

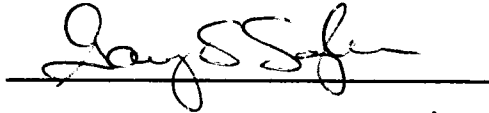
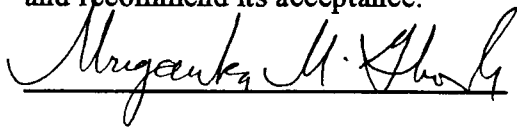
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I am submitting herewith a dissertation written by Hae-Jin Woo entitled "Cometabolic Oxidation of TCE (Trichloroethylene) by *Pseudomonas putida* F1 in Biofiltration." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Civil Engineering.



Chris D. Cox, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor and
Dean of The Graduate School

Cometabolic Oxidation of TCE (Trichloroethylene) by
***Pseudomonas putida* F1 in Biofiltration**

A Dissertation

Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Hae-Jin Woo

May 1999

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DEDICATION

This dissertation is dedicated to

my parents

Mr. Jeong-man and kyung-ja Woo

brother and sister

Sea-jin and Eun-sun Woo

my husband and daughter

Young-hyun, Margaret Eun-ha Kim

Whose love and sacrifice laid the foundation for my higher education.

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overcame together. She is always willing to give her valuable time to me. Her friendship is as precious as my degree.

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Finally, I thank God who completes everything with me.

ABSTRACT

Biofiltration is a technology that utilizes microorganisms supported on porous media such as compost, peat, or soil to remove gas-phase organic and inorganic compounds. The biodegradation of many chlorinated compounds requires a primary substrate to provide an energy and carbon source and to induce the necessary enzymes responsible for metabolism of the target compounds. In this research, a toluene-degrading bacterium, *Pseudomonas putida* F1, was used. Toluene induces the formation of the toluene dioxygenase enzyme (Tod) system, a non-specific enzyme capable of oxidizes TCE to formic and glyoxylic acid.

Batch and column experiments were performed to understand the biokinetics of TCE cometabolic oxidation with respect to competitive inhibition, NADH limitation, enzyme activity, and toxicity. A fluorescence -based assay was developed and used to monitor Tod activity during TCE degradation. Suspended-growth studies with pure cultures indicated that indoxyl, as measured by fluorescence, correlated with pure indigo production ($R^2=0.89$) as measured spectrophotometrically. In batch experiments, the normalized enzyme activity decreased without toluene, but remained relatively constant when toluene was present.

For the suspended cells, the maximum specific toluene utilization rate and half-saturation constant were $2.674 \mu\text{g toluene} (\text{min mg biomass})^{-1}$ and 3 mg L^{-1} , respectively. The degradation rate of toluene by suspended cells was not significantly affected by TCE. At toluene concentrations greater than 2.15 mg L^{-1} , significant inhibition of TCE

degradation was observed during the first 20 to 35 minutes of the experiment; however, once the toluene decreased below 2.15 mg L^{-1} , TCE degradation was rapid.

A differential volume reactor was constructed to measure the growth rate of immobilized cells and the rate of toluene utilization. The maximum specific growth rate (μ_{max}) of immobilized cells was estimated to be 0.029 hr^{-1} . Growth was strongly dependent upon toluene concentration up to $4000 \text{ } \mu\text{g L}^{-1}$. Growth did not increase significantly above $5000 \text{ } \mu\text{g/L}$ because the maximum specific growth rate was achieved. The maximum specific toluene utilization rate (k_T) and half saturation constant (K_{ST}) were found to be $1.250 \text{ } \mu\text{g toluene (min mg biomass)}^{-1}$ and $1988 \text{ } \mu\text{g toluene L}^{-1}$, respectively.

Normalized NADH was relatively unchanged when toluene and TCE were fed to the biofilter simultaneously. A steady-state NADH concentration of about $0.4 \text{ } \mu\text{mol (mg protein)}^{-1}$ was maintained whenever toluene was fed to the column. NADH concentrations in the biofilm were observed during TCE degradation with no toluene present. While the rate of TCE removal and the normalized enzyme activity decreased during each experiment, the average concentration of NADH remained above $0.477 \text{ } \mu\text{mol (mg protein)}^{-1}$.

The rate of enzyme inactivation in both suspended and immobilized cells appears to be correlated with the rate of TCE degradation, suggesting that enzyme inactivation is related to intermediates produced during TCE degradation. A dynamic biofiltration model was developed to predict the concentration profile and effluent history for the case when TCE alone is feed to the biofilter. The model demonstrated that the principal factor

resulting in the decrease in TCE removal with time is the inactivation of Tod enzyme by TCE metabolic intermediates.

The values of TCE reaction rate constant, $k_s' = 1428.6 \mu\text{g TCE (mg indigo L)}^{-1}$, TCE half-velocity constant, $K_s = 2620 \mu\text{g TCE L}^{-1}$ and inactivation constant of enzyme activity, $k_2 = 0.0004 \text{ mg indigo } (\mu\text{g TCE L})^{-1}$ were determined and used for prediction the changes in effluent TCE concentration and enzyme activity during TCE degradation via biofiltration.

In most cases, the model gives a reasonable prediction of TCE degradation in the biofilter. The model effectively predicted the diminishing rate of TCE degradation because of inactivation caused by TCE intermediates. Sensitivity testing of the model showed that TCE degradation increased with decreasing K_s and that the rate of increase in the TCE effluent concentration with time increased with decreasing K_s . The model became less sensitive to K_s as the value of this parameter increased. TCE degradation was not sensitive to k_2 , but enzyme activity was relatively sensitive to this parameter. The slope of the plot of effluent concentration as a function of time increased with TCE feed concentration. The normalized enzyme activity decreased rapidly for higher TCE feed concentrations.

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NOMENCLATURE

H : Henry's law constant ($\text{atm} \cdot \text{m}^3 \text{mol}^{-1}$)

T : Temperature (Kelvin's), 296.2 (at 23 °C)

A : 5.13 for toluene, 11.4 for TCE

B : 3.02×10^3 for toluene, 4.78×10^3 for TCE

P : pressure, atm

V : volume, L

T : Kelvin temperature, K

R : ideal gas law constant , $0.0821 \text{ L atm mol}^{-1} \text{ K}^{-1}$

n : number of moles

[E_c] : total concentration of enzyme in system, mass volume^{-1}

[ES] : enzyme/substrate complex concentration of enzyme in system, mass volume^{-1}

[E] : free enzyme concentration of enzyme in system, mass volume^{-1}

[P] : product concentration in system, mass volume^{-1}

[ER] : the enzyme-NADH complex concentration, mass volume^{-1}

[ERS] : the enzyme -NADH - TCE complex concentration, mass volume^{-1}

[ERT] : the enzyme -NADH - toluene complex concentration, mass volume^{-1}

[R] : NADH concentration, mass volume^{-1}

[S] : TCE concentration, mass volume^{-1}

[T] : toluene concentration, mass volume^{-1}

K₁, K₂, and K₃ : rate constants for the reactions designated

K_m : the saturation constant and is equal to the substrate concentration

μ : growth rate constant, hr^{-1}

K_d : decay rate constant, hr^{-1}

C_o : the influent toluene concentration, $\mu\text{g toluene L}^{-1}$

C_e : the effluent toluene concentration, $\mu\text{g toluene L}^{-1}$

X : cell mass, mg protein

$\mu_{\max}$: maximum specific growth rate, hr^{-1}

K_{sT} : saturation constant, $\mu\text{g L}^{-1}$

Q : flow rate of gas, L min^{-1}

T_o : influent toluene concentration, $\mu\text{g toluene L}^{-1}$

T_e : effluent toluene concentration, $\mu\text{g toluene L}^{-1}$

X : total biomass in reactor, mg protein

r_T : specific substrate utilization rate, $\mu\text{g toluene (mg protein min)}^{-1}$

k_T : maximum specific toluene utilization rate, $\mu\text{g toluene (mg protein min)}^{-1}$

K_{sT} : half-saturation constant for toluene, $\mu\text{g toluene L}^{-1}$

r_{TCE} : specific TCE utilization rate, $\mu\text{g TCE mg protein}^{-1}$

S_o : influent TCE concentration, $\mu\text{g TCE L}^{-1}$

S_e : effluent TCE concentration, $\mu\text{g TCE L}^{-1}$

R_c : the half reaction of cell material

R_a : the half reaction of the electron acceptor

f_e : the fraction of the electron donor for energy

f_s : the fraction of the electron donor for synthesis

V_x : gas velocity, cm min^{-1}

$[E_c]$: enzyme concentration, mg enzyme L^{-1}

E : enzyme activity, $\text{mg indigo min}^{-1}$

α : proportional constant, $\text{mg enzyme min (L mg protein)}^{-1}$

E_x : normalized enzyme activity, $\text{mg indigo mg protein}^{-1} \text{ min}^{-1}$

k_1 : decay constant, min^{-1}

k_2 : inactivation constant, $\text{mg indigo (min } \mu\text{g TCE)}^{-1}$

k_s' : TCE reaction rate constant, $\mu\text{g TCE (L mg indigo)}^{-1}$

CHAPTER 1

INTRODUCTION

1.1 Introduction

Air emissions of volatile organic compounds (VOCs) generated during treatment of contaminated soils via vapor vacuum extraction technologies (VVE) must often be treated before releasing into the environment. VOCs are also emitted from wastewater treatment plants and chemical process industries. Conventional technologies currently used for the removal of volatile organic compounds (VOCs) are activated carbon adsorption and incineration. Carbon adsorption does not result in the destruction of the contaminant but merely the transfer from the gas phase to the solid phase. Incineration may not be feasible for dilute waste streams. Both activated carbon and incineration involve high capital and operating costs. Biofiltration is a relatively new control technology which utilizes a biofilm supported on porous media to remove volatile organic compounds and odors (Hodge *et al.*, 1995, 1991; Tong *et al.*, 1994). In Europe, biofiltration has been used successfully to control odors, organic, and inorganic air pollutants from a variety of industrial and public sources (Leson *et al.*, 1991). The mechanism of biofiltration (Shareefdeen *et al.*, 1993; Deshusses *et al.*, 1995a, 1995b; Speitel *et al.*, 1993) involves sequential processes: the mass transfer of VOCs from the air stream to the biofilm and the subsequent biodegradation by microorganisms. As a contaminated vapor stream passes through the filter bed, pollutants are transferred from

the vapor phase to the biofilm by diffusion. Microbial utilization depletes the substrate in the liquid film, causing a concentration gradient that promotes the diffusion of more substrate from the gas to the biofilm.

The application of biofiltration (Seed *et al.*, 1994; Basil *et al.*, 1994; Zurlinden *et al.*, 1994) has been recently studied to control VOCs such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and other low molecular weight compounds that are highly soluble in water. Removal efficiencies for VOC often exceed 90 %. Wilson *et al.* (1988) demonstrated the cometabolism of TCE and TCA (1,1,1, -trichloroethane) by a mixed culture growing on n-butane. Initially, removals of 80 to 90 % of incoming contaminants were achieved. However, the efficiency of removal of both chemicals decreased over time. Speitel *et al.* extended the study of Wilson *et al.* (1988) to methanotrophs for biofiltration of TCE and 1,2-dichloroethane(DCA). These bacteria grow aerobically on methane as a carbon and energy source, and form monooxygenase (MMO) enzyme which enables the cometabolism of TCE and DCA. Removals of 20 to 80 % for influent concentrations of 300 to 1,000 µg/L at packed-bed gas-residence times of 5 to 12 min were shown. In the present research, *Pseudomonas putida* F1 was selected because of its ability to degrade TCE and toluene at relatively high rates (Heald *et al.*, 1994; Nelson *et al.*, 1988). Aerobic oxidation of toluene provides carbon and energy to the cell and also induces the formation of toluene dioxygenase enzyme to support cometabolism of TCE (Nelson *et al.*, 1987; Wackett, P. Lawrence *et al.*, 1988; Zylstra, J. Gerben, *et al.*, 1989).

Pseudomonas putida F1 initiates the oxidation of toluene, at the ortho and meta positions of the aromatic ring by incorporating both atoms of molecular oxygen into the

aromatic nucleus to form cis-toluene dihydrodiol. This reaction is catalyzed by the toluene dioxygenase enzyme system. Toluene dioxygenase requires both oxygen and NADH (Nicotinamide Adenine Dinucleotide) as substrates to perform the oxidation. Toluene dioxygenase is nonspecific and can catalyze the oxidation of a variety of chemicals. This property permits the cometabolism of a number of chlorinated aliphatic chemicals, including TCE (Gerben *et al.*, 1989).

To achieve a high degradation efficiency of TCE, competitive inhibition and toxicity effects of TCE to cell growth or/and dioxygenase enzyme should be minimized. The metabolic conditions should also maximize NADH production and enzyme activity. In most previous studies, total biomass concentration was used to describe microbial activity. The biomass concentration alone cannot directly express the status of biofiltration operating conditions because enzyme activity may be affected by TCE toxicity independent of a decrease in cell biomass. Therefore, the enzyme activity becomes an important factor to understand the biokinetics process of cometabolic degradation and to optimize operating conditions.

In this research, this fundamental concept of aerobic TCE cometabolism is extended to the biofiltration of VOC emissions. An attempt will be made to incorporate enzyme activity into the biokinetics of TCE cometabolic oxidation, and to develop a mathematical model for the design and operation of biofiltration.

1.2 Objectives

- 1) To understand the biokinetics of TCE cometabolic oxidation in suspended and immobilized culture with respect to:
 - a) competitive inhibition
 - b) NADH limitation
 - c) enzyme activity
 - d) toxicity
- 2) To evaluate the kinetic parameters of TCE and toluene oxidation by batch and column experiments.
- 3) To develop, calibrate, and validate a mathematical model for design and operating of biofiltration processes based on the biokinetic effects described above.

CHAPTER 2

LITERATURE REVIEW

2.1 Trichloroethylene (TCE)

2.1.1. General description

Trichloroethylene is a nonflammable, colorless liquid at room temperature. TCE is used in dry cleaning, metal degreasing, as a solvent, as a refrigerant, and as chain terminator for PVC production. The widespread use of this compound is declining due to its toxicity. Its use as a solvent is not permitted in some states and the FDA has prohibited its use in food, drugs and cosmetics (ATSDR, 1993).

TCE mostly affects the nervous system. Exposure to very high levels for short times has caused unconsciousness and death. People who breathe moderate levels may have headaches, dizziness, or impaired ability to perform. At high levels, dermal contact can cause rashes. Health effects from long-term exposures have been studied in animals, and animals exposed to moderate levels had enlarged livers. Higher exposures cause liver and kidney damage. The International Agency for Research on Cancer (IARC) has determined that TCE is not classifiable as to its carcinogenicity to humans because of the lack of strong evidence associating cancer in people with exposure to TCE and the mixed or flawed results from animal studies (ATSDR, 1993).

The U.S. Environmental Protection Agency (EPA) set a drinking water standard for TCE of 5 parts per billion (5 ppb) effective January 6, 1989. The Occupational Safety

and Health Administration (OSHA) limits exposure to 50 parts per million (50 ppm) for an 8-hour workday, 40-hour workweek. A level of 200 ppm has been set for a 15 minute average exposure in air any time during the workday (ATSDR, 1993)

The molecular weight of TCE is 131.4 and its specific gravity is 1.4649 @ 20 to 24 °C. The melting and boiling points are - 73 and 87°C, respectively. The solubility in water is less than 1 mg/ml @ 21°C, but it usually shows high solubility in solvents like ethanol, methanol, and acetone etc. The vapor pressure is 60 mmHg @20°C and 77 mmHg @ 25°C (Winholz, M., 1978).

2.1.2 Overview of TCE co-metabolism

TCE is a widely used industrial solvent and has become a significant environmental pollutant because of its toxicity and widespread occurrence in soil, air, and water. TCE is persistent in natural ecosystems and resist to degradation in conventional biological waste treatment systems. Anaerobic biodegradation of TCE produces harmful metabolites such as vinyl chloride (Kleopfer, *et al.*, 1985; Vogel, *et al.*, 1985). Many efforts has been made to find TCE degrading bacteria that can utilize TCE as a carbon and energy source but, these efforts have not been successful. Many additional studies have focused on utilizing oxygenase enzyme systems that are nonspecific and have the potential ability to catalyzes the oxidation of a number of chlorinated aliphatic chemicals, including TCE. Oxygen is needed for aerobic cooxidation of TCE both as a cosubstrate for the oxygenase reaction and as a terminal electron acceptor for cellular respiration. For cometabolism of TCE, one or more substrates must also be supplied as an inducer and as an exogenous electron donor capable of supplying NADH to the cell.

Laboratory studies have shown that TCE can be oxidized by several oxygenase-producing bacteria cultures such as *Methylosinus trichosporium* OB3b, *Pseudomonas ceppacia* G4, *Pseudomonas mendocina* KR-1, *Pseudomonas putida* F1, *Nitrosomonas europaea*, (Burrows *et al.*, 1984; Dalton, H., 1980; Folsom *et al.*, 1990; Higgins I. J., *et al.*, 1979; Landa *et al.*, 1994; 1989 Oldenhuis *et al.*, 1989; Shields *et. al.*, 1992; Stanley *et al.*, 1983; Tsien *et al.*; Vannelli *et al.*, 1991; Winter *et al.*, 1989; Wackett *et al.*, 1988; Arciero *et al.*, 1989) Oxygenase enzymes and the organisms that produce them are shown in Table 2.1. Simplified pathways for the degradation of the inducing substrates for these organisms are shown in Figure 2.1.

Table 2.1. The oxygenases and organisms for TCE oxidation

Organism	Enzyme
<i>Pseudomonas putida</i> F1	toluene dioxygenase
<i>Methylosinus trichosporium</i> OB3b	soluble methane monooxygenase
<i>Nitrosomonas europaea</i>	ammonia monooxygenase
<i>Pseudomonas cepacia</i> G4	toluene 2-monooxygenase
<i>Pseudomonas mendocina</i> KR-1,	toluene 4-monooxygenase

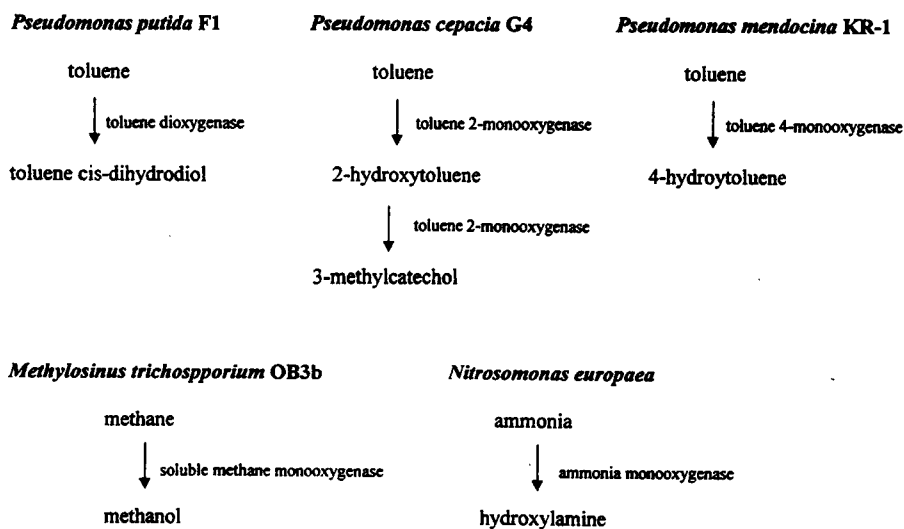


Figure 2.1 The metabolic pathways of primary substrates for oxygenase-producing organisms

2.1.2.1 *Methylosinus trichosporium* OB3b

Methanotrophs are a group of microorganism which grow on methane as a energy and carbon source. They can oxidize a variety of compound such as alkanes, alkenes, and aromatic hydrocarbons (Dalton 1980; Higgins *et al.*, 1979). Methanotrophs utilize the methane monooxygenase (MMO) system to catalyze the incorporation of one oxygen atom from molecular oxygen into methane to produce methanol. Two forms of the enzymes can be distinguished on the basis of their intracellular localization as a soluble (sMMO) and a particulate (membrane-bound) form (pMMO). The sMMO consists of three proteins including the hydroxylase, the reductase, and component B. The sMMO is produced under copper-limited growth conditions and degrades a wider range of substrates than pMMO. (Burrows *et al.*, 1984; Stanley *et al.*, 1983).

Methanotrophs have a much higher rate of TCE conversion than other cometabolic TCE degraders. The methanotroph *Methylosinus trichosporium* OB3b is a type II methanotroph and degrades TCE only when grown under copper limiting conditions such that soluble methane monooxygenase was expressed (Oldenhuis *et al.*, 1989; Tsien, *et al.*, 1989). Tsien *et al.* (1989) found that the expression of soluble methane monooxygenase occurred in culture medium containing 0.25 M or less copper sulfate. Oldenhuis *et al.* (1989) reported that TCE degradation was described by a first-order rate with a rate constant of $2.14 \text{ ml (mg cell min)}^{-1}$ in the concentration range of 0.1 to 0.0002 mM TCE.

TCE and its degradation products have been found to be toxic for cells. The cause of TCE toxicity is the nonspecific reaction of conversion products (epoxide or acylchlorides) with cell components, such as the sMMO hydroxylase component

(Oldenhuis *et al.*, 1989). Wackett and Gibson (1988) reported that the methanotroph *Methylosinus trichosporium* OB3b oxidized TCE at a high initial rate but the rate decrease rapidly with time due to toxicity. TCE epoxide is readily forms covalent bonds with cellular nucleophiles (protein, nucleic acids), thereby disrupting the metabolic functions of the cell. Other possible reactive metabolites are chloral, dichloroacetyl chloride, and formyl chloride. (Miller *et al.*, 1982).

2.1.2.2 *Nitrosomonas europaea*

Ammonia-oxidizing denitrifying bacteria such as *Nitrosomonas europaea* oxidize ammonia to nitrite via an enzyme system known as ammonia monooxygenase (AMO). Ammonia is oxidized to the intermediate, hydroxylamine by AMO: $2\text{H}^+ + 2\text{e}^- + \text{O}_2 + \text{NH}_3 \rightarrow \text{NH}_2\text{OH} + \text{H}_2\text{O}$. Two electrons for the reaction originate in the subsequent reaction by hydroxylamine oxidoreductase (AMO); $\text{H}_2\text{O} + \text{NH}_2\text{OH} \rightarrow 4\text{e}^- + 5\text{H}^+ + \text{NO}_2^-$ (Arciero, *et al.*, 1989). These microorganisms can catalyze the oxidation of carbon monoxide, methane, methanol, ethylene, and propylene through the AMO system (Vannelli *et al.*, 1990). *Nitrosomonas europaea* also is capable of degrading TCE by ammonia monooxygenase. *Nitrosomonas europaea* unlike all other TCE-degrading bacteria, does not require the addition of an exogenous inducer substrate such as toluene, methane, or phenol to induce the enzyme for the TCE degradation (Shields *et al.*, 1992).

Arciero *et al.* (1989) reported that the AMO system has a broad-specificity and catalyzes the degradation of TCE. They reported that TCE degradation occurred at a rate $1 \text{ nmol (mg protein min)}^{-1}$ and that this degradation rate was sustained for many hours.

TCE oxidation by was stimulated by ammonia and inhibited by acetylene, a known ammonia monooxygenase inhibitor.

2.1.2.3. *Pseudomonas cepacia* G4

Nelson *et al.* (1986, 1988) isolated an aerobic gram-negative and rod-shaped bacterium, designated *Pseudomonas cepacia* G4 from a water sample of the Pensacola, Fla. area that had a history of contamination with organochlorine compounds. *Pseudomonas cepacia* G4 requires the addition of an exogenous inducer substrate such as toluene, *o*-cresol, *m*-cresol or phenol for TCE degradation activity because TCE oxidation is a result of the induction of enzymes that catalyze an aromatic degradation pathway. This organism oxidizes toluene in the *ortho*- and *meta*-positions to form 3-methylcatechol. This enzyme system is named toluene 2-monooxygenase and is capable of the non-specific degradation of TCE.

Landa, *et al.* (1994) reported that *Pseudomonas cepacia* G4 was more resistant to high TCE concentrations and degraded TCE at a similar rate to methanotrophic TCE degraders. Folsom *et al.* (1990) reported that the $V_{\max}$ and K_s values for TCE were 8 nmol (min mg protein)⁻¹ and 3 μ M, respectively. Mars *et al.* (1996) found *Pseudomonas cepacia* G4 to be much less sensitive to TCE toxicity than methanotrophs.

2.1.2.4 *Pseudomonas mendocina* KR-1

Pseudomonas mendocina KR-1 initially oxidizes toluene in the *para*-position to form *para*-cresol and also has been observed to oxidize TCE. This enzyme inserts a single atom of oxygen at the *para*-position of toluene and is designated toluene *para*-

monooxygenase (TMO) (Wited, 1986). Winter *et al.* (1989) showed that toluene *para*-monooxygenase degrades TCE when *Pseudomonas mendocina* KR-1 is grown in the presence of toluene. They confirmed the role of the toluene *para*-monooxygenase system by transferring the gene that encode for this enzyme into *E. coli*. They found that the recombinant *E. coli* also degraded TCE. The rate of TCE degradation was 1 to 2 nmol (min mg protein)⁻¹, and this rate decreased with reaction time. Toluene acts both as a growth substrate and an inducer of enzyme synthesis. They reported a threshold concentration of toluene required to induce the enzyme, but found that when toluene concentration is higher than 1 mM, no TCE degradation occurred. This implies that the higher toluene concentration inhibits TCE oxidation reaction by competitive inhibition.

2.2 Toluene dioxygenase enzyme

2.2.1 Toluene metabolic pathway

Pseudomonas putida F1 (PpF1) utilizes toluene as a sole source of carbon and energy for growth. The genes coding for toluene degradation span 9.3 kb in the order Tod FC1C2BADEGIH (Gerben *et al.*, 1989; Ying *et al.*, 1995; Peter *et al.*, 1994). The catabolic pathway of toluene is shown in Figure 2.2. The toluene is first oxidized to *cis*-toluene dihydrodiol through the addition of both atoms of molecular oxygen to the *ortho*- and *meta*-positions on the aromatic ring. This addition occurs through the transfer of electrons from NADH mediated by components A, B, C₁, and C₂ of the Tod system. (Yeh *et al.*, 1977). *Cis*-toluene dihydrodiol is dehydrogenated by NAD⁺ mediated by Tod D to form 3-methylcatechol. Extradiol (*meta*) cleavage by (TodE) leads to the formation of 2-

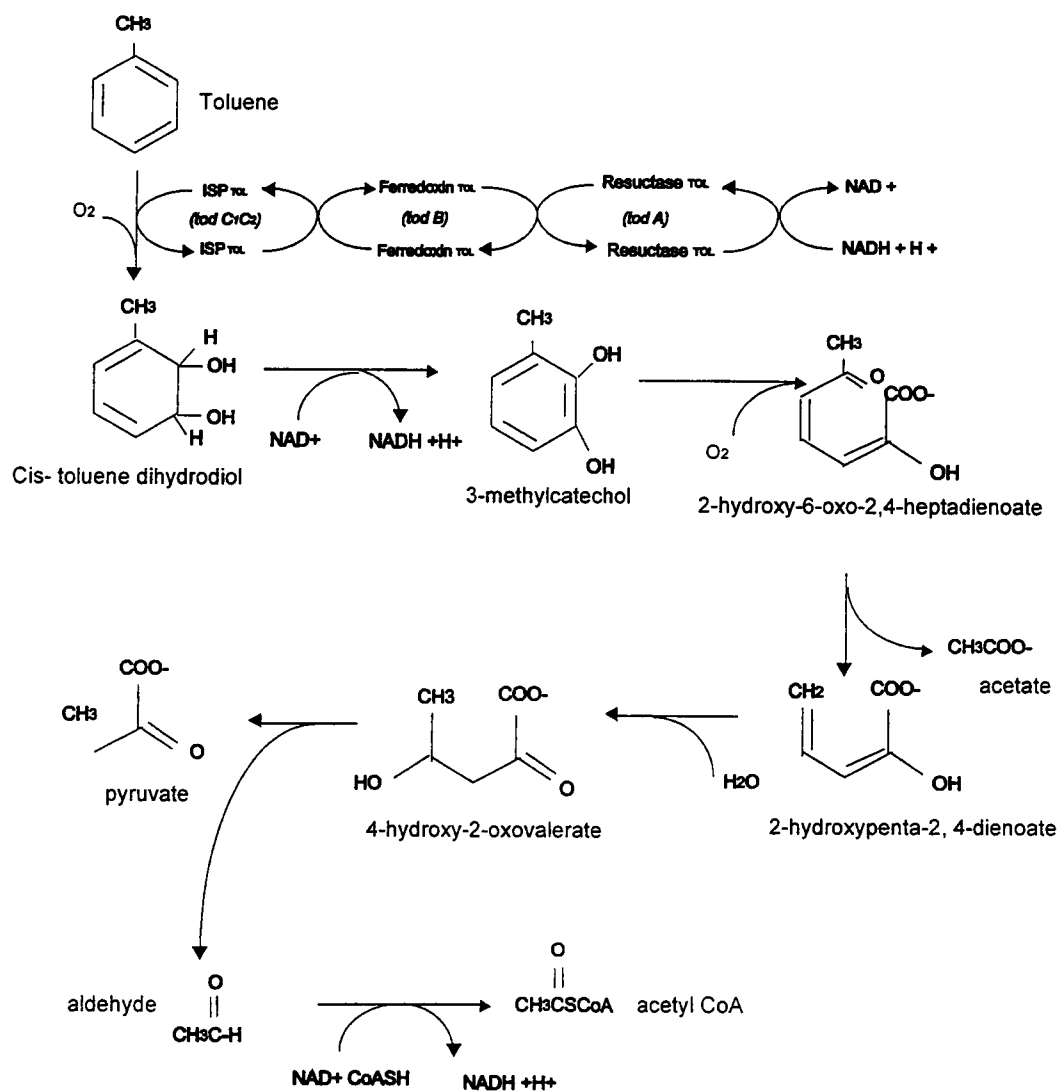


Figure 2.2 Biological steps in conversion of toluene to tricarboxylic cycle intermediates by *Pseudomonas putida* F1 (Lau *et al*, 1994)

hydroxy-6-oxo-2,4-heptadienoate (HOHD), which is further transformed by TodF to form 2-hydroxypenta-2, 4-dienoate (HPD). HPD is further metabolized to tricarboxylic-acid-cycle intermediates by the action components G, H, and I of the Tod system. The final products of toluene metabolism are acetate, pyruvate and acetyl-CoA. The acetate is converted to acetyl CoA using CoA as cofactor (Lubert Stryer, 1988) and pyruvate is also converted to acetyl-CoA using NAD⁺ and CoA as cofactors (Shingler. V. *et al.*, 1992).

2.2.2 TCE metabolic pathway by toluene dioxygenase enzyme

Oxygenase enzymes have been shown to be responsible for the aerobic conversion of TCE, including the methane monooxygenase of methanotrophs (Tsien, *et al.* 1989), the toluene-*ortho*-monooxygenase of *Pseudomonas cepacia* G4 (Shields *et al.*, 1989), and toluene dioxygenase of *P. putida* F1(Wackett *et al.*, 1988). Nonspecific enzymes that catalyze the first step in the oxidation of toluene also oxidize TCE. Simplified metabolic pathways of TCE oxidation by these oxygenase enzyme systems are shown in Figure 2.3.

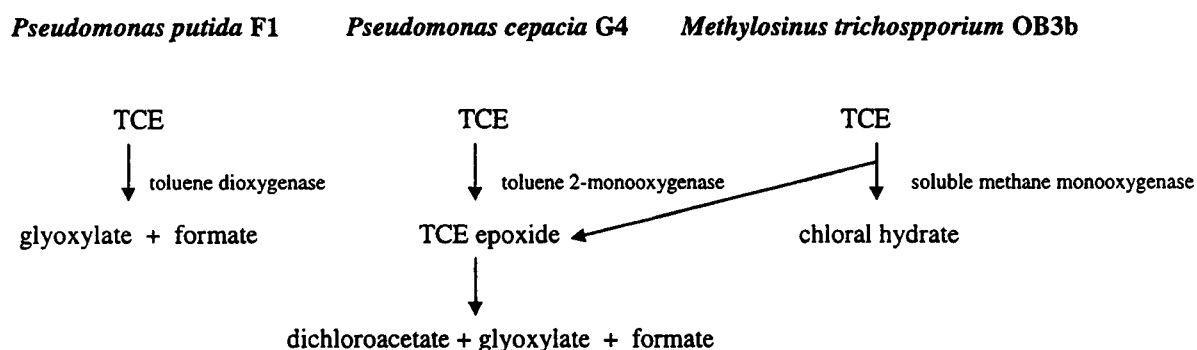
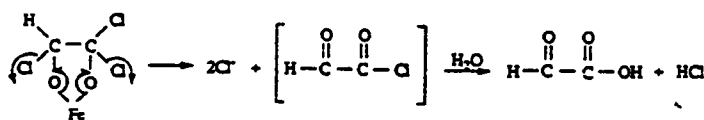


Figure 2.3 The metabolic pathways of TCE by oxygenase enzyme systems (Li *et al.*, 1992)

Toluene dioxygenase catalyzes the dioxygenation of toluene to form cis-toluene dihydrodiol and is implicated in TCE degradation. TCE oxidation by *P. putida* F1 results in cytotoxicity and loss of toluene dioxygenase activity (Wackett *et al*, 1988 and 1989). TCE co-metabolism by *P. putida* F1 is marked by a time-dependent inactivation of toluene dioxygenase. These inhibitory effects stem from the metabolic activation of TCE by toluene dioxygenase to form reactive intermediates that modify intracellular molecules. A higher rate of toxic oxidation product formation may account for the severe inhibition of growth and cell death (Heald *et al*, 1994). Also, the presence of toluene inhibits TCE oxidation through competitive inhibition. Because toluene induces toluene dioxygenase and is the growth substrate for *P. putida* F1, an appropriate balance must be maintained between the enzyme competition and the requirements for cell growth and energy supply. The proposed pathway and intermediates in TCE oxidation by toluene dioxygenase are shown in Figure 2.4. The major products of TCE oxidation are formic and glyoxylic acids, which are derived from glyoxylic chloride and formyl chloride (Li *et al*, 1992).

Reactions leading to glyoxylate



Reactions leading to formate derived from C₁ and C₂

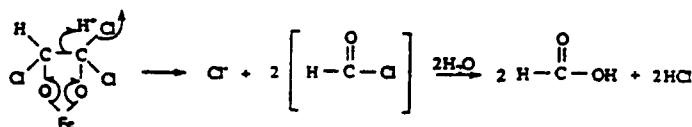
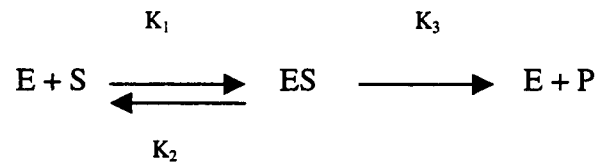


Figure 2.4 Proposed mechanism of TCE oxidation by toluene dioxygenase (Li, et al, 1992)

2.3 Biokinetics of TCE cometabolic oxidation

2.3.1 Michaelis-Menten enzyme kinetics

Michaelis and Menten developed an equation to describe simple enzyme catalyzed reactions (Shuler *et al*, 1992). They assumed that an enzyme-catalyzed reaction involves the reversible combination of enzyme, E, and substrate, S, to form an enzyme/substrate complex, ES, which, in turn, undergoes irreversible decomposition to form free enzyme and product, P. The latter reaction is the rate-limiting step of the overall reaction:



$$V = \frac{dP}{dt} = K_3[ES] \quad (2.1)$$

$$[E] + [ES] = [E_c] \quad (2.2)$$

Where $[E_c]$: total concentration of enzyme in system, mass volume⁻¹

$[ES]$: enzyme/substrate complex concentration of enzyme in system, mass volume⁻¹

$[E]$: free enzyme concentration of enzyme in system, mass volume⁻¹

$[P]$: product concentration in system, mass volume⁻¹

K_1 , K_2 , and K_3 : rate constants for the reactions designated

Application of a quasi-steady state approximation and the assumption of rapid equilibrium between the enzyme and substrate and the $[ES]$ complex, allow equations

(2.1) and (2.2) to be combined to give the Michaelis-Menten equation (Bailey *et al*, 1986):

$$V = \frac{dP}{dt} = \frac{V_{\max} [S]}{(K_m + [S])} = \frac{K_3 [E_c] [S]}{(K_m + [S])} \quad (2.3)$$

Where V : the rate of product formation, mass (volume time)⁻¹

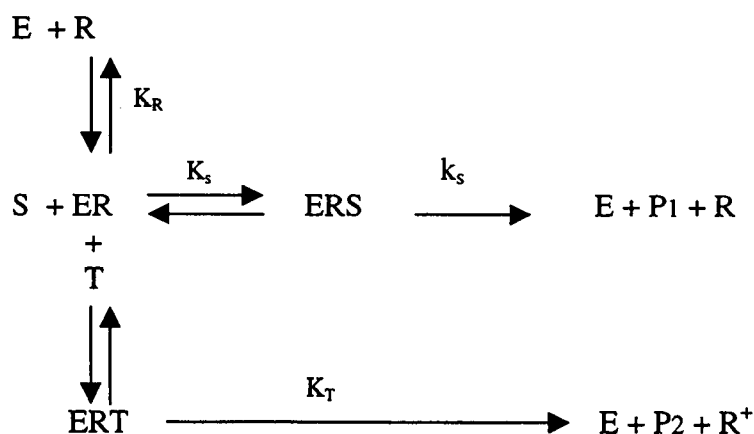
$V_{\max}$: maximum specific substrate utilization rate,
mass (volume biomass time)⁻¹

K_m : the saturation constant and is equal to the substrate concentration when the reaction rate is equal to $V_{\max} / 2$, mass volume⁻¹

$[S]$: aqueous substrate concentration, mass volume⁻¹

2.3.2 Enzyme kinetics for multiple competing substrates

The enzyme kinetics of TCE and toluene cometabolism can be explained by the kinetics of multiple competing substrates in the presence of a co-factor (e.g. reductant). A competitive inhibitor is a substrate that combines with free enzyme in a manner that prevents substrate binding. That is, inhibitor and the substrate cannot bind to the same active site of the enzyme. Bailey (1986) describes the reaction sequence and enzymatic kinetics:



$$[E_o] = [E] + [ER] + [ERT] + [ERS] \quad (2.4)$$

$$K_R = \frac{[ER][S]}{[ERS]} \quad K_T = \frac{[ER][T]}{[ERT]} \quad K_R = \frac{[E][R]}{[ER]} \quad (2.5)$$

Where [S] : TCE concentration, mass volume⁻¹
 [T] : toluene concentration, mass volume⁻¹
 [R] : the NADH concentration, mass volume⁻¹
 [ER] : the enzyme-NADH complex concentration, mass volume⁻¹
 [ERS] : the enzyme -NADH - TCE complex concentration, mass volume⁻¹
 [ERT] : the enzyme -NADH - toluene complex concentration, mass volume⁻¹
 [R] : the NAD⁺ concentration, mass volume⁻¹

Assuming equilibrium between the enzyme and substrate to form [ER], [ERS] and [ERT] complexes, the final equations for TCE oxidation and toluene by toluene dioxygenase enzyme are:

$$V_s = -\frac{dS}{dt} = \frac{k_s[E_c][S][R]}{([R][S] + K_s^{app}[R] + K_s K_R)} \quad (2.6)$$

$$V_T = -\frac{dT}{dt} = \frac{k_T[E_c][T][R]}{([R][T] + K_T^{app}[R] + K_T K_R)} \quad (2.7)$$

$$K_s^{app} = K_s \left(1 + \frac{[T]}{K_T}\right) \quad (2.8)$$

$$K_T^{app} = K_T \left(1 + \frac{[S]}{K_S}\right) \quad (2.9)$$

2.4 Biofiltration

2.4.1 General description

Biofiltration is a technology that utilizes microorganisms supported on a porous solid media such as compost, peat, or soil to remove air contaminants such as volatile organic and inorganic compounds and odors. As a gas stream containing biodegradable contaminants passes through the filter bed, contaminants are transferred from the gas phase to the biofilm and are metabolized by the microorganism in the biofilm. (Tahraoui *et al.*, 1994; Yavorsky *et al.*, 1993). This technology is relatively recent compared to the conventional methods such as activated carbon adsorption, wet air scrubbers and thermal processes, catalytic combustion and fluidized bed oxidation. Activated carbon is the most predominant control technology to remove unwanted VOCs from waste gases, but this process only entails the transfer of the contaminant from the gases phase to the solid phase and does not lead to the destruction of the pollutants. This means that a secondary disposal or treatment technology will be needed. Conventional technologies are characterized by high operating and maintenance cost. Biofiltration may be a promising new technology that not only removes but also destroys the contaminants (Hodge *et al.*, 1995, 1991; Tong *et al.*, 1994).

Biological treatment technologies in the field of wastewater treatment and bioremediation have been studied intensively in the United States, but there are a few studies and applications of biological processes for the removal inorganic and organic air contaminants from waste gases (Leson, 1991).

In Europe, biofiltration has been used widely to remove air pollutants and odors from various sources. The first application of biofiltration was reported in the control of

H₂S emissions from sewage treatment plants in Germany (Bach, 1923). Specially, Germany and The Netherlands have developed biofiltration since the 1960s to be used to control VOC and odors. In the 1960s, most research was focused on the empirical approaches to determine the efficiency of biofiltration and selection of design parameters (Dartwa, 1968). Since the early 1980s, new federal regulations requiring the control emissions of VOC and air toxics emissions has resulted in biofiltration being used increasingly to control odors, and VOCs from a variety of sources such as industrial plants (Luft 1986).

Due to the lack of air quality regulation programs and governmental financial support for research, biofiltration has not been studied widely in the U. S. However, the 1990 Amendments to the Clean Air Act have caused the strong interest in reducing VOCs from waste air streams. Interest in biofiltration as an economical air pollution control technology for removing VOCs and in meeting the statutory emission regulations is increasing.

2.4.2 Biofiltration for volatile organic compounds

Biofilters have been studied extensively for degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) and other low molecular weight compounds. They are constructed of one or more beds of support media such as peat, compost, wood chips, or soil, ceramic, plastic, or celite. The biofilm grows on the support media.

Ottengraf et al. (1983) evaluated the degradation capacity of a biofilter for a mixture of ethyl acetate, butylacetate, toluene, and butanol. The maximum zero-order biodegradation rate for these compounds ranged from $g\ 20\ to\ 40\ HC\ (m^3\ hr)^{-1}$. Hodge *et*

al. (1991) presented an experimental investigation of biofiltration for purification of waste gases contaminated with hydrocarbon vapors (JP-5 jet fuel and diesel fuel vapors). Four filter media were tested: a soil mixture, granular activated carbon (GAC), sintered diatomaceous earth, and a mixture of activated carbon and diatomaceous earth (50% each, by volume). They found that jet fuel was degraded much faster than diesel fuel. For the JP-5 fuel, a mixture of activated carbon and diatomaceous earth bed showed the greatest biodegradation rate ($5.4 \text{ g HC (m}^3 \text{ hr)}^{-1}$) while diatomaceous earth was least effective $0.4 \text{ g HC (m}^3 \text{ hr)}^{-1}$. For the diesel fuel, the degradation rate in the mixed media column (activated carbon and diatomaceous earth) was similar to that in the column of activated column ($1.3 \text{ g HC (m}^3 \text{ hr)}^{-1}$).

Peter *et al.* (1993) investigated the feasibility of treating a fuel-contaminated gas stream with flow through, bench-scale biofilters. The biofilter columns were 36 inch deep beds of biofilter material in 60 inch tall sections of 6 inch diameter clear PVE pipe. The influent loading was about 400 ppm/ft^2 expressed as total organic carbon. During the period of study, organic carbon removal efficiencies ranged from 60 to 99 %. During peak efficiency, removal exceeded 99% for BTEX compounds and n-alkanes between C_6 and C_8 , and 80 to 95 % for n-pentane and other C_5 to C_7 range hydrocarbons.

Zurlinden *et al.* (1994) used a pilot-scale biofilter to determine the feasibility of biofiltration for the treatment of airborne VOCs and developed design parameters for full-scale operation. The bioreactor consisted of a 3.5 feet diameter by 7 feet tall bed with an internal sprinkling system. The initial TPH (total petroleum hydrocarbon) concentration was about 200 ppm and the efficiency of VOC removal (measured as TPH concentration) varied from 55 to 90 %. They also found that the vapor pressure of the

compound was inversely proportional to the removal efficiency while the solubility of the compound was proportional to the removal efficiency.

Togna (1994) used a biofilter packed with conventional scrubber packing material to treat air contaminated with gasoline vapors containing 20% BTEX. The removal of BTEX was up to 0.5 kg COD (m³ d)⁻¹.

Sorial *et al.* (1997) used a pilot-scale biofilter packed with celite pellets (1.14 m bed height, 0.146 m internal diameter) to treat BTEX compounds. They studied operational parameters such as BTEX loading, empty bed contact time (EBCT), backwashing frequency and duration, recovery of biofilter removal efficiency after back washing, and nutrient-phosphorous limitation as a mean of biomass control. They reported a removal efficiency of 99% for BTEX loadings of 1.8 kg COD (m³ d) and 88% for loading of 6.2 kg COD(m³ d)⁻¹.

2.4.3 Biofiltration for TCE

The application of biofiltration to treat the chlorinated compounds has been intensively studied at the laboratory scale. The biodegradation of chlorinated compounds requires a primary substrate to provided an energy/carbon source and to induce the necessary enzymes responsible for metabolism of target compounds.

Wilson *et al.* (1988) demonstrated the first bioreactor study for the cometabolism of TCE and TCA(1,1,1,-trichloroethane) by a mixed culture growing on n-butane. Initially, 80 - 90 % removals of incoming contaminants were achieved. However, the efficiency of removal of both chemicals decreased over time. Speitel *et al.* (1993) studied the treatment of TCE and 1,2-dichloroethane(DCA) using methanotrophs and

developed a mathematical model for gas-phase biofilm reactor design. This bacteria utilizes the monooxygenase (MMO) enzyme for the aerobic catabolism of methane and to fortuitously degrade TCE and DCA. Removal efficiencies of 20 to 80 % for influent concentrations of 300 to 1,000 ug/L at packed-bed gas-residence times of 5 to 12 min were achieved.

Watwood *et al.* (1995) studied the effect of packing materials for TCE removal. Small reaction flasks were used to investigate the capability of gaseous TCE biodegradation in finished compost materials. Also, the enhancement of TCE degradation by enriching the compost materials with propane and methane was studied. Removal efficiencies of 85 to 95 % at 5 ppmv initial TCE concentration were achieved with and without enrichment with propane gas, respectively. Sukesan *et al.* (1997) extended the study of Watwood *et al.* (1995) using a biofilter packed with two different packing materials: deciduous leaf debris compost and woodchip and bark compost. They found that there were significant differences in TCE removal efficiency between the two composts and that TCE removal increased with propane and methane stimulation.

Devinny *et al.* (1995) used two biofilters (7.9-cm in diameter and 100-cm deep) to test whether biofilters are an effective technology for treating the perchloroethylene (PCE) and trichloroethylene (TCE). One biofilter was filled with carbon and the other one with compost as the support medium. They found that the carbon media biofilter performed better for removal of PCE than the compost media biofilter, but for TCE removal, the two biofilters showed essentially equal performance. They concluded that the bench-scale biofilters can provide partial removal of PCE and TCE. If it is applied in

conjunction with traditional activated carbon treatment, carbon consumption will be reduced.

Kim (1997) succeeded in treating TCE and tetrachloroethylene (PCE) using two activated carbon biofilters with phenol-oxidizing microorganisms. Virtually complete removal of gaseous TCE and PCE were obtained at residence times of 4 to 7min, and at average inlet concentrations of 47 to 85 ppm, respectively. The ranges of transformation yield for TCE and PCE were about 8 to 48 g TCE/ g phenol and 6 to 25 g PCE/g phenol.

A field test for in-situ biofiltration for the remediation of groundwater contaminated with TCE was conducted. A mixture of 1800 L groundwater and methanotrophic bacteria containing about 5.4 kg dry weight of methane monooxygenase (MMO) was injected into an aquifer through a single well. The TCE concentration in the extracted groundwater decreased to 10 ppb during the first 50 hr of withdrawal (Duba *et al.*, 1996).

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of *Pseudomonas putida* F1

Pseudomonas putida F1 was a gift from David T. Gibson (University of Iowa). *P. putida* F1 was routinely cultivated in modified Hunters medium. The medium consisted of three solutions; the inorganic constituents for each solution are shown in Table 3.1 and 3.2. The media was prepared by combining 20 ml of solution B and 5 ml of solution C in 1 L phosphate buffer solution. The phosphate buffer of Hunters medium was replaced with KH_2PO_4 (2.15 g/L) and K_2HPO_4 (5.3 g/L). The final pH was 7.0. Solution B and C were sterilized by micro-filtration and preserved by refrigerator at 5°C. Toluene (100 μl) was supplied in the vapor phase in a glass bulb suspended above the media.

Table 3.1. Nutrient solutions

Compound	Solution B (mg/L)	Solution C
Nitrilo triacetic acid	10	60 g / 300 ml H_2O
MgSO_4 or $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	14.45	
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	3.33	
$(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	9.25	
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	99	
Metals 44	50 ml	
$(\text{NH}_4)_2 \text{SO}_4$		

Table 3.2 Metals 44 Solution

Compound	mg /100 ml
EDTA	250
ZnSO ₄ 7H ₂ O (250 mg Zn)	109.5
MnSO ₄ H ₂ O (50 mg Mn)	154
FeSO ₄ 7 H ₂ O (100 mg Fe)	500
CuSO ₄ 5 H ₂ O (10 mg Cu)	39.20
Co(NO ₃) ₂ 6 H ₂ O (5 mg Co)	24.80
Na ₂ B ₄ O ₇ 10 H ₂ O (2 mg B)	17.10

3.1.1 preparation of cells for batch experiments

Pseudomonas putida F1 was inoculated from frozen stock cultures and grown in 50 ml of toluene-Hunters medium. Flasks were incubated at room temperature with shaking (200 rpm). When growth reached late exponential phase ($OD_{600} = 0.8- 1.0$), 0.5 ml was transferred to 50 ml fresh medium. Cells were harvested ($OD_{600} = 0.8$) by centrifugation at $10,000 \times g$ for 10 minutes at 4 °C. Cells were washed twice with phosphate buffer (pH 7.0), resuspended in Hunters medium to a cell density of 1.0, and placed on ice for batch experiments.

3.1.2 Inoculum for column experiment

Pseudomonas Putida F1 was grown in 500 ml of medium with toluene supplied in the vapor phase at room temperature. After an yellow color appeared in the culture, the culture was transferred into fresh growth medium containing diatomaceous earth pellets (Celite) in a 2 L flask. The flask was then placed on a shaker to provide mixing and an opportunity for microbial attachment to the diatomaceous earth, but a low rpm was maintained to avoid erosion of the pellets. The diatomaceous earth pellets were

transferred to the column and an air -toluene gas mixture was supplied to the biofilter for an acclimation period of 4 days.

3.2 Reactor design

3.2.1 Biofiltration reactor

Figure 3.1 shows the experimental arrangement. Three different glass biofiltration columns were used during the course of the research. Initially, a column 36 cm long by 2.5 cm in diameter was used. Five sampling ports sealed with Mininert caps were distributed along the length of the column. Gas samples could be taken through the cap with a gas-tight syringe. The caps could be removed for collecting biomass samples from the filter bed. To achieve longer EBCTs using the same mass flow controllers, this column was replaced with a column 100 cm in height with an inner diameter of 10 cm. Five sampling ports were supplied along the length of the column.

The third reactor was a differential volume reactor (DVR) constructed to measure immobilized cell growth and substrate utilization kinetic parameters. Shreve *et al.*(1993) suggested that the rates of growth and substrate utilization in immobilized cells may not be identical to those of suspended cultures. The DVR was designed to allow kinetic parameters to be measured for immobilized cells. The DVR was 10 cm in diameter by 10 cm high; a bed depth of 1 cm was used. The effluent gas and nutrient lines of the DVR were connected to the 100 cm long column to further degrade toluene and TCE, and to keep the larger column active for future biofiltration experiments.

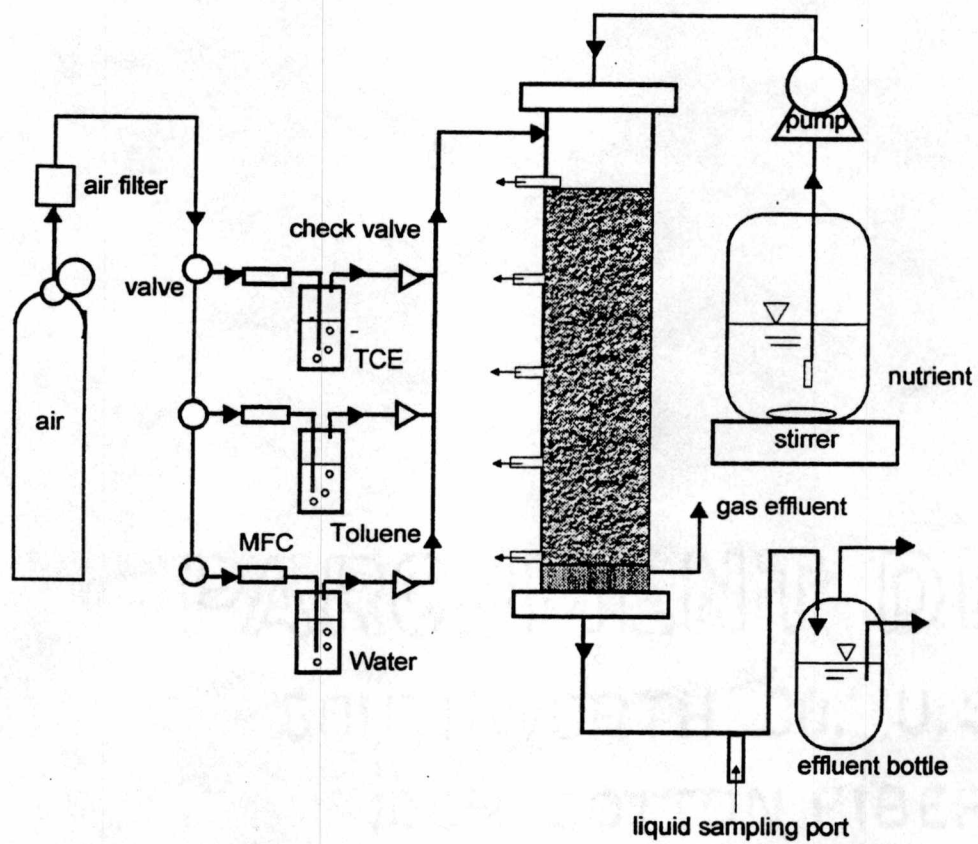


Figure 3.1 The experimental arrangement

In each column gaseous and aqueous media flowed cocurrently from top to bottom. Each column was packed with Celite Biocatalyst Carrier R-635 (Manville, Denver, Colo.), which provided the physical support for the biofilm. The Celite was washed several times with deionized water and dried in an oven at 105 °C over night. An aqueous solution was introduced at the top of column using a peristaltic pump fitted with Tygon tubing; the solution also provided moisture to the biofilm. Except for the pump tubing, all tubing was Teflon or stainless steel. The fittings were ¼ inch stainless steel. The three flasks contained pure TCE and toluene for generating contaminated gas, and sterilized deionized water for humidifying the air stream. The flow rate to each flask was controlled independently by individual mass flow controllers. The range of volumetric flow rate for the mass flow controllers supplying air to the flasks containing TCE, toluene, and water were 0 to 10, 0 to 250, and 0 to 1000 mL min⁻¹, respectively. The influent concentration of TCE and toluene were controlled by the flow rate of the respective gas streams and the amount of pure solvent in each flask. All apparatus including tubing, reactor, Celite and flasks were autoclaved prior to start-up.

3.2.2. Steady-state condition in biofiltration

Enzyme activity, NADH concentration, toluene and TCE removal were measured after the biofilter column reached steady-state. Steady-state conditions were rapidly achieved for each feed concentration once the biofilm was fully developed about two month after the column was inoculated with *Pseudomonas putida* F1. To determine when steady-state conditions had been achieved, the biomass and toluene concentrations were measured each day. When two consecutive daily biomass measurements differed by less

than 10 %, the biofilm was assumed to have reached steady-state (Shreve et al, 1993). In addition, it was required that two consecutive measurements of toluene removal efficiency must differ by less than 5 %. Each time the toluene feed concentration was changed, steady-state conditions had to be reestablished. Usually, 3 to 4 days were required to reach the steady-state conditions.

