to soil organic matter, the solvent could promote the extraction of the hydrophobic pollutants from the soil. The ensuing increased surface area for microbial contact and mass-transfer would result in enhanced rates of biodegradation. More investigations are required before the actual potential of this procedure is determined. Our research group has performed promising preliminary work in this direction. Using a PAH-adapted mixed culture, we have observed rapid mass-transfer from an artificially contaminated soil to the NAPL followed by enhanced biodegradation in a slurry TLPB with silicone oil as the NAPL (A. Benachenhou 1998, unpublished results).

Since the important biodegradative microorganisms usually adhere and develop at the interface between the two liquid phases in TLPBs, the recovery of the NAPL with the attached microflora could be used as an enriched inoculum for subsequent biotreatments. Solvents with low costs could be used and minimal environmental hazard is expected since they would be non-toxic and reusable.

Conclusion

The addition of appropriate water-immiscible solvents in biodegradation processes can be beneficial. TLPSs offer the possibility to control the bioavailability of NAPL-dissolved compounds by: (1) increasing the interfacial area between the pollutants and the microorganisms, which enhances the mass-transfer rate of hydrophobic compounds; and (2) sequestering toxic molecules into a separate phase, thus keeping their aqueous concentration below inhibitory levels. Difficulties may arise, however, if the NAPL is not carefully selected. It must be inert, innocuous and non-biodegradable. The ratio of NAPL added should allow the largest interfacial area with the aqueous phase, thus promoting the mass-transfer rate and the contact area for the adhering microorganisms. Most biological

activity in such culture systems will occur near the interface between the two liquid phases. Microorganisms possess some features helping them to access and metabolize substrates in TLPBs. They benefit from their ability to produce surface-active agents, which emulsify the NAPL and therefore increase the interfacial area. This promotes the partitioning of the target compounds in the aqueous phase and also favors adhering microorganisms. Furthermore, hydrophobicity of the cell surface assists in the attachment of microorganisms at the NAPL surface, which promotes the formation of a biofilm and the uptake of pollutant molecules directly from the NAPL.

There are a large number of publications about applications of TLPBs in commercial bioconversion processes, but until now few studies have focused expressly on biodegradation of water-insoluble/toxic compounds of environmental concern. Future investigations should be conducted on identification of new solvents with the required characteristics, especially low biodegradability in the conditions of use. A better understanding of microbial substrate uptake mechanisms involved in TLPSs could provide useful information on the design of TLPBs. Moreover, new applications of TLPBs such as improved treatment of contaminated soils deserve attention. In this context, key operational and design parameters require more research, especially in the later steps of a treatment scheme such as enriched microflora recovery, final phase separation and solvent recycling.

The potential benefits of TLPBs justify further exploration of this technology as a novel biotreatment strategy.

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References

Abe A, Inoue A, Usami R, Moriya K & Horikoshi K (1995) Degradation of polyaromatic hydrocarbons by organic solvent-tolerant bacteria from deep sea. Biosci. Biotech. Biochem. 59: 1154–1156

Alexander M (1994) Biodegradation and bioremediation. Academic Press, Inc., San Diego, CA

Allard A-S & Neilson AH (1997). Bioremediation of organic waste sites: a critical review of microbiological aspects. Internat. Biodeter. Biodegr. 39: 253–285

Amin PM, Nigam, JN, Lonsane, BK, Baruah, B, Singh HD, Baruah JN & Iyengar MS (1973) Microbial biomass production on solid hydrocarbons. Folia Microbiol. 18: 49–55

Aronstein BN & Alexander M (1992) Surfactants at low concentrations stimulate biodegradation of sorbed hydrocarbons in samples of aquifer sands and soil slurries. Environ. Toxicol. Chem. 11: 1227–1133

Ascon-Cabrera M & Lebeault J-M (1993) Selection of xenobiotic-degrading microorganisms in a biphasic aqueous-organic system. Appl. Environ. Microbiol. 59: 1717–1724

