Evaluation of Transformation Capacity for Degradation of Ethylene Chlorides by *Methylosinus trichosporium* OB3b

Eun Yeol Lee[1]*, Jung Mee Kang[2], and Sung Hoon Park[2]

[1] Department of Food Science and Technology, Kyungsung University, Busan 608-736, Korea
[2] Department of Chemical Engineering, Pusan National University, Busan 609-735, Korea

Abstract The transformation capacity (T_c) of *Methylosinus trichosporium* OB3b in the degradation of ethylene chlorides was determined by measuring the decrease of soluble methane monooxygenase (sMMO) activity of resting cells in batch experiments. All measurements of sMMO activity were taken in the presence of 20 mM formate to avoid the deficiency of reducing power, and within 2 hrs to avoid the effect of natural inactivation from instability of the resting cells. The constant T_c values of 0.58 ± 0.132 and 0.80 ± 0.17 μmol/mg cell were obtained for trichloroethylene (TCE) and 1,2-dichloroethylene (*cis* and *trans*-1,2-DCE), respectively, regardless of their concentrations. The transformation capacity measured by this method can be used to predict the amount of cells that should be stimulated in *in-situ* bioremediation.

Keywords: ethylene chlorides, *Methylosinus trichosporium* OB3b, transformation capacity, trichloroethylene

Ethylene chlorides such as trichloroethylene (TCE) and dichloroethylene (DCE) pose serious environmental problems through the widespread contamination of soil and groundwater [1,2]. Since these chlorinated hydrocarbons are very toxic to humans [3], various treatment methods have been investigated [4-8]. Biodegradation using methanotrophic bacteria is a promising solution for the clean up of ethylene chlorides [9-12]. In the first step of biodegradation, ethylene chlorides are oxidized to the corresponding epoxides by soluble methane monooxygenase (sMMO) [13]. This key reaction requires NADH as a reducing power regenerated in the degradation pathway of the growth substrate to CO_2.

Ethylene chlorides and their degradation products are known to exert toxicity to enzymes and cells [14]. This inactivates cells and decreases the biodegradation rate. The toxic effects of ethylene chlorides and their degradation products on cells can be described quantitatively by the concept of transformation capacity (T_c), which is defined as the ratio of the amount of degraded ethylene chloride to that of inactivated cells [15-18]. The maximum amounts of ethylene chlorides by a given mass of cells prior to inactivation can be determined from the transformation capacity. T_c is a very important parameter for quantitatively describing the degradation abilities of microorganisms for various ethylene chlorides. It can also be used to predict the required amount of biomass in a bioreactor operation or *in-situ* bioremediation for treatment of ethylene chlorides. Therefore, an accurate determination of T_c is requisite for the successful operation of bioreactor and *in situ* bioremediation. Current methods for determining the transformation capacity for TCE are based on measuring the amounts of degraded TCE until the rate falls below 5% of the initial degradation rate. Whenever TCE was exhausted, more TCE was added to the reaction mixture repetitively [15]. T_c is determined by the total amount of degraded TCE divided by the amount of cells in the reaction mixture. Two important factors, however, have been ignored in the current method for measuring T_c. First, under resting conditions methanotrophs could easily lose their sMMO activity even without toxic ethylene chlorides (referred to as the problem of resting-cell instability). This natural inactivation is not distinguished from the inactivation caused by TCE degradation. Second, the depletion of endogenous reducing power like NADH could decrease the sMMO-mediated degradation rate of ethylene chlorides by the resting cells of methanotrophs (referred to as the problem of reducing power depletion).

In the present study, we report a more accurate way to measure T_c during batch degradation of ethylene chlorides, such as TCE and DCE, by *Methylosinus trichosporium* OB3b. In order to avoid the effect of natural inactivation due to the instability of resting cells, the measurements were conducted within a short experimental time. Also, we measure T_c in the presence of a NADH regenerant, formate, to avoid the problem of reducing power depletion. In conclusion, to determine a more accurate T_c, the amounts of degraded ethylene chloride were repetitively measured with repetitive addition of formate.

