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Biodegradation of xylene and butyl acetate using an aqueous-silicon oil two-phase system

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Abstract

A stable microbial population, consisting of seven bacterial strains and three yeast strains, was selected in batch cultures on a mixture of ortho and meta-xylene and butyl acetate as the sole source of carbon and energy. This population can completely degrade up to 10 g/L of a mixture of these xenobiotics (70% xylene and 30% butyl acetate wt/wt) in a two-phase aqueous-silicone oil system (70%/30% vol/vol) within 96 h, while for the usual one-phase system very low growth degradation rates were observed. Further organic solvents were tested and finally, silicon oil was selected as the best organic phase for such a two-phase system. With periodical pH adjustments to 6.0 in fed-batch mode, the culture showed a global degradation rate of 63 mg L^{-1} h^{-1}.

Introduction

Numerous organic synthetic compounds are discharged into the environment by industrial and domestic human activities in too large quantities to be efficiently degraded by indigenous microbial populations. Their persistence in the environment or their recalcitrance to biodegradation could be due to their insolubility in water, their high concentration, their chemical structure, their physical state (solid or liquid), ... or their toxicity (Leisinger 1983; Thomas et al. 1986).

Butyl acetate and xylene isomers are commonly used as solvents (essentially in the paint industry) and significant waste enters the environment. Those compounds, present in low concentration (<0.05% wt/vol) in the microbial environment, can be biodegraded. As early as 1968, Davis et al. selected two strains of *Pseudomonas* able to grow specifically on para or meta-xylene and proposed a degradation metabolic pathway (Davis et al. 1968). Davey and Gibson have confirmed this pathway: the degradation occurs by the oxidation of a methyl substituent or by the direct oxidation of the aromatic ring (Davey & Gibson 1974; Gibson et al. 1974). The ortho-xylene can be degraded by *Pseudomonas stutzeri* via a supposed direct oxygenation of the aromatic nucleus followed by meta pathway reactions (Baggi et al. 1987). Oldenhuis et al have also selected a strain of *Nocardia* able to grow on ortho-xylene as the sole source of carbon and energy (Oldenhuis et al. 1989). The butyl acetate degradation metabolic pathway has not been specifically resolved, but this compound is usually degraded in mixtures of pollutants (Vandenberg 1988).

When present in higher concentration, however, these compounds are resistant to biodegradation (Stucki & Alexander 1987; Thomas et al. 1986). Xylene, as well as some organic aromatic compounds, is usually toxic to microorganisms. Toxicity is apparently due to the interaction of the aromatic compounds with the cytoplasmic membrane by loss of the cations Mg^{2+} and Ca^{2+} as well as other small molecules (de Smet et al. 1978). Nevertheless, some *Pseudomonas* species can grow in the presence of high concentrations of toluene (above 95% vol/vol) or xylene (5 to 50% vol/vol) as the sole source of carbon and energy (Cho et al. 1989; Cruden et al. 1992). Under these conditions, growth is observed but some changes appear

in the cytoplasmic membrane and the cell viability decreases.

This kind of culture may be called a two-phase aqueous-organic system: the mineral medium containing the microorganisms is the aqueous phase and the xenobiotic compounds constitute the organic phase. But, more generally, a two-phase aqueous-organic system is a culture medium where the organic phase corresponds to an organic solvent added to the aqueous phase and solubilizing the lipophilic compound. Several studies show that such an aqueous-organic system can be used in the bioconversion of substrates with low solubility in water. This system reduces the toxic effects of the substrates and/or the products (Bar & Gainer 1987; Brink & Tramper 1985). In such a two-phase system, the substrate is solubilized in the organic phase and diffuses into the aqueous phase. The microorganisms transform or degrade the substrate at the interface and/or in the aqueous phase. This system can be successfully used for biodegradation processes. Prokop et al. have studied the *n*-hexadecane degradation in a water-dewaxed gas oil medium (Prokop et al. 1971). Wodzinski and Larocca have demonstrated the degradation of naphtalene in a water-heptamethylnonane medium (Wodzinski & Larocca 1977). Efroymson and Alexander have also used the same culture medium for the degradation of naphtalene and n-hexadecane (Efroymson & Alexander 1991). Silicone oil has been used as an organic phase for: (i) the biodegradation of styrene or 2, 4, 6-trichlorophenol in a continuous reactor (Ascon-Cabrera & Lebeault 1995a; El Aalam et al. 1993), (ii) the selection of mixed microbial populations degrading some chlorinated and non chlorinated xenobiotic compounds (Ascon-Cabrera & Lebeault 1993) and (iii) the biodegradation of polynuclear aromatic hydrocarbons (Vanneck et al. 1994). More recently, 2-undecanone has been used as the organic phase in a two-phase system for the degradation of phenol in batch and fed-batch bioreactors (Collins & Daugulis 1997a,b).