Ascon-Cabrera M & Lebeault J-M (1995a) Interfacial area effects of a biphasic aqueous/organic system on growth kinetic of xenobiotic-degrading microorganisms. Appl. Microbiol. Biotechnol. 43: 1136–1141

Ascon-Cabrera M & Lebeault J-M (1995b) Cell hydrophobicity influencing the activity/stability of xenobiotic-degrading microorganisms in a continuous biphasic aqueous-organic system. J. Ferment. Technol. 80: 270–275

Atlas RM (1988) Biodegradation of hydrocarbons in the environment. Basic Life Sci. 45: 211–222

Bailey JE & Ollis DF (1986) Biochemical engineering fundamentals, 2nd ed. McGraw-Hill Publishing Co., New York

Bajpai RK, Prokop A & Ramkrishna D (1975) Dispersion in hydrocarbon fermentation. A retrospective study. Biotechnol. Bioeng. 17: 541–556

Barkovskii A & Adriaens P (1995) Biological and abiotic dechlorination of highly chlorinated dioxins in biphasic microcosms In: Hinchee RE, Anderson DB & Hoeppel RE (Eds) Bioremediation of recalcitrant organics, Vol. 7. (pp 73–82), Battelle Press, Columbus OH

Bar R (1987) Phase toxicity in water-solvent two-liquid phase microbial system. In: Laane C, Tramper J & Lilly MD (Eds) Biocatalysis in Organic media (pp 147–153), Elsevier, Amsterdam

Bell GH (1973) The metabolism of long-chain fatty acids and alcohols by *Candida tropicalis* and *Saccharomyces cerevisiae*. Antonie Van Leeuwenhoek 39: 137–149

Bestetti G, Galli E, Benigni C, Orsini F & Pelizzoni F (1989) Biotransformation of styrenes by a *Pseudomonas putida*. Appl. Microbiol. Biotechnol. 30: 252–256

Boethling RS (1984) Biodegradation testing of insoluble chemicals. Environ. Toxicol. Chem. 3: 5–7

Boethling RS & Alexander M (1979) Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. 37: 1211–1216

Bosma TNP, Middeldorp PJM, Schraa G & Zehnder AJB (1997) Mass-transfer limitation of biotransformation: quantifying bioavailability. Environ. Sci. Technol. 31: 248–252

Bouchez M, Blanchet D & Vandecasteele J-P (1995) Substrate availability in phenanthrene biodegradation: transfer mechanism and influence on metabolism. Appl. Microbiol. Biotechnol. 43: 952–960

Bouchez M, Blanchet D, Besnainou B, Leveau J-Y & Vandecasteele J-P (1997a) Kinetic studies of biodegradation of insoluble compounds by continuous determination of oxygen consumption. J. Appl. Microbiol. 82: 310–316

Bouchez M, Blanchet D & Vandecasteele J-P (1997b) An interfacial uptake mechanism for the degradation of pyrene by a *Rhodococcus* strain. Microbiology. 143: 1087–1093

Brink LES & Tramper J (1985) Optimization of organic solvent in multiphase biocatalysis. Biotechnol. Bioeng. 27: 1258–1269.

Brink LES, Tramper J, Luyben KCAM & Van't Riet K (1988) Biocatalysis in organic media. Enzyme Microb. Technol. 10: 736–743

Bruce LJ & Daugulis AJ (1991) Solvent selection strategies for extractive biocatalysis. Biotechnol. Prog. 7: 116–124.