3.3 Analytical methods

3.3.1 Toluene and TCE measurement

TCE and toluene concentrations were determined by GC. Gas samples were taken with a 25 μ l gas-tight syringe from the sampling ports on the column and from the headspace of vials for the batch experiments. Triplicate gas samples for each sample were taken and injected into a Shimadzu GC AM gas chromatogram (Shimadzu Analytical Instruments CO., Kyoto, Japan) equipped with a capillary column. An electron capture detector (ECD) and flame-ionization detector (FID) were conneted in series. Helium (6.8 ml/min) and nitrogen (49.62 ml/min) were used as the carrier and make up gas, respectively. The flow rate of hydrogen and oxygen for the FID detector were 35.5 ml/min and 425.5 ml/min, respectively. The GC oven was operated isothermally at 150 $^{\circ}$ C and both the detector and the injector were maintained at 250 $^{\circ}$ C. TCE and toluene concentrations were determined by comparison of the peak areas of the samples to the peaks areas of standards. Toluene and TCE had an average retention time of 4.2 and 3.9 min, respectively. The gas-phase standards of TCE and toluene were prepared by making 5 ml aqueous standards in 25 ml vials sealed with Mininert caps using Henry's law determine the gas-phase concentration (Speitel Jr. *et al.*, 1993;

Tschantz, 1996). Aqueous samples were collected into 5 ml Mininert cap vials and analyzed by injecting gas samples from the headspace of the vials into GC (Tschantz, 1996).

The Henry's law constant is expressed as a function of temperature (LaGrega *et al.*, 1994):

$$H = \exp\left(A - \frac{B}{T}\right) \quad (3.1)$$

Where H : Henry's law constant (atm · m³ mol⁻¹)
T : Temperature (Kelvin), 296.2 (at 23 °C)
A : 5.13 for toluene, 11.4 for TCE
B : 3.02 x 10³ for toluene, 4.78 x 10³ for TCE

The room temperature during batch and reactor experiments was at 23 °C throughout all experiments. According to the above equation, a Henry's law constant of 6.309 x 10³ atm m³ mol⁻¹ (dimensionless mass units 0.2656) was used for toluene calculations. A Henry's law constant of 8.758 x 10⁻³ atm m³ mol⁻¹ (dimensionless mass units 0.3687) was used for TCE calculations.

Units of µg L⁻¹ and ppmv were used. The relationship between these units are given by the ideal gas law (Masterton, *et. al.*, 1997).

$$PV = nRT \quad (3.2)$$

Where P : pressure, atm
V : volume, L
T : Kelvin temperature, K

R : ideal gas law constant , 0.0821 L atm mol⁻¹ K⁻¹
 n : number of moles

The equation (3.2) was rearranged to equation (3.3).

$$\begin{aligned}
 P &= \frac{nRT}{V} \\
 &= \left(\frac{\text{mol number}}{L} \right) \cdot \left[\frac{0.0821 \text{ atm L}}{\text{mol } ^\circ\text{K}} \right] \cdot 290 \text{ } ^\circ\text{K} \cdot \left[\frac{1 \text{ mol}}{M_{wt}} \right] \\
 &= \left(\frac{\text{mol number}}{L} \right) \cdot \left[\frac{24.466 \text{ atm L}}{M_{wt} 1000 \text{ mg}} \right] \quad (3.3)
 \end{aligned}$$

Because a partial pressure of 1 atm is equivalent to 10⁶ ppmv, equation (3.3) was rewritten as follows:

$$\begin{aligned}
 P &= \left(\frac{X \text{ mg}}{L} \right) \cdot \left[\frac{24.466 \text{ atm L}}{M_{wt} \cdot 1000 \text{ mg}} \right] \cdot \left[\frac{10^6 \text{ ppmv}}{\text{atm}} \right] \\
 P &= \left(\frac{X \text{ mg}}{L} \right) \cdot \left[\frac{24.466 \times 10^6 \text{ ppmv L}}{M_{wt} \cdot 1000 \text{ mg}} \right] \quad (3.4)
 \end{aligned}$$

Where M_{wt} = molecular weight

A sample calculation for toluene is shown below. Conversion of 0.191 mg/L toluene to ppmv is performed using equation (3.4):

$$\begin{aligned}
 P &= \left(\frac{X \text{ mg}}{L} \right) \cdot \left[\frac{24.466 \times 10^6 \text{ ppmv L}}{M_{wt} \cdot 1000 \text{ mg}} \right] \\
 &= \left(\frac{0.191 \text{ mg}}{L} \right) \cdot \left[\frac{24.466 \times 10^6 \text{ ppmv L}}{92.14 \times 1000 \text{ mg}} \right] \\
 &= 50.72 \text{ ppmv}
 \end{aligned}$$

3.3.2 Absorbance, fluorescence, and protein

Optical density (OD₆₀₀) and indigo production (A₆₀₀) were measured on a Beckman Spectrophotometer (model DU-70; Fullerton, CA). NADH and indoxyl were measured by fluorescence (Model 240, Perkin-Elmer, Inc., Buckinghamshire, England).

A BCA protein assay reagent kit was used for protein determination. BAC reagent was a 1000 ml solution containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2N sodium hydroxide. BAC reagent B was a 25 ml solution containing 4 % cupric sulfate. Albumine serum albumin (BSA) (2 mg/ml in 0.9 % sodium chloride and 0.05 % sodium azide) was used as a protein standard. The principle of this assay is the reduction of Cu⁺² to Cu⁺¹ by protein in an alkaline medium. Cuprous cation (Cu⁺) was detected colorimetrically using a unique reagent containing biocinchoninic acid. A purple-colored reaction product was formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentrations over a range of 20 µg/ml to 2000 µg/ml. The BCA-protein reaction scheme is:

Protein (peptide bonds) + Cu^{+2} $\longrightarrow$ tetradentate- Cu^{+1} complex

Cu^{+1} + 2Bicinchoninic acid (BCA) $\longrightarrow$ BCA- Cu^{+1} ternary complex (purple colored)

Figure 3.2 shows the relationship between protein concentration and OD_{600} of *P. putida* F1 in the nutrient rich medium. *Pseudomonas putida* F1 was grown in 50 ml of toluene-Hunters medium with toluene. Toluene was supplied to the vapor-phase of the flask by diffusion from a glass tube suspended in the headspace of the flask. Flask was incubated at room temperature with shaking (200 rpm). One milliliter of sample was taken regularly for measuring protein and OD_{600} . The protein concentration and OD_{600} of *P. putida* F1 in suspended culture is characterized by a linear relationship with a slope of 192.56 and a correlation coefficient of 0.99. This relationship was used to convert OD_{600} to protein concentration.

3.3.3 Determination of NADH

One milliliter of sample culture was transferred into a microcentrifuge tube. The cells were harvested and washed with phosphate buffer solution (pH=7.2) using a centrifuge for one minute at 14000 rpm at 4 °C in order to remove intermediates that may affect fluorescence readings. A mixture of 100 μl of cells with 900 μl of deionized water was injected into the fluorescence detector, utilizing 340 nm and 425 nm for the excitation and emission wavelengths, respectively. The concentration of NADH in whole cells was expressed as $\mu\text{mol NADH/ mg protein}$.

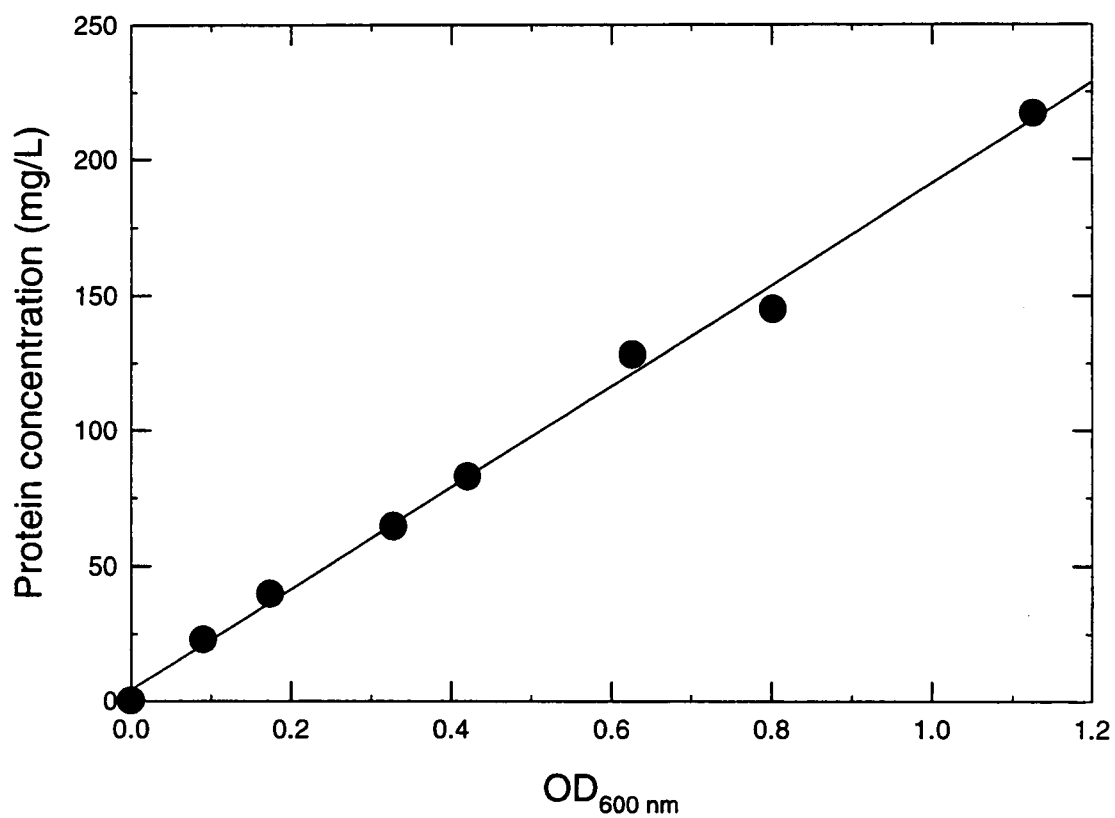


Figure 3.2 The relationship between protein concentration and OD₆₀₀ of *Pseudomonas putida* F1 in nutrient rich medium

3.3.4 Preparation of immobilized cells samples.

The assays for the normalized enzyme activity, NADH and protein concentration in whole cells were applied to immobilized cells from the biofiltration reactor. Two pellets taken from the each stage of the biofiltration column were mixed with 2 ml of sterile 0.1 % $\text{Na}_2\text{P}_2\text{O}_7$ and vortexed to detach immobilized cells from the pellets. The cell mixture was centrifuged and washed with phosphate buffer solution (pH=7.2). The final cell pellets were resuspended with 5 ml of fresh medium. The cells were sonicated three times for 3 seconds, each with a 30 second rest period between each. The prepared cell culture was used to measure the normalized enzyme activity, NADH and protein concentration.

3.4 Preparation of batch experiments

Batch aqueous-phase degradation experiments were performed to understand TCE and toluene cometabolic degradation and inhibition effects by both compounds, to investigate the relationship between TCE degradation and enzyme activity, and the inactivation of enzyme activity during TCE degradation.

Fifty milliliters of medium were inoculated with *Pseudomonas putida* F1 from a frozen stock culture in a 300 ml flask. Toluene was supplied to the vapor-phase of the flask by diffusion from a glass tube suspended in the headspace of the flask. Upon reaching late exponential growth ($\text{OD}_{600} = 0.8$ to 1.0) 500 μl of cells were transferred to 50 ml of fresh media. The cells were harvested again at late-exponential growth phase by centrifuging (15 ml at 1000rpm), resuspending them in fresh media at the desired OD, and preserving them in ice until needed. Batch experiments were conducted in 25 ml

Mininert cap vials. Five milliliters of cell suspension were dispensed into 25 ml vials with Mininert caps and the desired amount of toluene and/or TCE were added via syringe injection of saturated aqueous solutions. Experiments were conducted in triplicate. The vials containing the cells were placed on the shaker for continuous mixing. Killed-cells controls were used to verify losses due to volatilization or sorption. Killed-cells were prepared in vials boiled in a water bath at 100 °C for one hr.

CHAPTER 4

ENZYME ACTIVITY EXPERIMENTS

Yeh *et al.* (1977) measured the toluene dioxygenase activity in cell extracts by using [methyl- ^{14}C] toluene. Toluene dioxygenase is known to oxidize indole to indigo via indoxyl, a soluble yellow dye (Figure 4.1) (Ensely, *et al.* 1983). Jenkins *et al.* (1985) measured the toluene dioxygenase activity in cell extracts via the rate of increase in absorbance of indoxyl at 400 nm by spectrophotometer. However, this method is inadequate to measure the whole cells enzyme activity when interference by suspended biomass is present. A fluorescence-based method of measuring Tod activity based on the fluorescence of nidoxyl is developed in the present work. The principle is similar to that used in to quantify the nerve gas sodium perborate based on its reaction with indole to form the highly fluorescence compound indoxyl (Gehauf, *et al.*, 1957). The potential advantages of the fluorescence-based method compared to spectrophotometric methods are less interference by particles of suspended biomass and greater sensitivity. In this chapter, a fluorescence-based assay is described which measures intracellular Tod activity in a biofilm culture of *Pseudomonas putida* F1 as a tool for assessing the cometabolic degradation in fixed-film reactors. Other goals are to characterize the whole-cell enzyme activity of *Pseudomonas putida* F1 during growth, to determine if NADH is a potential limiting factor in the expression of whole-cell Tod enzyme activity, and to determine the half-life of the enzyme.

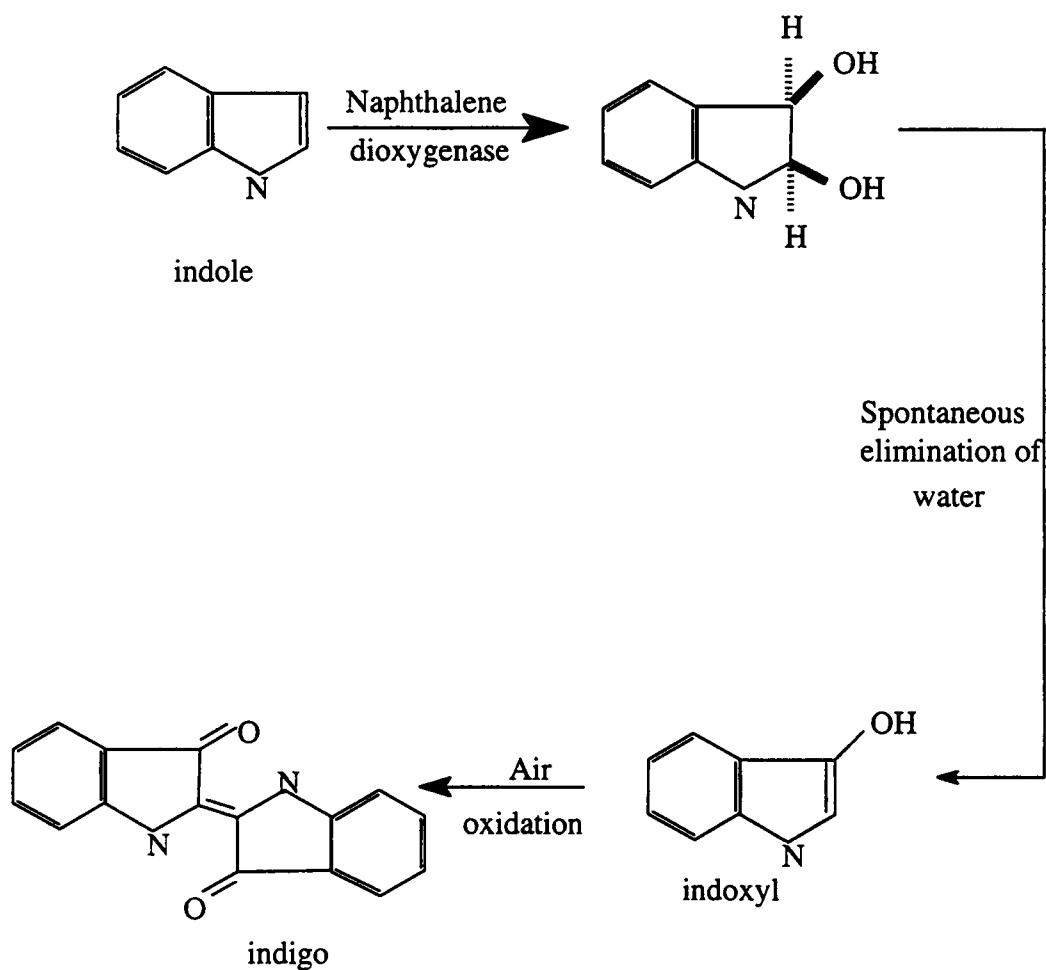


Figure 4.1 Proposed pathway for indigo biosynthesis by dioxygenase enzyme (Ensley *et al.*, 1983)

4.1 Determination of optimum emission wavelength for indoxyl.

To determine the optimum emission wavelength for indoxyl, emission scanning for indoxyl was required. Gehauf *et. al.*(1957) reported that the emitted fluorescent radiation of indoxyl ranged from about 460 to 490 nm. An optimum emission wavelength for indoxyl might be between that range. Because indoxyl was not available as a commercial product, a culture of *Pseudomonas putida* F1 with addition of indole solution was used to produce indoxyl from indole.

P. putida F1 was grown in 50 ml of toluene-hunters medium. When growth reached late exponential phase ($OD_{600} = 0.8$ to 1.0), the cells were harvested ($OD_{600} = 0.8$) by centrifugation at $10,000 \times g$ for 10 minutes at $4^{\circ}C$. Cells were washed twice with phosphate buffer (pH 7.0) then resuspended in Hunter medium to a cell density of 1.0. One milliliter of sample culture was transferred into a 1.5 ml microcentrifuge tube. The enzyme reaction was initiated by addition of $5 \mu l$ of 10 mM of indole solution in DMF (*N,N*-dimethylformade). After 2 minutes incubation at room temperature, the cells were centrifuged at $4^{\circ}C$. The supernatant containing the indoxyl was introduced to the fluorescence detector. Emission scanning was performed over the range 400 to 700 nm, using an excitation wavelength of 365 nm. The emission spectrum for indoxyl is shown in Figure 4.2. The peak emission occurred in the range of 460 to 490 nm; these results were in agreement with those reported by Gehauf *et al* (1957). A wavelength of 470 nm was chosen as the emission wavelength for further experiments.

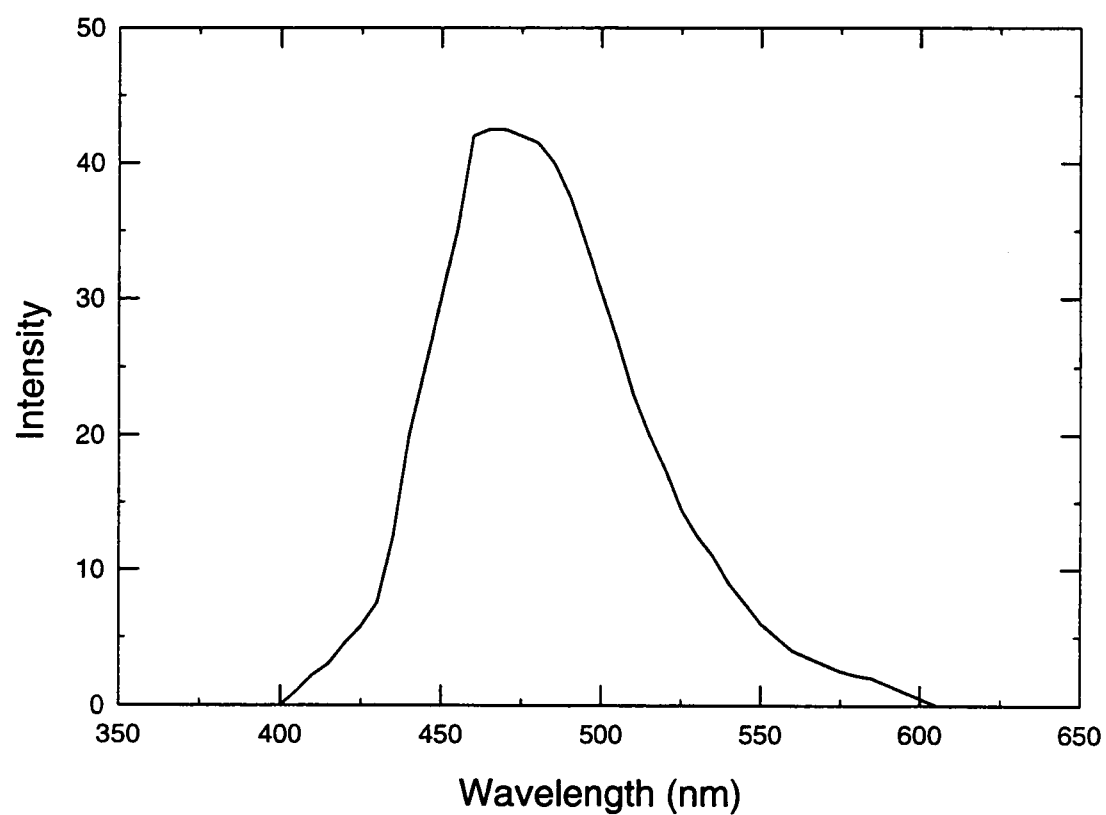


Figure 4.2 Indoxyl emission spectra for excitation at 360 nm

4.2 Comparison of enzyme activity assay.

Because indoxyl was not available as a commercial chemical, a method was required to convert indoxyl fluorescent intensity to enzyme activity. Figure 4.1 shows that toluene dioxygenase oxidizes indole to indigo, a blue dye, via indoxyl, a yellow dye. Indigo production, which can be quantified using standards made from commercial available chemical, can be related to indoxyl production as described below.

P. putida F1 was grown as described in section 3.3.1. After the cells were washed twice with phosphate buffer (pH 7.0), they were resuspended in Hunter medium to an OD₆₀₀ of 1.0. One milliliter of samples culture were transferred into 1.5 ml microcentrifuge tubes and into 1.5 ml cuvettes (1 cm path length). The cells in cuvette were used for determining the optical density of the culture at 600 nm, and to determine spectrophotometrically the indigo formation as a function of time. Five micromilliliter of 100 mM indole in DMF (*N,N*-dimethylformade) was added to the cells and the enzyme reaction initiated. The formation of indigo was monitored spectrophotometrically at 600 nm over the reaction time against a cell blank. The absorbance was plotted as a function of time to determine the initial rate of indigo formation. The enzyme activity was defined as the initial rate of indigo formation. The value of OD₆₀₀ was converted to protein concentration (mg L⁻¹) using a conversion factor (192.56) determined in section 3.3.2. The normalized enzyme activity was calculated from the ratio of the enzyme activity to the protein concentration of the cell suspension.

The cells in the microcentrifuge tubes were used to determine indoxyl formation by fluorescence detection. Excitation and emission wavelengths for indoxyl were 365 nm and 470 nm, respectively. The enzyme reaction was initiated by addition of five µl of

100 mM indole and incubated at room temperature for the desired reaction time. The incubated cells were immediately centrifuged and the cell supernatant was injected to the fluorescence detector. Blanks consisted of cell supernatant combined with 5µl of DMF and were measured for an identical time.

Production of indigo, measured spectrophotometrically at 600 nm, and indoxyl, measured fluorometrically at 470 nm, are shown as a function of time in Figure 4.3. Both results may be considered as a measure of Tod activity in whole cells. Absorbance increased gradually over the 10 minute reaction time while fluorescence decreased after 6 minutes of incubation. This pattern is consistent with the conversion if indole first to indoxyl and then to indigo.

The fluorescence increased linearly to a value of 437.5 ± 31.82 during the first 2 minutes; this value was approximately 76.5 % of the maximum fluorescence intensity of 571.5 ± 44.54 . A 2 minute reaction time was chosen for the fluorescence method of determining toluene dioxygenase activity in whole cells. The fluorescence readings of the blank were virtually zero over the 10 minutes reaction time.

Fluorescence intensity was correlated with Tod activity expressed as the rate of indigo formation. *P. putida* F1 was grown as described in section 3.3.1. After the cells were washed twice with phosphate buffer (pH 7.0), they were resuspended in Hunter medium to yield a range at different cell densities. Both the spectrometric and fluorometric enzyme activity assays were performed with each suspension using a 2 minutes reaction time. Figure 4.4 shows the Tod activity in whole cells of *P. putida* F1, as measured by fluorescence, correlated ($R^2 = 0.90$) with indigo production as measured by spectrophotometer. Thus, it can be described that 90 % of the variability in the Tod

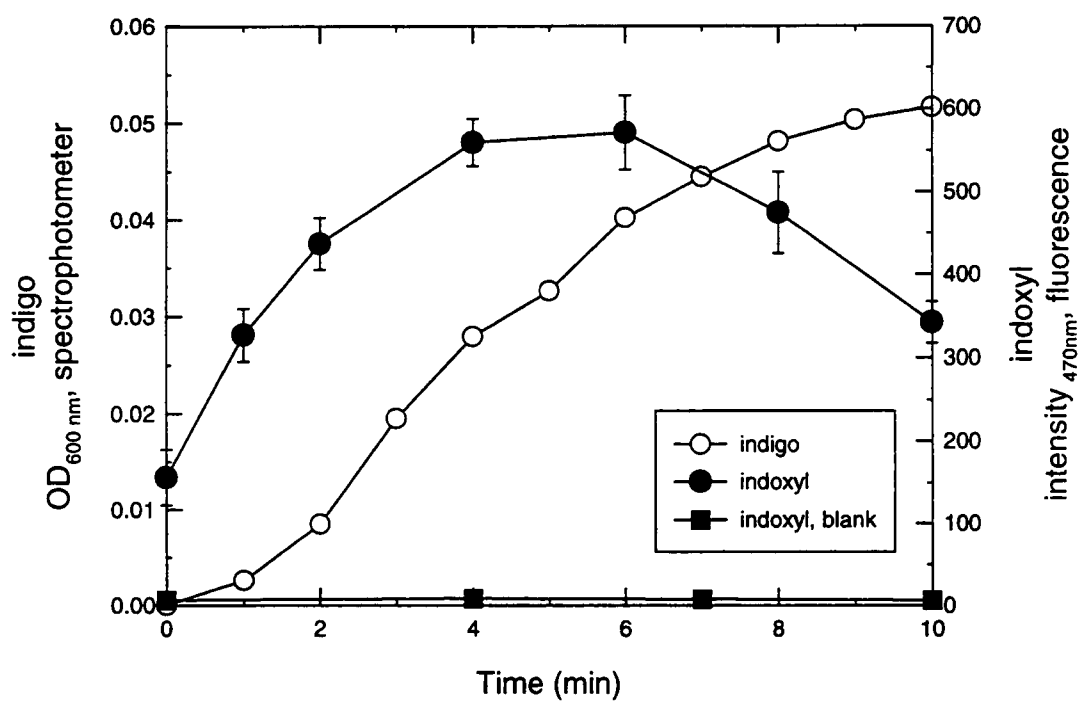


Figure 4.3 Absorbance and fluorescence intensities as a function of time for indole oxidation by *Pseudomonas putida* F1

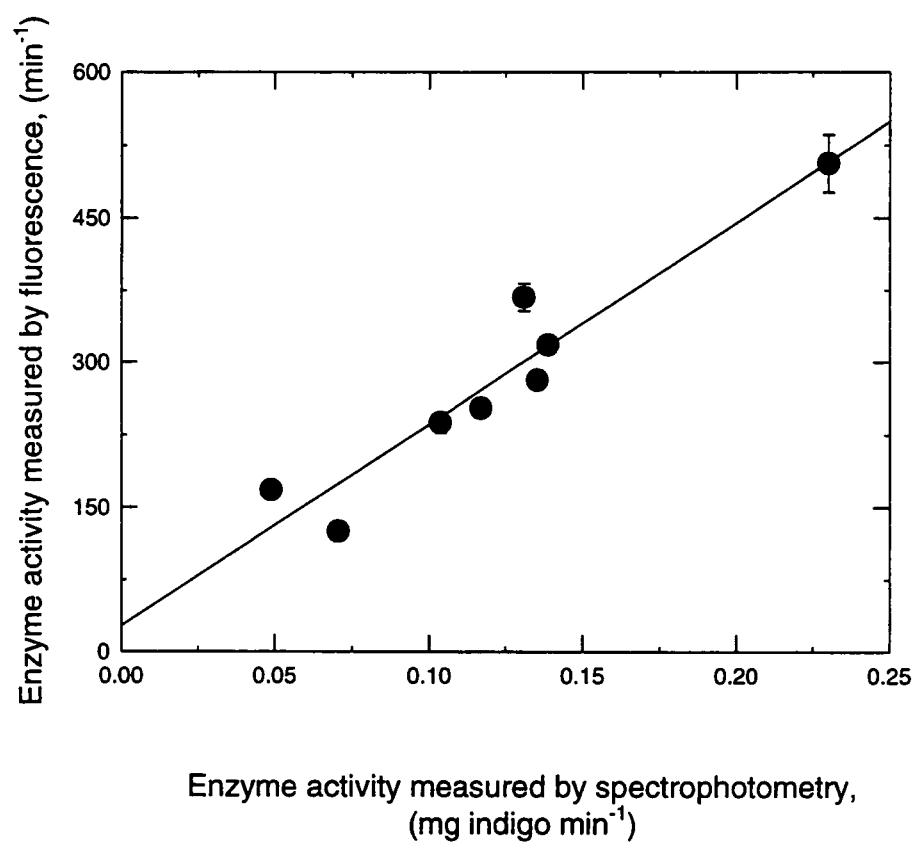


Figure 4.4 Correlation of Tod activity as measured by indoxyl and indigo formation

activity measured by fluorescence is accounted for by indigo production as measured by spectrophotometer.

A correlation factor of $2277.6 \text{ mg indigo}^{-1}$ was determined. Enzyme activity, measured by the change in fluorescence intensity as a function of time (R_I) can be converted to units of $\text{mg indigo} (\text{min})^{-1}$ by dividing by the correlation factor:

$$\frac{\text{mg indigo}}{\text{min}} = \frac{R_I (\text{min})^{-1}}{2277.6 (\text{mg indigo})^{-1}}$$

4.3 Specific enzyme activity and NADH concentration profiles in growth phase.

Because toluene dioxygenase activity is dependent on the presence of reduced nicotinamide adenine dinucleotide (NADH) and oxygen, monitoring of NADH is necessary. When cells are irradiated by ultraviolet light at about 340 nm, NADH fluorescence at about 420 nm. Duysens *et al.* (1957) made the first attempting to apply this property for the characterization of cultures. Zabriskie *et al.* (1978) used fluorescence to evaluate the concentration of cells by proving the existence of linear relationship between fluorescence and biomass of culture. Li *et al.* (1991) used the multiple excitation fluorometric system to monitor NAD(P)H, pyridoxine, tryptophan, and riboflavin in cells. Angell *et al.* (1993) and Rice *et al.* (1995) reported that fluorescence measurement can be applied to monitor tryptophan and NAD(P)H of an active growing bacteria biofilm.

Enzyme activity was observed during batch growth of *P. putida* F1 on mineral medium with toluene supplied in the vapor phase. An inoculum of *P. putida* F1 in late

exponential phase ($OD_{600} = 0.8-1.0$) was added to 50 ml of toluene-Hunters medium. The flask was incubated at room temperature with shaking (200 rpm). OD_{600} , enzyme activity, and NADH concentration were measured as a function of time. The experiment was conducted in triplicate.

Normalized Tod enzyme activity during growth is shown in Figure 4.5. Tod activity was observed to be relatively constant (3.84 to 7.73×10^{-4}) in the growth phase ($OD_{600} < 0.9$). In the stationary phase ($OD_{600} > 0.9$) the enzyme activity increased to (9.00 to 19.8×10^{-4}). Hugouvieux-Cotte-Pattat *et al* (1990) reported the greatest specific enzyme activity occurred at early-stationary phase for *Pseudomonas putida* containing the TOL plasmid pWWO. They concluded that the TOL catabolic operons were more strongly induced at late exponential phase than at early exponential phase. To obtain a high level of toluene dioxygenase enzyme activity for optimum cometabolic TCE degradation, cells should be maintained in the stationary phase.

Li *et al* (1991) monitored NADH during batch fermentation of ethanol by *Candida utilis*. They found NADH fluorescence to be a good indicator of the cellular metabolic state because the NADH fluorescence signal changed according to the utilization of ethanol and production of the metabolic intermediate, acetic acid. The specific NADH concentration of *P. putida* F1 grown on a mineral medium with toluene supplied in vapor phase is shown in Figure 4.6. Normalized NADH was maintained at a constant value of $0.102 \pm 0.005 \mu\text{mol (mg protein)}^{-1}$ during the exponential growth phase, ($OD_{600} < 0.9$) and increased continuously to $0.29 \pm 0.02 \mu\text{mol / mg protein}$ after the early-stationary-growth phase ($OD_{600} > 0.9$). The maximum normalized NADH was

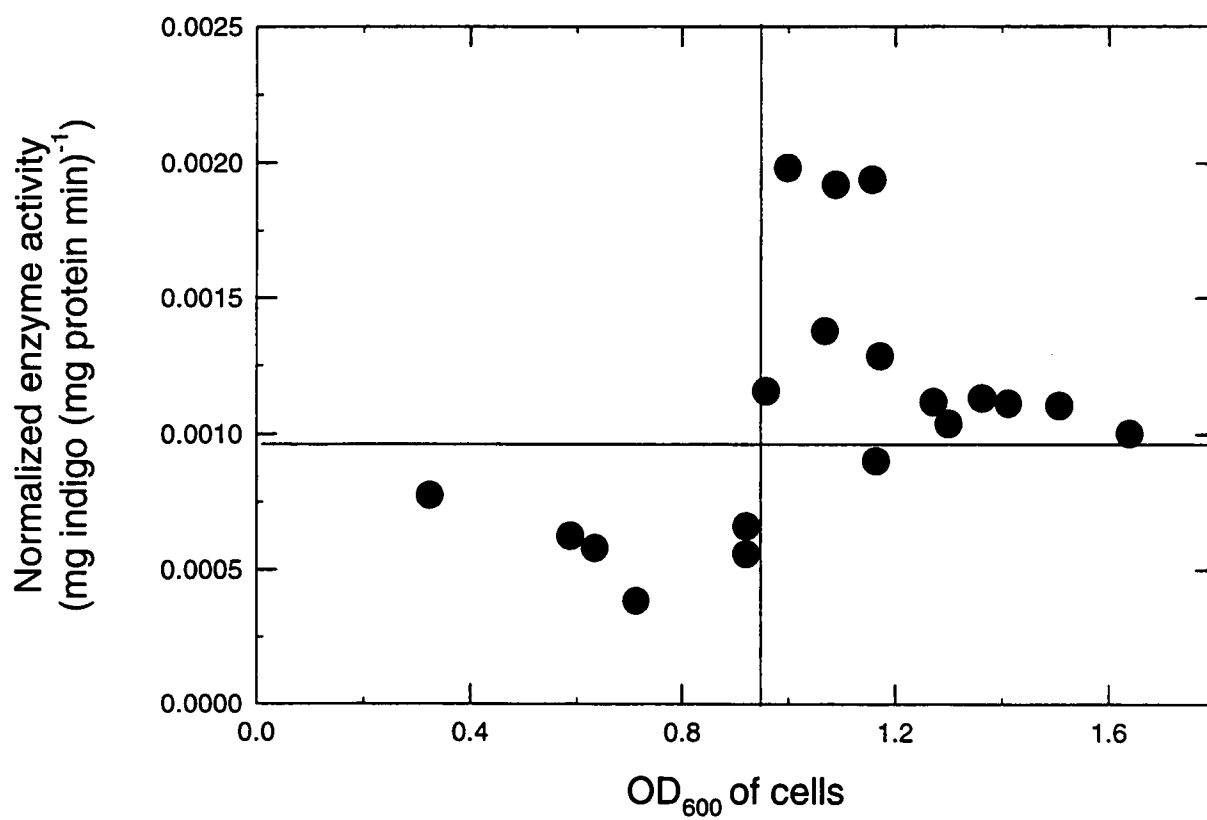


Figure 4.5 Normalized Tod activity in whole cells during growth

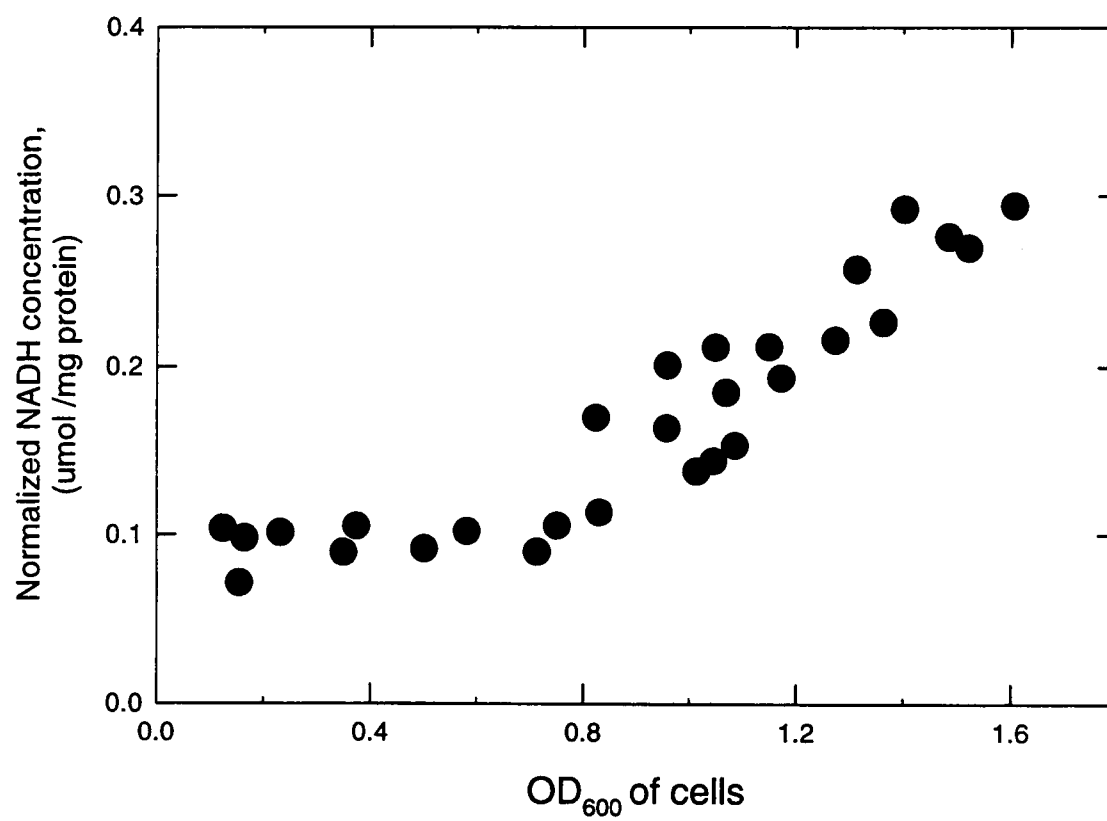


Figure 4 6 Normalized NADH concentration profile during growth

not found in this experiment; it was expected to be around the value of $0.29 \pm 0.02 \mu\text{mol} / \text{mg protein}$.

4.4 The estimation of normalized Tod activity half-life in whole cells.

Toluene dioxygenase enzyme activity naturally decays over time in the absence of toluene. To understand the enzyme activity behavior and to establish a biokinetic model for biofiltration, quantification of the rate of decay in enzyme activity was required. *P. putida* F1 was grown to OD of 1.0 at 600 nm as previously described. Fifty milliliters of cell suspension was dispensed into two 300 ml flasks after determining the enzyme activity and OD of cells. Only one flask was provided with toluene. Both flasks were placed on a shaker operating 180 rpm at room temperature. Five-milliliter samples were taken periodically for measurement of enzyme activity and OD₆₀₀. The time course of normalized enzyme activity in absence and presence of toluene was shown in figure 4.7(a) and (b) for two different experiments. In the absence of toluene, the initial normalized enzyme activity 1.62×10^{-3} to $0.77 \times 10^{-3} \text{ mg indigo (mg protein min)}^{-1}$ during 7 hr (Figure 4.7(a)). However, the normalized enzyme activity in the presence of toluene remained relatively constant at $1.99 \pm 0.21 \times 10^{-3} \text{ mg indigo (mg protein min)}^{-1}$.

The rate of specific enzyme decay was well described by the first order equation.

$$E_{x,t} = E_{x,0} \exp(-kt) \quad (4.1)$$

where $E_{x,t}$, $E_{x,0}$ are the final and initial specific enzyme activities, t is incubation time, and k is a decay constant. The R^2 and k value of 0.92 and -0.1228 hr^{-1} , respectively,

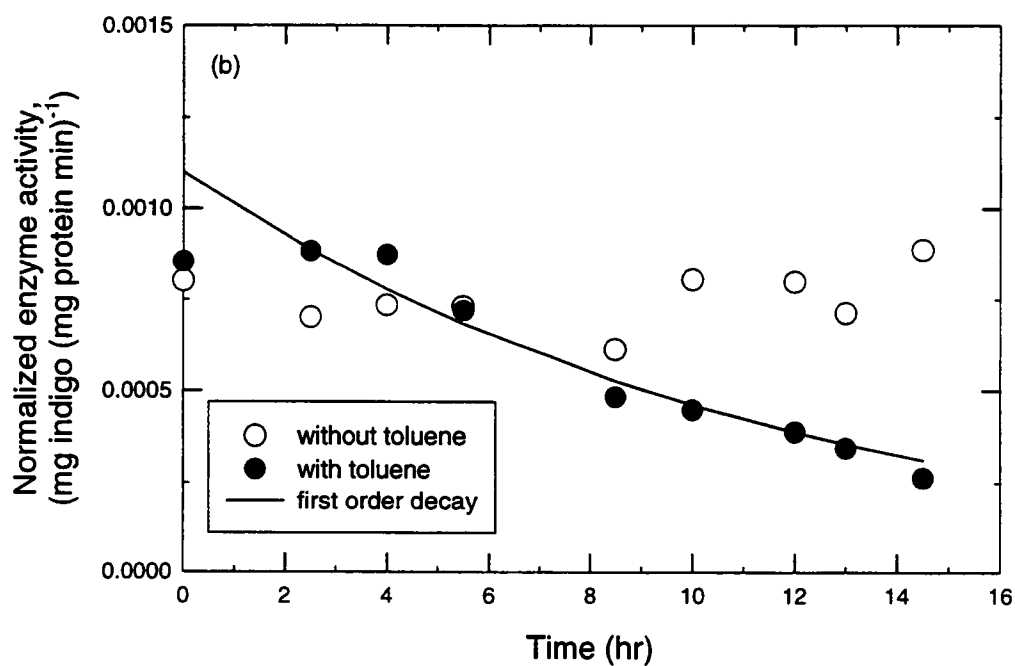
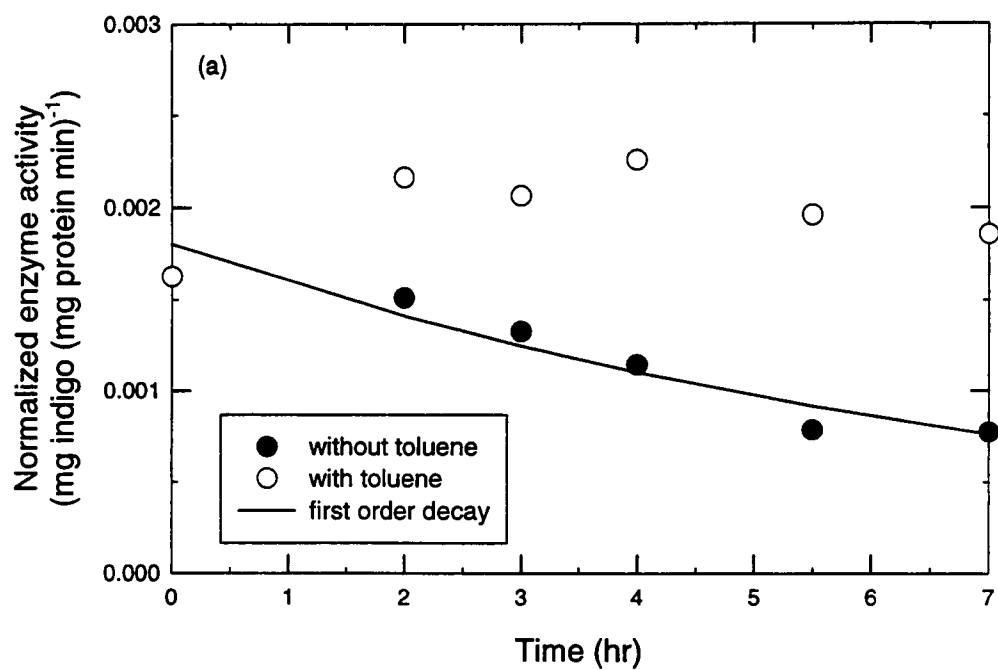


Figure 4.7 Decay of normalized enzyme activity in whole cells in batch culture:
(a) 7 hr experiment, (b) 15 hr experiment

were determined by fitting the model to the data. Thus, 92 % of variation in normalized enzyme activity is explained by the model equation. A similar analysis performed with data taken over a period of 14.5 hrs is shown in Figure 4.7(b). The initial normalized enzyme activity of 8.54×10^{-4} mg indigo (mg protein min)⁻¹ decreased to 2.63×10^{-4} mg indigo (mg protein min)⁻¹ after 14.5 hr. The R² and k value of 0.94 and -0.0869 hr⁻¹, respectively, were determined. Thus, 94 % of variation in normalized enzyme activity is explained by the model equation. The normalized enzyme activity in the presence of toluene remained constant at $19.93 \pm 2.27 \times 10^{-4}$ mg indigo (mg protein min)⁻¹.

The definition of the Tod half-life is the time required for the activity to decrease to half of the initial activity. The values of enzyme half-life determined from the decay constants are 5.64 hr and 7.98 hr for 7 and 15 hr experiments, respectively.

4.5 The estimation of normalized Tod activity half-life in immobilized cells.

The natural decay of enzyme activity was also observed in immobilized cells. The natural decay constant in immobilized cells was obtained and compared with that in suspended cultures; it is also used as a parameter of the biofiltration model presented later. The differential volume reactor (DVR) was used for this experiment. Toluene was continuously introduced to the differential immobilized cell reactor at a concentration of 1000 µg/L during the growth of the biofilm. The normalized whole cell enzyme activity was measured periodically after stopping the toluene feed. The decay in normalized enzyme activity is shown in Figure 4.8. The initial normalized enzyme activity of 4.14×10^{-4} mg indigo mg protein⁻¹ min⁻¹ decreased to 1.27×10^{-4} mg indigo mg protein⁻¹ min⁻¹ over a period of 12.28 hour. The R² and k values were determined to be 0.92 and -0.0982

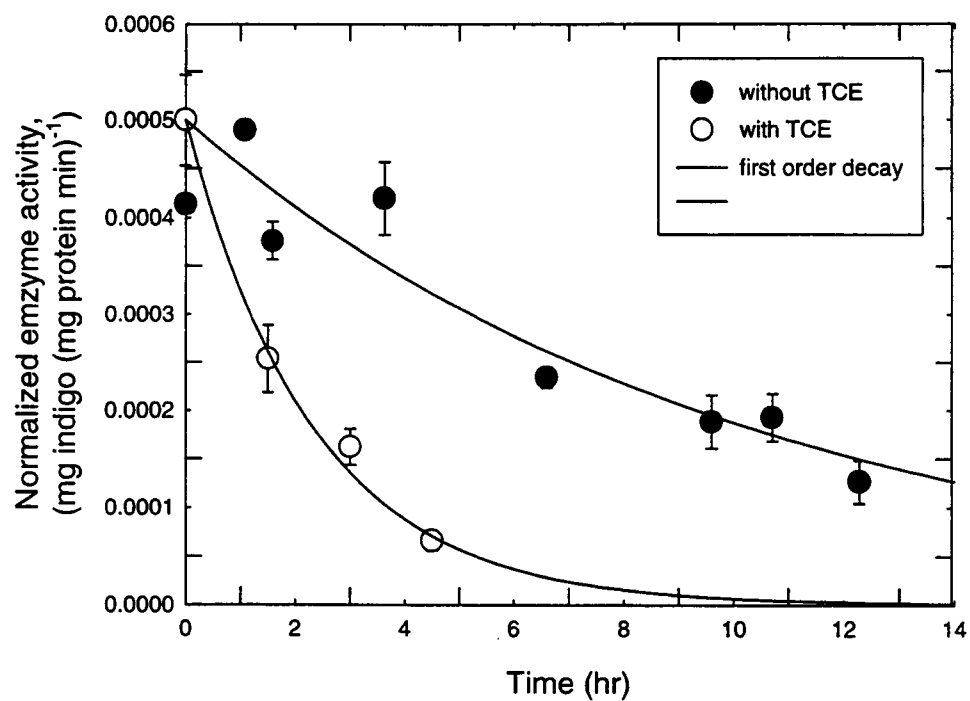


Figure 4.8 The decay of normalized enzyme activity as a function of time

hr⁻¹, respectively. A half-life of 7.06 hr was calculated from the decay constant. No significant difference was observed in the natural decay of normalized enzyme activity between the immobilized and suspended cells.

To evaluate the effect of TCE on the natural decay of enzyme activity, a TCE was applied to the DVR system. Prior to the experiment, toluene was continuously fed to the differential immobilized cell reactor at a concentration of 1000 µg/L to recover the enzyme activity lost during the previous decay experiment. After toluene feeding was stopped, TCE concentration of 1800 µg/L was introduced to the DVR and normalized whole cells enzyme activity was measured periodically. The decay was much faster than that without TCE (Figure 4.8).

The normalized enzyme activity decreased from 3.00×10^{-4} to 1.16×10^{-4} mg indigo (mg protein min)⁻¹ after only 8.8 hr. The half-life in the presence of TCE was only 1.59 hr compared to 7.06 hr in the absence of TCE. It can be concluded that TCE increased the rate of enzyme deactivation.

CHAPTER 5

BATCH EXPERIMENTS

5.1 Growth and decay of *Pseudomonas putida* F1

Figure 5.1 shows the growth pattern of *Pseudomonas putida* F1 over time in nutrient rich medium. *P. Putida* F1 was inoculated from late exponential phase ($OD_{600} = 0.8-1.0$), cultures and grown in 50 ml of toluene-Hunters medium. Toluene was supplied to the vapor-phase of the flask by diffusion from a glass tube suspended in the headspace. The flask was incubated at room temperature with shaking (200 rpm). One ml of sample was taken regularly for measuring OD_{600} .

The exponential growth phase is a period of balanced growth in which the cells grow at the same rate. Cell mass increases exponentially with time. The growth rate is independent of nutrient concentration and the growth rate per cell is a constant (Shuler *et al.*, 1992):

$$\frac{dX}{dt} = \mu X \quad (5.1)$$

$$X = X_0 \exp(\mu t) \quad (5.2)$$

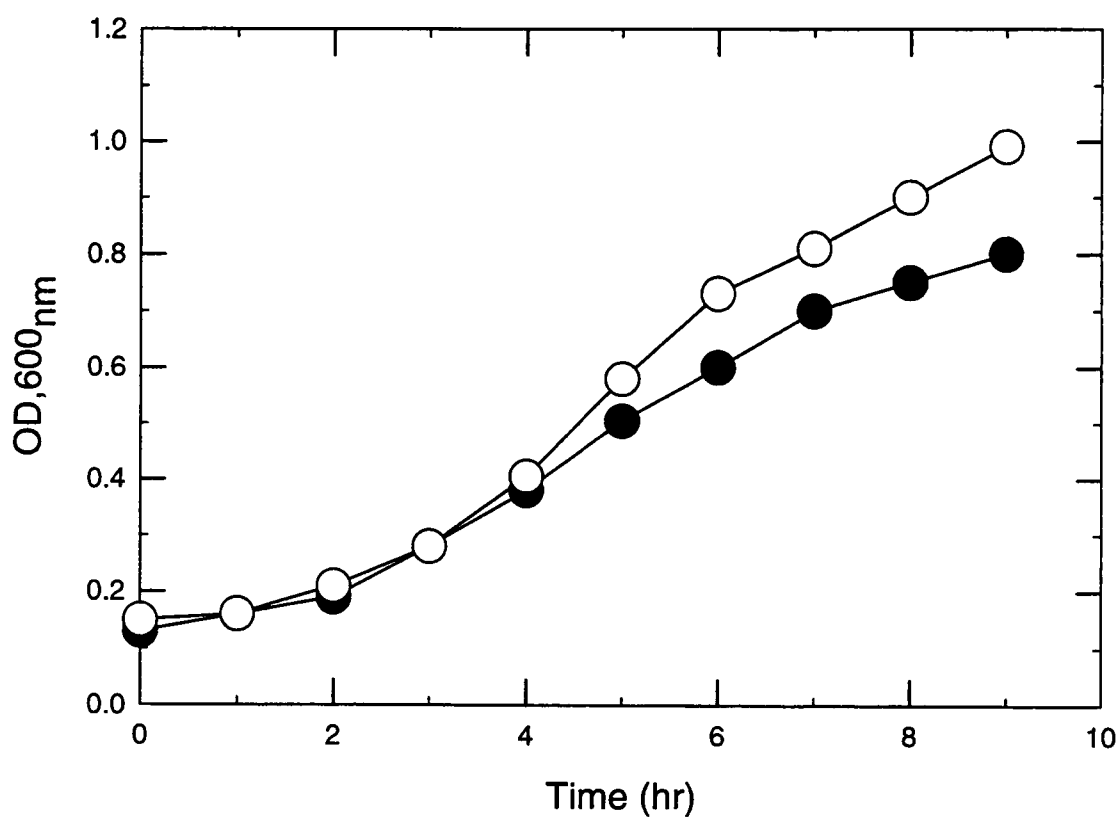


Figure 5.1 The growth curve of *Pseudomonas putida* F1

Where X : cell concentration at time t (hr)
 X_0 : cell concentration at time 0 (hr)
 μ : growth rate constant (hr^{-1})

The doubling time (τ_d) is defined as the time required for the microbial mass to double. The growth constant and doubling time determined from the data in Figure 5.1 were 0.34 hr^{-1} and 2.038 hr, respectively.

Figure 5.2 shows the decay pattern of *P. putida* F1 over time in the nutrient rich medium. *P. putida* F1 was grown in 50 ml of toluene-Hunters medium with vapor phase toluene until late exponential phase ($\text{OD}_{600} = 0.8-1.0$). The cells were harvested by centrifuging (15 ml at 1000rpm) and suspended at an OD of 0.6 in fresh media. The flask was then placed on a shaker to provide mixing and one ml of cell sample was taken regularly for measuring OD_{600} . No carbon or energy source was supplied to the flask during this time to avoid synthesizing new cells. During the first 7.6 hr of incubation, the cell density decreased rapidly to OD_{600} of 0.4; thereafter, the OD_{600} remained relatively constant. The cell mass decreases during endogenous respiration according to the following equation (Benefield *et al.*, 1980):

$$\frac{dX}{dt} = k_d X \quad (5.3)$$

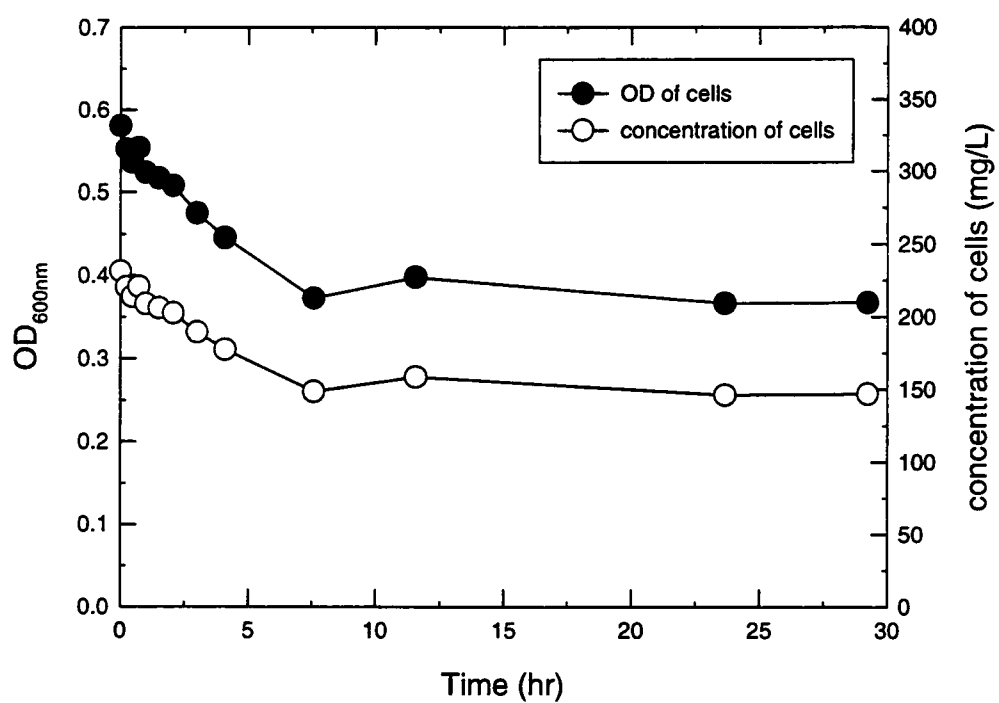


Figure 5.2 The endogenous decay of *Pseudomonas putida* F1

$$X = X_o \exp(K_d t) \quad (5.3)$$

Where X : cell concentration at time t (hr)
 X_o: cell concentration at time 0 (hr)
 K_d: decay rate constant (hr⁻¹)

The decay constant was calculated to be 0.0583 hr⁻¹ during the 7.6 hr period of decay.