The present paper reports a new application of a two-phase system. After the selection of the best organic phase among five organic solvents, the biodegradation kinetics of a mixture of butyl acetate and xylene were assessed in batch and fed-batch reactors.

Materials and methods

Synthetic organic compounds

Xylene and butyl acetate are industrial solvents and were kindly offered by the Air Industry System society (Courbevoie, France). Xylene is a mixture of meta and ortho isomers (30% meta and 70% ortho).

Microorganisms

The microorganisms used as an inoculum were isolated from various samples of soil, water and activated sludge. They were sampled on the industrial site. A high concentration of biomass was obtained by cultivating alicots (10 ml) of soil, water and activated sludge samples in a rich culture medium (100 ml; 10 g of pepton, 10 g of yeast extract, 5 g of NaCl, per liter of distilled water).

Culture media

- Aqueous phase: Microorganisms were grown in a mineral basic salts (MBS) medium containing (per liter of distilled water): 100 mg of $(NH_4)_2SO_4$; 350 mg of KH_2PO_4; 775 mg of K_2HPO_4; 100 mg of $MgSO_4$, $7H_2O$; 40 mg of $CaCl_2$; 1.00 mg of $FeSO_4$, $7H_2O$; 1.00 mg of $MnSO_4$, H_2O and 0.21 mg of $NaMoO_4$. pH is equal to 6.8.
- Organic phase: Various organic solvents were used for the experiments. They are described in Table 1.
- Microorganisms were enumerated by the spread plate technique (medium = 15 g of agar, 5 g of sodium chloride, 5 g of meat papain pepton and 15 g of tryptone, per liter of medium).

Culture conditions

Three different systems were used for the experiments: (i) the two-phase system contained silicon oil and the MBS and was called the two-phase system in our work, (ii) the cultures operated without an organic phase were called one-phase cultures and would highlight the interest of the two-phase process, (iii) the tests operated in two-phase system without substrates would provide information about the degradability and the toxicity of the organic phase (tested by the enumeration of microorganisms by plating on agar media).

Efficient microorganisms were selected at room temperature in 500-ml erlenmeyer flasks containing 100 ml of aqueous-organic medium. To maintain the

Table 1. Physico-chemical characteristics of organic compounds tested as organic phases in two-phase aqueous-organic systems.

Organic solvent	Pristane	Isopar V	Isopar L	Perfluored hydrocarbons	Silicon oil
Formula	Tetra 2,6,10,14-pentadecane	Isoparaffine C11-C12	Isoparaffine >C12	$F_{13}C_6$-CH—CH-C_6F_{13}	Dimethylpolysiloxane
Density (15 °C)	0.785	0.755	0.819	1.700	0.965
b.p. (°C)	298	190–207	278–305	–	>300
Miscibility with water	None	None	None	None	None
Solubility of xylene and butyl acetate	+++	+++	+++	+++	+++

good oxygenation and the emulsion of the medium, the flasks were placed on a rotary shaker (agitation =120 rpm). Every 96 h, the biomass contained in 20 ml of the medium (obtained by centrifugation at 12100 g for 20 min) was sub-cultured. After ten sub-cultures, the selected microorganisms were transferred into a 2-liter reactor (LSL Biolaffite S.A., Saint Germain en Laye, France) containing 1.2 liter of medium and were cultivated at 500 rpm and at room temperature in batch and fed-batch culture conditions.