Carpenter JC, Cella JA & Dorn SB (1995) Study of the degradation of polydimethylsiloxanes. Environ. Sci. Technol. 29: 864–868

Chakravarty M, Singh HD & Baruah JN (1975) A kinetic model for microbial growth on liquid hydrocarbons. Biotechnol. Bioeng. 17: 399–412

Chatzi EG, Gavrielides AD & Kiparissides C (1989) Generalized model for prediction of the steady-state drop size distribution in batch stirred vessels. Ind. Eng. Chem. Res. 28: 1704–1711

Collins LD & Daugulis AJ (1996) Use of two phase partitioning bioreactors for the biodegradation of phenol. Biotechnol. Tech. 10: 643–648

Collins LD & Daugulis AJ (1997a) Biodegradation of phenol at high initial concentrations in two-phase partitioning batch and fed-batch bioreactors. Biotechnol. Bioeng. 55: 155–162

Collins LD & Daugulis AJ (1997b) Characterization and optimisation of a two-phase partitioning bioreactor for the biodegradation of phenol. Appl. Microbiol. Biotechnol. 48: 18–22

Collins AM, Woodley JM & Liddell JM (1995) Determination of reactor operation for the microbial hydroxylation of toluene in a two-liquid phase process. J. Ind. Microbiol. 14: 382–388

Cookson JT (1995) Bioremediation engineering: design and application. McGraw-Hill, New York, NY

Cruden DL, Wolfram JH, Rogers RD & Gibson DT (1992) Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. 58: 2723–2729

Daugulis A (1997) Partitioning bioreactors. Curr. Opin. Biotechnol. 8: 169–174

Daugulis A (1994) Integrated fermentation and recovery processes. Curr. Opin. Biotechnol. 5: 192–195

Desai JD & Banat IM (1997) Microbial production of surfactants and their commercial potential. Microbiol. Mol. Biol. Rev. 61: 47–64

Dunn IJ (1968) An interfacial kinetics model for hydrocarbon oxidation. Biotechnol. Bioeng. 10: 891–894

Edwards DA, Liu Z & Luthy RG (1994) Surfactant solubilization of organic compounds in soil/aqueous systems. J. Environ. Eng. ASCE. 120: 5–22

Efroymson RA & Alexander M (1991) Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl. Environ. Microbiol. 57: 1441–1447

Efroymson RA & Alexander M (1994) Role of partitioning in biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. Environ. Sci. Technol. 28: 1172–1179

Efroymson RA & Alexander M (1995) Reduced mineralization of low concentrations of phenanthrene because of sequestering in nonaqueous-phase liquids. Environ. Sci. Technol. 29: 515–521

El Aalam S, Pauss A & Lebeault J-M (1993) High efficiency styrene biodegradation in a biphasic organic/water continuous reactor. Appl. Microbiol. Biotechnol. 39: 696–699

Erickson LE, Humphrey, AE & Prokop, A (1969) Growth models of cultures with two liquid phases. I. Substrate dissolved in dispersed phase. Biotechnol. Bioeng. 11: 449–466

Erickson LE & Nakahara T (1975) Growth in cultures with two liquid phases: hydrocarbon uptake and transport. Process Biochem.(June): 9–13

Favre-Bulle O, Schouten T, Kingma J & Witholt B (1991) Bioconversion of *n*-octane to octanoic acid by a recombinant *Escherichia coli* cultured in a two-liquid phase bioreactor. Bio-Technol. 9: 367–371

Freeman AJ, Woodley M & Lilly MD (1993) *In situ* product removal as a tool for bioprocessing. Bio/Technology. 11: 1007–1012.

Fugita M, Ike M & Kamiya T (1993) Accelerated phenol removal by amplifying the gene expression with recombinant plasmid encoding catechol-2,3-oxygenase. Water Res. 1: 9–13

Furuhashi K, Shintani M & Tagaki M (1986) Effects of solvents on the production of epoxides by *Nocardia corallina* B-276. Appl. Microbiol. Biotechnol. 23: 218–223

Gamerdinger AP, Achin RS & Traxler RW (1995) Effect of aliphatic nonaqueous phase liquids on naphthalene biodegradation in multiphase systems. J. Environ. Qual. 24: 1150–1156

Georgiou G, Lin SC & Sharma MM (1992) Surface-active compounds from microorganisms. Bio/Technology. 10: 60–65

