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EFFECTS OF TRANSFORMATION CAPACITY ON COMETABOLIC DEGRADATION OF TRICHLOROETHENE

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Abstract : The effects of transformation capacity on cometabolic degradation of trichloroethene (TCE) were evaluated using TCE-degrading actinomycetes pure and mixed culture under various culture conditions. The TCE transformation capacity of the actinomycetes enrichment culture in a batch test with phenol addition was 1.0 mg of TCE/mg of volatile suspended solids (VSS). The resting cell TCE transformation capacity of the actinomycetes pure culture cell was 0.75 mg TCE/mg VSS, which increased to 2.0 mg TCE/mg VSS when phenol was added as an external substrate. When the pure culture had an internal substrate in the form of poly-β-hydroxybutyrate (PHB) at 19% of the cell mass, the resting cell TCE transformation capacity increased from 0.47 to 0.6 mg TCE/mg VSS. The presence of PHB increased transformation capacity by 57%, whereas, the addition of phenol caused more than two fold increase in transformation capacity. The actinomycetes culture showed the highest transformation capacity.

Key Words : transformation capacity, actinomycetes, TCE

INTRODUCTION

Trichloroethene (TCE) is one of the most common contaminants found at hazardous waste sites in both saturated and vadose zones of the subsurface environment. Biological treatment of TCE has received much interest as a potential lower cost remediation alternative and has been investigated for in situ subsurface treatment, treatment of liquids from pump and treat operation, or treatment of contaminated gases from soil vapor extraction (SVE). Biological treatment of TCE can occur by either aerobic oxidation or anaerobic dechlorination. Anaerobic degradation of TCE to ethene has been demonstrated, but in some cases, these transformations may be incomplete and occasionally result in the accumulation of vinyl chloride, a carcinogenic metabolite.[1] The aerobic process may be preferable for above-ground operations treating SVE gases because of the oxygen content in the gas. Aerobic degradation of TCE is cometabolic, in which the bacteria are grown on another substrate which induces oxygenase or dioxygenase enzyme with broad substrate specificity that catalyze TCE oxidation.

There are numerous reports on TCE degradation studies using pure culture or mixed enrichments grown on methane,[2,3] toluene,[4,5] phenol,[6,7] ammonia,[8,9] propane,[10,11] butane,[12] 2-methyl-1,3-butadiene (isoprene)[13] and isopro-

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pylbenzene (cumene).[14] The focus of these studies included determining the ability to degrade TCE or other chlorinated compounds, TCE degradation kinetics, competitive inhibition between TCE and the growth substrate, and possible effects of metabolite toxicity.

TCE degradation efficiency in subsurface or reactor designs can be negatively impacted due to competitive inhibition between TCE and the growth substrate for the oxygenase enzyme and due to metabolite toxicity from TCE degradation. An example is the TCE reaction catalyzed by methane monooxygenase (MMO). MMO is known to catalyze formation of an epoxide ring from TCE which covalently binds with protein in MMO to inactivate the enzyme system.[15] The effect of metabolite toxicity has been described by a term called a transformation capacity (T_C), which is the maximum mass of TCE that can be degraded by a given amount of cells before complete cell inactivation occurs.[16]

Table 1 summarizes T_C values for TCE transformation reported in the literatures. Various T_C values have been obtained for different types of substrates, cultures and conditions of cell cultivation. Higher T_C values for methane-oxidizer were obtained with cultures grown under copper limited conditions, so that more soluble methane monoxygenase (sMMO) was expressed. Methane-oxidizers grown under nitrogen-limited conditions expressed a high T_C. This has been associated with nitrogenase activity and poly-β-hydroxybutyrate (PHB) production.[17,18]

