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# Enzymatic Degradation of Trichloroethylene Using Enzyme Extracts Isolated From a Bacterial Consortium

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## Abstract

Degradation of trichloroethylene (TCE) using crude enzyme extracts from a bacterial consortium was examined for wastewater treatment. The effects of pH, chemical induction, and cofactor were investigated. Enzyme extracts showed an optimal activity (3.03 ± 0.03 mg of TCE/[mg of protein·d]) at neutral pH (6.5–7.5). In an attempt to increase the production of effective enzymes for TCE degradation, chemical induction using both toluene and TCE in the growth of the bacterium consortium was conducted. Although the induction increased the overall production of protein by about fourfold, the activity of the extracts was only slightly improved (up to 3.40 mg of TCE/[mg of protein·d]), indicating that the induction did not specifically enhance the production of TCE-degrading enzymes. Interestingly, the addition of a cofactor (up to 0.02 mg/mL), NADH, led to an initial reaction rate of 5.30 ± 0.05 mg of TCE/(mg of protein·d). This observation demonstrated that the availability of the cofactor played an important role in determining the overall degradation reaction rates. The observations with NADH were in agreement with the assumption that toluene monooxygenases (which are NADH dependent) are the key enzymes for the degradation reactions.

**Index Entries:** Enzymatic degradation; trichloroethylene; NADH; bioremediation; toluene monooxygenase.

## Introduction

Trichloroethylene (TCE) is one of the most widely distributed industrial contaminants of water resources. Compared to other technologies such

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as catalytic oxidation and incineration, biologic treatment of TCE is attractive owing to its low energy consumption and environmentally benign nature *(1,2)*. However, the success rate of bioremediation at full-scale waste minimization applications has been limited *(3)*. Several factors can be attributed: First, the biotic systems that have been used predominantly for the treatment of municipal wastewater are dramatically different from those that would be effective for chlorinated solvents. As such, it is not surprising that several researchers have shown that chlorinated solvents pass through conventional wastewater treatment plants unaltered *(4,5)*. Second, most of the studied processes applied a single bacterium; however, most chlorinated solvents cannot be completely mineralized by a single microbe, and one bacterium can rarely effectively treat multiple contaminants. Third, the use of whole-cell degradation at an industrial scale can be complicated by limitations associated with continuous nutrient amendments, temperature control, pH adjustment, cell washout, dilution factors, and toxicity posed by the solvents themselves.

Recent advances in enzymatic biocatalysis have demonstrated the feasibility of using enzymes to conduct biotransformations in harsh environments including high organic solvent concentrations *(6–8)*. In many cases, isolated enzymes can provide faster reactions than the microbial processes. In addition, environmental concerns about using genetically engineered cells also make enzymatic processes more attractive. Accordingly, there has been a growing research effort in exploring enzymatic biodegradation as a promising alternative to the microbial approach for large-scale and cost-effective treatment of organic pollutants.

Traditional enzymatic biodegradation studies, however, have focused on the use of individual enzymes. This usually leads to simple one-step conversions, such as dechlorination, generating intermediates that may be quite toxic *(6,9,10)*. Studies have documented that microorganisms usually achieve mineralization through interactions of a series of enzymes and cofactors. For instance, depending on the microbes used, TCE degradation can occur via four enzymatic steps involving catechol-2,3-dioxygenase, toluene dioxygenase, and *cis*-dihydrodiol dehydrogenase. Although such mechanisms have been proposed for the living microorganisms, the complete degradation of organic pollutants by using isolated enzyme systems has not been demonstrated. The present study evaluates the efficiency of using enzyme systems isolated from biologic sources to carry out TCE biodegradation.

## Materials and Methods

### Chemicals

Bovine serum albumin (BSA), lysozyme (EC 3.2.1.17) from chicken egg white, reduced nicotinamide-adenine dinucleotide (β-NADH), and HCl were purchased from Sigma (St. Louis, MO). Sodium phosphate, hexane, and TCE were purchased in reagent-grade from Fisher (Fair Lawn, NJ).