Ghoshal S, Ramaswami A & Luthy RG (1996) Biodegradation of naphthalene from coal tar and heptamethylnonane in mixed batch systems. Environ. Sci. Technol. 30: 1282–1291

Goma G, Pareilleux A, & Durand G (1974) Aspects physicochimiques de l'assimilation des hydrocarbures par *Candida lipolytica*. Agr. Biol. Chem. 38: 1273–1280

Goswami P & Singh HD (1991) Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol. Bioeng. 37: 1–11

Guha S & Jaffé PR (1996) Bioavailability of hydrophobic compounds partitioned into the micellar phase of nonionic surfactants. Environ. Sci. Technol. 30: 1382–1391

Gutierrez JR & Erickson LE (1977) Hydrocarbon uptake in hydrocarbon fermentations. Biotechnol. Bioeng. 43: 1136–1141

Haferburg D, Hommel R, Claus R & Kleber HP (1986) Extracellular microbial lipids as biosurfactants. Adv. Biochem. Eng./Biotechnol. 33: 53–93

Harbron S, Smith BW & Lilly MD (1986) Two-liquid phase biocatalysis: epoxydation of 1,7-octadiene by *Pseudomonas putida*. Enzyme Microb. Technol. 8: 85–88

Harms H & Bosma TNP (1997) Mass-transfer limitation of microbial growth and pollutant degradation. J. Ind. Microbiol. Biotechnol. 18: 97–105

Harrop AJ, Hocknull MD & Lilly MD (1989) Biotransformation in organic solvents: a difference between Gram-positive and Gram-negative bacteria. Biotechnol. Lett. 11: 807–810

Head, IM (1998) Bioremediation: towards a credible technology. Microbiology. 144: 599–608

Ho CS, Ju LK & Baddour RF (1990) Enhancing penicillin fermentation by increased oxygen solubility through the addition of *n*-hexadecane. Biotechnol. Bioeng. 36: 1110–1118

Hocknull MD & Lilly MD (1988) The stability of the Δ^1-dehydrogenation system of *Arthrobacter simplex* in organic solvent/aqueous two-liquid phase environments. Enzyme Microb. Technol. 10: 669–674

Holliger C, Schraa G, Stams, AJM & Zehnder, AJB (1992) Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. 58: 1636–1644

Hommel RK (1990) Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation. 1: 107–119

Huang S-Y & Chou MS (1990) Kinetic model for microbial uptake on insoluble solid-state substrate. Biotechnol. Bioeng. 35: 547–558

Inoue A & Horikoshi K (1991) Estimation of solvent-tolerance of bacteria by the solvent parameter log P. J. Ferment. Bioeng. 71: 194–196

Jimenez I & Bartha R (1996) Solvent-augmented mineralization of pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. 62: 2311–2316

Ju LK & Ho CS (1989) Oxygen diffusion coefficient and solubility in *n*-hexadecane Biotechnol. Bioeng. 34: 1221–1224

Köhler A, Schüttoff M, Bryniok D & Knackmuss H-J (1994). Enhanced biodegradation of phenanthrene in a biphasic culture system. Biodegradation. 5: 93–103

Laane C, Boeren S & Vos K (1985) On optimizing organic solvents in multi-liquid phase biocatalysis. Trends Biotechnol. 3: 251–252

Laane C, Boeren S, Vos K & Veeger C (1987) Rules for optimization of biocatalysis in organic solvents. Biotechnol. Bioeng. 30: 81–87

Labare MP & Alexander M (1995) Enhanced mineralization of organic compounds in nonaqueous-phase liquids. Environ. Toxicol. Chem. 14: 257–265

Laha S & Luthy RG (1991) Inhibition of phenanthrene mineralization by nonionic surfactants in soil-water systems. Environ. Sci. Technol. 25:1920-1930

Lilly MD, Brazier AJ, Hocknull MD, Williams AC & Woodley JM (1987) Biological conversion involving water-insoluble organic compounds. In: Laane C, Tramper J & Lilly MD (Eds) Biocatalysis in organic media (pp 3–17), Elsevier Science Publishers, Amsterdam

