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Secondary Substrate Utilization of Methylene Chloride by an Isolated Strain of *Pseudomonas* sp.

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Secondary substrate utilization of methylene chloride was analyzed by using *Pseudomonas* sp. strain LP. Both batch and continuously fed reactors demonstrated that this strain was capable of simultaneously consuming two substrates at different concentrations: the primary substrate at the higher concentration (milligrams per liter) and the secondary substrate at the lower concentration (micrograms per liter). The rate of methylene chloride utilization at trace concentrations was greater in the presence of the primary substrate, acetate, than without it. However, when the substrate roles were changed, the acetate secondary substrate utilization rate was less when methylene chloride was present. Thus, substrate interactions are important in the kinetics of secondary substrate utilization. *Pseudomonas* sp. strain LP showed a preference toward degrading methylene chloride over acetate, whether it was the primary or secondary substrate, providing it was below an inhibitory concentration of ca. 10 mg/liter.

Methylene chloride (dichloromethane) is designated as a priority pollutant by the U.S. Environmental Protection Agency (20). It is widely used for cleaning, fumigation, refrigeration, paint removal, analytical applications, and as an aerosol propellant (23). Treated municipal wastewaters have been recorded to contain as much as 24 μg of methylene chloride per liter (14). Due to the versatility of methylene chloride and its high solubility in water (20 g/liter at 20°C), it is found in many aqueous environments (23).

Methylene chloride has been suspected of being carcinogenic because of positive responses in the Ames test (9). Stewart et al. (21) reported elevated carboxyhemoglobin levels in humans exposed to dihalomethane vapors.

The ability of microorganisms to degrade xenobiotic organic compounds when present at low concentrations typical of those in the environment is open to question. Organism growth rate is a function of substrate concentration. At sufficiently low concentrations, the organism growth rate may be exceeded by the organism decay rate so that no net growth can occur (1, 3, 11). The concentration at which growth is just balanced by decay is termed the minimum substrate concentration (S_{min}) (17, 18). It has been postulated (12, 19) that bacteria can, however, consume a xenobiotic compound at low concentrations if the population is supported through energy obtained from another substrate that is present in concentrations above its S_{min} level. The compound at high concentration is termed the primary substrate, and that at low concentration is termed the secondary substrate (12, 19). A corollary concept is that both the primary and secondary substrates can be used simultaneously. The purpose of this study was to evaluate this concept with a pure culture that is capable of using either methylene chloride or acetate as primary substrates for growth.

Although methylene chloride is ubiquitous in the environment, little is known about its biodegradation at trace concentrations. Rittmann and McCarty (16) evaluated secondary substrate utilization with methylene chloride. However, their study employed mixed cultures; therefore, the possibility exists that one species was responsible for utilizing the primary substrate and another was responsible for the simultaneous degradation of the secondary substrate that occurred. Confirmation of this finding with a pure culture was deemed desirable. Use of methylene chloride as one of the substrates was thought to be acceptable for this evaluation since both Stucki et al. (22) and Brunner et al. (4) isolated bacteria that were capable of utilizing methylene chloride at high concentrations (>10 mg/liter).

MATERIALS AND METHODS

Chemicals and radioisotopes. Reagent-grade methylene chloride and sodium [2-^{14}C]acetate (4.8 mCi/mM; ICN Chemical and Radioisotope Div., Irvine Calif.) were used in this study.

Isolation of methylene chloride-utilizing bacterium. A bacterium that utilizes methylene chloride was isolated from primary municipal wastewater from the Palo Alto, Calif., Water Quality Control Plant. Wastewater (10 ml) was placed in each of a series of 250-ml Erlenmeyer flasks with screw caps containing 90 ml of sterile mineral salts medium 1 plus 500 mg of methylene chloride per liter. Mineral salts medium 1 contained (milligrams per liter): KH_2PO_4, 500; $(NH_4)_2HPO_4$, 920; $CaOH_2$, 10; NaCl, 300; and $MgSO_4 \cdot 7H_2O$, 300. It also contained 10 ml of a trace metal solution of the following composition (milligrams per liter): $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot 5H_2O$, 20; $CuSO_4$, 40; $ZnSO_4 \cdot 7H_2O$, 20; H_3BO_3, 3; $CoCl_2$, 4; and $Na_2MoO_4 \cdot 2H_2O$, 4. The pH of the medium was then adjusted to 7.0.

