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Retaining and Recovering Enzyme Activity During Degradation of TCE by Methanotrophs**

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ABSTRACT

To determine if compounds added during trichloroethylene (TCE) degradation could reduce the loss of enzyme activity or increase enzyme recovery, different compounds serving as energy and carbon sources, pH buffers, or free radical scavengers were tested. Formate and formic acid (reducing power and a carbon source), as well as ascorbic acid and citric acid (free radical scavengers) were added during TCE degradation at a concentration of 2 m*M*. A saturated solution of calcium carbonate was also tested to address pH concerns. In the presence of formate and methane, only calcium carbonate and formic acid had a beneficial effect on enzyme recovery. The calcium carbonate and formic acid both reduced the loss of enzyme activity and resulted in the highest levels of enzyme activity after recovery.

Index Entries: TCE; methanotrophs; biodegradation; enzyme activity; formate.

INTRODUCTION

The cometabolic degradation of trichloroethylene (TCE) by methanotrophs utilizing the methane monooxygenase enzyme has become a major focus of study (e.g., *1–6*), since the discovery of methane induced TCE degradation by Wilson and Wilson *(7)*. The prime motivation for studies of the biodegradation of TCE is the high incidence of TCE presence

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at superfund sites, Department of Energy sites, and Department of Defense sites across the country.

Maintaining high rates of cometabolic degradation of TCE by methanotrophs is difficult because of reductions in enzyme activity. Loss of enzyme activity may be the result of free radical effects caused by the epoxidation of the TCE by the soluble methane monooxygenase (sMMO) enzyme *(8)*. Maintenance of high levels of sMMO is important, because cultures with high sMMO activity achieve the highest TCE degradation rates (e.g., *9,10*). The addition of formate is apparently one way to maintain sMMO activity. The effects of formate *(11,12)* on TCE degradation have been examined, and the effect of this added reducing power is beneficial to TCE degradation.

The goal of this study was to identify methods for the maintenance or recovery of high levels of enzyme for ultimate use in a multistage bioreactor for methanotrophic degradation of TCE *(13)*. The objective of these experiments was to determine if classes of compounds other than formate, e.g., antioxidant compounds, could either prevent enzyme destruction during TCE degradation or promote enzyme recovery.

MATERIALS AND METHODS

Culture Conditions

These experiments were performed with a mixed culture of *Methylosinus trichosporium* strain OB3b, a Type II obligate methanotroph and a heterotroph. A modification *(4)* of NATE medium *(14)* was used to grow the cultures. Further modifications of the medium in these experiments were substitution of additional nitrate for ammonia and elimination of copper from the trace metal formulation.

Cells for sMMO inhibition and recovery experiments were obtained from the mixed cultures maintained in an airlift bioreactor (Kontes) continuously flushed with 3% methane in air. Optical density ($\lambda = 600$ nm) and sMMO were measured prior to use, and allowed for the standardization and comparisons of experiments. Growth phase appeared to affect sMMO activity (unpublished data). Cultures having an OD of at least 0.8, but not higher than 1.0 expressed the highest activity. It appeared that methane and oxygen were in excess, since a plateau in optical density was reached, but optical density could be increased further with the addition of supplementary inorganic nutrients in the same proportions as in the original media (unpublished data).

Analytical Techniques

Relative sMMO levels were determined by the naphthalene oxidation assay *(9)*. The initial OD (OD_i) is used as a biomass indicator. The change in OD during the sMMO assay (ΔOD) is used as an indicator of the total

sMMO activity and the change in OD divided by the initial OD ($\Delta OD/OD_i$) is used as an indicator of biomass specific activity. These units of optical density can be converted to mol of naphthol/h/mg of cells using the following relationships. We worked in a range where there was a linear relationship between OD cell concentration with an OD_i of 0.1, which is equivalent to approx 110 mg cells/L (unpublished data). The relationship between ΔOD and naphthol was also linear with an extinction coefficient of 38,000 mol/cm. Thus, dividing ΔOD by 38,000 and dividing again by the cell concentration (in our experiments, usually about 100 mg/L) and the incubation time (usually 1 h) gives the biomass specific naphthol production rate. Using these relationships, an approximate conversion factor of 18 can be used to convert $\Delta OD/OD_i$ to nmol of napthol/mg cells, and these converted figures are used in this article.