Protein assay reagent was obtained from Bio-Rad (Hercules, CA). Other chemicals used for making buffers and nutrient solutions were purchased in biotechnical grade from Fisher.

## *Growth of Bacterial Consortium*

The consortium comprised bacteria initially isolated from soils contaminated with chlorinated solvents and petroleum products. It was cultivated from pure cultures of the isolated strains purchased form the American Type Culture Collection (ATCC) to ensure that each of the active species of the consortium (i.e., responsible for degradation and enzyme generation) could be accurately and expeditiously identified. The specific cultures used included *Pseudomonas putida* (ATCC 17484), *Pseudomonas fluorescens* (ATCC 13525), *Mycobacterium* sp. (ATCC 2676), *Nocardia paraffinae* (ATCC 21509), and *Nitrosomonas europeae* (ATCC 25978).

Revival and growth of each microbial strain occurred in separate, standard basal nutrient solutions comprising 1.33 g of $KH_2PO_4$, 2.67 g of $K_2HPO_4$, 1 g of $NH_4Cl$, 2 g of $Na_2SO_4$, 2 g of $KNO_3$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot 7H_2O$, and 1 mL of a trace metal solution in 1 L of distilled water. The trace metal solution contained 3.7 g/L of $CaCl_2 \cdot 2H_2O$, 2.5 g/L of $H_3BO_3$, 0.87 g/L of $MnCl_2$, 0.65 g/L of $FeCl_3$, 0.44 g/L of $ZnCl_2$, 0.29 g/L of $Na_2MoO_4 \cdot 2H_2O$, 0.01 g/L of $CoCl_2$, and 0.001 g/L of $CuCl_2$. Glucose (1 g/L) was employed as the carbon source for sustaining the microbes until acclimated to the target compound. At that time, TCE was used as the primary carbon source. After obtaining an adequate cell density, the individual strains were transferred to both single agar slants and 250-mL flasks. The individual strains (and subsequent mixed colony) were maintained on the slants to provide a long-term bacterial supply. Erlenmeyer flasks were used for normal subculturing activities and development of the consortium. The flasks were maintained in an orbital environmental shaker at 25 ± 3°C and 125 rpm. Cultivation of the mixed consortium required 6 mo.

## *Isolation of Enzyme*

Extracellular enzymes were isolated from the microbial growth medium by removing the living cells via filtration. The bacterial solution (800 mL each batch) was first filtered with 11-µm filter paper (Whatman) to remove visible suspensions, followed by filtration with polystyrene low-protein-binding membrane filter (0.22 µm; Corning). The enzyme concentration of the filtrate solution was measured using the Bradford protein assay. The enzyme solutions were then concentrated by evaporating water to a final volume of 200 mL using a rotoevaporator (Yamoto RE500) at 35°C. Intracellular enzymes were obtained by adding lysozyme to the medium before enzyme isolation, followed by the same filtration procedure. Typically, 10 mg of lysozyme was used for each 800-mL bacterial solution, and the solution was stirred at room temperature for 3 h to break the cell walls.

### Protein Assay

The total protein concentration was analyzed using the Bradford-Coomassie protein assay *(11)*. A 2-mL 1:10 (reagent:water) solution was prepared and transferred to a quartz cuvet. Then 50 µL of enzyme solution was added to the cuvet followed by gentle mixing. The absorbance was measured at 590 nm via a spectrophotometer (Shimadzu UV-1601 UV-vis). A calibration curve (using BSA as the standard) was then used to quantify the amount of enzymes in the solution.

### TCE Analysis

A gas chromatograph (Shimadzu GC-17A) equipped with a flame ionization detector and a Restek RTX-5 column (15 m × 0.35 mm × 1.0 µm) with 5% diphenyl and 95% dimethyl polysiloxane was used. The analytical method was similar to that reported previously *(12)*. The initial column temperature was kept at 40°C for 18 min, then ramped at 40°C/min to 120°C. The injector and detector temperatures were both 250°C. Nitrogen was the carrier gas at an inlet pressure of 18 kPa (~0.4 mL/min).