The addition of energy sources generally enhances both the rate of TCE transformation and the T_C.[19] Higher T_C is also associated with external energy supplies (formate, for example) that provide reducing power to drive the cometabolic transformations. Most of this work has been performed with methane-oxidizers with formate addition. Formate is known as an energy substrate and provides only reducing power to methanotrophs. No substrate has been identified for phenol-degraders that serves only as an energy substrate as formate does for methanotrophs. Enhanced T_C values have been observed with phenol-oxidizing cultures when formate is added as growth substrate.[20]

Addition of growth substrate during TCE degradation may result in the repair of cells damaged by TCE transformation or growth of new cellular components. Walter *et al.*[21] showed cell recovery when methane was present during TCE degradation. In a complete-mix methanotrophic enrichment reactor with intermittent TCE feeding and continuous methane feeding, the culture was able to use methane to recover from the metabolite toxicity effect during the recovery period with only methane feeding.

As shown in the above, a wide range of values has been reported for different types of cometabolic substrates, microorganisms, and conditions of cell cultivation. The studies include tests with resting cells with and without energy

Table 1. Comparison of TCE transformation capacity values (T_C)

Growth substrate	Energy substrate, mM	Culture	Culture growth condition	T_C * without Energy, (mg/mg)	T_C with Energy(mg/mg)	Reference
Methane	Formate(20)	Mixed	Batch	0.056	0.14	9)
Methane	Formate(20)	Mixed	Chemostat	-	0.54	6)
Methane	Formate(20)	OB3b	Batch	0.15	0.42	26)
Phenol	Phenol(0.1)	Mixed	Chemostat	0.09	0.11	11)
Toluene	Toluene(0.1)	Mixed	Chemostat	0.0073	0.0085	5)
Phenol	Phenol(0.21)	Mixed	Batch	0.51	-	3)
Phenol	Formate(10)	Mixed	Chemostat	0.24	0.38	17)
Methene	Formae(20)	OB3b	Batch	0.12	0.22	8)

* When necessary, conversion factor of 0.5 mg protein/mg dry cell was used

sources and studies with the cometabolic growth substrate present in both batch and continuous reactor system.[22] However, batch tests for determining resting cell T_C values may work only for culture with very low T_C values.[23] If a culture has high tolerance to metabolite toxicity, the observed T_C may be due to electron donor depletion and not to cell inactivation from metabolite toxicity. If electron donor is added the T_C value in these test may be higher than that from only metabolite toxicity, as the carbon addition may provide energy and carbon for cell repair to overcome the effect of metabolite toxicity.

In this study, the effects of transformation capacity on cometabolic TCE degradation were evaluated under batch experiments in the presence of both internal and external substrate. Modest amount of phenol was added to evaluate the effect of external substrate on the actinomycetes culture with high transformation capacity. The effect of internal substrate was investigated by measuring the internal PHB concentrations. The culture used was phenol-degrading actinomycetes bacteria in both mixed and pure culture. Previous studies showed that this culture was able to outcompete another phenol-degrading culture and exhibited an unusually high TCE transformation capacity at 1.0 mg TCE/mg VSS.[22,23]

MATERIALS AND METHODS

Phenol Nutrient Medium

The nutrient solution contained 1,000 mg/L phenol and the following nutrient concentrations: 700 mg/L KH_2PO_4, 1000 mg/L K_2HPO_4, 200 mg/L NH_4Cl, 30 mg/L $MgSO_4$, 66.5 mg/L $CaCl_2 \cdot 2H_2O$, 300 mg/L $NaHCO_3$, 55 μg/L $CuCl_2 \cdot 2H_2O$, 150 μg/L $ZnCl_2$, 20 μg/L $NiCl_2 \cdot 6H_2O$, 880 μg/L $FeSO_4 \cdot 7H_2O$, 135 $\mu Al_2(SO_4)_3 \cdot 18H_2O$, 280 μg/L $MnCl_2 \cdot 4H_2O$, 55 μg/L $CoCl_2 \cdot 6H_2O$, 30 μg/L $NaMoO_4 \cdot 2H_2O$, and 50 μg/L H_3BO_3.

