AN ABSTRACT OF THE THESIS OF

Christina Blatchford for the degree of Master of Science in Civil Engineering presented on September 22, 2005.

Title: Aerobic Degradation of Chlorinated Ethenes by *Mycobacterium* Strain JS60 in the Presence of Organic Acids

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Abstract approved:

This study evaluated the potential of the aerobic *Mycobacterium* strain JS60 to grow on a variety of organic acid substrates, and the possible effects an organic acid would have on the degradation rate of vinyl chloride (VC). A series of batch growth tests were designed to determine the time it took to consume the substrate and the overall increase in biomass. Strain JS60 was found capable of growth on acetate, propionate, and butyrate, but could not grow on formate or lactate. Acetate was chosen for further study because strain JS60 consumed acetate the most rapidly of all the organic acids tested, and acetate is a common product of fermentation reactions in the subsurface.

Strain JS60 was confirmed to grow on both ethylene and vinyl chloride as the sole carbon and energy source. Comparatively, strain JS60's rate of growth on VC is much slower than that of ethylene. With acetate as an augmenting growth substrate, ethylene and VC utilization rates increased by 30% and 48%, respectively. Since acetate and VC

are often found together in contaminated chlorinated ethene plumes, this makes a strong case for natural attenuation of VC by strain JS60.

A series of kinetic tests were implemented to determine the K_S and k_{max} of strain JS60 for ethylene, VC, and c-DCE. The K_S and k_{max} for ethylene determined through NLSR methods was similar to the values published in Coleman et al. (2002), supporting the maintenance of a pure culture throughout the experimental work.

When strain JS60 was exposed to the isomers of DCE (trans-1,2-dichloroethylene (t-DCE), cis-1,2-dichloroethylene (c-DCE), and 1,1-dichloroethylene (1,1-DCE)) the cells were unable to grow on these compounds. However, when growing on acetate, strain JS60 cometabolized c-DCE and t-DCE, but not 1,1-DCE, with c-DCE transformed more rapidly than t-DCE. Transformation of c-DCE was also observed with growth on VC and ethylene. The presence of c-DCE was shown to partially inhibit VC degradation, but had no effect on ethylene degradation. The cometabolism results with acetate further indicate that strain JS60 is a good candidate for natural attenuation of multiple chlorinated ethenes in the subsurface.

Aerobic Degradation of Chlorinated Ethenes by *Mycobacterium* Strain JS60
in the Presence of Organic Acids

by

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CONTRIBUTION OF AUTHORS

Dr. Semprini assisted in the conception and writing of this thesis and the design of all laboratory experiments. Dr. Dolan provided many helpful comments throughout the development of the laboratory experiments and thesis write-up. Mohammad Azizian, Anne Taylor, and Andy Seblowski all provided significant laboratory support. Anne Taylor also provided help with all PCR and DNA analysis.

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Aerobic Degradation of Chlorinated Ethenes by *Mycobacterium* strain JS60 in the Presence of Organic Acids

CHAPTER 1

Introduction

Contamination of groundwater with vinyl chloride (VC) occurs primarily via anaerobic reductive dechlorination of tetrachloroethene, trichloroethene, and 1,1,1-trichloroethane (Vogel et. al., 1987). The US EPA maximum contaminant level (MCL) of VC in drinking water is 2 μg/L. This is lower than any other volatile organic compound due to the fact that VC is a known human carcinogen (Pontius et. al., 1996; Verce et. al., 2000). Reductive dechlorination of VC to ethene (Distefano, 1999; Freedman and Gossett, 1989) and anaerobic oxidation of VC under iron-reducing and methanogenic conditions (Bradley et al., 1997; 1999) often occur at relatively slow rates. The potential for persistence of VC has long been a concern with the exclusive reliance on anaerobic dechlorination as a method for groundwater remediation.

In contrast, it is generally accepted that VC is readily biodegradable under aerobic conditions. Cometabolism of VC has been demonstrated with numerous primary substrates, including ethene, ethane, methane, propane, propylene, isoprene, 3-chloropropanol, and ammonia (Freedman and Hertz, 1996; Koziollek et al., 1999; Castro et al., 1992; Dolan and McCarty, 1995; Malachowsky et al., 1994; Phelps et al., 1991; Ensign et al., 1992; Ewers et al., 1990; Rasche et al., 1991; Vannelli et al., 1990). Aerobic biodegradation of VC during microcosm studies has also been widely reported

(Bradley and Chappelle, 1998; Davis and Carpenter, 1990; Edwards and Cox, 1997). However, only a few organisms have been isolated that have the ability to use VC as the sole growth substrate (Coleman et al., 2002; Hartmans et al., 1992; Hartmans and de Bont, 1992; Hartmans et al., 1992; Verce et al., 2000). Some isolates of *Mycobacterium* sp., *Xanthobacter* sp., and *Nocardioes* strain JS616 have shown that VC starvation can result in complete loss of their ability to resume VC biodegradation (Coleman et al, 2002; Hartmans et al., 1992). However, there are also isolates that are more robust in the VC degradation abilities (Verce et al., 2000; Coleman et al., 2002), including *Mycobacterium* strain JS60. Strain JS60 can be grown on either VC or ethylene as the sole carbon source and shows no signs of starvation causing irreversible loss of VC utilization ability. Additionally, strain JS60 shows favorable growth rates, yields, and colony behavior when grown on ethylene and VC.

OBJECTIVES

The main goal of this thesis was to evaluate *Mycobacterium* strain JS60's ability to utilize ethylene and VC in the presence of organic acids. This goal is pursued through the specific objectives listed below;

1) To verify that JS60 is capable of growth on both ethylene and VC as the sole carbon and energy source.

2) To evaluate JS60 for growth on a variety of organic acids.

3) To determine the change in growth rate and biomass production of JS60 when exposed to both ethylene and acetate, or both VC and acetate.

4) To conduct kinetic tests on JS60 to determine K_S for ethylene.

5) To determine if strain JS60 is capable of cometabolising c-DCE using ethylene, VC, or acetate as growth substrates.

6) To determine if strain JS60 is capable of cometabolising t-DCE or 1,1-DCE using acetate as the growth substrate.

7) To conduct simple kinetic inhibition tests to determine the favorability of one substrate over another among ethylene, VC, and c-DCE.

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CHAPTER 2

Literature Review

Chlorinated ethenes have become widely distributed environmental contaminants as a result of discharge from industrial wastewaters, seepage from landfills, leakage from underground storage tanks, and historically improper disposal at some manufacturing sites. Analysis of volatile organic compounds (VOCs) in water supplies from groundwater sources showed that the five most frequently observed chlorinated ethenes were trichloroethylene (TCE), cis-1,2-dichloroethylene (c-DCE), trans-1,2-dichloroethylene (t-DCE) and 1,1-dichloroethylene (1,1-DCE) (Westrick, 1984). The United States Environmental Protection Agency (EPA) registers most of the chlorinated C1 and C2 aliphatic hydrocarbons as major pollutants due to their adverse effects on human health. The specify toxicity of chlorinated ethenes varies between compounds. Vinyl chloride (VC) is a known human carcinogen, with a maximum contaminant level in drinking water of 2 μg/L, and the maximum contaminant level goal of 0 μg/L (EPA, 2005).

Contamination of groundwater with VC occurs primarily via anaerobic reductive dechlorination of tetrachloroethylene (PCE), TCE, and 1,1,1-trichloroethane (1,1,1-TCA), see Figure. 2.1. (Vogel et. al., 1987). However, there is also 15 billion lb of PVC produced annually in Canada and the Untied States in 2002 (Sass et al., 2005). VC is used in PVC production. Reductive dechlorination of VC to ethene (Distefano, 1999; Freedman, Gossett, 1989) and anaerobic oxidation of VC under iron-reducing and