### Enzymatic Degradation of TCE

Typically, 200 mL of the concentrated enzyme solution was transferred to a 250-mL Erlenmeyer flask. Degradation was initiated by adding TCE (85.5–171 µL) to the flask. The solution was then distributed into 9-mL aliquots (using 10-mL vials capped with a Teflon lid) and placed in an orbital environ-shaker operated at 25 ± 3°C and 125 rpm. Three aliquots were removed for gas chromatography (GC) analysis every 24 h by extracting the remaining TCE in the reaction solution with hexane (with a phase ratio of hexane:water of 60:40). Control reactions were conducted and analyzed in the same way except that pure buffer solution (50 m*M* phosphate buffer, pH 6.8) was used instead of enzyme solution. Bacterial growth curves (using only the headspace oxygen) demonstrated both exponential and stationary growth phases during the experimental duration for TCE concentrations <1500 mg/L, indicating sufficient oxygen supply to the bacteria in the reactors. Reactions with additional NADH were also conducted following the same procedure with enzyme solutions with added NADH (5 mg for each 200 mL of enzyme solution). The reaction solutions maintained optically clear during the course of the reactions, indicating no bacterial growth in the reaction solutions.

### Bacterial Degradation of TCE

Destructive batch degradation experiments were conducted in liquid phase in glass vials (45 mL) over a 1-wk period. To each reactor, 15 mL of the acclimated consortium was transferred followed by the addition of TCE at the desired concentration with a 0.5-µL syringe (Hamilton, Reno, Nevada). The reactors were sealed and kept in an orbital environ-shaker operated at 25 ± 3°C and 125 rpm. Three reactors were removed for GC

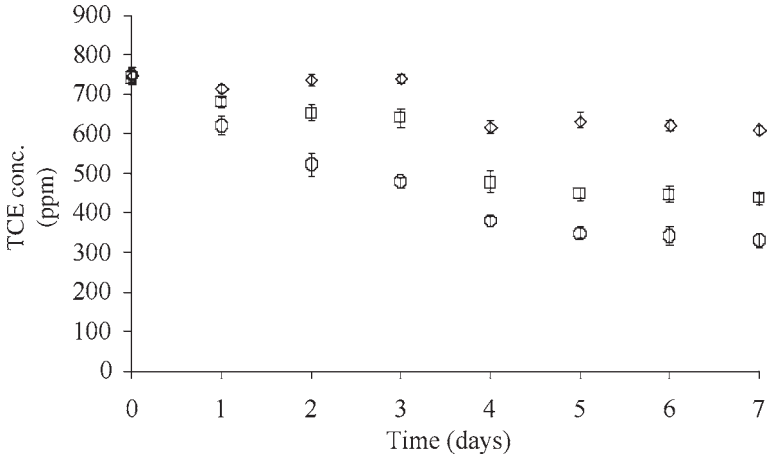


Fig. 1. Degradation of TCE using extra- and intracellular enzyme extracts: (◇) abiotic loss; (□) extracellular extract; (○) mixed extra- and intracellular extract. The initial TCE concentration was 750 ppm.

analysis every 24 h. Triplicate reactors were used for each sampling interval for reproducibility and reliability. Parallel control reactors were utilized to assess abiotic losses.

