Biofilter performance and characterization of a biocatalyst degrading alkylbenzene gases

M. C. Veiga, M. Fraga, L. Amor, C. Kennes

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Abstract

A biofilter treating alkylbenzene vapors was characterized for its optimal running conditions and kinetic parameters. Kinetics of the continuous biofilter were compared to batch kinetic data obtained with biofilm samples as well as with defined microbial consortia and with pure culture isolates from the biofilter. Both bacteria and fungi were present in the bioreactor. Five strains were isolated. Two bacteria, Bacillus and Pseudomonas, were shown to be dominant, as well as a Trichosporon strain which could, however, hardly grow on alkylbenzenes in pure culture. The remaining two strains were most often overgrown by the other three organisms in liquid phase batch cultures μ max, KS, KI values and biodegradation rates were calculated and compared for the difterent mixed and pure cultures. Since filter bed acidification was observed during biofiltration studies reaching a pH of about 4, experiments were also undertaken to study the influence of pH on performance of the different cultures. Biodegradation and growth were possible in all cases, over the pH range 3.5–7.0 at appreciable rates, both with mixed cultures and with pure bacterial cultures. Under certain conditions, microbial activity was even observed in the presence of alkylbenzenes down to pH 2.5 with mixed cultures, which is quite unusual and explains the ability of the present biocatalyst to remove alkylbenzenes with high efficiency in biofilters under acidic conditions.

Keywords:

Biodegradation, biofiltration, bioremediation, extremophile, VOC, waste gas

Introduction

Benzene and alkylbenzenes are common air pollutants originating, among others, from direct contamination by flue gases or from specific soil remediation processes. They are known to be degradable biologically (Smith 1990). Besides conventional physico-chemical treatment technologies, biological techniques have been developed and optimized more recently for VOC and (alkyl)benzene contaminated air obtaining very encouraging results (Kennes & Thalasso 1998; Van Groenestijn & Hesselink 1993). Among the different biological alternatives, biofiltration is the most widely used technique mainly as a result of the ease with which reactors can be set-up and run, and because of the relatively low cost of the technology (Kennes & Thalasso 1998; Leson &Winer 1991; Van Groenestijn & Hesselink 1993). Nevertheless, much work is still needed in order to optimize biofiltration processes.

The biocatalyst is one important element among many others playing a key role in biofilter performance. A direct relationship has been found between the nature of the biocatalyst as well as its kinetic characteristics and parameters as, for instance, pressure drop or elimination capacity (Holubar& Braun 1995; Kennes et al. 1996)

The nature of microbial systems developing in biofilters has in many cases not been elucidated and little information has been published regarding biocatalyst characterization and activity of specific organisms in biofilters. Even when defined pure cultures are seeded on starting-up the reactors, biofilters are run under nonsterile conditions and new unidentified strains may grow and end up playing a key role which cannot be neglected (Fritsche & Lechner 1992). According to the few recent data reported in the literature, microorganisms most frequently encountered in biofilms during treatment of alkylbenzene vapors in bioreactors belong to the genus Pseudomonas, either because of their natural growth or as a result of initial inoculation (Ergas et al. 1994, 1995; Mirpuri et al. 1997; Pedersen et al. 1997; Veir et al. 1996). That widespread genus has also been identified as an efficient alkylbenzene degrader in groundwater remediation studies (Alvarez& Vogel 1991; Chang et al. 1993).

Some kinetic characteristics of microorganisms found in bioreactors used for waste gas treatment have recently been reported (Pedersen et al. 1997). No doubt other bacteria might be dominant in some cases or even eukaryotes which were shown to be present at high concentrations in biofilters treating alkylbenzene vapors (Páca & Koutský 1994; Veiga et al. 1997). In most cases consortia of microorganisms instead of individual strains are involved in pollutant removal (Ergas et al. 1994).

In the present study, an alkylbenzene (toluene, ethylbenzene, xylene) treating biofilter was characterized from a kinetic point of view. The biocatalyst from that biofilter was analyzed for its microbial composition, characterization of its activity and determination of kinetic parameters in batch systems. Biofilm samples, defined consortia and pure cultures obtained from the biofilter were studied. Activities and removal performances were compared in order to try and elucidate the role of the different strains in the biofiltration process. The presence of both prokaryotes and eukaryotes was checked and quantified. The biofilter was characterized by a relatively strong acidification of the

medium, with pH dropping down to values around 4, although under such conditions high EC could be maintained. In the present work, the influence on microbial activity of working at different pHs (pH 1.5–7.0) was therefore also analyzed with biofilm samples and pure cultures.