methanogenic conditions (Bradley et al., 1997; 1999) often occur at relatively low rates. The potential for persistence of VC has long been a concern with the exclusive reliance on anaerobic dechlorination as a method for groundwater remediation (Verce et al., 2000).
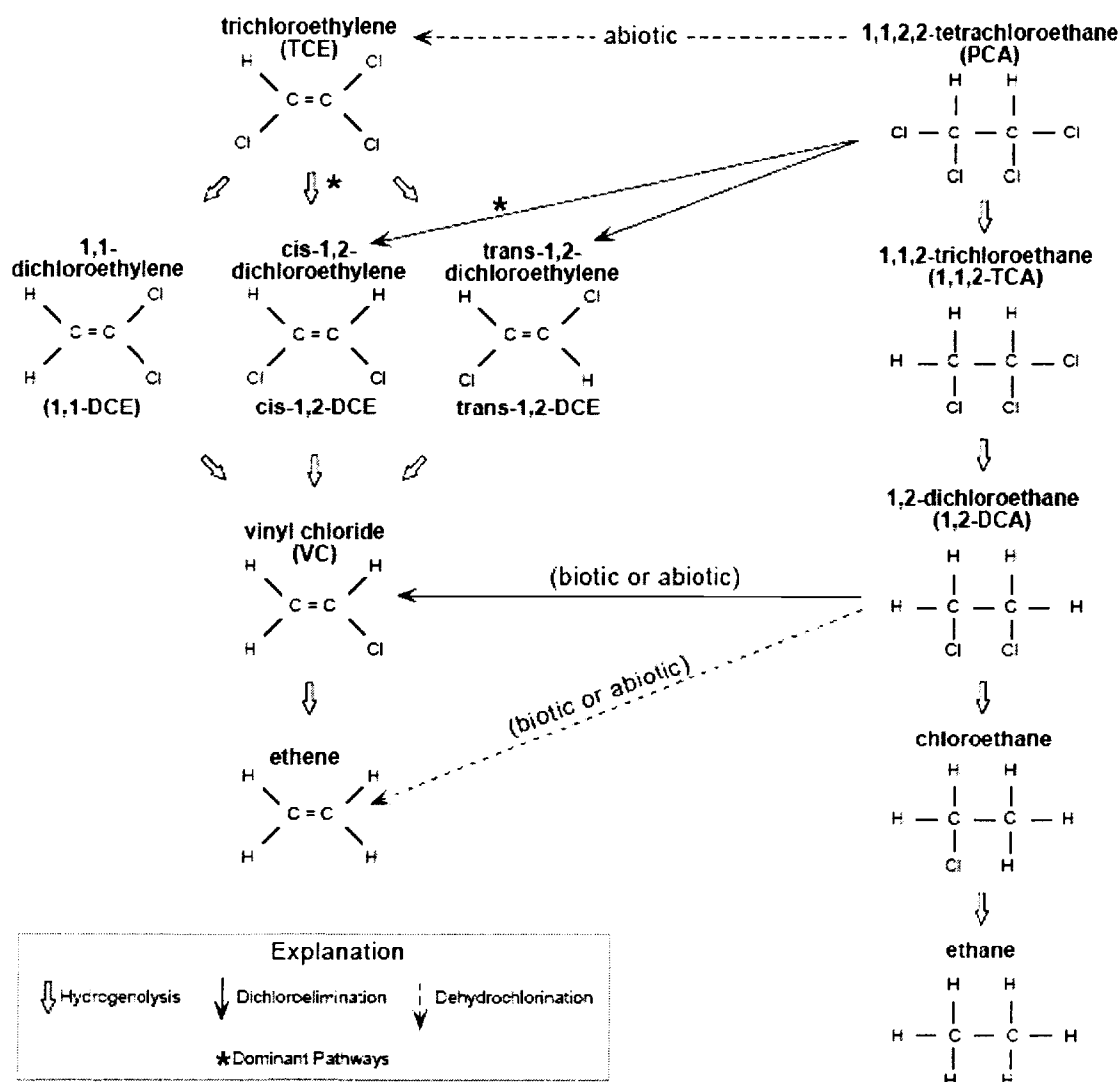
Figure. 2.1. Anaerobic degradation pathways for trichloroethylene and 1,1,2,2-tetrachloroethane. (Modified from Chen et al., 1996; McCarty, 1996; Nyer and Duffin, 1997; and Vogel et al., 1987).

AEROBIC MICROORGANISMS THAT COMETABOLIZE CHLORINATED ETHENES

Bioremediation of chlorinated solvents, especially trichloroethylene (TCE) and tetrachloroethylene (PCE), has been successful under anaerobic conditions. The breakdown products of PCE and TCE under anaerobic conditions are the DCE isomers (c-DCE, t-DCE, and 1,1-DCE) and VC (Figure 2.1). At some sites there has been limited success in the anaerobic degradation of DCE and VC under intrinsic conditions (Coleman et al. 2002).

Aerobic cometabolism is an emerging technology in which microbes cometabolize chlorinated ethenes to inorganic constituents. With the exception of VC and c-DCE (Coleman et al., 2002) cometabolism is currently the only means for aerobic degradation of most chlorinated ethenes, including TCE, 1,1-DCE, and t-DCE. In cometabolism, a non-specific oxygenase catalyses the transformation of the chlorinated ethenes without providing either energy or carbon to the organism (van Hylckama Vlieg and Janssen, 2001). Cometabolism of VC has been demonstrated with numerous primary substrates, including ethene, ethane, methane, propane, propylene, isoprene, 3-chloropropanol, and ammonia (Freedman and Hertz, 1996; Koziollek et al., 1999; Castro et al., 1992; Dolan and McCarty, 1995; Malachowsky et al., 1994; Phelps et al., 1991; Ensign et al., 1992; Ewers et al., 1990; Rasche et al., 1991; Vannelli et al., 1990).

AEROBIC MICROORGANISMS THAT GROW ON CHLORINATED ETHYLENES

Aerobic bioremediation of many chlorinated ethenes by monooxygenase expressing microorganisms is often limited by the toxic epoxides produced as intermediates (Oldenhuis et al., 1991). Under aerobic conditions, epoxides are generally formed by monooxygenases when they initiate attack on chlorinated ethenes (Fox et al., 1990). The compound formed is a chlorinated epoxyethane which is electrophillic and may covalently bond to cellular components. This bond could modify cellular components such as; DNA, RNA, lipids and proteins, causing a turnover dependant inactivation of the biocatalyst and the subsequent death of the cell (Oldenhuis et al., 1991). Microbes that can deal with the toxic intermediates effectively are of particular interest in the research community.

The electrons produced in a transformation reaction must be transferred to a terminal electron acceptor. During the electron transfer process, energy is produced which is utilized by the cell. Aerobic bacteria are characterized by the fact that they can use oxygen as the terminal electron acceptor for degradation reactions (Azadpour-Keeley et al., 1999). It is generally accepted that VC is readily biodegradable under aerobic conditions. However, only a few organisms have been isolated that have the ability to use VC as the sole growth substrate; isolates from *Mycobacterium*, *Nocardiodes*, and *Pseudomonas* have shown the ability to utilize VC (Coleman et al., 2002; Hartmans et al., 1992; Verce et al., 2000). VC degrading microorganisms are fairly widespread and arise wherever conditions are appropriate for their growth. The most favorable niches for the

growth of aerobic VC degraders are in aerobic fringe areas down gradient of chlorinated ethene plumes, where anaerobic electron donors are depleted and the end products of anaerobic chloroethene metabolism (VC and ethylene) accumulate (Coleman et al, 2002). In these same areas, organic acids resulting from fermentation reactions might also be present.

Mycobacterium Strain JS60

Several strains of mycobacterium which are capable of growth on VC as the sole carbon source have been isolated. The first *Mycobacterium* strains were identified approximately 30 years ago (de Bont, 1976). More recently, strains of *Mycobacterium*, *Nocardiodes*, and *Pseudomonas* capable of growth on both VC and ethylene have been isolated (Coleman et al., 2002; Hartmans et al., 1992; Verce et al., 2000).

Many of the strains of *Mycobacterium* isolated by Coleman et al. (2002) from groundwater, aquifer solids, and surface water samples are clustered in a loose group that includes *M. rhodesiae*, *M, sphagni*, *M. aichiensse*, *M. fortuitum*, and *M. mucogenicum* (Pitulle et al., 1992; Tsukamura and Mizuno, 1977). This group also includes the ethylene degrader strain K1 (Koziollek et al., 1999). Based on 16S rDNA sequencing using 1420 base pairs, Coleman et al. (2002) tentatively assigned *Mycobacterium* strain JS60 to *M. rhodesiae*.

All of the VC degrading isolates identified by Coleman et al. (2002) were gram-

positive bacteria. Verce et al. (2000) showed that the *Pseudomonas* MF1 VC degrader was gram-negative bacteria. These differences are most likely due to the fact that Coleman et al. (2002) used VC for isolate selection, whereas Verce et al. (2000) used ethane and (2001) ethylene in the initial enrichments. It appears that mycobacteria are more likely candidates than pseudomonads for the natural attenuation of VC that occurs in chlorinated ethene contamination sites (Coleman et al., 2002). However, MF1 is not sensitive to VC starvation like various mycobacteria, which lost their ability to degrade VC after brief periods in the absence of VC (Verce et al, 2000). The *Mycobacterium* strain JS60, however, is a robust culture capable of maintaining VC degradation capabilities even after periods of VC starvation. Strain JS60 can be grown on either 1/10-TSA plates (1 week) or MSM-VC plates (1 month). On 1/10-TSA plates the colonies formed are cream colored, smooth, and circular.

Strain JS60 can be grown on either ethylene or VC and readily degrade both, suggesting that the same monooxygenase enzyme is active on VC and ethylene and is inducible by both substrates. A growth curve reported by Coleman et al. (2003) is shown in Figure. 2.2. The growth rates were determined by fitting the data set to an exponential trend line of equation $X = X_o e^{kt}$, where k is the growth rate. The growth rates for ethylene and VC were 0.080 hour^{-1}and 0.017 hour^{-1}, respectively. The ethylene growth rate is approximately four times faster than the VC growth rate. The kinetics of strain JS60 degradation of ethylene and VC are shown in Table. 2.1. (Coleman et al., 2002). The kinetic parameters were determined by fitting the data to the Michaelis-Menten model by using the AQUASIM software program (Coleman et al., 2002). Both the availability of the kinetics and the fact that strain JS60 is not sensitive to VC starvation,

are the main reasons that strain JS60 was chosen as the *Mycobacterium* strain to study.

Table. 2.1. A summary of the kinetic parameters for strain JS60 on ethylene and VC found in Coleman et al. (2002).