## Results and Discussion

### *Activities of Extracellular and Intracellular Enzymes*

Toluene monooxygenases (TMOs), which are believed to be the key enzymes in TCE degradation, may exist as either extracellular or intracellular enzymes *(13,14)*. To evaluate the distribution of the enzymes within or outside the living cells in the bacterium consortium, TCE degradation with extracellular extracts and extracts consisting of mixed extra- and intracellular components was conducted. As a result, the initial reaction rate was doubled for extracts containing the intracellular components (Fig. 1). For experiments with a 700-ppm initial TCE concentration, approx 8% degradation was achieved within 1 d by extracellular extract, whereas the extract of mixed extra- and intracellular components achieved approx 17% degradation (Fig. 1). Within 7 d, the mixed extract reached 56% TCE degradation vs 41% observed for the extracellular extract. Control experiments without using enzyme extract showed abiotic losses of TCE in the range of 11–22% within 7 d. Control experiments with solutions containing only lysozyme showed the same abiotic loss.

These observations indicated that both the extracellular and the mixed extracts contained enzymes that were effective for TCE degradation. The difference in degradation rates may simply reflect the effect of enzyme concentration. The total protein concentration increased by approximately twofold in the extracts when the cells were ruptured using

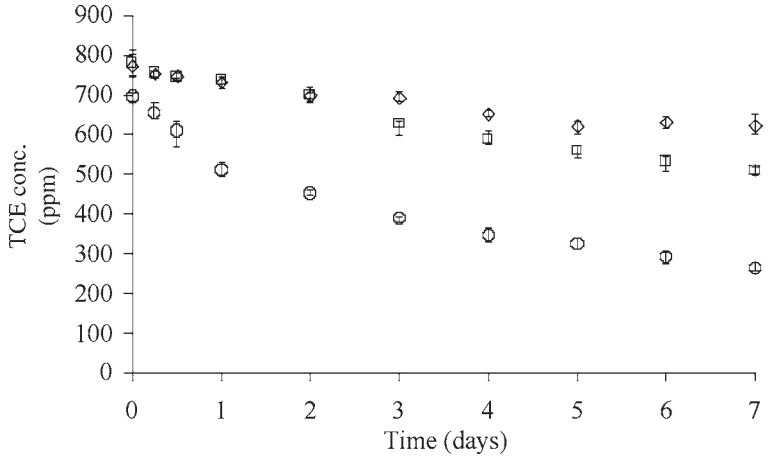


Fig. 2. TCE degradation with enzyme extracts of different protein contents: (◇) abiotic loss; (□) enzyme solution with total protein concentration of 0.015 mg/mL; (○) enzyme solution with total protein concentration of 0.057 mg/mL. Both enzyme solutions were mixed extra- and intracellular extracts. The initial TCE concentration was 750 ppm.

lysozyme (excluding the amount of lysozyme) before the isolation of enzymes (protein concentration changed from 0.011 to 0.026 mg/mL). This is in agreement with the increase in the initial reaction rate. For a further confirmation, an enzyme extract of 0.015 mg/mL of protein was concentrated to 0.057 mg/mL by evaporating water. The concentrated enzyme solution degraded TCE about fourfold faster than its parent solution (Fig. 2). Evaluation of the degradation achieved within the first day reflected an initial degradation rate of 3.03 ± 0.03 mg of TCE/(mg of protein·d).

## *Effect of Chemical Induction on Growing Bacterial Consortium*

One important method to improve the production of effective enzyme components is chemical induction. For whole-cell bioremediation, the chemical induction may introduce toxicity toward the cells, and that usually leads to decreased efficiency for TCE degradation *(15)*. In the present work, both toluene and TCE were used to induce the production of TMOs, which were assumed to be the key catalysts for TCE degradation. The results showed that although both chemicals improved the protein concentration in the crude extracts, toluene led to a lower protein concentration (0.031 mg/mL) compared with that achieved with TCE (0.068 mg/mL). TCE degradation rates of 3.24 and 3.40 mg of TCE/(mg of protein·d) were observed for the toluene-induced and the TCE-induced extracts, respectively (Fig. 3), which are only slightly higher than those achieved without inductions. This result indicated that although overall protein production was improved by the inductions, the concentration of effective enzyme components (TMOs) was not effectively elevated specifically (relative to other protein components) by the inductions.