All the cultures were carried out in triplicate and the given results correspond to the average.

Analytical methods

The microbial growth was measured by changes of optical density (OD) in the aqueous phase at 540 nm with a WTW Mikroprozessor model MPM 1500 photometer (Prolabo, Paris, France). It was also quantified by dry weight: 10 ml of the medium were centrifuged at 12100 g for 20 min and the pellet set at 110 °C for 24 h. pH was measured with a combined WTW pH/temperature probe and a WTW 537 pHmeter (Prolabo, Paris, France). The pO_2 was recorded during the culture in the Biolaffite fermentor with a combined pO_2 probe.

The residual concentrations of substrates were measured by gas chromatography with an INTERSMAT IGC 121 DFL chromatograph equipped with a flame ionization detector (Touzart et Matignon, Vitry sur Seine, France), under isothermic conditions (180 °C column and 210 °C injector and detector). The column was a 80/120 carbopac B/3% SP 1500, 3 meter length (Supelco, Saint Germain en Laye, France). One microliter of the organic phase or aqueous phase was injected into the column for analysis. To preserve the column, a glass wool filter was placed into the injector to capture the organic phase.

Results and discussion

Choice of the organic phase

The organic phase must possess the following characteristics: good solubility of xylenes and butyl acetate, no biodegradability, no toxicity to microorganisms, low volatility, low cost for its future use in industrial processes. Of course, the compound must not be an Environmental Protection Agency (EPA) priority pollutant as well.

Five organic compounds were selected for their physico-chemical characteristics (Table 1). Their biodegradability was tested by cultures of microorganisms in the two-phase medium in the absence of substrates. Figures 1a and 1b give the results. The variations of OD and pH values showed that tetra 4,6,10,14-pentadecane (pristane) and Isopar L and V (isoparaffines) could be used by the microorganisms as a source of carbon and energy. Variations in pH during the culture confirmed the degradation of the organic phase by the microorganisms. No significant variation appeared with silicone oil and perfluored hydrocarbons. Their toxicity was established. Culture of the microbial populations on agar plates during these experiments showed that no considerable mortility occurred with silicone oil while perfluored hydrocarbons were toxic to microorganisms (Guénard 1989). Other authors have already observed such effects. Bar and Gainer have tested a large variety of organic solvents

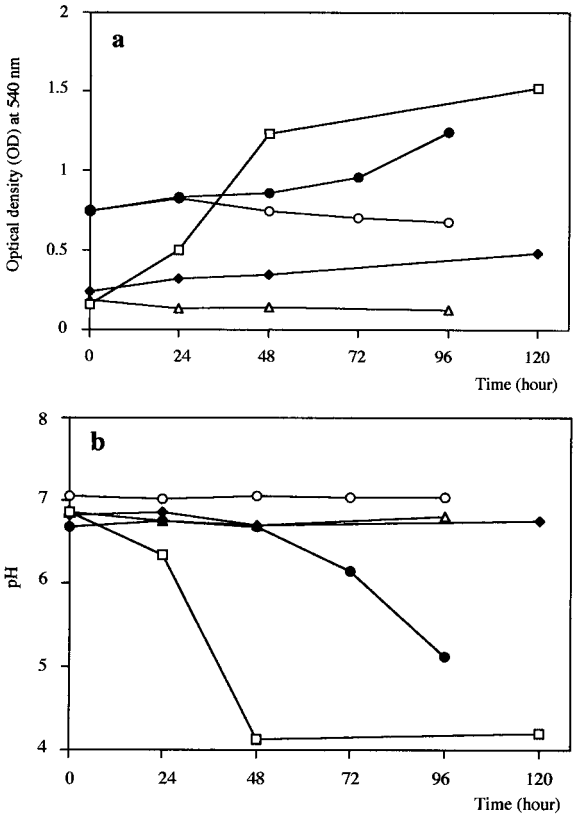

Figure 1. Optical density (a) and pH (b) courses versus time, for cultures realized with the selected microbial population in presence of five organic solvents as organic phases in two-phase media, in absence of xylene and butyl acetate: (□) = pristane, (●) = Isopar V, (○) = silicon oil, (♦) = Isopar L and (△) = perfluored hydrocarbons.