Methylosinus trichosporium OB3b was provided by Dr. R. T. Taylor at the Biomedical Sciences Division of the

***Corresponding author**
Tel: +82-51-620-4716 Fax: +82-51-622-4986
e-mail: eylee@star.ks.ac.kr

Lawrence Livermore National Laboratory (USA). The culture medium was Higgins nitrate minimal salt medium, and there was not enough copper for the efficient expression of sMMO [8]. Cultivation of *M. trichosporium* OB3b was performed in a 300-mL side-arm flask in a shaking incubator (Model KMC-8480sf, Vision Scientific Co., Korea). Flow rates of methane and air were controlled by a gas proportioner (Cole-Parmer II 60648, USA), and the gases were injected through the sampling septum. Cell concentration was determined by measuring the absorbance at 660 nm in a Spectronic 20 spectrophotometer (Milton Roy Inc., USA) with the use of calibration curve. Analytical grade TCE was obtained from Junsei Chemical Co. (Japan), and DCEs were obtained from Aldrich Chemical Co. (USA).

Batch degradation experiments were performed using 40 mL amber glass vials with Teflon-lined rubber septum in a shaking water bath (Model SWB 02, JEIO Tech., Korea) at 30°C. The volume of the total reaction mixture was 4.0 mL and cell density was adjusted to about 0.2 mg/mL. We began T_c measurement by adding ethylene chlorides, after which gas samples (200 - 400 μL) were withdrawn periodically using a 500 μL Precision-lock gas tight syringe to analyze concentrations of ethylene chloride. The TCE concentration in the headspace of the reaction vial was analyzed using gas chromatography (Hewlett Packard 5890 GC, USA) with a packed column (inner diameter 1/8 inch, length 6 ft, stainless steel column with 80/100 mesh graphac C powder covered with 0.1% AT 1000, Alltech Inc., USA), nitrogen carrier gas (30 mL/min), and FID (oven temperature, 150°C). The other ethylene chlorides were analyzed under the same conditions, except for the oven temperature, which was at 110°C. Methane concentration in a gas phase was determined from 300 μL of gas sample by GC (Young-In GC, Korea) with a thermal conductivity detector, helium (as carrier gas; 30 mL/min), and a packed column (6 ft stainless steel with Porapak Q, Alltech, USA; oven temperature, 40°C).

It was known that sMMO activities of resting cells in a shaking water bath decreased during incubation even without contacting toxic compounds like ethylene chloride [15]. The effect of incubation time on resting-cell stability in the presence and absence of formate was investigated to determine the appropriate experiment time. As shown in Fig. 1, the trends of inactivation of resting cells in the presence and absence of formate were almost the same, and initial activity of sMMO was maintained for 2 h only. Therefore, it is recommended that experiments measuring T_c be completed within 2 h, before resting-cell stability becomes problematic.

Substrate toxicity may limit the amount of degraded ethylene chloride by substrate inhibition. Therefore, the effect of initial TCE concentration on the TCE degradation rate was tested to determine the maximum allowable TCE concentration in determining T_c. As shown in Fig. 2, TCE degradation kinetics followed the Michalis-Menten model, with no TCE inhibition from up to 200 μM meaning that high initial TCE concentrations of up to 200 μM can be used. To determine T_c of *M. trichosporium* OB3b

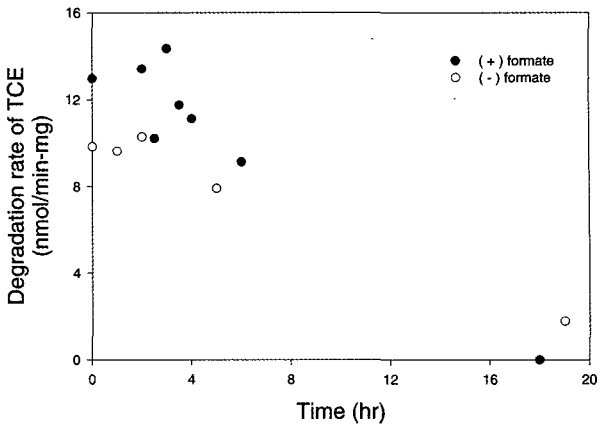

Fig. 1. Effect of incubation time on resting cell stability in the presence and absence of formate. The trends of inactivation of resting cells with or without formate were almost the same, and initial activity of sMMO was maintained for only 2 h.