Kinetic Parameter	Ethylene	Vinyl Chloride
K_S (µM)	0.9±0.1	0.5±0.1
k_{max} (day^{-1})	0.58±0.05	0.22±0.02
k (nmol/min/mg)	25.4±0.8	9.7±0.2
Y (g protein/mol substrate)	11.2±0.6	6.6±0.7

In aerobic degradation reactions VC and ethylene are catalyzed by monooxygenase, transforming it into the reactive chlorooxirane and epoxyethane, respectively. Coleman and Spain (2003) conducted enzyme assays in which alkene monooxygenase and EaCoMT enzymes of strain JS60 are active in both VC and ethylene assimilation pathways. The finding of EaCoMT adds strain JS60 to the small group of bacteria, including *Xanthobacter* and *Rhodococcus*, known to use CoM in catabolic reactions (Krum and Ensign, 2000). Also, genes (*etnABCD*) encoding a four-component monooxygenase and two genes possibly involved in acyl-CoA ester metabolism were found. NAD and CoA are likely responsible in regenerating the reduced form of CoM, see Figure. 2.3 (Coleman and Spain, 2003). The fact that acytl-CoA is present as an enzyme might suggest that acetate would be a good growth substrate and that VC degradation rates could be enhanced. However, Coleman et al. (2002) indicate that after long periods of growth on acetate, the ability to oxidize ethylene to epoxyethane was lost.
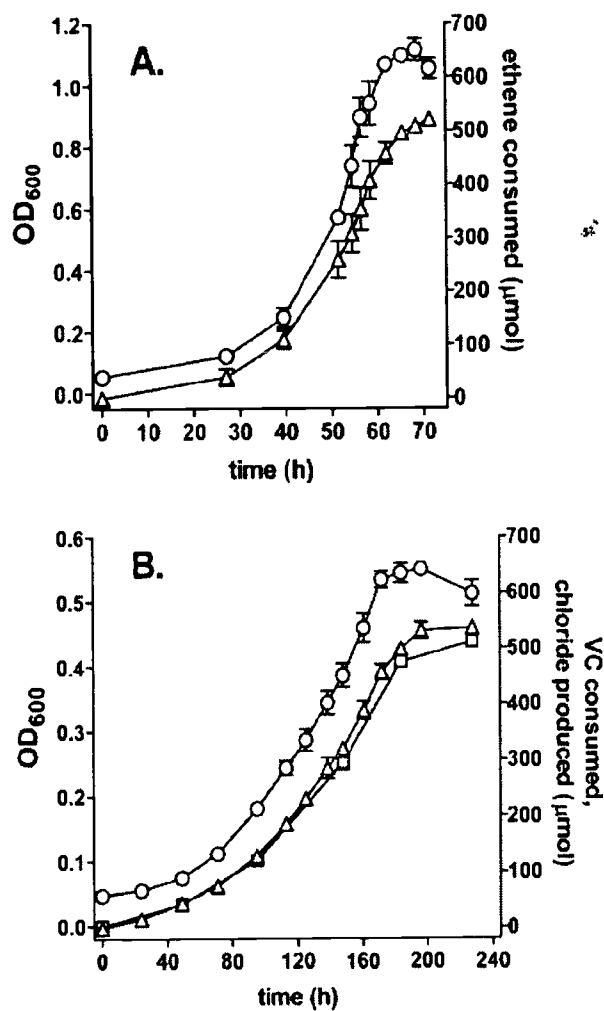
Figure. 2.2. Growth of Mycobacterium strain JS60 on (a) ethene, and (b) VC. o-biomasss measured, □-cumulative chloride produced, and Δ-cumulative amount of substrate consumed. Growth rates are 0.080 hour^{-1} with ethene, 0.017 hour^{-1} with VC (Coleman and Spain, 2003).

$$CH_2=CH_2 \qquad\qquad CH_2=CH\text{-}Cl$$

NADH, H⁺, ½O₂

NAD⁻, H₂O

alkene monooxygenase

NADH, H⁺, ½O₂

NAD⁻, H₂O

$$\underset{CH_2\text{-}CH_2}{\overset{O}{\triangle}} \qquad\qquad \underset{CH_2\text{-}CH\text{-}Cl}{\overset{O}{\triangle}}$$

CoM-SH

epoxyalkane:CoM transferase

CoM-SH

$$\underset{CH_2\text{-}CH_2}{\overset{CoM\text{-}S\quad OH}{|\qquad\quad|}} \qquad\qquad \left[\underset{CH_2\text{-}CH\text{-}Cl}{\overset{CoM\text{-}S\quad OH}{|\qquad\quad|}}\right]$$

$$\left[\underset{CH_2\text{-}CH}{\overset{CoM\text{-}S\quad O}{|\qquad\quad||}}\right]$$

HCl

CoA-SH

CoM-SH

$$\underset{CH_3\text{-}C\text{-}S\text{-}CoA}{\overset{O}{||}}$$
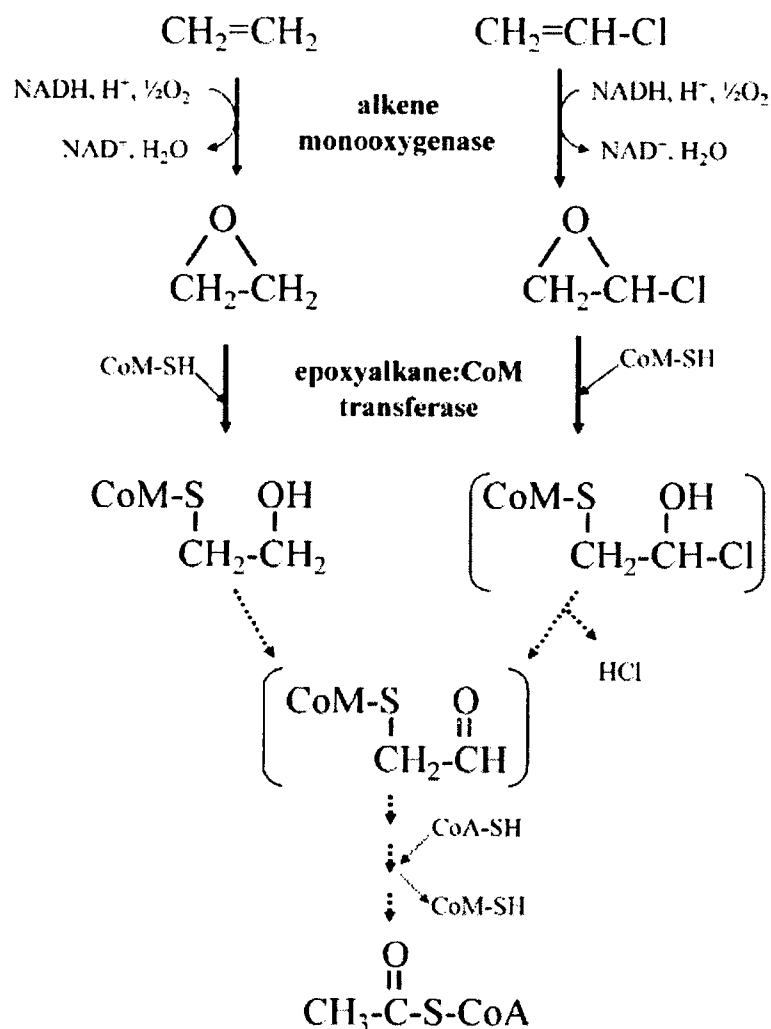
Figure. 2.3. Proposed pathways of VC and ethene assimilation in Mycobacterium strains. Intermediates that have not been identified are in brackets [ ], and hypothetical reactions are indicated by dotted lines ... (Coleman and Spain, 2003).

Acetate as a Substrate

Aerobic cometabolism of chlorinated ethenes has been demonstrated using microbes stimulated by methane, phenol, toluene, and other regulated organic compounds (US EPA, 2000). Acetate, a non-regulated organic compound, is common in contaminated groundwater plumes because it is generated as a byproduct of anaerobic metabolism. Acetate is commonly found in BTEX contaminated sites, and in landfill leachates (Cozzarelli et al., 1995). Some pathways of acetate production are shown in Figure 2.4. The first group of microorganisms secrete enzymes which hydrolyze polymeric materials to monomers, and then to higher volatile fatty acids, hydrogen, and acetic acid. Acetogenic bacteria then convert the fatty acids, such as propionic and butyric acids, to hydrogen, CO_2, and acetic acid. In addition to the decomposition of long-chain fatty acids, ethanol and lactate are also converted to acetate and hydrogen by acetogens. Acetogenic bacteria are obligate hydrogen producers, some of which include *Syntrophobacter wolinii* a propionate decomposer (Boone et al., 1980) and *Sytrophomonos wolfei*, a butyrate decomposer (McInerney et al., 1981). Due to H_2 inhibition, these bacteria perform well when in symbiosis with a hydrogen consumer (Boone et al., 1980).

COMPLEX ORGANICS — 76% → HIGHER ORGANIC ACIDS

4% → H_2 — 28%

24%

52%

H_2 → CH_4

HIGHER ORGANIC ACIDS → ACETIC ACID

20%

ACETIC ACID — 72% → CH_4

HYDROLYSIS AND ACIDOGENESIS

ACETOGENESIS AND DEHYDROGENATION

METHANO-GENESIS

STAGE 1   STAGE 2   STAGE 3
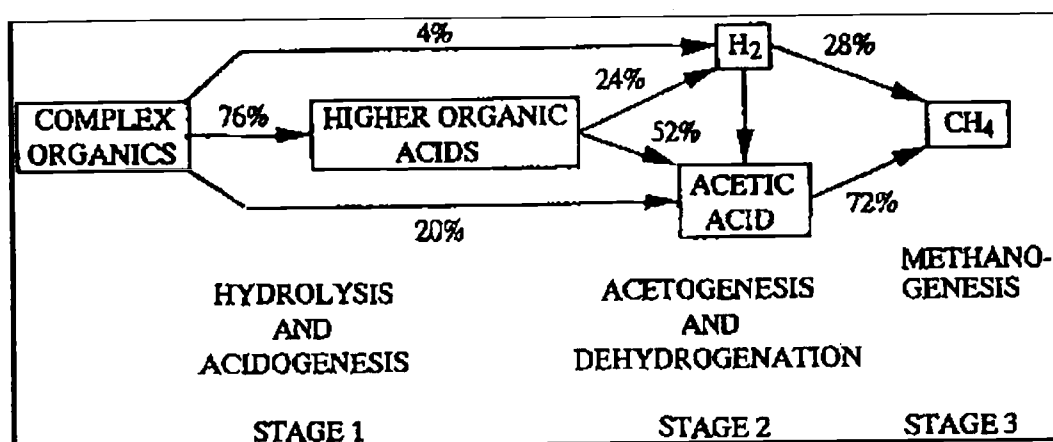
Figure. 2.4. Methane fermentation pathways, based on an analysis of bacteria isolated from sewage sludge digesters and from the rumen of some animals (McCarty, 1982).

