Trichloroethylene Mineralization in a Fixed-Film Bioreactor Using a Pure Culture Expressing Constitutively Toluene *ortho*-Monooxygenase

Adam K. Sun, Thomas K. Wood

Department of Chemical and Biochemical Engineering, University of California, Irvine, Irvine, California 92697-2575; telephone: 714-824-3147; fax: 714-824-2541; e-mail: tkwood@uci.edu

Abstract: An aerobic, single-pass, fixed-film bioreactor was designed for the continuous degradation and mineralization of gas-phase trichloroethylene (TCE). A pure culture of *Burkholderia cepacia* PR1$_{23}$(TOM$_{23C}$), a Tn5 transposon mutant of *B. cepacia* G4 that constitutively expresses the TCE-degrading enzyme, toluene *ortho*-monooxygenase (TOM), was immobilized on sintered glass (SIRAN™ carriers) and activated carbon. The inert open-pore structures of the sintered glass and the strongly, TCE-absorbing activated carbon provide a large surface area for biofilm development (2–8 mg total cellular protein/mL carrier with glucose minimal medium that lacks chloride ions). At gas-phase TCE concentrations ranging from 0.04 to 2.42 mg/L of air and 0.1 L/min of air flow, initial maximum TCE degradation rates of 0.007–0.715 nmol/(min mg protein) (equivalent to 8.6–392.3 mg TCE/L of reactor/day) were obtained. Using chloride ion generation as the indicator of TCE mineralization, the bioreactor with activated carbon mineralized an average of 6.9–10.3 mg TCE/L of reactor/day at 0.242 mg/L TCE concentration with 0.1 L/min of air flow for 38–40 days. Although these rates of TCE degradation and mineralization are two- to 200-fold higher than reported values, TOM was inactivated in the sintered-glass bioreactor at a rate that increased with increasing TCE concentration (e.g., in ~2 days at 0.242 mg/L and <1 day at 2.42 mg/L), although the biofilter could be operated for longer periods at lower TCE concentrations. Using an oxygen probe and phenol as the substrate, the activity of TOM in the effluent cells of the bioreactor was monitored; the loss of TOM activity of the effluent cells corroborated the decrease in the TCE degradation and mineralization rates in the bioreactor. Repeated starving of the cells was found to restore TOM activity in the bioreactor with activated carbon and extended TCE mineralization by ~34%. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 55: 674–685, 1997.

Keywords: fixed-film bioreactor; biofilter; trichloroethylene; mineralization; toluene *ortho*-monooxygenase

INTRODUCTION

Since trichloroethylene (TCE) has been the subject of much environmental and public health concern, it has become an important compound for hazardous waste remediation (McFarland et al., 1992; Nyer and Morello, 1993; Wilcox et al., 1995), and numerous bioreactor designs have been studied for its degradation (Fathepure and Vogel, 1991; Folsom and Chapman, 1991; McKay et al., 1994; Phelps et al., 1990; Shields et al., 1994). The most commonly studied microorganisms are *Methylosinus trichosporium* OB3b (Brusseau et al., 1990; Eng et al., 1991; Ensley and Kurisko, 1994; Fox et al., 1990; McFarland et al., 1992; Oldenhuis et al., 1989, 1991, Strand et al., 1991) and *Burkholderia cepacia* G4 (formerly *Pseudomonas cepacia* G4) (Folsom and Chapman, 1991; Hecht et al., 1995; Landa et al., 1994; Mars et al., 1996; Nelson et al., 1986; Shields et al., 1989, 1991). Although *M. trichosporium* OB3b has the highest aerobic TCE degradation rate (Oldenhuis et al., 1989, 1991), this organism grows extremely slowly (Park et al., 1991; Shah et al., 1995), and the expression of the TCE-degrading enzyme soluble methane monooxygenase (sMMO) is repressed by the presence of low copper ions (0.25 μM; Tsien et al., 1989). In addition, methane, which is required for sMMO expression, can compete with TCE for the active site of the sMMO enzyme (competitive inhibition). A two-stage bioreactor design overcomes the competitive inhibition problem of *M. trichosporium* OB3b at the expense of increased reactor cost and complexity (Alvarez–Cohen and McCarty, 1991b; McFarland et al., 1992).

With induction of an aromatic compound such as phenol or toluene, *B. cepacia* G4 (G4) expresses toluene *ortho*-monooxygenase (TOM) which can cometabolize TCE in bioreactors (Ensley and Kurisko, 1994; Folsom and Chapman, 1991; Landa et al., 1994; Mars et al., 1996). However, the use of these aromatic inducers is not an attractive method because they may be considered hazardous pollutants, and the problem of competitive inhibition for the monooxygenase remains. *B. cepacia* PR1$_{23}$(TOM$_{23C}$) (henceforth PR1$_{23}$), a mutant of G4, has the ability to degrade TCE in a bioreactor without an inducer (Shields et al., 1994). Based on its maximum TCE degradation rate, extent of TCE degradation and

mineralization, and growth rate, PR1$_{23}$ is one of the best microorganisms for TCE remediation (Sun and Wood, 1996). However, the most attractive advantage of PR1$_{23}$ is its ability to express TOM constitutively. Because an inducer is no longer required for PR1$_{23}$, TOM can be expressed in various media (Luu et al., 1995; Shields et al., 1991). Shields et al. (1994) have used PR1$_{23}$ attached to crushed oyster shells for TCE degradation in a bioreactor for a short period (72 h).

Fixed-film bioreactors contain a loose, porous, solid matrix upon which a moist, fixed film of bacteria is developed; for a given volume of bioreactor, much higher amounts of biomass can be achieved (Mol et al., 1993). Gas containing volatile organic compounds, such as TCE, is passed through the matrix, and the immobilized bacteria degrade the organic compounds. Because fixed-film bioreactors can treat the contaminant in the gas phase in a contained reactor, introduction of TCE-degrading organisms (either the wild-type or genetically engineered microorganisms) into the environment is minimized, and indigenous microorganisms from the polluted sites may be filtered from the incoming gas to avoid contamination (Ensley and Kurisko, 1994). Fixed-film bioreactors also have the advantage that they can withstand shock loading. When a transient high contaminant concentration enters the bioreactor, the outer layers of biomass may die due to the local toxicity, but the inner layers will be protected and will be capable of degrading the contaminant (Hardman, 1991). In addition, fixed-film bioreactors have the advantage that they can handle high gas and liquid (either nutrient or pollutant) flow rates (King et al., 1992; Ulonska et al., 1995).

This study examined the feasibility of TCE degradation using a pure culture that constitutively expresses TOM in a fixed-film bioreactor with activated carbon or chemically inert open-pore sintered glass (SIRAN™ carriers) as the supporting matrix. Due to the high adsorption capacity of the activated carbon, distinguishing the difference between the removal of TCE by carbon adsorption and biodegradation of TCE can be extremely difficult. Therefore, the inert glass matrix, which has a diameter similar to the activated carbon, was used to determine the TCE degradation rate in the reactor. TCE breakthrough curves were measured and compared between biologically active reactors and control systems (abiotic and biotic), and high concentrations of TCE were used to study TOM inactivation.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

B. cepacia G4 (Folsom and Chapman, 1991) and *B. cepacia* PR1$_{23}$ (TOM$_{23C}$; Shields and Reagin, 1992) were provided by Professor Reagin at the University of West Florida. G4 is the parent strain of PR1$_{23}$ and expresses TOM only in the absence of glucose and in the presence of phenol or toluene; hence, G4 was used as the negative control (biotic) for this study. G4 and PR1$_{23}$ were grown in a 0.4% (w/v) glucose chloride-free minimal medium (MCl: Luu et al., 1995) by inoculating from −85°C stocks (with 50 μg/mL of kanamycin for PR1$_{23}$). All pseudomonads were grown in 1-L cultures at 30°C in 2-L Erlenmeyer flasks by shaking at 250 rpm for 16–24 h (New Brunswick Scientific Co. Inc., Edison, NJ). The final optical density of the seed culture was greater than 2.0 as measured at 600 nm with a Milton Roy Spectronic 20D spectrophotometer (Fisher Scientific, Tustin, CA). For preparation of the cell inoculum for biofilm formation, cells were centrifuged at room temperature (Beckman, Fullerton, CA) for 10 min at 5000g. The supernatants were decanted and cell pellets were resuspended using 1 L of sterile MCl medium (with 50 μg/mL of kanamycin for PR1$_{23}$) and were transferred to a sterile 2-L flask to yield a concentrated inoculum.