TCE was analyzed using a Sigma 2000 Model (Perkin Elmer, Norwalk, CT) gas chromatograph (GC). The GC was equipped with a capillary column and an electron capture detector (T = 300°C). The oven temperature was set at 150°C. TCE had a retention time of 3.4 min, and was measured in 30-μL samples of the headspace gas. Standards in triplicate consisted of NATE plus TCE added for a final concentration of 0.5, 1.0, and 5.0 ppm. Autoclaved cells plus TCE (1.0 ppm) were used as a control for adsorption to the cellular biomass.

Experimental Design

The enzyme inhibition and recovery experiments were run at 20°C, and consisted of exposing OB3b cells containing high levels of sMMO to TCE in the absence of methane using 40 mL EPA vials with Teflon-lined septa (Supelco, Bellefonte, PA) and a liquid volume of 5 mL. TCE was added as a saturated aqueous solution, and concentrations given are nominal concentrations because all the added TCE did not remain in the liquid phase. Actual concentrations in the liquid phase were lower because of partitioning into the gas phase. Vials were incubated inverted on a shaker. A series of preliminary experiments were performed to determine the appropriate concentrations of formate and TCE and exposure times for use in these recovery experiments. A total of 10 cm^3 of methane (100%) was added to the vials in the recovery experiments. Since the headspace was 35 mL, the methane concentration in the liquid phase approached saturation.

A scoping experiment was performed to document enzyme loss on exposure to 10, 20, and 50 ppm (nominal concentrations) TCE. Vials were incubated inverted on a shaker for 4 h, and residual headspace TCE was measured. Recovery of enzyme activity was initiated in the presence of 10 cm^3 added methane (100%) and 4 m*M* formate.

In the primary experiment, cells were grown to densities specified above, and sMMO activity measured. Cultures were exposed to 10 ppm TCE for 6 h in the presence of the chemicals being tested for their effects on enzyme levels and recovery (Table 1). Treatments included bacteria (b)

Table 1
Treatments During Primary Experiment

Treatment	Bacteria	Methane 10 m*M*	TCE, *degradation*	Addition during TCE
A	Yes	No	No	None
B	Yes	Yes	No	None
C	Yes	Yes	Yes	None
D	Yes	Yes	Yes	2 m*M* formate
E	Yes	Yes	Yes	2 m*M* citric acid
F	Yes	Yes	Yes	2 m*M* ascorbic acid
G	Yes	Yes	Yes	2 m*M* formic acid
H	Yes	Yes	Yes	$CaCO_3$ (saturated)

[a]Because there was no methane or TCE in treatment A, it is a control examining reduction in sMMO caused only by starvation. Treatment B is a control in which there is no treatment to reduce sMMO. Treatment C represents the baseline TCE effect of 10 m*M* on sMMO, and the remaining treatments examine the effect of the additions on the sMMO activity and its recovery. Formate and formic acid were added as a source of reducing power and energy. Citric acid and ascorbic acid were added as free radical scavengers, and calcium carbonate was added as a pH buffer.

alone, b + methane, b + TCE, and b + treatment chemicals. The chemical additions were 2 m*M* formate, 2m*M* citric acid, 2 m*M* ascorbic acid, 2 m*M* formic acid, and $CaCO_3$ to yield a saturated solution. Residual TCE (headspace) was measured, and cultures were then air-sparged to removed from contact with residual TCE. Activity of sMMO was assayed, and pH was measured. Recovery of enzyme activity was initiated in the presence of 4 m*M* formate and 10 cm^3 methane (100%), and continued for 32 h. At 16 and 32 h, enzyme activity was measured.