Acetate has been studied as an option for biological enhancement as an in situ remediation option at contaminated groundwater sites. One study includes using acetate to stimulate the removal of soluble U(VI) in Gunnison, CO. Push-pull tests indicated that U(VI) concentrations decreased approximately 60% after injection of an acetate solution relative to a control well receiving no acetate (Anderson et al., 2002). Another study involves the removal of nitrate from groundwater using acetate.

Acetate has also been shown to be an electron donor in chlorinated ethene reactions. The microbial reductive dechlorination of PCE to ethylene using acetate as an electron donor has been shown in microcosms (He et al., 2002). Also, at a PCE contaminated groundwater site in Weston, Ontario, methanol and acetate were detected in the groundwater in the source area, and appeared to be promoting PCE biotransformation to ethene (Major, 1995). Methanol was likely released during facility operations, and acetate was produced by the conversion of methanol by acetogenic microorganisms

(Cozzarelli et al., 1995).

Little research has been done with acetate or other organic acids concerning aerobic cultures degrading chlorinated ethenes either cometabolically or as an enhancement of ethylene or VC as a carbon and energy source. The research conducted in this thesis is intended to expand this information/

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CHAPTER 3

Aerobic Growth on Ethylene, Vinyl Chloride, and Organic Acids

SUMMARY

Growth experiments were performed to confirm that *Mycobacterium* strain JS60 was capable of growth on vinyl chloride or ethylene as the sole carbon and energy source. Additionally, multiple growth experiments were performed to identify, out of several organic acids, which would support growth for strain JS60. Acetate, propionate, and butyrate were found to support growth, while lactate and formate could not. Strain JS60 grew the fastest on acetate, followed by propionate, then butyrate. Finally, a series of batch experiments were designed to identify the effects of acetate on the degradation rate of ethylene and vinyl chloride. Ethylene and VC degradation rates were found to increase in the presence of acetate.

INTRODUCTION

Anaerobic reductive dechlorination of tetrachloroethylene (PCE) and trichloroethylene (TCE) often leads to the accumulation of *cis*-1,2-dichloroethylene (c-DCE) and vinyl chloride (VC) (Verce et al., 2002). As the contaminated groundwater containing c-DCE and VC migrates away from the source area, electron-acceptor conditions may transition from anaerobic to aerobic (Verce et al., 2002). Disappearance of VC at the aerobic fringe due to natural attenuation has been documented in the field (Cox et al., 1995; Edwards and Cox, 1997). One possible explanation of this occurrence is that VC is degraded by aerobic bacteria that are capable of using VC as a growth substrate.
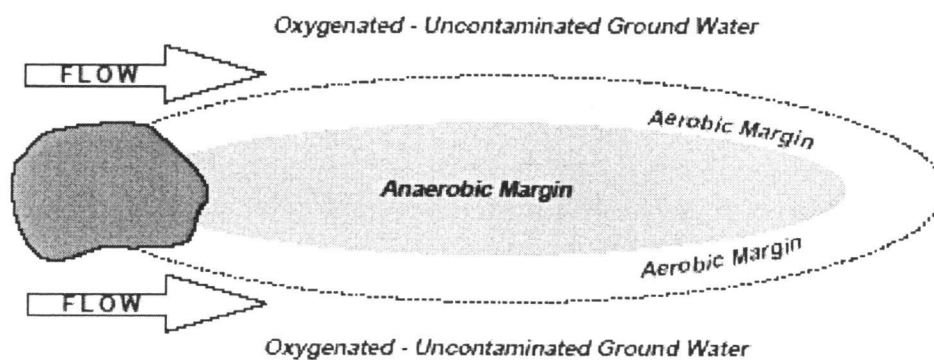
Figure. 3.1. A plan view of a typical contaminated groundwater plume undergoing natural attenuation (Azadpour-Keeley, 1999).

While aerobic VC-assimilating bacteria may be responsible for the natural attenuation of VC in aerobic down gradient portions of chlorinated ethylene plumes, the rates and extents of in situ VC oxidation are largely unknown. This is partly due to the large variation in the kinetic parameters of the few VC degraders that have been studied (Coleman et al., 2002). Some of the VC degraders previously studied include *Mycobacterium* (Hartmans et al., 1992), and *Pseudomonas* (Verce et al., 2001) which were isolated from soils, surface water, and activated sludge. Specifically, the *Mycobacterium* strain JS60, isolated from a groundwater sample identified in Coleman et al. (2002), was studied in detail because it was a robust strain that was capable of growth on both vinyl chloride (VC) and ethylene as the sole carbon and energy source and it was not sensitive to VC starvation.

The possibility of additional compounds being present in the contaminated groundwater plume that could affect the rate of VC degradation is an important aspect to explore when considering natural attenuation as a feasible bioremediation option. Organic acids are often byproducts of microbial metabolic processes and are therefore commonly found in groundwater plumes (Cozzarelli et al., 1995).

Through multiple batch growth studies, we verified that *Mycobacterium* strain JS60 was capable of growth on ethylene and VC as the sole carbon source. We also determined that strain JS60 is capable of growth on several organic acids. Finally, the effect of organic acids on the degradation rate of VC and ethylene was analyzed through a series of different batch growth tests each designed to show a distinct aspect of strain JS60 behavior.

MATERIALS AND METHODS

Chemicals

Ethylene gas (99.5%), and vinyl chloride gas (99.5+%) was purchased from Aldrich (St. Louis, MO); sodium acetate anhydrous (99.3%) and lactic acid (85%) were purchased from Mallinckrodt Chemical (Paris, KT); sodium propionate (99%), sodium butyrate (99%) and sodium formate (99+%) were purchased from Aldrich (St. Louis, MO).

In order to achieve the desired concentrations for growth experiments; sodium acetate, sodium propionate, sodium butyrate, and sodium formate were prepared by dissolving a measured amount of each solid in 1000 mL of autoclaved deionized water. Lactate was added as a syrup directly from the stock bottle. Vinyl chloride gas was transferred directly from the stock bottle to the 125 mL batch bottles used for the growth experiments. Ethylene gas was transferred into a double-flask container. Measured quantities of ethylene gas were then filtered with a Pall 0.2 μm PTFE Acrodisc prior to injection into the 125 mL batch bottles used for the growth experiments.

Analysis

Consumption of ethylene and vinyl chloride were monitored by gas chromatograph (GC) analysis of headspace samples by headspace analysis (Kampbell at al., 1989). 100-μl samples were injected into a HP6890 series GC using a DB 624 capillary column, 30-m long, I.D. 0.25 mm narrowbore, 1.4 μm film (Agilent Technologies) operated at $50^{\circ}C$. The gas chromatograph response was calibrated to give the gas phase concentration per sample. The Henry's constant (Gossett, 1989) was used to convert the gas phase concentration to a liguid phase concentration. Equation 3.1 was then used to determine the total mass of the compound in the reactor based on the liquid phase concentration. This conversion assumed that the gas phase and the liquid phase are at equilibrium. The total mass is given by:

$$M_T = C_L (V_L + H_{CC}V_G)$$
Equation. 3.1.

where C_L is the liquid phase concentration, V_L is the volume of liquid in the reactor, V_G is the headspace volume in the reactor, M_T is the total mass of the substrate, and H_{CC} is the Henry's constant (Gossett, 1987) (ethylene 7.64, and VC 0.97).

Consumption of organic acids were monitored by high pressure liquid chromatograph (HPLC). A 1 mL liquid phase sample was taken daily from the batch growth reactors for acids analysis. The concentrations of the organic acids; acetate, formate, propionate, and lactate were measured using a Dionex 500 series high-pressure

liquid chromatograph (HPLC) with a Prevail Organic Acid 5-μm column (Altech), operated at 25°C, with a AD 20 series Absorbance Detector. The HPLC was calibrated by determining a response factor for each organic acid by dividing the known acid concentration in the reactor by the peak area response from the HPLC. The response factor was used to determine unknown organic acid concentration by using the HPLC peak area response. Butyrate concentrations were determined using total organic carbon TOC analysis. The 1 mL samples were centrifuged and filtered using a 0.22 μm membrane filter (Osmonics Inc., Minnetonka, MN) then analyzed for butyrate using a Rosemount Analytical total carbon analyzer, series Dohrmann DC-190. Calibration curves for all compounds were developed as presented in Appendix B.

Optical density (OD) was measured at 600 nm using a HP8453 UV-Visible spectrophotometer. The biomass concentration was determined by measuring TSS (American Public Health Association, 1985). A 10 mL sample was filtered using a 0.22 μm membrane filter (Osmonics Inc., Minnetonka, MN), then placed in a drying oven at 105°C for over 24 hours.

The culture protein was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL), method (Bardawill, 1949). A mixture of 150 μL cell sample suspended in 150 μL of 3M NaOH, were prepared by heating at 65°C for at least 30 min. The samples were then centrifuged for 5 min, and 50 μL of supernatant was removed from each sample in duplicate. The samples were brought to a volume of 400 mL through addition of deionized water, then 100 mL of NaOH, and 500 mL of biuret reagent was added. After 30 min, the absorbance at 540 nm was measured.

Culture Maintenance

Mycobacterium strain JS60 was originally isolated from a groundwater sample, as described in Coleman et al. (2002). The pure culture for our study was obtained from Dr. Jim Spain. The culture was grown in 125 mL batch media bottle capped with butyl rubber septa (Wheaton, Millville, NJ), containing 50 mL of MSM and 15 mL of ethylene gas. See Appendix A for the MSM mixture. The bottles were shaken at 200 rpm on a rotary shaker and kept at $20^{o}C$. After one week of incubation, 1% of the culture was back-transferred into a new bottle containing fresh MSM growth media and 15 mL of ethylene gas.