After initial growth on the above medium, the bacteria were transferred and fed methylene chloride at lower concentrations (10 to 100 μg/liter); henceforth; the mineral salt concentrations (medium 2) were (milligrams per liter); KH_2PO_4, 8.5; K_2HPO_4, 28.5; Na_2HPO_4, 33.4; NH_4Cl, 1.7; $MgSO_4 \cdot 7H_2O$, 46; and $CaCl_2$, 27; it also contained 10 ml of trace metal solution.

The bacteria were fed daily and mixed on a shaker at about

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TABLE 1. Comparison of *Pseudomonas* sp. strain LP and strain DM1[a]

Property	LP				DM1			
	Growth[b]	Observed after h	Pigment formation[c]	Description	Growth[b]	Observed after h	Pigment formation[c]	Description
Substrate (0.5%; incubation temp, 30°C)								
Acetate	+	24	++		+	168	−	
Lactate	+	24	++		+	168	−	
Succinate	+	24	++		+	168	−	
Pyruvate	+	24	−		+	168	−	
Glucose	+	24	−		−	168	−	
L-Alanine	+	24	++		−	168	−	
Ethanol	+	24	−		+	168	−	
Methanol	+	24	±[d]		+	168	−	
Methylamine	+	168	−		+	168	−	
Dimethylamine	−	24	−		−	168	−	
	+	168	−					
Trimethylamine	−	24			+	168	−	
	+	280	[d]					
Dichloromethane	+	168	−		+	168	−	
Methane	−	168	−		−	168	−	
L-Arginine	+	144	+		−	144	−	
Denitrification								
On acetate	+	144			−			
On methylamine	−				−			
Growth at 41°C								
On acetate	+	120			−			
On methylamine	−				−			
Formation of β-hydroxybutyric acid	+	144			−			
Motility				Yes				Yes
Capsule or slime layer				No				Yes
Gram reaction				Negative				Negative
Length (μm)				0.7–1				1–1.4
Diameter (μm)				0.5				0.5–0.7

[a] From Brunner et al. (4).
[b] +, Easy visible growth; −, no growth.
[c] Diffusible green fluorescent pigment. Types of formation: ++, strong; +, normal and well recognizable; ±, questionable; −, none.
[d] Cells slightly rose in color.

175 rpm at 23°C in the dark. Each week, 1 ml of the liquid culture was transferred to a second flask containing 100 ml of fresh medium 2. After eight transfers, serial dilutions were plated onto Difco Bacto-Agar plates (10) containing 10 mg of methanol per liter as substrate (W. B. Brunner, Ph.D. dissertation, Eidgenössische Technische Hochschule, Zurich, Switzerland, 1981) and incubated for at least 36 h at 35°C. Colonies were isolated from the plates and streaked onto fresh sterile agar plates. Isolated colonies were transferred to the liquid medium, and the process was repeated to ensure pure culture isolation.

Analytical techniques. (i) Methylene chloride. Methylene chloride concentration was determined by solvent extraction and gas chromatography (7, 13). With concentrations greater than 100 μg/liter, a 1-ml sample was injected into a 0.5-dram (ca. 1.85-ml) vial; 0.1 ml of octane was added, and the vial was capped and shaken vigorously for 1 min (15). After phase separation, 5 μl of the octane phase was injected into a column (height, 2 m; inside diameter, 4 mm) packed with 10% squalane on Chromosorb W/AW (80-100 mesh) at 67°C. Argon-methane was used as carrier gas, and a linearized ^{63}Ni detector (Tracor Instruments, Austin, Tex.) with a Sigma 10 reporting integrator (The Perkin-Elmer Corp., Norwalk, Conn.) was used for detection and quantification. With concentrations of less than 10 μg/liter, pentane extraction was employed (7). Pentane (1 ml) was directly injected into the 60-ml vial and vigorously shaken for 15 min, and 5 μl of the pentane phase was used for gas chromatography analysis.