E. coli JM109/pMS64 contains the *tom* metabolic pathway cloned into plasmid pGEM4Z and expresses TOM and catechol 2,3-dioxygenase constitutively (Shields et al., 1995). This strain was used as the positive control for the assay of phenol oxidation activity, and *E. coli* JM109 and G4 (grown in the presence of glucose only) were used as the negative controls. *E. coli* JM109 and *E. coli* JM109/pMS64 were inoculated from −85°C stocks and grown in a 30-mL culture of Luria–Bertani (LB) medium (Rodriguez and Tait, 1983) (with 100 μg/mL of ampicillin for JM109/pMS64) at 37°C in a 250-mL Erlenmeyer flask by shaking at 250 rpm.

Fixed-Film Bioreactor

The bioreactor (Fig. 1) consists of a glass cylinder (4.75 cm in diameter and 63 cm long) containing a solid matrix (suspended by a perforated Teflon disk), and the reactor bed (volume of the supporting matrix) was maintained at 0.7 L unless specified otherwise. All fittings and connectors were constructed of either Teflon, glass, or Viton tubing to prevent TCE adsorption. Heating tape (Fisher Scientific) was used to provide a uniform temperature within the bioreactor (30°C). Using TCE as the pulse tracer, the empty bed contact time was approximately 10 min at 100 mL/min of air flow rate (time required for detecting the maximum TCE concentration at the outlet sampling ports). The support materials used in this study were open-pore sintered glass (SIRAN™ carriers), particle size 2–5 mm (Jaeger Biotech Engineering, Inc., Costa Mesa, CA), and BPL 6 × 16 granular activated carbon, particle size 1.19–3.36 mm (Calgon Carbon Corp., Huntington, WV). The granular activated carbon has a smaller pore size distribution and higher total surface area than SIRAN™.

The bioreactor was operated continuously with respect to both the nutrient and TCE flow rates. The biofilm was seeded by recirculating the inoculum culture
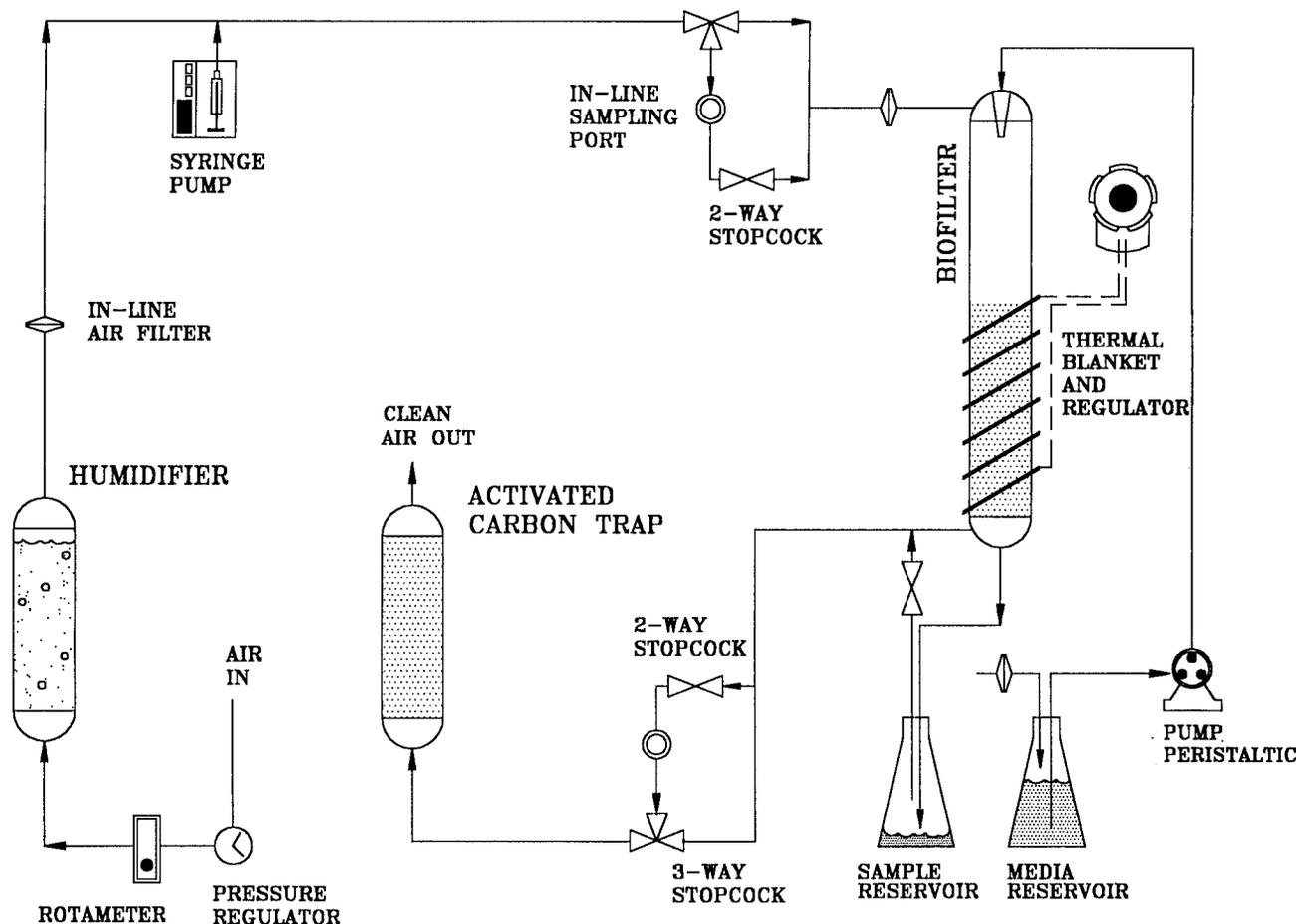
Figure 1. Schematic of the fixed-film bioreactor for TCE bioremediation.

for a period of 24 h. The inoculum was completely drained from the reactor before the sterile MCl medium (with 50 µg/mL of kanamycin for $PR1_{23}$) was added continuously for biofilm development.

After 5–10 days of biofilm formation, TCE was pumped into the bioreactor's inlet air by a syringe pump (Cole–Parmer Instrument Company, Chicago, IL). TCE concentrations were controlled by adjusting the syringe pump setting (4–240 µL/day), and the air flow rate (100 mL/min) was controlled by a rotameter (Matheson Gas Company, Montgomeryville, PA). The incoming air was humidified to prevent drying of the solid matrix in the bioreactor. The TCE-laden air was fed from the top of the bioreactor through two 0.22-µm sterile bacterial air vents (Gelman Sciences, Ann Arbor, MI), and nutrients (MCl) were fed into the top of the bioreactor (0.74–1.19 L/day) through a 0.2-µm sterile nitrocellulose membrane (Gelman Sciences). The TCE-laden air was introduced into the top of the bioreactor to minimize the retention of medium in the bioreactor and for uniform gas flow (no burping).