Bacterial Culture

The actinomycetes mixed culture was taken from the gas treatment bioreactor treating TCE-contaminated air.[23] The bacterial pure culture was isolated form the mixed culture.[24] Serial dilutions of mixed culture were smeared onto mineral medium agar plates containing 14 mg/L of phenol. From these plates a colony was isolated and plated onto another similarly prepared agar plate. Single colony was then transferred into a 160-mL serum bottle containing 1% yeast extract. Yeast extract-grown culture was transferred onto an agar plate containing 14 mg/L of phenol. After three more transfers onto agar plates, an actinomyctes pure culture was isolated.

Gaseous TCE Concentration

The gaseous concentration of TCE in the headspace of serum bottles were measured by gas chromatograph (GC) headspace analysis. Serum vials containing headspaces of known TCE concentrations were used as standards. Primary TCE standard (approximately 1,000 ppm TCE in methanol) was gravimetrically measured and added to 160-mL serum vials with 80 mL deionized water to achieve various aqueous TCE concentrations. Vials were then capped with aluminum crimp tops and Teflon/rubber septa and placed on a 25°C shaker table overnight to archive equilibrium between gas and aqueous phase.

Headspace gas (0.5 mL) was injected into a SRI 8610B GC equipped with a flame ionization detector (FID) and a 30 m × 0.53 mm Restek RTX-1 column. The gas chromatograph conditions were as follows: temperature isothermal at 131 °C, helium carrier gas flow 45 mL/min, air flow 21 mL/min, and hydrogen flow 8 mL/min.

Poly-β-hydroxybutyrate (PBH) Measurement

PHB was measured using GC-FID analysis. PHB or centrifuged cells were added to a mixture of 2 mL methanol acidified with H_2SO_4 (3% v/v) and 2 mL chloroform.[25] The prepa-

ration was heated at 100°C for 4 hours and then cooled. One-mL distilled water was then added and the sample shaken for 10 minutes. The organic phase was injected into the GC. The GC oven temperature was held for 2 minutes at 70 °C, ramped at 8°C/min and then held for 1 minute at 131°C.

Biomass Measurement

Phase contrast examinations at 1000 x magnification showed that the culture developed mycelium and other cell types such as rods and cocci were observed at only trace levels in the mixed culture.

Biomass was routinely measured by dry weight. Because of fully developed filamentous characteristic and its large size, glass fiber filters with large pore size at 0.45 μm (Gelman Sciences) were used for filtration of biomass. The filters were placed in aluminum trays and baked at 550°C for 15 minutes, then cooled in a desiccator for more than 30 minutes before weighing. A 30 mL sample volume was used for filtration. Drying occurred overnight in a 105 °C oven, followed by cooling in a desiccator before weighing. Finally, the organic fraction was incinerated for 15 minutes in a 550°C oven. After cooling in a desiccator, the filter and trays were weighed again. Volatile suspended solid (VSS) was calculated as the dry weight (105°C) minus the final weight after 550°C divided by the sample volume. Total suspended solid (TSS) was calculated as the dry weight (105°C) minus filter weight divided by the sample volume.

Measurement of TCE Transformation Capacity

Batch TCE degradation experiments were conducted using 160-mL serum bottles. Diluted volatile suspended solids (80 mL) from the phenol-fed TCE gas treatment bioreactor (42.3 mg/L) were added to 160-mL duplicate serum bottles and pre-aerated for an hour to volatilize residual TCE and its metabolic intermediates in the liquid medium. The bottles were then capped with septum valves (Mininert), and amended with TCE-saturated water (1.6 mL, 1,100±200 mg/L TCE). The TCE-amended bottles were vigorously shaken by hand for 1 min before the first TCE headspace measurement and then incubated on a rotary shaker at 200 rpm at 25°C. When necessary, 5 mg/L of phenol and/or TCE-saturated water were spiked into the bottles.