The cells were harvested for growth experiments at an optical density (OD_{600}) between 0.0025 and 0.004. A 5 mL aliquot of active cell suspension was added to 50 mL of 1/5 MSM mixture and 120 mL of headspace containing air, in 175 mL capped-bottles. The batch growth experiments were conducted over 5-10 days, depending on the substrate.

In order to maintain a pure culture, all work was done with autoclaved media bottles and MSM, and filtered ethylene gas. Reactors were constructed and all samples were obtained under a laminar flow hood with a burner to continuously flame bottle necks, and caps. When obtaining samples, new needles were used each time the bottle cap septa was pierced, after heating the cap for 10 passes through the flame.

Batch Growth Reactors

Experimental growth reactors were consisted of 125 mL serum bottles containing 50 mL of 1/5 MSM growth media and deionized water, and 125 mL of headspace containing air. See Appendix A for the MSM mixture. The reactors were incubated at $20^{\circ}C$, and 200 rpm on a rotary shaker to achieve effective mass transfer between the gas and liquid phases.

Either ethylene, VC, or an organic acid were added to the batch growth reactors. The initial concentrations of substrates were determined after 30 minutes of rotary shaking at 200 rpm. The batch growth bottles were monitored daily by obtaining a 100 μL sample for GC head-space analysis, and a 1 mL liquid sample used for OD spectrophotometer analysis, and HPLC organic acid analysis. All experiments were conducted in duplicate or triplicate, and also included a biotic control, which contained cells but no substrate. The reactors provided substrate were compared to the biotic control reactor in order to determine if growth occurred.

The amounts of substrate added in the batch growth reactors were based on an equivalent electron donor addition, permitting comparisons between compounds and experiments. The half reactions used to calculate electron equivalents are provided in Table 3.1. Oxidation of the electron donor to CO_2 was assumed in the calculation. This approach was used to provide direst comparison of cell yields for the different substrates used.

An initial mass of ethylene added was chosen based on the oxygen availability in the bottle, and the 15 mL of ethylene gas provided to grow the stock culture. The amount

of ethylene chosen, at 2.3 mL, is approximately 15% of that used to grow the stock culture, and would consume approximately 33% of the available oxygen in a batch reactor based on complete oxidation to CO_2 and H_2O. Using the ideal gas law, 2.3 mL of ethylene is equivalent to 9.53×10^{-5} moles of ethylene. Using a ratio of 12 e$^-$ moles of ethylene/Y e$^-$ moles, the mass of the other compounds were determined. The calculated values are shown in Table 3.1.

Table. 3.1. A table of the electron transfer half reactions that were used in determining the mass of substrate addition based on total electron equivalent basis.

Substrate	Reaction	Amount Added	Moles Added (mmol)	e- equiv
Ethylene	$C_2H_4 + 4H_2O \rightarrow 2CO_2 + 12H^+ + 12e^-$	2.3-mL	0.094	1.13
VC	$C_2H_3Cl + 4 H_2O \rightarrow 2CO_2 + Cl^- + 11H^+ + 10 e^-$	2.8-mL	0.125	1.25
Acetate	$NaCH_3COO + 3H_2O \rightarrow CO_2 + HCO_3^- + 8H^+ + 8e^-$	11.8-mg	0.144	1.15
Propionate	$NaCH_3CH_2COO + 5H_2O \rightarrow 2CO_2 + HCO_3^- + 14H^+ + 14e^-$	7.8-mg	0.074	1.12
Butyrate	$NaCH_3CH_3CH_2COO + 9H_2O \rightarrow 4CO_2 + HCO_3^- + 25H^+ + 25e^-$	5.5-mg	0.057	1.20
Lactate	$CH_3CHOHCOO^- + 4H_2O \rightarrow 2CO_2 + HCO_3^- + 10H^+ + 10e^-$	21.1-µL	0.114	1.14
Formate	$NaHCOO + H_2O \rightarrow HCO_3^- + 2H^+ + 2e^-$	39.5-mg	0.057	1.14

RESULTS AND DISCUSSION

Growth Experiments on Ethylene, Vinyl Chloride, and Organic Acids

The growth curves of ethylene or vinyl chloride as the sole carbon and energy source are shown in Figure 3.2. Given the same amount of substrate, based on electron equivalencies, strain JS60 did not grow as quickly or produce as much biomass on VC as compared to ethylene. The result indicated a lower yield than expected on VC. Due to the presence of the chlorine atom in VC, the substrate is more difficult to metabolize and may present some low level toxicity to strain JS60.

Coleman and Spain (2003) reported a growth rate for ethylene of 0.08 hour^{-1}, while the growth rate determined in our study was 0.014 hour^{-1}. The growth rate for VC reported by Coleman and Spain (2003) was 0.017 hour^{-1}, while our growth rate was determined to be 0.006 hour^{-1}. The ethylene growth rate computed for our experiment was approximately six times lower than the value reported by Coleman et al. (2002), while the VC value was three times lower. This difference might result from both our initial cell concentrations and the total amount of substrate provided being much lower in our experiments. However, in Coleman et al. (2002) the maximum growth rates for ethylene and VC were 0.58 day^{-1} and 0.22 day^{-1}, respectively, which compare more closely to our growth rates of 0.33 day^{-1} and 0.15 day^{-1}, for ethylene and VC, respectively.

The results from the growth tests on the organic acids are shown in Figures 3.3a,

and 3.3b, while the results from a second test are shown in Figures 3.4a, 3.4b, and 3.4c (where M_t is total mass). Acetate, propionate, and butyrate tested positively as growth substrates for strain JS60. The rates of growth on the organic acids were: acetate $\geq$ propionate > butyrate. It is interesting to note that the rate of growth decreased with an increase in the number of carbons in the organic acids. When the growth rates of the organic acids were compared to the growth rates of the gaseous substrates: acetate $\geq$ ethylene $\geq$ propionate > butyrate $\geq$ VC. The growth curves for lactate and formate closely compared to the biotic control, indicating no growth on these substrates. There was close agreement between the two experiments conducted on two different weeks, and the duplicates in each experiment as well. The growth rates for these substrates are summarized in Table 3.2. Based on these results, the experiments were shown to be very reproducible.

Table. 3.2. A summary of growth rates determined by fitting an exponential trend line of $X = X_o\, e^{\,kt}$, through a sub-set of the OD_{600} data. This data can be found in Appendix C.