Analytical Methods

TCE concentrations were determined by periodically sampling 5 µL of the gas from the inlet and outlet air streams using a 50-µL gas-tight syringe (Hamilton, Reno, NV) by puncturing a Teflon-coated septum attached to glass sampling ports, and samples were injected into a gas chromatograph (GC) equipped with an electron capture detector (Hewlett–Packard 5890 Series II, Wilmington, DE) and analyzed as described previously (Luu et al., 1995). The initial maximum TCE degradation rates (for the bioreactor with inert SIRAN™ carriers) were determined when the biofilter was first exposed to TCE from the average of two to seven air samples (measured over a 5–20 minute period) of the outlet and inlet TCE concentrations. Variability in the inlet TCE concentration was less than 10%. With the chloride-free MCl medium, the chloride ion concentration of the reactor effluent from TCE mineralization was measured using a chloride ion specific electrode (Jahng and Wood, 1994; Sun and Wood, 1996).

As an indicator of $PR1_{23}$ in the reactor, effluent cells were collected and phenol oxidation activities by TOM were determined by measuring the dissolved oxygen uptake rates using a biological oxygen monitor (YSI Model 5300, YSI Incorporated, Yellow Springs, Ohio) with data acquisition by a Vectra VL2 computer (Hewlett–Packard, Wilmington, DE). The oxygen removal rate in the absence of phenol (background respi-

ration rate) was determined by 5-min monitoring of a reaction vial containing 2 mL of air-saturated 20 mM potassium phosphate buffer, pH 7.0, and 1 mL of the effluent cells from the bioreactor (0.8 μmol dissolved oxygen at 25°C). The activity of TOM can be determined from the increase in oxygen removal rate (above background respiration) when phenol is added to the reaction vial (final concentration of 0.17 μM). The phenol oxidation activity was normalized with the total amount of cellular protein in the reaction vial (determined from a calibration curve of optical density vs. protein/mL) and reported as nanomoles of oxygen removed per minute per milligram of protein.

Biomass concentrations on the solid matrix (from the top to bottom sections) were removed at the end of the experiments with 1N NaOH and were boiled for at least 10 min (Nelson et al., 1987). Total cellular protein for the entire bioreactor was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) by averaging the samples from various sections of the reactor (four to five sections tested). Bovine serum albumin diluted in 1N NaOH was used as the protein standard.

RESULTS AND DISCUSSION

Biofilm Development

To determine the optimum nutrient flow rate, TCE mineralization was monitored using a 0.37-L bioreactor while maintaining a constant TCE concentration (0.484 mg/L of air). TCE mineralization performance as the function of MCl nutrient flow rate (0.74–1.19 L of medium/day) was used to determine the optimal nutrient flow rate. With 1.0 L/day of MCl flow rate, an average TCE mineralization rate of 10 ± 2 mg TCE/day was obtained, whereas a MCl flow rate of 0.74 and 1.19 resulted in mineralization rates of 7 ± 1 and 5 ± 2 mg TCE/day, respectively; hence, an MCl flow rate of 1.0 L/day was chosen. With this MCl flow rate and 0.1 L/min of air flow, an average time of 5–10 days was required for the development of a robust G4 or PR1$_{23}$ biofilm on SIRAN™ carriers (after seeding with concentrated inoculum for 24 h), and a similar time was required for biofilm development on activated carbon. The development of biofilm was quickest at the top of the bioreactor where it was the closest to the nutrient inlet. The distribution of biofilm on SIRAN™ carriers was relatively uniform as determined by examining five different sections (average values shown in Table I), indicating no significant channeling of nutrient flow. For example, for the PR1$_{23}$ fixed-film bioreactor in the absence of TCE, the biofilm distribution in the presence of glucose [0.4% (w/v)] as the sole carbon and energy source ranged from 7 to 8 mg protein/mL carriers after 19 days of culture time (in a single reactor).

Comparing the average biofilm values in Table I, similar amounts of PR1$_{23}$ colonized the activated carbon and SIRAN™ carriers (2–8 mg protein/mL vs. 4 ± 1 mg protein/mL), even though the pore size distributions were vastly different from these two matrices. This smaller than expected difference is probably due to the fact that most of the biofilm was present in the interstitial space between similar-size matrix particles.

TCE Breakthrough

Granular activated carbon provides high surface area and fine pore structures for PR1$_{23}$ biofilm development; however, its high adsorption capacity precluded measuring TCE degradation by enzymatic attack. Therefore, chloride ion generation (with a chloride-free medium) was used to indicate complete mineralization of TCE. The PR1$_{23}$ bioreactor generated significantly higher amounts of chloride ion as compared to the control (G4) bioreactor (Fig. 2) which verifies the feasibility of TCE mineralization using the reactor design. The variability of TCE mineralization over time could be due to the interaction of different adsorption processes and TCE mineralization by the biofilm.

To measure the TCE degradation rate independent of TCE adsorption, chemically inert SIRAN™ carriers were chosen because they do not adsorb TCE and the actual biological degradation of TCE can be determined directly from the breakthrough curves. To reduce the experimental time for each reactor run a high TCE concentration range was used. With 0.1 L/min air flow and a TCE concentration of 0.242 mg/L of air, complete TCE breakthrough time was approximately 40 min for both the abiotic (wet SIRAN™ carriers only, Fig. 3B) and biotic negative control (G4 grown on glucose and immobilized on SIRAN™ carriers, Fig. 3B); however, the time required for the complete TCE breakthrough for a TCE-degrading PR1$_{23}$ fixed-film bioreactor was approximately 120 h (Fig. 3B), an 180-fold increase. At a higher TCE concentration (2.42 mg/L of air) the TCE breakthrough time for the PR1$_{23}$ fixed-film bioreactor was approximately 21 h (Fig. 3C), and at a lower TCE concentration (0.04 mg/L) only 40% of inlet TCE was detected at the outlet sampling port for the PR1$_{23}$ bioreactor after 14 days of operation (Fig. 3A). Thus, at lower TCE concentrations the reactor could be operated for extended periods with SIRAN™ carriers without complete TCE breakthrough. TCE breakthrough occurring in PR1$_{23}$ fixed-film bioreactor is probably due to the inactivation of TCE-degrading enzyme caused by the toxicity of the TCE by-products.

TCE Degradation and Mineralization

Due to the difference in the amount of biomass obtained in the reactors, TCE degradation rates were normalized using the concentration of total cell protein measured at the end of each bioreactor experiment [nmol TCE/(min · mg protein)]; hence, the degradation rates are conservative because biomass increased during the

Table I. Initial TCE degradation rates, amounts of TCE degraded and mineralized, and biomass levels of the *B. cepacia* PR1$_{23}$(TOM$_{23C}$) fixed-film bioreactor.

| Strain	TCE concn., mg/L of air	Initial TCE deg. rate, nmol/min/mg protein (mg TCE/L of reactor vol/day)	Total TCE deg., mg	Total TCE miner., mg	Ave. final biomass amt., mg protein/mL of packed vol	Length of exp., days	Support matrix
PR1$_{23}$	0.000	ND	ND	ND	8 ± 1	19	SIRAN
		ND	ND	ND	4 ± 1	23	Activ. carbon (0.3 L)
PR1$_{23}$	0.040	0.007 (8.6)	64.5	ND	6 ± 4	22	SIRAN
PR1$_{23}$	0.121	ND	ND	261.1	ND	38	Activ. carbon (0.3 L)
PR1$_{23}$	0.242	0.157 (44.9)	56.5	92.1	2 ± 1	10	SIRAN
		0.082 (46.5)	68.4	74.0	3 ± 2	24	SIRAN
PR1$_{23}$	0.242	ND	ND	410.0	ND	40	Activ. carbon
PR1$_{23}$	0.242	0.289 (26.9)	28.0	ND	0.481[a]	4	Suspended
PR1$_{23}$	1.210	0.717 (213.0)	112.1	133.5	2 ± 1	11	SIRAN
PR1$_{23}$	2.420	0.715 (392.3)	130.5	130.9	3 ± 2	7	SIRAN
G4	0.242	ND	ND	ND	3 ± 2	5	SIRAN
		ND	ND	ND	5 ± 1	53	SIRAN

ND, not determined. The bioreactor had 0.7 L of supporting matrix and an air flow rate of 0.1 L/min.