The T_C values were calculated from the change in the measured aqueous TCE concentration, $\triangle C_L$, the gas and liquid volumes, V_G and V_L, the dimensionless Henry's constant, H (0.42 at 25 °C), and biomass concentration, X using equation 1, which assumed that the aqueous and vapor phase TCE were in continuous equilibrium.

$$T_C = \frac{(V_L + HV_G)\cdot \Delta C_L}{V_L \cdot X} \quad (1)$$

RESULTS AND DISCUSSION

Many investigators have shown that TCE degradation rates decline and eventually stop during batch tests with cometabolic bacteria. Though depletion of stored electron donor that is essential for the oxygenase reaction with TCE may affect TCE degradation rates, the main cause of rate inhibition appears to be metabolic toxicity from TCE degradation intermediates.

Figure 1 shows the results of a batch TCE degradation test with the actinomycetes enrichment culture that occurred over a 16-day period at an attempt to measure the T_C value. The actinomycetes enrichment culture was taken from the gas treatment bioreactor treating TCE-contaminated air and added into duplicate vials. Duplicate vials were initially exposed to 1.87 mg TCE. After 24 hr, the duplicate vials were respiked with 1.85 mg TCE and 5.0 mg/L phenol. Additional phenol (5 mg/L) and TCE (1.67 mg) were respiked at 5 days, as shown in Figure 1. Because abiotic loss of TCE was demonstrated to be negligible from a set of several uninoculated batch bottle experiments, all TCE removal was due to the biological

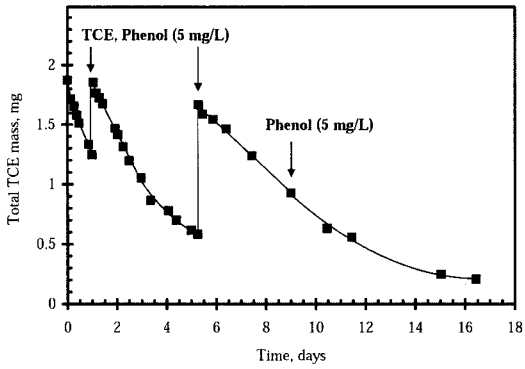

Figure 1. Total TCE mass change over time with actinomycetes enrichment at 3.38 mg VSS.

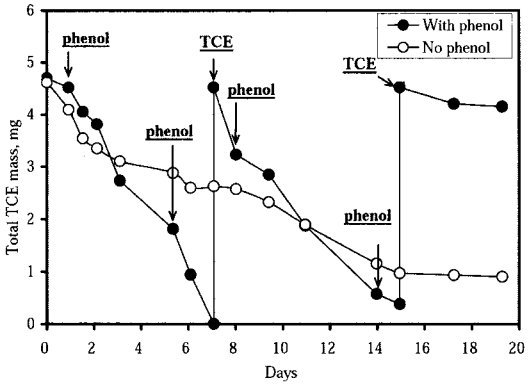

Figure 2. Total TCE mass change over time with actinomycetes pure culture at 4.63 mg VSS.

activity.[22]

The transformation capacity (T_C) was 1.0 mgTCE/mgVSS. It should be noted that the actinomycetes culture used for the batch bottle test was from the treatment reactor and thus exposed to TCE constantly before the batch test so that the culture probably had attained a higher T_C value than observed in the batch test. By days 15 - 16, the TCE degradation rate was significantly reduced probably due to depletion of the electron donor supply within the cells.

Similar TCE degradation batch tests were conducted on actinomycetes pure culture isolated from the mixed culture for measuring T_C under the presence and absence of added phenol. Figure 2 shows the TCE batch experiment for measuring TCE transformation capacity. The resting cell culture (open circle) degraded TCE without phenol addition and culture amended with phenol (closed circle) received phenol at 10 mg/L at day 1, 5.4, and 8. The initial VSS mass was 4.63 mg and temperature was 22°C. The dimensionless Henry's constant was 0.329 at 22°C. The only endogenous dissolved oxygen (DO) depletion was observed when phenol (10 mg/L) was added after the end of TCE degradation rate, confirming that the cells remained in the bottle were completely inactivated after the completion of TCE degradation.[22] The T_C value of pure culture resting cells for TCE was 0.75 mg TCE/mg VSS. When a modest amount of phenol was added, the T_C value increased more than twice at 2.0 mg TCE/mg VSS. Table 2 shows the summary of transformation capacity results of actinomycetes mixed and pure culture.