Materials and methods

Microbial cultures and medium

The nondefined microbial culture used originally was obtained from biofilter samples withdrawn from an alkylbenzene degrading biofilter (see below) operating under steady-state conditions. Pure cultures and defined consortia were obtained after enrichments of the nondefined mixed culture. Growth and biodegradation assays were performed with a synthetic mineral medium containing per liter demineralized water: 4.5 g KH_2PO_4, 0.5 g K_2HPO_4, 2 g NH_4Cl, and 0.1 g $MgSO_4.7H_2O$ as well as 2 mL each of a vitamin solution and a trace mineral solution (Kennes et al. 1996). Culture bottles containing the mineral medium were autoclaved. The vitamin and trace mineral solutions were filter-sterilized and added before inoculating the bottles. A calculated amount of pure alkylbenzene(s) was added to the culture medium to reach the desired final substrate concentration.

Plates containing the above mentioned synthetic medium solidified with agar were used for microbial characterization of the mixed culture. A mixture of alkylbenzenes (toluene, ethylbenzene, xylene) was used as carbon source. Several different colonies were obtained after serially diluting the original biofilm samples. They were plated on solid medium until reaching complete purification. In some cases specific inhibitors of prokaryotes and eukaryotes were added to the medium (Kennes et al. 1995). The latter addition as well as observations under the microscope allowed evaluation of the ratio of eukaryotic to prokaryotic cells. Samples of most of the isolated colonies at the two highest positive dilutions were used for microscopic observations allowing one to distinguish between bacteria, yeasts and filamentous fungi. The dominant microorganisms, identified by conventional techniques, were used as pure culture inocula or were mixed together in order to obtain defined consortia. Whenever working with defined consortia, the initial cell concentration was the same for each strain.

Biofilter operation

The biofiltration equipment and operation were as described elsewhere (Kennes et al. 1996). The feed was composed of a mixture of toluene, ethylbenzene and o-xylene at similar concentrations. Two biofilters were used, obtaining highly reproducible results with both reactors. Perlite was used as inert carrier.

Growth and biodegradation experiments

The bottles for batch assays were maintained in a thermostated rotary shaker at 30 ºC and were continuously shaken at 200 rpm. All bottles were inoculated with the same amount of biomass estimated by measuring the initial absorbance at 660 nm, dry weight

or protein concentration. Substrate depletion was followed by analyzing alkylbenzene concentrations in headspace samples. Concentration in the liquid phase could be determined using Henry's coefficient for each specific compound. Growth rates were calculated by measuring the absorbance at 660 nm at regular time intervals. Autoclaved controls allowed checking for any possible abiotic removal. All experiments were performed, at least in duplicate or triplicate. Purity of the cultures or microbial composition of the consortia was checked by observation under the microscope and eventually by plating samples at the end of the experiments.

Kinetic parameters calculated from biodegradation experiments were estimated by performing assays with different alkylbenzene concentrations and using the method of initial biodegradation rates. Other kinetic parameters (μ_{max}, K_S, K_I) were calculated from growth curves.

Analytical methods

Concentrations of alkylbenzenes in the gas phase were followed by gas chromatography on a Hewlett Packard 5890, Series II chromatograph equipped with a 50 m TR-Wax capillary column and a FID. Analyses were carried out isothermally at 175 ºC. Concentration of the samples was calculated by comparing the GC response to the one obtained with external standards used for calibration.

In order to check the possible presence or accumulation of biodegradation products, HPLC analyses were performed on a HP 1100 chromatograph, with a C-18 column using different methanol :water mixtures.

Protein concentrations were determined with the Coomassie Assay Reagent (Pierce, USA) after boiling samples for 10 minutes in 1 M NaOH. Bovine serum albumin was used as a standard.