5.2 Whole-cell toxicity experiment

Bench-scale experiments were conducted to determine the effects of TCE on the growth of *Pseudomonas putida* F1. The experiments were designed to exclude toxicity by toluene dioxygenase mediated TCE metabolism. *P. putida* F1, in the late exponential phase (OD₆₀₀ = 0.8- 1.0) of growth was inoculated into 50 ml of Hunters medium. Succinate was added at a concentration of 10 mM as the growth substrate since it cannot induce toluene dioxygenase enzyme (section 5.4.2). The flask was incubated at room temperature with shaking (200 rpm). The cells were harvested at the late-exponential growth phase by centrifuging (15 ml at 1000 rpm), and suspended in fresh media (including succinate) at OD₆₀₀ = 0.1. Batch experiments were conducted in 25 ml Mininert cap vials. Five milliliters of cell suspension were placed into 25 ml Mininert caps vials and the desired amount of TCE added via syringe injection of TCE saturated water (1100 mg/L TCE). The vials were placed on the shaker for mixing during experiments. After 15 hrs, the caps of the vials were removed to allow the measurement of OD₆₀₀. The results of the experiment are shown in Table 5.1. It can be concluded that.

Table 5.1. Whole-cell toxicity batch experiment results.

Tube number	TCE mass added (mg TCE L ⁻¹)	Final cells concentration (mg protein L ⁻¹)
1	0	146.8
2	0	166.0
3	0	147.8
4	10	148.1
5	10	164.8
6	20	162.4
7	20	152.9
8	40	159.7
9	40	158.9
10	50	168.0
11	50	148.3

the biomass was independent of TCE concentration ($p = 0.48$). For this reason, toxicity caused by the direct action of TCE on the cells was not included in the biofiltration model

In contrast, Tschantz (1996) using a similar experiment, found TCE to have a direct effect on *Methylosinus trichosporium* OB3b. TCE was added to several tubes resulting in final TCE concentrations of 10, 20, 40, and 80 mg /L. The investigator reported that the rate of cell growth decreased when TCE was added to the system. For the lowest TCE concentration tested (10 mg L⁻¹), the final biomass reached only 43 % of that in the absence of TCE.

5.3 TCE and toluene degradation experiments

5.3.1 Toluene degradation experiments

Batch aqueous-phase toluene degradation experiments using 5 ml of *Pseudomonas putida* F1 culture were performed in 25 ml Mininert caps vials. Toluene

degradation rates for different initial toluene concentrations were determined. Prior to the batch experiments, killed cell tests were performed to quantify the losses of TCE and toluene due to volatilization and/or sorption (Figure 5.3). Sufficient quantities of TCE and toluene saturated aqueous solutions were injected into each vial containing 5 ml of culture ($OD_{600} = 1.0$) to yield a final concentrations 8 mg/L of toluene and 1 mg/L TCE. Recoveries of TCE and toluene were in the range of 90 to 95 %, verifying that losses from the system were negligible.

Batch toluene degradation experiments were conducted in duplicate or triplicate for each toluene concentration tested. A toluene saturated aqueous solution was injected through the septa of the sealed vials containing cells at $OD_{600} = 1.0$ with a gas-tight syringe to make the desired toluene concentration in the aqueous phase. A 25 μ l gas sample from the headspace of the vials was routinely taken and injected into the GC for analysis. Figure 5.4(a) and (b) show that biotransformation of toluene by *P. putida* F1 for initial concentrations of 2.43, 3.88, 7.76, 8.73, 15.52, and 18.43 mg/L in the aqueous phase. The time required for complete degradation of toluene was 10 min at toluene concentrations less than 10 mg/L and 20 min at toluene concentrations greater than 15 mg/L.

The OD_{600} of the culture used for batch experiments was 1.0, corresponding to 398.994 mg /L of *P. putida* F1 biomass (Yu, 1998). The specific utilization rate for each Toluene concentration was calculated and shown in Figure 5.5. The relationship is described by typical enzyme saturation kinetics (Lawrence and McCarty, 1970). The maximum specific substrate utilization rate of $0.002674 \text{ mg toluene (min mg biomass)}^{-1}$, and half-saturation constant (K_s) of 2.5 to 3.5 mg/L was estimated from the curve.

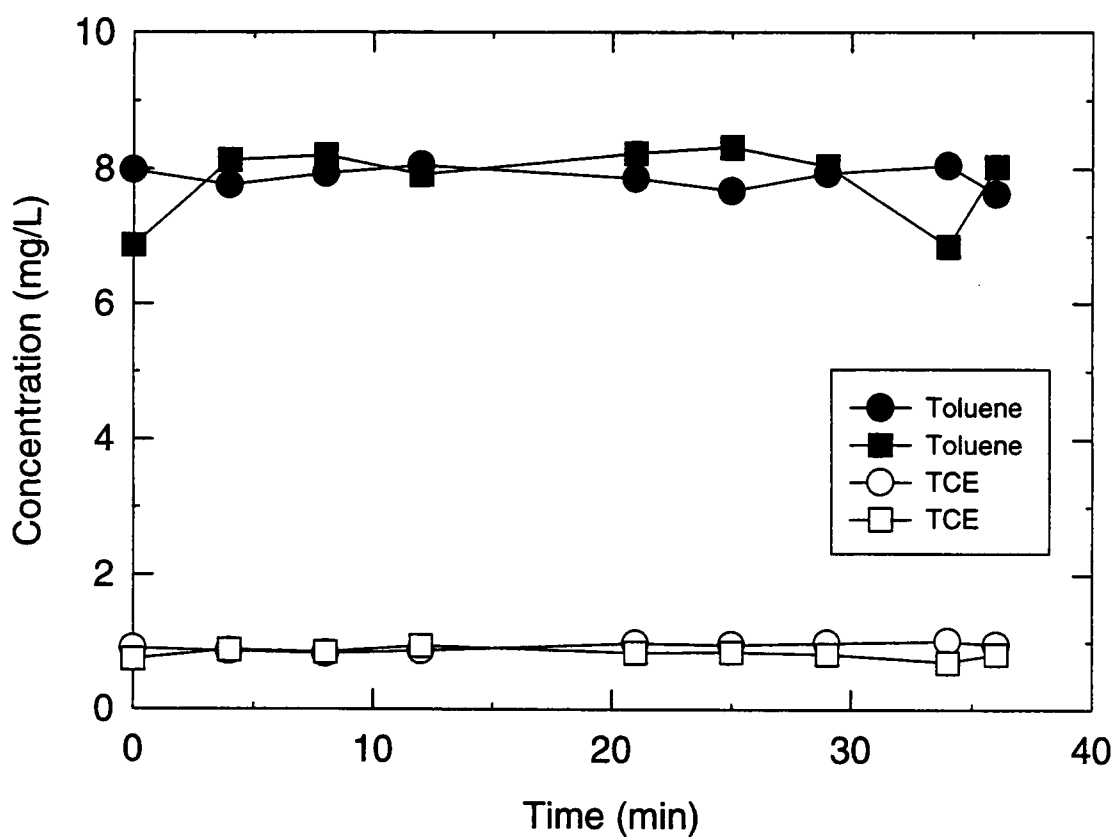


Figure 5.3 Loss of toluene and TCE from aqueous phase batch experiments using killed cells

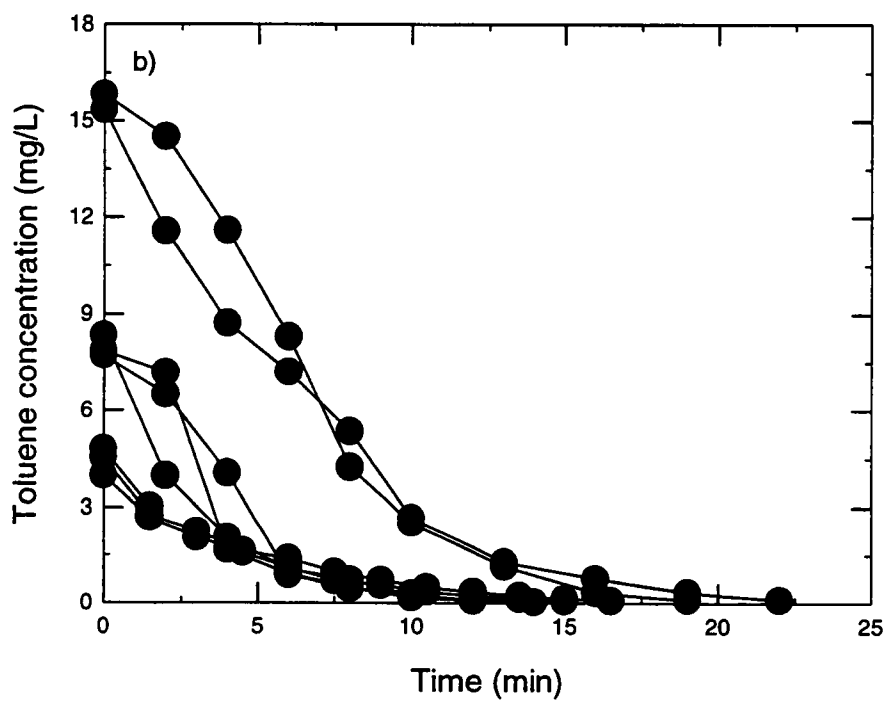
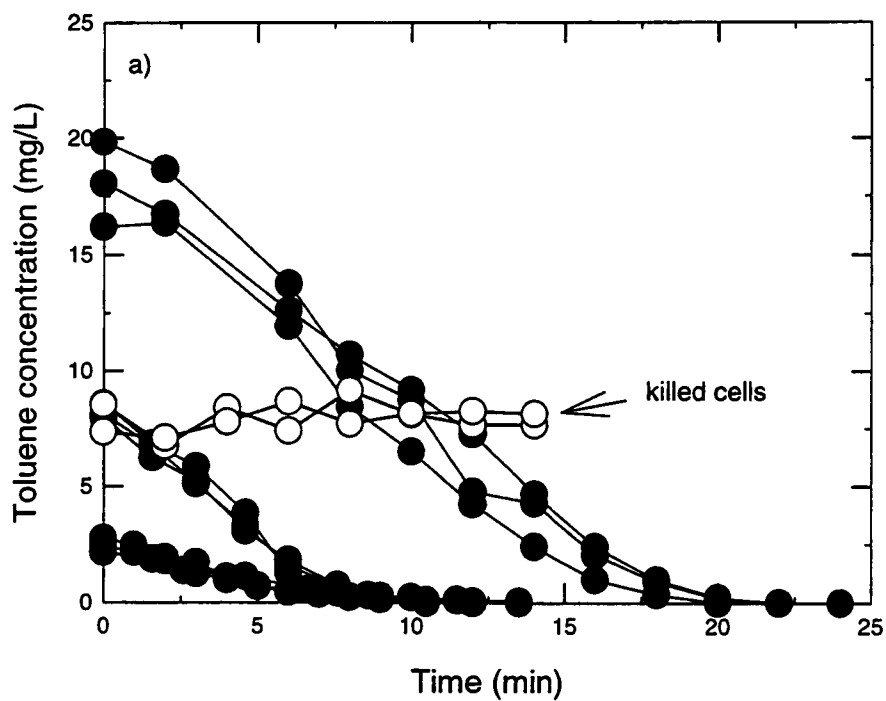


Figure 5.4 Biodegradation of toluene in the absence of TCE by *Pseudomonas putida* F1 in aqueous phase batch experiments

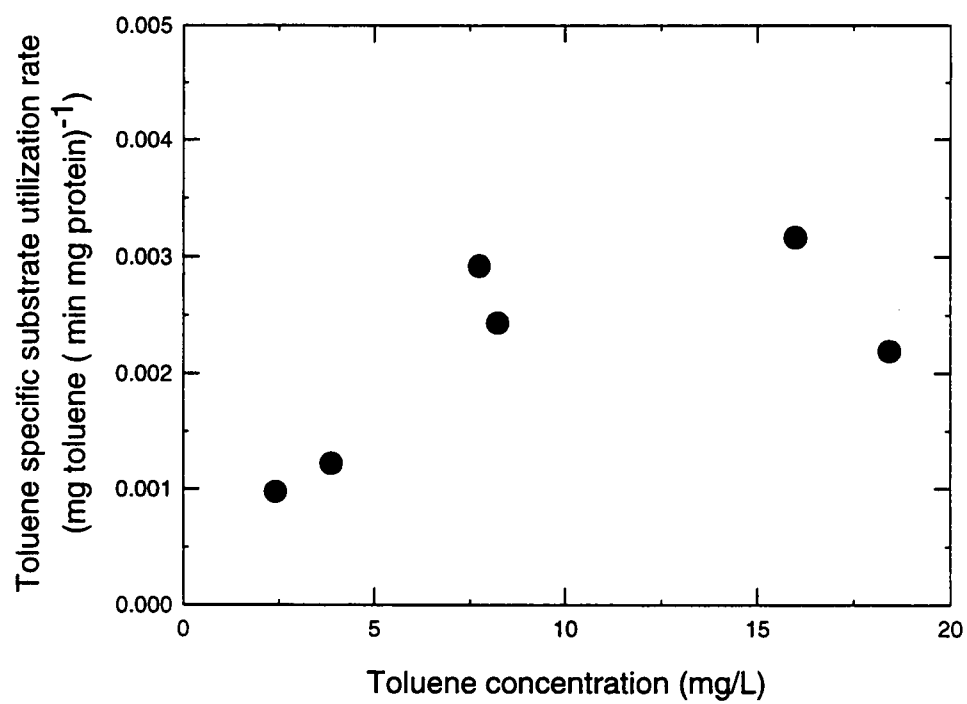


Figure 5.5 The toluene specific utilization rate as a function of toluene feed concentration

5.3.2 Toluene inhibition effect on TCE co-oxidation

Aqueous-phase batch experiments were conducted to quantify the extent of competitive inhibition in the *Pseudomonas putida* F1. The experiments were performed in the same manner as the batch aqueous-phase toluene degradation experiments. A series of experiments were conducted in which the mass of TCE added to the vials was held constant at 0.005 mg. The mass of toluene added varied; experiments were conducted using 0.025 mg, 0.01 mg, 0.15 mg, and 0.2 mg toluene. The results are shown in Figure 5.6(a) to (d). The time required to degrade the toluene increased from about 10 minutes at the lowest toluene concentration to about 20 minutes at the highest concentration. The specific toluene utilization rate were 0.00087, 0.00291, 0.00291, and 0.00194 mg toluene (min mg biomass)⁻¹ in order from the lowest to the highest toluene concentration tested. These results showed that toluene degradation was not inhibited by TCE addition (0.005 mg). Additional experiments were performed to investigate the possibility of TCE inhibition on toluene degradation (Figure 5.7). A series of vials containing *P. putida* F1 were prepared in which 0.005mg of TCE and varying amounts of toluene (0.025 mg, 0.01 mg, and 0.2 mg) were added to the vials. The degradation rates of toluene in the presence of TCE were not significantly different than in absence of TCE.

The TCE degradation rate was observed to be a function of toluene concentration. At the lowest toluene concentration tested (Figure 5.6(a)), competitive inhibition was limited and the initial rate of TCE oxidation was high. At toluene concentrations greater than 2.43 mg/L (0.025mg toluene), significant inhibition was observed during the first 20 to 25 minutes of the experiment. However, once the toluene decreased below 2.43 mg/L

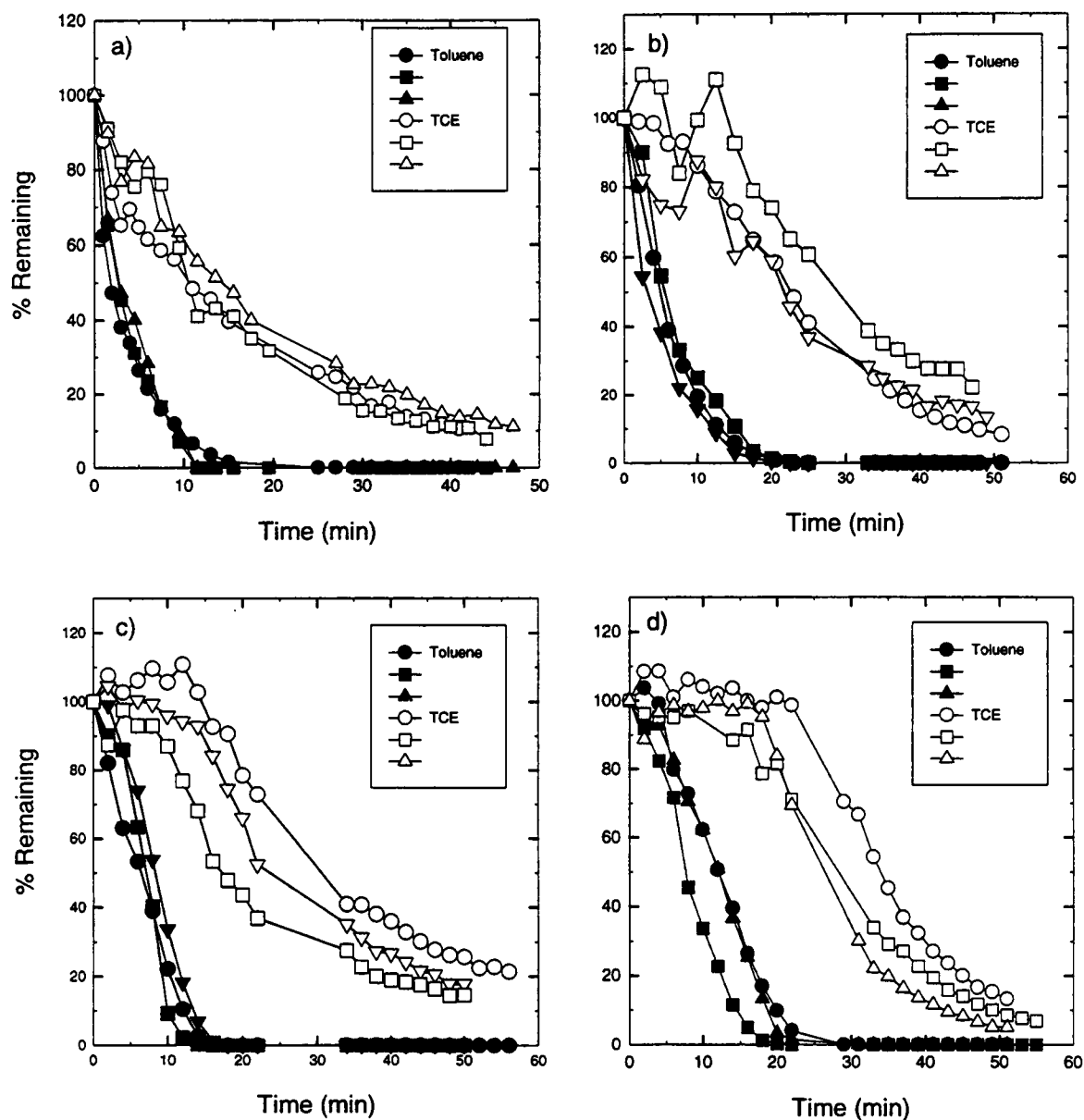


Figure 5.6 Biodegradation of toluene and TCE by *Pseudomonas Putida* F1 in aqueous phase batch experiments
(a) toluene: 0.025mg, TCE : 0.005mg
(b) toluene: 0.1 mg, TCE: 0.005mg
(c) toluene: 0.15mg, TCE : 0.005 mg
(d) toluene: 0.20mg, TCE: 0.005mg.

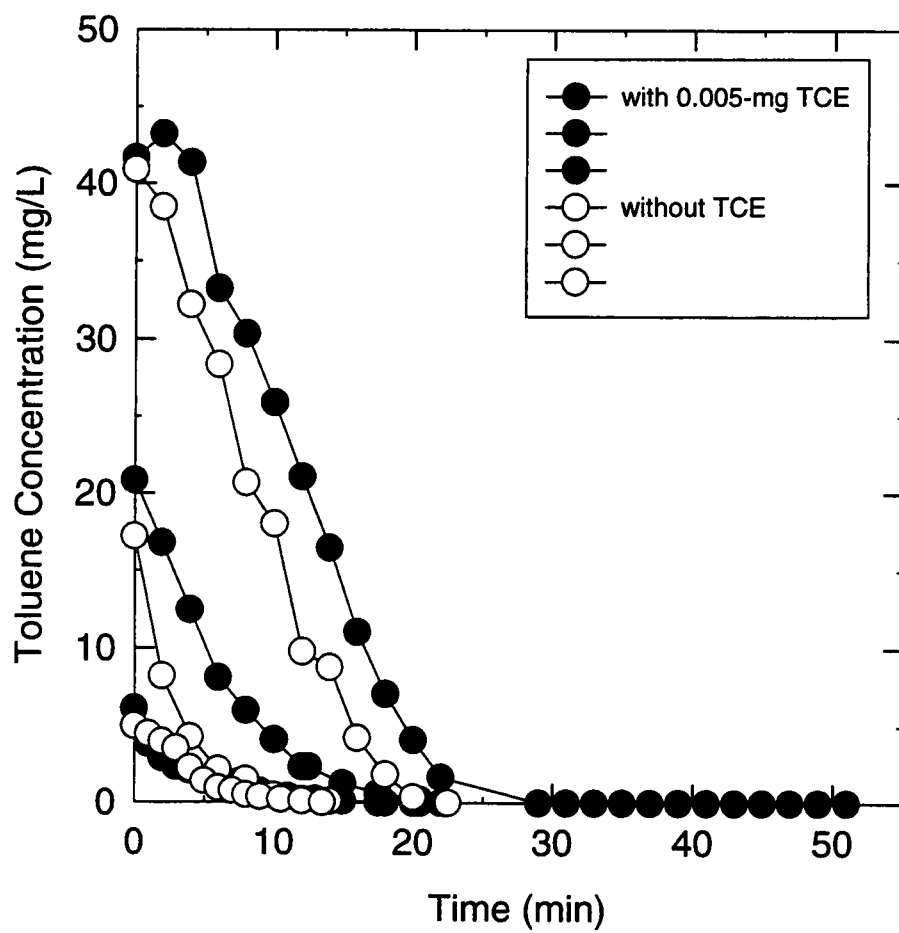


Figure 5.7 Effect of TCE (0.005 mg) on biodegradation of toluene

(0.025 mg toluene), TCE degradation was rapid. In every case, about 90 % of TCE was oxidized after 50 minutes. Comparison of TCE oxidation at the lowest (2.43 toluene mg/L) and highest (19.4 toluene mg/L) toluene concentrations is shown in Figure 5.8(a). Although the lag period increased with toluene concentration, the subsequent degradation rate also increased. The net result was a similar removal efficiency at the end of the experiment. In order to further investigate toluene inhibition, an experiment using a higher TCE mass (0.025mg) and toluene mass (1 mg) was performed (Figure 5.8(b)). Similar results were achieved, in that TCE degradation was not significant until most of the toluene had been degraded.

5.3.3 Effect of TCE degradation on Tod activity

Aqueous-phase batch experiments were conducted to observe the change in Tod activity during TCE degradation in *Pseudomonas putida* F1. Five milliliters of pre-grown culture ($OD_{600} = 1.0$) was dispensed in 25 ml Mininert-caps vials and a constant amount of TCE added by injection of TCE-saturated aqueous solution to make a final TCE concentration of 0.25 mg/L in the aqueous phase. Duplicate sets of sealed cultures were placed on a shaker operating at 180 rpm. A 25 μ l gas sample from the headspace of each vial was routinely collected. At each time point, vials were sacrificed for analysis of biomass and enzyme activity.

TCE degradation and the normalized enzyme activity as a function of time are shown in Figure 5.9. Normalized enzyme activity decreased during TCE degradation. The initial enzyme activity of 15.11×10^{-4} mg indigo mg protein⁻¹ min⁻¹ decreased to

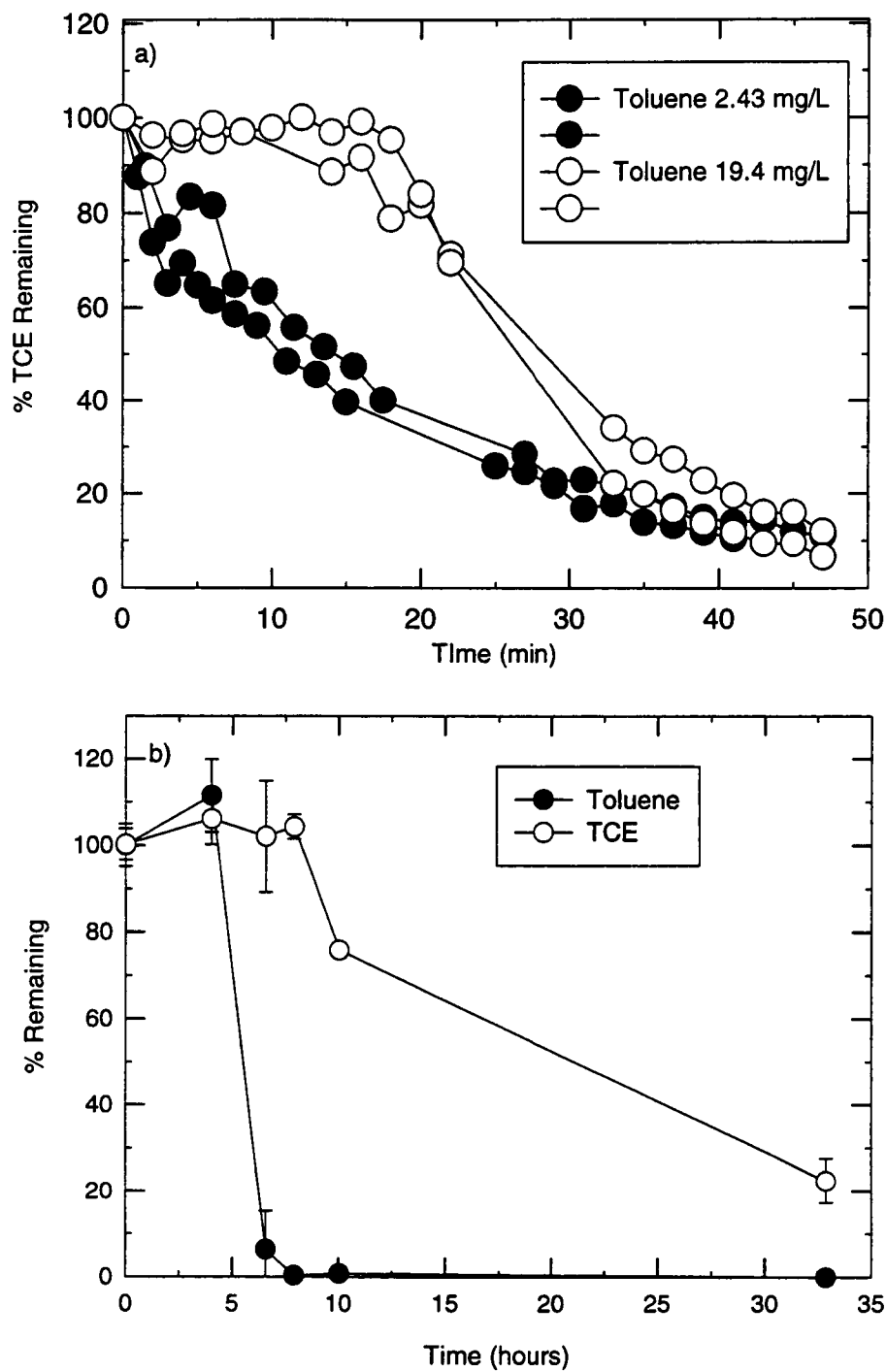


Figure 5.8 Degradation of TCE and toluene by *Pseudomonas putida* F1 at optical density (OD) of 1.0: (a) toluene: 2.43 mg/L

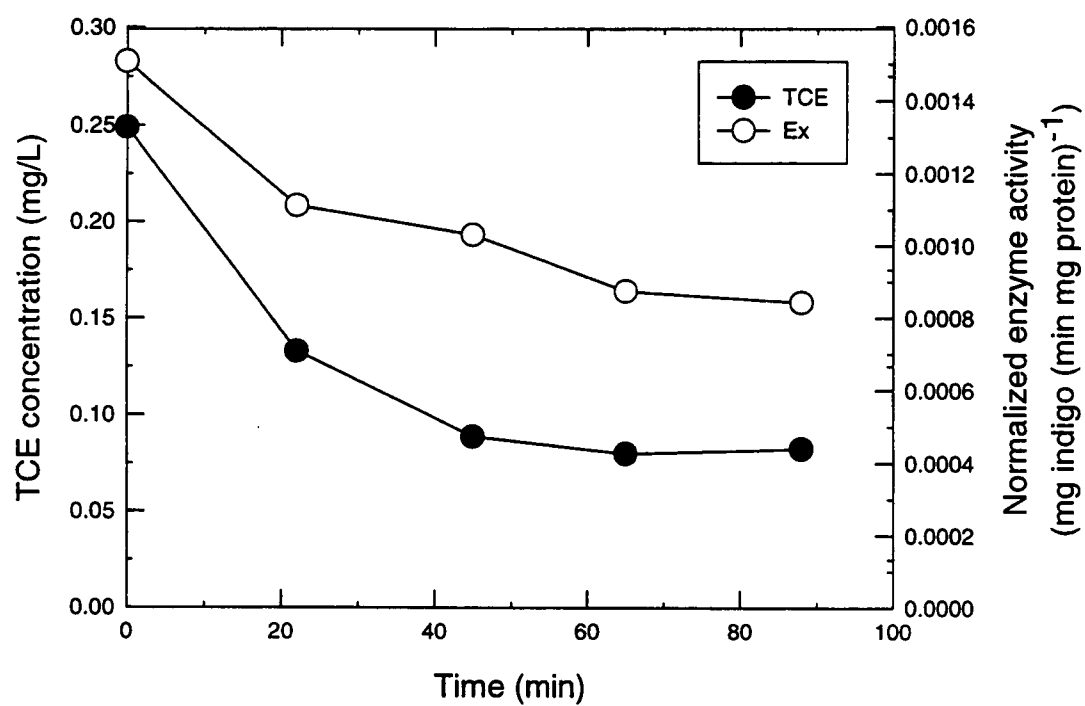


Figure 5.9 TCE removal and normalized enzyme activity as a function of time

11.11×10^{-4} mg indigo mg protein⁻¹ min⁻¹ within 20 minutes. The rate of enzyme inactivation appears to be correlated with the rate of TCE degradation, suggesting that enzyme inactivation is related to intermediates produced during TCE degradation (Wackett *et al.*, 1988 and 1989). The TCE degradation rate decreased to virtually to zero. This was somewhat unexpected because over 50 % of the initial enzyme activity remained. The possibility that NADH limitation was responsible for the interruption in TCE degradation, was investigated in experiments described later in this chapter.

5.4 Enzyme activity recovery experiments

The experiments in the previous section demonstrate that loss of Tod activity can be expected during TCE degradation. Practical application of TCE cometabolism necessitates that the enzyme activity be regenerated. In this section, the relationship between Tod activity and toluene degradation is investigated.

5.4.1 Effect of toluene on Tod activity

Pseudomonas putida F1 was grown in 50 ml flasks with of toluene-Hunters medium. Flasks were incubated at room temperature with shaking (200 rpm). When growth reached late exponential phase ($OD_{600} = 0.8-1.0$), cells were harvested ($OD_{600} = 0.8$) by centrifugation at $10,000 \times g$ for 10 minutes at 4 °C. Cells were washed twice with phosphate buffer (pH 7.0), resuspended in fresh media, and placed on the shaker. *P. p.* F1 was incubated without any carbon source for one week. The enzyme activity and optical density of the culture decayed during the incubation time. After one week, the cells were harvested and washed twice with phosphate buffer. Cells were resuspended in

Hunters medium to a cell density of 0.5 and placed on ice for batch experiments.

Five milliliters of culture were dispensed into 25 ml Mininert cap vials. The initial normalized enzyme activity and NADH were measured. Selected volumes of toluene-saturated aqueous solution were added through the septa with a gas-tight syringe and the initial toluene concentration was measured by GC. After incubation for 10 minutes, a 25 μ l gas sample taken from the head space of the vial was analyzed for toluene concentration and the enzyme activity was determined by assay. Triplicate determinations were conducted for each condition. The change in normalized enzyme activity as a function of initial toluene concentration is shown in Figure 5.10(a). Similar results are shown at a higher range of toluene concentration in Figure 5.11(a). The average value of normalized enzyme activity at steady state (section 3.2.2) was $3.67 \pm 0.49 \times 10^{-4}$ mg indigo (mg protein min) $^{-1}$ and $6.56 \pm 0.51 \times 10^{-4}$ mg indigo (mg protein min) $^{-1}$ in the two experiments. These activities are only 19.5 % and 33.1 % of the maximum specific enzyme activity of 19.8×10^{-4} mg indigo (mg protein min) $^{-1}$ found during the early-stationary growth phase in section 4.3. Two reasons may account for the difference. First, some cells may become inactive during the one week incubation because of the starvation condition under which the experiment was performed. Second, the reaction time of the enzyme recovery test may not be long enough to allow full expression of the enzyme. Toluene removal for different initial concentrations are shown in Figures 5.10(b) and 5.11(b). The average toluene utilization rates were $16.15 \pm 7.4 \times 10^{-4}$ and $13.72 \pm 6.0 \times 10^{-4}$ mg toluene mg protein $^{-1}$ min $^{-1}$. The toluene utilization rates were similar in the two experiments. These rates were only 50 to 60 % of the maximum

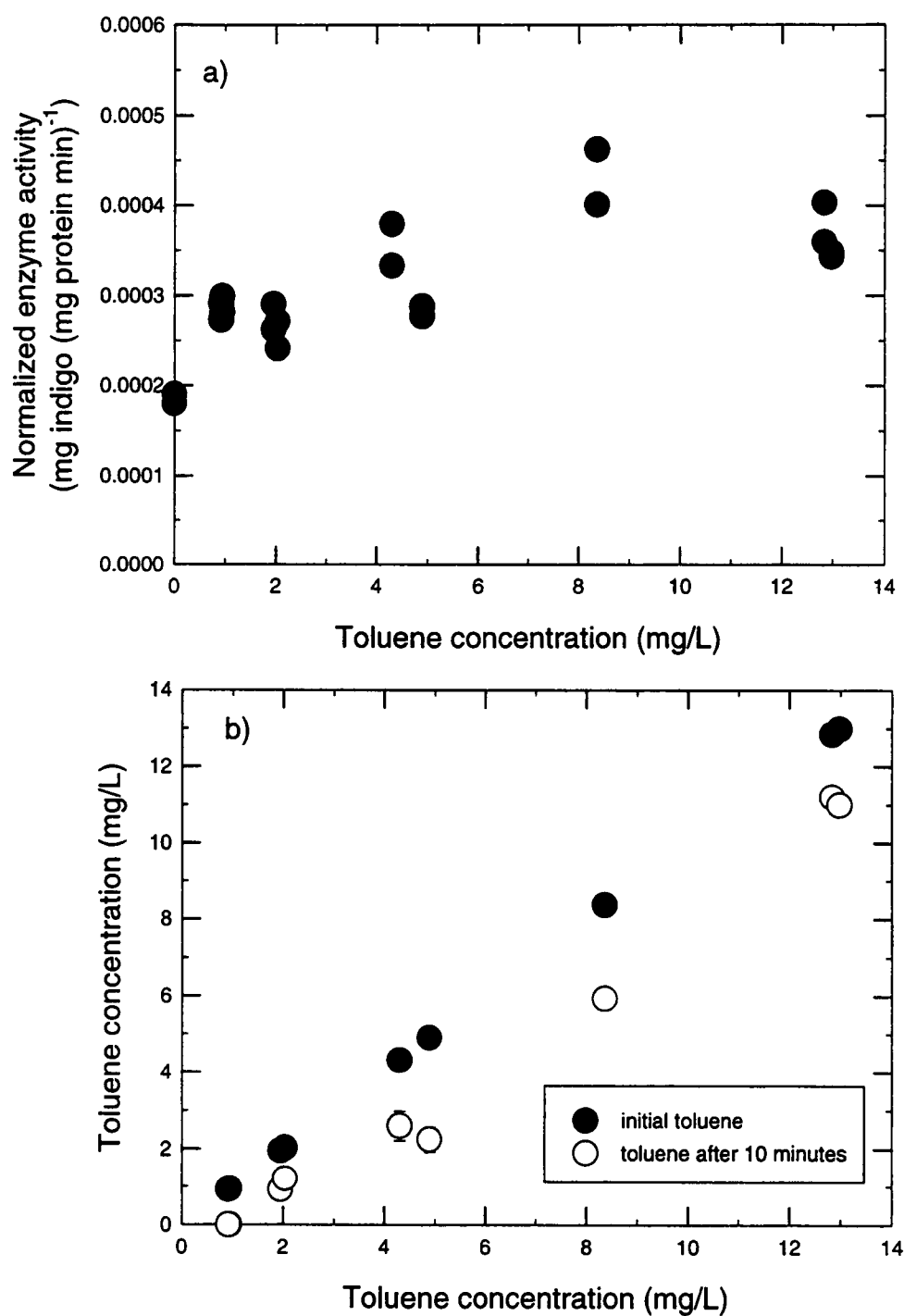


Figure 5.10 Normalized enzyme activity and toluene removal for initial toluene concentrations in the range of 0 -12 mg/L for a 10 minute reaction time: (a) normalized enzyme activity (b) toluene concentration

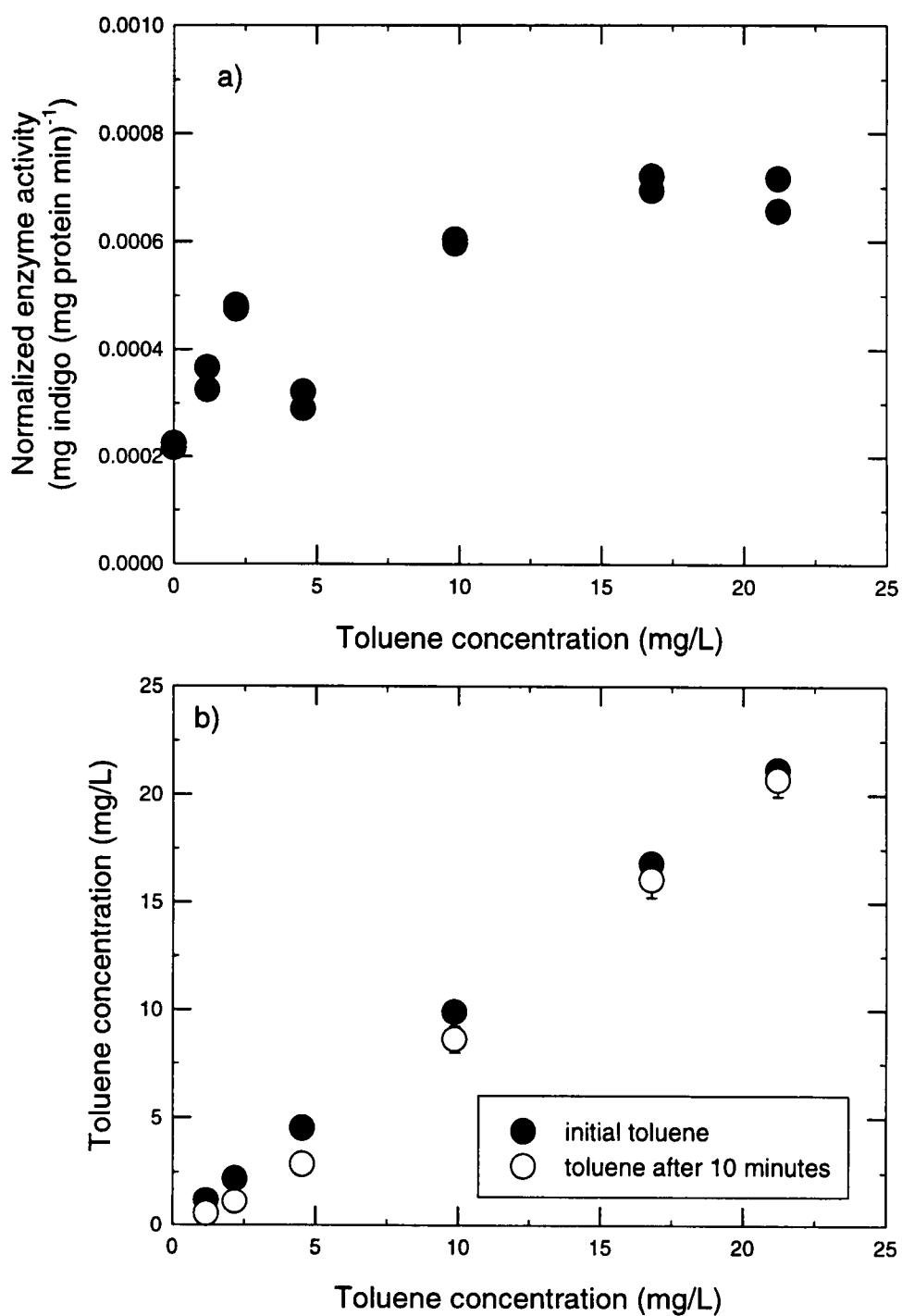


Figure 5.11 Normalized enzyme activity and toluene removal for initial toluene concentrations in the range of 0 - 20 mg/L for a 10 minute reaction time: (a) normalized enzyme activity (b) toluene concentration

utilization rate of toluene (26.7×10^{-4} mg toluene (mg protein min)⁻¹) reported in section 5.3.2.

To determine the effect of pre-exposure of the cells to toluene on the Tod activity the experiment was repeated with cells grown on 10 mM succinate (no toluene). After incubating for one week without a carbon source, experiments using the same amount of toluene saturated aqueous solution as used previously were conducted under identical conditions. The normalized enzyme activity and toluene removal as a function of toluene concentration is shown in Figure 5.12(a) and (b). The initial normalized enzyme activity of 0.42×10^{-4} mg indigo (mg protein min)⁻¹ was much smaller than that in toluene-grown cells, demonstrating that succinate metabolism did not produce Tod enzyme. During the 10 minutes reaction time, virtually no additional Tod was produced and virtually no toluene oxidation occurred. Clearly enzyme synthesis occurs much faster in cells that have been previously exposed to toluene.

Growth experiments were conducted in which 50 mL samples of toluene-hunters medium were inoculated with succinate-grown cultures of *P. putida* F1; a 12 hr lag time occurred before the cells began to grow. In contrast, cells grown on toluene continued to grow when transferred to toluene-Hunters medium. These results supported the conclusion that a certain period of time was required to initiate the toluene metabolic pathways for cells that had not been previously exposed to toluene. On the other hand, the rapid increase in Tod activity observed in cells that had been preexposed to toluene is of great practical value in TCE cometabolic biodegradation processes.

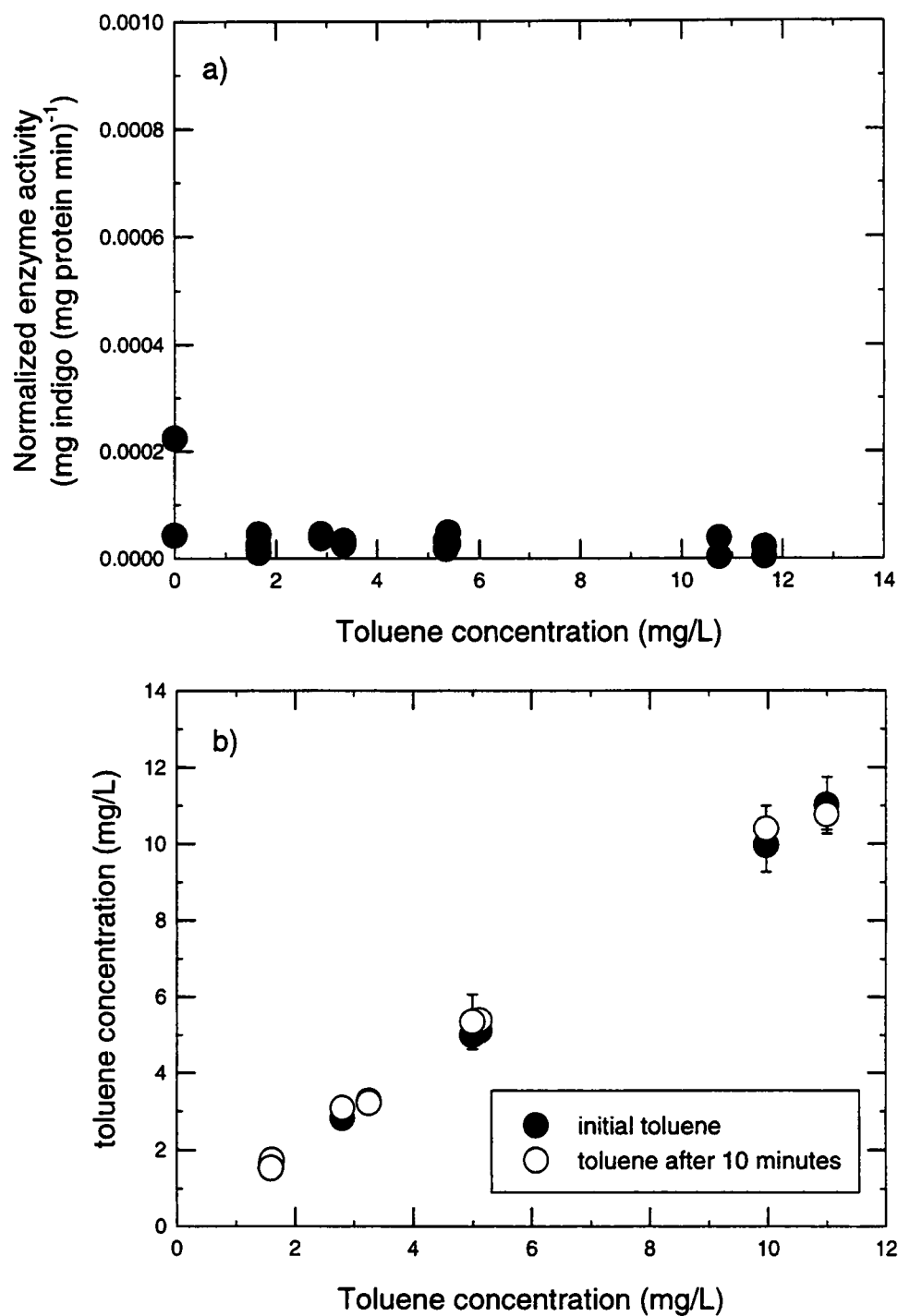


Figure 5.12 Normalized enzyme activity and toluene removal as a function of initial toluene concentration by succinate grown cells for a 10 minute reaction time: (a) normalized enzyme activity (b) toluene concentration

5.4.2 Effect of succinate on Tod activity

Pseudomonas putida F1 was grown in 50 ml of toluene-Hunters medium. The cells were prepared and incubated for one week without toluene addition. After one week, cells were harvested and washed twice with phosphate buffer. Cells were resuspended in Hunters medium to a cell density of 0.5 and dispensed into 25 ml Mininert cap vials. Various amounts of succinate stock solution were injected into each vial. The vials were incubated for 10 minutes on the shaker. The change of normalized enzyme activity as a function of succinate concentration is shown in Figure 5.13. The normalized enzyme activity did not increase above its initial value of 3.1×10^{-4} mg indigo (mg protein min)⁻¹. Succinate metabolism did not produce the toluene dioxygenase enzyme, as previously demonstrated in section 5.4.1.

5.4.3 Induction of Tod by TCE

Heald *et al.* (1994) reported that TCE can induce TCE-degrading activity (toluene dioxygenase) in *Pseudomonas putida*. They demonstrated that indole was converted to the blue dye indigo during growth of the organism on arginine. Toluene degradation rate by TCE-induced cells reached approximately 40 % of that observed in toluene-induced cultures. McClay *et al.* (1995) showed that toluene oxidation activity was induced by TCE in *P. mendocina* KR1 and *Pseudomonas* sp. Strain ENVPC5. When supplied with an energy source such as glutamate, the degree of induction was 86 % of that induced by toluene itself. However, induction by TCE in *B. cepacia* G4, *P. putida* F1, *Pseudomonas* sp. Strain ENV110 and, *Pseudomonas* sp. Strain ENV113 were not observed. Leahy *et al.* (1996) also investigated TCE induction of toluene

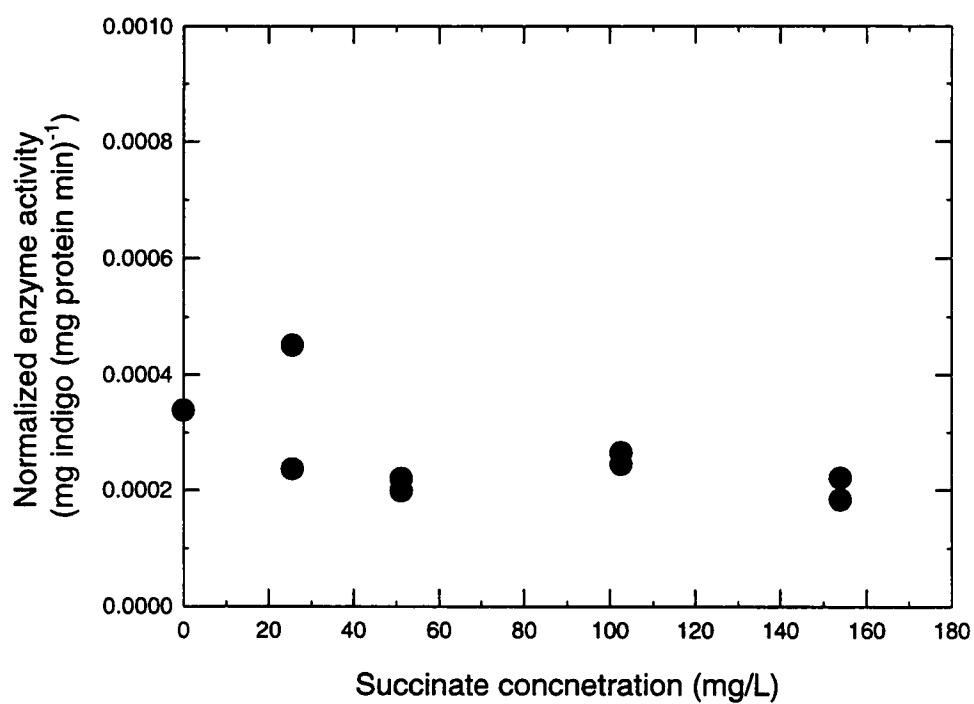


Figure 5.13 Normalized enzyme activity as a function of succinate concentration by toluene grown cells during 10 minute reaction period

degradation activity. They reported that TCE could induced the toluene-degradative activities in *B. cepacia* G4, *P. putida* F1, *P. mendocina* KR1, and *B. pickettii* PKO1 grown on lactate and TCE. The level of activity was observed to be 36 to 105 % of that in toluene-induced cultures. *B. cepacia* G4, *B. pickettii* PKO1, and *P. mendocina* KR1 grown on lactate and TCE showed a significant amount of TCE degradation, but *P. putida* F1 did not. However, McClay *et al.*, (1995) suggested that *B. cepacia* G4 and *P. putida* F1 did not induced toluene-degrading activity, which is a clear discrepancy with the findings of Leahy *et al.*, (1996) and Shingleton (1998).

According to the above studies, there is the possibility that TCE might induce toluene dioxygenase enzyme activity in *Pseudomonas putida* F1. If TCE induces considerable levels of toluene-degradative activity during biofiltration, it must be included in the biofiltration model. In order to determine whether TCE could induced the toluene-degrading activity in *Pseudomonas putida* F1, simple batch experiments were conducted.

Pseudomonas putida F1 was grown in a 500 ml flask with succinate-Hunters medium. When growth reached late exponential phase ($OD_{600} = 0.8-1.0$), cells were harvested and washed twice with phosphate buffer (pH 7.0). Cells were resuspended in fresh media to an OD_{600} of 0.5. The resuspended cells were transferred to three 250 ml of flasks: the first one included 10 mM of succinate, the second one was supplied with TCE using a glass tube suspended in the headspace of the flask, and the third one was supplied with both of succinate and TCE. All flasks were placed on a shaker operating at 200 rpm and incubated for 9 hours. Samples were routinely taken for determination of enzyme activity. The change in normalized enzyme activity as a function of time is

shown in Figure 5.14. The initial normalized enzyme activity was 0.1×10^{-4} mg indigo (mg protein min)⁻¹. The normalized enzyme activity in the control flasks, which contained succinate or TCE only, did not increase. In fact, the enzyme activity decreased in the flask with succinate only. In the flask containing both succinate and TCE, the enzyme activity increased after a 1 hr lag period to 0.85×10^{-4} mg indigo (mg protein min)⁻¹ after 5 hr of incubation. Further incubation did not result in further increases in enzyme activity, which implied the cells had reached maximum induction for these conditions. The activity measured for TCE induction was a small fraction (4.2 %) of the activity observed for toluene-induced cells (19.8×10^{-4}) (section 4.3). It was concluded that TCE induction could be neglected from the biofiltration model.

5.5 Effect of NADH on TCE oxidation

Toluene dioxygenase enzyme reactions with toluene (growth substrate) and TCE (cometabolic substrate), require NADH as the electron donor and O₂ as the electron acceptor. While NADH is regenerated during toluene degradation, NADH is not generated during TCE oxidation. Eventually, NADH may become limiting during TCE oxidation. In Figure 5.9, it was observed that TCE degradation became negligible even while Tod activity remained high. In this section, the hypothesis that such behavior could be explained by NADH limitation was tested. Acetate was selected as an alternate reducing energy substrate for TCE oxidation. Acetate is one of the final intermediate compounds produced during toluene metabolism. Acetate binds with coenzyme (CoA) to produce acetyl coenzyme A. Acetyl CoA produces three moles of NADH in the Kerbs

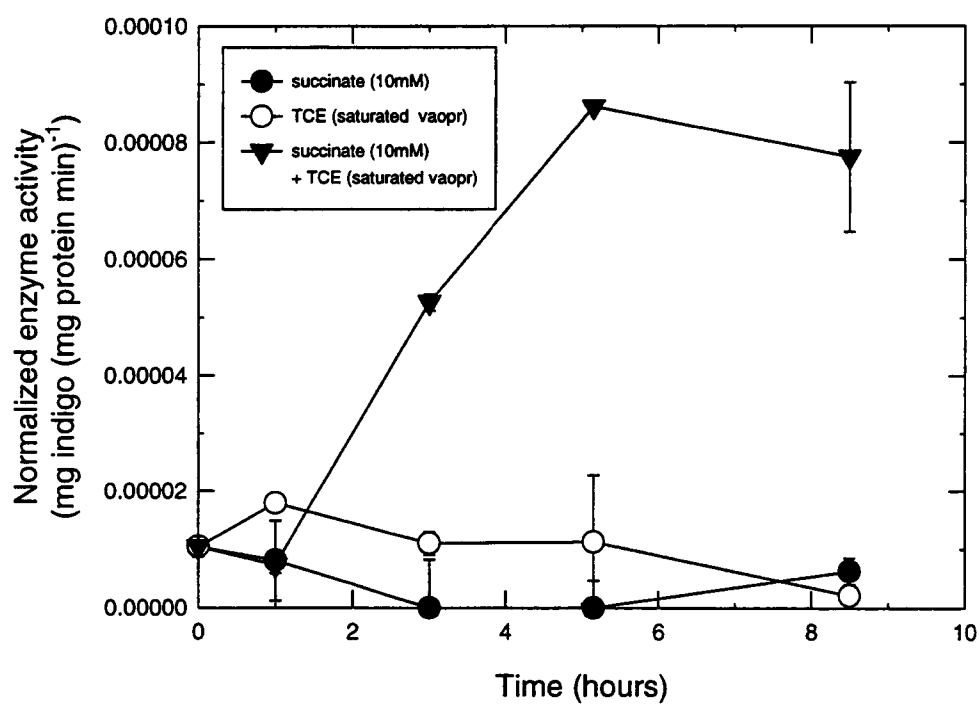


Figure 5.14 Normalized enzyme activity as affected by various substrates

cycle. Batch aqueous-phase TCE degradation experiments using 5 ml of *Pseudomonas putida* F1 culture were performed in 25 ml Mininert caps vials. *P.putida* F1 was grown in 50 ml of toluene-Hunters medium. Acetate was injected into two vials to yield acetate concentrations of 20 mM and 80 mM. Two additional control vials, without acetate, were prepared. TCE was injected into vials through the septa using a gas-tight syringe, the vials were incubated on a shaker at 200 rpm, and the remaining concentration of TCE monitored as a function of time (Figure 5.15). In each case, 50 % of TCE was degraded within 15 minutes. There was no significant difference in TCE degradation efficiencies for any of the vials, implying that NADH was not limiting.