(ii) Acetate measurement. Acetate concentration was measured with sodium [2-^{14}C]acetate (2). Samples were filtered through a 0.45-μm membrane filter. The filtrate (1 ml) was added to a glass scintillation vial acidified with ca. 0.1 ml of concentrated HCl and subsequently bubbled with nitrogen for 5 min to remove CO_2. ACS scintillation fluid (10 ml; Amersham Corp., Arlington Heights, Ill.) was added, and the sample was counted in a Packard Tri-Carb model 3330 liquid scintillation spectrometer (Parkard Instrument Co., Inc., Downers Grove, Ill.). Quench corrections were made by the channel ratio method (2). Since the counting variation was less than 1%, the analytical standard deviation was determined only by the sampling error. When a 1-ml syringe was used, it was ca. ±5%.

(iii) Bacterial counts. The epifluorescent method described

by Hobbie et al. (8) was employed for direct counting of bacteria.

Batch and immobilized cell studies. Initial studies with the isolated bacterium were conducted, using batch-feed conditions. Subsequently, a study was conducted with a continuously fed column containing immobilized cells on glass beads. Details of procedures used for each are described below.

RESULTS

Characterization of *Pseudomonas* sp. strain LP. A comparison between *Pseudomonas* sp. strain LP and *Pseudomonas* sp. strain DM1 (4), which can also use methylene chloride, is given in Table 1. The physiology and biochemistry of strain LP are clearly different from strain DM1. The ability of LP to grow aerobically on acetate and its being mobile and gram negative strongly suggest that this organism belongs to the genus *Pseudomonas* (6). Accumulation of poly-β-hydroxybutyrate, the use of arginine as sole carbon source, and growth at 41°C (Table 2) indicate that *Pseudomonas* sp. strain LP belongs to section II. Its reactions to various tests compare best with no. 14 *Pseudomonas caryophylli* (6).

Batch studies. To test the ability of this organism to simultaneously utilize both substrates, a series of batch experiments was performed. Sixty-milliliter hypovials (Pierce Chemical Co., Rockford, Ill.) with Teflon-sealed caps containing three sterile 3-mm glass beads (to facilitate mixing) were completely filled with mineral salts medium 2 containing various concentrations of acetate and methylene chloride. The initial dissolved oxygen concentration was 8 to 9 mg/liter and was sufficient to maintain aerobic conditions throughout the incubation period. The hypovials were, except for the controls, inoculated equally with bacteria from a liquid culture and incubated at 23°C on a reciprocating shaker at 175 rpm. The controls served to monitor a possible evasion of methylene chloride through the Teflon seals. With this experimental setup, no loss of methylene chloride was observed. Increasing acetate concentrations up to 1 mg/liter were stimulatory for methylene chloride removal (Fig. 1). *Pseudomonas* sp. strain LP was able to utilize methylene chloride and acetate simultaneously, even at initial methylene chloride concentrations as low as 10 μg/liter (Fig. 2). Up to 1 mg of methylene chloride per liter was used preferentially by the organism (Fig. 3). However, at higher concentra-

TABLE 2. Comparison of *Pseudomonas* sp. strain LP with *Pseudomonas* strains in Bergey's manual[a]

Test	*P. caryophylli*	LP
Diffusible pigments	±[b]	+
Denitrification	+	+
Gelatin liquefaction	−	−
Growth on		
Glucose	+	+
Xylose	+	+
D-Ribose	+	+
Arabinose	+	+
Fucose	+	−
L-Rhamnose	+	+
Cellobiose	+	+
Maltose	−	−
Saccharate[c]	+	+
Levulinate	−	+
D(−)Tartrate[d]	−	+
Mesotartrate[d]	+	
Erythrital	−	+
m-Hydroxybenzoate	−	+
p-Hydroxybenzoate	+	+
Glycollate	+	+
Malonic acid	−	+
Succinic acid	−	+
Glutaric acid	−	+
D-Alamine	−	NT[e]
L-Alamine	NI[f]	+
Acetamide	−	−

[a] Reference 6.
[b] +, Positive reaction or growth on corresponding substrate; −, no reaction or growth; ±, reaction or growth uncertain.
[c] Saccharose.
[d] Tartrate.
[e] NT, Not tested.
[f] NI, Not indicated.