Cell dry weight was determined by filtering a given amount of medium through a 0.22 μm filter and leaving the filtered material overnight at 105 ºC or until reaching constant weight. Growth was followed by measuring the absorbance on a UV/V Perkin Elmer spectrophotometer.

Results

Biofiltration kinetic parameters

Biofiltration kinetic studies undertaken under steadystate conditions, at biofilter inlet concentrations in the range 0.3–2.0 g m^{-3} and at a volumetric load of 68 m^3 m^{-3} h^{-1}, indicated that reaction limiting conditions were prevailing in such cases. As a result of the natural acidification of the medium during biofiltration, the pH of the system dropped naturally to 4.3 ± 0.2 under steady-state conditions. However, removal efficiencies did not drop with time at such relatively low pH value reaching a maximum EC of 70 gTEX m^{-3} h^{-1}. When lowering the alkylbenzene inlet concentration while maintaining a constant substrate loading, diffusion limiting conditions started being observed at substrate concentrations below 0.06–0.07 gm^{-3}.

Table 1. Biofilter characteristics when removing biofilm samples for microbial characterization of the biocatalyst

Feed composition	Toluene : Ethylbenzene : *o*-Xylene (1 : 1 : 1)
Elimination capacity	70 g m^{-3} h^{-1}
% Removal	>95
Volumetric load	68 m^3 m^{-3} h^{-1}
Gas residence time	57 s
$pH_{drain\ water}$	4.3 ± 0.2
Protein concentration	0.71 g L^{-1}

Experiments undertaken under reaction limiting conditions showed that the reaction followed zeroorder kinetics at inlet substrate concentrations above 0.3 g m^{-3}. The zero order rate constant was evaluated by following the substrate removal profile along the column and by plotting (C_0- C_x) versus time. A zero order rate constant of 0.022 g m^{-3} s^{-1} was obtained.

Microbial characterization of the biocatalyst

Representative biofilm samples were removed from the biofilter after the bioreactor had reached steadystate conditions. Biofilter running parameters reached when undertaking the present biocatalyst characterization are summarized in Table 1. The ratio of colony forming units of eukaryotes to prokaryotes was 0.27, showing the non negligible concentration of fungi in the biofilm.

After serially diluting suspended biofilm samples, dominant strains were isolated and identifications were performed. Different colonies were obtained at the highest positive dilutions. Several of these colonies corresponded to the same species, according to morphology, gram-staining, metabolic tests, and oxidase and catalase assays. Five dominant strains were clearly present, two fungal strains and three bacterial strains. From several batch experiments in aqueous medium it appeared that two of the five strains were repeatedly overgrown by the other three, meaning that, at least in liquid phase, the later three strains were more active in the presence of alkylbenzene substrates. These dominant strains were identified as *Trichosporon beigelei*, *Pseudomonas sp*. and *Bacilluslike sp*., hereafter called *Trichosporon*, *Pseudomonas* or *Bacillus*. The other two strains were *Rhodococcus* sp. and a non-identified brownish fungus. The three-culture consortium was reconstituted by mixing identical concentrations of each strain based on optical density. At initial substrate concentrations up to 120 ppm, when working close to neutral pH, *Trichosporon* became often overgrown by the two bacterial strains, but usually not at lower pH values corresponding to the conditions prevailing in the biofilter, as shown in Table 1. That fungus often failed to grow on alkylbenzenes in pure culture suggesting the possible existence of trophic relationships between that strain and the two dominant bacteria, although this was not investigated further.

Growth and biodegradation kinetics with mixed cultures

Since one of the major goals of this study was to elucidate the role of the dominant microorganisms found in the biofilm of the alkylbenzene treating biofilter, mixtures of ethylbenzene, toluene and o-xylene were used as substrate in the present experiments. As for the feed of the biofilter, equal concentrations of the pollutants were used. The

maximum biodegradation rates obtained with the nondefined mixed culture from biofilm samples and with the defined consortium of the five above mentioned microorganisms were respectively 0.80 and 0.82 ppmTEX h^{-1}, starting with the same biomass concentration. These results were obtained with an aqueous medium at pH 5.8, which was approximately the original pH of the filter bed in biofiltration experiments. This shows that, from a kinetic point of view, the non defined mixed culture and the defined consortium are very similar. The maximal biodegradation rates of toluene, ethylbenzene and oxylene with the defined consortium were respectively, 0.91, 0.87 and 0.60 ppm h^{-1}, meaning that xylene removal was the slowest (Figure 1) which was also concommitant with biofiltration data since o-xylene was the first contaminant to be detected at the reactor outlet when working with substrates overloads. Toluene and ethylbenzene removal rates were quite similar in all vials. No significant removal was observed in control bottles. Basically identical results were obtained with the three-culture defined consortium composed of the two dominant bacteria and the fungus mentioned above.