[a]0.7 L of suspended culture, biomass (mg protein/mL culture).

course of the experiments (Table I). The initial maximum degradation rates for the PR1$_{23}$ fixed-film bioreactor packed with SIRAN™ carriers were 0.157 and 0.082 nmol/(min·mg protein), equivalent to 44.9 and 46.5 mg TCE/L of reactor volume/day at 0.242 mg/L TCE concentration. A fivefold increase in TCE concentration, 1.21 mg/L of air, produced a 4.6–8.7-fold increase in the TCE degradation rate of 0.717 nmol/(min·mg protein) (213.0 mg TCE/L/day). A further twofold increase in TCE concentration to 2.42 mg/L resulted in a TCE degradation rate of 0.715 nmol/(min·mg protein) (392.3 mg TCE/L of reactor volume/day). When the TCE concentration was decreased to 0.04 mg/L (sixfold decrease), the initial maximum degradation rate was lowered to 0.007 nmol/(min·mg protein) (8.6 mg TCE/L of reactor volume/day).

To compare the biofilm reactor to a suspension-cell reactor, a bubble column reactor was evaluated under the same conditions (0.242 mg TCE/L of air, 0.7 L of reactor volume, and 100 mL/min of air flow rate). The performance of the fixed-film bioreactor was superior because it provided greater active biomass (2–8 mg protein/mL volume for the fixed-film bioreactor vs. 0.481 mg protein/mL volume for the suspended culture) and sustained TCE degradation (67% longer based on TCE breakthrough times).
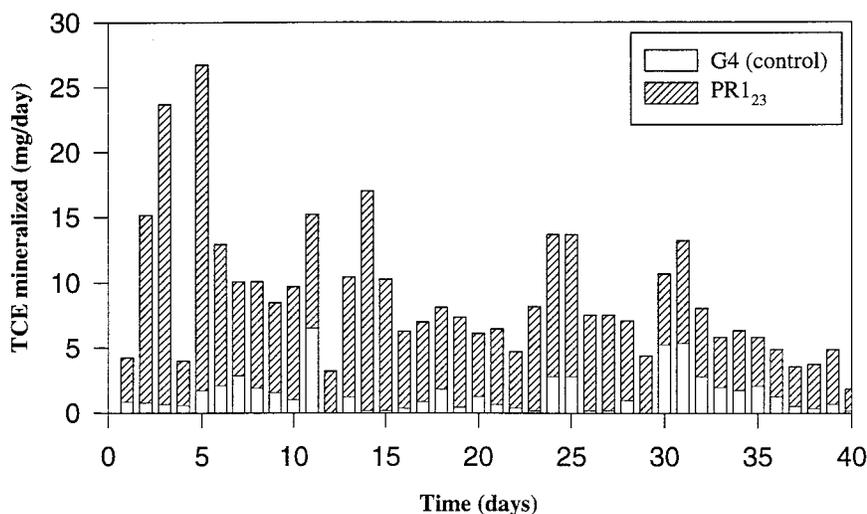

Figure 2. Comparison of TCE mineralization by *B. cepacia* G4 (negative control, empty bar) and *B. cepacia* PR1$_{23}$ (filled bar) in bioreactors filled with activated carbon. Air flow rate at 0.1 L/min, TCE concentration at 0.242 mg/L, and 0.7 L of reactor bed were used.
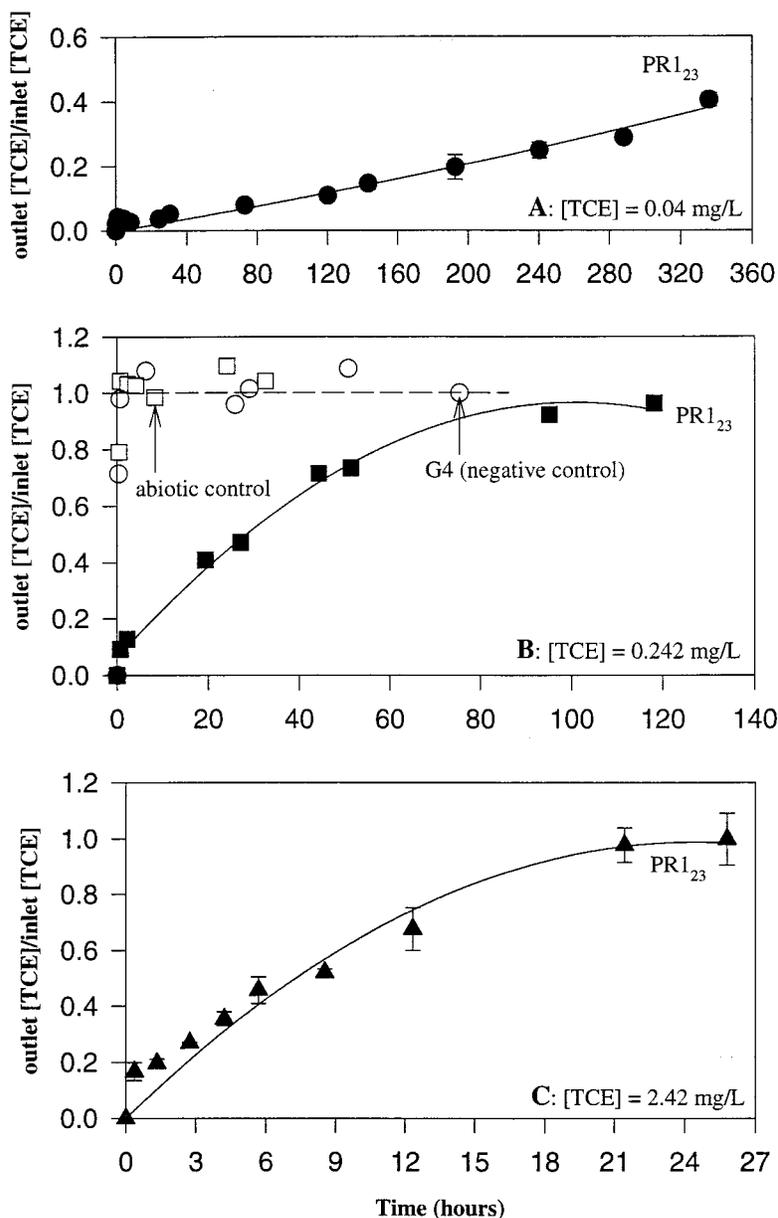
Figure 3. TCE breakthrough curves of the *B. cepacia* PR1$_{23}$ bioreactor with SIRAN™ carriers at different TCE concentrations. Air flow rate at 0.1 L/min and 0.7 L of reactor bed were used. (A): 0.04 mg/L, (B) 0.242 mg/L, and (C) 2.42 mg/L of TCE. Data are average of two to seven samples (error bars indicate 1 standard deviation).

As shown in Figure 4, in independent experiments the TCE degradation rates decreased as TCE addition continued due to the toxic effect of TCE breakdown products, as seen with other TCE-degrading microorganisms (Oldenhuis et al., 1991; Wackett and Gibson, 1988; Wackett and Householder, 1989; Zylstra et al., 1989). Assuming complete TCE breakthrough occurs when the TCE-degrading enzyme is completely deactivated and the biofilm distribution in the bioreactor does not change significantly, the percentage of TCE breakthrough corresponds to the same percentage of TOM enzyme deactivation. From the linear portion of five TCE breakthrough curves [0.04, 0.242 (n = 2), 1.21,