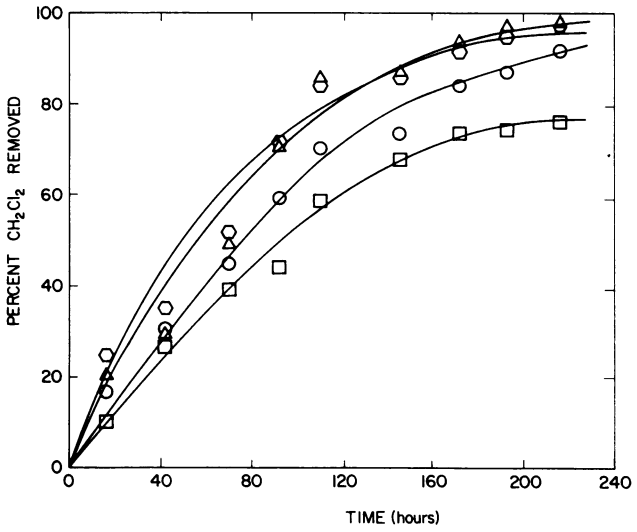

FIG. 1. Effect of various initial acetate concentrations on the removal of methylene chloride. Concentrations consisted of 100 μg of methylene chloride per liter alone (□) or plus 0.5 (○), 1.0 (◌), or 5 (△) mg of acetate per liter.

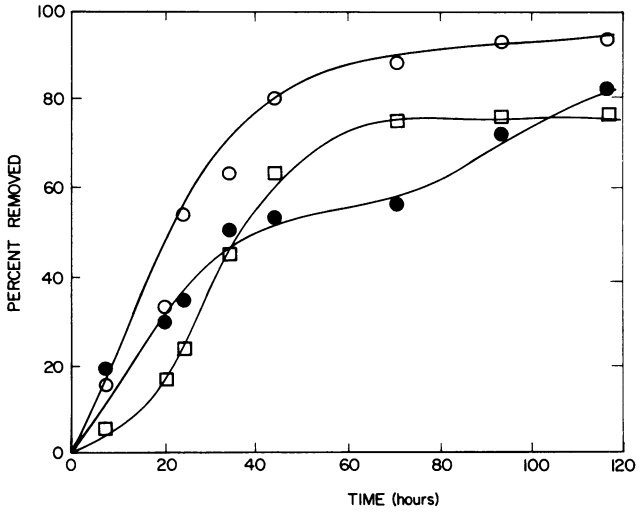

FIG. 2. Simultaneous removal of 1-mg/liter acetate (●) and 10-μg/liter methylene chloride (○) compared with removal of 10-μg/liter methylene chloride alone (□).

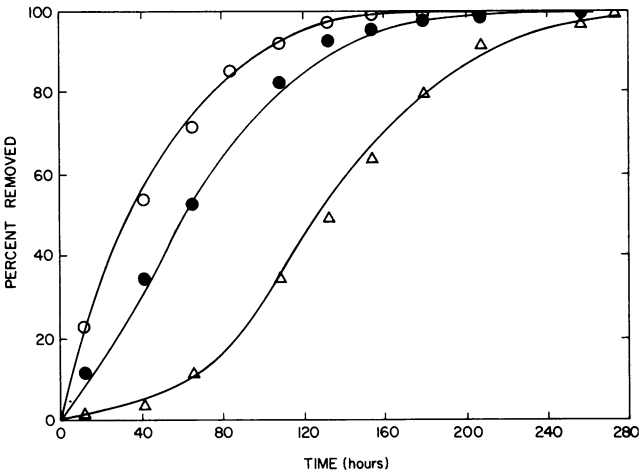

FIG. 3. Simultaneous removal of 1-mg/liter acetate (△) and 1-mg/liter methylene chloride (○) compared with removal of 1-mg/liter methylene chloride alone (●).

tions (up to 5 mg of methylene chloride per liter), a preference of one substrate over another was found to be less clear.

When methylene chloride became the primary substrate and acetate became the secondary substrate, the acetate utilization rate was less than that of methylene chloride (Fig. 4). Also, acetate utilization was greater in the absence of methylene chloride. The apparent behavior of the organism toward acetate as secondary substrate was opposite to that found when methylene chloride served as the secondary substrate. *Pseudomonas* sp. strain LP showed a preference toward degrading methylene chloride over acetate when both substrates were present.

Figure 5 shows the increase in bacterial numbers as a function of initial methylene chloride concentrations of 20, 50, and 100 μg/liter in batch cultures. Degradation continued until there was less than 2 μg of methylene chloride per liter remaining. However, the number of bacteria increased only as long as the methylene chloride concentration was above 9 μg/liter, the aparent S_{min} for *Pseudomonas* sp. strain LP.