The addition of phenol would competitively inhibit TCE degradation. In this experiment, however, the effect of competitive inhibition on T_C value would be minimal. Because the specific degradation rate for phenol is about 70 times higher than the value with TCE, phenol (0.4 mg) will be completely depleted in a few minutes.[23]

The T_C values observed for the batch test with actinomycetes pure and mixed culture are much higher than T_C values reported for other batch tests with electron donor addition. For methane-oxidizers the highest T_C value was reported by Chang and Alvarez-Cohen[26] and was 0.54 mg TCE/mg cells for mixed-chemostat culture with 20 mM formate addition. For phenol-degraders the highest value was reported by Hopkins *et al.*[20] at 0.38 mg TCE/mg cell for

Table 2. Summary of transformation capacity measured using actinomycetes mixed and pure culture

Actinomycetes mixed culture		Actinomycetes pure culture	
Resting cell	Phenol-amended cell	Resting cell	Phenol-amended cell
-	1.0 ± 0.16	0.75 ± 0.12	2.0 ± 0.22

a mixed culture with 10 mM formate addition.

The addition of energy sources generally enhances both the rate of TCE transformation and the T_C value.[19] Previously, similar determination of the effect of external carbon sources on T_C was performed with methane-oxidizers with formate addition.[27] Formate is not carbon for cell synthesis and provides only reducing power to methanotrophs. The T_C values of methane and propane oxidizers were also doubled by the addition of growth substrates, methane and propane, respectively. However, the T_C values of toluene and phenol oxidizers increased by only 10 - 15% when toluene or phenol were added at low concentration (0.1 mM).[28] In other case, however, increasing the concentration of growth substrate significantly decreased TC values for unknown reason.[20,28] The growth substrate, on the other hand, would competitively inhibit the degradation of cometabolic substrate.

Batch TCE degradation tests have been performed by numerous investigators with and without electron donor addition to observe the effects of metabolite toxicity. Figure 3 is a schematic diagram showing the effects of electron donor addition on transformation capacity. In batch tests without electron donor addition, if the effect of metabolite toxicity is great, TCE degradation may cease due to cell inactivation before the depletion of stored electron donor supply limits TCE degradation (Figure 3(a)). In these cases, a low T_C value is observed. However, for cultures with high tolerance to metabolite toxicity, the observed T_C value may be due to electron donor depletion and not to cell inactivation from metabolite toxicity. Therefore, even if there is enough active cells capable of degrading more TCE, the similar T_C value can be observed as shown in Figure 3(b). If electron donor is added the T_C value in these tests may be higher than that from only metabolite toxicity since the carbon addition may provide energy and carbon for cell repair to overcome the effect of metabolite toxicity, as shown in Figure 3(c). Such tests are at best a

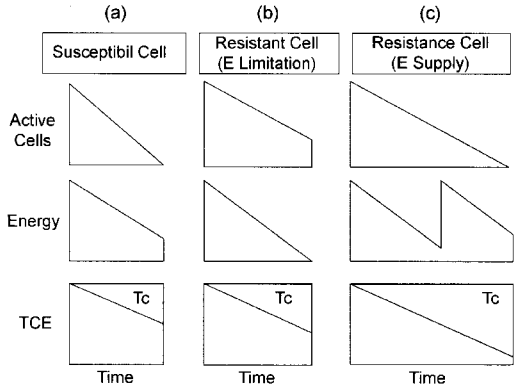

Figure 3. Schematic diagram showing the effects of electron donor addition on transformation capacity.