Liu, Z., Jacobson AM and Luthy RG (1995) Biodegradation of naphthalene in aqueous nonionic surfactant systems. Appl. Environ. Microbiol. 61: 145–151

Luthy RG, Aiken GR, Brusseau ML, Cunningham SD, Gschwend PM, Pignatello JJ, Reinhard M, Traina SJ, Weber Jr. WJ & Westall JC (1997) Sequestration of hydrophobic organic contaminants by geosorbents. Environ. Sci. Technol. 31: 3341–3347

Marin M, Pedregosa A & Laborda F (1996) Emulsifier production and microscopical study of emulsions and biofilms formed by the hydrocarbon-utilizing bacteria *Acinetobacter calcoaceticus* MM5. Appl. Microbiol. Biotechnol. 44: 660–667.

McAllister KA, Lee H & Trevors JT (1996) Microbial degradation of pentachlorophenol. Biodegradation 7: 1–40.

McLee AG & Davies SL (1972) Linear growth of a *Torulopsis* sp. on *n*-alkanes. Can. J. Microbiol. 18: 315–319

Mihelcic JR, Lueking DR, Mitzell RJ & Stapleton JM (1993) Bioavailability of sorbed- and separate-phase chemicals. Biodegradation. 4: 141–153

Miller TL & Johnson MJ (1966) Utilization of normal alkanes by yeasts. Biotechnol. Bioeng. 8: 549–565

Miura Y, Okazaki M, Hamada S-I, Murakawa S-I & Yugen R (1977) Assimilation of liquid hydrocarbon by microorganisms. I. Mechanism of hydrocarbon uptake. Biotechnol. Bioeng. 19: 701–714

Morrison DE & Alexander M (1997) Biodegradability of nonaqueous-phase liquids affects the mineralization of phenanthrene in soil because of microbial competition. Environ. Toxicol. Chem. 16: 1561–1567

Munro DR & Daugulis AJ (1997) The use of an organic solvent and integrated fermentation for improved xenobiotic degradation. Res. Environ. Biotechnol. 1: 207–225

Nakahara T, Erickson LE & Gutierrez JR (1977) Characteristics of hydrocarbon uptake in cultures with two liquid phases. Biotechnol. Bioeng. 19: 9–25

Neu TR (1996) Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol. Rev. 60: 151–166

Neufeld RJ, Zajic JE & Gerson DF (1980) Cell surface measurements in hydrocarbon and carbohydrate fermentation. Appl. Environ. Microbiol. 39: 511–517

Ng TK & Hu WS (1989) Adherence of emulsan-producing *Acinetobacter calcoaceticus* to hydrophobic liquids. Appl. Microbiol. Biotechnol. 31: 480–485

Nikolova P & Ward OP (1993) Whole cell biocatalysis in nonconventional media. J. Ind. Microbiol. 12: 76–86

Ortega-Calvo J-J & Alexander M (1994) Roles of bacterial attachment and spontaneous partitioning in the biodegradation of naphthalene initially present in nonaqueous-phase liquids. Appl. Environ. Microbiol. 60: 2643–2646

Ortega-Calvo J-J, Birman I & Alexander M (1995) Effects of varying the rate of partitioning of phenanthrene in nonaqueous-phase liquids on biodegradation in soil slurries. Environ. Sci. Technol. 29: 2222–2225

Osswald P, Baveye P & Block JC (1996) Bacterial influence on partitioning rate during the biodegradation of styrene in a biphasic aqueous-organic system. Biodegradation. 7: 297–302

Preusting H, Hazenberg W & Witholt B (1993) Continuous production of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans* in a high-cell-density, two-liquid phase chemostat. Enzyme Microb. Technol. 15: 311–316

Prichanont S, Leak DJ & Stuckey DC (1998) Alkene monooxygenase-catalysed whole cell epoxidation in a two-liquid phase system. Enzyme Microb. Technol. 22: 471–479