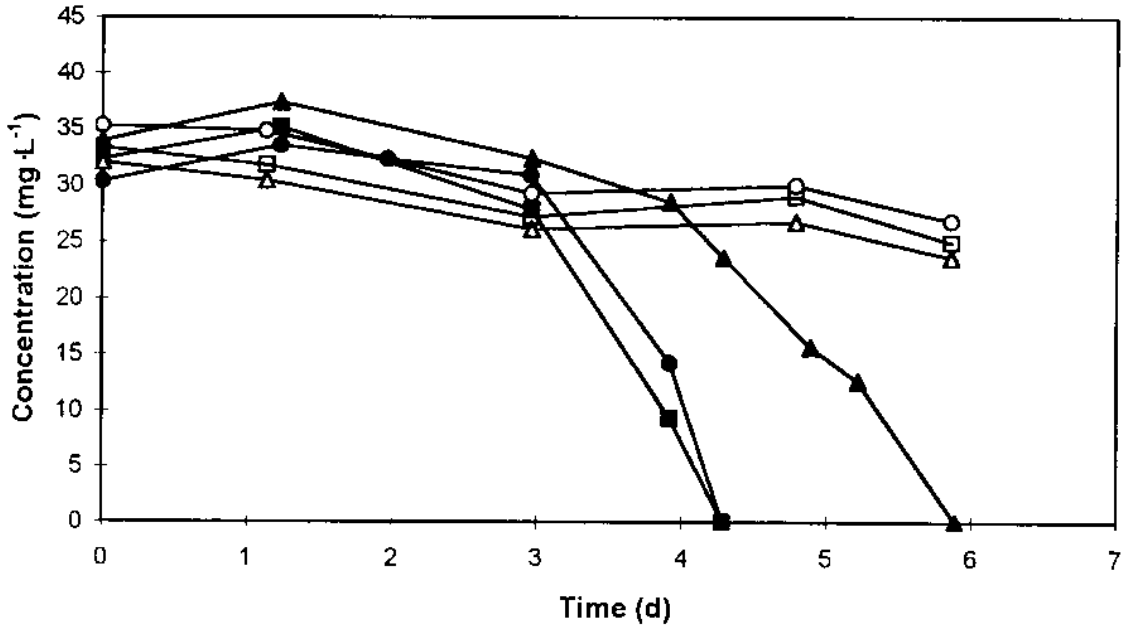

Figure 1. Removal of a mixture of toluene (●), ethylbenzene (■) and *o*-xylene (▲) with a defined consortium of microorganisms. The unfilled symbols correspond to the same compounds in controlbottles.

In order to compare batch and biofiltration kinetic data, the maximal specific substrate biodegradation rate was calculated from experimental batch results, reaching 0.62 mg_{TEX} h^{-1} mg^{-1} protein. Considering that the maximum elimination capacity of the biofilter used to treat the TEX mixture corresponds to 70 g_{TEX} m^{-3} h^{-1} and, taking into account the total average biofilter protein concentration, one can estimate the expected theoretical microbial activity or batch biodegradation rate based on biofiltration experiments. The latter rate would be 0.10 mg_{TEX} h^{-1} mg^{-1} protein, which is much lower than the experimental value reported above and obtained during batch assays.

