Tetrachloroethylene Degradation by Culture Supernatant of the Anaerobic Bacterium, Strain T

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Degradation of tetrachloroethylene (PCE) by a gram negative, anaerobic bacterium strain T isolated from sludge in drainage from a laundry was examined.

The activity of degradation was obtained in the culture supernatant of strain T, but not in the cell-extract by sonication.

The reaction was optimal between pH 7.0 and 8.0 and at a temperature of 37°C. The activity was unstable in oxygen, heat labile and inhibited not only by trypsin and chymotrypsin but also by thiol inhibitors such as N-ethylmaleimide and p-hydroxymercuribenzoic acid.

The substance degrading PCE in the culture supernatant of a gram negative anaerobic bacterium strain T might be a protein with the thiol group as an active site.

**Keywords** — tetrachloroethylene; biodegradation; anaerobic bacterium; culture supernatant

**Introduction**

Tetrachloroethylene (PCE) and trichloroethylene (TCE) are solvents often used for dry cleaning, metal degreasing and cleaning. The contamination of groundwater by these compounds has been reported in several areas of the world. The compounds are suspected of being carcinogenic and, since their degradation in nature is difficult, it is quite unlikely that groundwater, once contaminated by them, will ever be free of contamination. This has made the contamination of groundwater by these compounds recognized as a serious problem for public health and the environment.

We previously isolated a gram negative anaerobic bacterium (strain T) from drain pipe sludge which was highly contaminated by PCE. We also reported the conditions supporting the growth of strain T and showed that this bacterium degrades PCE to TCE and cis-1,2-dichloroethylene (cDCE).

Bacteria including *Pseudomonas*, methanotrophs, and an ammonia-oxidizing bacterium have been isolated which degrade TCE. There are also a few reports on PCE-degrading bacteria; for example, a 3-chlorobenzoic acid-assimilating bacterium by Fathepure *et al.* and PER-K23 isolated by Holliger *et al.* The degradation of TCE and PCE by these bacteria has been studied in *vivo*, but no investigation has been done with the active substances involved.
For the purpose of determining the substances involved in the degradation of PCE, we have examined the PCE-degrading activity in both cell-free extract and culture supernatant of strain T and have found this activity only in the culture supernatant. We describe the effects of various factors on the degradation of PCE by this culture supernatant.

Materials and Methods

1. Organism — The PCE-degrading gram negative anaerobic bacterium (strain T), which we earlier isolated from sludge, was used.

2. Culture Conditions — Strain T was incubated in medium consisting of 7 g of KH₂PO₄, 2 g of K₂HPO₄, 0.1 g of MgSO₄, 1 g of (NH₄)₂SO₄, 0.5 g of sodium citrate, 2 g of yeast extract, 0.3 g of L-cysteine HCl and 1000 ml of distilled water. The pH was adjusted to 7.2. Yeast extract was purchased from DIFCO, and the other chemicals were special-grade reagents by Wako Pure Chemical Industries, Ltd.

Eighty ml of the meidum was placed in a 125 ml vial and sterilized for 15 min at 121°C. The medium was subsequently inoculated with 0.1 ml of strain T culture. The vial was sealed with a butyl rubber septa and an aluminum crimp cap. To replace the air inside the vial with anaerobic gas for the culture, the rubber septa was punctured with an injection needle fitted with a membrane filter (pore size: 0.45 μm), and then the vial was set in an anaerobic culture jar, and the air within the vial was removed with a suction pump and the vial filled with an anaerobic gas mixture (N₂ : CO₂ : H₂ = 8 : 1 : 1). The jar was then incubated for 3 d at 30°C.

3. Preparation of Culture Supernatant and Cell-Free Extract of Strain T —— The culture supernatant was prepared as follows. After 3 d incubation, the culture was transferred to a centrifuge tube with a lid. After the air within the centrifuge tube was purged with N₂ gas, centrifugation was performed for 20 min at 15000 g at 4°C. To ensure there were no viable bacteria in the supernatant, 500 μg/ml of kanamycin, to which strain T is sensitive, was added to the supernatant.

The cell-free extract was prepared as follows. The bacteria after the above-mentioned centrifugation were washed twice in a phosphate buffer (Na₂HPO₄ 0.12%, KH₂PO₄ 0.07%, pH 7.0), then subsequently suspended again in the same buffer and sonicated for 10 min at 20 KHz, using a Model UR-200P sonicator (Tomy Seiko). This was followed by 20 min centrifugation at 15000 g at 4°C and then by removal of residues, to obtain the cell-free extract.

During sonication, the sample was cooled with ice and N₂ gas was used for purging. This purging and cooling was continued on the cells and culture supernatant during these preparations.

4. Analytical Methods —— Ten milliliters of the culture supernatant or cell-free extract was placed in a 20 ml sterile vial. After 0.1 ml of saturated aqueous solution of PCE was added, the vial was sealed with a butyl rubber septa and an aluminum crimp cap. Reaction was then continued for 24 h at 35°C. TCE and cis-1,2-dichloroethylene (cDCE) were products of PCE degradation by strain T, and molar quantities of TCE and cDCE produced were equal to molar quantities of PCE degraded. The total amount of PCE degraded was believed to be transformed into TCE and cDCE. Consequently, the PCE-degrading activity was expressed in units of the total amounts of TCE and cDCE produced.