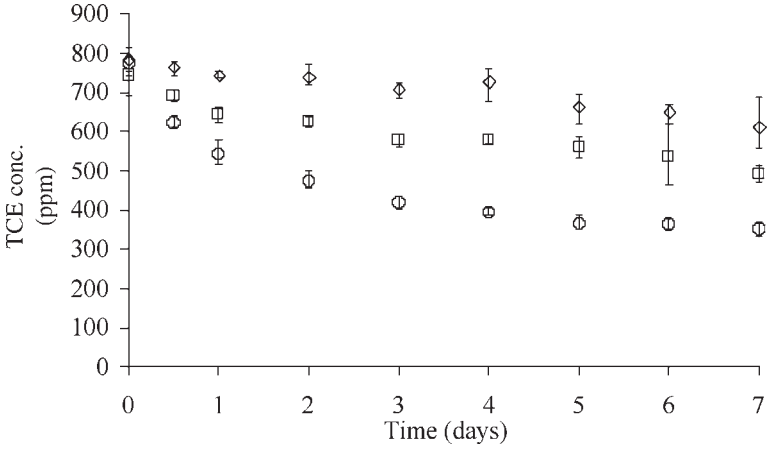


Fig. 3. Effect of different inducers on activity of enzyme extracts: (◇) abiotic loss; (□) enzymes extract from bacterial consortium induced with toluene; (○) enzyme extract from bacterial consortium induced with TCE. Enzyme solutions were mixed extra- and intracellular extracts. The initial TCE concentration was 750 ppm.

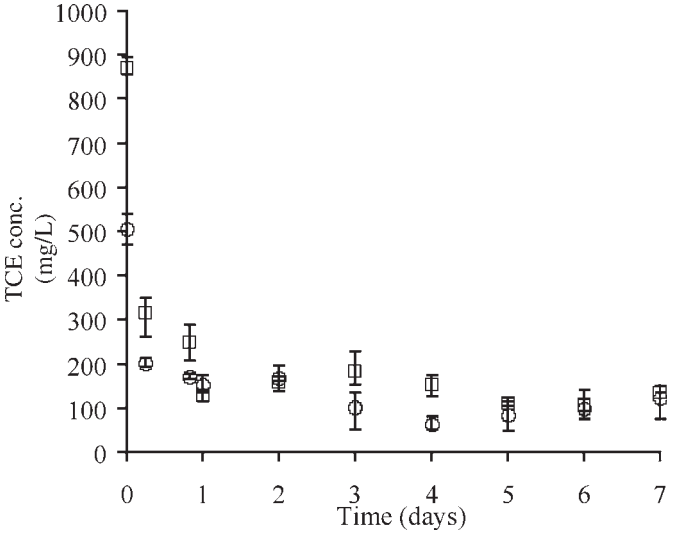


Fig. 4. Aerobic whole-cell degradation of TCE. TCE concentration: (○) 425 ppm; (□) 869 ppm.

## *Degradation by Bacterial Consortium*

Experiments with living cells of the microbial consortium demonstrated faster initial reaction rates (Fig. 4). Interestingly, all the TCE disappearance for bacterial degradation happened almost within the first day. It is suspected that as degradation products that were consumed competitively with TCE were generated, TCE was no longer the preferred substrate to the living cells *(14,16)*.

Table 1
Effect of pH on Enzymatic Degradation of TCE[a]

| pH | Degradation (%) (1 d) | Degradation (%) (7 d) |
|---|---|---|
| 5.5 | 11 | 39 |
| 6.5 | 19 | 45 |
| 7.5 | 21 | 40 |
| 8.5 | 17 | 30 |

[a]The initial TCE concentration was 500 ppm, protein concentration was 0.025 mg/mL, and NADH concentration was 0.02 mg/mL.