and 2.42 mg TCE/L], PR1$_{23}$ oxidized an approximate average of 0.09 ± 0.04 mg TCE/mg protein before TOM activity was completely inactivated. Values for inactivation of other TCE-degrading enzymes range from 0.0079 mg TCE/mg protein for the ammonia-oxidizer *Nitrosomonas europaea* (Hyman et al., 1995) to 0.031 mg TCE/mg cells [or 0.062 mg TCE/mg protein, assuming 50% of the total dried cell weight is cellular protein (Bailey and Ollis, 1986)] for a consortium of phenol oxidizers (Chang and Alvarez–Cohen, 1995). Slightly higher values of protein inactivation were reported for some methane oxidizers (0.04–0.06 mg TCE/mg cells; Alvarez–Cohen and McCarty,
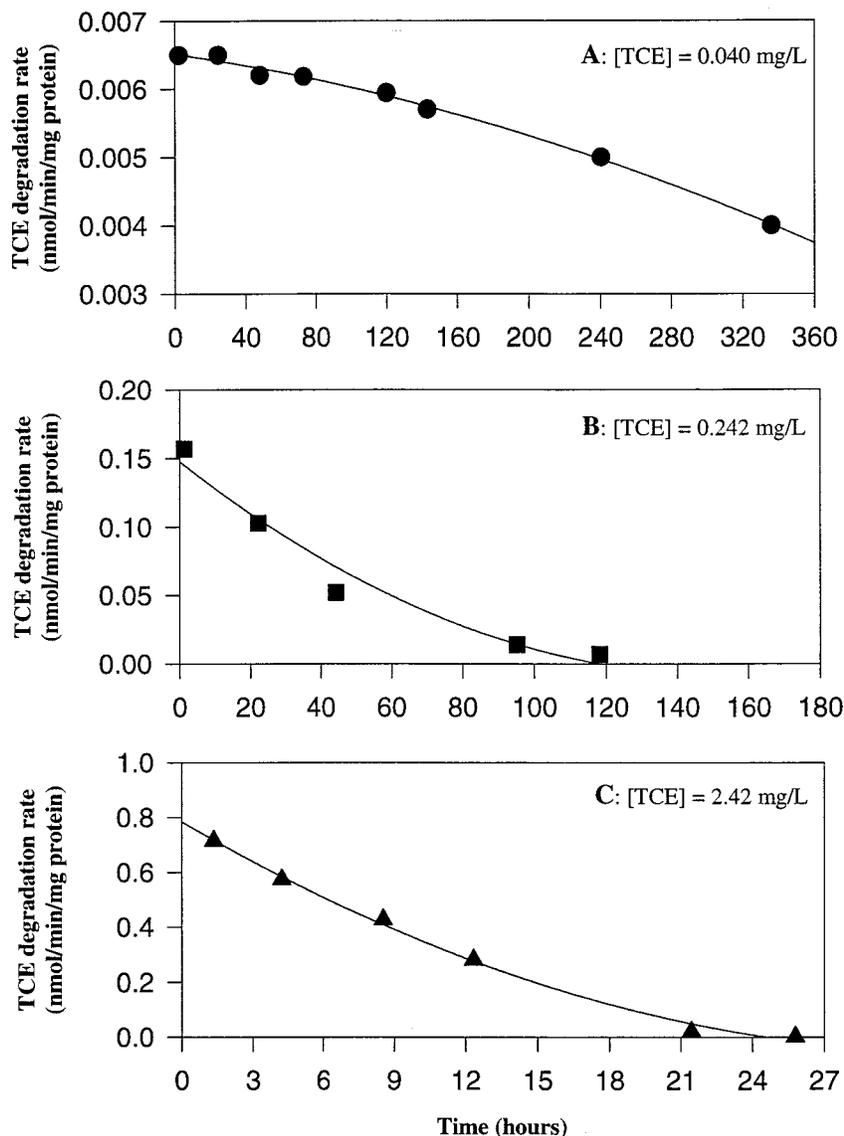
Figure 4. TCE degradation rates for the *B. cepacia* PR1₂₃ bioreactor with SIRAN™ carriers at different TCE concentrations. Air flow rate at 0.1 L/min and 0.7 L of reactor bed were used. (A) 0.04 mg/L, (B) 0.242 mg/L, and (C) 2.42 mg/L of TCE.

1991a; Chang and Alvarez–Cohen, 1995; Chu and Alvarez–Cohen, 1996).

Based on the chloride ions generated, the extent of TCE mineralization by the PR1₂₃ bioreactor with SIRAN™ carriers agrees well with the total TCE degradation by the bioreactor (Table I). These data indicate that complete mineralization of TCE can be achieved using the fixed-film bioreactor. For the bioreactor packed with activated carbon, the TCE mineralization rate was also monitored using the chloride ion concentration in the reactor effluent. The PR1₂₃ bioreactor with 0.3 L of activated carbon and 0.121 mg TCE/L of air mineralized TCE at an average rate of 7 ± 1 mg/L of reactor volume/day for 38 days and 10 ± 6 mg/L of reactor volume/day for 40 days with 0.7 L activated carbon and 0.242 mg TCE/L of air.

**Biofilter TOM Activity and Long-Term Performance**

Using an oxygen probe TOM activity can be gauged from oxygen removal rates above background respiration by measuring oxygen uptake by the cells in the presence and absence of the specific substrate for TOM (phenol). Using the negative controls G4 grown on glucose and *E. coli* JM109 grown on LB, the addition of phenol did not increase oxygen uptake. However, for the positive control *E. coli* JM109/pMS64 (containing constitutively expressed TOM) grown on LB with ampicillin, the oxygen removal rates (phenol oxidation rates) increased (15%) in the presence of phenol; hence, phenol addition to the PR1₂₃ cells can serve to directly indicate TOM activity. Note that oxygen removal rates above background respiration are even higher in PR1₂₃

because TOM is much more active in the pseudomonad.

When no TCE was supplied to the fixed-film bioreactor with either SIRAN™ carriers or activated carbon, the oxygen removal rates by $PR1_{23}$ effluent cells in the presence of phenol were >100% above background respiration, indicating the consistent activity of TOM (TOM activity was sustained in the biofilter for at least 19 days of operation with SIRAN™ carriers and at least 24 days for activated carbon in the absence of TCE). However, upon addition of TCE to the bioreactor, TOM activity of $PR1_{23}$ gradually decreased (Fig. 5). For 0.242 mg/L of TCE, the oxygen removal rate by the $PR1_{23}$ cells in the presence of phenol was reduced below the background respiration after 2–4 days of TCE exposure (Fig. 5B), indicating the complete inactivation of the TOM enzyme. Increasing the TCE concentration accelerated this inactivation of TOM for at higher TCE concentrations 2.42 mg TCE/L, TOM was inactivated after only 1 day of TCE exposure (Fig. 5C).