Prokop A, Erickson LE & Parades-Lopez O (1971) Growth models of cultures with two liquid phases. V. Substrate dissolved in dispersed phase-Experimental observations. Biotechnol. Bioeng. 13: 241–256

Prokop A, Ludvik M & Erickson LE (1972) Growth models of cultures with two liquid phases. VIII. Experimental observations on droplet size and interfacial area. Biotechnol. Bioeng. 14: 587–608

Providenti MA, Lee H & Trevors JT (1993) Selected factors limiting the microbial degradation of recalcitrant compounds. J. Ind. Microbiol. 12: 379–395.

Rekker, RF (1977) The hydrophobic fragmental constant. Nauta WT & Rekker RF (Eds) Elsevier, Amsterdam

Rezessy-Szabó JM, Huijberts GNM & de Bont JAM (1987) Potential of organic solvents in cultivating microorganisms on toxic water-insoluble compounds. In: Laane C, Tramper J & Lilly MD (Eds) Biocatalysis in organic media (pp 295–302), Elsevier Science Publishers B. V., Amsterdam

Rosenberg E (1989) Biofilms on water-insoluble substrates. In: Characklis WG & Wilderer PA (Eds) Structure and function of biofilms (pp 59–71), John Wiley & Sons, Ltd, New York

Rosenberg M (1991) Basic and applied aspects of microbial adhesion at the hydrocarbon:water interface. Crit. Rev. Microbiol. 18: 159–173

Rosenberg M & Rosenberg E (1981) Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. J. Bacteriol. 148: 51–57

Rouse JD, Sabatini DA, Suflita JM & Harwell JH (1994) Influence of surfactants on microbial degradation of organic compounds. Crit. Rev. Environ. Sci. Technol. 24: 325–370

Schaeffer TL, Cantwell SG, Brown JL, Watt DS & Fall RR (1979) Microbial growth on hydrocarbons: terminal branching inhibits biodegradation. Appl. Environ. Microbiol. 38: 742–746

Schmid A, Kollmer A & Witholt B (1998) Effect of biosurfactant and emulsification on two-liquid phase *Pseudomonas oleovorans* cultures and free-cell emulsions containing *n*-decane. Enzyme Microb. Technol. 22: 487–493.

Schwartz RD & McCoy CJ (1977) Epoxidation of 1,7-octadiene by *Pseudomonas oleovorans*: fermentation in the presence of cyclohexane. Appl. Environ. Microbiol. 34: 47–49

Shennan JL (1984) Hydrocarbons as substrates in industrial fermentation. In: Atlas RM (Ed) Petroleum Microbiology (pp 643–683), Macmillan Publishing Company, New York, NY

Sikkeman J, de Bont JAM & Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59: 201–222

Singer ME & Finnerty WR (1984) Microbial metabolism of straight-chain and branched alkanes. In: Atlas RM (Ed) Petroleum Microbiology (pp 1–59), Macmillan Publishing Company, New York, NY

Spain JC & van Veld PA (1983) Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum, and chemical structure. Appl. Environ. Microbiol. 45: 428–435

Stanlake GJ & Finn RK (1982) Isolation and characterization of a pentachlorophenol-degrading bacterium. Appl. Environ. Microbiol. 44: 1421–1427

Stevens C (1998) Environmental degradation pathways for the breakdown of polydimethylsiloxanes J. Inorg. Biochem. 69: 203–207

Stucki G & Alexander M (1987) Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl. Environ. Microbiol. 53: 292–297

Thomas JM, Yordy JR, Amador JA & Alexander M (1986) Rates of dissolution and biodegradation of water-insoluble organic compounds. Appl. Environ. Microbiol. 52: 290–296