for TCE, the amounts of TCE degraded were measured for 2 h at various initial concentrations of TCE. Whenever the degradation rates were determined, 20 mM formate was added to every sample of reaction mixture to avoid the problem of reducing power depletion. The amount of inactivated cells was determined by comparing the degradation rate at 2 h with initial degradation rate: the degradation rate of TCE by fresh cells and by the 2 h-incubated cells in the presence of formate were compared, and then the amount of inactivated cells was calculated by subtracting remaining activity from initial activity. Finally, T_c was simply calculated by dividing the amount of degraded TCE by the calculated amount of the inactivated cells. The T_c value of *M. trichosporium* OB3b for TCE was determined to be constant at 0.58 ± 0.132 μmol/mg regardless of change in initial TCE concentration (Table 1).

To investigate whether TCE T_c is dependent on the presence of reducing power or not, TCE degradation experiments were performed in the presence and absence of formate, since formate is a NADH regenerant that does not exert competitive substrate inhibition. Table 1 shows the effect of formate on TCE transformation capacity of *M. trichosporium* OB3b. In previous reports, the addition of formate caused an increment of TCE T_c by 50 to 100% [15]. In our study, however, the presence of formate didn't affect TCE T_c, and the average T_c value was determined to be 0.58 ± 0.132 μmol/mg. This discrepancy was probably due to the fact that previous studies did not discriminate between an authentic decrease in TCE degradation rate due to the toxic effects of ethylene chlorides and a decrease in TCE degradation rate due to the depletion of internal reducing power. In our experiments, whenever the degradation rates were determined, 20 mM formate was added to every sample of reaction mixture to avoid the decrease in TCE degradation rate due to the depletion of the reducing agent, while no additional formate was added except the initial addition of 20 mM formate in previous studies.

Table 1. Batch transformation capacity (T_c) for various ethylene chlorides*

	This study[1]	Taylor[2]		Alvarez-Cohen[3]	
		- Formate	+ Formate	- Formate	+ Formate
TCE	0.58 ± 0.132	1.90 ± 0.228	2.74 ± 0.304	0.27	0.56
Cis- DCE	0.80 ± 0.17	1.65 ± 0.103	1.96 ± 0.103	NA[4]	NA
Trans-DCE	0.80 ± 0.17	2.68 ± 0.206	4.85 ± 0.619	NA	NA
1,1-DCE	NA	0.15 ± 0.031	0.21 ± 0.031	NA	NA

* This study and Taylor used single culture of *M. trichosporium* OB3b, while Alvarez-Cohen used mixed culture of methanotrophs. Unit of T_c is μmol/mg of cell. [1] For both (+) and (-) formate, the same value was obtained, [2] From reference no. 17, [3] From reference no. 15 and 18, [4] Not available

Table 2. Effect of formate, methane or methanol on TCE transformation capacity by *M. trichosporium* OB3b at 62.2 ± 7.15 μM TCE

Methanol (mM)	0	0.4	1.0	2.0
(-) formate	0.84	0.84	0.50	0.56
(+) formate	0.65	0.55	0.54	0.49
Methane (μM)	0	70	200	300
(-) formate	0.43	0.39	0.43	0.51
(+) formate	0.72	0.49	0.67	0.63

[1] Unit of T_c is μmol/mg of cell, and the average value of T_c is constant at 0.58 ± 0.132 μmol/mg of cell.