Enzyme activity and NADH were also monitored during TCE degradation. Vials containing acetate and control vials were prepared as above. Vials were sacrificed at each time point to measure enzyme activity and NADH (Figure 5.16). Addition of acetate did not increase NADH or normalized enzyme activity. An additional experiment in which commercial NADH (20 mM) was added after TCE degradation stopped was also conducted (Figure 5.17). TCE removal was relatively constant after 60 minutes. NADH was injected to the vial at $t = 70$ minutes. Although a slight decrease in TCE concentration occurred immediately upon injection, complete degradation of TCE did not occur. It could not be concluded that NADH was limiting TCE degradation. No clear explanation of the incomplete batch degradation of TCE could be determined. The major emphasis of this work was cometabolic degradation of TCE in biofiltration; therefore, the reason for this anomaly were not pursued further.

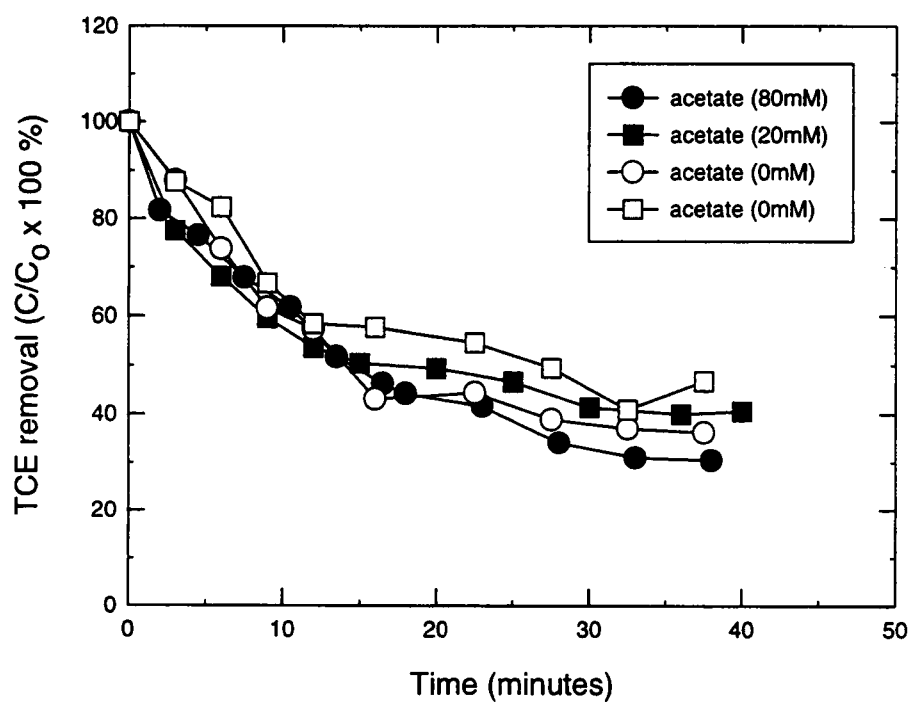


Figure 5.15 Effect of acetate on TCE removal

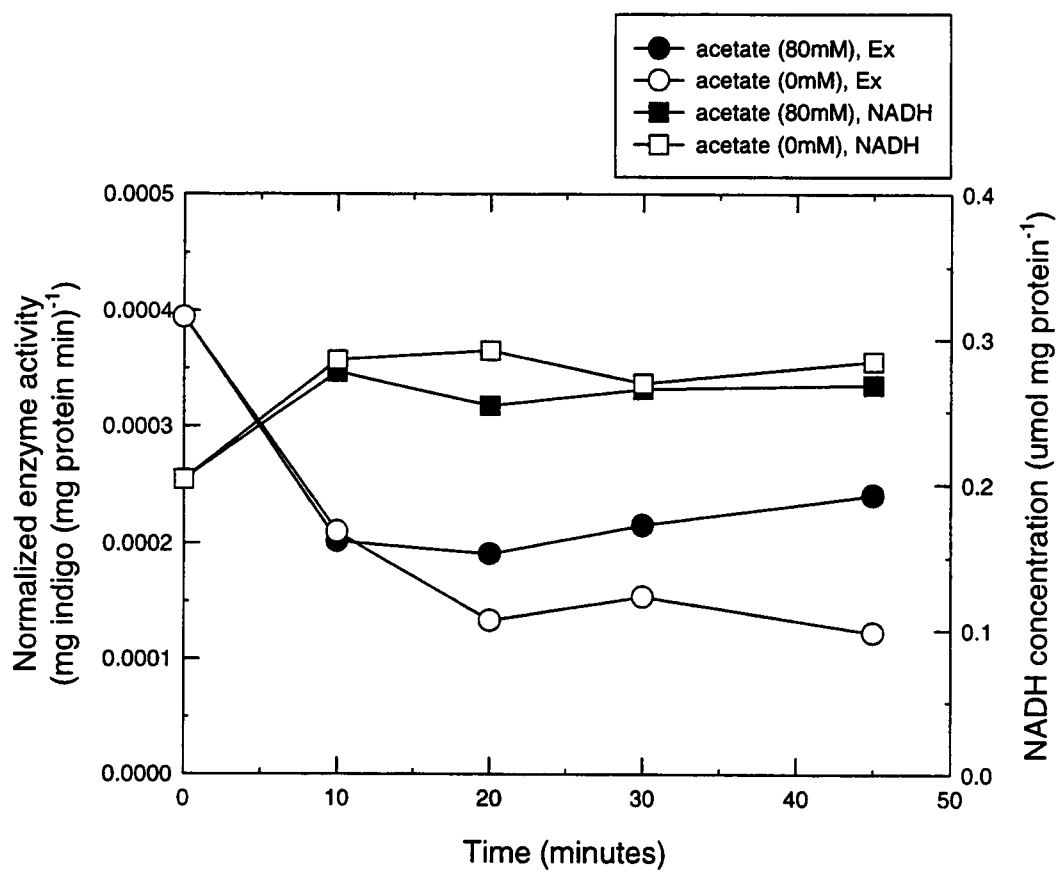


Figure 5.16 Effect of acetate on enzyme activity and NADH concentration

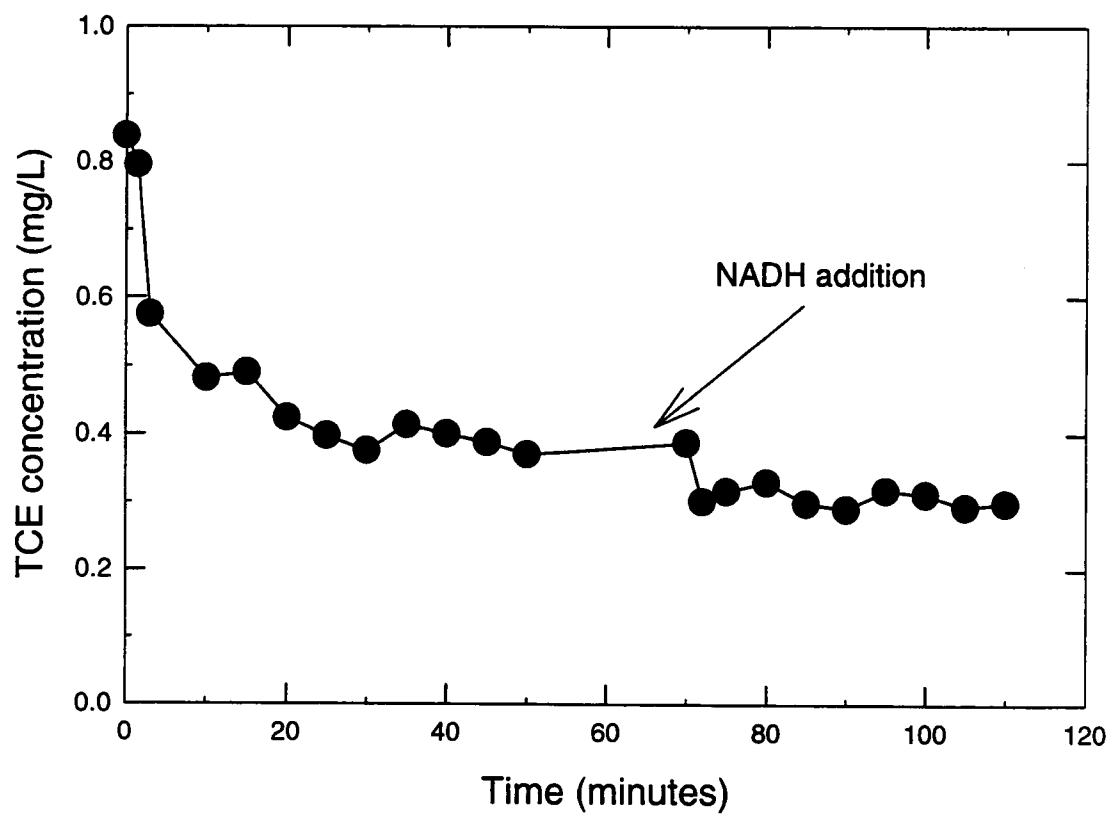


Figure 5.17 The effect of NADH addition on TCE removal

CHAPTER 6

TCE AND TOLUENE DEGRADATION EXPERIMENTS IN BIOFILTRATION

In this chapter, the application of *Pseudomonas putida* F1 in a biofiltration column for the aerobic degradation of toluene and cometabolic degradation of TCE was investigated. The focus of the experiments was to understand the role of enzyme activity in TCE cometabolism and to formulate a mathematical model to predict TCE removal rates as function of enzyme activity during biofiltration.

6.1 Preliminary biofiltration experiments

Preliminary experiments using a small-scale biofiltration column were conducted to verify the feasibility of removal of gas-phase TCE by cometabolism and to gather preliminary data for designing the remaining experiments.

6.1.1 Adsorption losses to diatomaceous earth (Celite) media

The ability of Celite to adsorb TCE and toluene was determined to distinguish this effect from biodegradation. TCE and toluene were individually added to three sets of five 20 ml Mininert-cap vials containing 2g of Celite. Different volumes of TCE and toluene gas were added to the vials. No liquid phase was present. The vials were shaken (180

rpm) at room temperature. Adsorption losses for TCE and toluene were less than 3 and 4 %, respectively, over a 5 day period. This demonstrates that adsorption was not a significant loss mechanism during biofiltration.

6.1.2 Abiotic losses during biofiltration

A differential volume reactor (DVR), consisting of a 10 cm long glass column (10 cm ID), was inoculated with *Pseudomonas putida* F1; the bed height was 1 cm. Air containing 5000 $\mu\text{g L}^{-1}$ of toluene was fed at rate of 500 ml min^{-1} for a period of one month to establish the biofilm. At the end of the one month period, about 9.67 % of the toluene fed to the reactor was removed. The bioreactor was then autoclaved for 20 minutes to kill the cells in the reactor. The reactor was cooled to room temperature and connected to the biofiltration system again. The column was then fed with 500 ml min^{-1} of air containing 5000 $\mu\text{g L}^{-1}$ of toluene to quantify abiotic losses of toluene. Also liquid nutrients were fed to the column during this experiment. The toluene profile for this experiment is shown in Figure 6.1. There is no significant difference between the influent and effluent toluene concentrations across the reactor. This result verified that sorption and other losses were negligible.

6.1.3. Toluene degradation via biofiltration

In initial experiments, a glass biofiltration reactor, 36-cm long with an inner diameter of 2.5-cm was used. The gas stream to be treated and an aqueous nutrient solution flowed from the top to the bottom of the column. *Pseudomonas Putida F1* was grown in a 1-L flask at room temperature in 500-ml of medium with toluene supplied in

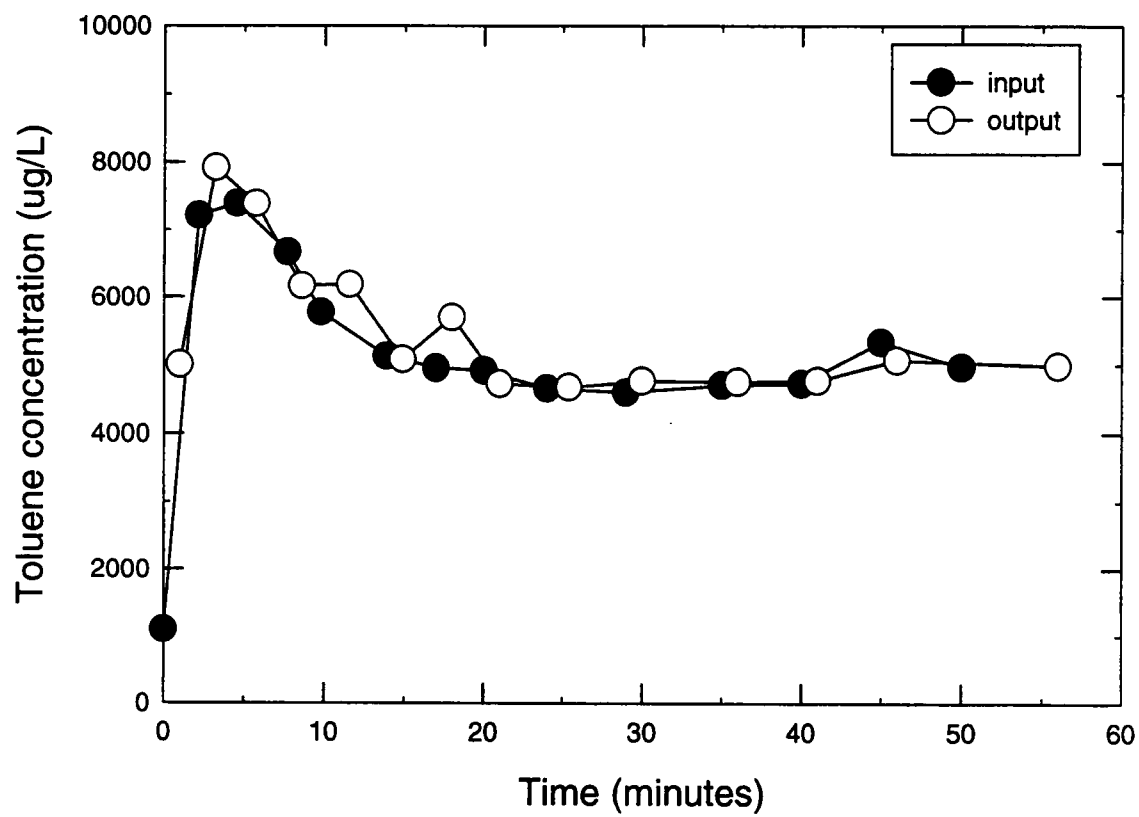


Figure 6.1 Toluene removal from sterilized biofilter

the vapor phase. After a yellow color appeared in the culture, the culture was transferred into fresh growth medium containing diatomaceous earth pellets (Celite) in a 2-L flask. The flask was then placed on a shaker to provide mixing and an opportunity for microbial attachment to the diatomaceous earth. A slow shaking intensity was used to avoid erosion of the diatomaceous earth pellets. The pellets were then transferred to the column and a mixture of air and toluene gas was supplied for an acclimation period of 4 days.

The biofilm was observed to be thickest near the influent end of the column. The toluene concentration profile through the column during the first 5 days after the acclimation period is shown in Figure 6.2. The column was operated at an EBCT of 1.5 minutes. Toluene was fed to the column at a concentration of 150 ppmv (0.565 mg/L) yielding a bed loading rate of $1.7 \text{ kg COD m}^{-3} \text{ day}^{-1}$. When the column was start-up, about 30 % of the toluene was removed, corresponding to degradation rate of $0.51 \text{ kg COD m}^{-3} \text{ day}^{-1}$. Within one day, the effluent concentration of toluene in the column decreased to 5 ppmv (0.0188 mg/L). Most of the toluene was removed in the front half of the biofiltration column. On subsequent days, virtually completely removal of the toluene occurred in the first 60 % of the column. The toluene degradation rate in this portion of the column was $2.9 \text{ kg COD m}^{-3} \text{ day}^{-1}$. Smith *et al.* (1996) reported similar toluene removal performance at a loading of $2.27 \text{ kg COD m}^{-3} \text{ day}^{-1}$. Toluene concentration profiles after initiation of TCE feed are shown in Figure 6.3. Feed concentrations of toluene and TCE were 300 ppmv and 50 ppmv, respectively. Toluene degradation was less efficient upon initiation of TCE feeding. However, after 4 days, toluene degradation was near complete in the first half of the column. The degradation

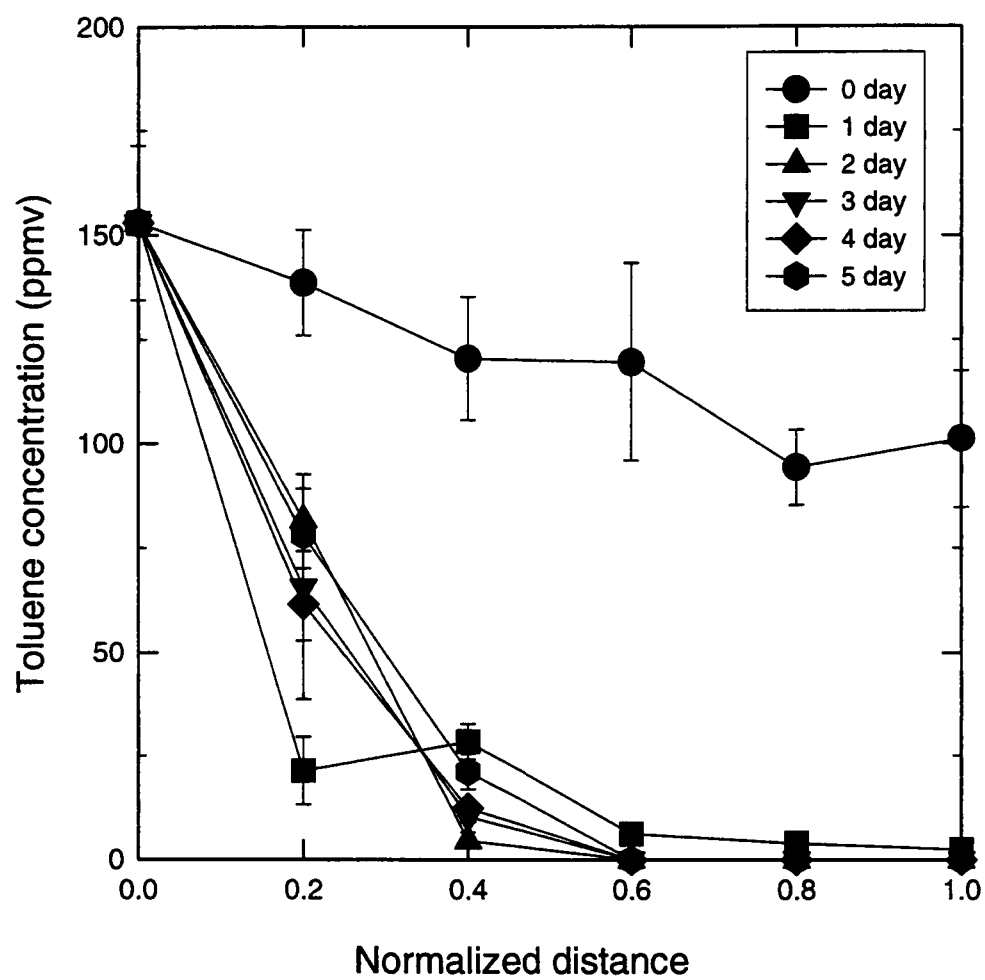


Figure 6.2 Removal of toluene by biofiltration as a function of time after start-up.

of TCE was not significant during the experiment, probably because of competitive inhibition by toluene (Speitel *et al.*, 1993; Segar *et al.*, 1995; Smith, et al., 1996 ; Yu, 1998).

6.1.4 TCE degradation via biofiltration in the absence of toluene

In the previous section, no TCE removal occurred when TCE and toluene were fed simultaneously at the concentrations tested. An attempt was made to avoid competitive inhibition effects and improve TCE degradation efficiency by feeding toluene alone for a period of 48 hrs to induce dioxygenase formation, followed by feeding TCE alone. Concentration profiles of TCE as a function of operating time are shown for two different empty bed contact times (EBCT): 1.9 minutes (Figure 6.4) and 3.1 minutes (Figure 6.5). About 175 and 50 minutes were needed to reach the steady-state conditions for these two conditions. TCE removal across the column was about 40 to 50 % for an EBCT of 1.9 min and about 50-60 % for an EBCT of 3.1 min. TCE degradation decreased markedly after longer exposure of TCE due to depletion of dioxygenase. TCE removal reduced to less than 5 % after 24 hrs. Factors that may contribute to the reduction of TCE biodegradation over time include NADH limitation and inactivation of Tod enzyme activity by toxic intermediates of TCE co-oxidation. These effects were investigated further in later experiments.

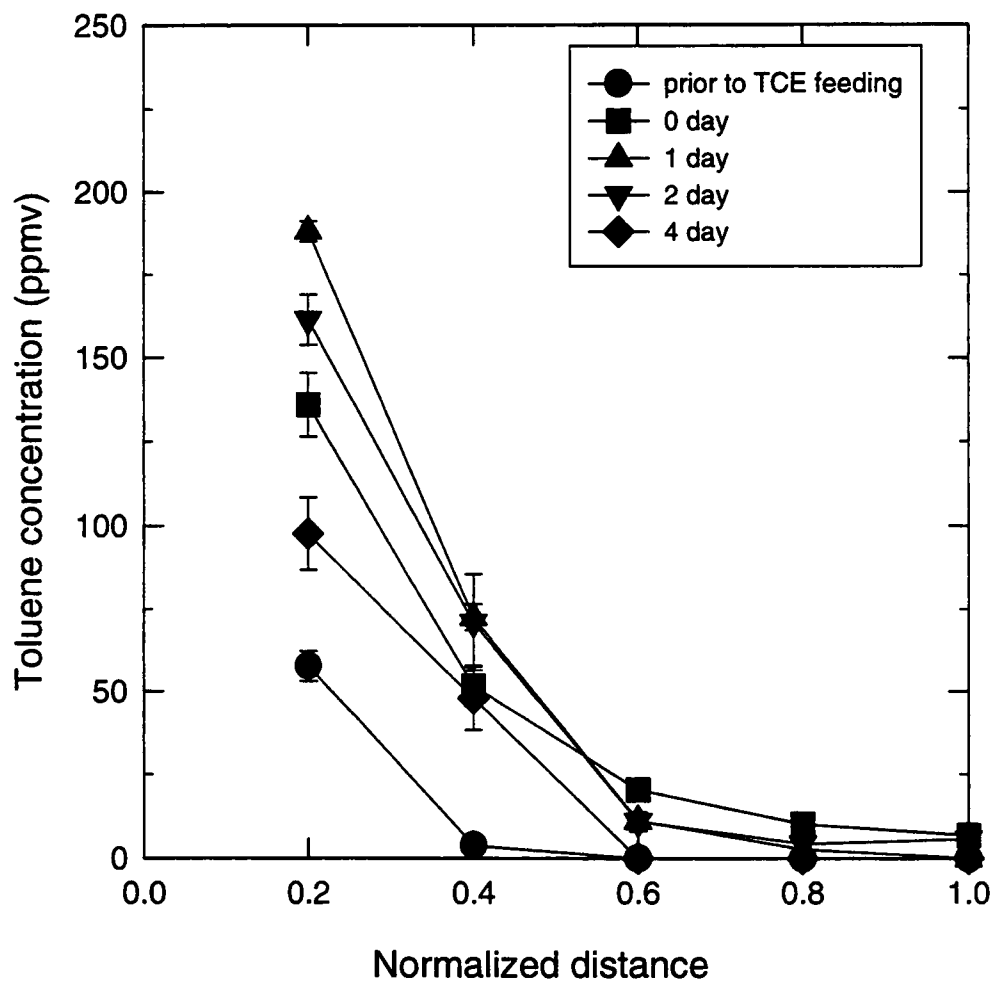


Figure 6.3 Removal of toluene by biofiltration as a function of time after introduction of TCE to column feed (toluene = 300 ppmv, TCE = 50 ppmv)

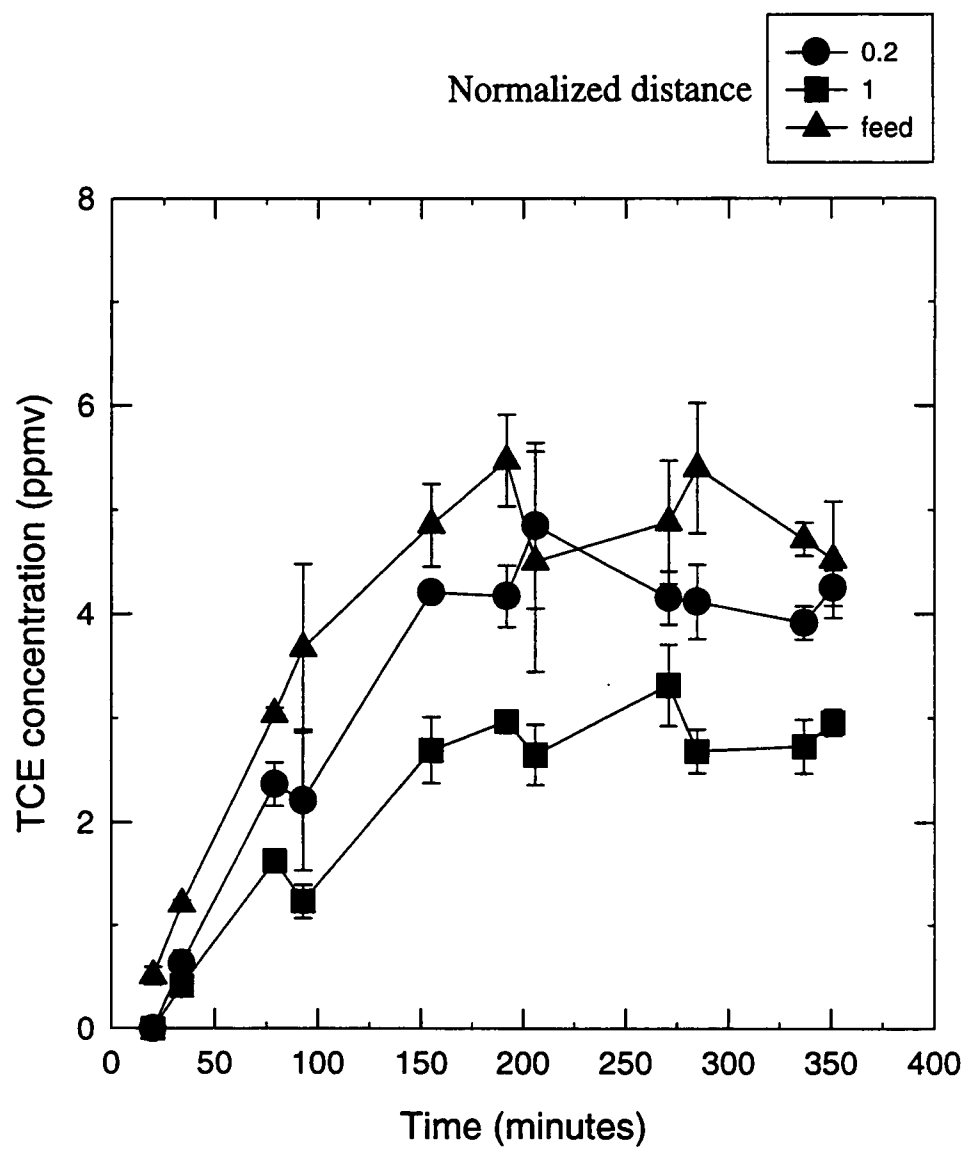


Figure 6.4 Removal of TCE during biofiltration (EBCT = 1.9 min)

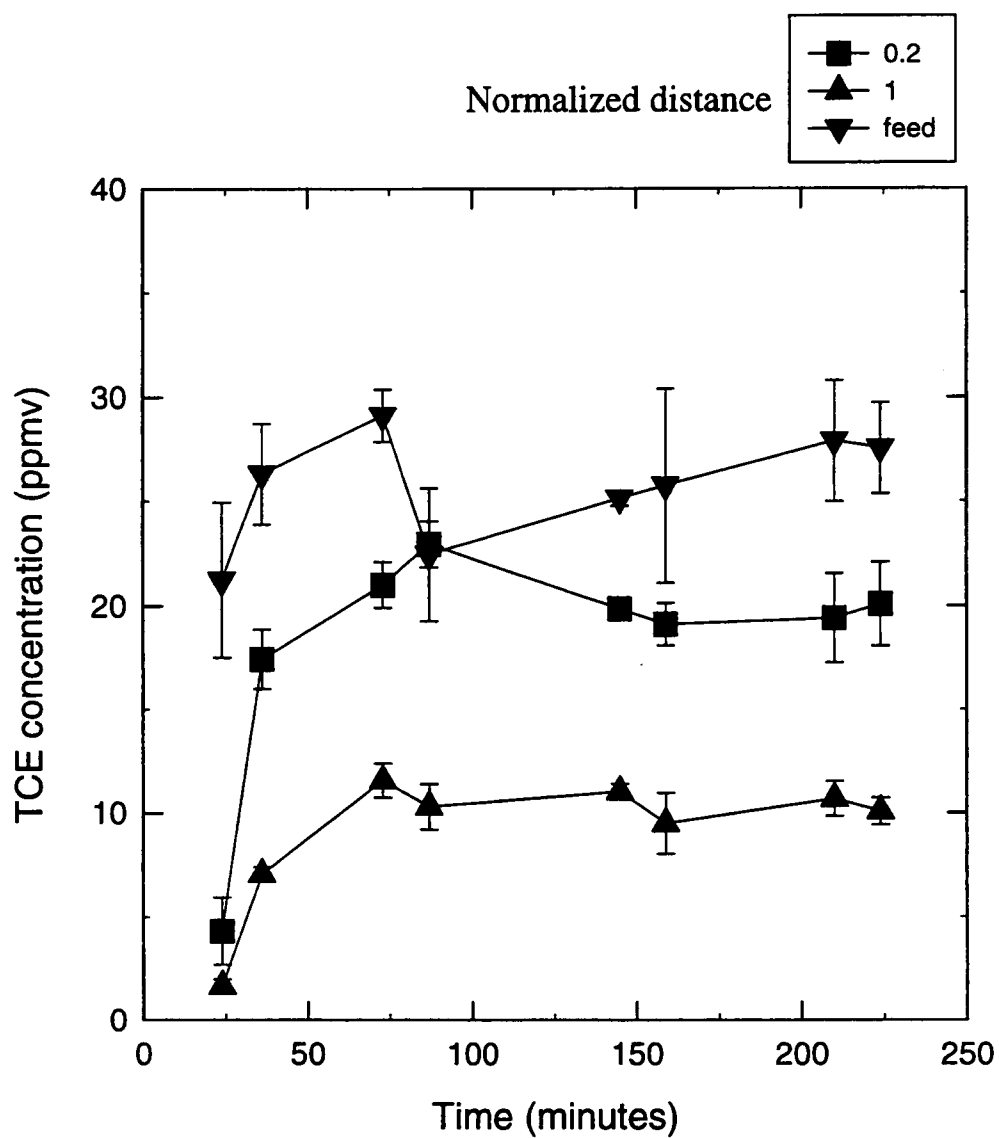


Figure 6.5 Removal of TCE during biofiltration (EBCT=3.1 min)

6.1.5 Toluene and TCE degradation via biofiltration

Given the capacities of the mass flow controllers, the 2.5-cm diameter by 36-cm long biofiltration column was limited in the range of initial TCE and toluene concentrations that could be tested. A larger column, 10 cm in diameter by 100-cm long, was designed. The bed height was 64-cm with sample ports placed every 16-cm. This bioreactor was used to conduct experiments to determine if lower levels of TCE could be treated in a biofilter in which toluene was fed continuously. Figure 6.6 shows the toluene and TCE concentration profiles across the column during seven days of operation. The feed concentration of toluene and TCE were $100 \mu\text{g L}^{-1}$ and $60 \mu\text{g L}^{-1}$, respectively and an EBCT of 5.02 minutes was used. Toluene and TCE concentrations were measured on the first and seventh days after start-up. The toluene and TCE loading rates were $28.66 \mu\text{g toluene cm}^{-3} \text{ day}^{-1}$ and $17.20 \mu\text{g TCE cm}^{-3} \text{ day}^{-1}$, respectively. Overall removal of TCE ranged from 30 to 40 %. Performance of the column was stable over the 7-day period of the experiment. As in previous experiments, most of the toluene was degraded in the first half of the column. TCE removal also occurred in the same part of the column. The average removal rate of toluene and TCE were $51.59 \mu\text{g toluene cm}^{-3} \text{ day}^{-1}$ and $6.02 \mu\text{g TCE cm}^{-3} \text{ day}^{-1}$, respectively. This experiment demonstrates that toluene is needed to induce the Tod enzyme in order for TCE degradation to occur. Toluene was not present in the second half of the column, hence significant quantities of Tod enzyme were not produced and little TCE degradation occurred.

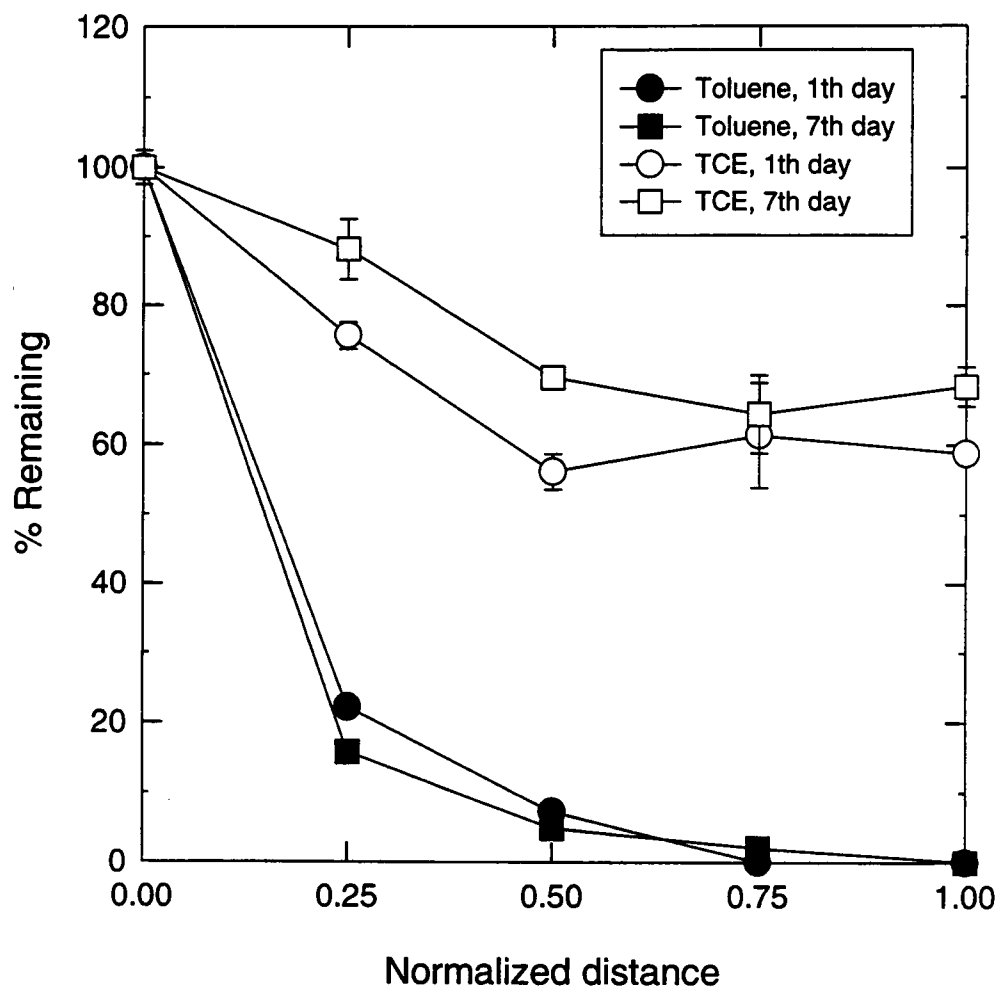


Figure 6.6 Degradation of TCE (60 ug/L) and toluene (100 ug/L) simultaneous via biofiltration

The biofiltration experiments described thus far illustrate the difficulty in obtaining high TCE removal efficiencies in cometabolic systems. When toluene and TCE are fed simultaneously, competitive inhibition limits the TCE degradation rate. The rate can be increased by sequential feeding of TCE and toluene, but *Tod* enzyme and NADH reservoirs are depleted over time. To obtain high TCE removal efficiencies, reactor design and operation strategies are needed which maximize *Tod* enzyme production while minimizing competitive inhibition. Examples of such strategies include pulsed feeding of toluene to a single reactor or multiple reactors that are alternately fed either toluene or TCE. Better quantitative understanding of competitive inhibition, NADH limitation, enzyme activity, and product toxicity is needed to form a rational basis for formulating such strategies.

Biokinetic parameters are required to understand the growth and substrate utilization kinetics of *Pseudomonas putida* F1. The different physical and chemical environments of suspended and immobilized cultures are likely to yield variations in cellular physiology and biokinetic parameters (Shreve *et. al.*, 1993). If the kinetics of suspended and immobilized cells are different, the parameters estimated from suspended cell experiments may not be directly applicable to the immobilized cell experiments. The kinetic studies for suspended cells were described in chapter 5. A differential volume reactor (DVR) was used for kinetic studies of immobilized cells.

6.2 Growth of immobilized *Pseudomonas putida* F1

The growth rate of immobilized *P. putida* F1 was measured in a DVR at various concentrations of toluene. The data were fitted to the Monod equation.

6.2.1 Growth rate of immobilized cells

The differential volume reactor (DVR) was constructed to measure the growth rate of immobilized cells and the substrate utilization rate parameters. The DVR consisted of a 10-cm diameter by 10-cm long glass column; the bed height was 1 cm. The DVR was operated with the same flow rate as used in the biofiltration column: 500 ml/min, yielding an EBCT of 0.157 min. The reactor was inoculated with *Pseudomonas putida* F1 cells as described in section 3.1.2. The DVR was operated at four toluene feed concentrations: 500, 1400, 1700, and 3000 µg/L. After the first three days of operation, the biomass in the column was quantified daily. Two Celite pellets were removed from the column and mixed with 2 ml of sterile 0.1 % Na₂P₂O₇ and vortexed to detach immobilized cells from pellets. The cell mixture was centrifuged and washed with phosphate buffer solution (pH=7.2). The final cell pellets were resuspended with 2 ml of fresh medium. The cells were detached and suspended homogeneously by alternatively sonicating for three burst of 3 seconds followed by interval for 30 seconds. The protein concentration of the prepared cell culture was determined using a BCA protein assay reagent kit. When two consecutive biomass measurements differed by less than 10 %, the biofilm was assumed to have reached steady-state (Shreve et al, 1993) and the toluene utilization rate was calculated. In this experiments, when the DVR was operated at the four toluene feed concentrations of 500, 1400, 1700, and 3000 µg/L, the total biomass in the reactor reached its steady-state level after 216, 189, 170 and 192 hrs of toluene feeding, respectively.

The advantage of a DVR for measurement of kinetic parameter is that constant conditions prevail throughout the bed. Shreve *et al.* (1993) indicated that this condition can be met if the substrate conversion is less than 5 %.

$$\% \text{ conversion} = ((C_o - C_e) / C_o) \times 100 \quad (6.1)$$

where C_o : the influent toluene concentration
 C_e : the effluent toluene concentration

In the present experiments, substrate conversion was about 5 % in all experiments. Another consideration is that the difference in influent and effluent concentration should be significantly greater than the standard error of the measured influent and effluent concentrations. A summary of the immobilized cells growth experiments is shown in Table 6.1.

6.2.2 Mathematical model for cells growth

The Monod equation describes the relationship between the concentration of the growth-limiting substrate and the specific growth rate of biomass:

$$dX/dt = \mu_{\max} X C / (K_{sT} + C) \quad (6.2)$$

where C : toluene concentration, $\mu\text{g L}^{-1}$
 X : cell mass, mg protein
 $\mu_{\max}$: maximum specific growth rate, hr^{-1}
 K_{sT} : saturation constant, $\mu\text{g L}^{-1}$

Table 6.1 Summary of Immobilized Cells Growth Experiments

RUN #	toluene influent concentration ($\mu\text{g toluene L}^{-1}$)	standard deviation for influent concentration ($\mu\text{g L}^{-1}$)	toluene effluent concentration ($\mu\text{g toluene L}^{-1}$)	standard deviation for effluent concentration ($\mu\text{g toluene L}^{-1}$)	Difference between influent and effluent concentration ($\mu\text{g toluene L}^{-1}$)	percent substrate conversion (%)	toluene utilization rate ($\mu\text{g toluene (mg protein min)}^{-1}$)
1	495.3	7.37	455.5	4.03	39.8	8.03	8.94
2	1402.3	28.17	1321.6	22.73	80.7	5.75	15.71
3	1700.8	20.52	1632.4	14.35	68.4	4.02	14.71
4	3018.8	63.12	2864.0	33.66	154.8	5.13	31.07

Integration of equation (6.2) yields in the following equation:

$$\ln X = \{ \mu_{\max} X C / (K_{sT} + C) \} t + \ln X_0 \quad (6.3)$$

where X : cell mass at time t , mg protein
 X_0 : cell mass at time 0, mg protein

In each experiment, a lag period of approximately 20 - 40 hrs was observed prior to the onset of biomass growth. For modeling purposes, $t=0$ was taken to be 72 hrs after the initiation of toluene feed to the column. The value of the maximum specific growth rate ($\mu_{\max}$) was estimated by fitting equation (6.3) to the immobilized cell growth data. The value of K_{sT} ($1988 \mu\text{g L}^{-1}$) was determined in section in 6.3. Toluene feed concentrations of 500, 1400, 1700, and $3020 \mu\text{g toluene L}^{-1}$ were used in the experiments. The maximum specific growth rate ($\mu_{\max}$) was estimated to be 0.029 hr^{-1} by fitting the data to the model equation (6.3). The model results are shown superimposed with the experimental data in Figure 6.7(a) to (d). Figure 6.8 shows the predicted growth of immobilized cells for toluene concentration ranging from 100 to $12000 \mu\text{g L}^{-1}$. Growth strongly depends on the toluene concentration up to $4000 \mu\text{g L}^{-1}$. Growth did not increased significantly above $5000 \mu\text{g/L}$ because the maximum specific growth rate was already attained (in section of 6.3). In Figure 6.8, it appears that biomass grows to infinite mass because only growth is considered. In reality, biomass eventually reaches a steady-state level when the growth rate is balanced by removal of biomass by fluid shear. As the biofilm grows thicker, limitations of substrate, oxygen, and nutrients within the

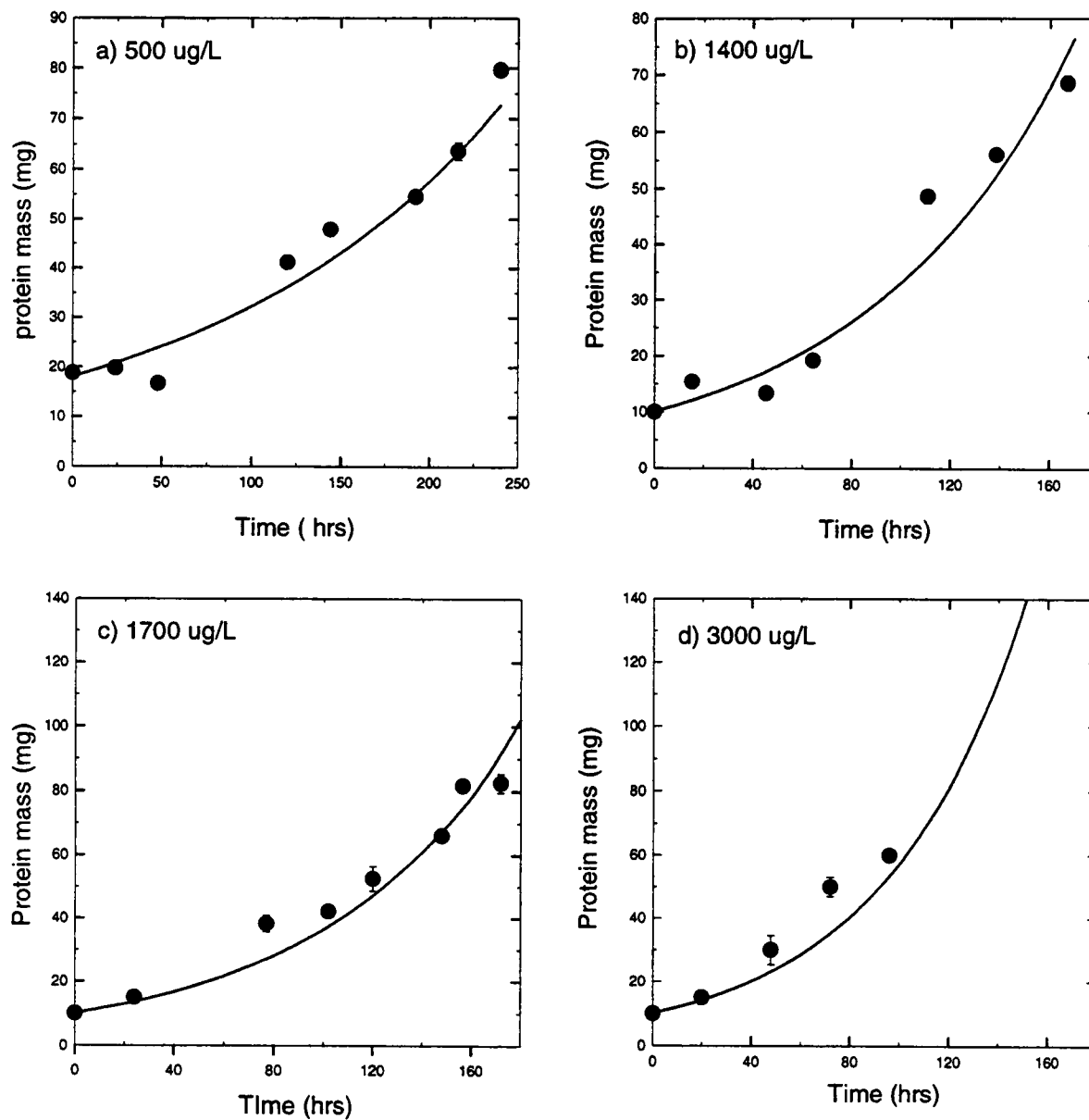


**Figure 6.7 Growth of immobilized *P.p.* F1 at various toluene concentrations:
(a) 500 ug/L (b) 1400 ug/L (c) 1700 ug/L (d) 3000 ug/L**

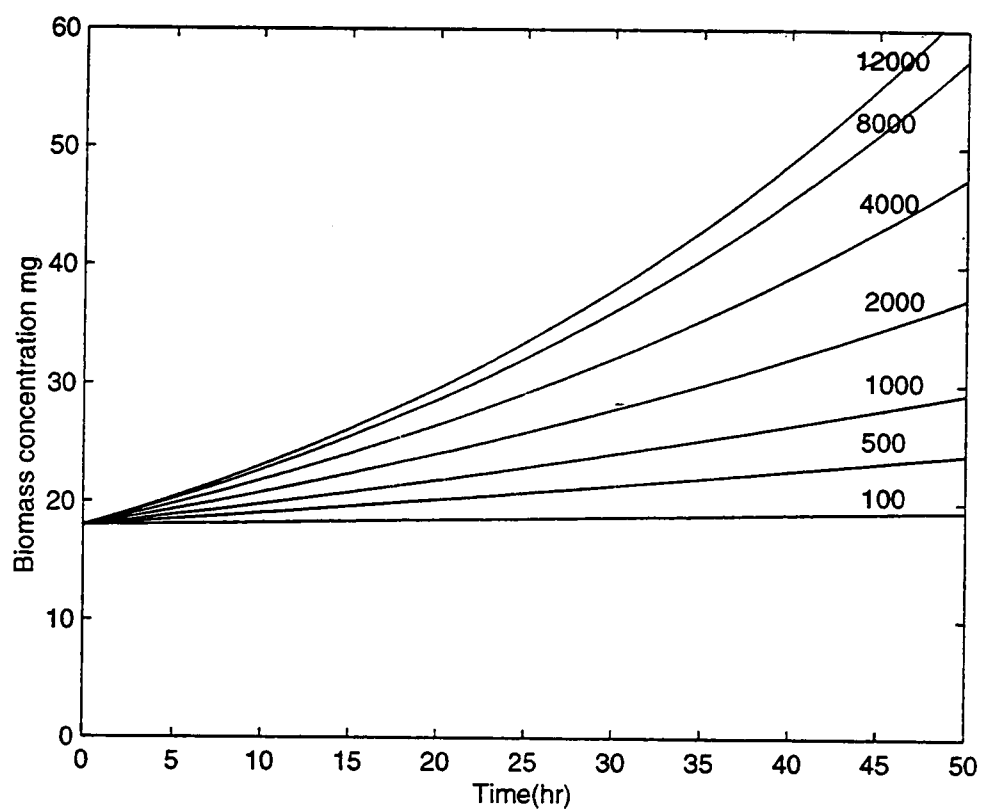


Figure 6.8 predicted growth of immobilized cells for various toluene concentrations

biofilm become more likely (Trulear et. al., 1979). Characklis (1981) described biofilm development as a function of transport of organic molecules and microbial cells to the wetted surface, adsorption of organic molecules to the wetted surface resulting in a conditioned surface, adhesion of microbial cells to the conditioned surface, metabolism, detachment of portions of the biofilm. He has also showed that an S-shaped curve results when biofilm thickness is plotted as a function of time. At steady-state, the biofilm thickness remains constant. Biofilm development is expressed by following equation:

$$\begin{aligned} &\text{Net rate of biofilm growth} \\ &= \text{growth rate} - \text{detachment rate} \\ &\quad + \text{cell adsorption rate} - \text{endogenous respiration} \end{aligned}$$

6.3 Toluene utilization experiments

When two consecutive biomass measurements differed by less than 10 %, the biofilm was assumed to have reached steady-state and the toluene utilization rate experiments were initiated. Experiments were conducted at influent toluene concentrations of 500, 700, 1400, 1700, 3000, 4000, 4700, 5100, 5700, and 6000 µg/L. Each feed concentration was sustained for approximately 2 - 3 days until a stable effluent concentration was achieved. Substrate utilization rates for immobilized cells were evaluated from the steady-state DVR mass balance equation:

$$-\frac{d[T]}{dt} \frac{1}{X} = r_T = \frac{Q(T_o - T_e)}{X} \quad (6.4)$$

Where r_T : specific substrate utilization rate, $\mu\text{g toluene mg protein}^{-1} \text{ min}^{-1}$

Q : flow rate of gas, L min^{-1}

T_o : influent toluene concentration, $\mu\text{g toluene L}^{-1}$

T_e : effluent toluene concentration, $\mu\text{g toluene L}^{-1}$

X : total biomass in reactor, mg protein

The specific toluene utilization rate of immobilized cells as a function of toluene concentration is shown in Figure 6.9. This is a typical substrate utilization curve as described Lawrence and McCarty (1970). The relationship between utilization rate and toluene concentration was represented by typical saturation kinetics.

$$r_T = k_T \frac{C_o}{(K_{sT} + C_o)} \quad (6.5)$$

where r_T : specific substrate utilization rate, $\mu\text{g toluene mg protein}^{-1} \text{ min}^{-1}$

C_o : influent toluene concentration, $\mu\text{g toluene L}^{-1}$

k_T : maximum specific substrate utilization rate,
 $\mu\text{g toluene mg protein}^{-1} \text{ min}^{-1}$

K_{sT} : half-velocity constant, $\mu\text{g toluene L}^{-1}$

The half -velocity constants and maximum specific substrate utilization rate were evaluated by constructing a double-reciprocal plot (Figure 6.10) (Shuler *et. al.*, 1992).

The linearized form of equation (6.5) is:

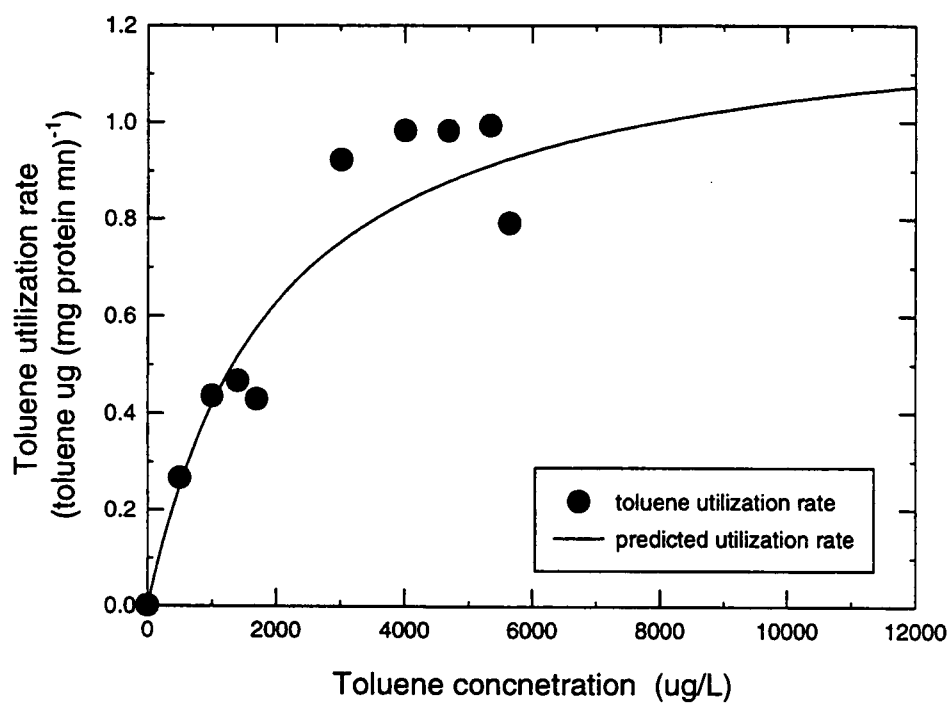


Figure 6.9 Toluene utilization rate as a function of toluene concentration

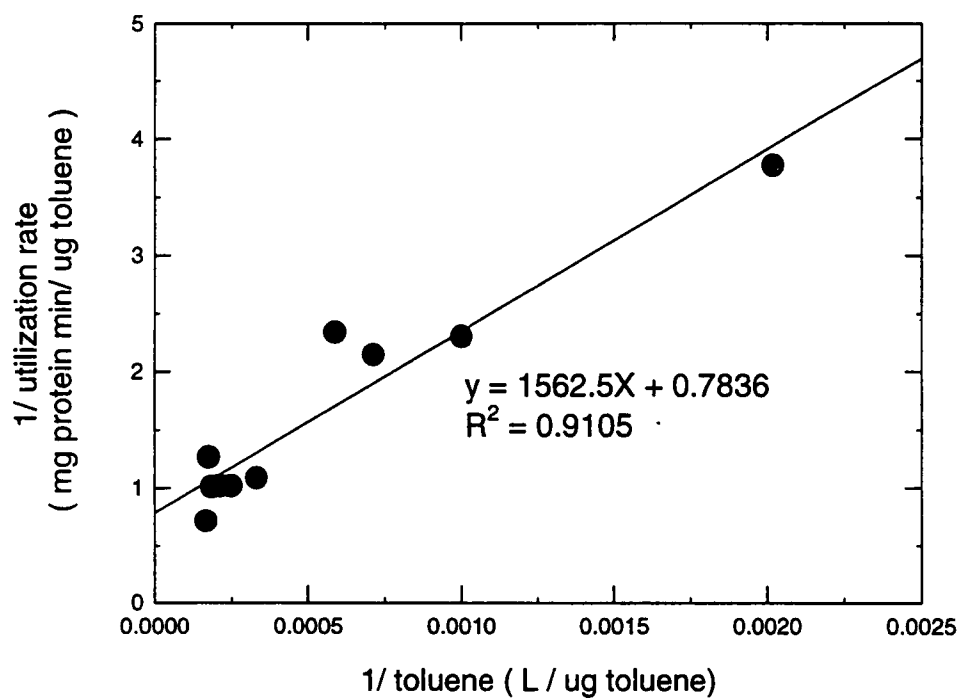


Figure 6.10 Determination of growth constants for the Michaelis-Menten equation

$$\frac{1}{r_T} = \frac{1}{k_T} = \frac{K_{sT}}{(k_T \cdot [T])} \quad (6.6)$$

A plot of $1/r_T$ versus $1/[T]$ yields a straight line with a slope of K_{sT}/k_T and a y-axis intercept of $1/k_T$. The slope and y-axis intercept were found by linear least-squares fit of the experimental data.