Substrate	Growth Rate Week 1 (hour^{-1})	Growth Rate Week 2 (hour^{-1})
Ethylene	0.013	0.015
Vinyl Chloride	0.006	--
Acetate	0.018	0.023
Propionate	0.011	0.013
Butyrate	0.007	0.006
Lactate	0.0	--
Formate	0.0	--
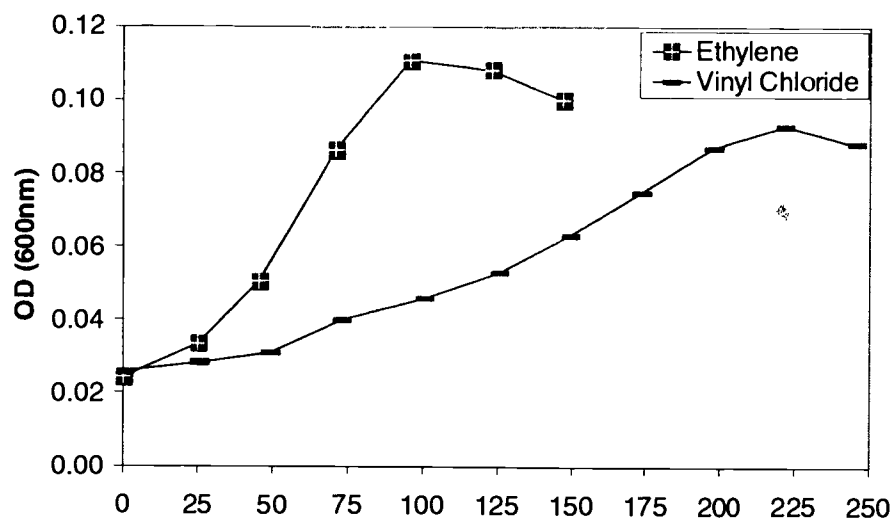
Figure. 3.2. *Mycobacterium* strain JS60 growth curves on ethylene and vinyl chloride.
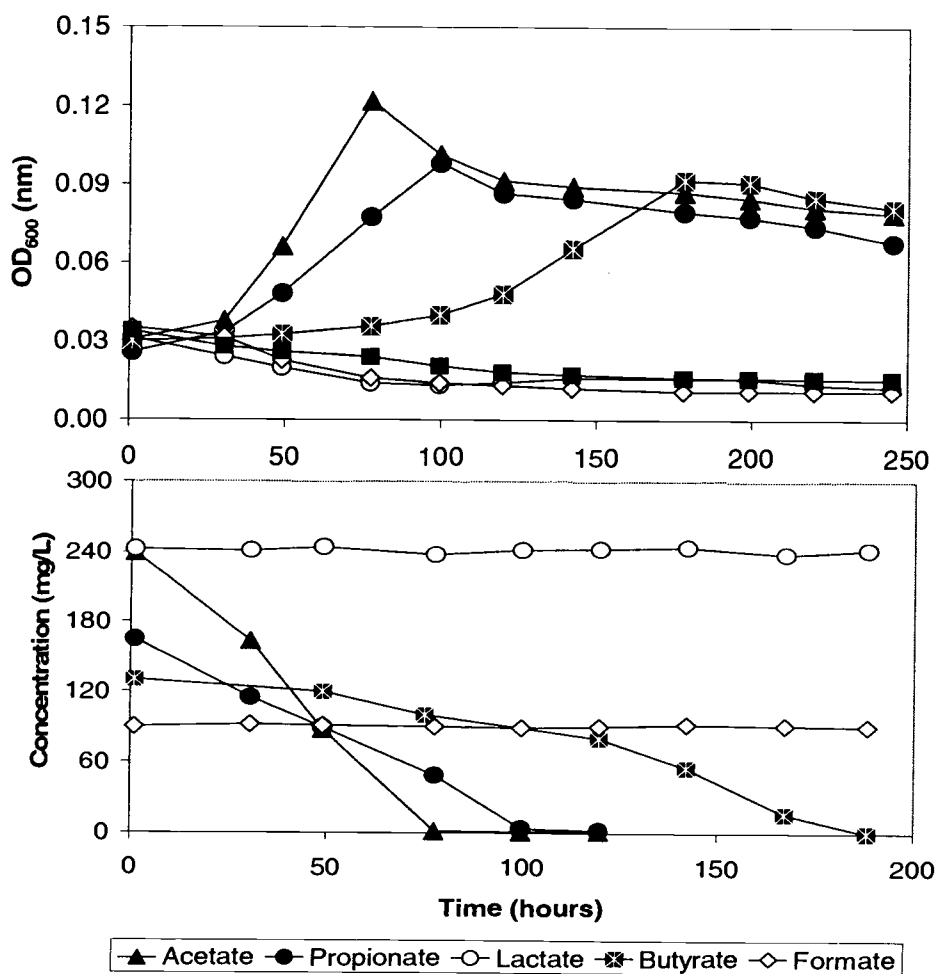
Figure. 3.3 Growth experiment on organic acids and a negative control. (a) growth curves, (b) organic acid consumption.
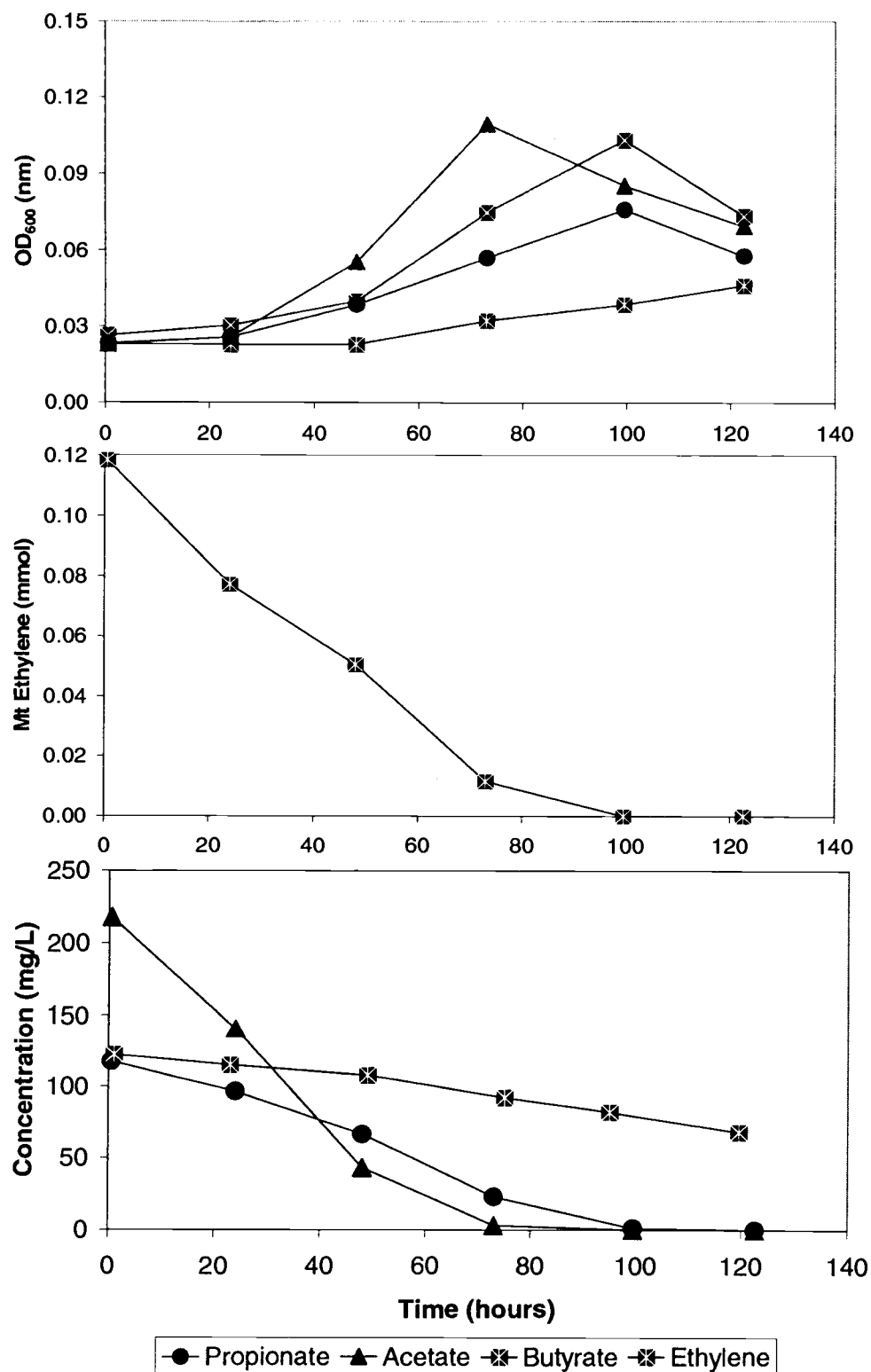
Figure. 3.4. Data from the second experiment conducted under the same conditions. (a) growth curves, (b) ethylene consumption, (c) organic acid consumption.

Growth Experiments on Ethylene in the Presence of an Organic Acid

Our previous results showed that the growth rate on acetate was faster than on ethylene. Thus we decided to test whether the presence of an organic acid substrate would increase ethylene utilization by JS60, and also increase overall growth rates. The growth experiments were designed to contain both an organic acid along with ethylene as a substrate. Both propionate and acetate were tested. The reactors containing both ethylene and an organic acid contained the same amount of total substrate on an electron equivalent basis as previous experiments. They each had 50% ethylene and 50% organic acid on an electron donor basis. The reactors which contained only 50% of the total substrate was indicated, i.e. ½ ethylene. The purpose of these test reactors was to compare ethylene and organic acid substrate utilization to the reactors which contained both substrates. The substrate additions made for these experiments are shown in Table 3.3.

Table. 3.3. Substrate additions made for the growth experiments on ethylene in the presence of an organic acid.

Reactor	Ethylene Added	Organic Acid Added	e- Equiv Added
Ethylene	94 μmol	--	1.13
½ Ethylene	47 μmol	--	0.56
½ Acetate	--	120 mg/L	0.58
½ Propionate	--	70 mg/L	0.56
Ethylene/Acetate	47 μmol	120 mg/L	1.14
Ethylene/Propionate	47 μmol	70 mg/L	1.12

Substrate consumption results were consistent with OD measurements of growth (Figure 3.5a). In the reactors that contained both ethylene and an organic acid the OD peak approached 0.12. In the reactors that contained ½ the amount of substrate the OD reached a peak of 0.06. At the OD peak, all of the growth substrates were consumed (Figures 3.5b and 3.5c). This was especially noticeable with the propionate concentration in the HPLC acids analysis. At a time of 75 hours, all of the propionate in the bottle containing both ethylene and propionate had been consumed, however the bottle containing propionate alone was not fully consumed until closer to 100 hours. Therefore, the presence of propionate or acetate decreased the time taken to consume all of the ethylene in the batch growth reactor, and the rates of growth were also increased.

The growth rates of each reactor were computed by fitting the exponential growth phase of the OD curve to the equation;

$$X = X_o \, e^{\,kt} \qquad\qquad \text{Equation 3.2}$$

where is k is the growth rate.

The growth rate on ethylene was 0.038 hour^{-1}. The growth rate on ethylene and acetate, or ethylene and propionate together was 0.043 hour^{-1} and 0.042 hour^{-1}, representing an increase of 12% and 10% in overall growth rates, respectively (Table 3.4). Results indicating that the presence of propionate increased the growth rate were surprising since the growth rate on propionate was found to be slightly slower than on ethylene determined in previous experiments.

All of the growth rates obtained in this experiment were approximately double the rate achieved earlier growth experiments. During part of the incubation, the air

conditioning in the 20°C incubation room was broken and the temperature increased to

25°C. This increase in temperature might have contributed to the increase in the growth

rates for this experiment.

Table. 3.4. A summary of mixtures of acetate and ethylene, and propionate and ethylene growth rates compared to the experiments including propionate, acetate, and ethylene.