Growth and biodegradation kinetics with pure cultures

Kinetic studies were undertaken with the dominant strains isolated from the defined consortium. The biodegradation rates observed at pH 5.8 with the two dominant strains, *Pseudomonas* and *Bacillus*, are summarized in Table 2. Kinetic studies were also undertaken with these dominant bacteria to allow comparing μ_{max}, K_S and K_I values of both microorganisms. The substrate inhibition model proposed by Haldane (Andrews 1968; Haldane 1930) was used to calculate the apparent inhibition constant (KI):

$$\mu = \mu_{max}(S/K_S + S + S^2/K_I)$$

As appears in Table 2, K_S values are quite similar for both strains although Bacillus shows a slightly higher substrate affinity. On the other hand, the maximum growth rate of Pseudomonas at pH 5.8 is about 1.5 times higher than observed for Bacillus, which seems logical when comparing global (toluene + ethylbenzene + xylene) biodegradation rates which are higher for *Pseudomonas*, although the difference between both organisms is not as large as for their μ_{max} (Table 2). The inhibition constants for *Pseudomonas* and *Bacillus* reached values of respectively 161.2mg L^{-1} and 117.5 mg L^{-1}. Typical biodegradation curves for both bacterial strains are plotted in Figures 2 and 3. These figures give relevant information on the performance and biodegradation characteristics of each of these two dominant strains. Although *Bacillus* is able to fully degrade all three contaminants, *Pseudomonas* does completely degrade toluene and ethylbenzene but only partly degrades o-xylene. The reason for the accumulation of a fraction of the initial o-xylene concentration with that strain remains unknown, although it is worth noting that microbial growth slows down or even stops, while o-xylene disappearence is still going on (data not shown). Formation or accumulation of potentially inhibitory biodegradation products by any one strain was not detected by HPLC.

Table 2. Kinetic parameters of the three dominant strains isolated from the biocatalyst at pH 5.8

	Strain		
	Pseudomonas	*Bacillus*	*Trichosporon*
Biodegradation rate (ppm h^{-1})	0.85	0.72	Negligible in pure culture
μ_{max} (h^{-1})	0.18	0.12	
K_S (mg L^{-1})	18.9	18.0	
K_I (mg L^{-1})	161.2	117.5	
$Y_{X/S(max)}$ (g g^{-1})	0.55	0.38	

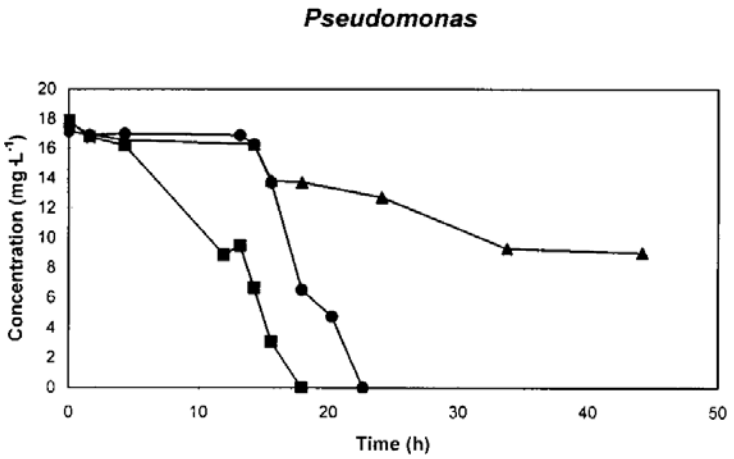

Figure 2. Typical removal pattern of a mixture of toluene (●),ethylbenzene (■) and *o*-xylene (▲) by pseudomonas.