These results of declining TOM activity coincided with the decline in TCE degradation rates shown in
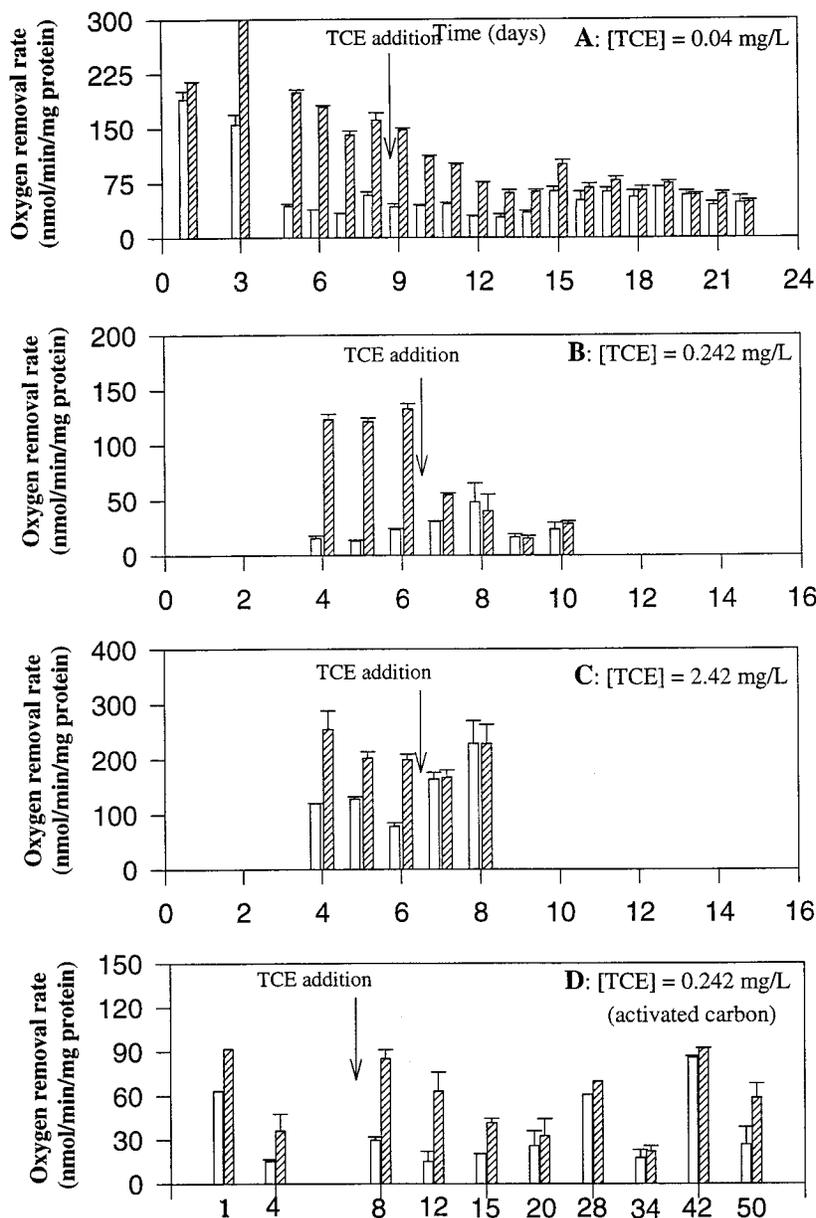

Figure 5. TOM activity indicated by phenol oxidation of effluent cells from the *B. cepacia* $PR1_{23}$ bioreactor at different TCE concentrations. (A) 0.04 mg/L with SIRAN™ carriers, (B) 0.242 mg/L with SIRAN™ carriers, (C) 2.42 mg/L of TCE with SIRAN™ carriers, and (D) 0.242 mg/L with activated carbon. Empty bar, background respiration; filled bar, oxygen removal rate in the presence of phenol as the substrate. Data are the average of three samples (error bars indicate 1 standard deviation).

Figure 4; hence, TOM activity in the effluent cells was indicative of TOM activity in the biofilm. This phenomenon of enzyme inactivation (TOM) was also observed by Ensley and Kurisko (1994) using G4 for TCE degradation in a bioreactor (even with continuous phenol induction). At low TCE concentrations (0.04 mg/L) TOM activity still decreased with continuous TCE addition, although significant TOM activity was measurable 14 days after TCE addition (Fig. 5A). Thus, at lower TCE concentrations the fixed-film bioreactor can be operated for extended periods. Furthermore, with the bioreactor packed with activated carbon and evaluated under the same conditions (air flow rate of 0.1 L/min, TCE concentration of 0.242 mg/L, and 0.7 L of reactor volume), the phenol oxidation activity of TOM in $PR1_{23}$ effluent cells was sustained much longer and was much more robust (cf. Fig. 5B,D), even though the average TCE mineralization rates of the bioreactors with activated carbon were lower than the bioreactor with SIRAN™ carriers (10 ± 6 mg/L of reactor volume/

day for activated carbon and 21 ± 15 mg/L of reactor volume/day for SIRAN™ carriers).

To further enhance the long-term performance of the bioreactor, a period of nutrient starvation (1–3 days) resulted in significant improvement in TCE mineralization. With 1 day of nutrient starvation in the presence of TCE, the TCE mineralization increased from 3.2 mg TCE/day to 5.5 mg/day for 2 days (Fig. 6). Using the oxygen probe, TOM activity of effluent cells before nutrient starvation ranged from 17% below to 29% above the background respiration rate; but, after 1-day of nutrient starvation, the oxygen removal rate of the effluent cells increased to 123% above the background respiration rates corroborating the increase in TCE mineralization. Under these same conditions, the negative control G4 mineralized only 0.8 mg TCE/day with no increase in oxygen removal rate. Repeated attempts to cause nutrient starvation (turning off the nutrient pump) to restore TCE mineralization performance were successful (TCE mineralization increased from 1.4 to 5.5 mg/
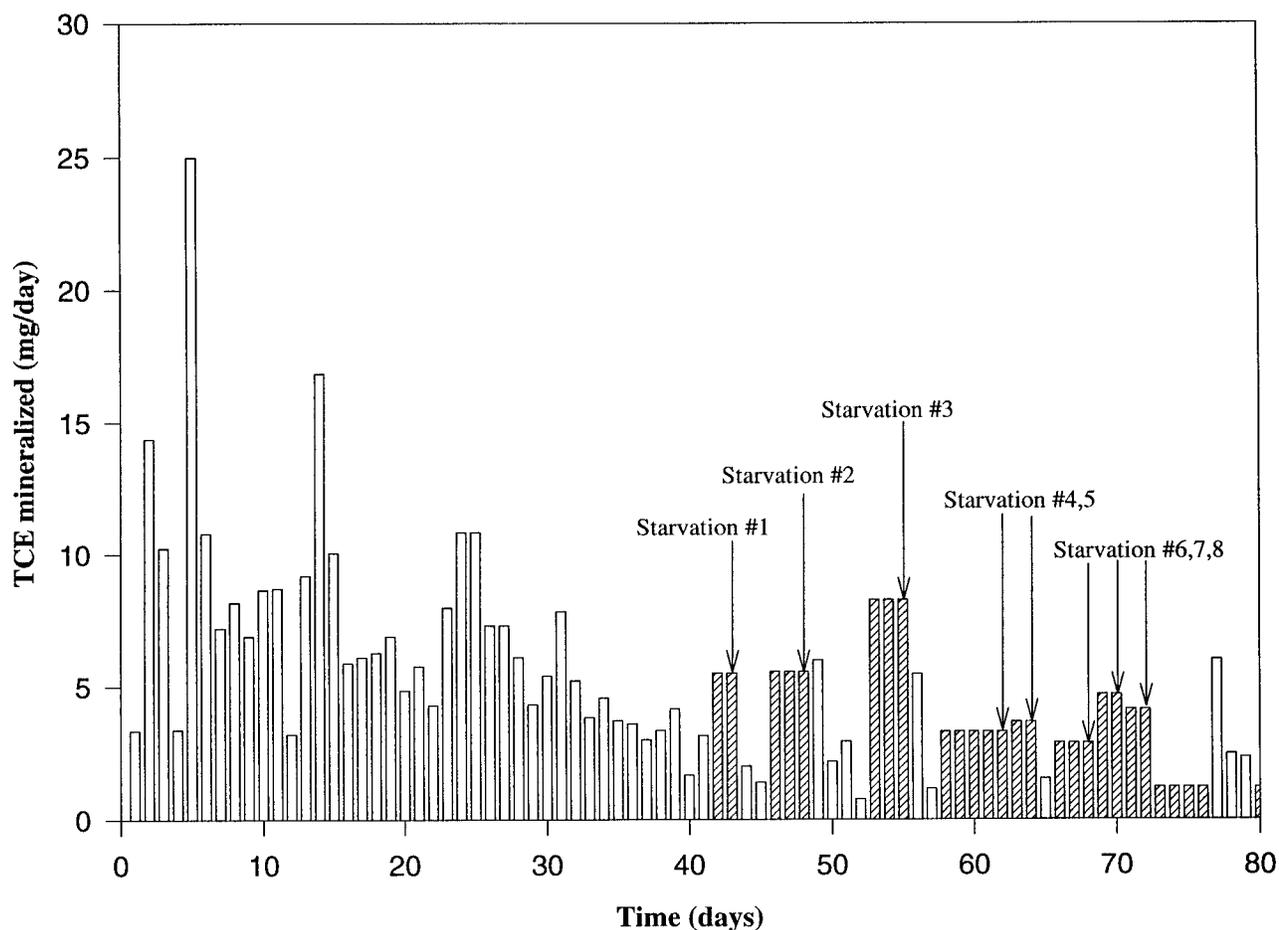
Figure 6. Long-term TCE mineralization in the *B. cepacia* $PR1_{23}$ bioreactor with activated carbon. Empty bar, TCE mineralization without nutrient starvation; filled bar, TCE mineralization with nutrient starvation. Air flow rate of 0.1 L/min, TCE concentration of 0.242 mg/L, and a reactor volume of 0.7 L were used.

day for 3 days and 0.8 to 8.3 mg/day for 3 days); however, the improved mineralization performance could not be sustained (TOM activity decreases when monitored up to 100 days, data not shown). The mechanism for this behavior is not yet well understood; however, a study conducted by McCann et al. (1991) showed that *E. coli* under carbon starvation can induce a set of genes that can enhance resistance to a variety of stresses. Such a response might allow the bacteria to regain TOM activity; however, it is important to note that it is not clear whether the increase in TCE mineralization occurs during starvation or upon addition of fresh medium.