The maximum specific substrate utilization rate of toluene was found to be $1.250 \mu\text{g toluene min}^{-1} \text{mg biomass}^{-1}$ and the saturation constant (K_{sT}) was $1988 \mu\text{g toluene L}^{-1}$. The relationship between the specific toluene utilization rate and toluene concentration was calculated using equation (6.5) and compared to the experimental values in Figure 6.9. The predicted and measured values match well.

For each toluene concentration tested, the steady-state NADH concentration and enzyme activity were measured and reported in Figure 6.11 and 6.12, respectively. The samples were prepared in the same manner as described in section 3.3.5 except the final volume of cells was 5 ml. The normalized NADH concentration was independent of toluene concentration in the range of 500 to $6000 \mu\text{g L}^{-1}$, and had a mean value of $0.48 \pm 0.076 \mu\text{mole mg protein}^{-1}$. For cells grown in suspended culture, the normalized NADH concentration increased with increasing toluene concentration to a maximum value of increased $0.29 \pm 0.02 \mu\text{mol mg protein}^{-1}$ after the early-stationary-growth phase (section 4.3). The normalized NADH concentration observed for immobilized cells at steady state was almost twice the value measured for suspended cells at the early-stationary phase. As long as toluene was introduced to the

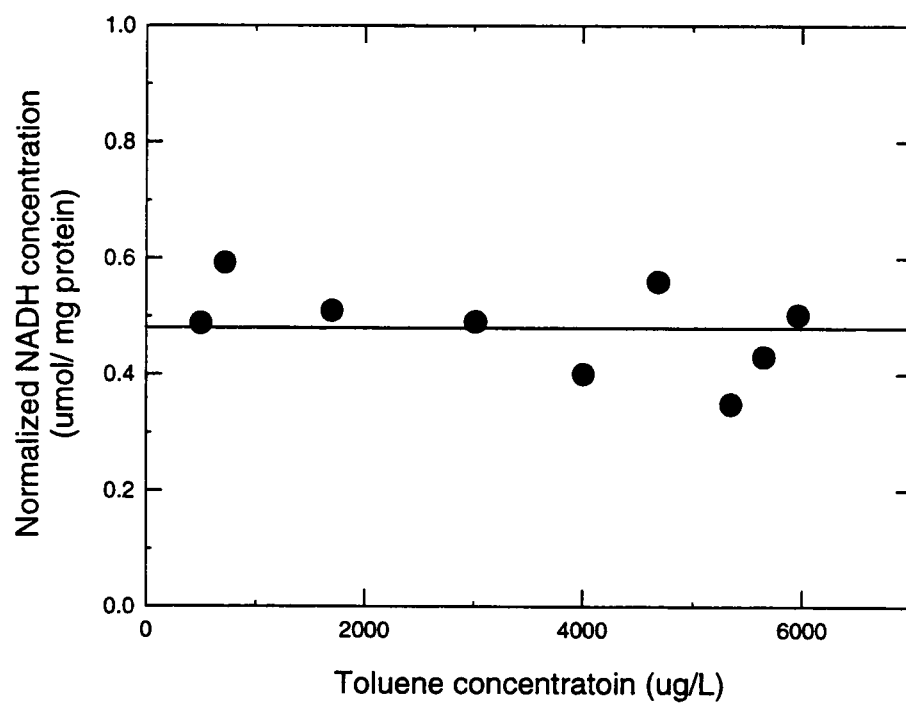


Figure 6.11 Normalized NADH concentration in immobilized cells as a function of toluene concentration

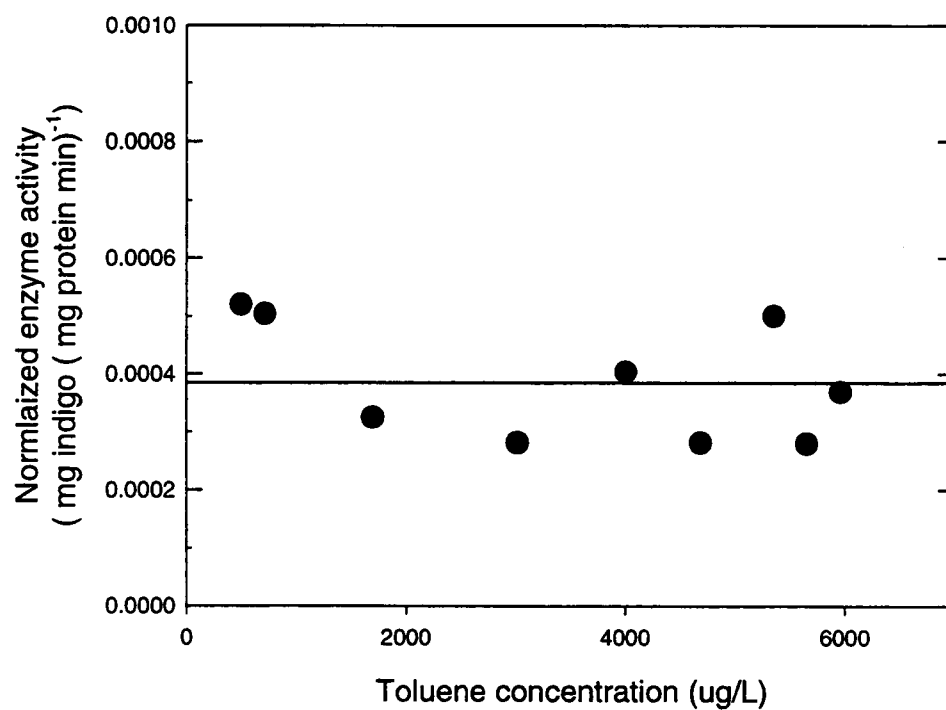


Figure 6.12 Normalized enzyme activity in immobilized cells as a function of toluene concentration.

immobilized reactor, cells maintained the same level of NADH. It can be also concluded that high levels of toluene are not needed to maintain NADH activity within the cells.

Figure 6.12 shows the relationship between toluene feed concentration and normalized whole-cell Tod activity in immobilized cells. Similar to NADH concentration, the activity is independent of toluene concentration in the range of 500 to 6000 $\mu\text{g/L}$. It can be concluded that the Tod enzyme system is fully induced at toluene concentrations as low as 500 $\mu\text{g L}^{-1}$. The mean whole -cell Tod enzyme activity is $3.85 \pm 1.0 \times 10^{-4}$ mg indigo mg protein⁻¹ min⁻¹ under the steady-state conditions. The maximum normalized enzyme activity in suspended culture grown was $13.5 \pm 1.5 \times 10^{-4}$ mg indigo mg protein⁻¹min⁻¹ during the early-stationary phase (section 4.3). The normalized Tod activity of immobilized cells at steady state was only about 28.5 % of the value for the suspended culture at early-stationary phase. In case of immobilized cells, mass transfer limitation may prevent induction of Tod enzyme through out the biofilm. For the suspended culture experiment, the enzyme activity was measured for growing cells, so each cell was very active and most of protein mass was considered to be cell mass.

6.4 Competitive inhibition between TCE and toluene

To simplify data collection, only the first quarter of the biofiltration column (length = 16-cm) was used. Gas samples were collected from the port at a bed depth of 16-cm and the concentrations of TCE and toluene were measured. Different TCE to toluene ratios in the feed stream were tested to determine the effect of toluene on

inhibition of TCE degradation. The summary of experimental conditions and result are shown in Table 6.2.

Table 6.2. Specific TCE utilization rates at various toluene loadings.

RUN	Average influent gas flow rate (mL min⁻¹)	Average influent toluene concentration (µg L⁻¹)	Average influent TCE concentration (µg L⁻¹)	specific TCE utilization rate (µg TCE mg protein⁻¹ min⁻¹)	Average biomass content (mg protein)
1	500	15263	271	0.000886	2257
2	280	12315	89	0.001867	2918
3	500	11190	166	0.000795	2956
4	280	8130	40	0.003538	2201
5	280	8007	178	0.002798	3136
6	280	7475	102	0.002222	2802
7	280	5549	261	0.006287	3042

The plot of toluene feed concentration (µg L⁻¹) versus TCE removal rate (µg TCE mg protein⁻¹ min⁻¹) is shown in Figure 6.13. The specific TCE utilization rate in immobilized cells was determined from the equation:

$$-\frac{d[S]}{dt} \frac{1}{X} = r_{TCE} = \frac{Q(S_o - S_e)}{X} \quad (6.7)$$

Where r_{TCE} : specific TCE utilization rate, µg TCE mg protein⁻¹

Q : flow rate of gas, L min⁻¹

S_o : influent TCE concentration, µg TCE L⁻¹

S_e : effluent TCE concentration, µg TCE L⁻¹

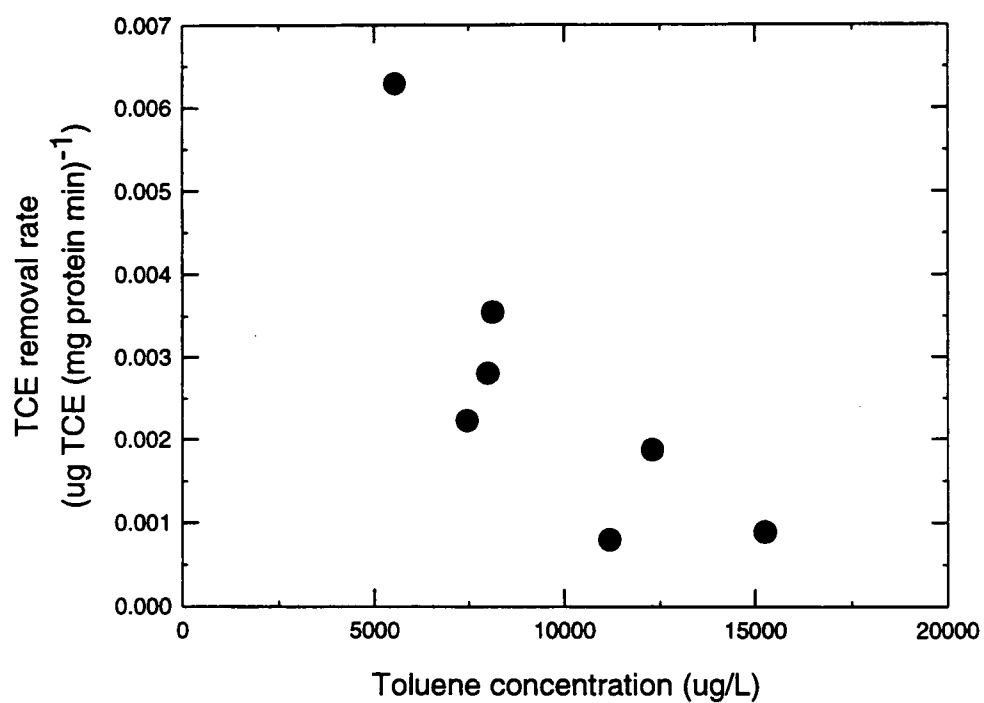


Figure 6.13 TCE removal rate as a function of toluene concentration

The specific TCE utilization rate decreases with increasing toluene concentration, which can be explained by competitive inhibition. TCE oxidation was almost completely inhibited at a toluene concentration of $15000 \mu\text{g L}^{-1}$. Similar behavior was observed in the suspended culture experiments. In section 5.3.2, competitive inhibition was limited and the initial rate of TCE oxidation was high at low toluene concentrations; however, at higher toluene concentrations, significant inhibition occurred until toluene was degraded.

6.5 TCE degradation experiments

The biofiltration column had been operating for about four-months before the experiments in this section were performed. As in previous experiments, only the first quarter of the bed (length 16-cm, volume 1.26 L) was used to simplify sampling. Effluent gas samples were collected through the second sampling port in the column at a depth of 10-cm. Initially, both toluene and TCE were supplied the column and the toluene and TCE influent and effluent concentrations were measured. The NADH and normalized enzyme activity were also quantified at the top of the column and at a depth of 16-cm. Two different flow rates, 500 and 280 ml/ min, were used, providing EBCTs of 2.3 and 4.5 minutes, respectively. During each TCE degradation experiment, toluene was discontinued at time = 0 to avoid competitive inhibition. The TCE influent and effluent concentrations, NADH, and normalized enzyme activity were measured at the two different sampling points over the course of the experiment. The summary of each experimental condition is shown in Table 6.3.

Table 6.3. Summary of experimental conditions in TCE degradation experiments.

Experiment	Average influent gas flow rate (mL min ⁻¹)	Average influent TCE concentration (µg L ⁻¹)	Empty bed contact time (EBCT, min)	Average loading rate (µg cm ⁻³ day ⁻¹)	Average biomass content (mg protein)
1	500	126	10.1	18.06	2957
2	500	99	10.1	14.19	2258
3	280	30	17.9	2.41	2201
4	280	725	17.9	58.18	2420
5	280	313	17.9	25.12	3042
6	280	218	17.9	17.50	3135
7	280	110	17.9	8.82	2802
8	280	93	17.9	7.46	2918

The TCE concentration and normalized enzyme activity during each experiment are shown in Figure 6.14(a) to (h). The summary of the experimental results is shown in Table 6.4.

Table 6.4 Summary of experimental results for TCE degradation experiments.

Experiment	Total operation time (min)	Average effluent TCE concentration (µg L ⁻¹)	Average TCE mass degraded (mg)	Average TCE degradation rate (µg mg protein ⁻¹ min ⁻¹)	Average TCE degraded (%)
1	443	66	27.063	0.01015	47.6
2	560	57	30.283	0.00930	42.4
3	406	7	8.956	0.00293	76.7
4	411	554	72.730	0.01978	23.6
5	342	222	29.760	0.00838	29.1
6	320	126	26.790	0.00822	42.2
7	360	74	14.132	0.00360	32.7
8	360	43	17.321	0.00480	53.8

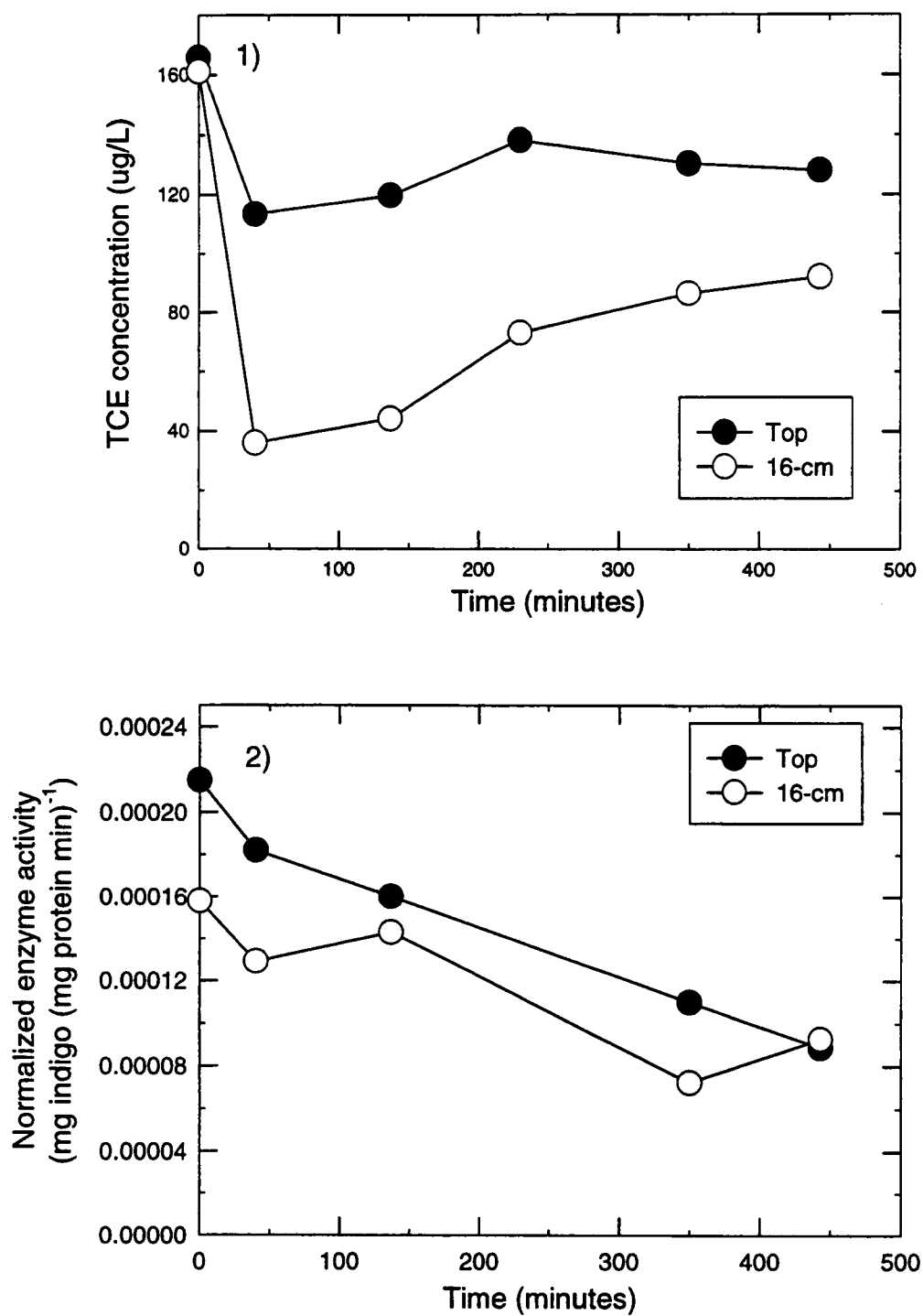


Figure 6.14 TCE concentration and normalized enzyme activity as a function of time during experiment 1 (a)

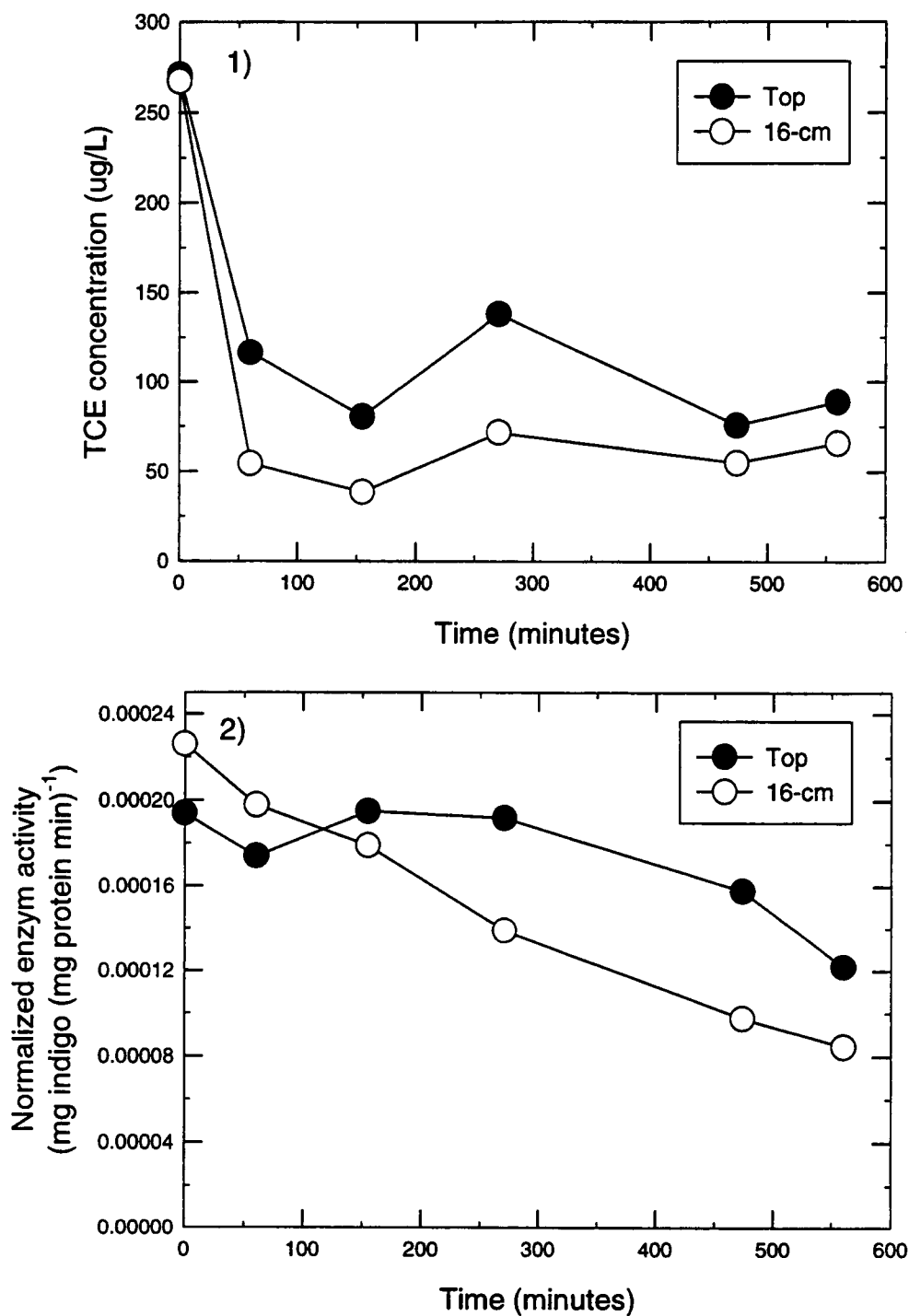


Figure 6.14 (continued)

**TCE concentration and normalized enzyme activity
as a function of time during experiment 2 (b)**

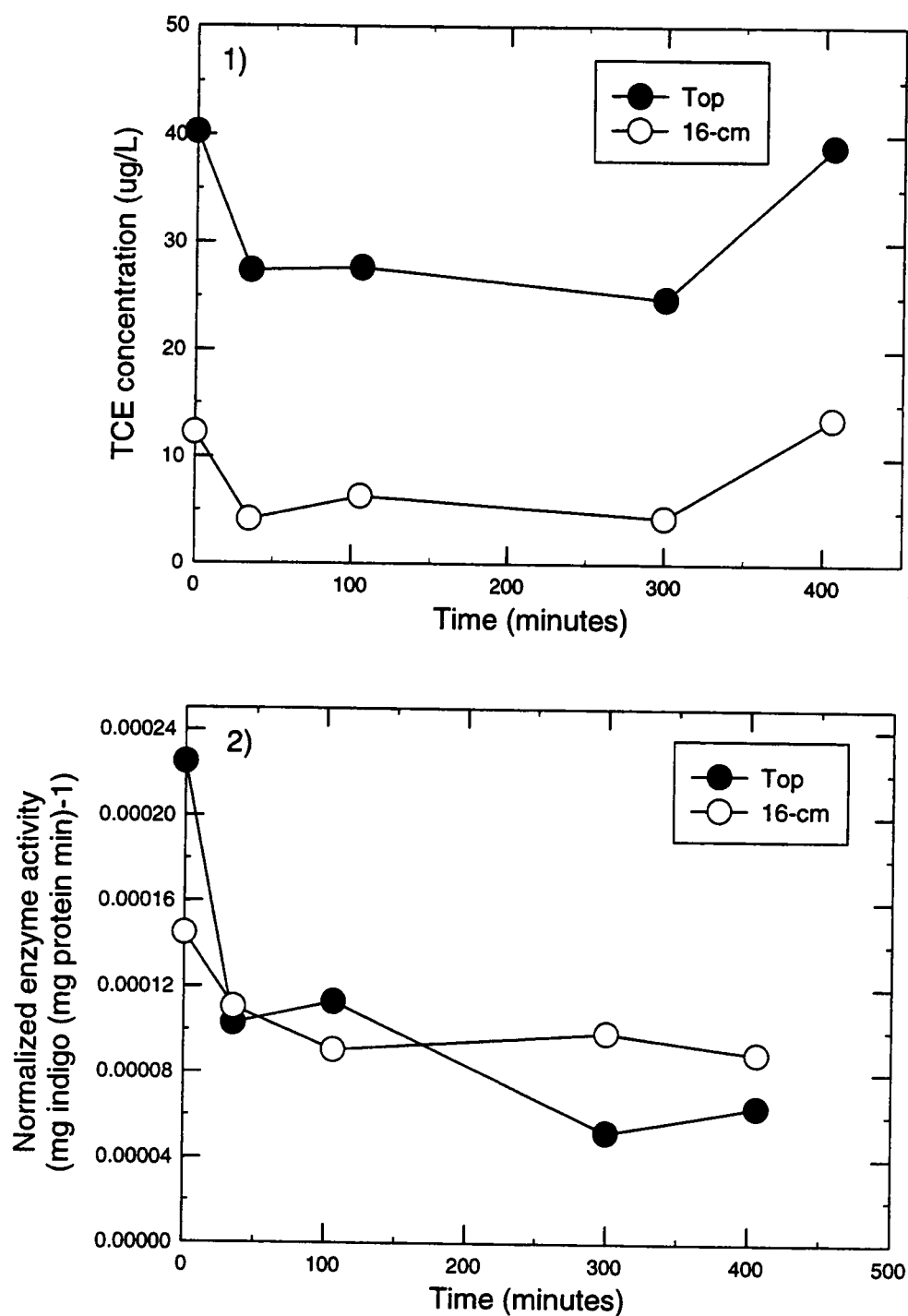


Figure 6.14 (continued)

TCE concentration and normalized enzyme activity
as a function of time during experiment 3 (c)

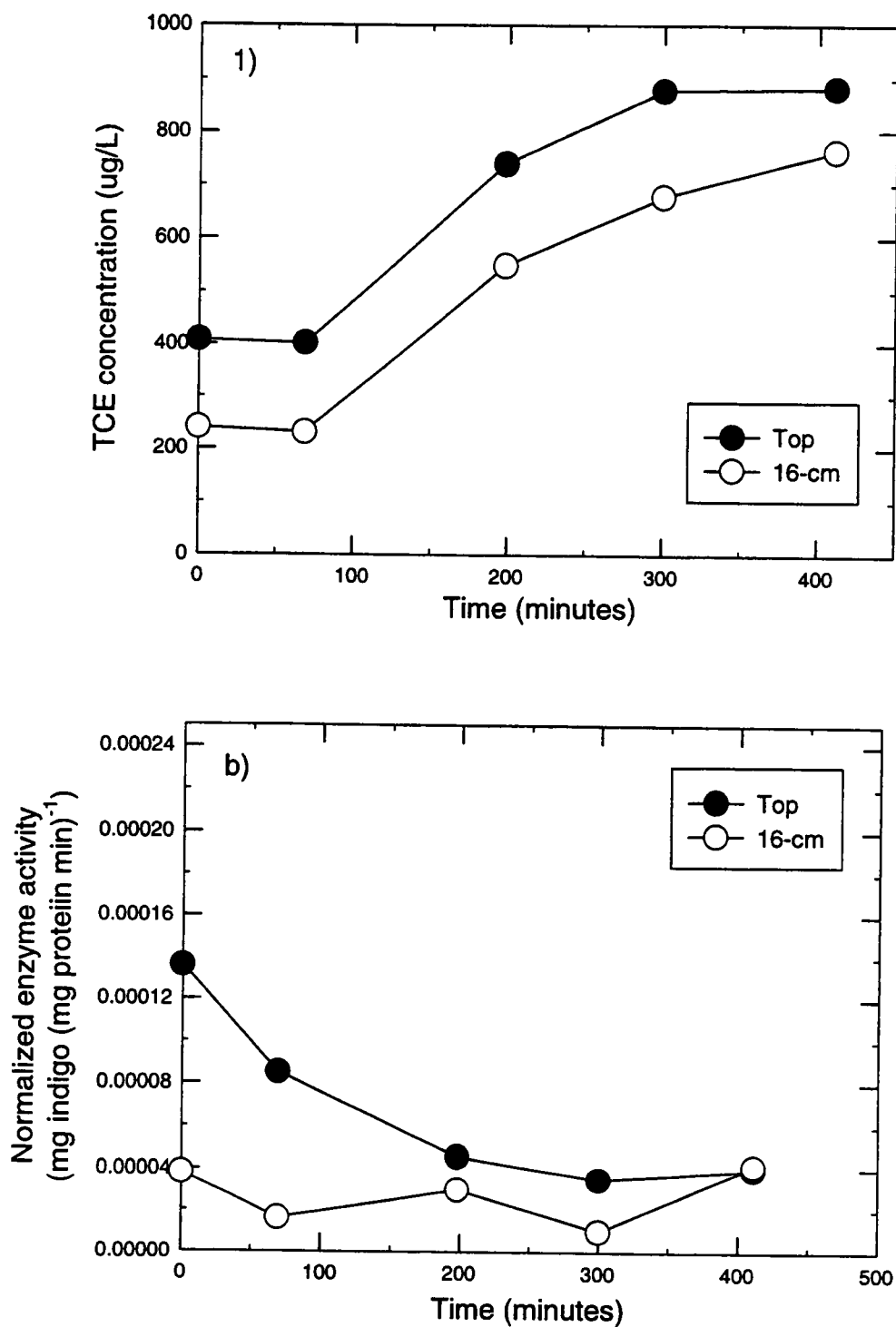


Figure 6.14 (continued)

TCE concentration and normalized enzyme activity
as a function of time during experiment 4 (d)

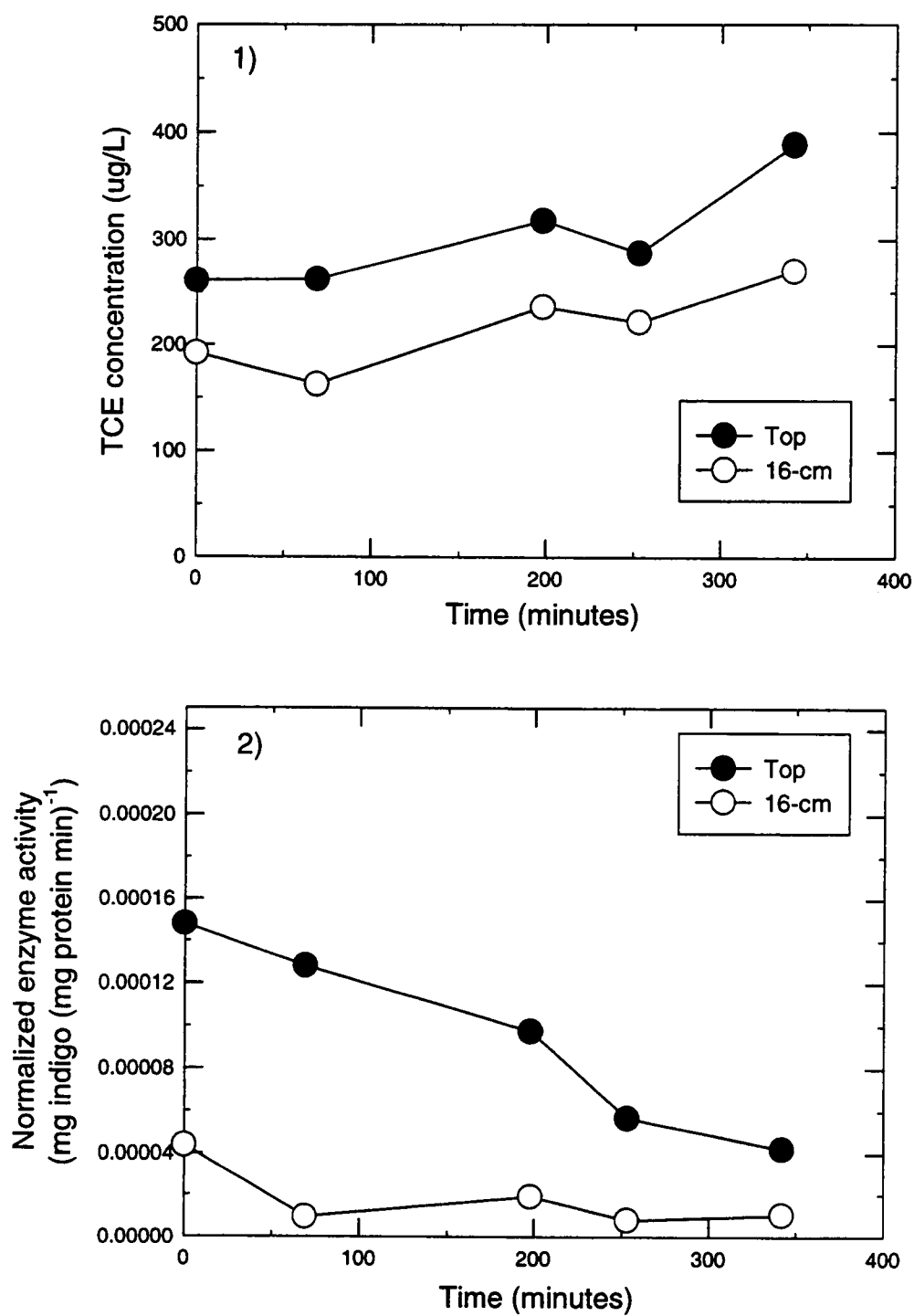


Figure 6.14 (continued)
TCE concentration and normalized enzyme activity
as a function of time during experiment 5 (e)

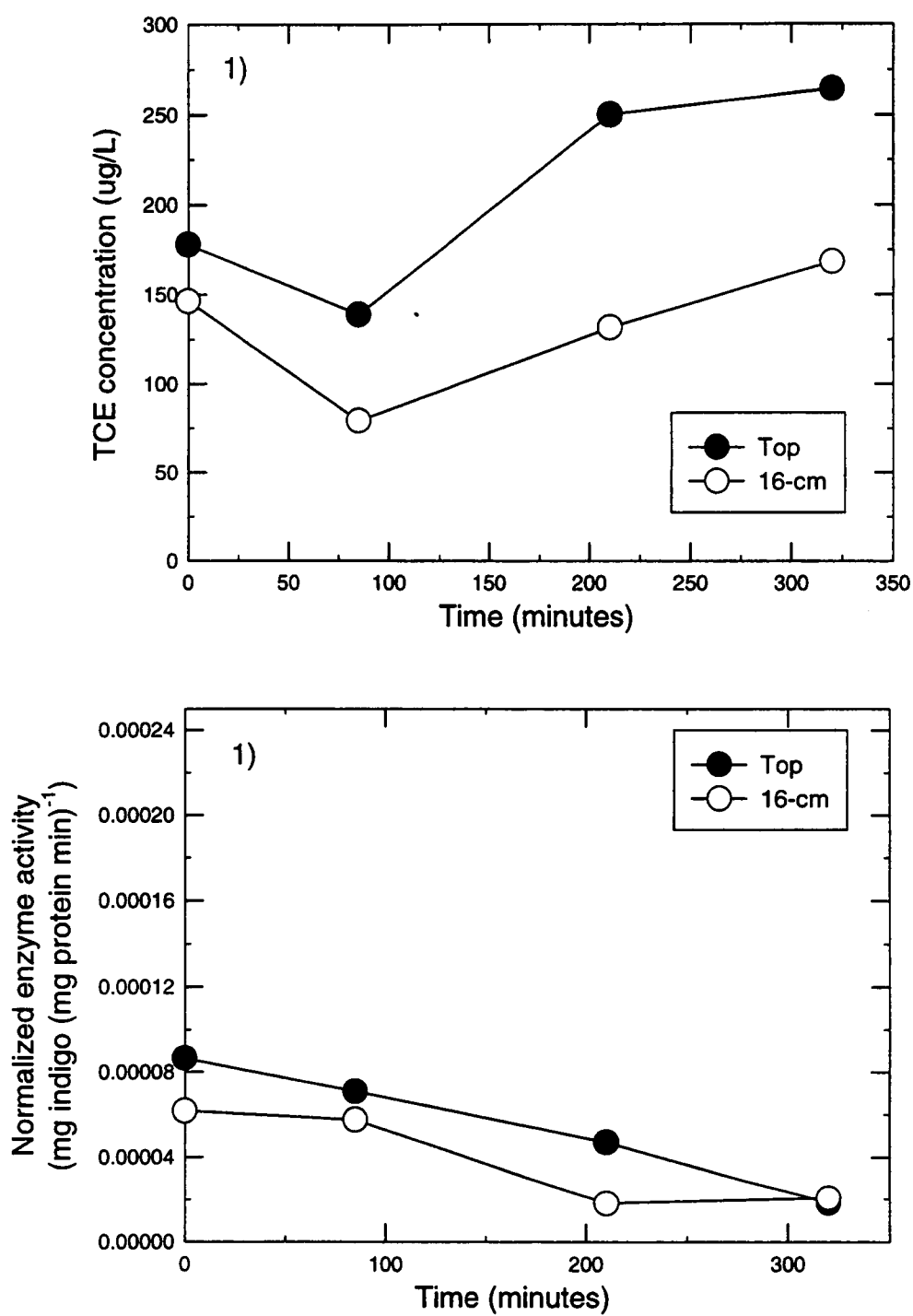


Figure 6.14 (continued)

TCE concentration and normalized enzyme activity
as a function of time during experiment 6 (f)

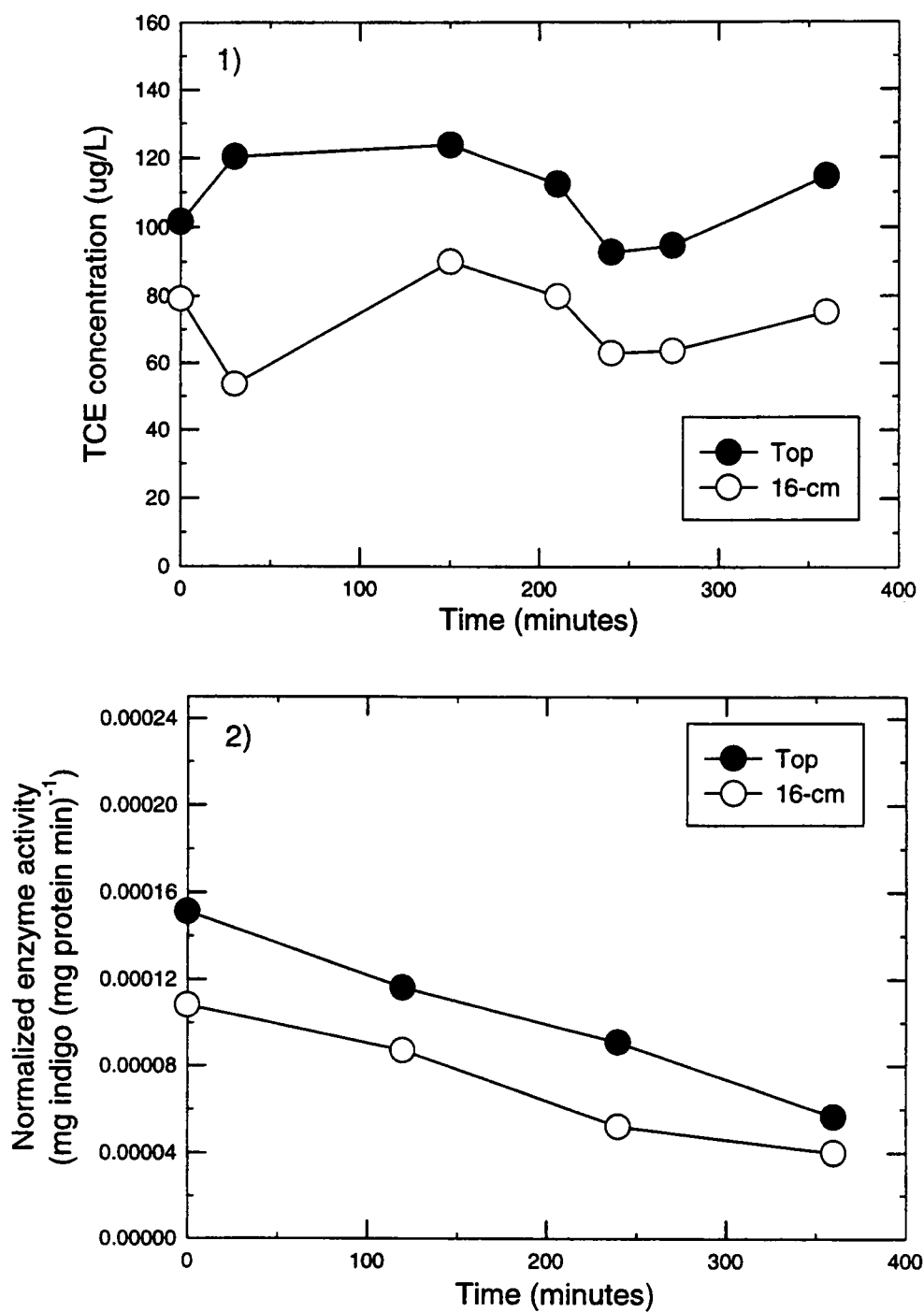


Figure 6.14 (continued)

**TCE concentration and normalized enzyme activity
as a function of time during experiment 7 (g)**

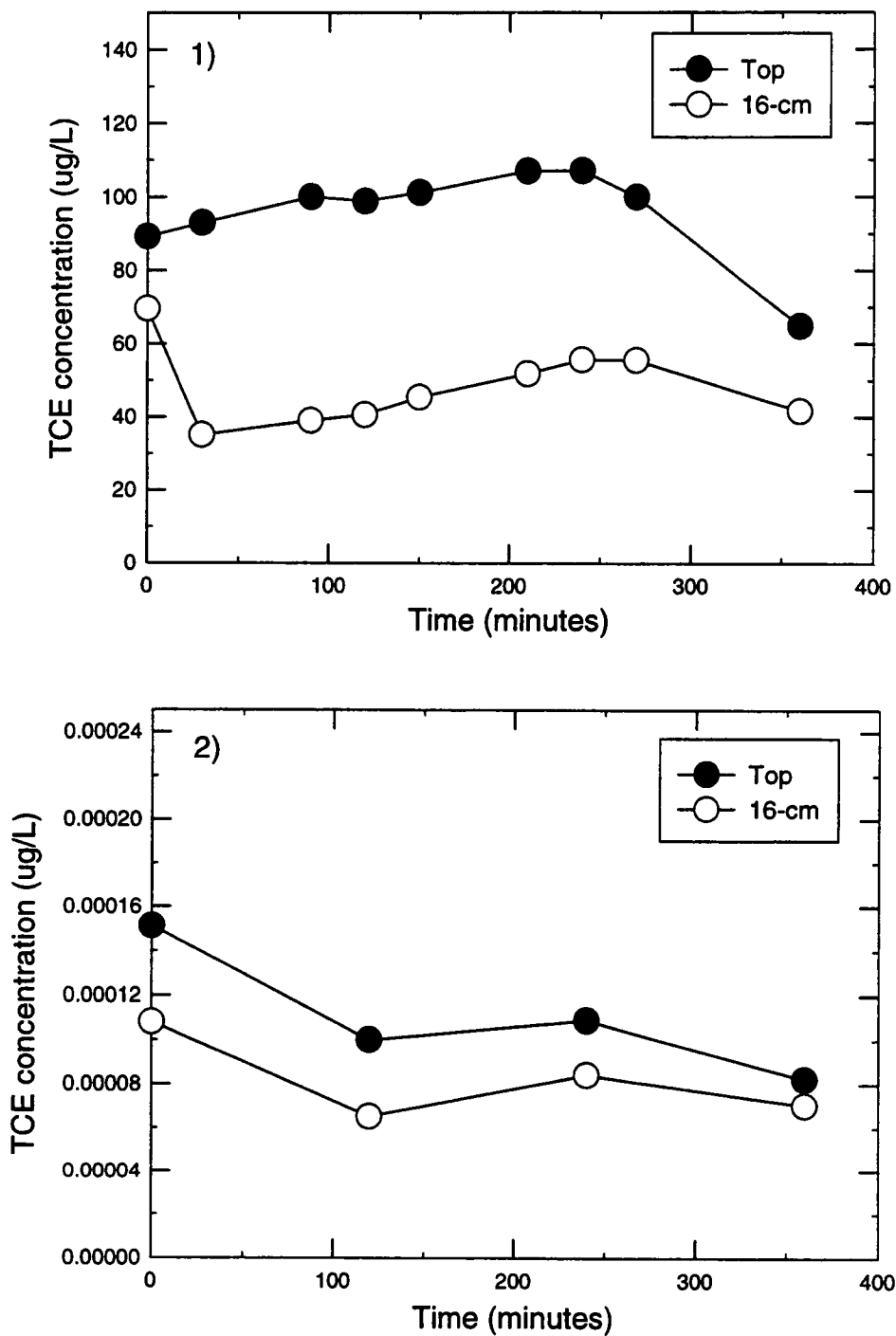


Figure 6.14 (continued)
TCE concentration and normalized enzyme activity
as a function of time during experiment 8 (h)

The total protein varied between 2200 and 3000 mg in the experiments. The feed concentration of TCE varied from 30 to 900 $\mu\text{g/L}$. There was some slight variation in feed concentrations during each experiment because of the limitations of the mass flow controllers. The highest removal efficiency occurred in experiment 3 (Figure 6.14(c)); a removal of about 80 % was sustained for about 300 minutes. The range of TCE feed concentration was 24 ~ 30 $\mu\text{g/L}$ in this experiment. The TCE removal rate was about $2.7 \times 10^{-3} \mu\text{g TCE (mg protein)}^{-1} \text{ min}^{-1}$. During the next 100 minutes, the TCE feed concentration increased to 40 $\mu\text{g L}^{-1}$ and TCE removal decreased to 65 %; however, the TCE removal rate increased to $3.19 \times 10^{-3} \mu\text{g TCE (mg protein)}^{-1} \text{ min}^{-1}$. The greatest TCE removal rate among the eight experiments occurred in experiment 4. In these experiments, the TCE feed concentration varied from 400 to 900 $\mu\text{g/L}$. The TCE removal rate was about $1.98 \times 10^{-2} \mu\text{g TCE (mg protein)}^{-1} \text{ min}^{-1}$ even though the TCE removal efficiency was only 23 to 43 %. In section 6.9.2.1, the half saturation constant K_s for TCE was shown to be about 2620 $\mu\text{g L}^{-1}$. The maximum concentration of TCE used in these experiments was about 900 $\mu\text{g L}^{-1}$. In this concentration range, a near first-order relationship between TCE concentration and TCE degradation rate is expected.

The TCE degradation rate decreased during the course of each experiment, as would be expected due to Tod inactivation byproduct toxicity. The extent of enzyme deactivation was consistent with this hypothesis. Enzyme activity was greatest at the top of the column; as a result, the local rate at the top of the column was greater than the local rate at a depth of 16-cm. A correspondingly greater decrease in enzyme activity was

observed over the time course of the experiment at the top of the column compared to at a depth of 16-cm (Figure 6.14(a)).

Wackett *et al.* (1989) has reported toxicity related to TCE oxidation by toluene dioxygenase in *Pseudomonas putida* F1. They inoculated toluene-grown cells of *Pseudomonas putida* F1 into the fresh salts medium plus L-arginine and exposed the cells to TCE for 0,1,2,3, and 9 hrs. They compared the growth curve of *Pseudomonas putida* F1 for different TCE exposure times and found out that the growth rate decreased with increasing exposure time. They explained the decrease in growth as a toxic effect which was caused by metabolic activation of TCE by toluene dioxygenase to form reactive intermediates. They indicated that the reactive intermediates can covalently modify intracellular molecules such as RNA, DNA and other proteins. For example, acyl chlorides may react with protein nucleophilic groups to form amides, ester, and thioesters.

According to the mechanism described by Wackett *et al.*, it is reasonable to assume that the degree of normalized enzyme deactivation might be proportional to the cumulative mass of TCE degraded. This hypothesis seems to be consistent with the measured change in enzyme activity during the TCE degradation experiments. In most experiments, normalized enzyme activity decreased more than 50 % after 300 minutes. In experiments 4,5,6, and 7, less than 30 % of the initial Tod activity remained after 300 minutes.

To further refine the hypothesis, it might be assumed that the decrease in normalized enzyme activity is proportional to the cumulative mass of TCE degraded. To test this hypothesis a scatter plot of the decrease in normalized enzyme activity as a function of cumulative mass of TCE degraded per unit biomass is shown in Figure

6.15. The total biomass in reactor did not change significantly during the course of the experiments. E_{x0} was the initial normalized enzyme activity while E_x was the normalized enzyme activity at various time periods in each experiment. Although the relationship is not perfect, it is clear that TCE degradation is a significant factor affecting Tod activity. The slope of the line in Figure 6.15 (0.0764) was significantly greater than zero ($p=0.0001$). Natural first -order decay in enzyme activity may be an additional mechanism that contributes significantly in these experiments. These two mechanisms were the basis for predicting the normalized enzyme activity profile during biofiltration in the mathematical model presented later. Explicit consideration of enzyme activity represents an advance in biofiltration modeling.

In previous models (Segar *et al.*, 1995; Speitel *et al.*, 1993), total biomass concentration or protein concentration were used to describe microbial activity. The above analysis has shown that biomass concentration alone cannot fully describe the decrease in cell activity because enzyme activity is affected by TCE toxicity. Further, total biomass includes a large fraction of the biofilm that is dormant or dead and does not contribute to contaminant oxidation. This is especially relevant in biofilters that have been operating for long periods of time and have established thick biofilms. In these cases, enzyme activity, rather than biomass, may best represent the ability of the system to degrade TCE. The effect of Tod activity on TCE oxidation rate is discussed below.

According to Michaelis-Menten kinetics, when the substrate concentration (S) is much smaller than K_s , S can be neglected in the denominator. For this limiting case, Michaelis-Menten kinetics reduce to a first-order reaction with respect to S (Benefield *et al.*, 1980). Detailed explanation of equation (6.8) is given in section 6.9.1.

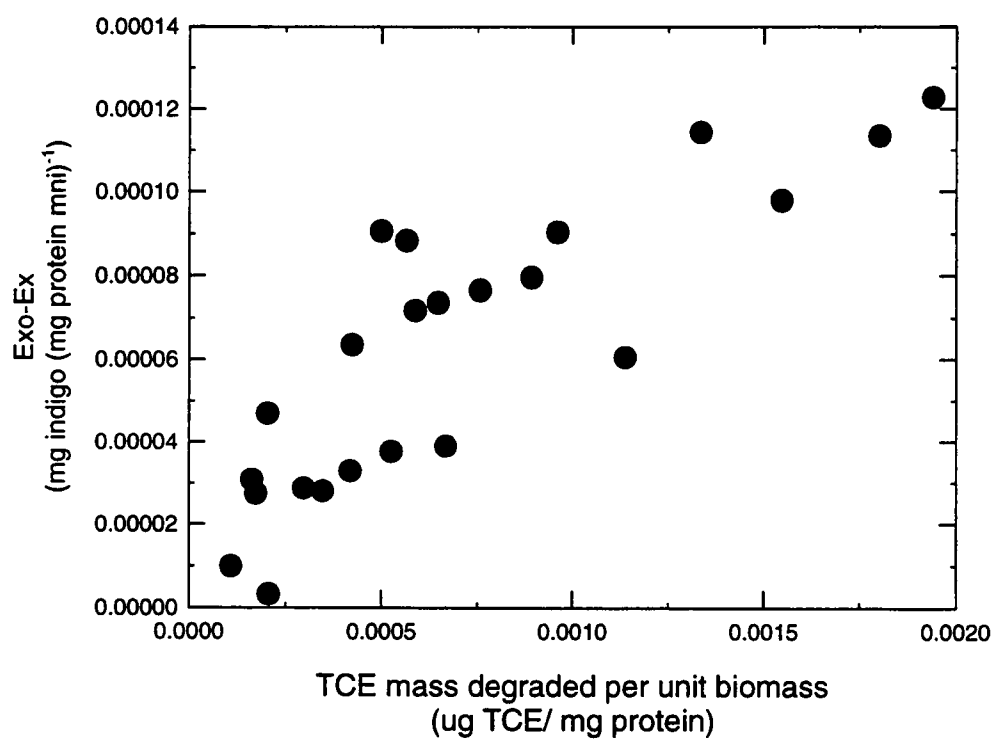


Figure 6.15 Tod enzyme deactivation as a function of TCE degraded

$$\frac{dS}{dt} = \frac{k_s' E[S]}{(K_s + [S])} = \frac{k_s' E}{K_s} \cdot \frac{1}{[S]} \quad (6.8)$$

$$dS/dt \propto [S] \quad \text{when } K_s \gg S, \text{ and } E \text{ is considered as a constant} \quad (6.9)$$

From section 6.9.2.1, TCE half-velocity constant (K_s) is $2620 \mu\text{g TCE L}^{-1}$. This K_s value is much greater than the range of TCE concentrations in these experiments; therefore, the TCE removal rate should be first order in used TCE concentration. The TCE removal rate ($\text{TCE } \mu\text{g (mg protein min)}^{-1}$) versus influent TCE concentration ($\text{TCE } \mu\text{g L}^{-1}$) is shown in Figure 6.16. The data collected at time periods greater than 1100 minutes (long term) are denoted by the open symbol in Figure 6.16. As the TCE influent concentration was increased, the TCE removal rate gradually increased, but long term data did not follow with this trend. While the coefficient of determination (R^2) for the data collected in the short term is 0.9127 compared to 0.3646 for the short-term (at the time periods less than 600 minutes) and long-term data combined. Equation (6.9) fails when the duration of TCE feeding is more than 1100 minutes. A better relationship is obtained when the enzyme activity E is considered.

If E is considered as a variable in equation (6.8), dS/dt is proportional to the product with E and S .

$$dS/dt \propto E[S] \quad \text{when } K_s \gg S, \text{ and } E \text{ is considered as a variable} \quad (6.10)$$

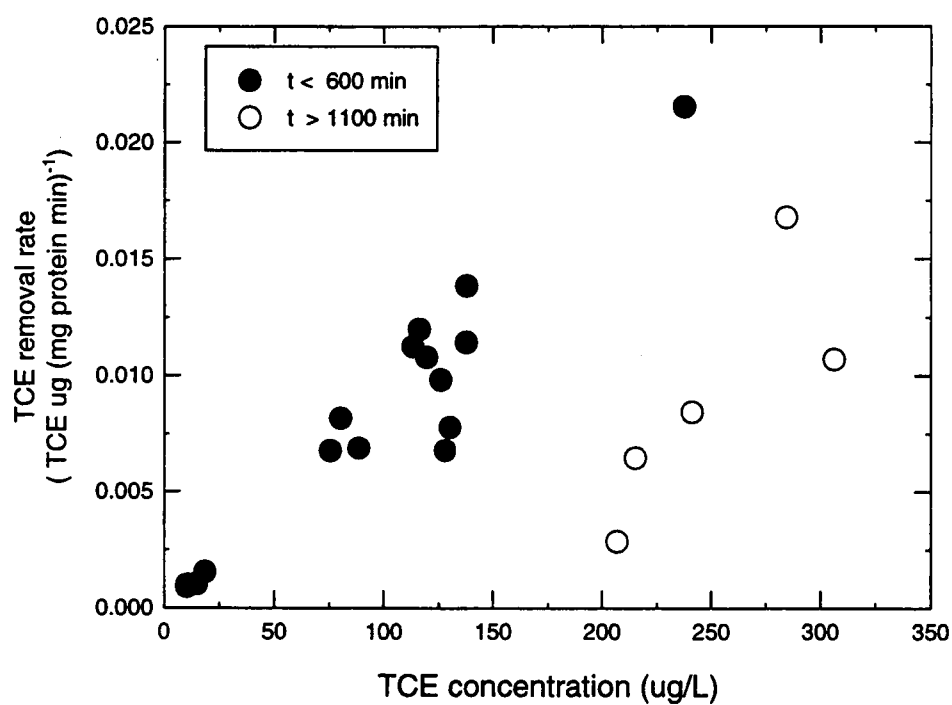


Figure 6.16 TCE removal rate as a function of TCE concentration

The TCE removal rate is plotted as a function of the product of the TCE influent concentration ($\mu\text{g L}^{-1}$) and enzyme activity ($\text{mg indigo min}^{-1}$) in Figure 6.17. When the enzyme activity data are combined with TCE data, the long term data follow the limiting form of the Michaelis-Menten kinetics. The coefficient of determination (R^2) for Figure 6.17 is 0.8396, suggesting that the enzyme activity is an important factor in biofiltration.

6.6 Recovery of Tod activity by toluene feeding.

The experiments described in this section were conducted after the biofilm had been fully developed over a 4 month period. Initially a mixture containing about $7800 \mu\text{g L}^{-1}$ of toluene and $100 \mu\text{g L}^{-1}$ of TCE was fed to the column at a flow rate of 280 ml min^{-1} . At time = 0, the toluene feed to the biofilter was discontinued while maintaining the TCE feed for a period greater than 6 hours; during this time the Tod activity was observed to decrease. Then, the toluene feed was reestablished and after 1 hr the Tod activity measured again, followed by a second period in which TCE alone was fed to the biofilter. Only the top 25% of the biofilter (16 cm) was used for these experiments, yielding an EBCT of 4.5 minutes. The total biomass in this part of the column was about 2750 mg protein. Gas and biofilm samples were collected at the top of the bed and at depth of 16 cm; the gas samples were analyzed for toluene and TCE concentration and the biofilm samples analyzed for NADH and Tod activity.