Substrate	Growth Rate (hour^{-1})	Growth Rate (hour^{-1}) Previous Tests
Ethylene	0.038	0.014
½ Ethylene	0.026	--
½ Acetate	0.028	0.018 (full acetate)
½ Propionate	0.028	0.011 (full propionate)
Acetate/Ethylene	0.043	0.0212
Propionate/Ethylene	0.042	--
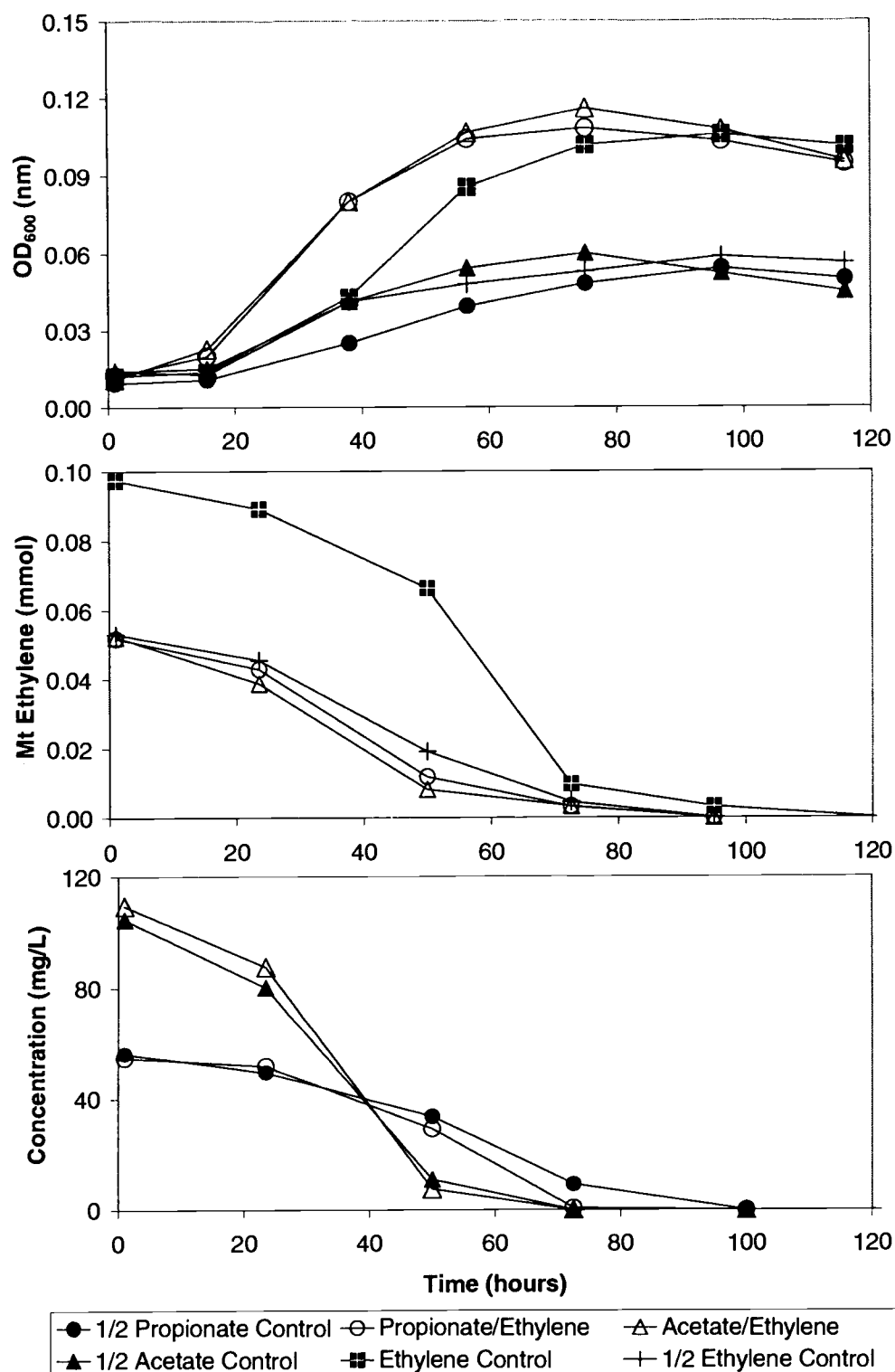
Figure. 3.5. *Mycobacterium* strain JS60 growth curves and substrate consumption curves on ethylene and an organic acid compared to either ethylene alone or the organic acid alone. (a) OD_{600} growth curves, (b) ethylene total mass, (c) organic acid concentration.

Growth Experiments on Ethylene or Vinyl Chloride in the Presence of Acetate

We chose to continue our experimentation using acetate, because the growth rate on acetate was faster than that of propionate, and acetate is commonly observed in contaminated groundwater plumes. Experiments were performed focusing on the effects of the presence of acetate on the growth of strain JS60 on both ethylene and VC. The experiment included a reactor containing acetate and ethylene, a reactor containing acetate and VC, and three control reactors each containing either acetate, ethylene, or VC. These control reactors where used for growth and utilization rate comparisons to the reactors which contained both substrates. The amount of each substrate added is shown in Table 3.5.

Table. 3.5. Substrate additions to the batch reactors.

Reactor	VC/Eth Added	Acetate Added	e- Equiv Added
Ethylene Control	94 μmol	--	0.56
Ethylene/Acetate	47 μmol	120 mg/L	1.14
Acetate Control	--	240 mg/L	1.15
½ Acetate Control		120 mg/L	0.58
Vinyl Chloride/Acetate	62.5 μmol	120 mg/L	1.20
Vinyl Chloride Control	125 μmol	--	1.25

The fastest rate of growth was observed in the ethylene/acetate reactor, while the slowest was observed in the reactor containing VC. The optical density peaks were similar in the acetate and ethylene/acetate occurring at around 75 hours. The maximum optical density peak of ethylene and VC/acetate reactors occurred at 100 hours. The

maximum optical density peaks for these reactors were comparable, indicating similar amounts of growth occurred. When acetate was present, the growth curves on ethylene and VC begin to look more like a growth curve on acetate alone (Figure 3.6 and 3.7). The time to peak OD is reduced from approximately 100 hours to around 75 hours for ethylene, and from 225 hours to 100 hours for VC. The growth rates of ethylene and VC were calculated as previously described. In the presence of acetate, the growth rates were increased by 32% and 65%, respectively (Table 3.6). In the previous experiment, the growth rate of ethylene and acetate together compared to ethylene alone was only increased by 12%.

The growth yield was calculated by taking the change in protein from start to OD peak, and dividing by the total amount of substrate added. The calculated yield on acetate was 0.63 mg protein/mg substrate, whereas the yield of ethylene was 0.87 mg protein/mg substrate. When these substrates are together, the calculated yield is slightly higher than that of ethylene, at 0.87 mg protein/mg substrate. Also, the yield for acetate and VC increased to 0.47, compared to 0.31 for VC alone.

The gross substrate utilization rates were calculated by computing the total amount of substrate consumed divided by the time until optical density peak and the maximum amount of protein produced. Based on the ethylene utilization data, the rate of ethylene disappearance is faster when acetate is not present. A result of particular interest is the large increase in the VC utilization rate for the batch reactor containing both acetate and VC. The utilization rate increased by 48% over the utilization rate for VC alone (Table 3.7).