for the lactic acid fermentation in a two-phase medium (Bar & Gainer 1987). They have also shown that different solvents (esters, halogenated hydrocarbons, aromatics, alcohols) inhibit the consumption of glucose by *Lactobacillus delbrueckii* and they have proved the toxic effects of the organic phase to microorganisms (cell wall disruption, blockage of nutrients from the medium to the cells by a solvent coating. . .). Brink and Tramper have studied the epoxidation of propene and 1-butene by *Mycobacterium* in two-phase systems and have demonstrated that "many solvents caused almost instant inactivation of the suspended cells" (Brink & Tramper 1985). It is therefore a *sine qua none* condition to work with an inert organic phase for the culture of microbial populations in two-phase systems. Silicone oil was then selected as organic phase for our work.

Selection of microorganisms

The microorganisms were cultivated in the two-phase system. A 70% MBS and 30% silicone oil (vol/vol) system was used as in previous works (Ascon-Cabrera & Lebeault 1993; Vanneck et al. 1994). Gradual concentrations of substrates (0.3 g/L to 3.0 g/L) were added to the subcultures in order to exert selective pressure on the microorganisms. In addition to each subculture, a culture in a two-phase system but without substrates served as control for the biodegradation of xylene and butyl acetate. There were a total of ten subcultures.

Soon, in the first subculture, a growth was observed in the two-phase system (data not shown). As the inoculum had been taken from the industrial site, the microorganisms had been in contact with the xenobiotic compounds in their natural environment and some probably possessed the enzymes required for the degradation of xylene and butyl acetate before being selected in our laboratory. The growth we detected in the one-phase system enhances this conclusion (see later). After 40 days, a mixed and stable population was selected. Figure 2 shows the variations of OD and pH values observed with the tenth subculture in the presence (3 g/L) or the absence of xylene and butyl acetate. These results show that the microbial population was able to use xylene and butyl acetate (70%/30% (wt/wt)) as the sole source of carbon and energy (no variation of OD and pH values appeared in the absence of substrates). The final microbial population was composed of three yeasts and seven bacteria, all gram negative. After the acclimation period, the maximum specific growth rate of the mixed population was equal to 0.040 h^{-1} and the system had a biodegradation rate of 31 mg L^{-1} h^{-1}, calculated from the data obtained between 2 and 72 hours of culture. No further identification, either of the microorganisms or of the role of each strain in the overall degradation, was made, as the experiments were focused on the microbial degradation performances in the two-phase system.

Comparison of growth between two-phase and one-phase systems

To quantify the action of the organic phase on the degradation rates, 0.3 to 10.0 g/L of xylene and butyl acetate (70%/30% wt/wt respectively) were added to the two-phase and one-phase systems. The microbial

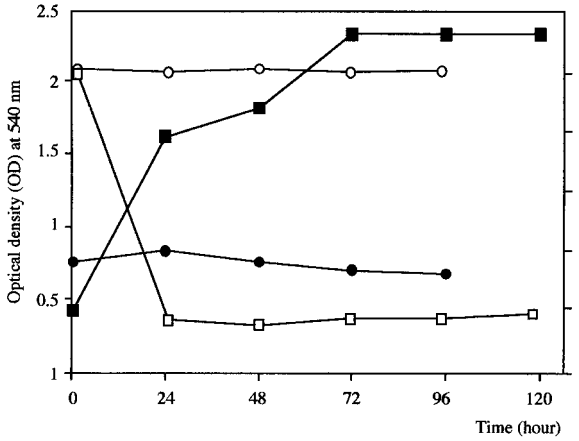

Figure 2. Changes in optical density value (black symbols) and pH value (white symbols) with time for cultures realized with (squares; substrates = 3 g/L) and without (circles) substrates in a two-phase system.