A similar study was conducted on the increase in bacterial numbers as a function of initial acetate concentrations of 0.20, 0.35, 0.60, 1.0, and 10.0 mg/liter in batch cultures. *Pseudomonas* sp. strain LP continued to degrade acetate until less than ca. 0.1 mg/liter remained. However, the number of bacteria increased only as long as the acetate concentration was above 0.1 mg/liter, the apparent acetate S_{min} for strain LP.

Immobilized cell (biofilm) studies. To test the hypothesis (12, 19) that the presence of a primary substrate (acetate) facilitated the breakdown of methylene chloride at trace concentration, two continuously fed biofilm reactors were established (Fig. 6). Serving as reactors were 25-ml Pyrex glass syringes containing 3-mm glass beads at 23°C. The reactor syringes, the 100-ml glass influent-containing syringes of the syringe pump (Beckman Instruments Altex, Berkeley, Calif.), the 3-mm glass beads, and all Teflon tubing were sterilized by autoclaving. Each time new medium was added, both the 100-ml syringes and Teflon tubing were sterilized by autoclaving. The porosity by water displacement was 33%.

Each flow rate reported was maintained for at least 1 week after biofilm development. Three reservoir volumes were allowed to pass through the effluent reservoir before sampling.

The reactors were inoculated with *Pseudomonas* sp. strain LP and fed continuously with mineral salts medium 2 and 100 μg of methylene chloride per liter at a flow rate that resulted in a hydraulic detention time (θ = pore volume/flow rate) of 4.0 h at 23°C. After 1 month, reactor 1 then received 10 μg of methylene chloride per liter, and reactor 2 received

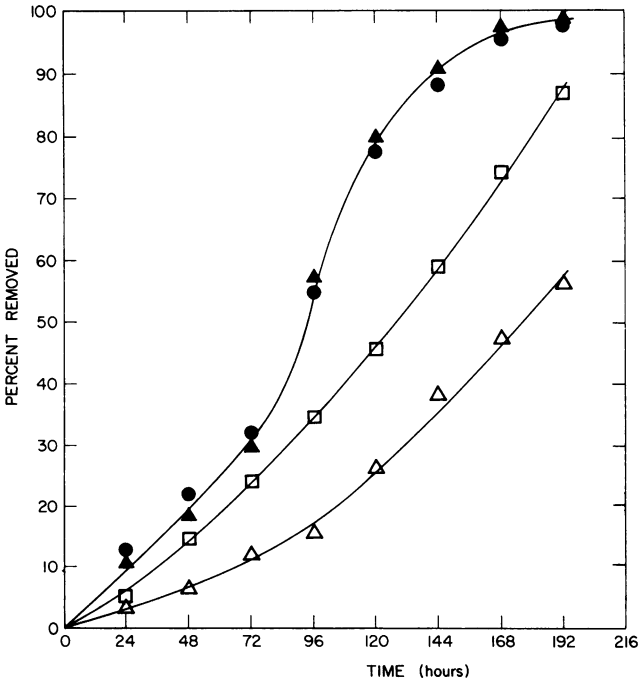

FIG. 4. Simultaneous removal of 25-μg/liter acetate (△) and 1-mg/liter methylene chloride (▲) compared with removal of 25-μg/liter acetate alone (□) and 1-mg/liter methylene chloride alone (●).

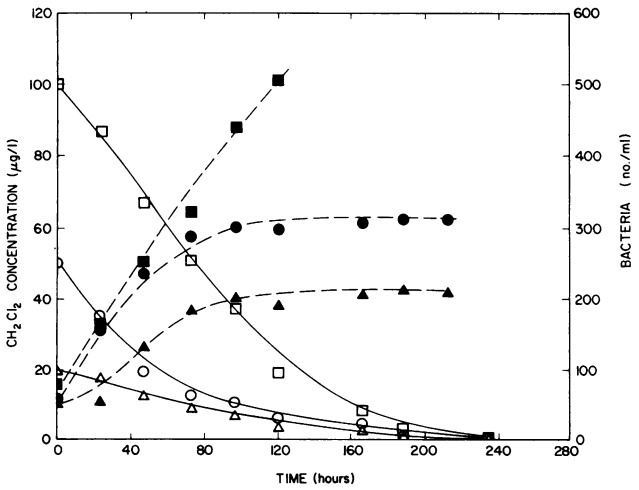

FIG. 5. Increase in bacterial numbers as functions of time and degradation of methylene chloride. Symbols: □, ○, △, initial concentrations of 100, 50, and 20 μg of methylene chloride per liter, respectively; ■, ●, ▲, bacteria in 100, 50, or 20 μg of methylene chloride per liter, respectively.