Primary growth substrate may exert dual effects on TCE T_c, enhancement of transformation capacity by NADH regeneration and diminishment of T_c by competitive inhibition [8]. To investigate the effect of growth substrates such as methanol or methane on TCE transformation capacity, methanol was directly added in the liquid phase and methane was added in the gas phase using a gas-tight syringe. Addition of methane (0.4 - 2.0 mM) or methanol (70 - 300 μM) also did not affect T_c when added alone or together with formate (Table 2). There were some variations in T_c values but no noticeable trend was observed. We conclude that the positive effect of growth substrates was offset by the negative effect within the range of concentrations tested in this study.

The T_c for 1,2-DCE and 1,1-DCE were also measured by the same method. As shown in Fig. 2, the degradation rates of *cis*-DCE and *trans*-DCE followed Michaelis-Menten kinetics without substrate inhibition of up to 300 - 400 μM. In the case of 1,1-DCE, however, the degradation rate was found to decrease significantly above the concentration of 15 μM due to severe substrate inhibition (data not shown). Therefore, predicting T_c for 1,1-DCE by this method was difficult, since the decrease of the degradation rate occurred not only to the toxicity of ethylene chlorides but also to substrate inhibition. As shown in Table 1, the T_c for *cis*-DCE and *trans*-DCE were determined to be about 0.80 ± 0.172 μmol/mg. These values obtained in this study were compared with the data from other literature, as indicated Table 1. The T_c values for *cis*-DCE and *trans*-DCE from the literature showed significant variation with or without formate, but T_c values from our experiments were constant regardless of the presence of formate. It was also found that T_c values were similar for *cis*-DCE and *trans*-DCE. These results were different from those of the literature showing noticeable differences in T_c values for *cis*-DCE and *trans*-DCE [16, 17]. The literature suggested *cis*-DCE to be more toxic than *trans*-DCE, since *cis*-DCE actively transforms into *cis*-DCE epoxide by sMMO. Ethylene chlorides were fortuitously oxidized to the corresponding epoxides by sMMO of *M. trichosporium* OB3b, and then the epoxides were

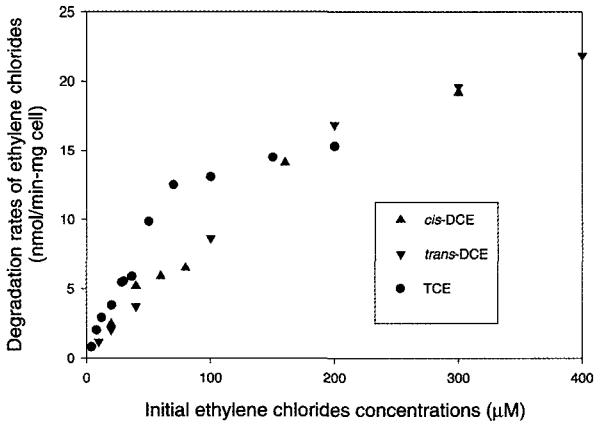

Fig. 2. Effects of initial TCE, *cis*-DCE, and *trans*-DCE concentrations on their degradation rates. High initial concentrations up to 400 μM of TCE, *cis*-DCE and *trans*-DCE can be used to determine the amount of degraded ethylene chlorides.

further metabolized to CO_2, H_2O and chloride. The toxic effect of ethylene chlorides was proportional to the amount of degraded ethylene chlorides, and the high reactivity of degradation products, mainly epoxides, was assumed to be the main cause of the toxicity of ethylene chlorides transformation by methanotrophs [16,17]. If the accumulation of the toxic epoxides is not observed and epoxides are easily metabolized to non-toxic end products, the T_c values for *cis*-DCE and *trans*-DCE could be at similar levels as described in this report. Further investigation is underway to explain the discrepancy between the literature and our results, and to provide a mechanistic understanding of the inactivation of enzymes and cells in the degradation of ethylene chlorides.

For an accurate measurement of T_c, batch experiments were finished within 2 h to avoid resting-cell instability, and the degradation rate of ethylene chlorides was measured in the presence of 20 mM formate to prevent the decrease in degradation rate due to the depletion of the reducing agent. The T_c determined in this study can be useful in predicting the minimal amount of cells that should be stimulated in bioreactor operations and *in-situ* bioremediations for treating ethylene chlorides.

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