Influent and effluent toluene and TCE concentrations are shown as a function of time in Figure 6.18 (a). About 20% of TCE was being removed just before the toluene feed to the biofilter was interrupted. TCE removal increased to 55.5% 30 min after the toluene was removed from the feed and competitive inhibition was eliminated. The TCE

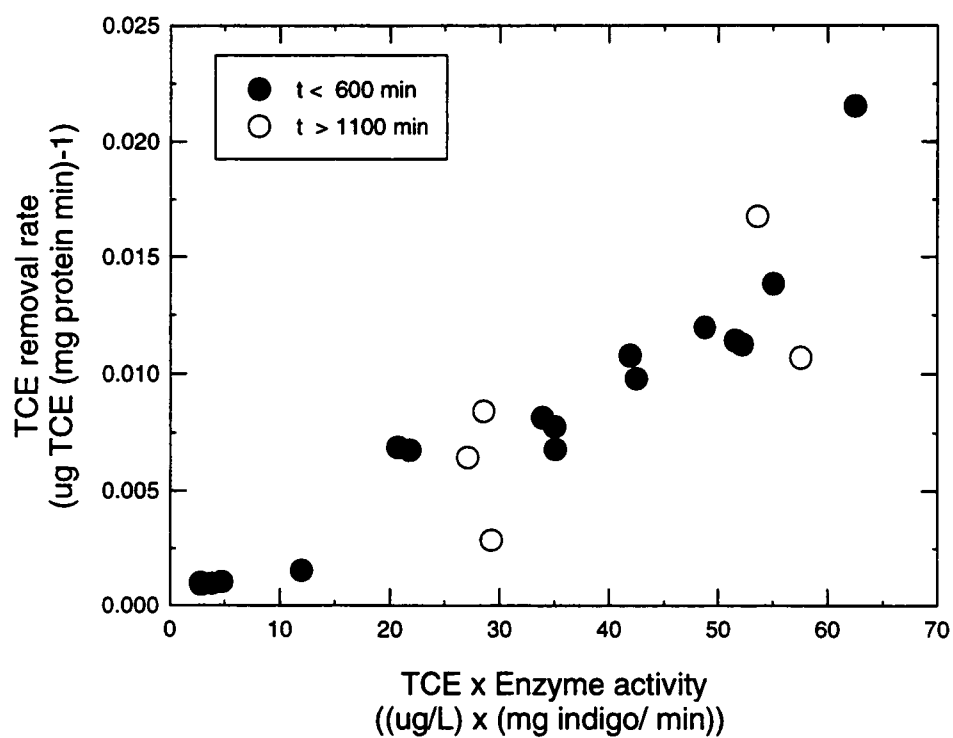


Figure 6.17 TCE removal rate as a function of the product of TCE concentration and enzyme activity

degradation rate was $6.78 \times 10^{-3} \mu\text{g (mg protein)}^{-1} \text{ min}^{-1}$, similar to those reported in Table 6.4 for other experiments in which TCE alone was fed to the biofilter. After operating for 373 min. the removal efficiency and rate had decreased to 34.4% and $3.99 \times 10^{-3} \mu\text{g (mg protein)}^{-1} \text{ min}^{-1}$, respectively. The decrease in removal rate can be attributed to the decrease in Tod activity that occurred during the period in which toluene was not fed to the column (Figure 6.18 (b)). Over the time period in which TCE alone was fed to the column, the Tod activity decreased from 1.3×10^{-4} to $0.39 \times 10^{-4} \text{ mg indigo (mg protein)}^{-1} \text{ min}^{-1}$. After the toluene feed was reestablished in the column, competitive inhibition caused the TCE removal efficiency and rate to decrease to 19.% and $2.09 \times 10^{-3} \mu\text{g (mg protein)}^{-1} \text{ min}^{-1}$, respectively. After 1 hr of feeding, the enzyme activity increased to the same level as observed before the toluene was removed from the feed (Figure 6.18 (b)). The recovery of enzyme activity resulted in an improvement of biofilter performance once the toluene was removed for the second time; TCE removal increased to 62.1% and the TCE removal rate was $7.53 \times 10^{-3} \mu\text{g (mg protein)}^{-1} \text{ min}^{-1}$. Enzyme activity and biofiltration performance decreased more rapidly during the second period in which TCE alone was fed to the reactor. Measurements of NADH taken during the experiment showed that it varied slightly with operation time, but that at no point during the experiment would it limit TCE oxidation (Figure 6.18(c) and 6.19(c)).

In a second enzyme recovery experiment the conditions were identical except the initial toluene feed concentration was increased to $12,300 \mu\text{g L}^{-1}$ and the toluene concentration during the recovery phase was increased to $21,600 \mu\text{g L}^{-1}$. The results are shown in Figure 6.19. The general trends observed during periods of TCE feeding and combined TCE and toluene feeding are the same as observed in the first enzyme recovery

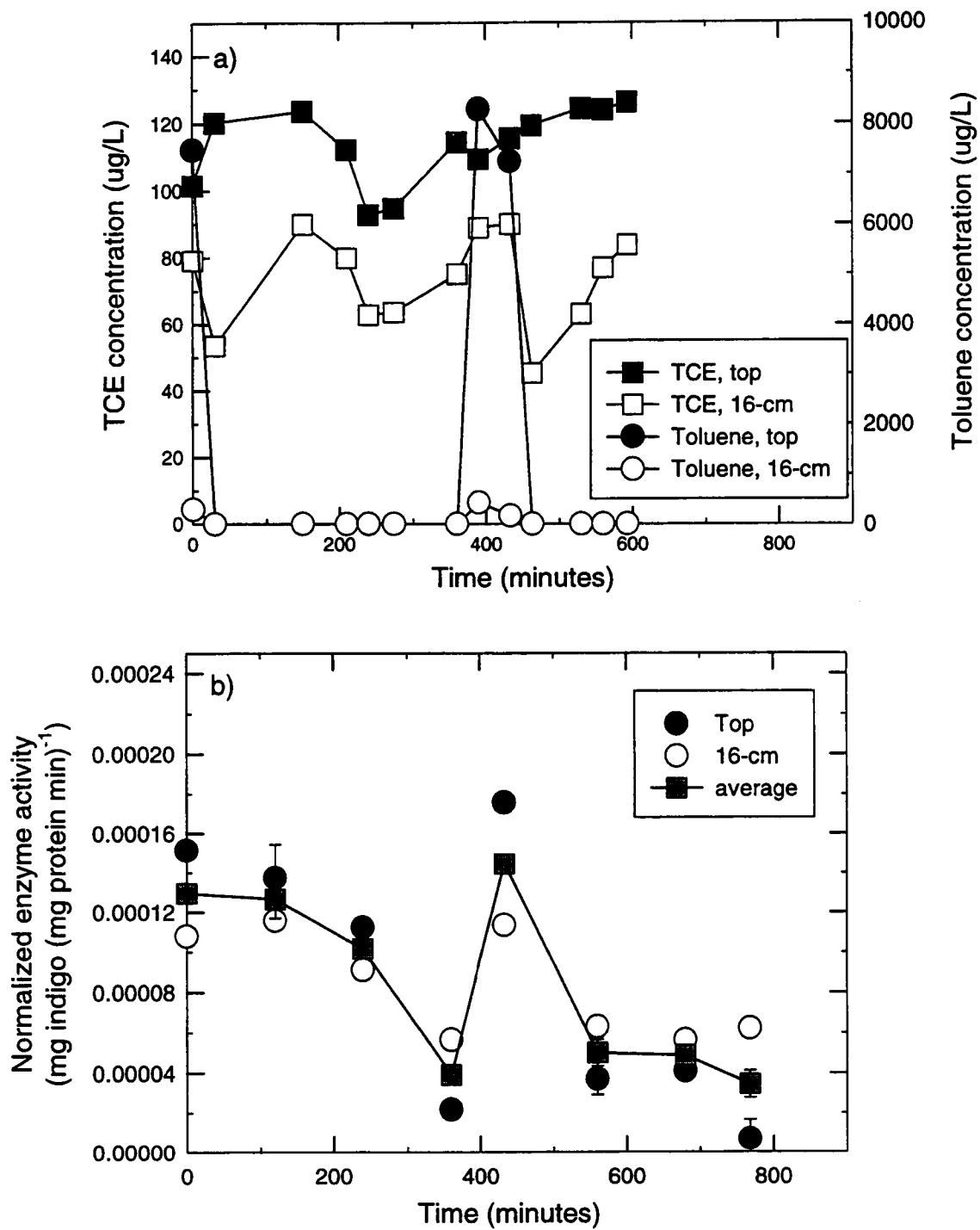


Figure 6.18 Biofiltration performance during cyclic toluene feeding:I
(a) toluene and TCE concentrations (b) normalized Tod activity

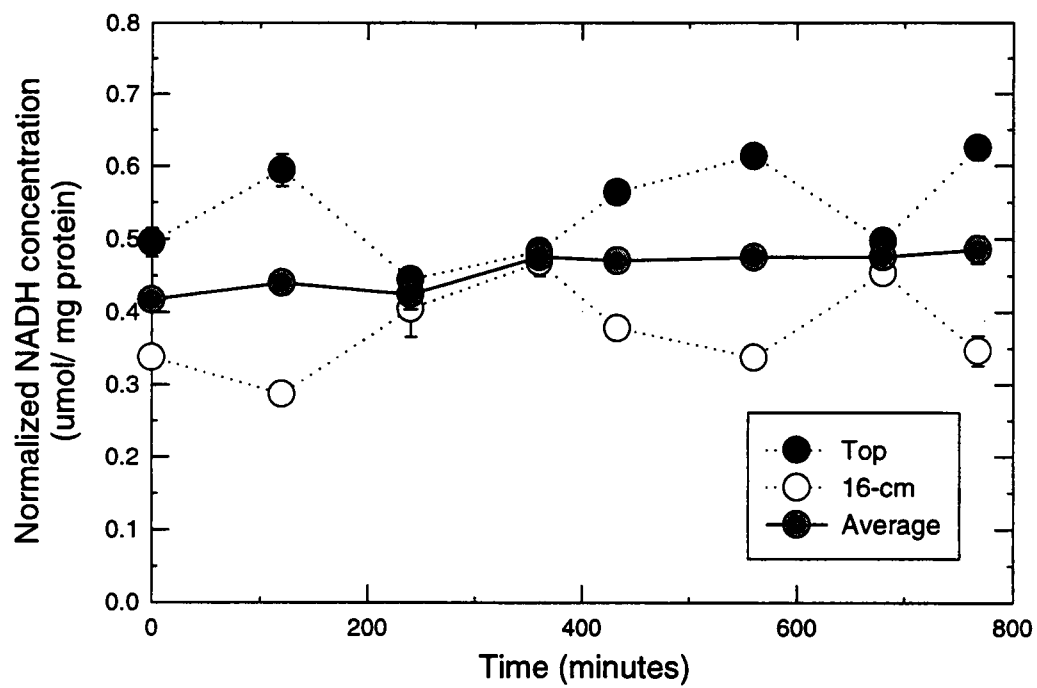


Figure 6.18 (continued)
Biofiltration performance during cyclic toluene feeding
(c) NADH concentration

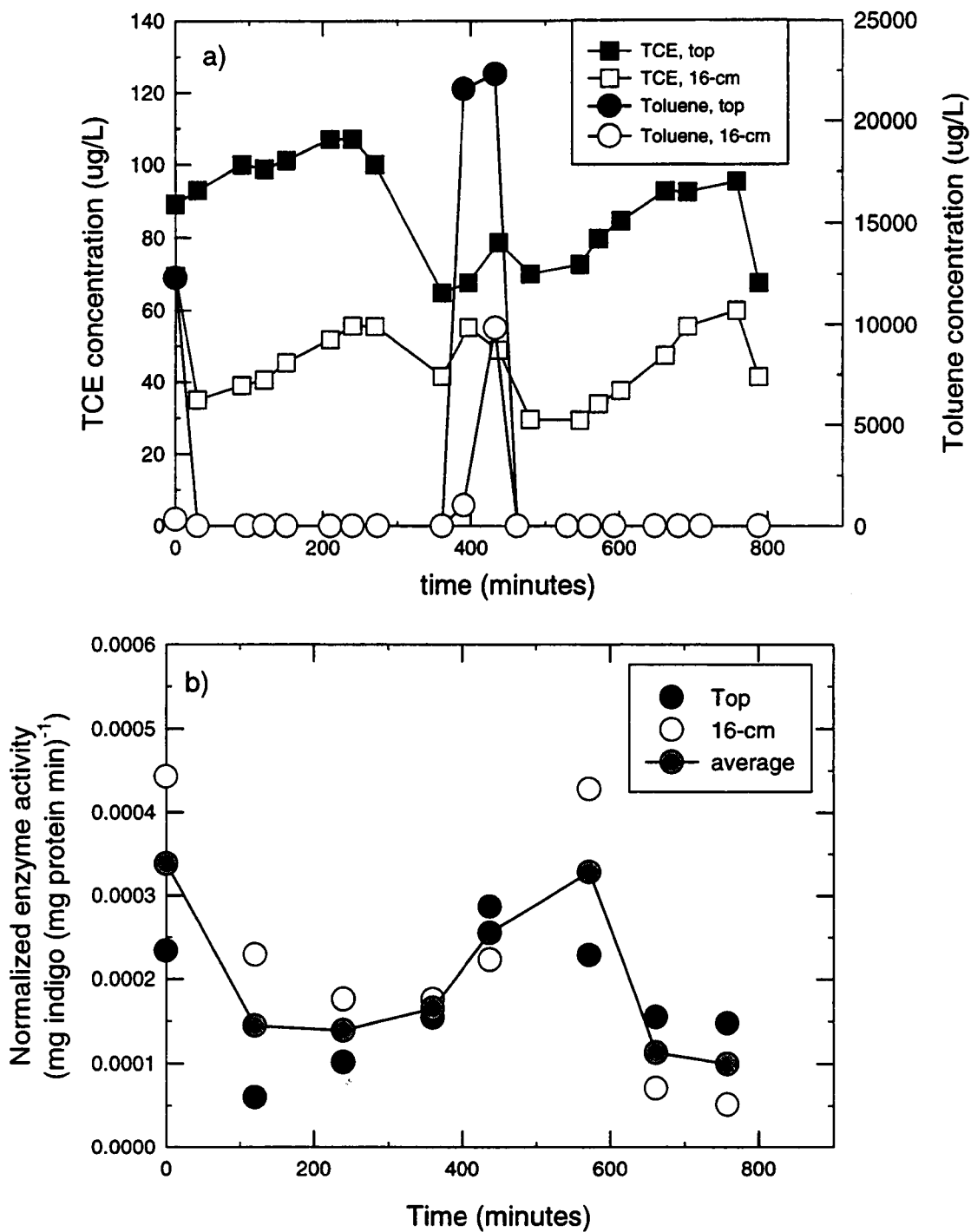


Figure 6.19 Biofiltration performance during cyclic toluene feeding:II
 (a) toluene and TCE concentrations (b) normalized Tod activity

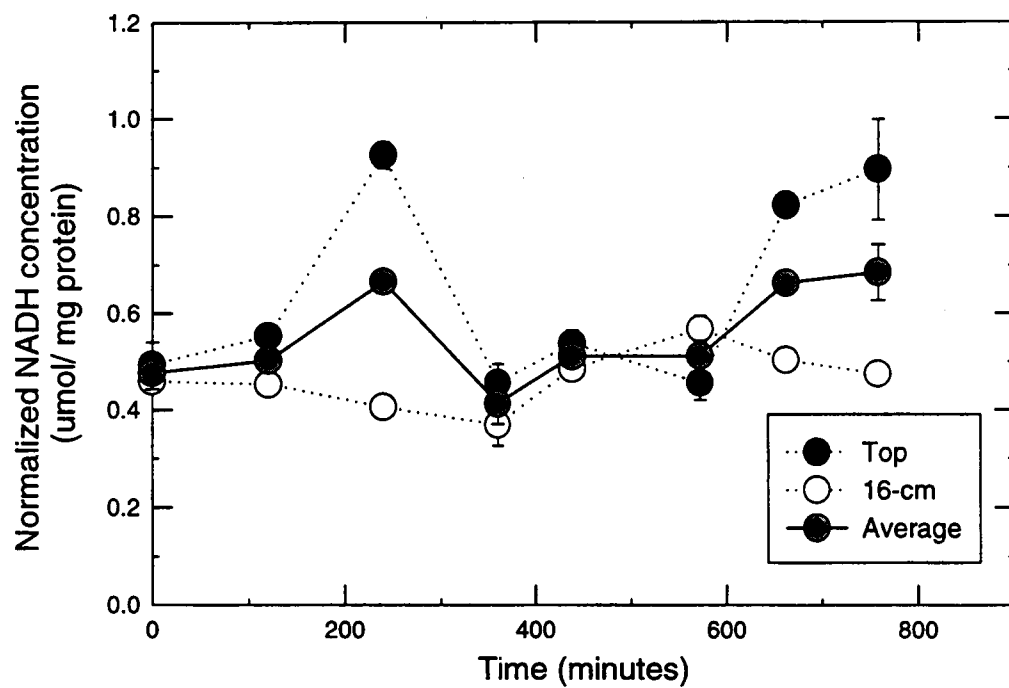


Figure 6.19 (continued)
Biofiltration performance during cyclic toluene feeding:
(c) NADH concentration

experiment. At the end of the recovery period, the Tod activity was about the same as it was at the beginning of the experiment (3×10^{-4} mg indigo (mg protein) $^{-1}$ min $^{-1}$), despite the fact that the toluene concentration was twice as high. This activity was also the same as the steady-state value observed when the toluene concentration was varied over the range of 500 to 6000 $\mu\text{g L}^{-1}$ in the DVR experiments. A Tod activity of 3×10^{-4} mg indigo (mg protein min) $^{-1}$ may be considered to be the maximum activity of the cells under ordinary conditions. The normalized Tod activity remained near its maximum value for a longer period when 21600 $\mu\text{g L}^{-1}$ of toluene was used for regeneration (Figure 6.19 (b)), compared to the first experiment when 2800 $\mu\text{g L}^{-1}$ of toluene was used for regeneration (Figure 6.18 (b)).

In the experiments above, Tod activity was observed to be restored to its original value after feeding toluene for a period of one hour. However, these experiments are not adequate to define the rate of increase in Tod activity during recovery. Prior to conducting the experiments described below, the column was back washed to remove inactive biomass and fed with toluene for a period of 7 weeks to reestablish the biofilm. Two experiments were conducted in which TCE alone, at a concentration of 300 to 500 $\mu\text{g L}^{-1}$, was fed to the column for a period of about 400 min to deplete the Tod enzyme activity. Then, toluene was fed to the reactor at a concentration of 21,600 $\mu\text{g L}^{-1}$ and the Tod activity measured as a function of time.

Results of the two experiments are shown in Figure 6.20 (a),(b). The Tod activity increases to near maximum within 20 minutes of toluene feeding. These results suggest that Tod activity can be fully restored by feeding toluene at high concentration for a relatively short period of time.

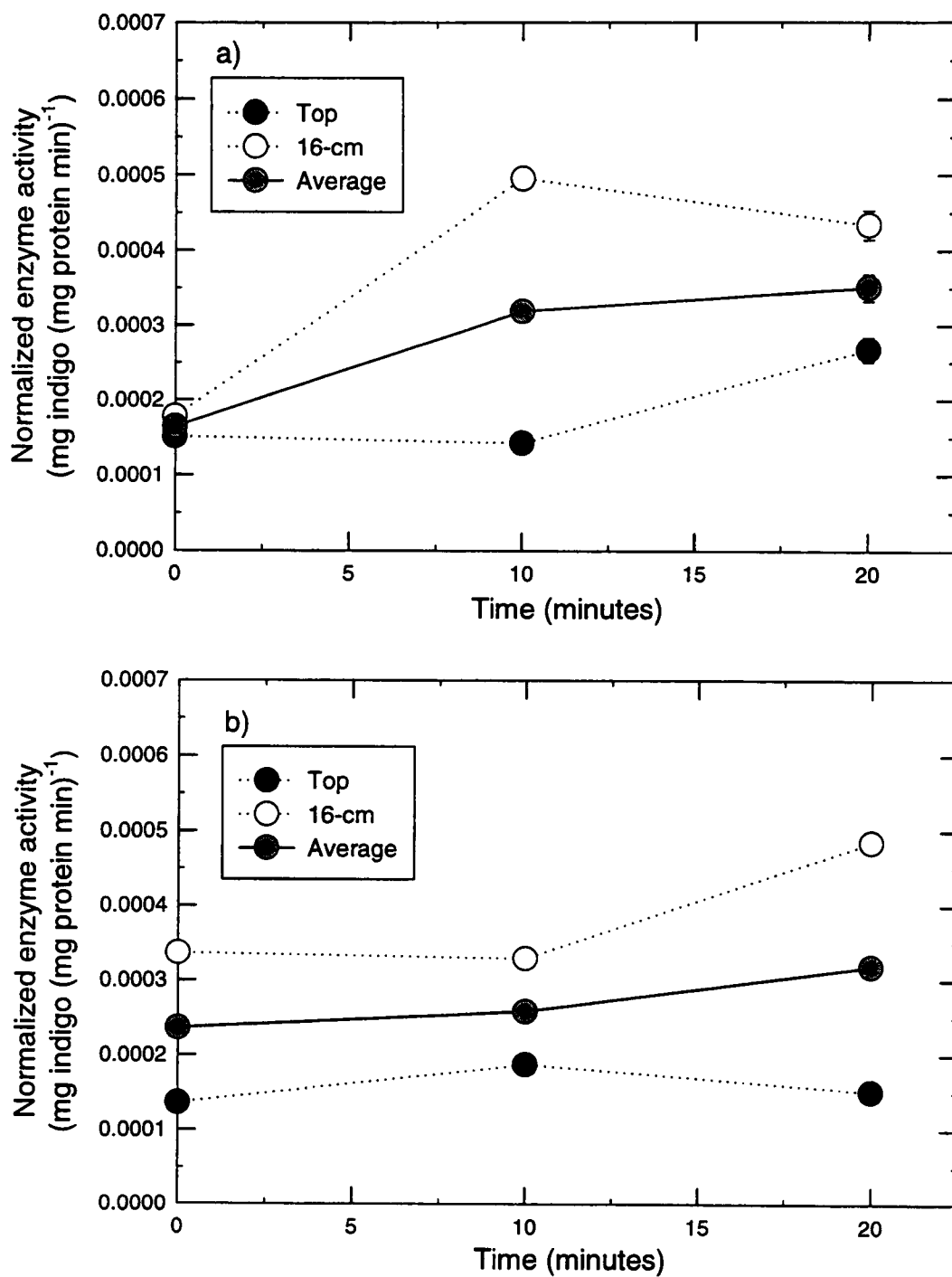


Figure 6.20 Normalized enzyme activity as a function of time during Tod activity recovery experiment

6.7 NADH concentration during TCE degradation

NADH concentration during TCE degradation was determined in batch experiments in section 3.3.4. Acetate and commercial NADH solutions were to supplement the ambient NADH level in the cell. Supplemental sources of NADH did not significantly affect TCE degradation rates. NADH concentrations did not limit TCE degradation.

In this section, NADH concentrations during biofiltration were measured under three conditions: 1) toluene only, 2) toluene and TCE, and 3) TCE alone. In the first experiment, toluene was fed to the 100-cm long (64-cm bed height) by 10-cm diameter biofiltration column at a concentration of $1000 \mu\text{g L}^{-1}$ and a flow rate of 500 mL min^{-1} . The EBCT was 10.05 minutes. After a steady-state condition in the column was achieved, the profiles of toluene, Tod activity, protein, and NADH concentration were measured (Figure 6.21).

The biofilm developed primarily were in the front half of column, corresponding to the region in which the toluene was degraded. Tod enzyme activity undetectable in the second of the column which received no toluene. However, the normalized NADH concentration (0.41 ± 0.066) was relatively constant over the entire column. Even though the normalized NADH concentration was same level over the column, the amount of protein in the second half of the column was less than 10 % of that in the front half of column. Therefore, the total NADH in the second half of the column was much smaller than that in the front half of column. Biomass was present in the second half of the column even toluene was not detected there. Aqueous phase toluene, degradation intermediates, and sloughed biomass are potential substrates accounting for this growth.

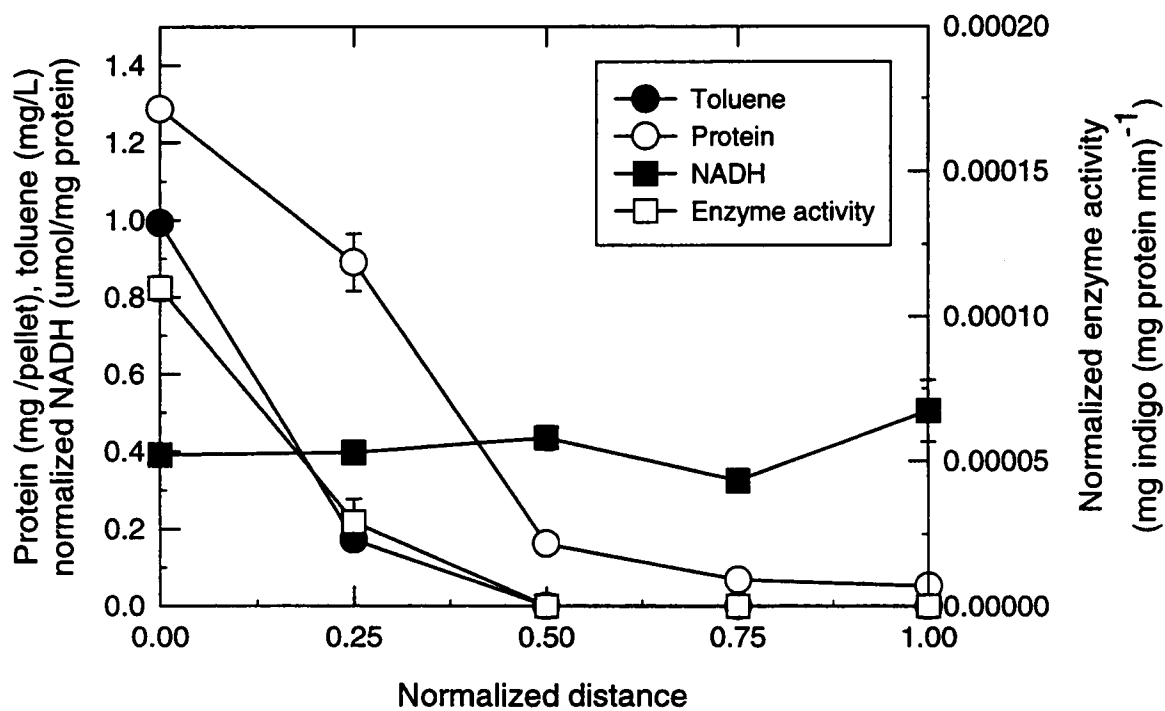


Figure 6.21 Profiles of toluene, protein, NADH, and enzyme activity in biofiltration column: TCE = 0 ug/L

The NADH concentration was measured for different toluene feed concentrations in the DVR experiments described in section 6.3. The normalized NADH concentration was independent for toluene concentration in the range of 500 to 6000 $\mu\text{g L}^{-1}$. The mean of normalized NADH concentration was $0.48 \pm 0.076 \mu\text{mole mg protein}^{-1}$. This is comparable to the level reported above for the biofiltration column. For cells grown in suspended culture, the normalized NADH concentration increased with increasing toluene concentration to a maximum value of $0.29 \pm 0.02 \mu\text{mol mg protein}^{-1}$ after the early-stationary-growth phase (section 4.3). The normalized NADH concentration observed for immobilized cells at steady state was almost twice the value observed for suspended cells at the early-stationary phase.

In the second case, NADH was observed in immobilized cells in the presence both of TCE and toluene. Toluene ($16800 \mu\text{g L}^{-1}$) and TCE ($170 \mu\text{g L}^{-1}$) were continuously introduced to the column over a period of four days. The profiles of toluene, TCE, protein, enzyme activity, and NADH were shown in Figure 6.22(a) - (e), respectively. Data taken at $t=0$ represent steady-state conditions during toluene feeding only, before TCE is introduced to the feed. After 2 hr of feeding TCE, toluene removal seemed to decrease slightly, but after 4 days, toluene removal was equivalent to that before TCE was fed to the biofilter. TCE removal efficiency was 30 % and 15 % after 2 hr and 4 days, respectively. Most of the TCE degradation occurred in the first 25% of the column. Biomass remained fairly constant during the course of the experiment with about 50 % of the total residing in the first 25 % of the column. Total enzyme activity is shown in Figure 6.22(d). Total enzyme activity was proportional to biomass, resulting in the greatest rate of occurring near the inlet.

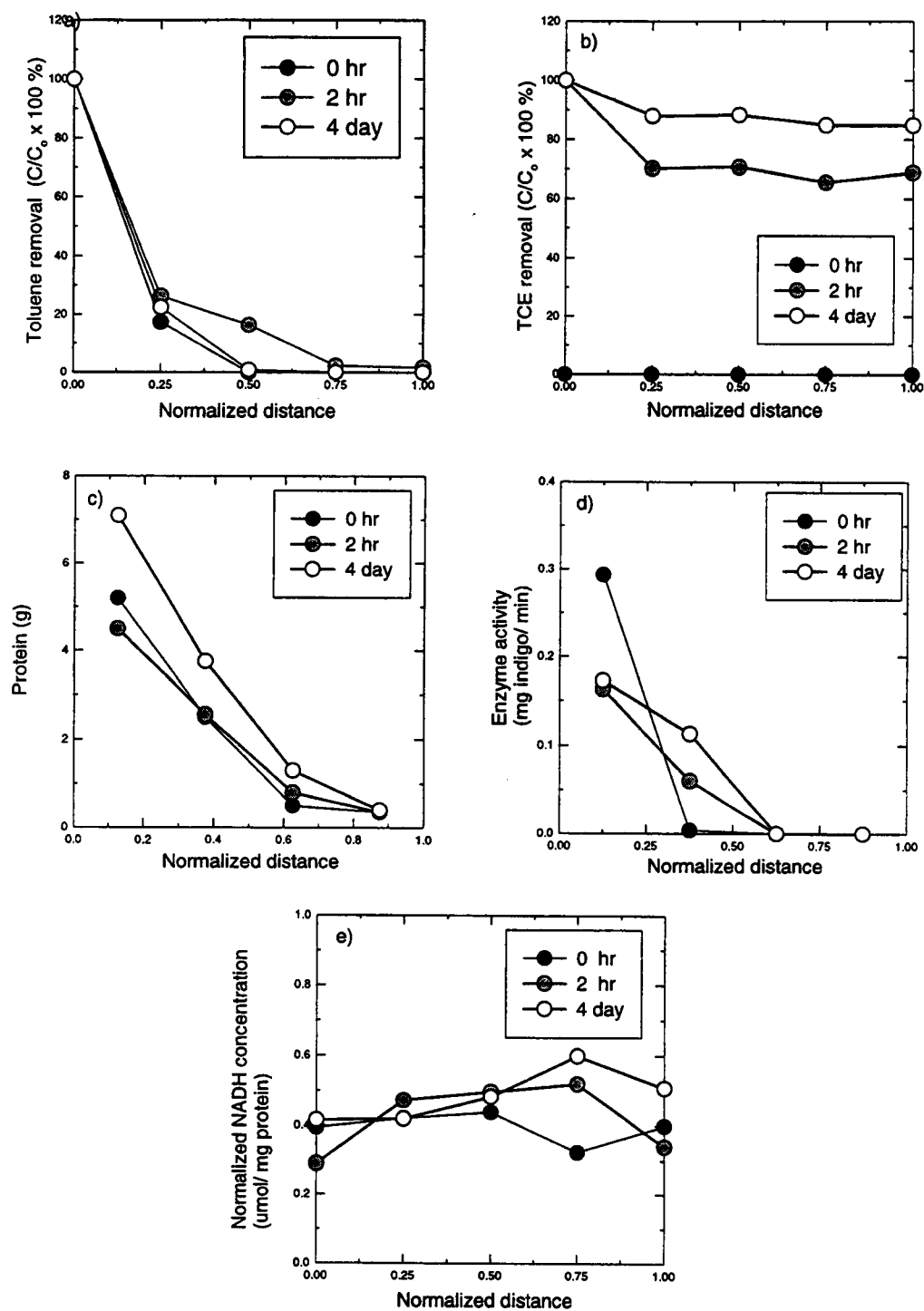


Figure 6.22 Profiles of (a) TCE, (b) toluene, (c) protein, (d) enzyme activity, and, (e) normalized NADH concentration as a function of time

The normalized NADH profile is shown in Figure 6.22(e). Normalized NADH was relatively unchanged, both along the length of the column and during the course of the 4-day experiment. The NADH concentration of about $0.4 \mu\text{mol (mg protein)}^{-1}$ was essentially the same as observed when toluene alone was fed to the column.

In the third group of experiments, NADH concentrations in the biofilm were observed during TCE degradation with no toluene present. These are the same experiments as described in Table 6.3 in section 6.5. The eight sets of NADH measurement in the course of TCE oxidation were shown in Figure 6.23(a) - (h). The average value of NADH in the each set was 0.415 ± 0.029 , 0.431 ± 0.020 , 0.512 ± 0.042 , 0.479 ± 0.031 , 0.502 ± 0.027 , 0.521 ± 0.021 , 0.446 ± 0.012 , and $0.513 \pm 0.025 \mu\text{mol (mg protein)}^{-1}$, respectively. While the rate of TCE removal and the normalized enzyme activity decreased during each experiment, there was no consistent trend in the change in NADH concentration during TCE degradation. In all cases, the average normalized NADH concentration remained near the normal value observed when toluene is present, about $0.477 \mu\text{mol (mg protein)}^{-1}$. This implied that TCE degradation was not limited by NADH concentration under any of the conditions tested. These results are consistent with those of section 6.6. NADH varied somewhat during the experiment, but the average concentration of NADH remained above $0.477 \mu\text{mol (mg protein)}^{-1}$.

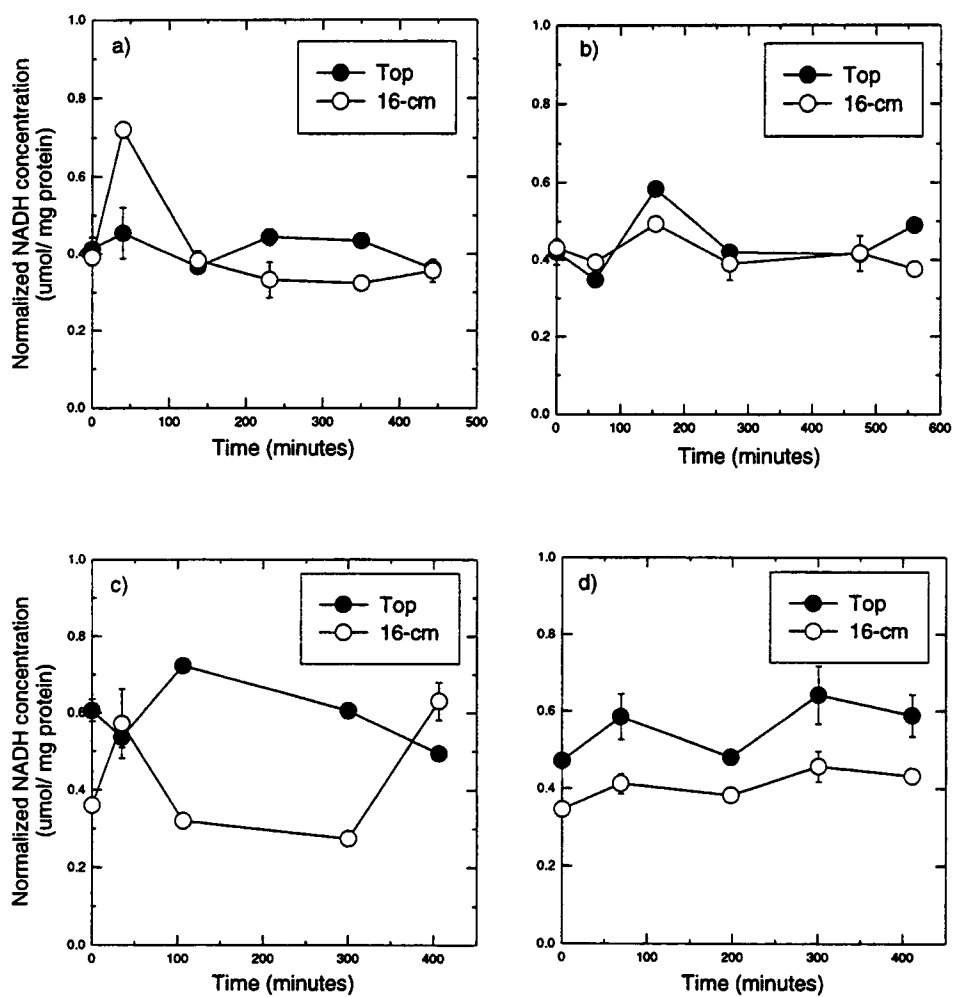


Figure 6.23 Normalized NADH concentration as a function of time during experiment 1(a) to 4(d)

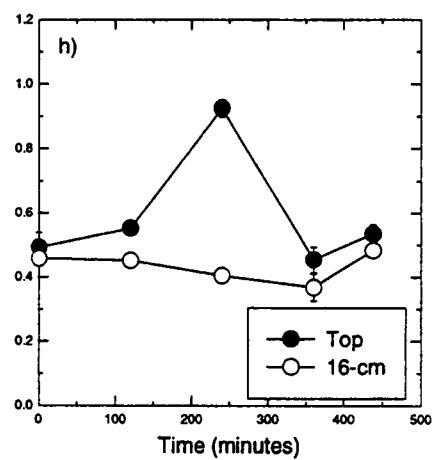
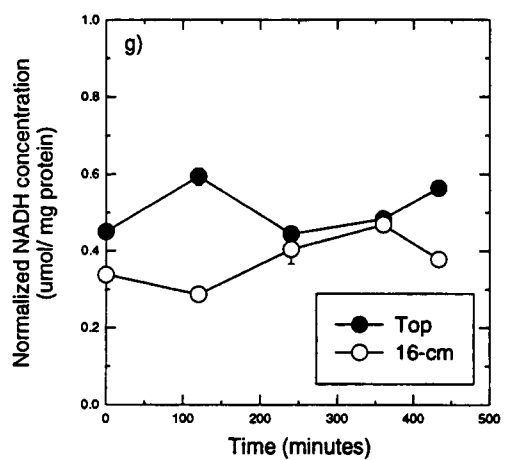
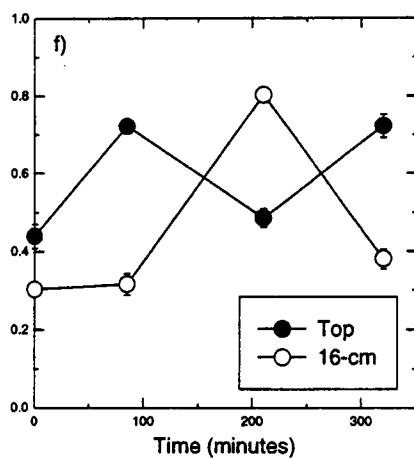
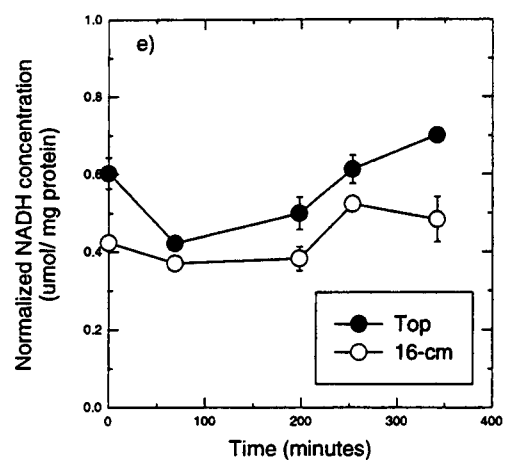


Figure 6.23 (continued)

Normalized NADH concentration as a function of time during experiment 5 (e) to 8 (h)

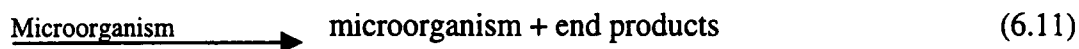
6.8 Calculation of theoretical NADH demand during TCE co-oxidation

6.8.1 The stoichiometry of microbial growth

6.8.1.1 The general equation for microbial growth.

A substrate serves as both carbon source for assimilation and energy source for growth and maintenance. The carbon in the synthesis component in the energy component ends up in cell materials, whereas the carbon in the energy component ends up as carbon dioxide (Grady *et al.*, 1980). The general equation for microbial growth can be written as:

Carbon source + energy source + terminal hydrogen acceptor + nutrients



Rapid growing cell populations use more substrate for assimilation and growth energy, while a cell population in a resting or stationary state consumes substrate for maintenance energy. Microbially mediated reactions are also oxidation-reduction reactions and thus involve the transfer of electrons from a donor, which is generally the substrate being oxidized, to an acceptor. Half reactions for the electron donor, electron acceptor and cell material can be combined to give the overall oxidation-reduction equation for microbial growth (Grady *et al.*, 1980).

$$R = R_d - f_e \cdot R_a - f_s \cdot R_c \quad (6.12)$$

Where R : the overall stoichiometric equation

R_d : the half reaction of the electron donor

R_a : the half reaction of the electron acceptor

R_c : the half reaction of cell material

f_e : the fraction of the electron donor for energy
 f_s : the fraction of the electron donor for synthesis
 $f_s + f_e = 1$

6.8.1.2 The empirical chemical formula for cell material

Various empirical formulas have been proposed to represent the organic composition of microbial cells. The cellular composition based on the four major elements C, N, O, and H for several different microorganisms are shown in Table 6.5. The empirical chemical formula of $\text{CH}_2\text{N}_{0.25}\text{O}_{0.5}$ is used for cell material in stoichiometry of microbial growth.

Table 6.5 Data on elemental composition of several microorganisms (Bailey *et al.*, 1987).

μ denotes specific growth rate = mass of cells formed per unit time (mass of cells)⁻¹

Microorganism	Limiting nutrient	μ (h ⁻¹)	Composition (% by wt)							Empirical chemical formula
			C	H	N	O	P	S	Ash	
Bacteria			53.0	7.3	12.0	19.0			8	$\text{CH}_{1.466}\text{N}_{0.20}\text{O}_{0.27}$
Bacteria			47.1	7.8	13.7	31.3				$\text{CH}_2\text{N}_{0.25}\text{O}_{0.5}$
<i>Aerobacter aerogenes</i>			48.7	7.3	13.9	21.1			8.9	$\text{CH}_{1.78}\text{N}_{0.24}\text{O}_{0.33}$
<i>Klebsiella aerogenes</i>	Glycerol	0.1	50.6	7.3	13.0	29.0				$\text{CH}_{1.74}\text{N}_{0.22}\text{O}_{0.43}$
<i>K aerogenes</i>	Glycerol	0.85	50.1	7.3	14.0	28.7				$\text{CH}_{1.73}\text{N}_{0.24}\text{O}_{0.43}$
Yeast			47.0	6.5	7.5	31.0			8	$\text{CH}_{1.66}\text{N}_{0.13}\text{O}_{0.40}$
Yeast			50.3	7.4	8.8	33.5				$\text{CH}_{1.75}\text{N}_{0.5}\text{O}_{0.50}$
Yeast				6.2	8.5	31.2	1.08	0.6		$\text{CH}_{1.64}\text{N}_{0.16}\text{O}_{0.52}\text{P}_{0.01}\text{S}_{0.005}$
<i>Candida utilis</i>	Glucose	0.08	50.0	7.6	11.1	31.3				$\text{CH}_{1.82}\text{N}_{0.19}\text{O}_{0.47}$
<i>C. utilis</i>	Glucose	0.45	46.9	7.2	10.9	35.0				$\text{CH}_{1.84}\text{N}_{0.5}\text{O}_{0.56}$
<i>C. utilis</i>	Ethanol	0.06	50.3	7.7	11.0	30.8				$\text{CH}_{1.82}\text{N}_{0.19}\text{O}_{0.46}$
<i>C. utilis</i>	Ethanol	0.43	47.2	7.3	11.0	34.6				$\text{CH}_{1.84}\text{N}_{0.2}\text{O}_{0.55}$

6.8.1.3 Determination of f_s

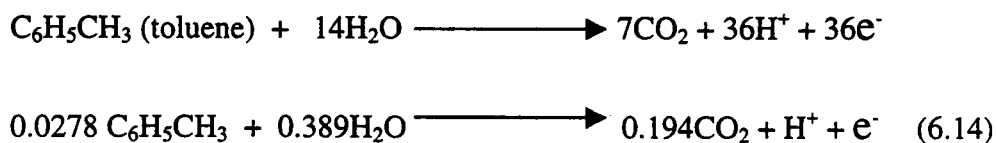
The fraction of energy used for synthesis (f_s) is related to the observed yield (Y) of the culture that must be measured in the laboratory for the particular growth conditions under consideration. The unit of f_s is equivalents of cells formed per equivalent of electron donor consumed, whereas the unit of Y is grams of cells formed per gram of substrate (toluene) removed. The Y can be converted to grams of cells formed per equivalent by multiplying by 36, because one mole toluene produces 36 electrons when it is oxidized. One mole of $\text{CH}_2\text{N}_{0.25}\text{O}_{0.5}$ cells weigh 25.5g and one equivalent is 1/4.25 of a mole. The relationship between f_s and Y is expressed by following equation:

$$f_s = \frac{\left(\frac{\text{Y grams cells formed}}{\text{gram toluene removed}} \right) \left(\frac{2.556 \text{ gram toluene}}{\text{equivalent}} \right)}{\left(\frac{6 \text{ grams cells}}{\text{equivalent}} \right)} = 0.426 \cdot Y \quad (6.13)$$

6.8.1.4 Estimation the microbial growth by stoichiometry

Pseudomonas putida F1 oxidizes toluene as carbon and energy source. Hsiao-Lung Chang *et al.* (1995) reported the yield coefficient (Y) of toluene oxidizers as 0.29 (mg mg^{-1}). Based on this yield a balanced equation for growth can be derived:

a) Electron donor reaction



b) Electron acceptor reaction

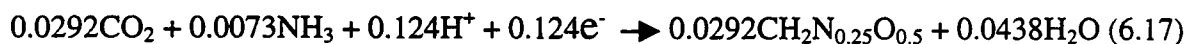
$$f_s + 0.426 \times Y = 0.426 \times 0.29 = 0.124 \quad (6.15)$$

87.6% of donor electrons ($f_e=0.876$) are used in energy production.

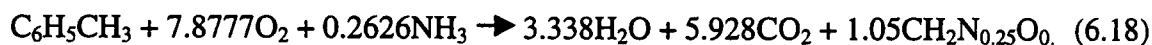


c) Cell synthesis reaction

12.4% of donor electrons are used in biosynthesis ($f_s = 0.124$).



Adding the three equations (6.14), (6.16), and (6.17) results in a final equation for microbial growth:



According to the equation (6.18), 0.29 mg of biomass produced per mg of toluene oxidized ($((25.5 \times 1.05)/92)$), which is the same yield as used in equation (6.15). Oxygen and NH_3 requirements are $2.74 \text{ mg O}_2 (\text{mg toluene})^{-1}$ and $0.0485 \text{ mg NH}_3 (\text{mg toluene})^{-1}$, respectively.

6.8.1.5 Estimation of net NADH production via toluene oxidation

The production of NADH productions during catabolism of toluene is illustrated in Figure 6.24.

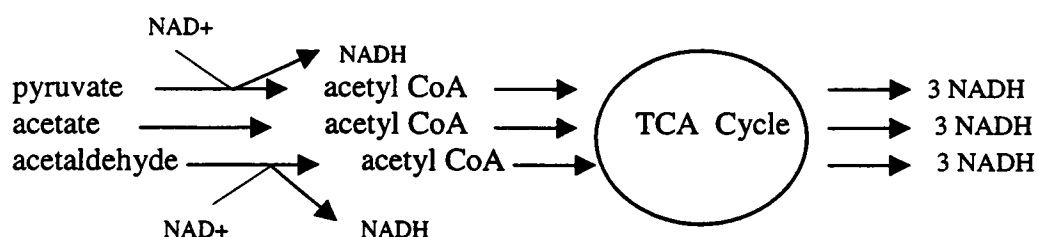
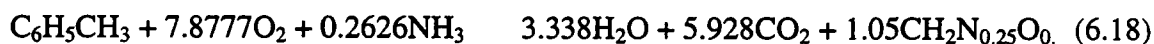


Figure 6.24 NADH production via toluene metabolic pathway and TCA cycle.

A total of eleven moles of NADH are produced for each mole of toluene degraded by *PpF1*. However, a certain amount of NADH may be utilized as an energy source for maintenance and biosynthesis, as illustrated below.

The fraction of energy used for synthesis (f_s) is related to the observed yield (Y) of the culture. Hsiao-Lung Chang *et al.* (1995) reported the yield coefficient (Y) of toluene oxidizers as 0.29 mg cell/mg toluene. Based on equation (6.18), a following sample calculation for the available amount of NADH for toluene degradation can be evaluated.



The carbon in the synthesis component ends up the cell material ($\text{CH}_2\text{N}_{0.25}\text{O}_{0.5}$), while the carbon in the energy component ends up as carbon dioxide:

The carbon ratio for energy production = $5.928 \times 12 / (12 \times 7) = 0.85$

The carbon ratio for biosynthesis = $1.05 \times 12 / (12 \times 7) = 0.15$

15% of total carbons are used in biosynthesis, so 15% of total NADH are utilized for biosynthesis.

$$0.15 \times 11 \text{ NADH} = 1.65 \text{ NADH} \quad (6.19)$$

86% of total carbons are used in energy production, so 85% of total NADH are used for energy production.

$$0.85 \times 11 \text{ NADH} = 9.35 \text{ NADH} \quad (6.20)$$

Under the steady state conditions, the fraction of carbon used for biosynthesis may be constant. Therefore, 9.35 NADH may be maximum available amount of NADH for TCE degradation.

The theoretical amount of NADH required to oxidize TCE by toluene dioxygenase enzyme was calculated from the TCE oxidation pathway proposed by Li *et al.* (1992). The pathway indicates that one mole of NADH is required per mole of TCE degraded:

$$\frac{1 \text{ mole NADH}}{1 \text{ mole TCE}} \times \frac{1 \text{ mole TCE}}{131.39 \text{ g TCE}}$$

$$= 7.61 \times 10^{-3} \text{ mole NADH/ g TCE} \quad (6.21)$$

The theoretical production of NADH during toluene oxidation by Tod enzyme was determined above. It was shown that 9.35 moles of NADH are used for TCE oxidation by *P. putida* F1:

$$\frac{9.35 \text{ mole NADH}}{1 \text{ mole toluene}} \times \frac{1 \text{ mole toluene}}{92.14 \text{ g toluene}}$$

$$= 1.05 \times 10^{-1} \text{ mole NADH/ g toluene} \quad (6.22)$$

If the same mass of TCE and toluene were degraded simultaneously, only 7.2 % of the NADH produced by toluene degradation would be utilized for TCE oxidation:

$$(7.61 \times 10^{-3}) / (1.05 \times 10^{-1}) \times 100 = 7.2 \% \quad (6.23)$$

This suggests that sufficient NADH for TCE degradation will always exist in the presence of toluene.

The theoretical requirement for NADH during TCE oxidation by resting cells (no growth substrate present) can be calculated to evaluate the possibility of NADH becoming the limiting factor for TCE degradation. Of the eight experiments described in

Table 6.3, the experiment with the greatest mass of TCE degraded was selected for an example calculation. This was experiment 4 in which the total TCE mass degraded was 20389 μg TCE over a period of 411 minutes.

The NADH required for TCE oxidation is:

$$\frac{7.61 \times 10^{-3} \text{ mole NADH}}{\text{g TCE}} \times 20389 \mu\text{g TCE} \times \frac{1 \text{ g}}{10^{-6} \mu\text{g}}$$

$$= 1.55 \times 10^{-4} \text{ mole NADH required.} \quad (6.24)$$

Because the average normalized NADH concentration remained about 0.477 $\mu\text{mol (mg protein)}^{-1}$ under various toluene concentration at steady state condition, the total NADH in the column was about:

$$0.477 \mu\text{mol (mg protein)}^{-1} \times 2420 \text{ mg protein}$$

$$= 11.54 \times 10^{-4} \text{ mol NADH} \quad (6.25)$$

Theoretically, in order to degrade 20389 μg TCE, about 13.43% of the NADH in biofilm was depleted. However, Figure 6. 23 does not indicate a decrease in NADH during TCE degradation. Also, the NADH in cells does not seem to increase much during toluene degradation. White (1995) reported that regulative processes within the bacteria maintain a constant NADH:NAD⁺ ratio. Tschantz (1996) reported that in the

presence of sufficient growth substrate, the NADH concentration reached a maximum steady-state value of 0.25 $\mu\text{mol MADH (mg cells)}^{-1}$. Assuming cell biomass is about 50% protein, the steady-state NADH concentration for *Methylosinus trichosporium* OB3b can be reported as 0.5 $\mu\text{mol MADH (mg protein)}^{-1}$, which is very close to the value found in this work for *Pseudomonas putida* F1.

In the experiments, the decrease in TCE degradation rate was not caused by NADH limitation, but by toxicity resulting from the formation of highly reactive intermediates during TCE degradation. This conclusion is consistent with the results reported in section 6.7.

Equation (2.6) and (2.7), describing the enzyme kinetics of TCE and toluene cometabolism, can be simplified by neglecting the term describing the variation in NADH concentration:

$$r_{\text{TCE}} = -\frac{dS}{dt} = \frac{k_s[E_c][S]}{([S] + K_s^{\text{app}})} \quad (6.26)$$

$$r_{\text{toluene}} = -\frac{dT}{dt} = \frac{k_T[E_c][T]}{([T] + K_T^{\text{app}})} \quad (6.27)$$

The simplified equations are similar to the conventional Michaelis-Menten kinetics. These were used as the basic equations for building a mathematical model to predict TCE concentration and normalized enzyme activity in the biofiltration column.

6.9 Mathematical model studies

6.9.1 Mathematical model for biofiltration

Biofiltration utilizes immobilized microorganisms to degrade the volatile organic compound (VOC) in contaminated gases. Often the mechanism of VOC degradation is not fully understood and empirical relationships are used for design and operation of biofilters because the complexity of the biodegradation and mass transfer mechanisms involved. Therefore, appropriate biofiltration models need to be developed and validated for improved process design and operation. In one of the first models of biofiltration, Ottengraf (1983) adapted a liquid-phase model to gas. Since then, more biofilter models have been developed for various systems and applications (Shareenfdeen *et al*, 1992; Hodge *et al*, 1992; Deshusses, 1995). Deshusses (1995) successfully explained the interaction between multiple pollutants using a dynamic biofiltration model. The basic concept of this mathematical model will be adapted and modified to describe cometabolic biofiltration of TCE. The model will be calibrated by comparing the simulations to the results of biofiltration experiments under various conditions.

6.9.1.1 Model assumptions

The basis of the model is a dynamic mass balance on an ideal-plug flow reactor. Several assumptions were made in constructing the biofiltration model; that is,

1. The gas flow through the filter can be described as plug flow. That is the gas flow pattern inside has uniform velocity and concentration in the radial direction at any point along the length of the reactor.

2. No longitudinal (axial) dispersion of TCE or toluene along the reactor.
3. The concentrations of toluene and TCE vary with the axial distance x only.
4. Biodegradation in the biofilm can be described by the Michaelis -Menten relationship.
5. Whole cell solvent toxicity effects are negligible.
6. The specific enzyme activity of cells was uniform in the radial direction
at any point along the length of the reactor.
7. NADH was not a limiting factor during TCE oxidation.
8. The natural decay rate of enzyme was proportional to the specific enzyme activity.
9. The rate of enzyme inactivation by TCE oxidation was proportional to the mass of
TCE degraded.
10. Mass transfer of TCE and toluene into the biomass is not limited.
11. The gas- and liquid-phase concentrations of each pollutant are always in equilibrium
at the phase boundary and can be related by Henry's Law.

The model will be limited to cases in which TCE only is fed to the biofilm. The extremely rapid rate at which T_{od} activity was restored upon feeding toluene was difficult to quantify and negated the need to include this phenomenon in the model.

6.9.1.2 Mass transfer limitations

Assumption 10 states that mass transfer of TCE and toluene in the biomass is not limiting. The purpose of this section is to demonstrate that this assumption is valid under the conditions tested. The following theoretical analysis of mass transfer is based on an

analysis of the influence of internal mass transfer resistance on reactions occurring within a flat fixed film by Grady *et al.* (1980).

Rittmann *et al.* (1981) developed a submerged biofilm model which accounts for liquid advection and dispersion, diffusion through a liquid film, and diffusion and reaction within a biofilm. Speitel *et al.* (1993) adapted Rittmann's work to gas phase applications to include bulk gas advection and dispersion, diffusion through a gas film, diffusion through a liquid film, and diffusion and reaction within the biofilm (Figure 6.25).