Toxic intermediates produced from TCE oxidation have been shown to inactivate cellular proteins in many microorganisms (Ensley and Kurisko, 1994; Fox et al., 1990; Hyman et al., 1995; Oldenhuis et al., 1991; Wackett and Householder, 1989), and these intermediates appear to have similar detrimental effects on $PR1_{23}$ as well. From the phenol oxidation activity measurement of effluent cells, TCE-exposed $PR1_{23}$ lost the ability to express active TOM; however, the TomA gene was present as shown by a colony lift assay (data not shown). Preliminary data from shake flasks experiments ruled out the possibility of changes in *tom* transcription because addition of TCE to $PR1_{23}$ reduced TOM activity but did not reduce transcription from the *tomAB* locus

as evidenced by sustained catechol 2,3 dioxygenase (TomB) activity (data not shown). Hence, $PR1_{23}$ lost the ability to make active TOM as a result of either a decrease in translation of Tom mRNA or increased protease activity due to the stress shock caused by TCE degradation. For long-term applications using $PR1_{23}$, TOM activity must be enhanced and the problems of enzyme inactivation must be overcome (e.g., by growth without TCE, by inducing another promoter, or regenerating by growth on phenol).

CONCLUSIONS

The reactor design in this research is the first to use a pure culture of $PR1_{23}$ immobilized on activated carbon or sintered glass for degrading and mineralizing TCE in the gas phase. From the results of TCE breakthrough curves and chloride ion generation, this fixed-film bioreactor design is clearly capable of degrading and mineralizing TCE continuously. To avoid the impact of carbon adsorption of TCE, TCE degradation rates were determined with a chemically inert material. Using the SIRAN™ carriers, the initial maximum TCE degradation rate of 0.007–0.715 nmol/(min · mg protein) was obtained at TCE concentrations ranging from 0.04 to 2.42 mg/L of TCE (which corres-

Table II. Performance comparison of bioreactors designed for TCE remediation.

Strain	Carbon source	[TCE] (μM)	Duration (day)	TCE deg. rate (mg TCE/L of reactor/day)	Reference
B. cepacia G4 $PR1_{23}$ (fixed-film)	Glucose	0.3–18.3 (gas)	1–22 4–40	8.6–392.3[a] 7–10[b]	This study
B. cepacia G4 $PR1_{23}$ (fixed-film)	Yeast extract, peptone, glucose	1.0–10.0 (gas)	4	0.7–2.1	Shields et al. (1994)
B. cepacia G4 (suspended)	Phenol	10–327 (liquid)	~14	28–78	Folsom and Chapman (1991)
B. cepacia G4 (suspended)	Phenol	2.3–30.4 (gas)	~14	10–156	Ensley and Kurisko (1994)
B. cepacia G4 (suspended)	Toluene	1.9–87.7 (gas)	—	22–126	Landa et al. (1994)
P. putida F1 and mixed culture	Phenol	~1.2 (liquid)	<1	1.7–3.0	Coyle et al. (1993)
Alcaligenes eutrophus JMP134	Phenol	~40 (liquid)	7–14	6.7–9.5	McKay et al. (1994)
Methanotrophs (fixed-film)	Methane	3.5–320.4 (liquid)	7–21	2.8–90.7	Fennell et al. (1993)
Methanotrophs (fixed-film)	Methane	6.8–8.4 (liquid)	—	2.4–13.8	Strandberg et al. (1989)
Methane/propane oxidizers (packed)	Methane, propane	3.4–13.2 (liquid)	—	0.2–0.9	Lackey et al. (1993)
M. trichosporium OB3b (PP358)	Methane	1.5–7.6 (liquid)	18	0.2–3.0	Tschantz et al. (1996)
Methanotrophs (suspended)	Methane	114–222 (liquid)	~3	33.8–41.4 (20 mM formate)	McFarland et al. (1992)
Methanotrophs	Methane	0.1–38.8 (liquid)	170	0.5–15.4	Strand et al. (1991)

[a]Maximum initial TCE degradation rate by the fixed-film bireactor with SIRAN carriers.
[b]Average TCE mineralization rate by the fixed-film bioreactor with activated carbon.

ponds to degradation rates of 8.6–392.3 mg TCE/L of reactor volume/day).

Compared to similar TCE bioreactors using pure cultures (Table II), the fixed-film bioreactor with a $PR1_{23}$ biofilm had higher TCE degradation rates (two- to 200-fold) that were sustained longer (two- to fivefold) (Ensley and Kurisko, 1994; Folsom and Chapman, 1991; Landa et al, 1994; Shields et al., 1994). The $PR1_{23}$ biofilter also did not require a toxic inducer; however, TOM activity decreased with continuous exposure to TCE. Based on the TCE degradation (breakthrough curves) and mineralization (chloride ion generation) achieved using the fixed-film bioreactor, the feasibility of this bioreactor design was demonstrated. However, the TCE degradation activity of $PR1_{23}$ declined rapidly in the presence of TCE as corroborated by the inactivation of phenol oxidation by TOM. For a long-term application using $PR1_{23}$, TOM activity must be enhanced and the problems of enzyme inactivation must be overcome. Preliminary results indicate nutrient starvation may help sustain TOM activity.

References

Alvarez–Cohen, L., McCarty, P. L. 1991a. A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity. Environ. Sci. Technol. **25**: 1381–1387.

Alvarez–Cohen, L., McCarty, P. L. 1991b. Two-stage dispersed-growth treatment of halogenated aliphatic compounds by cometabolism. Environ. Sci. Technol. **25**: 1387–1393.

Bailey, J. E., Ollis, D. F. 1986. Biochemical engineering fundamentals. McGraw–Hill, New York.

Brusseau, G. A., Tsien, H.-C., Hanson, R. S., Wackett, L. P. 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation **1**: 19–29.

Chang, H.-L., Alvarez–Cohen, L. 1995. Model for the cometabolic biodegradation of chlorinated organics. Environ. Sci. Technol. **29**: 2357–2367.

Chu, K.-H., Alvarez–Cohen, L. 1996. Trichloroethylene degradation by methane-oxidizing cultures growth with various nitrogen sources. Water Environ. Res. **68**: 76–82.

Coyle, C. G., Parkin, G. F., Gibson, D. T. 1993. Aerobic, phenol-induced TCE degradation in completely mixed, continuous-culture reactors. Biodegradation **4**: 59–69.

Eng, W., Palumbo, A. V., Sriharan, S. Strandberg, G. W. 1991. Methanol suppression of trichloroethylene degradation by *Methylosinus trichosporium* (OB3b) and methane-oxidizing cultures. Appl. Biochem. Biotechnol. **28/29**: 887–899.

Ensley, B. D., Kurisko, P. R. 1994. A gas lift bioreactor for vapor-phase destruction of chlorinated organics. Appl. Environ. Microbiol. **60**: 285–290.