As shown in Fig. 4, when intact cells were used, degradation efficiencies of 62 and 84% was achieved for initial TCE concentrations of 450 and 868 ppm, respectively, and enzyme extracts degraded mostly <50% (the amount of enzymes and reaction scale are equivalent to the bacterial reaction). A possible explanation for the lower degree of degradation achieved with the enzymes is the inhibitory effect of some degradation intermediates that can covalently bind to proteins *(16,17)*, and ultimately lead to inactivated enzymes.

### *Effect of pH*

The effect of pH on the degradation rate of TCE by the enzyme extracts was evaluated. The value of pH varied from 5.5 to 8.5 while the concentrations of enzyme and substrate were kept constant. Table 1 provides the initial degradation (based on the degradation of the first 24 h) at different pH values. As expected, the enzymes showed an optimal pH of about 7.0. This pH optimum is the same as observed for TMOs *(18)*.

### *Effect of Cofactor*

It has been proposed that the first step in the biodegradation pathway of TCE was the enzymatic oxidation of TCE *(19)*. As mentioned earlier, TMOs and other oxygenases are believed to be the key catalysts enabling the TCE oxidation. Owing to the dependence of TMOs on NADH, the concentration of the cofactor (NADH) may provide an alternative approach to optimizing the enzymatic TCE degradation. TCE degradation with enzyme extract was conducted with the addition of NADH (0.02 mg/mL). The addition of NADH increased the initial reaction rate significantly (Fig. 5) compared with the enzymatic degradation without the addition of NADH. Evaluation of the reaction rates revealed that the initial rate of TCE degradation was increased to 5.30 ± 0.05 mg of TCE/(mg of protein·d). The enhancement in degradation rate implied that NADH was a rate-limiting factor in the biodegradation pathway. However, the final degradation over the 7-d course was only slightly improved (64% with NADH vs 54.4%

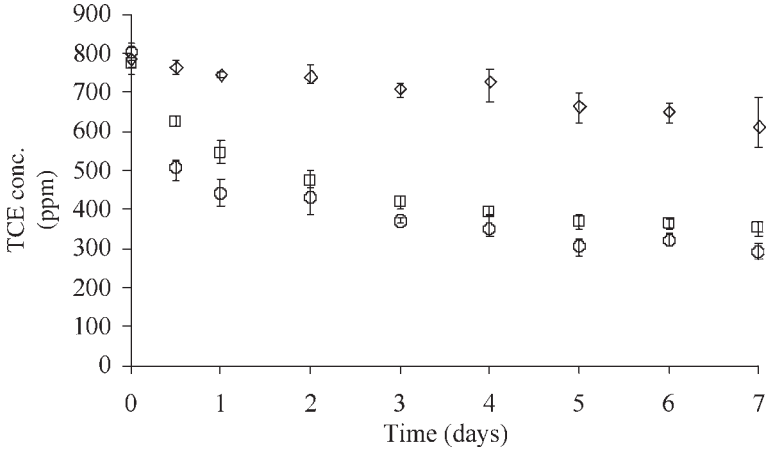


Fig. 5. Effect of NADH on enzymatic degradation of TCE: (◇) abiotic losses; (□) without addition of NADH; (○) with 0.02 mg/mL of NADH. Enzyme solutions were mixed extra- and intracellular extracts. The protein concentration was 0.068 mg/mL and the initial TCE concentration was 750 ppm.

without NADH). The slowed degradation rate in the later stage again suggested the possibility of product inhibition.

## Conclusion

TCE degradation using crude enzyme extracts isolated from a consortium of aerobic bacteria was demonstrated. Factors such as pH and cofactor showed noticeable effects on the enzymatic degradation rates. Manipulation of these factors will provide future optimization of the enzymatic degradation of TCE. It is expected that by combining recent advances in cofactor regeneration and enzyme stabilization/activation technologies, it is possible to develop efficient enzymatic degradation technologies for different organic pollutants using enzyme extracts. Compared to the bacterial approach, enzymatic degradation is promising in that it requires simple operations, can apply concentrated enzymes, can function at harsh reaction conditions, and is free of environmental concerns including those associated with genetically engineered microorganisms.

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