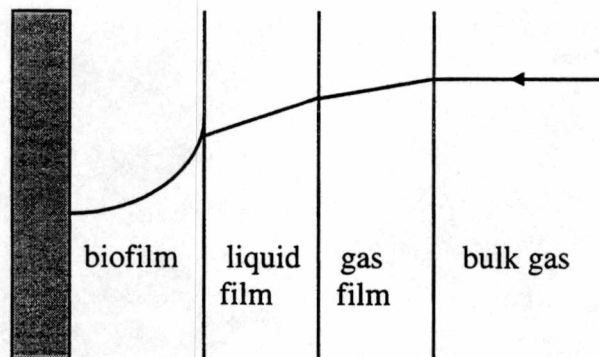
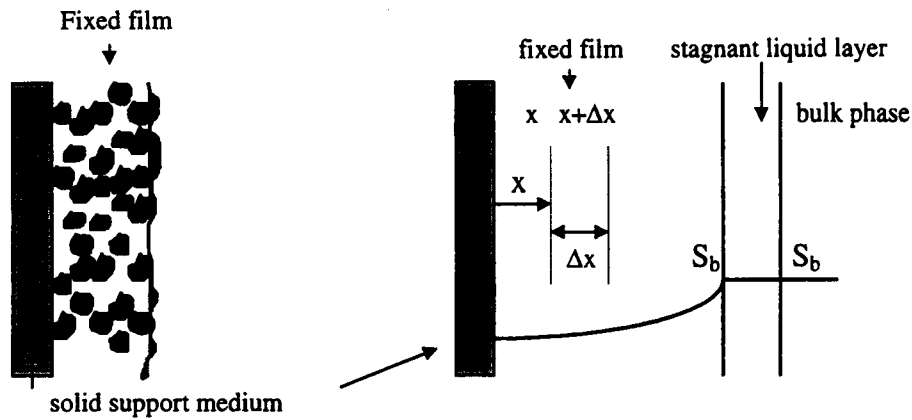


Figure 6.25 Substrate concentration profile in unsubmerged biofilm reactor (Speitel *et al.* (1993)).

In a biofiltration column, cells are assumed to be uniformly distributed throughout the film and the biofilm covers a flat solid support. It may also be assumed that there is no external mass transfer resistance limiting the transport of substrate from the bulk liquid to the liquid-solid interface (Grady *et al.*, 1980). Figure 6.26 shows the substrate concentration profiles for reaction within a thick biofilm.



a) Schematic view of fixed film b) idealized fixed-film

Figure 6.26 Substrate concentration profiles for reaction within a biofilm (Grady *et al.*, 1980)

Diffusion is assumed to be the only mechanism by which substrate can reach an organism within the biofilm because biofilm was very thin. This diffusion is described by Fick's law:

$$N_s = -D \frac{dS}{dX} \quad (6.28)$$

where N : flux, $M L^{-2} t^{-1}$
 D : diffusion coefficient, $L^2 t^{-1}$
 dS/dX : concentration gradient, $M L^{-4}$

The system is assumed to reach a steady state when substrate concentration is maintained at S_b , biofilm thickness, L_f is a constant and there is no accumulation within the film. A mass balance on substrate around a differential element in the film is:

$$-D \cdot A_s \frac{dS}{dx} \Big|_x + D \cdot A_s \frac{dS}{dx} \Big|_{x+\Delta x} + r_s \cdot L_f (1-\phi) \cdot V \cdot a_s \cdot \rho_s \cdot A_s \cdot \Delta x = 0 \quad (6.29)$$

where A_s : the total surface area normal to the direction of diffusion, L^2

x : the distance into the biofilm from the solid support, L

ρ_s : the biofilm density, $M L^{-3}$

r_s : the substrate utilization rate, t^{-1}

L_f : biofilm thickness, L

a_s : specific surface area, $L^2 L^{-3}$

ϕ : bed porosity

V : bed volume, L^3

$\delta_s : L_f (1-\phi) V a_s \rho_s$

Equation (6.29) is simplified by dividing both sides by $A_s \Delta x$, taking the limit as

Δx goes to zero, and assuming D to be constant:

$$D \frac{d^2 S}{dx^2} + r_s \cdot \delta_s = 0 \quad (6.30)$$

The rate r_s is given by equation (6.48), which when substituted into equation

(6.30) yield:

$$D \frac{d^2 S}{dx^2} + \delta_s k_s \frac{S \cdot E_x}{(S + K_s)} = 0 \quad (6.31)$$

The boundary conditions for the equation are

$$S = S_b \quad \text{at } x=L_f \quad (6.32)$$

$$dS/dt = 0 \quad \text{at } x = 0 \quad (6.33)$$

No analytical solution for equation (6.31) is possible due to the nonlinear nature of equation. However, most of our experiments for TCE degradation in biofiltration were conducted at TCE feed concentration ($S \leq 300 \text{ TCE } \mu\text{g L}^{-1}$) much less than K_s ($2620 \text{ TCE } \mu\text{g L}^{-1}$), so we can use the analytical solution for first-order kinetic under these conditions.

The equation (6.31) can rewritten for first-order kinetics;

$$D \frac{d^2 S}{dx^2} + \delta_s \cdot \left(\frac{k_s' E_x}{K_s} \right) \cdot S = 0 \quad (6.34)$$

Defining the dimensionless variables:

$$S' = S/S_b \quad (6.35)$$

$$z = x/L_f \quad (6.36)$$

The equation (6.34) may be rewritten as follows:

$$D \frac{d^2 S}{dx^2} + \Phi^2 \cdot S' = 0 \quad (6.37)$$

$$\Phi = \left(\frac{\delta_s \cdot k_s \cdot E_x \cdot L_f^2}{K_s \cdot D} \right)^{0.5} \quad (6.38)$$

$$S' = 1 \quad \text{at } z = 1 \quad (6.39)$$

$$dS'/dx = 0 \quad \text{at } z = 0 \quad (6.40)$$

The solution of the equation (6.37) is

$$S' = \frac{\cosh \Phi z}{\cosh \Phi} \quad (6.41)$$

The profiles of substrate concentration versus depth within the biofilm for several values of the parameter Φ is shown in Figure 6.27. The substrate concentration decreases with distance from the liquid-biofilm interface ($z = 1$). As the parameter (Φ) increases, the substrate concentration within the biofilm decreases much faster. The parameter Φ is the ratio of the rate of biodegradation to the rate of mass transfer. For values of $\Phi > 1$ the rate of degradation is faster than the rate of mass transfer and significant concentration gradients exist in the biofilm. For values of $\Phi < 1$, diffusion is much less than the rate of biodegradation and concentration gradients are less pronounced.

To determined the relative importance of biodegradation and mass transfer for

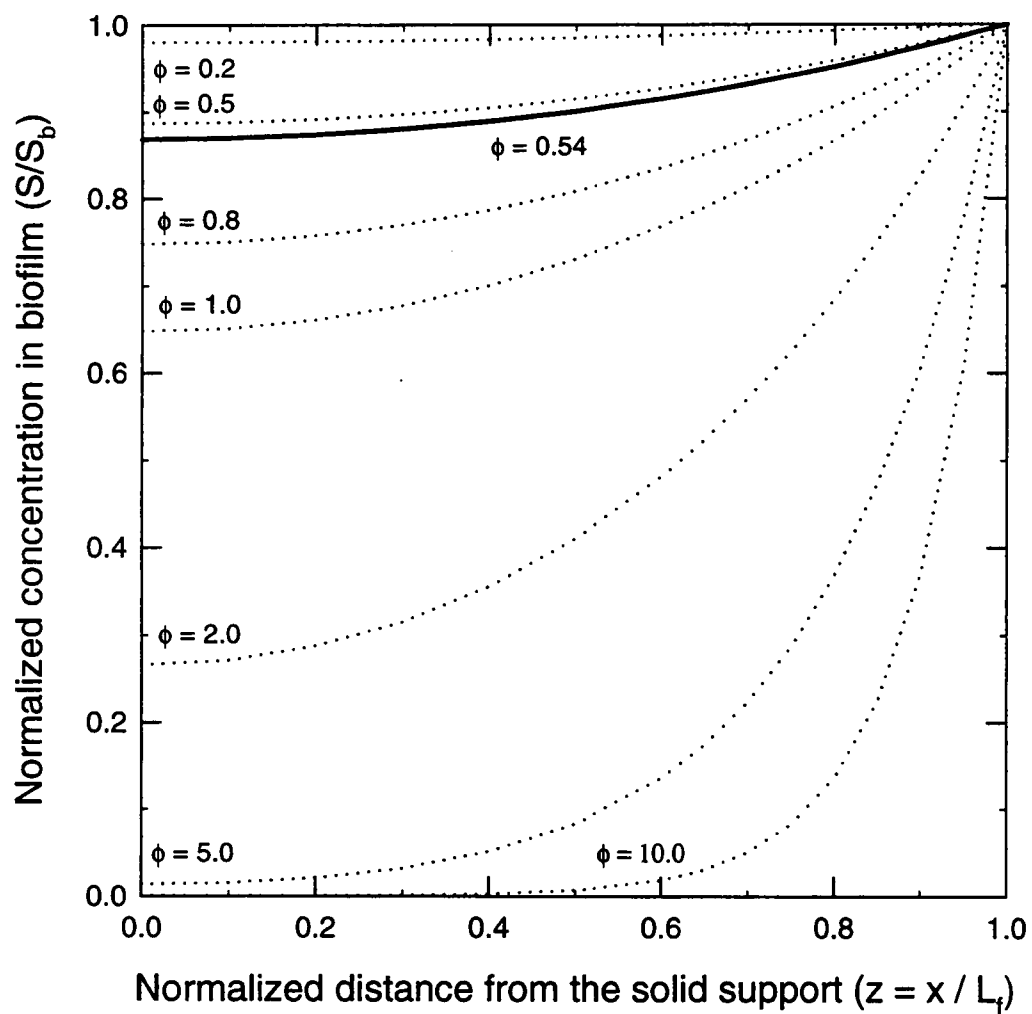


Figure 6.27 Calculated substrate concentration profile within the biofilm under a first-order reaction

TCE oxidation by *Pseudomonas putida* F1 within a biofilm, the parameter Φ was calculated using parameters from Table 6.6.

Table 6.6 Parameters used in analysis of TCE mass transfer within biofilm.

Parameter	value	unit	source
biofilm density (ρ_s)	10	mg protein cm ⁻³	Segar et al., 1995
TCE diffusivity in biofilm (D)	0.533	cm ² day ⁻¹	Speitel et al., 1993
biofilm thickness (L_f)	0.03	cm	Segar et al., 1995
TCE saturation constant (K_s)	2620	μg TCE L ⁻¹	section 5.9.2.1
TCE reaction rate constant (k_s')	1428.6	μg TCE (mg indigo L) ⁻¹	section 5.9.2.1
reactor bed volume (V)	1256	cm ³	section 5.5
porosity (θ)	0.32		Speitel et al., 1993
specific surface area (a_s)	10.5	cm ² (cm ³ media) ⁻¹	Speitel et al., 1993

$$\begin{aligned}
 \delta_s &= L_f (1-\theta) V a_s \rho_s \\
 &= (0.03\text{-cm}) \times (1-0.32) \times (1256\text{-cm}^3) \times (10.5\text{-cm}^2 \text{ cm}^{-3}) \times (10 \text{ mg protein cm}^{-3}) \\
 &= 2690 \text{ mg protein}
 \end{aligned}$$

$$\begin{aligned}
 \Phi &= [\delta_s (k_s' E_x / K_s) L_f^2 / D]^{0.5} \\
 &= [(2690 \text{ mg protein}) \times (1428 \text{ μg TCE (mg indigo L)}^{-1}) \times (E_x \text{ mg indigo (min} \\
 &\quad \text{mg protein)}^{-1})) \times (2620 \text{ μg TCE L}^{-1})^{-1} \times (0.03\text{-cm})^2 \times (0.533\text{- cm}^2 \text{ day}^{-1})^{-1} \\
 &\quad \times (24 \times 60 \text{ min})]^{0.5} \\
 &= [3564 \times E_x]^{0.5}
 \end{aligned}$$

The value Φ for TCE degradation in the biofiltration column was calculated using data from section 6.5. E_x is not constant value. For the range of E_x values measured in

our experiments, the value of Φ was found to be in the range of 0.24 to 0.81 with an average value of 0.54. The profiles of TCE concentration in a function of biofilm depth for the parameter Φ of 0.54 is superimposed as solid line in Figure 6.27.

For all conditions tested in this research $\Phi < 1$, suggesting that diffusion resistance is less than the rate of substrate utilization and the substrate concentration does not change radically within the biofilm. Therefore, the assumption that mass transfer does not limit the rate of biofiltration appears in the case of TCE oxidation by *Pseudomonas putida* F1.

6.9.1.3 Model equations

The mass balance for TCE around an arbitrary control volume is:

$$\begin{array}{l} \text{[molar flow rate of TCE into the control volume]} \\ \text{[molar flow rate of TCE out of the control volume]} \\ \text{[rate of TCE loss due to the cometabolic oxidation within the control volume]} \end{array} = \begin{array}{l} \text{[rate of change in the mass of TCE in the control volume]} \end{array}$$

$$n_{s,x} - n_{s,x+\Delta x} - r_s A_c \Delta x = \frac{\partial(A_c \Delta x C_s)}{\partial t} \quad (6.42)$$

Where A_c : the cross-sectional area of the reactor.

x : the distance from the reactor entrance.

r_s : TCE degradation rate, $\mu\text{g TCE L}^{-1} \text{ min}^{-1}$

$n_{s,x}$ and $n_{s,x+\Delta x}$ are the molar flow rates of TCE at the distances x and $x+\Delta x$ from the inlet of the reactor.

Taking the limit as Δx goes to zero, the following partial differential equation is found:

$$\frac{\partial(n_s)}{\partial x} + r_s A_c = \frac{A_c \partial(C_s)}{\partial t} \quad (6.43)$$

The molar flow rate is given by the product of the flow rate and molar concentration ($n_s = F C_s$), which when substituted into equation (6.43) yields:

$$V_x \frac{\partial S}{\partial x} - r_s = \frac{\partial S}{\partial t} \quad (6.44)$$

Where V_x : gas velocity, cm min^{-1}

In section 6.7, it was shown that under the conditions investigated that NADH never becomes the limiting reactant. Under these conditions, the enzyme kinetics of TCE oxidation by toluene dioxygenase enzyme in absence of toluene can be simplified to the following equation:

$$r_s = -\frac{dS}{dt} = \frac{k_s[E_c] \cdot S}{(S + K_s)} \quad (6.45)$$

The enzyme concentration $[E_c]$ can be expressed in terms of the enzyme activity E , because the actual concentration of enzyme difficult to measure. The enzyme concentration is assumed to be proportional to the enzyme activity:

$$[E_c] = \alpha E \quad (6.46)$$

Where $[E_c]$: enzyme cocncentration, mg enzyme L^{-1}
 E : enzyme activity, mg indigo min^{-1}
 α : proportional constant, mg enzyme min $(L \text{ mg protein})^{-1}$

Equation (6.47) describes the relationship between maximum TCE utilization rate and corresponding enzyme activity of cells:

$$k_s[E_c] = k_s\alpha E = k'_s X E_x \quad (6.47)$$

Where k'_s : $k_s\alpha$, $\mu g \text{ TCE } (L \text{ mg indigo})^{-1}$
 E : $X E_x$, mg indigo mg min^{-1}
 E_x : normalized enzyme activity, mg indigo mg protein $^{-1} min^{-1}$
 X : biomass, mg protein

Substituting equation (6.47) into equaiton (6.45) yields the following eqiation:

$$r_s = -\frac{dS}{dt} = \frac{k'_s \cdot X \cdot E_x \cdot S}{(S + K_s)} \quad (6.49)$$

When only TCE is present, the net rate of change in the enzyme activity is a function of the rate of natural decay of the enzyme and enzyme inactivation by toxicity (Wackett, L. P., *et al.*, 1988 and 1989).

$$\frac{dE_x}{dt} = \frac{dE_x}{dt} \text{decay} + \frac{dE_x}{dt} \text{toxicity} \quad (6.50)$$

It was shown in section 4.4 that the natural decay may be expressed as the product of the enzyme activity and decay constant:

$$\frac{dE_x}{dt} \text{decay} = -k_1 E_x \quad (6.50)$$

Where k_1 : decay constant, min^{-1}

Tod-mediated metabolic transformation of TCE to toxic intermediates is the second mechanism by which Tod is inactivated. The rate of enzyme deactivation is proportional to the total mass of TCE degraded:

$$\frac{dE_x}{dt} \text{toxicity} = -k_2 dS \frac{Q}{X} \quad (6.51)$$

Where k_2 : inactivation constant, $\text{mg indigo} (\text{min } \mu\text{g TCE})^{-1}$
 Q : flow rate, L min^{-1}

Substituting equation (6.51) and (6.50) into equation (6.49) yields the following equation describing the overall rate of Tod enzyme inactivation:

$$\begin{aligned}\frac{dE_x}{dt} &= \frac{dE_x}{dt} \text{decay} + \frac{dE_x}{dt} \text{toxicity} \\ &= -k_1 E_x - k_2 dS \frac{Q}{X}\end{aligned}\quad (6.52)$$

In the absence of a growth substrate, the degradation of TCE can be described by the following coupled differential equations:

$$-V_x \frac{\partial S}{\partial x} - k_s' X S \frac{E_x}{(S + K_s)} = \frac{\partial S}{\partial t} \quad (6.53)$$

$$\frac{dE_x}{dt} = -k_1 E_x - k_2 dS \frac{Q}{X} \quad (6.54)$$

Both TCE concentration and normalized enzyme activity are a function of reaction time (t) and column length (x). The dynamic, coupled equations are solved simultaneously by a finite difference method.

6.9.1.4 Solution procedure

Figure 6.28 shows a schematic description of the finite difference formulation of the problem. The biofilter is divided into n-layers. The gas phase and biofilm of each layer are assumed to be ideally mixed. Equilibrium partitioning of pollutants between the gas phase and biofilm is assumed. Simultaneous diffusion and biodegradation of the pollutant occurs within the biofilm.

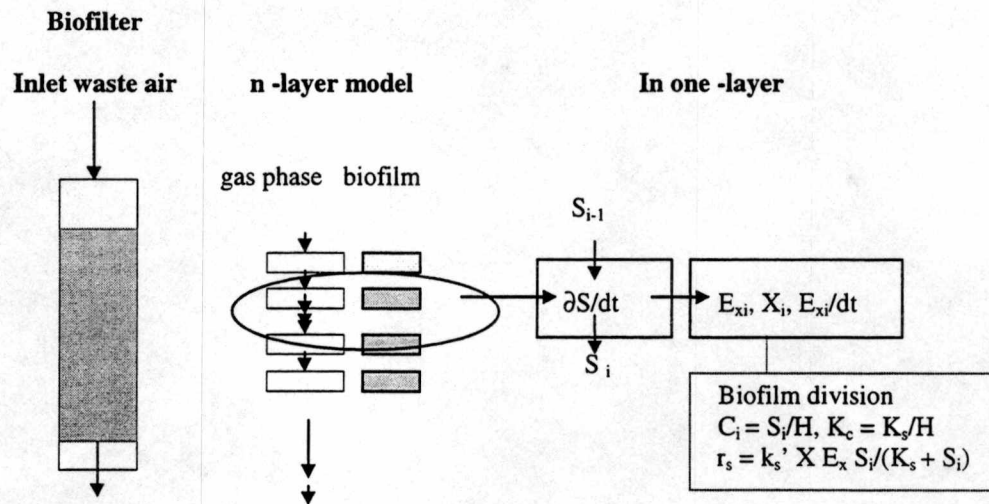


Figure 6.28 Schematic representation of the finite difference approach

Equation (6.53) can be transformed as follows to yield an analytical solution:

Let

$$\tau = t + \frac{1}{V_x} x \quad (6.55)$$

then

$$\frac{\partial \tau}{\partial t} = 1 \quad \frac{\partial \tau}{\partial x} = \frac{1}{V_x} \quad (6.56)$$

and

$$\frac{\partial t}{\partial x} = \frac{1}{V_x} \quad \frac{\partial x}{\partial \tau} = V_x \quad (6.57)$$

Applying the chain rule to determine $dS/d\tau$ yields:

$$\frac{dS}{d\tau} = \frac{\partial S}{\partial t} \cdot \frac{\partial t}{\partial \tau} + \frac{\partial S}{\partial x} \cdot \frac{\partial x}{\partial \tau} \quad (6.58)$$

Substituting equations (6.56) and (6.57) yield:

$$\frac{dS}{d\tau} = \frac{\partial S}{\partial t} + V_x \frac{\partial S}{\partial x} \quad (6.59)$$

Substituting equations (6.59) into (6.53) yields:

$$\frac{\partial S}{\partial \tau} = -k_s' X S \frac{E_x}{(S + K_s)} \quad (6.60)$$

Equations of (6.54) and (6.60) were solved numerically using the following initial and boundary conditions:

$$S = 0 \text{ for all } x \quad \text{at } t = 0$$

$$S = S_0 \text{ for } x = 0 \quad \text{at } t > 0$$

$$E_x = \text{known for all } x \quad \text{at } t = 0$$

The subscripts i and j present time and position, respectively. Integration of equation (6.54) and (6.60) over a finite difference in space and time yields the general equations for TCE ($S_{i,j}$) and normalized enzyme activity ($E_{x,i,j}$) in the biofiltration column:

$$\begin{aligned}
\tau_{i,j-1} - \tau_{i,j} &= (t_i + \frac{x_{j-1}}{V_x}) - (t_i + \frac{x_j}{V_x}) \\
&= \frac{1}{(k_s X E_{x,i-1,j-1})} [(S_{i,j} - S_{i,j-1}) + K_s \ln(\frac{S_{i,j}}{S_{i,j-1}})] \quad (6.61)
\end{aligned}$$

$$E_{x,i,j} = [E_{x,i-1,j} - \frac{k_2 Q}{k_1 X} (S_{i,j} - S_{i,j+1})] \cdot \exp[-k_1 (t_i - t_{i-1})] - \frac{k_2 Q}{k_1 X} (S_{i,j} - S_{i,j+1}) \quad (6.62)$$

The solution procedure for determining the TCE concentration and the normalized enzyme activity in biofiltration reactor are described below:

Step 1: For the first time step ($i = 1$), find $S_{1,j}$ for each position in the column using equation (6.61).

$$\begin{aligned}
\tau_{1,0} - \tau_{1,1} &= (t_1 + \frac{x_0}{V_x}) - (t_1 + \frac{x_1}{V_x}) \\
&= \frac{1}{(k_s X E_{x,0,0})} [(S_{1,1} - S_{1,0}) + K_s \ln(\frac{S_{1,1}}{S_{1,0}})]
\end{aligned}$$

Find $S_{1,1}$ using known $S_{1,0}$ and $E_{x,0,0}$.

$$\tau_{1,1} - \tau_{1,2} = (t_1 + \frac{x_1}{V_x}) - (t_1 + \frac{x_2}{V_x})$$

$$= \frac{1}{(k_s X E_{x,0,1})} [(S_{1,2} - S_{1,1}) + K_s \ln(\frac{S_{1,2}}{S_{1,1}})]$$

Find $S_{1,2}$ using known $S_{1,1}$ and $E_{x,0,1}$. Or in general:

$$\begin{aligned} \tau_{1,j-1} - \tau_{1,j} &= (t_1 + \frac{x_{j-1}}{V_x}) - (t_1 + \frac{x_j}{V_x}) \\ &= \frac{1}{(k_s X E_{x,0,j-1})} [(S_{1,j} - S_{1,j-1}) + K_s \ln(\frac{S_{1,j}}{S_{1,j-1}})] \end{aligned}$$

Find $S_{1,j}$ using known $S_{1,j-1}$. Finally, TCE concentration $S_{1,j}$ at different position (j) for the same time (i=1) are determined.

Step 2: TCE concentrations $S_{1,j}$ are used to find the normalized enzyme activity $E_{x,1,j}$ at different position (j) for the same time (i=1) using equation (6.62).

$$E_{x,1,0} = [E_{x,0,0} - \frac{k_2 Q}{k_1 X} (S_{1,0} - S_{1,1})] \cdot \exp[-k_1 (t_1 - t_0)] - \frac{k_2 Q}{k_1 X} (S_{1,0} - S_{1,1})$$

Find $E_{x,1,0}$ using known $E_{x,0,0}$, $S_{1,0}$ and $S_{1,1}$.

$$E_{x,1,1} = [E_{x,0,1} - \frac{k_2 Q}{k_1 X} (S_{1,1} - S_{1,2})] \cdot \exp[-k_1 (t_1 - t_0)] - \frac{k_2 Q}{k_1 X} (S_{1,1} - S_{1,2})$$

Find $E_{x,1,1}$ using known $E_{x,0,1}$, $S_{1,1}$ and $S_{1,2}$. Or in general:

$$E_{x,1,j} = [E_{x,0,j} - \frac{k_2 Q}{k_1 X} (S_{1,j} - S_{1,j+1})] \cdot \exp[-k_1 (t_1 - t_0)] - \frac{k_2 Q}{k_1 X} (S_{1,j} - S_{1,j+1})$$

Find $E_{x,1,j}$ using known $E_{x,0,j}$, $S_{1,j}$ and $S_{1,j+1}$. Consequently, the normalized enzyme activity $E_{x,1,j}$ at different position for the same time ($i=1$) are determined.

Step 3: Repeat step 1 and 2 for different time point (i) and position (j) to find

$S_{i,j}$ and $E_{x,i,j}$. The general equations for TCE $S(i,j)$ and $E_x(i,j)$ are :

$$\begin{aligned} \tau_{i,j-1} - \tau_{i,j} &= (t_i + \frac{x_{j-1}}{V_x}) - (t_i + \frac{x_j}{V_x}) \\ &= \frac{1}{(k_s X E_{x,i-1,j-1})} [(S_{i,j} - S_{i,j-1}) + K_s \ln(\frac{S_{i,j}}{S_{i,j-1}})] \end{aligned} \quad (6.61)$$

$$E_{x,i,j} = [E_{x,i-1,j} - \frac{k_2 Q}{k_1 X} (S_{i,j} - S_{i,j+1})] \cdot \exp[-k_1 (t_i - t_{i-1})] - \frac{k_2 Q}{k_1 X} (S_{i,j} - S_{i,j+1}) \quad (6.62)$$

6.9.2 Model parameter determination

The model variables are listed in Table 6.7.

Table 6.7. Biofiltration model variables.

Variables	Definition	Units
t	time	min
S	gas phase TCE concentration	$\mu\text{g L}^{-1}$
x	distance from top of column	cm
X	biomass concentration	protein mg
V_x	velocity	cm min^{-1}
E_x	Normalized specific enzyme activity	$\text{mg indigo (mg protein min)}^{-1}$

Model parameters derived from experimental data are listed in Table 6.8. All of the parameters were determined from immobilized cell experiments.

Table. 6.8 Biofiltration model parameters.

Parameter	Definition	Units
k_s'	TCE reaction rate constant	$\mu\text{g TCE (mg indigo L)}^{-1}$
k_1	natural decay rate constant for E_x	min^{-1}
k_2	inactivation constant of E_x	$\text{mg indigo (min } \mu\text{g TCE)}^{-1}$
K_s	TCE half-velocity constant	$\mu\text{g TCE L}^{-1}$

6.9.2.1 Determination of k_s' and K_s

The TCE half -velocity and reaction rate constants were evaluated by constructing a double-reciprocal plot (Shuler *et. al.*, 1992) using experimental data in biofiltration.

Equation (6.63) can be linearized in following form:

$$\frac{dS}{dt} = k_s X S \frac{E_x}{S + K_s} \quad (6.63)$$

$$\frac{E_x}{\left[\frac{(dS/dt)}{X}\right]} = \frac{E_x}{r_s} = \frac{1}{k_s'} + \frac{K_s}{(k_s' S)} \quad (6.64)$$

TCE degradation experiments were conducting in absence of toluene in order to avoid the competitive inhibition effects. The data from Experiments 1 to 8 were used for the determination of k_s' and K_s (Table 6.4). A plot of E_x/V_s versus $1/[S]$ yields a straight line with a slope of K_s/k_s' and y-axis intercept of $1/k_s'$ (Figure 6.29) The slope (1.834) and y-axis intercept (0.0007) were found from the linear least-squares fit of experimental data. The correlation coefficient was 0.89. The values of $k_s' = 1428.6 \mu\text{g TCE (mg indigo L)}^{-1}$ and $K_s = 2620 \mu\text{g TCE L}^{-1}$ were determined.

6.9.2.2 Determination of k_1

Toluene dioxygenase enzyme activity in both immobilized and suspended cells naturally decays over time in the absence of toluene. The natural decay of enzyme activity in immobilized cells was determined using the differential volume reactor (DVR) in section of 4.5. Toluene was continuously introduced to the DVR at a concentration of $1000 \mu\text{g L}^{-1}$. After toluene feeding was stopped, normalized whole cells enzyme activity was measured periodically. The decay of specific enzyme activity was described by the first order equation:

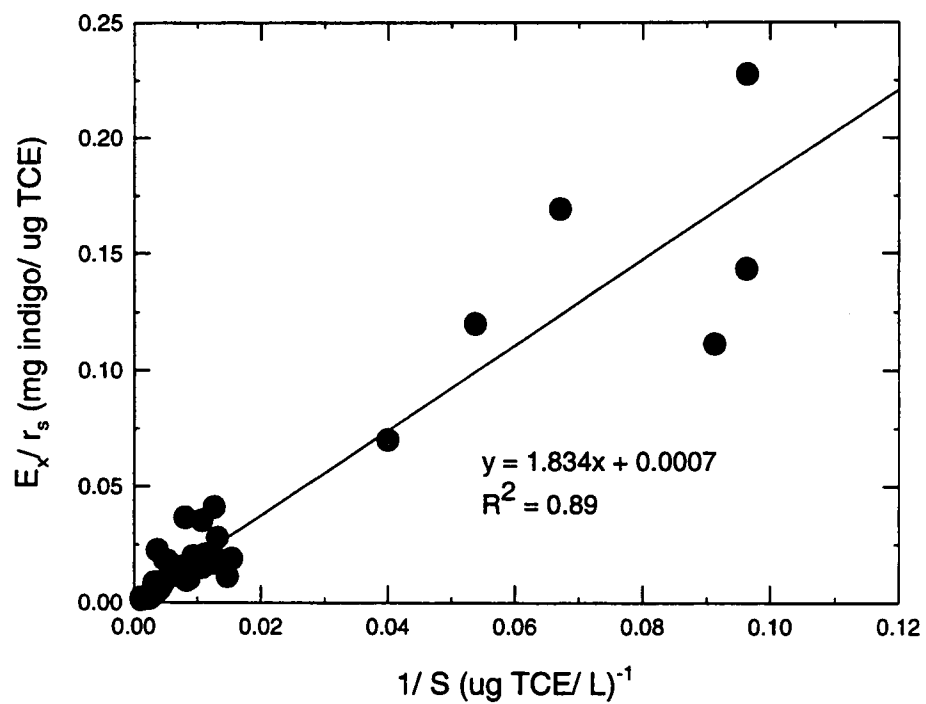


Figure 6.29 Determination of kinetic constants for the Michaelis-Menten equation

$$E_{x,t} = E_{x,0} \exp(-k_1 t) \quad (6.65)$$

The initial normalized enzyme activity of 4.14×10^{-4} mg indigo mg protein⁻¹ min⁻¹ decreased to 1.27×10^{-4} mg indigo (mg protein min)⁻¹ over a period of 12.28 hours. The correlation coefficient was 92 % and a rate constant of -0.0982 hr^{-1} (0.0016 min^{-1}) was found. An enzyme half-life of 7.06 hr was determined from the decay constant. The average decay constant in suspended culture was found to be 0.105 hr^{-1} in section 4.4. The decay constant for immobilized cells was between these values. No significant difference was observed in the natural decay of normalized enzyme activity between the immobilized and suspended cells.

6.9.2.3 The model calibration for determination of k_2

The model was calibrated using the data from experiment 1. The operating condition of biofiltration column in Experiment 1 is presented in Table 6.3. The TCE effluent gas samples were collected at a depth of 16-cm. The normalized enzyme activity was also quantified at the top of the column and at a depth of 16 cm.

The values of the model parameters K_s , k_s' and k_1 were determined above. The rate constant k_2 for the inactivation rate of normalized enzyme activity during TCE degradation was determined by fitting the model to the data. The parameters used for calibration were listed in Table 6.9.

Table. 6.9. Biofiltration model input parameters.

Parameter	Units	values
TCE reaction rate constant, (k_s')	$\mu\text{g TCE (L mg indigo)}^{-1}$	1428.6
natural decay rate of Ex, (k_1)	min^{-1}	0.0016
inactivation constant of Ex, (k_2)	$\text{mg indigo } (\mu\text{g TCE min})^{-1}$	unknown
TCE half-velocity constant, (k_s)	$\mu\text{g TCE L}^{-1}$	2620

The TCE concentration and the normalized enzyme activity data measured from Experiment 1 and predicted results after calibration are shown in Figure 6.30(a) and (b). The open symbols are data from the top portion of column and the star symbols are data from a depth of 16 cm. TCE influent concentration varied over time due to limitations in the TCE feed system; this variation was included as an input to the model.

The enzyme inactivation constant k_2 was evaluated through the model calibration. The value determined was $0.0004 \text{ mg indigo } (\mu\text{g TCE min})^{-1}$. The model describes the TCE effluent concentration at Tod enzyme activity as a function of time reasonably well. Further predictions for seven more experiments were performed using the parameters listed in Table 6.9.

6.9.2.4 Bioreactor simulation for TCE and normalized enzyme activity

Model concentration predictions of effluent TCE and normalized enzyme activity were compared with the experimental data at seven different operating conditions. The parameter values in Table 6.9 were used. The operating conditions for Experiments 1 through 8 are presented in Table 6.3.

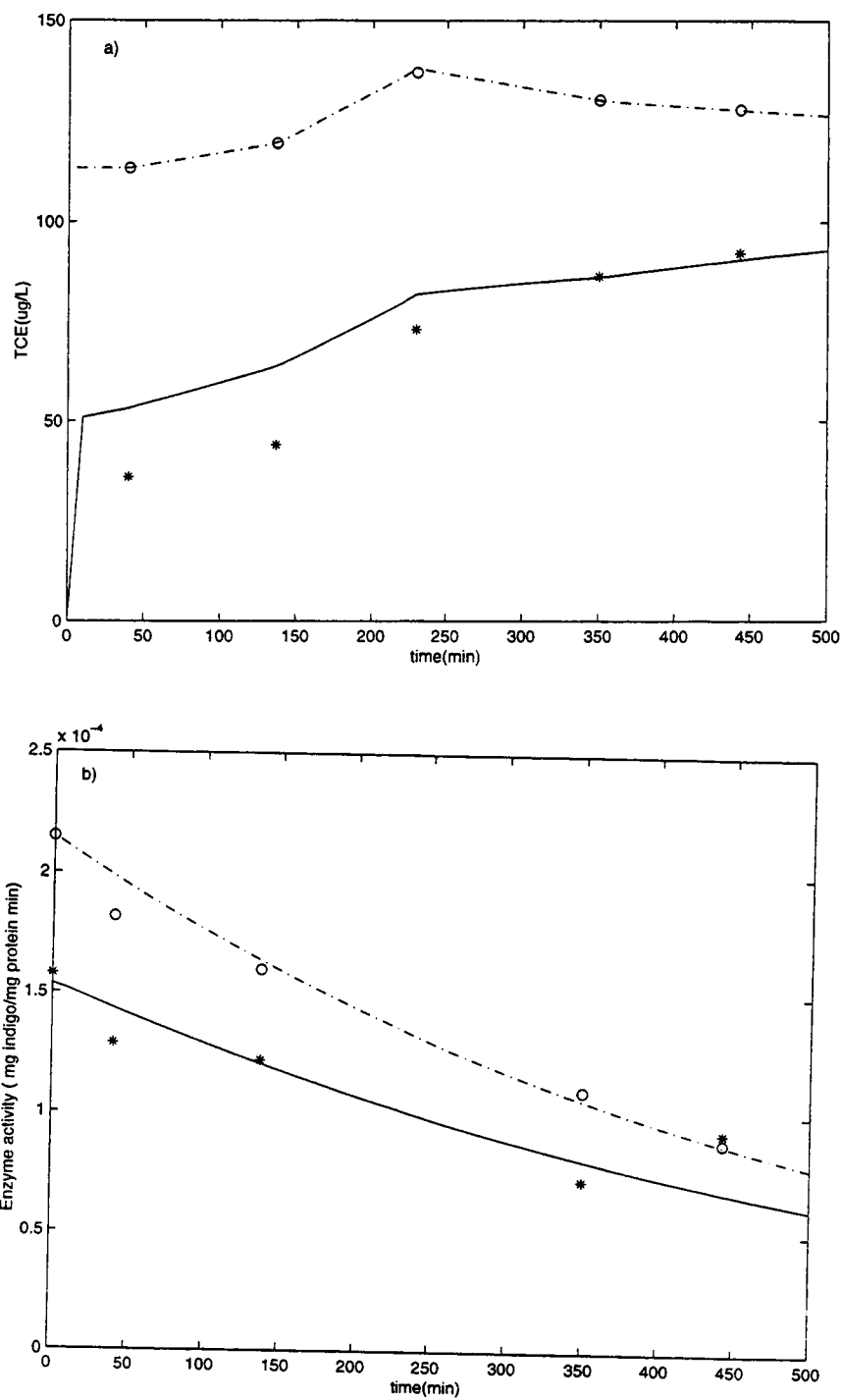


Figure 6.30 Model prediction for Experiment 1: (a) TCE (b) normalized enzyme activity (measured value; o: Top, * : 16-cm, predicted value; --- : Top, — : 16-cm)

The results are shown in Figure 6.31(a) to 6.37(a) for TCE concentration and 6.31(b) to 6.37(b) for the normalized enzyme activity. The open symbols represent the data from the top of the column and the star symbols represent the data from a depth of 16 cm. The summary of model predictions is shown in Table 6.10.

Table 6.10 The summary of model prediction results

Experiment No.	1	2	3	4
TCE	matches well	matches well	overestimated	matches well
normalized enzyme activity	matches well	underestimated	overestimated	matches well

Table 6.10 continued

Experiment No.	5	6	7	8
TCE	matches well	matches well	matches well	matches well
normalized enzyme activity	matches well	matches well	matches well	matches well

Inconsistencies between the experimental data and model predictions for normalized enzyme activity were observed in the data of two of the experiments. The model predictions for Experiment 2 (Figure 6.31(b)) underestimate the measured TCE activity. In Experiment 3 (Figure 6.32(b)), the model overestimates the measured values. The enzyme activity from the top of the column in Experiment 2 (Figure 6.31(b)) did not decrease over time and remained constant during the first 300 minutes.

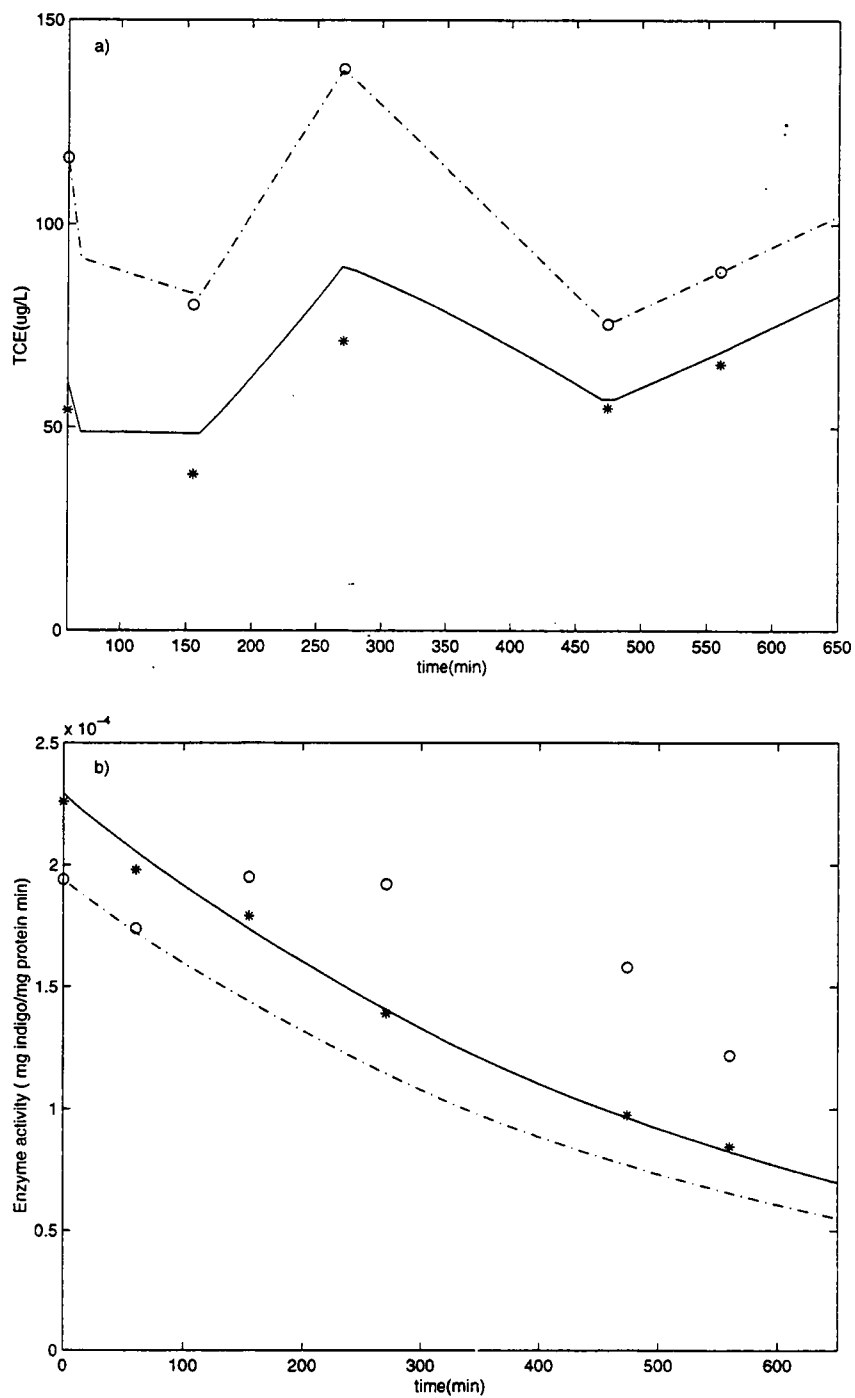


Figure 6.31 Model prediction for Experiment 2: (a) TCE (b) normalized enzyme activity (measured value; o: Top, *: 16-cm, predicted value; --- : Top, — : 16-cm)

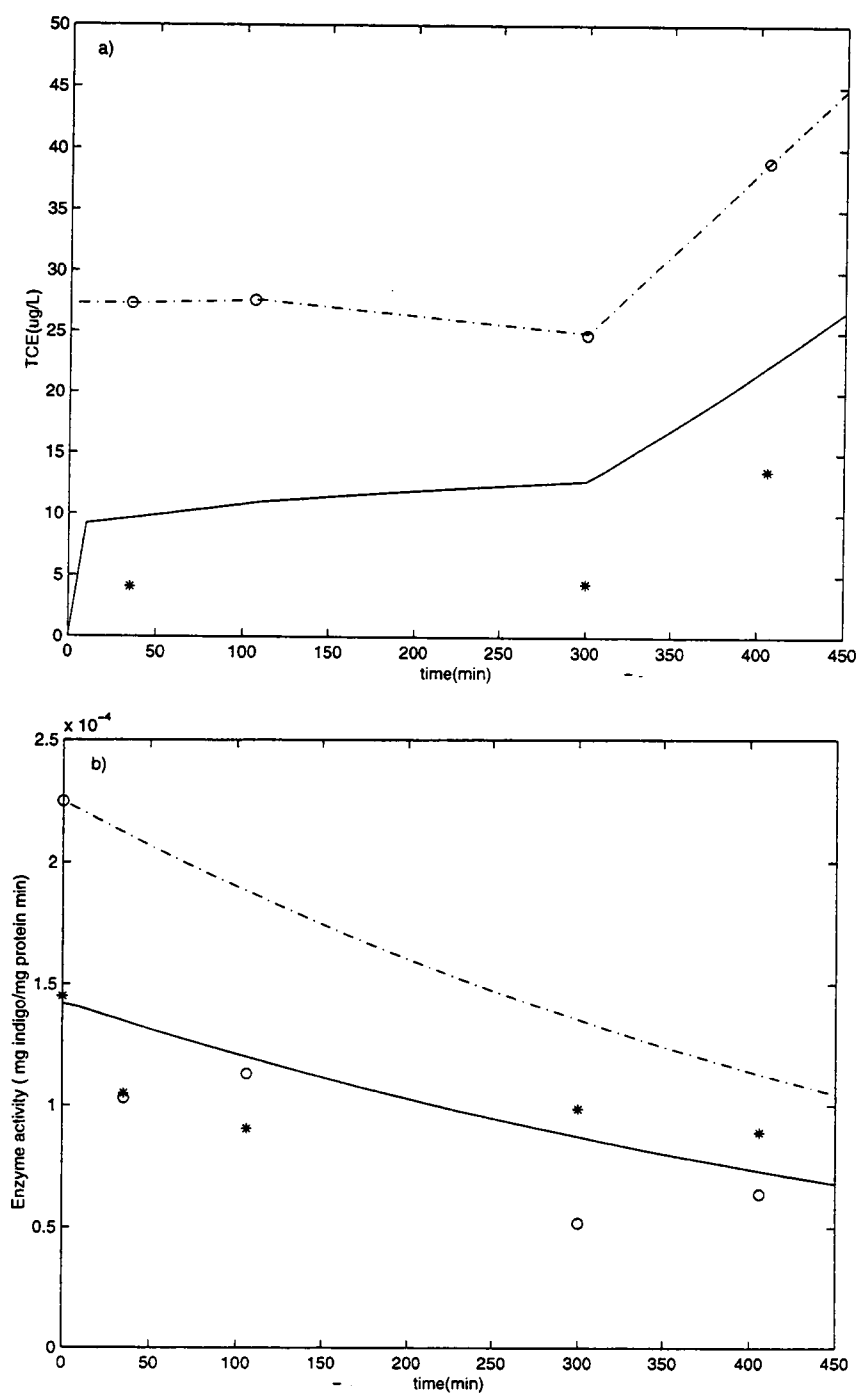


Figure 6.32 Model prediction for Experiment 3: (a) TCE (b) normalized enzyme activity (measured value; o: Top, *: 16-cm, predicted value; --- : Top, — : 16-cm)

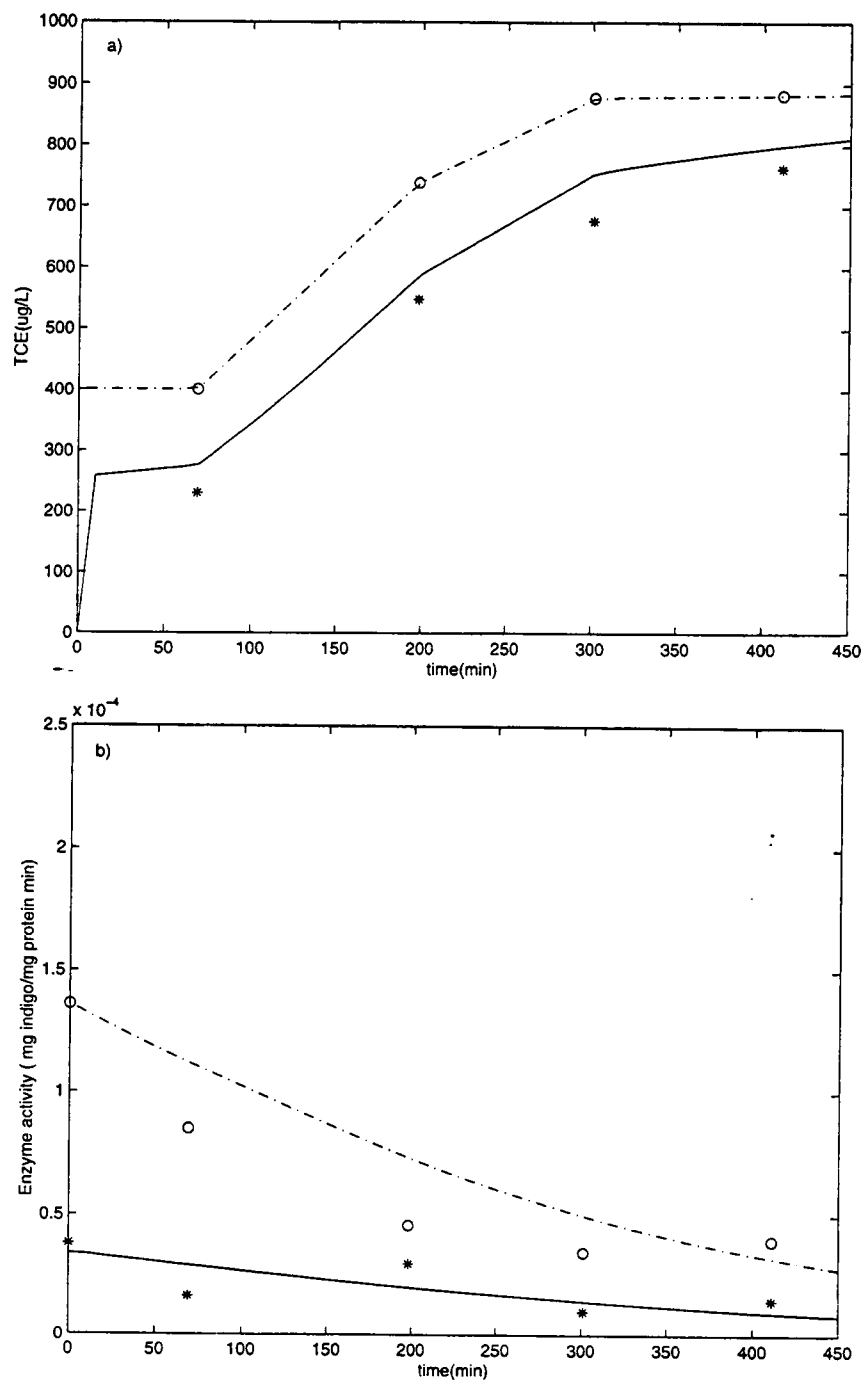


Figure 6.33 Model prediction for Experiment 4: (a) TCE (b) normalized enzyme activity (measured value; o: Top, *: 16-cm, predicted value; ---: Top, —: 16-cm)

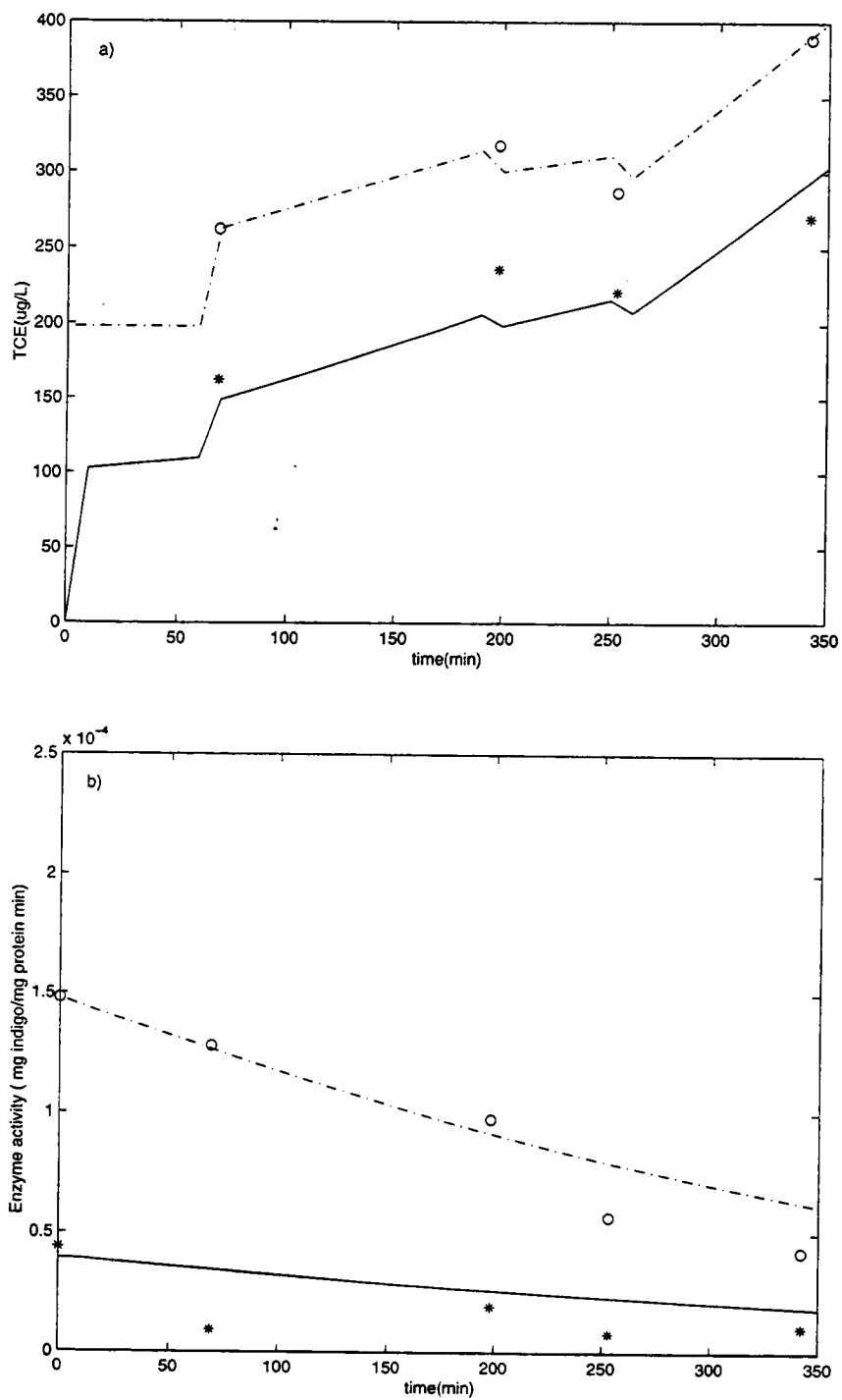


Figure 6.34 Model prediction for Experiment 5: (a) TCE (b) normalized enzyme activity (measured value; o: Top, * : 16-cm, predicted value; --- : Top, — : 16-cm)

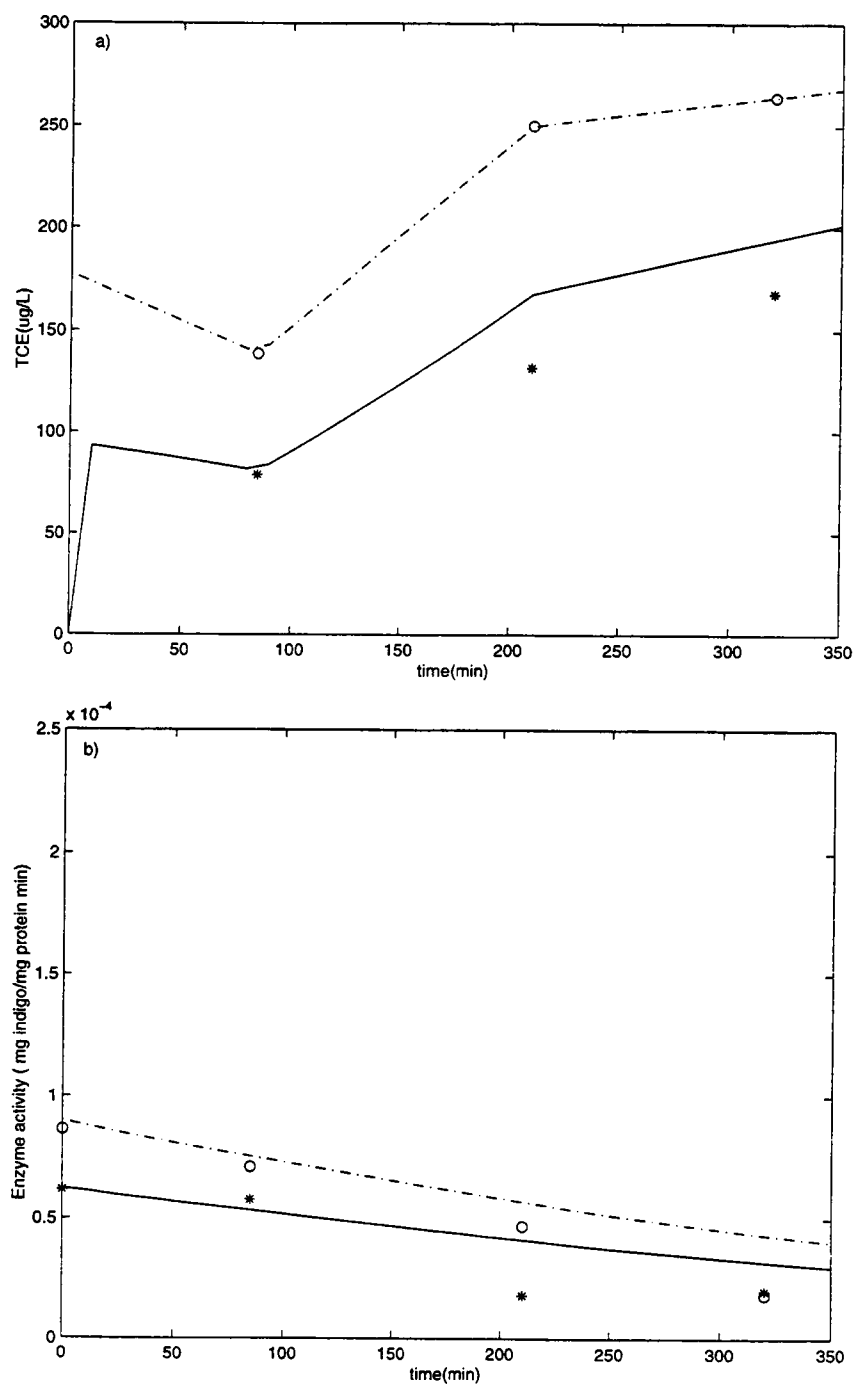


Figure 6.35 Model prediction for Experiment 6: (a) TCE (b) normalized enzyme activity (measured value; o: Top, * : 16-cm, predicted value; --- : Top, — : 16-cm)