Tiehm A (1994) Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60: 258–263

Tsai JT, Wahbi LP, Dervakos GA & Stephens GM (1996) Production of toluene cis-glycol by a recombinant *Escherichia coli* strain in a two-liquid phase culture system. Biotechnol. Lett. 18: 241–244

van der Meer AB, Beenackers AACM, Burghard R, Mulder NH & Fok JJ (1992a) Gas/liquid mass-transfer in a four-phase fermentor: effects of organic phase hold-up and surfactant concentration. Chem. Engng. Sci. 47: 2369–2374

van der Meer JR, de Vos WM, Harayama S & Zehnder AJB (1992b) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56: 677–694

van Loosdrecht MCM, Lyklema J, Norde W, Schraa G & Zehnder AJB (1987) The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol. 53: 1893–1897

Vanhaecke E, Remon J-P, Moors M, Raes F, Rudder D De & Van Peteghem A (1990) Kinetics of *Pseudomonas aeruginosa* adhesion to 304 and 316-L stainless steel: role of cell surface hydrophobicity. Appl. Environ. Microbiol. 56: 788–795

Vanneck P, Beeckman M, De Saeyer N, D'Haene S & Verstraete W (1995) Biodegradation of polycyclic aromatic hydrocarbons in a two-liquid-phase system. In: Hinchee RE, Anderson DB & Hoeppel RE (eds), Bioremediation of recalcitrant organics, Vol. 7 (pp. 55–62), Battelle Press, Columbus OH

Van Sonsbeek, HM, Beeftink HH, & Tramper J (1993) Two-liquid-phase bioreactors. Enzyme Microb. Technol. 15: 722–729

Velankar SK, Barnett SM, Houston CW & Thompson AR (1975) Microbial growth on hydrocarbons-some experimental results. Biotechnol. Bioeng. 17: 241–251

Vermuë M, Sikkema J, Verheul A, Bakker R & Tramper J (1993) Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* sp. and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnol. Bioeng., 42: 747–758

Vigon BW & Rubin AJ (1989) Practical considerations in the surfactant-aided mobilization of contaminants in aquifer. J. Water Pollut. Control Fed. 61: 1233–1240

Volkering F, Breure AM, Sterkenburg A & Van Andel JG (1992) Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. 36: 548–552

Wang DIC & Ochoa A (1972) Measurements on the interfacial areas of hydrocarbon in yeast fermentations and relationship to specific growth rates. Biotechnol. Bioeng. 14: 345–360

Weber FJ, Ooijkaas LP, Schemen RMW, Hartmans S & de Bont JAM (1993) Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. Appl. Environ. Microbiol. 59: 3502–3504

West CC & Harwell JH (1992) Surfactants and subsurface remediation. Environ. Sci. Technol. 26: 2324–2330

Westgate S, Bell G & Halling PJ (1995) Kinetics of uptake of organic liquid substrates by microbial cells: a method to distinguish interfacial contact and mass-transfer mechanisms. Biotechnol. Lett. 17: 1013–1018

Williams AC, Woodley JM, Ellis PA, Narendranathan TJ, & Lilly MD (1990) A comparison of pig liver esterase and *Bacillus subtilis* as catalysts for the hydrolysis of menthyl acetate in stirred two-liquid phase reactors. Enzyme Microb. Technol. 12: 260–265

Wodzinski RS & Larocca D (1977) Bacterial growth kinetics on diphenylmethane and naphthalene-heptamethylnonane mixtures. Appl. Environ. Microbiol. 33: 660–665

Wodzinski RS & Coyle JE (1974) Physical state of phenanthrene for utilization by bacteria. Appl. Microbiol. 27: 1081–1084

Woodley JM, Brazier AJ & Lilly MD (1991) Lewis cell studies to determine reactor design data for two-liquid-phase bacterial and enzymatic reactions. Biotechnol. Bioeng. 37: 133–140

Wubbolts MG, Favre-Bulle O & Witholt B (1996) Biosynthesis of synthons in two-liquid-phase media. Biotechnol. Bioeng. 52: 301–308

Zehnder AJ & Svensson BH (1986) Life without oxygen: what can and what cannot? Experientia 42: 1197–1205