PCE, TCE, and cDCE were determined by gas chromatography (GC), in a 2.5 ml headspace sample. Standards were prepared in 20 ml vials containing 10 ml of MMY. PCE, TCE, and cDCE were added from a methanol stock solution.

GC was carried out using the following apparatus and conditions: gas chromatograph, Hitachi GC163; detector, flame ionization detector; column, 3 mm inner diameter, 3 m length, glass column; packing, 20% TCP chromosorb W AW DMCS 60—80 mesh; column temperature, 80°C; injection temperature, 120°C.

5. Effects of Various Factors on PCE Degradation in the Culture Supernatant of Strain T —— 5.1 Effect of Air: One group of
the culture supernatants were stored in sterile reagent bottles with lids to be kept in contact with air. Another group was stored in sterile vials, the air of which had been replaced with anaerobic gas mixture (N₂ : CO₂ : H₂ = 8 : 1 : 1) to avoid contact with oxygen. Both groups were stored at 0°C and 10 ml of the supernatant was collected in a 20-ml vial to check for PCE degradation, according to the method described in 4.

5.2 Optimal Reaction Temperature: The culture supernatants of strain T and PCE were kept at various temperatures (20, 25, 30, 37, 40, 45, 50, 55 and 60°C) to examine optimal temperature of PCE degradation, using the method in 4.

5.3 Optimal pH: The culture supernatants of strain T at different pHs from 5 to 11 were prepared using 1N HCl and 1N NaOH. Their degradation of PCE was examined to learn the optimal pH by the method described in 4.

5.4 Thermal Inactivation: The culture supernatant (10 ml) of strain T was placed in a 20-ml vial. After the vial was sealed with a butyl rubber septa and an aluminum crimp cap, the gas in the vial was replaced with the anaerobic gas. The vials were heated for 30 min in a water bath at 25, 40, 50, 60 and 70°C. They were immediately returned to room temperature and treated with 0.1 ml of saturated aqueous solution of PCE to examine PCE degradation according to the method described in 4.

5.5 Inhibition by Proteinases: The culture supernatant (10 ml) of strain T was treated with trypsin of swine pancreas origin (Wako Pure Chemical Industries, Ltd.) at concentrations of 0.47—4700 USP unit/ml or with chymotrypsin of cattle pancreas origin (Wako Pure Chemical Industries, Ltd.) at concentrations of 0.1—1000 USP units/ml. After the vials were sealed with a butyl rubber septa and an aluminum crimp cap, air in the vials was replaced with the anaerobic gas mixture and the vials were allowed to stand at room temperature. Fifteen min after addition of the enzyme, 0.1 ml of saturated aqueous PCE solution was added to examine PCE degradation using the method described in 4.

5.6 Effects of Thiol Inhibitors: The culture supernatant (10 ml) of strain T was treated with N-ethylmaleimide (NEM; special grade reagent, Wako Pure Chemical Industries, Ltd.) or p-hydroxymercuribenzoic acid (Aldrich Chemical Company, Inc.), both at concentrations of 1—1000 µM. After sealing of the vials and gas replacement, the vials were left standing at room temperature. Fifteen minutes after addition of the thiol inhibitor, 0.1 ml of saturated aqueous solution of PCE was added to examine PCE degradation by the method described in 4.

Results

1. Effects of Various Factors on PCE Degradation in the Culture Supernatant of Strain F

1.1 PCE Degradation in the Culture Supernatant and the Cell-Free Extract of Strain T —— PCE was degraded to TCE and cDCE in the culture supernatant but not in the cell-free extract, similar to the degradation by viable bacteria studied previously. 3)

1.2 Effects of Air —— Figure 1 shows differences in the time course of TCE and cDCE production from PCE between the supernatant exposed to air and the anaerobic gas mixture (N₂ : CO₂ : H₂ = 8 : 1 : 1). About 80% of the TCE and cDCE production from

![Fig. 1](image)

**Fig. 1** Effect of Air on TCE and cDCE Production from PCE by Culture Supernatant of Strain T

The culture supernatant of strain T was kept in anaerobic gas (80% N₂ + 10% CO₂ + 10% H₂) : ●, air : ○
PCE was lost for 24 h when the supernatant was stored in air, but only about 20% was lost for 96 h in the anaerobic gas mixture.

1.3 Optimal Reaction Temperature — The optimal temperature for PCE degradation in the supernatant of strain T was examined between 20 and 80°C (Fig. 2). The degradation occurred between 20 and 45°C with the maximum at 37°C.

1.4 Optimal pH — Figure 3 shows PCE degradation in the supernatant of strain T between pH 5 and 11. Degradation occurred between pH 6.0 and 9.7 with the maximum pH 7.0—8.0.