a mixture of methylene chloride (10 μg/liter) and acetate (1 mg/liter). After 3 weeks, steady state was reached, and the bacterial film was visible in the first 10 ml of the syringe. The biofilm was most dense at the inlet and became gradually more faint toward the outlet. With these columns, the removal of methylene chloride and acetate at various detention times was tested (case 1). The results are summarized in Fig. 7. The percentage of removal of methylene chloride was consistently higher in reactor 2 with acetate than in reactor 1 without acetate over all detention times (0.1 to 4.5 h). Similar to the batch experiments, methylene chloride was also found to be removed more rapidly than acetate.

To determine whether the more efficient removal of methylene chloride in reactor 2 was the result of the higher number of bacteria in this column, the feed solutions to the two reactors were switched, and the degradation of the two organic substrates was followed again at various detention times (case 2; Fig. 8). Methylene chloride consumption was still markedly faster in the presence of acetate, verifying that the presence of acetate stimulated methylene chloride utilization.

DISCUSSION

Pseudomonas sp. strain LP is a newly recognized strain that is capable of utilizing methylene chloride. It differs from *Pseudomonas* sp. strain DM1 (4), another methylene chloride user, in several ways. Strain LP can utilize several substrates not used by strain DM1 (L-alanine, glucose, and L-arginine). It can utilize acetate with denitrification, can grow on acetate at 41°C, and forms β-hydroxybutyric acid. Also, growth with acetate, lactate, and succinate was observed much sooner with strain LP (24 h) than with strain DM1 (168 h).

Strain LP was also demonstrated to simultaneously utilize methylene chloride and acetate. This has significance in practical problems such as contamination of groundwaters with xenobiotic compounds. Here, contaminant concentrations may be sufficiently high to cause concern for consumers of the water but may be too low to support the growth of a bacterial population capable of transforming the contaminant. However, with the potential for simultaneous utilization of substrates, an adequate bacterial population might be supported in growth by a natural primary substrate, such as acetate, present at higher concentration, making it possible for the organism to simultaneously transform the xenobiotic compound at trace concentration levels while serving as the secondary substrate.

The potential for trace contaminant transformation through secondary utilization was previously demonstrated by Rittmann and McCarty (16), but with mixed populations. In additional studies, Rittmann (B. E. Rittmann, Ph.D. thesis, Stanford University, Stanford, Calif., 1979) used methylene chloride as the primary substrate (25 mg/liter) and acetate as the secondary substrate (0.072 mg/liter). However, it was not clear from the results obtained whether a single organism or different organisms were using the two substrates. With mixed populations it is also difficult to demonstrate conclusively the effect of one substrate on the utilization rate of another.

The results obtained with *Pseudomonas* sp. strain LP

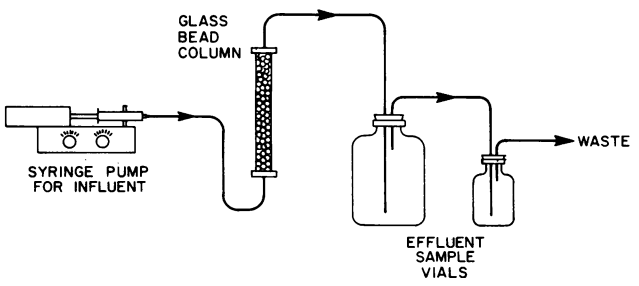

FIG. 6. Schematic diagram of continuous-flow feed system.