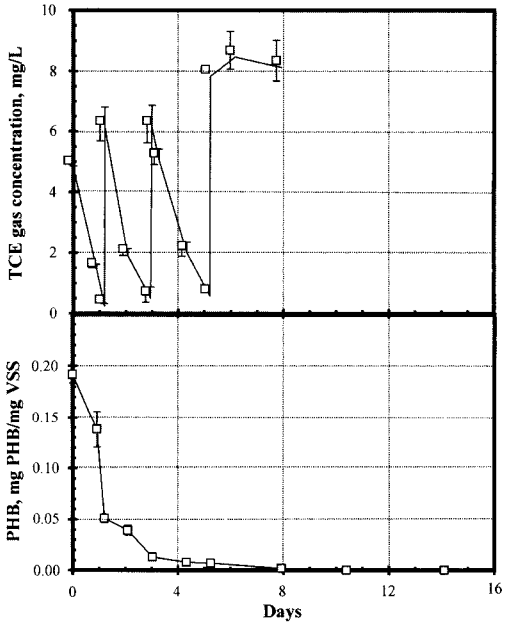

Figure 4. Degradation of TCE and internal PHB by actinomycetes pure culture.

qualitative indication of tolerance to metabolite toxicity, especially at high T_C.

To investigate the effect of internal PHB contents on the degradation of TCE, the PHB content of the pure culture was followed with time during TCE degradation (Figure 4). The cell suspension was taken from a chemostat reactor in which the phenol feed solution was batch-fed every two hours with the initial reactor phenol concentration at 14 mg/L. The initial

PHB content of the cells were about 19% of the cell mass. The PHB content of TCE-degrading culture decreased over the 8-day duration experiment, with the largest drop occurring in the first day. The calculated high T_C for cells with 19% initial PHB contents was 0.6 mg TCE/mg VSS, whereas the high T_C value measured after exhausting all internal PHB was 0.47 mg TCE/mg VSS. Several other studies also showed that the greater T_C was associated with the presence of internal PHB contents.[17,18] The internal storage product, PHB, can be used by TCE-degraders during starvation conditions as an endogenous, reducing power source to regenerate NADH for TCE degradation.[29]

The presence of PHB increased T_C value by 57%. In the other hand, the addition of phenol caused more than two fold increase in T_C. PHB is slowly degrading compound and degradation would not be fast enough to provide necessary carbon and energy to compensate the toxic inactivation of TCE degrading cell. Moreover, the addition of phenol not only provides the reducing power, carbon and energy source but also induces key enzymes, thus promote rapid enzyme repair and/or resynthesis.

As shown in this study, the T_C values measured in TCE batch experiments depend on several factors such as the type of culture used, history of culture cultivation and the availability of electron donor in the form of an internal or external substrate.

CONCLUSIONS

This research evaluated the effects of transformation capacity on cometabolic degradation of TCE using actinomycetes culture under various culture conditions. Both mixed and pure actinomycetes culture were used for TCE transformation capacity tests. After carrying out batch TCE degradation tests under several conditions, the following conclusions were made.

1. The TCE transformation capacity of the actinomycetes enrichment culture in a batch test with phenol addition was 1.0 mg of TCE/mg of volatile suspended solids (VSS).
2. The resting cell TCE transformation capacity of the actinomycetes pure culture cell was 0.75 mg TCE/mg VSS, which increased to 2.0 mg TCE/mg VSS when phenol was added three times at 10 mg/L as an external substrate.
3. When the pure culture had an internal substrate in the form of poly-β-hydroxybutyrate (PHB) at 19% of the cell mass, the resting cell TCE transformation capacity increased from 0.47 to 0.6 mg TCE/mg VSS.
4. The presence of PHB increased transformation capacity by 57%, whereas, the addition of phenol caused more than two fold increase in transformation capacity. The actinomycetes culture showed the highest transformation capacity.

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