1.5 Thermal Inactivation — Figure 4 shows TCE and cDCE production from PCE by the supernatant of strain T at various temperatures. This production was found to be heat labile, that is, it hardly decreased when the culture supernatant was heated at 40°C for 30 min, but at 50°C for 30 min about 70% of the production was lost, and at 60°C for 30 min production was lost almost completely.

1.6 Inhibition by Proteinases — Figure 5 shows the effects of proteinases (trypsin

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Fig. 2 Effect of Reaction Temperature on TCE and cDCE Production from PCE by the Culture Supernatant of Strain T

Fig. 3 Effect of pH on TCE and cDCE Production from PCE by the Culture Supernatant of Strain T

Fig. 4 Heat Stability of TCE and cDCE Production by the Culture Supernatant of Strain T
Fig. 5 Effect of Proteinase on the Culture Supernatant of Strain T

- trypsin, 100% of formed TCE and cDCE was 2.96 μM.
- chymotrypsin, 100% of formed TCE and cDCE was 3.5 μM.

Fig. 6 Effect of Thiol Inhibitors on TCE and cDCE Production by the Culture Supernatant of Strain T

- NEM (N-ethylmaleimide), ○: p-Hydroxymercuribenzoic acid. 100% of formed TCE and cDCE production on both thiol inhibitors was 0.2 μM.

and chymotrypsin) on the TCE and cDCE production from PCE by the supernatant of strain T. The TCE and cDCE production from PCE was inhibited markedly by treatment with 470 USP units of trypsin per ml or with 1000 USP units of chymotrypsin per ml.

1.7 Effects of Thiol Inhibitors —— Figure 6 shows the effects of thiol inhibitors, NEM (N-ethylmaleimide) and p-hydroxymercuribenzoic acid, on the TCE and cDCE production from PCE by the supernatant of strain T. Treatment of the culture supernatant with 1000 μM of either agent caused no PCE degradation.

Discussion

Although PCE and TCE do not degrade easily, it has been reported that they are degraded by some microorganisms. Studies of the pathway of PCE and TCE degradation by microorganisms were based on analyses of their degradation products.

For example, the degradation of TCE by bacteria of the *Pseudomonas* species has been presumed to involve enzymes related to cleavage of the aromatic ring on the grounds that TCE was degraded only when these bacteria were incubated in the presence of toluene, phenol, or cresol. Regarding the pathway of TCE degradation by methanotrophs, it has been speculated that TCE is degraded *via* trichloroepoxide into glyoxylic acid and chloroacetylate, and finally to carbon dioxide. Anaerobes are thought to degrade PCE into TCE by means of dechlorination and to further degrade TCE into cDCE. Shields *et al.* reported the presence of toluene-degrading enzymes in the extracts of TCE-degrading *Pseudomonas cepacia* G4 and its mutant.

Despite numerous reports on the degradation of PCE by bacteria, all of the research was involved viable cells. There has been no study regarding PCE degradation in cell-free extracts from bacteria or in culture supernatant of bacterial cultures.

Thus, we examined PCE-degrading activity of the cell-free extract and the culture supernatant of strain T. The effects of various factors on the PCE-degrading activity of this culture supernatant were also examined. Table 1 summarizes the results of this study. The culture supernatant of strain T was found to be capable of degrading PCE. This
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<th>Treatment</th>
<th>PCE degrading activity</th>
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<tr>
<td>Culture supernatant</td>
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<tr>
<td>Cell-free extract</td>
<td>−</td>
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<tr>
<td>Air</td>
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<tr>
<td>aerobic</td>
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<td>Optimum pH</td>
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<td>50°C for 30 min</td>
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<td>60°C for 30 min</td>
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<td>Thiol inhibitor</td>
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<td>ρ-hydroxymercuri-</td>
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<td>benzoic acid</td>
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Activity seems attributable to a metabolite which is produced outside this bacterium, because PCE-degradation did not occur in the cell-free extract of the bacterium. This active substance seems unstable against oxygen, because the PCE-degrading activity of the supernatant was easily lost in the presence of oxygen. Furthermore, the PCE-degrading activity of the supernatant was lost when the supernatant was heated at 60°C for 30 min. It was also lost or reduced when the supernatant was treated with trypsin or chymotrypsin. Therefore, the substance involved in the PCE-degrading activity appears to be a protein. This protein is suggested to possess thiol group because the PCE-degrading activity was eliminated by treatment with thiol inhibitors. The substance responsible for the PCE-degrading activity of strain T is thus considered to be a protein with a thiol group, which is heat labile and unstable in the presence of oxygen.

**Conclusion**

The PCE degradation was noted only in the culture supernatant of strain T, and did not occur in the cell-free extract of strain T, a PCE-degrading bacterium. Therefore, the PCE degradation by strain T is attributable to a substance which is produced outside this bacterium.

The active substance of the PCE-degrading bacterium was unstable in the presence of oxygen and was heat labile.

Since the PCE-degrading activity of the culture supernatant of strain T was inhibited by trypsin and chymotrypsin and by thiol inhibitors, this active substance seems to be a protein with a thiol group which is an essential component for its activity of PCE degradation.

**References**