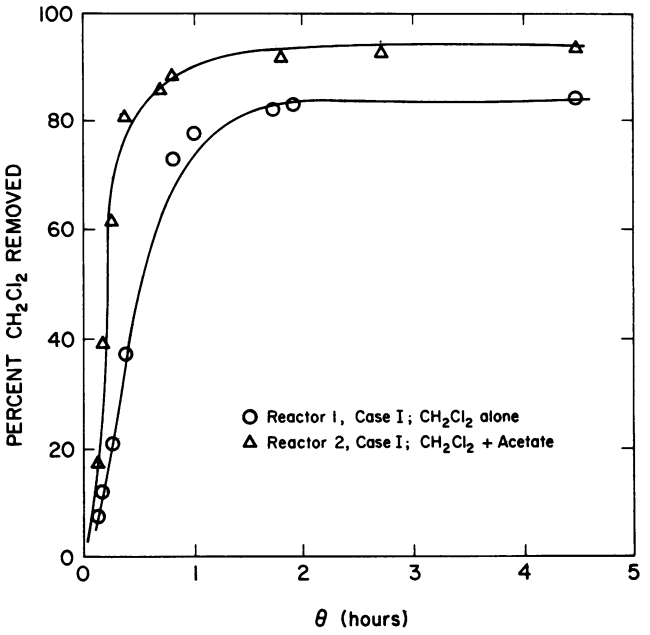

FIG. 7. Removal of methylene chloride by biofilm reactors with and without acetate (case 1).

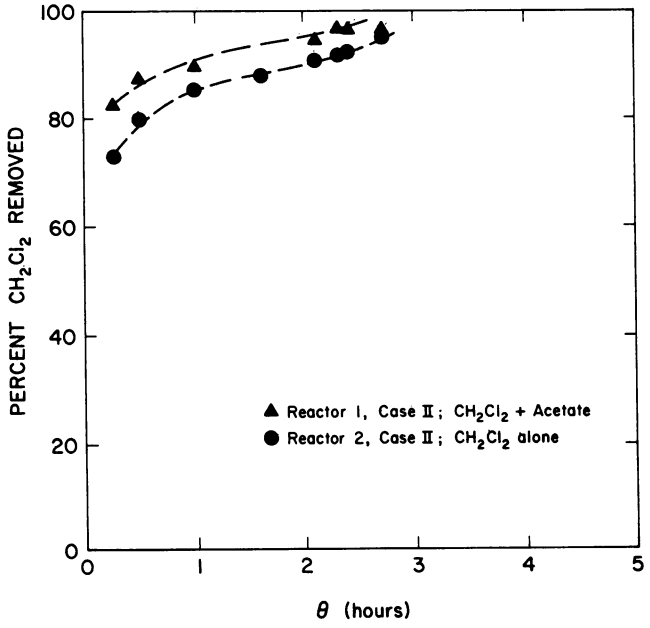

FIG. 8. Removal of methylene chloride by biofilm reactors with and without acetate after influent feeds have been switched (case 2).

demonstrate an interdependency between the rate of utilization of primary and secondary substrates. The relative rate of methylene chloride utilization was generally greater than that of acetate, regardless of which was the primary substrate. Also, the rate of methylene chloride utilization was enhanced in the presence of acetate, whereas the acetate utilization rate decreased when methylene chloride was present. Thus, *Pseudomonas* sp. strain LP demonstrated an unexpected preference for methylene chloride, a xenobiotic compound.

Others have demonstrated simultaneous utilization of substrates. Dijkhuizen and Harder (5) found that *Pseudomonas oxalaticus* could simultaneously utilize acetate and formate in continuous culture. However, acetate repressed the synthesis of ribulosebiphosphate carboxylase, forcing the organism to grow heterotrophically rather than autotrophically as it normally does with formate. The result was that the organism used acetate primarily for assimilation and energy production, resulting in higher growth yields than when grown on either substrate alone. (Brunner et al. [4] found that *Pseudomonas* sp. strain DM1 appears to utilize methylene chloride by dehalogenation to formaldehyde which is assimilated by the serine pathway.) If *Pseudomonas* sp. strain LP grew autotrophically on methylene chloride in a similar way, then the enhanced utilization rate in the presence of acetate might result from a change to heterotrophic growth. Further studies would be needed to evaluate this effect.

This study has demonstrated through batch- and continuous-feed column studies that a pure culture was capable of using a xenobiotic substrate in trace concentration in the presence of a common substrate in relatively high concentrations that served as the primary substrate for growth and maintenance. This finding has significant implications with respect to the fate of hazardous chemicals in the environment, especially with respect to contaminated groundwaters where processes for transforming compounds are limited. It helps to elucidate the environmental factors that affect the rate of biotransformation and may help to suggest methods to enhance biotransformation when this is thought to be desirable.

ACKNOWLEDGMENT

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