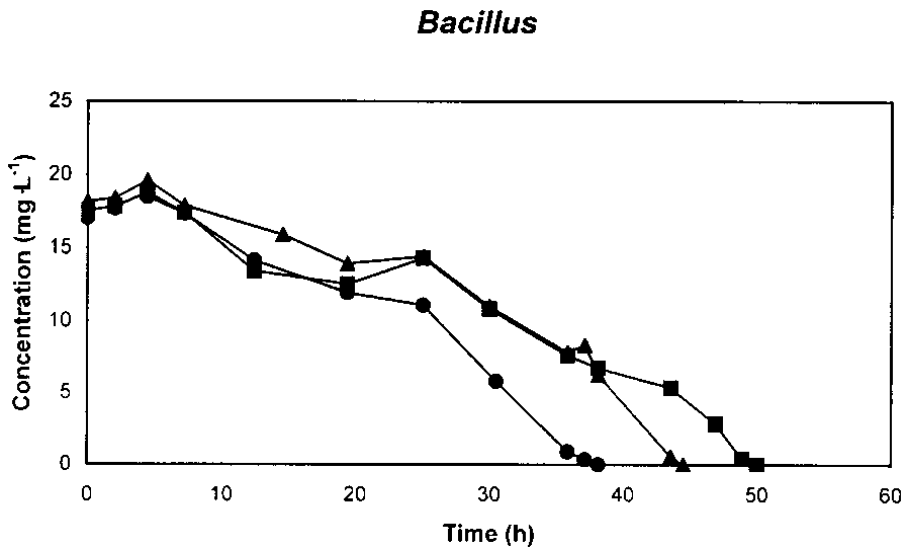

Figure 3. Typical removal pattern of a mixture of toluene (●), ethylbenzene (■) and *o*-xylene (▲) by *Bacillus.*

It is also worth observing that toluene and o-xylene biodegradation by *Pseudomonas* are after inoculation irrespective of the initial concentration, the highest concentration tested being about 200 mg L^{-1}. *Bacillus* always degrades toluene slightly faster than ethylbenzene whereas the opposite is observed with *Pseudomonas*.

Regarding biomass yields, the amount of biomass produced with *Pseudomonas* on alkylbenzenes is higher than with *Bacillus* (Table 2).

Influence of pH and vitamins/trace minerals on growth and removal efficiencies.

From data of biofiltration studies where acidification of the filter bed was systematically preceeded by a lag phase, whereas ethylbenzene removal started almost immediately observed, with pH variations in the range 4 to 6, the bacterial isolates are expected to be efficient alkylbenzene degraders at quite low pH values. Therefore, the influence of that parameter on biodegradation rates and growth was studied. Experiments were undertaken in the pH range 1.5 to 7.0. Basically no data have been reported on bacterial degradation of alkylbenzenes under extreme acidic conditions. In the pH range 4–6, again *Trichosporon* became gradually overgrown by the two bacterial strains in batch

experiments. Both bacterial strains were able to grow down to pH values of 3.5 but only the consortium grew and degraded the alkylbenzene mixture at pH 2.5. Biodegradation of the alkylbenzene mixture at pH 1.5 was impossible in all cases, except when using biofilm samples which allowed slow substrate removals, meaning that probably at least one other nonidentified microorganism might be present in the original biocatalyst, although not dominant in biofilm samples under less acidic conditions and/or that trophic relationships (for instance, release of some growth factors, etc.) might play a key role in the biofilm. At pH 1.5, biodegradation started after several days lag phase.

Maximum specific growth rates of both *Pseudomonas* and *Bacillus* grown on the TEX mixture at different pH values are given in Table 3. Since the media were buffered, pH fluctuations during the course of the experiments were basically negligible and always less than 0.2 unit respective to the initially adjusted pH value. Although Pseudomonas presented higher maximum specific growth rates than Bacillus near neutral pH or in slightly acidic mediumthe differences between the μmax of both strains decreased steadily when lowering the pH of the medium. μmax of *Bacillus* at pH 3.5 was even higher than for *Pseudomonas*.

Table 3. Maximum specific growth rates (h^{-1}) of the two dominant bacterial strains at different pHs

pH	Strain	
	Pseudomonas	*Bacillus*
7.0	0.20	0.13
5.8	0.18	0.12
4.5	0.15	0.10
3.5	0.07	0.09
2.5	Negligible	Negligible

Regarding the influence of vitamins/trace minerals, batch biodegradation experiments were conducted with the defined consortium and with the vitamins and trace minerals concentrations recommended in the literature and detailed in Material and methods. The same experimentswere performed under the following conditions: (i) vitamins and trace minerals diluted by a factor of twenty, (ii) vitamins (not diluted) and traces minerals diluted by a factor of twenty, (iii) vitamins diluted by a factor of twenty and trace minerals (not diluted), (iv) tap water. In all four cases a similar biodegradation rate of 0.80□0.07 ppm h^{-1} was obtained, demonstrating that the amount of vitamins/trace minerals could be drastically reduced, maintaining equally high biodegradation rates, i.e., EC, allowing reduction of biofilter operation costs.