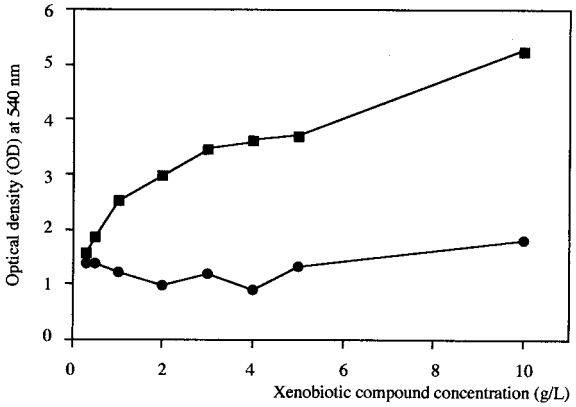

Figure 3. Evolution of the microbial growth with the substrate concentration for batch cultures in two-phase (■) and one-phase (●) systems after 96 h of batch cultures.

growths were compared through the optical density obtained after 96 h of batch culture. The initial conditions were OD = 0.50 at 540 nm and pH = 7.0. The results (Figure 3) show that the microbial growth was greatly enhanced in the two-phase system. For each concentration of substrate, the maximum optical density was obtained in the two-phase system and the cell concentration increased progressively with the concentration of substrate. The final optical density increased from 1.5 to 5.0 for respective initial substrate concentrations of 0.3 to 10 g/L. This increase was not observed in the one-phase system. The maximum optical density obtained in the one-phase system was still constant at around 1.0 to 1.5. In these conditions, the microorganisms were in direct contact with the xenobiotic compounds and the microbial growth seemed to be limited or weakened. However, the detected growth shows that the microorganisms possessed the metabolic pathway for the degradation of xylene and/or butyl acetate. They degraded a constant part of the substrate whatever the concentrations in the culture medium. This demonstrates that the nature and the origin of the inoculum are as important as the culture conditions in a biodegradation process.

Comparative cultures in two-phase and one-phase media emphasize the role of the organic phase. Xylene and butyl acetate rapidly weaken the microbial growth when they are present in high concentration (0.5 to 10 g/L) in the one-phase system while great degradations occur in the two-phase system as shown in the Figure 3. In the two-phase system, the silicone oil seems to act as a substrate tank. Xylene and butyl acetate are solubilized and dispersed in the organic phase. By vigorous shaking, the latter is emulsified and largely dispersed as droplets in the continuous aqueous phase, creating many zones of contact between both phases; that creates a large interfacial area. No substrate was ever detected in the aqueous phase by the gas chromatography analysis during the cultures. However, in the absence of microorganisms, Ascon-Cabrera and Lebeault have shown that the xenobiotics diffuse in the aqueous phase according to their water solubility and K_{ow} (octanol/water partition coefficient) (Ascon-Cabrera & Lebeault 1995b). Thus, the xenobiotic compounds diffuse from the organic phase to the aqueous one and are degraded either there or more probably at the interface, as soon as they are transfered from the silicone oil to the MBS. So, the degradation rate of xenobiotic compounds, in a two-phase organic-aqueous system, appears to be controlled by the xenobiotic diffusion rates at the two-phase interface which in turn depends (i) on the interfacial area size, (ii) on the compound's solubilisation rate in water and (iii) on the microorganisms' xenobiotic consumption rate.

Influence of pH

The degradation of xylene and butyl acetate by microorganisms led to the production of acidic compounds (Figure 4). After 24 h of culture, the pH fell to values of about 2.5 to 3.0. In such conditions, microbial enumerations on agar plates indicated that the three yeast strains and two bacteria had become dominant. To allow the survival of, or at least the activity

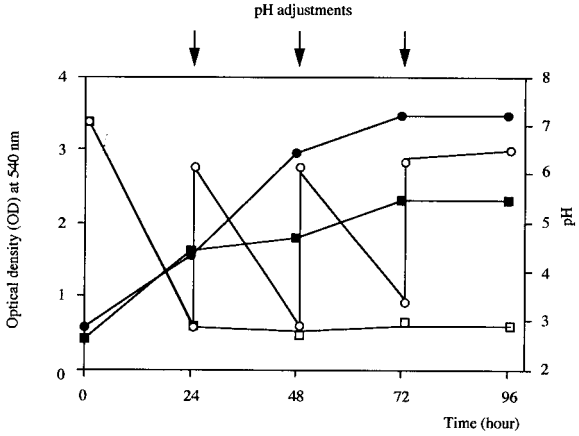

Figure 4. Comparison between pH-adjusted (circles) and pH-free (squares) two-phase cultures, followed by the optical density (black symbols) and pH (white symbols).