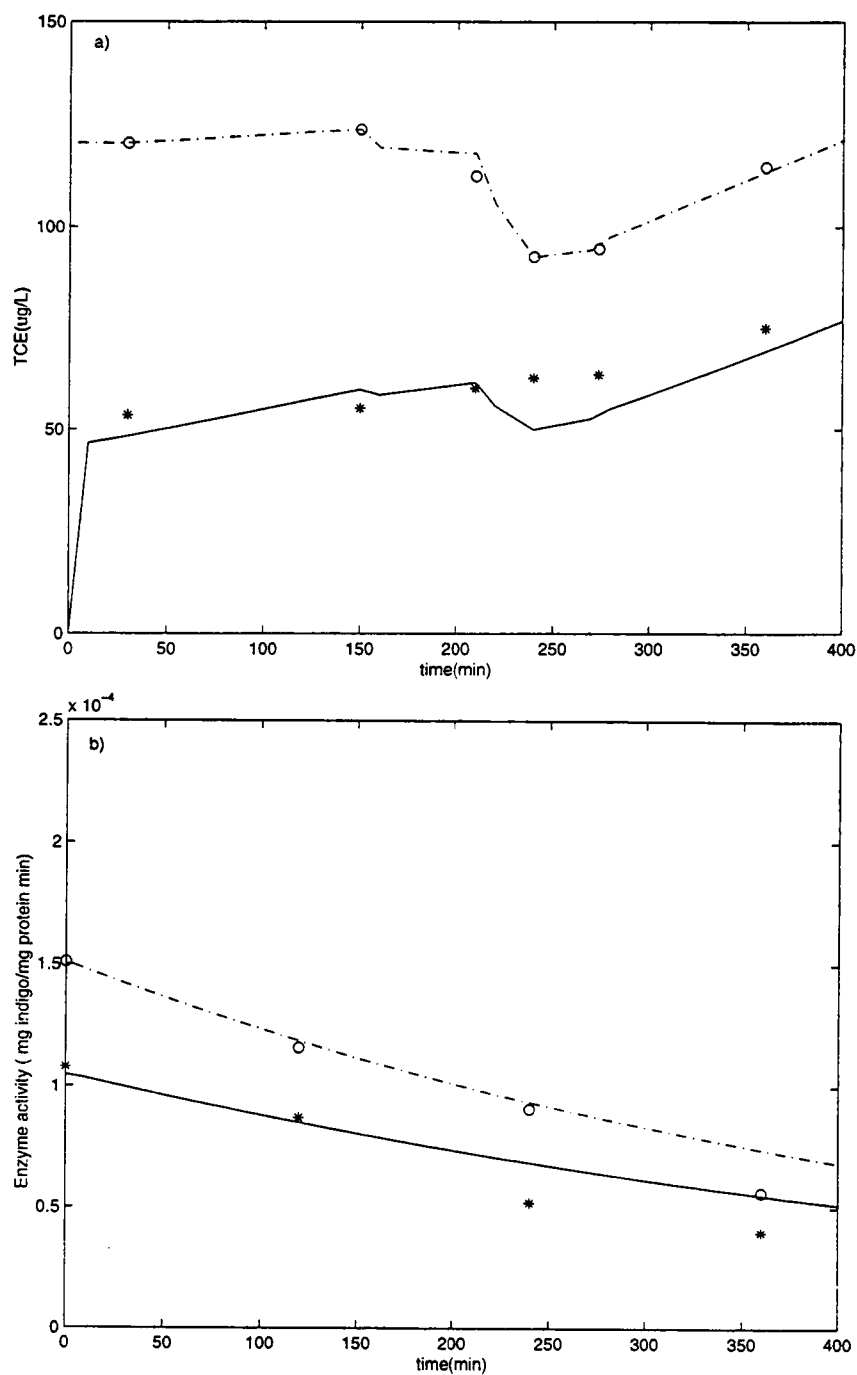


Figure 6.36 Model prediction for Experiment 7: (a) TCE (b) normalized enzyme activity (measured value; o: Top, *: 16-cm, predicted value; --- : Top, — : 16-cm)

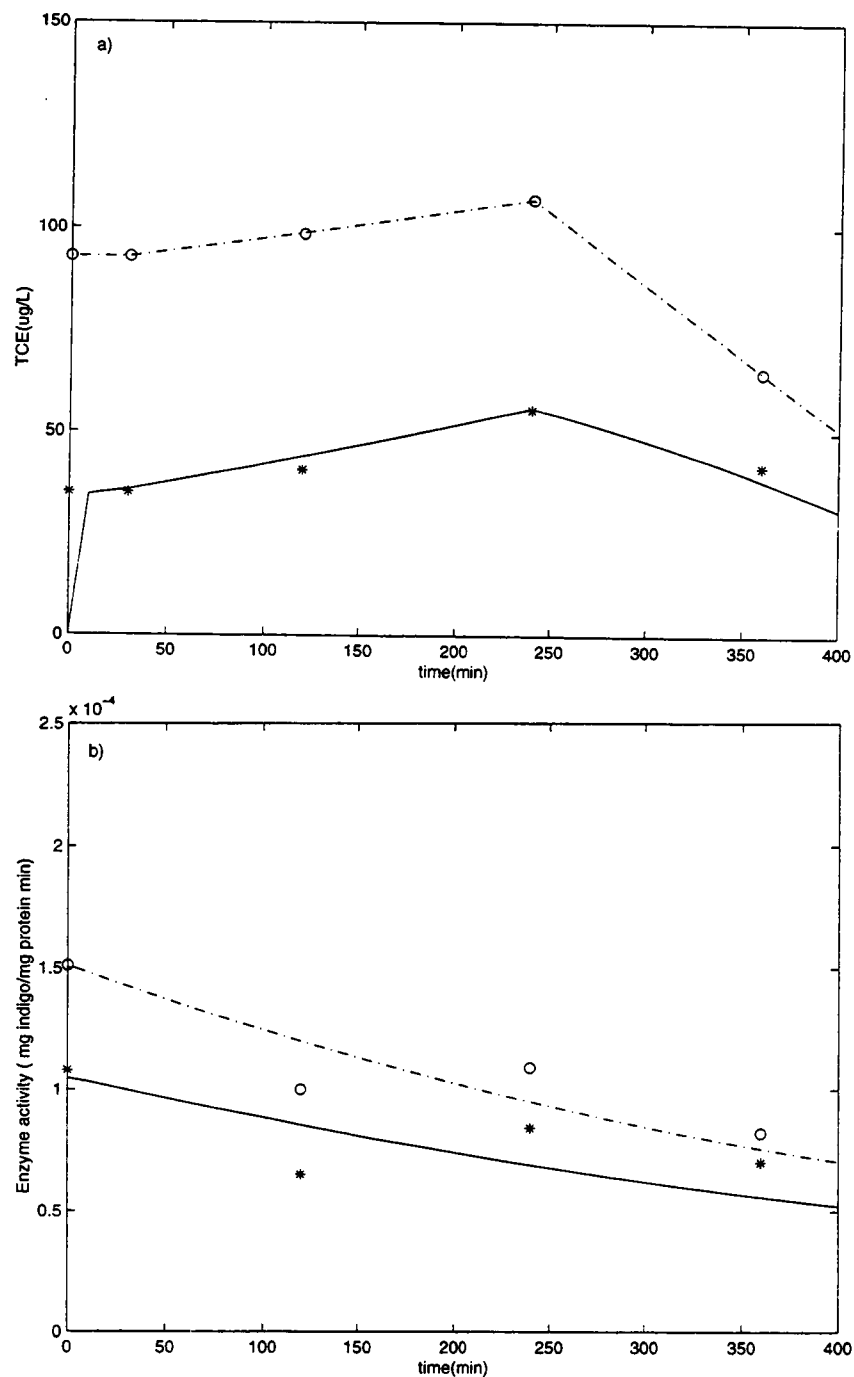


Figure 6.37 Model prediction for Experiment 8: (a) TCE (b) normalized enzyme activity (measured value; o: Top, *: 16-cm, predicted value; --- : Top, — : 16-cm)

In most cases, the model gives a reasonable prediction of TCE degradation in the biofilter. The model predicted effectively the diminishing rate of TCE degradation because of inactivation caused by TCE intermediates. Speitel *et. al.* (1993) assumed that TCE degradation could be described by a pseudo-first order reaction, and used a non-steady state model. The pseudo-first order rate constant for TCE degradation was determined by model calibration. They found that the pseudo-first order rate constant was needed to fit the data, suggesting that product toxicity was occurring. The model in this work represents a substantial advance in that the inactivation of Tod activity could be predicted in a parsimonious model.

The modeling effort neglected enzyme competition for the sake of mathematical simplicity. Therefore, the TCE concentration and enzyme activity including enzyme competition, enzyme inactivation, and enzyme production in presence of toluene were not predicted in this research. However, if strategies of pulsed feeding of TCE and growth substrate or multiple reactors that are alternately fed either toluene and TCE are used to avoid competitive inhibition for enhancing TCE degradation rate (Segar *et. al.*, 1995, Speitel, *et. al.*, 1993), this model might be useful to understand TCE degradation and enzyme activity in biofiltration reactor.

6.9.2.5 Model sensitivity test

Sensitivity analysis to the TCE degradation rate constant (k_s') and TCE half-velocity constant (K_s) were determined. The model was run for k_s' values in 500, 1000, 1500, 2000, and 3000 $\mu\text{g TCE (L mg indigo)}^{-1}$ at a constant K_s of 2620 $\mu\text{g TCE L}^{-1}$ and constant TCE feed concentration of 100 $\mu\text{g TCE L}^{-1}$. The predicted TCE concentration

and normalized enzyme activity are presented in Figure 6.38(a) and (b), respectively. TCE degradation increases with k_s' . The rate of increase in TCE effluent with time also increased with k_s' . Model sensitivity to k_s' decreased slightly as the parameter increased. The rate of normalized enzyme activity inactivation increased with k_s' because of the greater mass of TCE degraded. Enzyme activity was not very sensitive to changes in k_s' .

The model was run for K_s values of 500, 1000, 2000, 2500, and 3500 $\mu\text{g TCE L}^{-1}$ at a constant k_s' value of 1428.6 $\mu\text{g TCE (L mg indigo)}^{-1}$ and TCE feed concentration of 100 $\mu\text{g TCE L}^{-1}$. The predicted TCE concentration and normalized enzyme activity are presented in Figure 6.39(a) and (b), respectively. TCE degradation increased with decreasing K_s . The rate of increase in TCE effluent concentration with time increased with decreasing K_s . The model became less sensitive to K_s as the value of the parameter increased. The rate of enzyme inactivation increased with decreasing K_s because of the greater mass of TCE degraded.

The model was run for k_2 values of 0.0002, 0.0004, 0.0008, 0.0012, and 0.0016 $\mu\text{g TCE mg indigo}^{-1}$ at constant k_s' value of 1428.6 $\mu\text{g TCE (L mg indigo)}^{-1}$, K_s of 2620 $\mu\text{g TCE L}^{-1}$, and TCE feed concentration of 100 $\mu\text{g TCE L}^{-1}$. The results of sensitivity test for k_2 in TCE concentration and normalized enzyme activity were presented in Figure 6.40(a) and (b), respectively. TCE degradation was not sensitive to k_2 , but enzyme activity was relatively sensitive to this parameter.

The model was run for different TCE feed concentrations in 100, 250, 500, 750, and 1000 $\mu\text{g TCE L}^{-1}$ at constant k_s' value of 1428.6 $\mu\text{g TCE mg indigo}^{-1}\text{min}^{-1}$, K_s of 2620 $\mu\text{g TCE L}^{-1}$, and k_2 of 0.004 $\mu\text{g TCE mg indigo}^{-1}$. The results of sensitivity

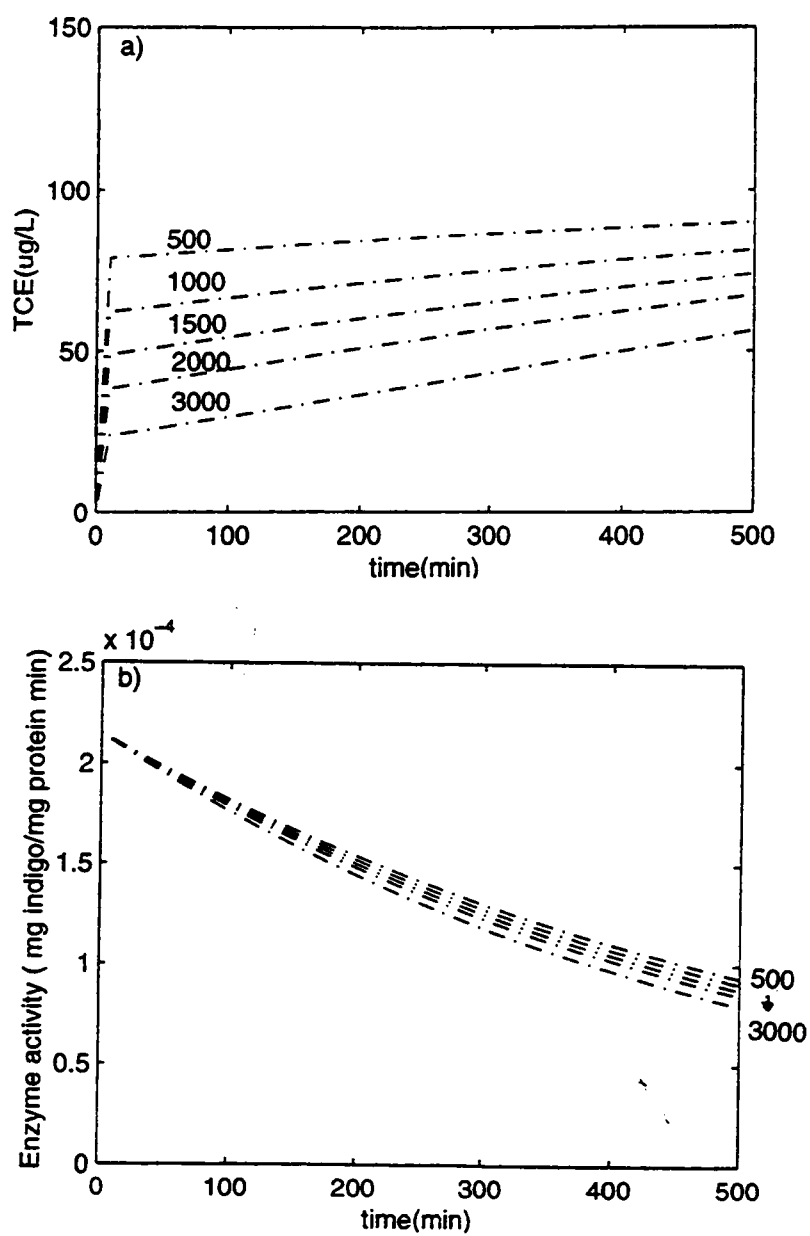


Figure 6.38 Model sensitivity test for k_s' : (a) TCE (b) normalized enzyme activity ($K_s = 2620 \mu\text{g/L}$, TCE = $100 \mu\text{g/L}$, $k_s' = \text{various}$, $k_2 = 0.0004 \text{ mg indigo } (\mu\text{g TCE min})^{-1}$)

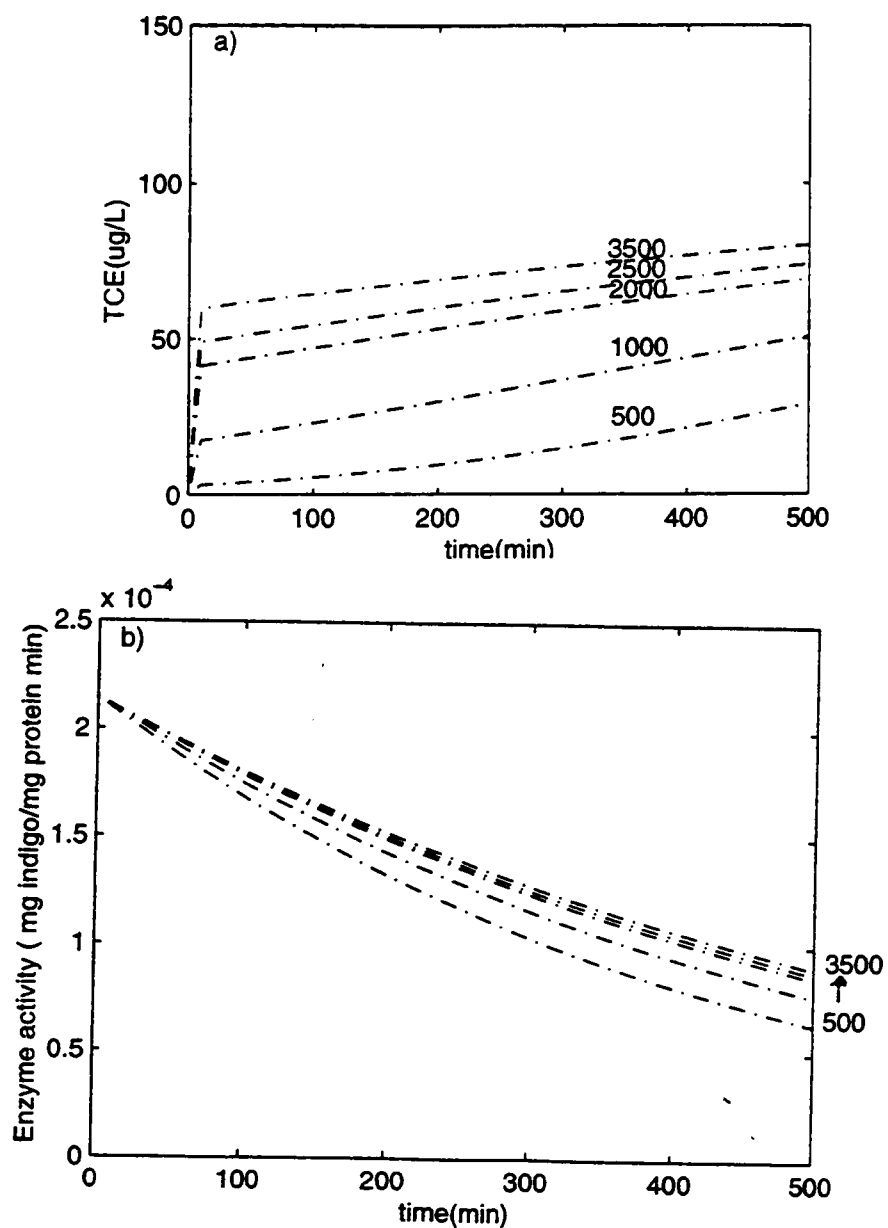


Figure 6.39 Model sensitivity test for K_s : (a) TCE (b) normalized enzyme activity ($K_s = \text{various}$, TCE = $100 \mu\text{g/L}$, $k_s' = 1428.6 \mu\text{g TCE (L mg indigo)}^{-1}$, $k_2 = 0.0004 \text{ mg indigo } (\mu\text{g TCE min})^{-1}$)

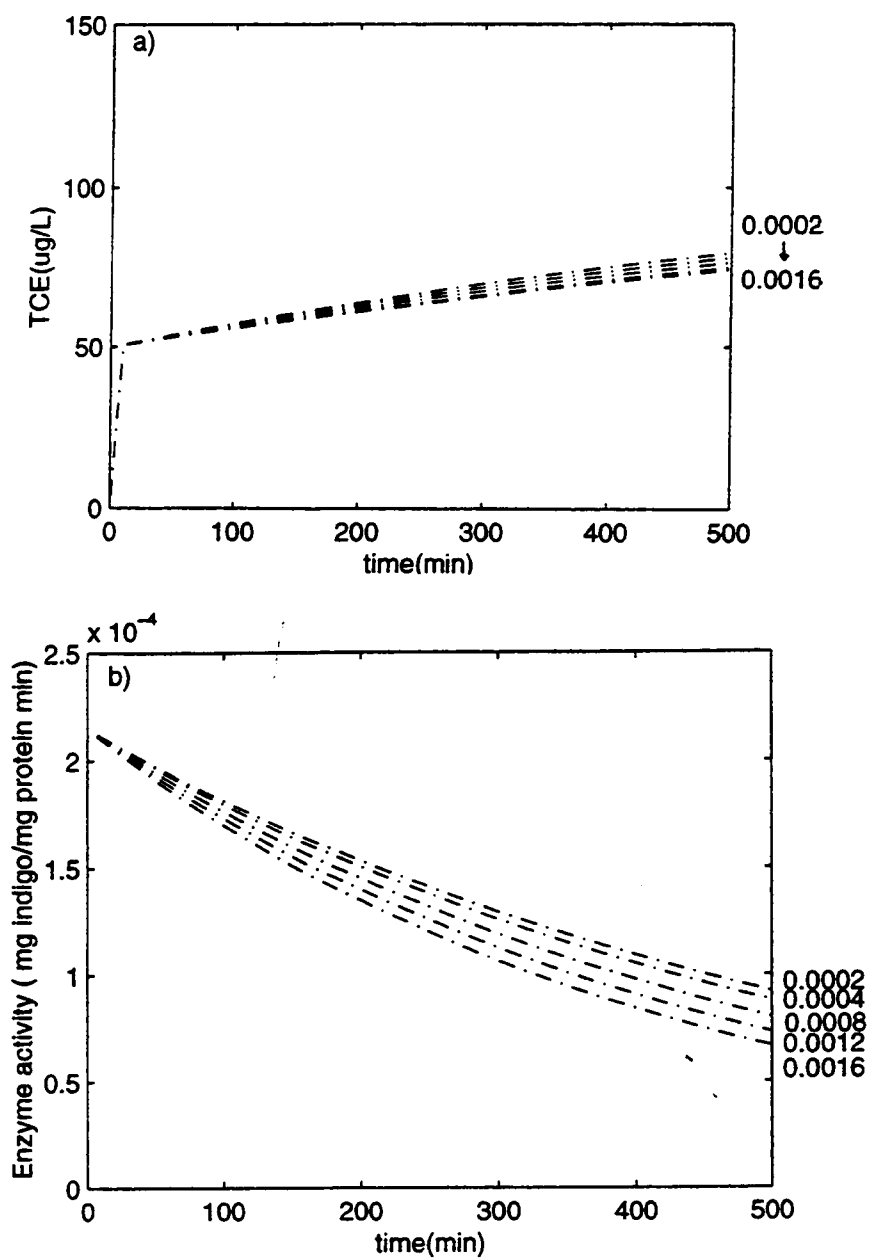


Figure 6.40 Model sensitivity test for k_2 : (a) TCE (b) normalized enzyme activity
 $(K_s = 100 \mu\text{g/L}, \text{TCE} = 100 \mu\text{g/L}, k_s' = 1428.6 \mu\text{g TCE (L mg indigo)}^{-1},$
 $k_2 = \text{various})$

test for TCE feed concentration in TCE concentration and normalized enzyme activity were presented in Figure 6.41(a) and (b), respectively. The slope of effluent concentration increment in the course of time was much higher at the higher TCE feed concentration. The normalized enzyme activity decreased rapidly for higher TCE feed concentrations.

6.9.2.6 Comparison of immobilized and suspended cells

The growth and substrate utilization kinetics parameters for suspended and immobilized cells are summarized in Table 6.11.

Table 6.11 The kinetics parameters of suspended and immobilized cells

Parameters	Suspended cells	Immobilized cells
K_{sT} (toluene saturation constant), $\mu\text{g L}^{-1}$	3000	1988
K_T (toluene maximum specific substrate utilization constant), $\mu\text{g (min mg biomass)}^{-1}$	2.647	1.250
K_s (TCE saturation constant), $\mu\text{g L}^{-1}$		2620
k_s' (TCE reaction rate constant), $\mu\text{g TCE (L mg indigo)}^{-1}$		1428.6
k_1 (natural decay rate of E_x), min^{-1}	0.1048	0.0982
k_2 (TCE inactivation constant of E_x), $\text{mg indigo } (\mu\text{g TCE min})^{-1}$		0.0004
$\mu_{\max}$ (maximum growth rate constant), hr^{-1}	0.34	0.029

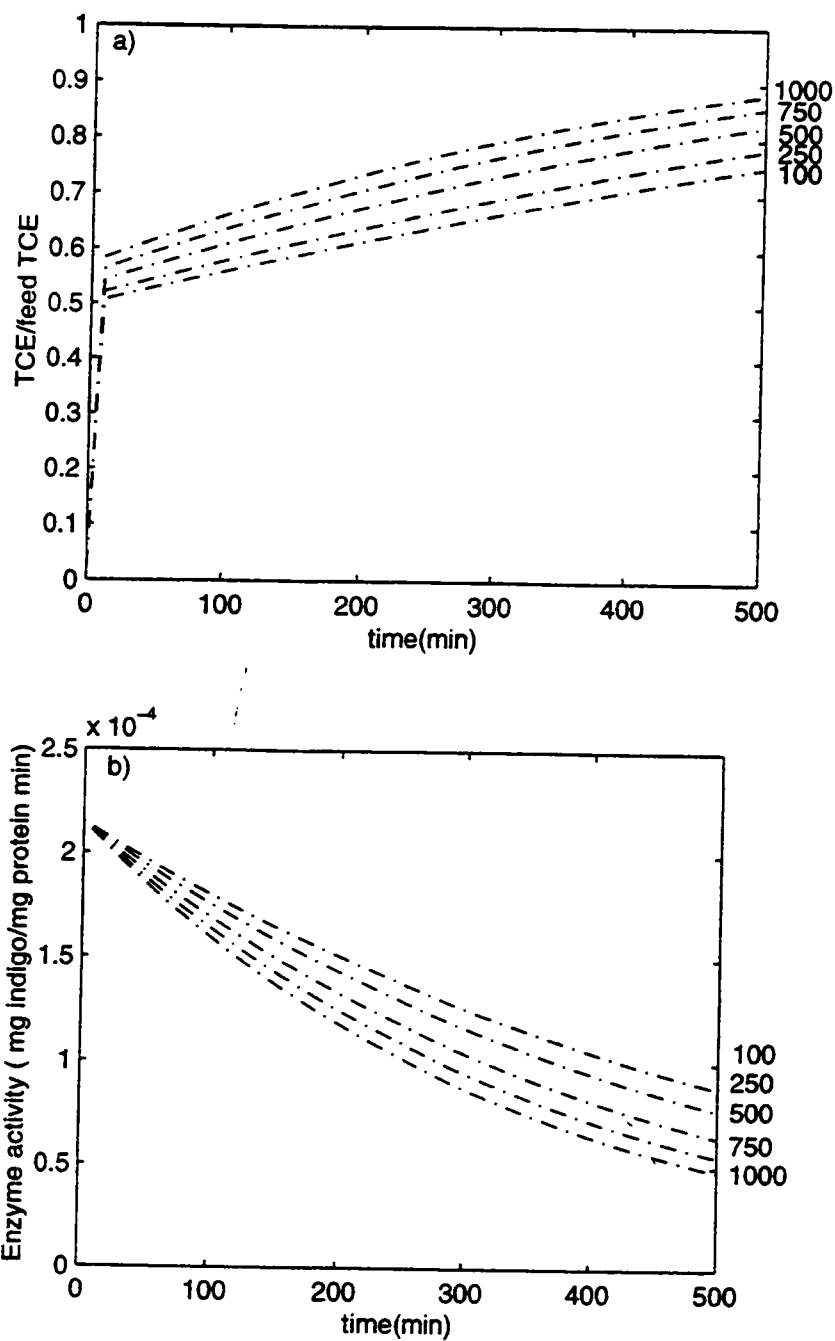


Figure 6.41 Model sensitivity test for TCE: (a) TCE (b) normalized enzyme activity
 $(K_s = 100 \mu\text{g/L}, \text{TCE} = \text{various}, k_s' = 1428.6 \mu\text{g TCE (L mg indigo)}^{-1},$
 $k_2 = 0.0004 \text{ mg indigo } (\mu\text{g TCE min})^{-1})$

Shreve et al. (1993) compared substrate utilization and growth kinetics parameters for suspended and immobilized cells of *Pseudomonas* strain K3-2 while growing on toluene. They demonstrated that the growth kinetics were much faster for suspended cells, while substrate utilization kinetics remained relatively unchanged. Similar conclusions are supported by a comparison of the parameters in Table 6.11. Values of K_{sT} , K_T , and k_1 are similar for suspended and immobilized cells; however, the value of μ_{max} for suspended cells is more than ten times greater than for immobilized cells.

6. 10 Application

Biofiltration of TCE vapors would have many applications in industrial facilities and in the vapor extraction fields used for subsurface remediation. However, TCE is resistant to biodegradation and does not induce the enzymes required for its degradation. Biofilter performance is related to several factors including, degradation capacity of the filter material, contaminant loading rate, and detention time. Design parameters such as moisture content and microbial activity can be optimized to increase biofilter efficiency (Hodge, *et al.*, 1992). Several groups of microorganisms have been shown to be capable of cometabolism of TCE in laboratory studies, including: *Methylosinus trichosporium* OB3b, *Pseudomonas cepacia* G4, *Pseudomonas mendocina* KR-1, *Pseudomonas putida* F1, *Nitrosomonas europaea*. These microorganisms produce various oxygenase enzyme systems and are candidates for TCE cometabolism. The microbial activity can be described by the enzyme activity if a proper assay for the specific enzyme activity is available for immobilized cells. The growth and metabolic activity of the microorganisms in a biofilter depend on the dissolved oxygen in the biofilm, the availability of nutrients,

moisture level, temperature and pH. The control of these factors is important to obtain high levels of TCE removal in a biofilter.

In this research, bench-scale studies of a biofilter using the toluene dioxygenase system in *Pseudomonas putida* F1 were conducted for the treatment of TCE vapor. Tod activity, influent and effluent TCE concentrations, biomass concentration, and NADH concentration were monitored during biofilter operation. Results in this study indicated that Tod deactivation is related to the cumulative mass of TCE degraded. Tod deactivation in turn decreases the removal rate of TCE. A biofiltration model based on this concept was developed to predict the TCE effluent concentration as a function of various operating conditions such as the TCE influent concentration and influent gas flow rate.

The filter material must provide an optimum environmental condition for the microorganisms and its particle size distribution should yield low pressure drops through the column (Leson, *et al.*, 1991). The control of pH in biofilter is essential because pH can affect its microbial activity. Most microorganisms prefer a pH range of between 7 and 8. Biodegradation occurs in liquid phase, so pH control is easily made by addition a buffer to the nutrient solution. The pressure drop across the filter bed needs to be monitored because compaction of filter material increases gradually and eventual back washing is required.

The design and operation of a biofilter should maximize TCE degradation. A sequencing biofilm reactor (Speitel, *et al.*, 1992) is available to solve problems of competitive inhibition to achieve high TCE degradation rates. A sequencing reactor is operated with separate degradation and rejuvenation stages. Growth substrate is supplied

to stimulate enzyme activity during the rejuvenation stage, while only TCE is fed during the degradation stage. The feeding strategy, that is, time of rejuvenation per time of degradation, would be decided from bench-scale experiments. Alternatively, an optimum feeding strategy can be determined using a calibrated biofiltration model such as the one developed in this research.

Another possible solution is a multiple inlet biofilter. In this case, growth substrate is fed into a biofilter at different stages to sustain high levels of enzyme activity and TCE degradation. At an each stage, TCE and growth substrate feed rate must be balance to avoid competitive inhibition. This research indicated that significant inhibition occurred at toluene concentrations greater than 2.5 mg L^{-1} and that a small amount toluene can stimulate Tod activity.

6.10.1 Sample calculation for biofiltration design

A pilot-scale biofilter can be designed to control TCE from contaminated gas streams. In the example to be evaluated, TCE-contaminated gas is emitted from a soil vapor extraction well operating at TCE contaminated site. A biofilter system is to be designed to treat soil vapor. A sequencing biofilm reactor is chosen. It is operated with two separate stages: degradation and rejuvenation stages. Initially, the biofilter is inoculated and grown with *Pseudomonas putida* F1 using toluene as a growth substrate. After the biofilm is well formed through columns, TCE-contaminated gas from a soil extraction well is introduced to the biofilter for TCE degradation; TCE is degraded without competition inhibition. The Tod activity and TCE removal rate decrease as TCE

is degraded. When TCE effluent concentration exceeds the limit, the biofilter is supplied with toluene to rejuvenate the enzyme activity in the biofilter.

Design criteria for the biofilter are:

$$\begin{aligned}\text{Average biomass per filter bed } (X_b) &= 2690 \text{ mg protein } (1256 \text{ cm}^3 \text{ bed volume})^{-1} \\ &= 2.14 \text{ mg protein } (\text{cm}^3 \text{ bed volume})^{-1}\end{aligned}$$

$$\text{Diameter of biofilter } (D) = 30 \text{ cm}$$

$$\text{Biofilter bed area } (A) = 3.14 \times D^2 / 4 = 3.14 \times 30^2 / 4 = 706.5 \text{ cm}^2$$

$$\text{Biofilter height } (H) = \text{variable}$$

$$\text{Average biomass concentration } (X)$$

$$= 2.14 \text{ mg protein } (\text{cm}^3 \text{ bed volume})^{-1} \times 706.5 \text{ cm} \times H$$

$$\text{Flow rate } (Q) = 4.78 \text{ ft}^3 \text{ minute}^{-1} = 135.4 \text{ L minute}^{-1}, (\text{Zurlinden } et al., 1994)$$

$$\text{Gas velocity } (V_x) = Q/A = 135.4 \times 10^3 / 706.5 \text{ cm}^2 = 191.66 \text{ cm } (\text{min})^{-1}$$

TCE vapor pressure in soil = 5×10^{-5} atm, the range of concentration for TCE vapor in soil at one site was 2×10^{-5} to 14×10^{-5} atm (Wickramanayake *et al.*, 1998)

$$\text{TCE influent concentration } (S_i) = 5 \times 10^{-5} \times 131.5 \times 10^3 / (24.466 \times 10^6) = 268 \text{ } \mu\text{g L}^{-1}$$

$$\text{Desired TCE concentration} = 5 \text{ } \mu\text{g TCE L}^{-1}$$

$$\text{TCE reaction rate constant, } (k_s') = 1428.6 \text{ } \mu\text{g TCE } (\text{L mg indigo})^{-1}$$

$$\text{Natural decay rate of } E_x (k_1) = 0.0016 \text{ min}^{-1}$$

$$\text{Inactivation constant of } E_x, (k_2) = 0.0004 \text{ mg indigo } (\mu\text{g TCE min}^{-1})$$

$$\text{TCE half-velocity constant, } (k_s) = 2620 \text{ } \mu\text{g TCE L}^{-1}$$

$$\text{Toluene degradation rate} = 31.07 \text{ } \mu\text{g toluene } (\text{mg protein min})^{-1} \text{ (Table 6.1)}$$

$$\text{Time for rejuvenation stage} = 20 \text{ minute (Figure 6.20)}$$

The model was run for filter heights of 60, 80, 100, 120, and 130 cm using the above design parameters. The change in TCE effluent concentration and normalized enzyme activity during TCE degradation is shown in Figure 6.42. The filter bed volume and total biomass of protein decrease with filter bed height. The enzyme activity at the influent decreases dramatically with time and is almost depleted after 20 minutes. When TCE effluent concentration reaches the desired value ($5 \mu\text{g L}^{-1}$), toluene is supplied to the filter for recovery of the enzyme activity. When the height is less than 100 cm, TCE concentration exceeds $5 \mu\text{g L}^{-1}$ in less than 5 minutes. A bed height of 130 cm is selected and TCE degradation continues for 7.5 minutes before the effluent TCE concentration exceeds $5 \mu\text{g L}^{-1}$.

Toluene mass degraded during rejuvenation stage

$$\begin{aligned}
 &= 31.07 \mu\text{g toluene (mg protein min)}^{-1} \times 2.14 \text{ mg protein (cm}^3\text{)}^{-1} \\
 &\quad \times 706.5 \text{ cm}^2 \times 130 \text{ cm} \times 20 \text{ minutes} \\
 &= 1.22 \times 10^8 \mu\text{g toluene}
 \end{aligned}$$

If toluene is desired to be degraded completely

Toluene influent concentration (C_0)

$$= 1.22 \times 10^8 \mu\text{g toluene} / ((135.4 \text{ L minute}^{-1}) \times (20 \text{ minute})) = 4.51 \times 10^4 \mu\text{g toluene L}^{-1}$$

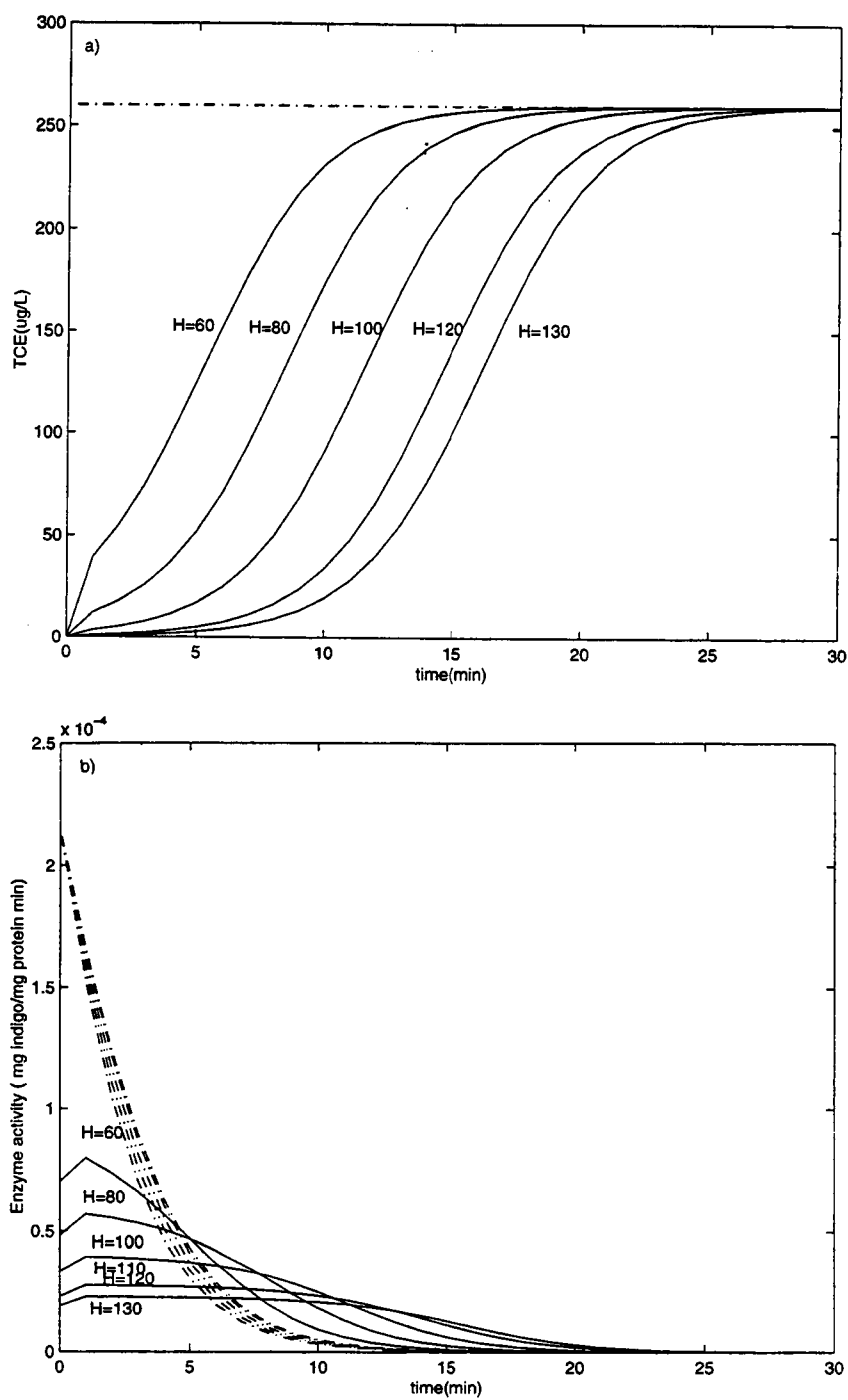


Figure 6.42 Model prediction of TCE effluent concentration and enzyme activity history as a function of bed depth (a) TCE (b) normalized enzyme activity, : - - - - - Top, — :bottom)

Toluene concentration of $4.51 \times 10^4 \mu\text{g L}^{-1}$ is fed to filter for 20 minute. Toluene is completely degraded and the enzyme activity is restored for the next biodegradation stage. The summary of the design and operation of the biofilter is given below:

Diameter of biofilter (D) = 30 cm

Biofilter filter height (H) = 130 cm

Flow rate (Q) = $4.78 \text{ ft}^3 \text{ min}^{-1}$

TCE influent concentration (S_o) = $268 \mu\text{g TCE L}^{-1}$

Desired TCE concentration (S_e) = $5 \mu\text{g TCE L}^{-1}$

Toluene influent concentration (C_o) = $4.51 \times 10^4 \mu\text{g L}^{-1}$

Time for rejuvenation stage = 20 minutes

Total of three columns - at any given time two are being rejuvenated while once is degrading TCE.

TCE influent and effluent concentration, biomass concentration, and enzyme activity govern biofiltration performance. In this calculation, the filter diameter and gas flow rate are fixed and the remaining design parameters are determined from the model.

CHAPTER 7

RESULTS AND CONCLUSIONS

7.1 Results

The fundamental concept of aerobic trichloroethylene (TCE) cometabolism was extended to biofiltration. Biofiltration experiments with the toluene-degrading organism, *Pseudomonas putida* F1, were conducted. TCE removal by biofiltration was influenced by competitive inhibition, product toxicity, and enzyme activity (toluene dioxygenase). An attempt was made to develop a fluorescence-based assay to monitor Tod (toluene dioxygenase enzyme) activity in whole cells of *Pseudomonas putida* F1 immobilized on support media during biofiltration. Aqueous-phase batch experiments were conducted to understand competitive inhibition and the effect of toluene and TCE on toluene dioxygenase enzyme activity. Enzyme activity was incorporated in the biokinetic model of TCE cometabolic oxidation and applied to a biofilter.

7.1.1 Batch experiments

The following conclusions were determined from the batch experiments with suspended cultures of *Pseudomonas putida* F1:

1. The Tod activity in whole cells of *P. putida* F1, as measured by fluorescence, was correlated with indigo production ($R^2 = 0.90$), as measured spectrophotometrically, with a correlation factor of $2277.6 \text{ mg indigo}^{-1}$.
2. In batch suspended-cell culture the Tod activity was measured to be $3.84 \text{ to } 7.73 \times 10^{-4} \text{ mg indigo (mg protein min)}^{-1}$ in the growth phase ($\text{OD}_{600} < 0.9$) and $9.00 \text{ to } 19.8 \times 10^{-4} \text{ indigo (mg protein min)}^{-1}$ in the stationary phase ($\text{OD}_{600} > 0.9$).
3. Normalized NADH remained constant during the exponential growth phase, ($\text{OD}_{600} < 0.9$) at a value of $0.102 \pm 0.005 \text{ } \mu\text{mol (mg protein)}^{-1}$ and increased continuously to $0.29 \pm 0.02 \text{ } \mu\text{mol mg protein}^{-1}$ after the early-stationary-growth phase ($\text{OD}_{600} > 0.9$).
4. In batch experiments the Tod enzyme activity remained relatively constant in the presence of toluene but decayed according to first-order kinetics in the absence of toluene. The enzyme half-life determined from the first-order decay constants (-0.1228 hr^{-1} , -0.0869 hr^{-1}) were 5.64 and 7.97 hr, respectively.
5. The growth constant and doubling time of *P. putida* F1 were 0.34 hr^{-1} and 2.038 hr, respectively. The decay constant of the cells was 0.058 hr^{-1} during the first 7.6 hr of endogenous respiration. TCE concentrations of up to 50 mg L^{-1} did not effect the growth of the cells on succinate, demonstrating that TCE had no direct toxic effect.
6. For the suspended cells, the maximum specific toluene utilization rate and half-saturation constant were $2.674 \text{ } \mu\text{g (min mg biomass)}^{-1}$ and 3 mg L^{-1} , respectively. The degradation rate of toluene by suspended cells was not significantly affected by the presence of TCE.

7. At toluene concentrations greater than 2.43 mg L^{-1} , significant inhibition of TCE degradation was observed during the first 20 to 35 minutes of the experiment; however, once the toluene decreased below 4.85 mg L^{-1} , TCE degradation was rapid.
8. The rate of enzyme inactivation appears to be correlated with the rate of TCE degradation, suggesting that enzyme inactivation is related to intermediates produced during TCE degradation.
9. Cells grown on toluene but starved for 7 days showed a significant increase in Tod activity within 10 minutes of reexposure to toluene; maximum activity was observed at toluene concentrations above 4 mg L^{-1} . No significant increase in Tod activity was observed for succinate-grown cells under similar conditions. Tod activity was not induced by succinate.
10. The Tod activity of *Pseudomonas putida* F1 increased after a 1 hr lag period when cells were incubated in the presence of both succinate and TCE; however, the activity was a small fraction of that observed for toluene-induced cells.
11. Addition of acetate (80mM, 20mM) and commercial NADH (20mM) did not enhance the TCE degradation and enzyme activity, suggesting that NADH was not limiting.

7.1.2 Column experiments

The following conclusion were determined from the biofiltration experiments:

1. In the absence of toluene, the natural decay of Tod enzyme in immobilized cells was found to be first order with a rate constant and half life of -0.0982 hr and 7.06 hr , respectively. No significant difference was observed in the natural decay of

normalized Tod enzyme activity between the immobilized and suspended cells. The immobilized-cell half-life of Tod enzyme. Tod in the presence of TCE was only 1.59 hr demonstrating the inactivation of Tod during TCE metabolism.

2. Toluene could be reduced from 150 ppmv to below the detection limit in a biofilter operating of a loading of $1.7 \text{ kg COD (m}^3 \text{ day)}^{-1}$ and an EBCT of 1.5 minutes.
3. The maximum specific growth rate (μ_{max}) of immobilized cells was estimated to be 0.029 hr^{-1} . Growth is dependent on the toluene concentration up to $4000 \mu\text{g L}^{-1}$. Growth did not increase significantly above $5000 \mu\text{g/L}$ because the maximum specific growth rate was achieved.
4. For immobilized cells, the maximum specific toluene utilization rate (k_T) and saturation constant (K_{sT}) were found to be $1.25 \mu\text{g toluene (mg biomass min)}^{-1}$ and $1988 \mu\text{g toluene L}^{-1}$, respectively.
5. The normalized NADH concentration of immobilized cells was independent of toluene concentration in the range of 500 to $6000 \mu\text{g L}^{-1}$, and had a mean value of $0.48 \pm 0.076 \mu\text{mole (mg protein)}^{-1}$.
6. The specific TCE utilization rate decreased with increasing toluene concentration, which can be explained by competitive inhibition. TCE oxidation was almost completely inhibited at a toluene concentration of $15000 \mu\text{g L}^{-1}$.
7. When TCE alone was fed to the biofilter, the TCE degradation rate and Tod enzyme activity decreased during the course of each experiment.
8. Tod activity depleted during TCE degradation could be restored to its initial level by feeding toluene at concentrations of 12300 to $21600 \mu\text{g L}^{-1}$. This activity was also

the same as the steady-state value observed when the toluene concentration was varied over the range of 500 to 6000 $\mu\text{g L}^{-1}$ in the DVR experiments. The Tod activity increased to near maximum within 20 minutes of toluene feeding. These results suggest that Tod activity can be fully restored by feeding toluene at high concentration for a relatively short period of time.

9. Normalized NADH was relatively unchanged when toluene and TCE were fed simultaneously. The NADH concentration of about 0.4 $\mu\text{mol (mg protein)}^{-1}$ was essentially the same as observed when toluene alone was fed to the column. NADH concentrations in the biofilm were observed to remain above 0.477 $\mu\text{mol (mg protein)}^{-1}$ during TCE degradation with no toluene present.
10. A dynamic biofiltration model was developed to predict the concentration profile and effluent history for the case when TCE alone is feed to the biofilter. The model demonstrated that the principal factor resulting in the decrease in TCE removal with time is the inactivation of Tod enzyme by TCE metabolic intermediates.
11. The values of the TCE reaction rate constant, $k_s' = 1428.6 \mu\text{g TCE (mg indigo L)}^{-1}$, the TCE half-velocity constant, $K_s = 2620 \mu\text{g TCE L}^{-1}$, and enzyme activity inactivation constant $k_2 = 0.0004 \text{ mg indigo } (\mu\text{g TCE L})^{-1}$ were determined.
12. In most cases, the model gives a reasonable prediction of TCE degradation and enzyme activity in the biofilter. The model predicted effectively the diminishing rate of TCE degradation because of inactivation caused by TCE intermediates.
13. A sensitivity test of the model indicated that TCE degradation increased with decreasing K_s and the rate of increase in TCE effluent concentration with time increased with decreasing K_s . The model became less sensitive to K_s as the value of

the parameter increased. TCE degradation was not sensitive to k_2 , but enzyme activity was relatively sensitive to this parameter. The slope of the plot of effluent concentration as a function of time increased with TCE feed concentration. The normalized enzyme activity decreased rapidly for higher TCE feed concentrations.

7.2 Conclusions

This work demonstrates the feasibility of incorporating Tod enzyme activity into the biokinetics of TCE cometabolic oxidation by *Pseudomonas putida* F1. Tod enzyme activity in suspended and immobilized culture can be measured by a fluorescence-based assay. The enzyme activity describes directly the microbial activity and provides a basis to predict biofiltration performance. The decrease in TCE removal rate with time was explained by the inactivation of Tod activity. A mathematical model incorporating this principal gives a reasonable prediction of the diminishing rate of TCE degradation in a biofilter. This work demonstrates that the enzyme activity is an important factor to understand the biokinetics process of cometabolic degradation.

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410.

APPENDICES

Appendix 1

Main program for Biofiltration

Clear all

% Condition input

x = 0:0.2:18; % position

t = 0:10:500; % time

[a,n] = size(x);

[b,m] = size(t);

S=zeros(m,n);

Ex=zeros(m,n);

tau=zeros(n);

$E_x(1,:) = 0.000215 \cdot \exp(-0.0187 \cdot x);$

S(:,1) = 100; TCE influent concentration, $\mu\text{g TCE L}^{-1}$

v=6.37; % gas velocity, cm min^{-1}

k=500; % TCE reaction rate constant, $\mu\text{g TCE } (\mu\text{g indigo L})^{-1}$

ks=2620; %TCE concentration, $\mu\text{g L}^{-1}$

$k_1 = 0.0016$; % natural decay constant for E_x , min^{-1}

$k_2 = 0.0004$; % inactivation constant of E_x , $\text{mg indigo } (\text{min } \mu\text{g TCE})^{-1}$

Q=0.278; % Flow rate. ml min^{-1}

X=2500; % protein concentration, mg protein

for I = 1:m;

if t(i) <= 60;

S(i,1) = 116.25;

elseif (60 < t(i)) & (t(i) <= 155);

S(i,1) = 98.85 - 0.1011*t(i);

```

elseif (155 < t(i)) & (t(i) <= 271);
S(i,1) = 3.777+0.307*t(i);

elseif (271 < t(i)) & (t(i) <= 474);
S(i,1) = 271.1-0.307*t(i);

else t(i) > 474;
S(i,1) = 4.0837+0.1510*t(i);

end;
end;

for i=l:m-1;
for j=l:n-1;

tau(j)=t(i)+x(j)/v;
tau(j+1)=t(i)+x(j+1)/v;

S1 = S(i+1,j);
Ex1 = Ex(i,j);

taul=tau(j);
tau2=tau(j+1);

K=0;
Sold = 0.5;
Snew = 1;
delta = abs(Snew-Sold);

while delta >= 0.001;
Sold=Snew;
Snew = Sold-(ks*log(Sold)+Sold-(taul-tau2)*k*X*Ex1-S1-ks*log(S1)) / (ks*l/Sold +1);

delta = abs(Snew-Sold);
K = K+1;

end

S(i+1,j+1) = Snew;

S;

Ex(i+1,j)=(Ex(i,j)+(1/X)*k2/kl*Q*(S(i+1,j)-S(i+1,j+1)))*exp(-kl*(t(i+1)-t(i)))

```

```

        -(1/X)*k2/kl*Q*(S(i+1,j)-S(i+1,j+1));
Ex;

S(i+1,n) = S(i+1,n-1);

Ex(i+1,n) =Ex(i+1,n-1);

end
end

figure(1)
plot(x,S(1,:), '-.')
hold on

plot(x,S(m,:), 'g-')
xlabel ('column length (cm)')
ylabel ('TCE (µg/L)')
axis([0 16.5 0 150])

figure(2)
plot(x,Ex(1,:), '-.')
hold on

plot(x,Ex(m,:), 'g-')
xlabel ('column length (cm)')
ylabel ('Enzyme activity (mg indigo (mg protein min))-1')
axis([0 16.5 0.000 0.0003])

data1=[116.25 80.35 137.96 96.75 75.68 88.67];
data2=[54.3 38.41 41.71 54.87 65.88];
t1=[60 155 474 560];

figure(3)
plot(t,S(:,1), '-.')
hold on

plot(t,S(:,n));
plot(t1,data1,'o');
plot(t1,data2,'*');
xlabel ('time(min)')
ylabel ('TCE (µg/L)')
axis ([60 650 0 150])
legend ('predicted influent', 'predicted effluent', 'measured influent', 'measured effluent')

```

```

data3={0.000194 0.000174 0.000195 0.000192 0.000158 0.000122};
data4=[0.000226 0.000198 0.000179 0.000139 0.0000976 0.0000846];
t2=[0 60 155 474 560];

figure(4)
plot(t,Ex(:,1) , '-.')
hold on

plot(t,Ex(:,n));
plot(t2,data1,'o');
plot(t2,data2,'*');
xlabel ('time(min)')
ylabel('enzyme activity (mg indigo (mg protein min)-1)')
axis([0 650 0.00025])
legend ('predicted Ex', 'predicted Ex', 'measured Ex', 'measured Ex');

```

Appendix 2

Program for Biofilm Growth

```
mumax = 0.008575; % maximum growth rate, hr-1
Kg=1994; % half saturation constant, µg L-1
T =2000;% toluene concentration, µg/L

Xo = 18;% initial biomass in the reactor, mg protein

t=0:1:240;

X = exp(mumax*T/(Kg + T)*t + log(XO));

Xdata=[0 24 28 120 144 192 216 240];
Ydata=[18.8 19.8 16.72 41.22 47.87 54.52 35.57 79.66];
L=[0.2 0.398 0.211 0.32 0.5998 1.21 1.723 0.599];
U=[0.2 0.389 0.211 0.32 0.5998 1.21 1.723 0.599];

figure(1)
errorbar(Xdata, Ydata, L, U, '*');
xlabel('Time (hr)')
ylabel('Biomass concentration mg protein')
title('Figure 1 Biomass increases with time in the reactor')
hold on

plot(t,X);
axis ([0 240 0 90]);
```

VITA

Hae-jin Woo was born in Republic of Korea, Pusan on January 30, 1966. She graduated with a Bachelor of Engineering degree in Environmental Engineering from the Pusan National University, Pusan in 1988. She entered the Master's program in Environmental Engineering at the Pusan National University and received the Master's degree in 1990. She worked for two years as a teaching assistant at program in Department of Environmental Engineering at the Pusan National University. She pursued the Doctorate of Civil Engineering at the University of Tennessee, Knoxville in fall semester, 1993.