RESULTS AND DISCUSSION

TCE at 10, 20, and 50 ppm significantly reduced sMMO activity over that which was seen in unexposed cells (Fig. 1). The oxidation of TCE is apparently detrimental to methanotrophs and results in loss of TCE degradation capacity *(15)*. This toxicity is, in part, likely owing to the inhibition of the sMMO by the TCE epoxide. TCE toxicity is much lower for cells grown on methanol that do not express MMO than it is for cells grown on methane *(10)*, which do express MMO. Although some substrates (i.e., acetylene) appear to be suicide substrates *(15)* and specifically result in toxicity to methane-oxidizing activity, there is also evidence that TCE toxicity can be nonspecific *(15)* and thus damage other enzyme systems. In these studies run with 10 ppm TCE, complete degradation was measured (detect limits < 5 ppb).

When added before TCE degradation, the citric acid actually had a negative effect on sMMO activity remaining after the degradation period

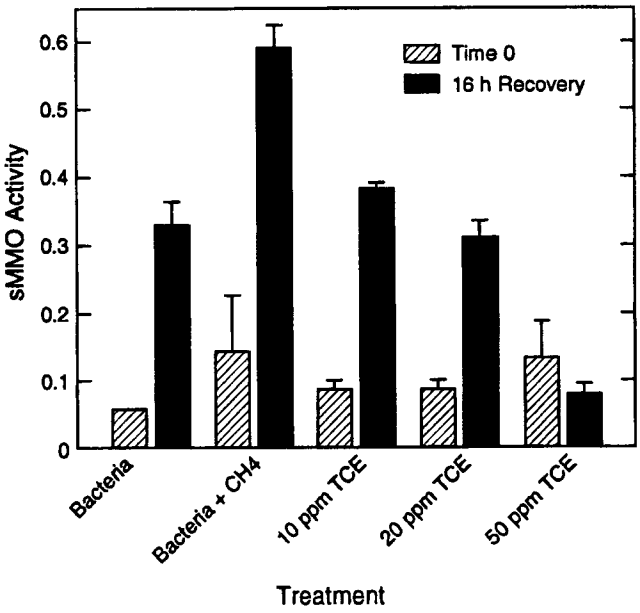

Fig. 1. Results of preliminary experiment showing sMMO activity immediately after a 6 h of exposure to 10, 20, and 50 ppm TCE (Time 0) and 16 h after initiation of recovery with addition of 4 m*M* formate, compared to levels in cells without TCE exposure, but with added methane (Bacteria + CH_4) and cells starved for methane, but not exposed to TCE (Bacteria).

(Fig. 2A) and prior to the recovery period. After a 16-h recovery period, sMMO activity in the citric acid treatment was still lower than in any other treatment (Fig. 3). During TCE degradation, pH in this treatment fell to 4.9 (Fig. 2B). Measurements in previous experiments had indicated there was no effect by the chemical additions on initial pH. In all other treatments, the pH remained at ~7 (Fig. 2B). Published data have shown that pH can affect TCE degradation *(16)* and pH can decline during TCE degradation in response to release of chloride ions *(17)*.

From our previous experiments and the literature (e.g., *12*), both formate and formic acid promoted recovery of the sMMO to higher levels than with methane alone (Fig. 3). Also, with the formate and formic acid present, sMMO activities did not fall as low during TCE degradation as it did in their absence (Fig. 2). The effect of formate on TCE degradation has been examined in a number of studies and often has shown a beneficial effect *(10,18,19)*. The positive effect has been attributed to provision of reducing equivalents believed to overcome rate limitations *(19)*.

Although it did not affect the loss of enzyme activity during TCE degradation (Fig. 2), the addition of calcium carbonate apparently had a beneficial effect on recovery of sMMO activity (Fig. 3). Ascorbic acid apparently had a slightly positive effect in reducing enzyme loss during