of all of the ten microbial strains, adjustment of the pH to a more neutral value was attempted. Every 24 h, the pH value was adjusted to 6.0 in order to improve the overall efficiency of the process. The results (Figure 4) show that the microbial growth as well as the degradation efficiency, were greatly improved by the pH readjustment. The whole substrate seemed to be transformed in 72 h of fermentation (either the pH was constant or slowly increased at t = 96 h), while in an unadjusted pH system, some carbon source seemed to remain after 96 h of culture (in the absence of pH adjustments, the optical density after 96 h was smaller than in pH adjustment conditions for the same initial concentration of xenobiotics). Following these results, the pH adjustments to 6.0 was then applied to other cultures (see Figure 5).

The acidification of the medium (from 7.0 to 2.5) corresponds to the production of acidic intermediates during the degradation of xylene (Baggi et al. 1987; Davey & Gibson 1974; Davis et al. 1968; Gibson et al. 1974) and butyl acetate (Figure 5: the first pH drop corresponds to the butyl acetate degradation). Only a few specific strains are able to grow in such extreme acidic conditions (three yeasts and two bacteria in our culture). With periodical readjustments of pH to 6.0, a better degradation efficiency was obtained. The drops of pH and high oxygen consumption rates observed just after the readjustments of pH to 6 in Figure 5 show that these readjustments boosted the degradation activities and favored the maintenance of the ten microbial strains in the population (enumeration on agar plates).

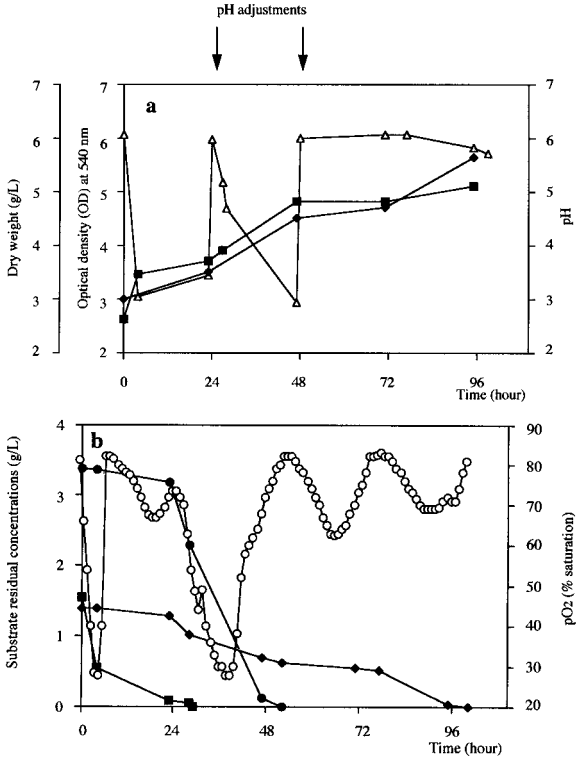

Figure 5. Microbial growth and kinetics of xylenes and butyl acetate degradations in a two-phase system, with pH-adjustments. Figure 5a: (♦) dry weight biomass, (■) optical density at 540 nm and (△) pH variations versus time. Figure 5b: (■) butyl acetate, (●) ortho xylene, (♦) meta xylene residual concentrations and (○) pO_2 variations (% saturation) versus time. Top arrows indicate the pH readjustments to 6.0.

Kinetics of degradation

Several fermentation cycles were realized in the reactor, in fed batch conditions. Three g/L of substrate were added to the medium every 96 h. The mixed population was able to degrade the substrates in these conditions and a high concentration of biomass was produced. The ability to degrade the xenobiotics was retained by the mixed population after many fermentation cycles.