Discussion

Little information is available in the literature on microbial characterization of biocatalysts developing in alkylbenzene degrading biofilters. Bacteria are the most frequently found microorganisms in such systems, although it appears from the present and a few other studies (Cox et al. 1994; Kennes et al. 1995, Veiga et al. 1997; Páca & Koustký 1994) that both prokaryotes and eukaryotes may be present at high concentrations, mainly when the system is not run at near neutral pH value.

Three bacteria and two fungi isolated from an alkylbenzene treating biofilter were dominant in the consortium described in this paper. Our results allowed highlighting the fact that biofilter performance was most often reaction limited and that alkylbenzene mass transfer limitation was only significant at inlet concentrations below 0.06–0.07 g m^{-3}. Two bacteria of the dominant microbial consortium, *Pseudomonas* and *Bacillus*,

grewmore efficiently on a toluene, ethylbenzene and o-xylene mixture than the other three strains, in a slightly acidic aqueous medium under batch conditions. Both strains were able to growat significant rates and degrade the alkylbenzenes over the pH range 3.5–7.0, which includes the pH variations 4– 6 observed in biofiltration studies (Kennes et al. 1996), although only the consortium of the isolated strains as well as the nondefined mixed culture showed significant growth and biodegradation rates at the lower pH of 2.5, suggesting the importance of trophic relationships between the different organisms, mainly under extreme conditions. Biodegradation of alkylbenzenes at significant rates by pure bacterial cultures under acidic conditions has, according to our knowledge, not been reported before. Kinetic characteristics of the two dominant strains were shown in Table 2. K_S values of both strains were quite similar, while a more significant difference was observed for μmax values. Although *Pseudomonas* presents a higher μmax near neutral pH, its maximum specific growth rate drops faster than for *Bacillus* when lowering the pH of the medium, which might explain the ability of the latter to compete against *Pseudomonas* in the biofilter where acidification was observed. *Bacillus* could also better grow on xylene than the *Pseudomonas* strain. From a technological point of view, *Bacillus* would represent the best candidate as pure culture inoculum in biofiltration since it completely degrades each one of the contaminants and it is characterized by a lower biomass yield, although it presents lower biodegradation rates than *Pseudomonas*.

The μmax values obtained in the present work are similar to though slightly lower than those reported recently by other authors (Alonso et al. 1997; Arcangeli & Arvin 1992; Chang et al. 1993; Choi et al. 1992; Elmén et al. 1997; Pedersen et al. 1997;Wübker et al. 1997), with different strains in the presence of toluene or other alkylbenzenes as substrate. The K_S values reported by other authors are in the range of 2–6 mg L^{-1}, which is lower than the 18 and 18.9 mg L^{-1} obtained in this work. However, a higher K_S value of 0.38 mM was recently reported for an Azoarcus tolulyticus strain grown on toluene with nitrate as electron acceptor (Elmén et al. 1997). The authors reported a K_I value of 1.3 mM similar to the 117.5 and 161.2 mg L^{-1} reported in this work, but much lower than the 1.98 g L^{-1} reported by Choi and collaborators (1992).

In order to try and determine if the isolates and the consortium are representative of the biocatalyst found in biofiltration experiments, biodegradation rates obtained with the defined consortium in batch assays with a defined aqueous medium were compared to those observed in gas phase biofilters. When comparing batch and continuous reactor kinetic characteristics, rates were nevertheless higher in batch assays with the defined consortium than in biofilters. This is not totally suprising. Similar observations were reported recently for toluene removal (Arcangeli & Arvin 1992; Pedersen et al. 1997). It was concluded that part of the biomass in the bioreactors was most probably inactive, which might also have been the case in the present experiments. It should nevertheless also be pointed out that other factors may affect the discrepency between batch and continuous kinetic data among which the difference in experimental conditions such as using an almost perfectly mixed aqueous medium in batch assays and a not perfectly mixed gas phase in continuous biofilters. The dropping pH value in continuous biofilters as opposed to the constant pH value in buffered batch assays is another relevant parameter, as is the excess of vitamins and trace minerals in batch assays.

Acknowledgments

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Nomenclature

EC: elimination capacity
C_0 inlet concentration
C_x concentration or different times or biofilter height
μ_{max} maximum specific growth rate
K_S Monod constant
K_I inhibition constant
$Y_{x=s}$ biomass yield

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