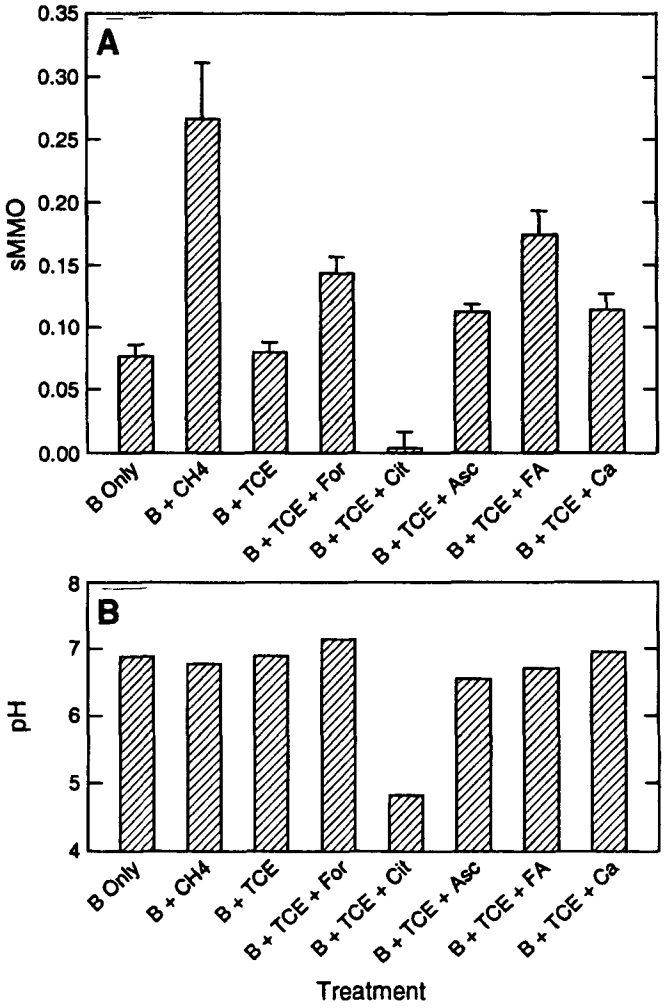

Fig. 2. The effect of TCE degradation on sMMO activity prior to recovery period **(A)** and pH in vials after period of TCE degradation **(B)** in the presence of compounds as noted in Table 1.

degradation (Fig. 2A) and little effect on final enzyme levels after the recovery period (Fig. 3). These results for calcium carbonate and ascorbic acid may be owing to physiological effects on the bacteria.

Addition of calcium carbonate and formate (or formic acid) appears to result in either reduced loss of sMMO activity or enhanced recovery of sMMO activity to higher levels after TCE exposure than without these compounds. However, addition of antioxidant compounds (citric and ascorbic acid) appeared to have no beneficial effect on the recovery of sMMO or in protecting against loss of sMMO. Based on these results, formate addition was included in the bioreactor project *(13)* to promote recovery of the enzyme activity. The addition of calcium carbonate and ascorbic acid should be considered for future efforts.

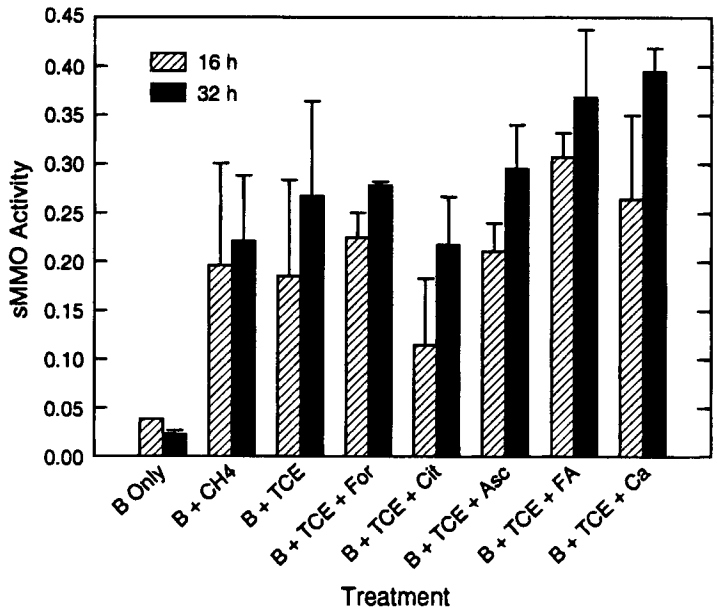

Fig. 3. The effect of 10 ppm TCE on sMMO activity 16 and 32 h after initiation of recovery with the addition of 4 m*M* formate. Treatments as noted in Table 1.

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