Finally, the degradation kinetics of xylene and butyl acetate were studied in a 2-liter reactor (Figure 5). The two-phase system (70% MBS and 30% silicone oil vol/vol) was used; the pH was adjusted to 6.0 every 24 h. The pO_2 variations appeared to be clearly correlated to the pH adjustment and to the consumption of each substrate (the residual substrate concentrations were measured by gas chromatography in the organic phase – no substrate was detected in

the aqueous phase at all) (Figures 5a and 5b). Butyl acetate was first degraded (65% after 4 h of culture) and completely disappeared in 29 h. Its degradation generated a drop in the pH value (pH = 2.7 after 7 h of culture). The microbial oxygen consumption was stopped. That showed the acidification of the environmental medium had reduced the microorganisms' biodegradation ability. The readjustment of pH to 6.0 after 24 h of culture enabled the microorganisms to degrade completely the ortho-xylene in the next 29 h. The degradation of meta-xylene appeared less affected by the environmental pH value. Its degradation kinetic remained almost linear during the fermentation.

The specific degradation rates are 14 mg L^{-1} h^{-1}, 53 mg L^{-1} h^{-1} and 65 mg L^{-1} h^{-1} for meta-xylene, butyl acetate and ortho-xylene respectively. The global rate of the xylene and butyl acetate degradation reached 63 mg L^{-1} h^{-1}. These degradation rates are largely higher than those obtained by several authors in one-phase systems with very low concentrations (<0.05% wt/vol) of substrates. In their works, the xylene degradation (meta, ortho and para isomers) was only correlated to a detected growth or a weak consumption of oxygen. No degradation rate has ever been calculated (Baggi et al. 1987; Davey & Gibson 1974; Davis et al. 1968; Gibson et al. 1974). Vandenberg studied the degradation of butyl acetate by two strains of *Pseudomonas putida* and measured the disappearance of 6.8 μg/L of substrate in seven days (= 0.04 μg L^{-1} h^{-1}) (Vandenberg 1988). On the other hand, these degradation rates can be compared to those obtained with some others xenobiotic compounds, degraded in presence of silicon oil as an organic phase. A specific degradation rate of 12.5 mg L^{-1} h^{-1} for some chlorinated compounds and 34 mg L^{-1} h^{-1} for some non chlorinated compounds were obtained by Ascon Cabrera and Lebeault with two different mixed populations (Ascon-Cabrera & Lebeault 1993). Higher specific degradation rates were obtained (230 mg L^{-1} h^{-1}) for the degradation of styrene in batch culture at pH = 6 (El Aalam et al. 1993). Thus, it clearly appears, that the organic phase enables the xenobiotic load and then the degradation rate to increase greatly.

Finally, during culture, the medium turned yellow. Two separate cultures were grown with xylene or butyl acetate as an unique substrate. The results showed that the medium had turned yellow during the degradation of xylene. No change in the medium color appeared when butyl acetate was degraded. The apparition of a yellow color during the degradation of xylene would highlight the metabolic pathway developed by the microorganisms. Several authors who have studied the metabolic pathways of xylene isomers have observed the same change of color in the medium (Baggi et al. 1987; Davey & Gibson 1974; Davis et al. 1968; Gibson et al. 1974). This color corresponds to the production of a semi-aldehyde synthetized during the meta-cleavage of meta or ortho xylene. So a total mineralization of the substrate can be expected in our work.

Conclusion

The two-phase aqueous-organic system efficiency has been demonstrated in this paper for the degradation of some apolar xenobiotic compounds. This appears to be an interesting and easy method for a selection of microorganisms which are able to degrade this kind of recalcitrant molecules. Further works with xylene and butyl acetate might deal with the identification of each strain and its role in the degradation pathway, and with the process optimization. Chemostat cultures may permit the treatment of a continuous flow of pollution. The organic phase acts as a pollutant tank and this process should enable microorganisms to support variations in alimentation flow rates and unexpected high toxic concentrations. Some new processes are now in development for liquid and gaseous pollution treatment with two-phase aqueous-organic systems using silicon oil as an organic phase.

Acknowledgments

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