Fathepure, B. Z., Vogel, T. M. 1991. Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Appl. Environ. Microbiol. **57**: 3418–3422.

Fennell, D. E., Nelson, Y. M., Underhill, S. E., White, T. E., Jewell, W. J. 1993. TCE degradation in a methanotrophic attached-film bioreactor. Biotechnol. Bioeng. **42**: 859–872.

Folsom, B. R., Chapman, P. J. 1991. Performance characterization of model bioreactor for the biodegradation of trichloroethylene by *Pseudomonas cepacia* G4. Appl. Environ. Microbiol. **57**: 1602–1608.

Fox, B. G., Borneman, J. G., Wackett, L. P., Lipscomb, J. D. 1990, Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: Mechanistic and environmental implications. Biochemistry **29**: 6419–6427.

Hardman, D. J. 1991. Biotransformation of halogenated compounds. Crit. Rev. Biotechnol. **11**: 1–40.

Hecht, V., Brebbermann, D., Bremer, P., Deckwer, W.-D. 1995. Cometabolic degradation of trichloroethylene in a bubble column bioscrubber. Biotechnol. Bioeng. **47**: 461–469.

Hyman, M., Russell, S. A., Ely, R. L., Williamson, K. J., Arp, D. J. 1995. Inhibition, inactivation, and recovery of ammonia-oxidizing activity in cometabolism of trichloroethylene by *Nitrosomonas europaea.* Appl. Environ. Microbiol. **61**: 1480–1487.

Jahng, D., Wood, T. K. 1994. Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **60**: 2473–2482.

King, R. B., Long, G. M., Sheldon, J. K. 1992. Practical environmental bioremediation. Lewis Publishers, Boca Raton, FL.

Lackey, L. W., Phelps, T. J., Bienkowski, P. R., White, D. C. 1993. Biodegradation of chlorinated aliphatic hydrocarbon mixtures in a single-pass packed-bed reactor. Appl. Biochem. Biotechnol. **39**: 701–713.

Landa, A. S., Sipkema, M. E., Weijma, J., Beenackers, A. A. C. M., Dolfing, J., Janssen, D. B. 1994. Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl. Environ. Microbiol. **60**: 3368–3374.

Luu, P. P., Yung, C. W., Sun, A. K., Wood, T. K. 1995. Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1. Appl. Microbiol. Biotechnol. **44**: 259–264.

Mars, A. E., Houwing, J., Dolfing, J., Janssen, D. B. 1996. Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Appl. Environ. Microbiol. **62**: 886–891.

McCann, M. P., Kidwell, J. P., Matin, A. 1991. The putative σ factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli.* J. Bacteriol. **173**: 4188–4194.

McFarland, M. J., Vogel, C. M., Spain, J. C. 1992. Methanotrophic cometabolism of trichloroethylene (TCE) in a two stage bioreactor system. Water Res. **26**: 259–265.

McKay, D. J., Morse, J. S., Hazen, T. C. 1994. Biodegradation of trichloroethylene by *Alcaligenes eutrophus* JMP134 in a laboratory scale bioreactor. Hazardous Waste Hazardous Mater. **11**: 491–499.

Mol, N., Kut, O. M., Dunn, I. J. 1993. Adsorption of toxic shocks on carriers in anaerobic biofilm fluidized bed reactors. Water Sci. Technol. **28**(2): 55–65.

Nelson, M., Montgomery, S., Mahaffey, W., Pritchard, P H. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**: 949–954.

Nelson, M. J. K., Montgomery, S. O., O'Neil, E. J., Pritchard, P. H. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. **52**: 383–384.

Nyer, E. K., Morello, B. 1993. Trichloroethylene treatment and remediation. Ground Water Monit. Remed. **13**: 98–103.

Oldenhuis, R., Oedzes, J. Y., Waarde, J. J. V. D., Janssen, D. B. 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. **57**: 7–14.

Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B., Witholt, B. 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55**: 2819–2826.

Park, S., Hanna, M. L., Taylor, R. T., Droege, M. W. 1991. Batch cultivation of *Methylosinus trichosporium* OB3b. I: Production of soluble methane monooxygenase. Biotechnol. Bioeng. **38**: 423–433.

Phelps, T. J., Niedzielski, J. J., Schram, R. M., Herbes, S. E., White, D. C. 1990. Biodegradation of trichloroethylene in continuous-recycle expanded-bed bioreactors. Appl. Environ. Microbiol. **56**: 1702–1709.

Rodriguez, R. L., Tait, R. C. 1983. Recombinant DNA techniques: An introduction. Benjamin/Cummings Publishing, Menlo Park, CA.

Shah, N. N., Hanna, L. M., Jackson, K. J., Taylor, R. T. 1995. Batch cultivation of *Methylosinus trichosporium* OB3b. IV. Production of hydrogen-driven soluble or particulate methane monooxygenase activity. Biotechnol. Bioeng. **45**: 229–238.

Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskey, S. M., Pritchard, P. H. 1989. Novel pathway of toluene catabolism in trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. **55**: 1624–1629.

Shields, M. S., Montgomery, S. O., Cuskey, S. M., Chapman, P. J., Pritchard, P. H. 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. **57**: 1935–1941.

Shields, M. S., Reagin, M. J. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58**: 3977–3983.

Shields, M. S., Reagin, M. J., Gerger, R. R., Campbell, R., Somerville, C. 1995. TOM, a new aromatic degradative plasmid from *Burkholderia* (*Pseudomonas*) *cepacia* G4. Appl. Environ. Microbiol. **61**: 1352–1356.

Shields, M. S., Reagin, M. J., Gerger, R. R., Somerville, C., Schaubhut, R., Campbell, R., Hu–Primmer, J. 1994. Constitutive degradation of trichloroethylene by an altered bacterium in a gas-phase bioreactor, pp. 50–65. In: R. E. Hinchee, A. Leeson, L. Semprini, and S. K. Ong, (eds.), Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Lewis Publishers, Boca Raton, FL.

Strand, S. E., Wodrich, J. V., Stensel, D. H. 1991. Biodegradation of chlorinated solvents in a sparged, methanotrophic biofilm reactor. Res. J. WPCF. **63**: 859–866.

Strandberg, G. W., Donaldson, T. L., Farr, L. L. 1989. Degradation of trichloroethylene and *trans*-1,2-dichloroethylene by a methanotropic consortium in a fixed-film, packed-bed bioreactor. Environ. Sci. Technol. **23**: 1422–1425.

Sun, A. K., Wood, T. K. 1996. Evaluation of trichloroethylene degradation and mineralization by pseudomonads and *Methylosinus trichosporium* OB3b, Appl. Microbiol. Biotechnol. **45**: 248–256.

Tschantz, M. F., Bowman, J. P., Evans, F., Bienkowski, P. R., Donaldson, T. L., Sayler, G. S. 1996. Development of a fully suspended, multi-stage bioreactor system for trichloroethylene degradation based on soluble monooxygenase cometabolism. J. Environ. Sci. Health Part A Environ. Sci. Eng. Toxic Hazard. Subst. Control **31**: 249–266.

Tsien, H. C., Brusseau, G. A., Hanson, R. S., Wackett, L. P. 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55**: 3155–3161.

Ulonska, A., Deckwer, W.-D., Hecht, V. 1995. Degradation of quinoline by immobilized *Comamonas acidovorans* in a three-phase airlift reactor. Biotechnol. Bioeng. **46**: 80–87.

Wackett, L. P., Gibson, D. T. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ.Microbiol. **54**: 1703–1708.

Wackett, L. P., Householder, S. R. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenae. Appl. Environ. Microbiol. **55**: 2723–2725.

Wilcox, D. W., Autenrieth, R. L., Bonner, J. S. 1995. Propane-induced biodegradation of vapor phase trichloroethylene. Biotechnol. Bioeng. **46**: 333–342.

Zylstra, G. J., Wackett, L. P., Gibson, D. T. 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55**: 3162–3166.