The results from the duplicate test are very similar to the original test (Figures 3.7 and 3.9). The comparisons of the growth rates and utilization rates are found in Tables 3.8 and 3.9 and are nearly identical between the two trials. There is no more than a 5% difference between the rates it any of the duplicate reactors.
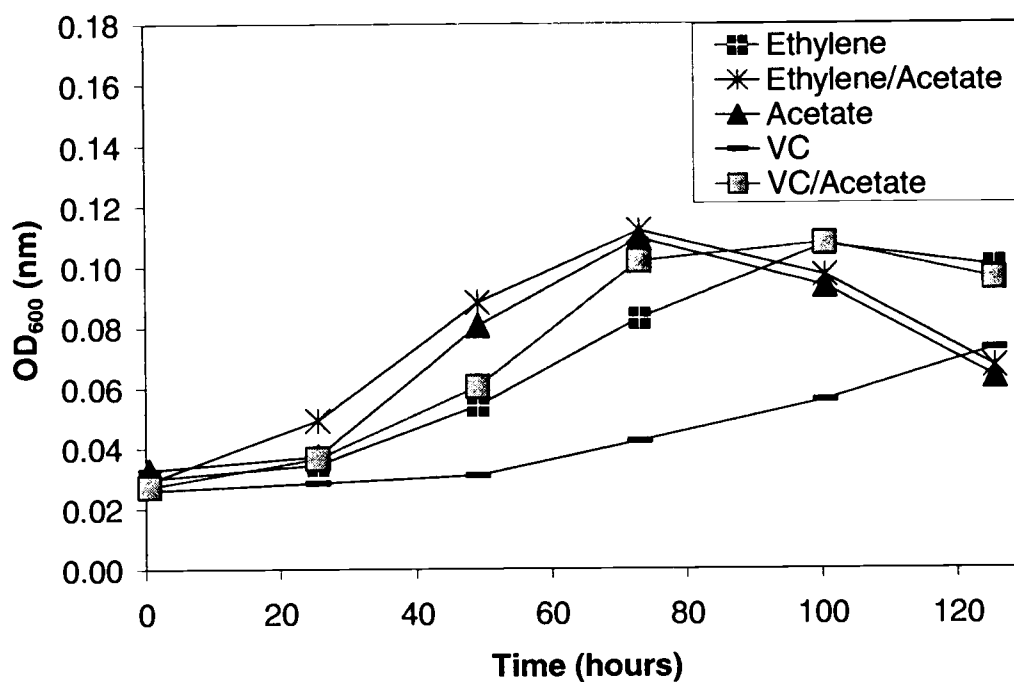
Figure. 3.6. Growth curves of ethylene and VC in the presence of acetate. The experiment was plotted against the controls of each substrate.
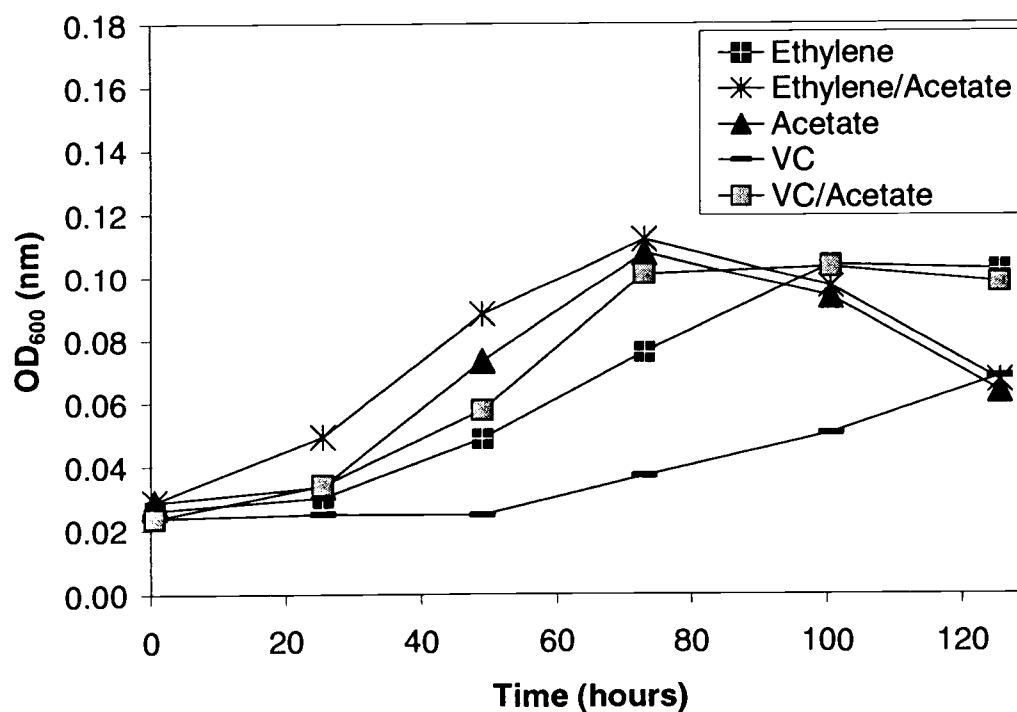
Figure. 3.7. Growth curves of ethylene and VC in the presence of acetate. The experiment was plotted against the controls of each substrate.
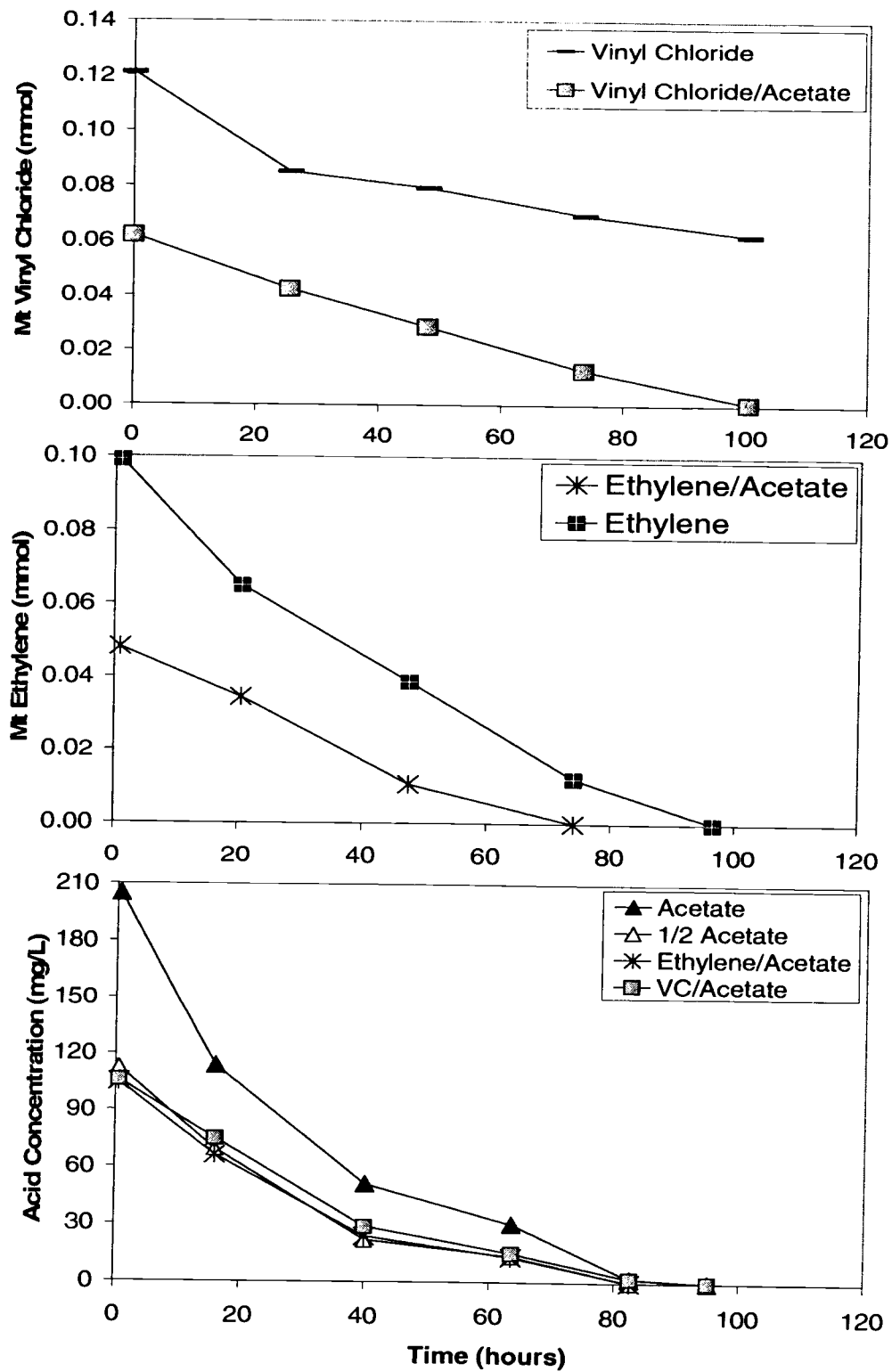
Figure. 3.8. Gas chromatograph data for (a) ethylene and (b) vinyl chloride utilization alone and in the presence of acetate. (c) High pressure liquid chromatograph (HPLC) data for acetate alone, and in the presence of VC or ethylene.
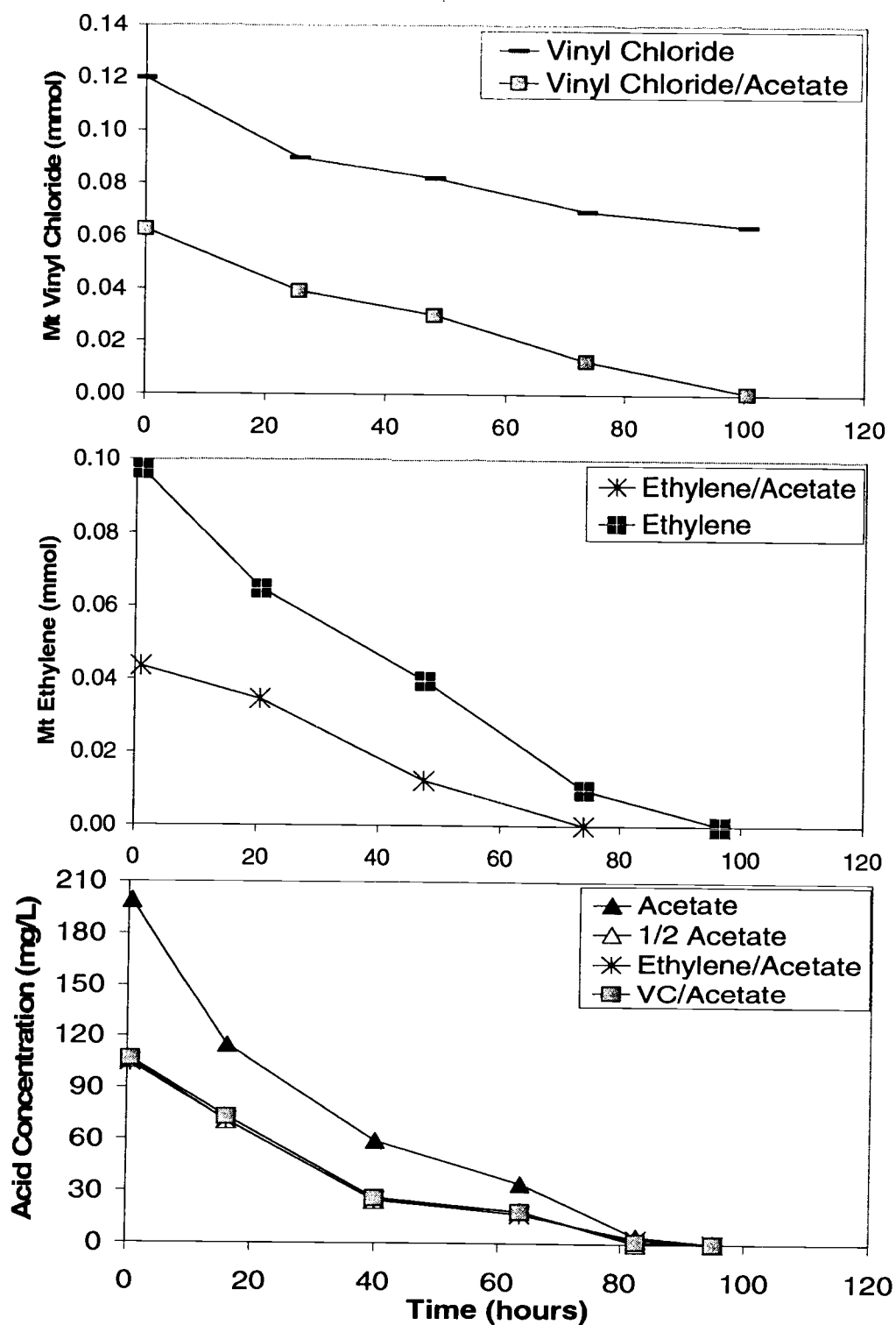
Figure. 3.9. Gas chromatograph (GC) data for the duplicate trial (a) ethylene and (b) vinyl chloride utilization alone and in the presence of acetate. (c) High pressure liquid chromatograph (HPLC) data for acetate alone, and in the presence of VC or ethylene.

Table. 3.6. The growth rate comparisons based on OD_{600} analysis for this experiment compared to the previous two experiments.

Substrate	Growth Rate (hour^{-1})	Growth Rate (hour^{-1})* Previous Tests	Growth Rate (hour^{-1}) Previous Tests
Ethylene	0.015	0.038	0.015
VC	0.006	0.026	0.006
Acetate	0.018	0.028 (1/2 acetate)	0.017
Acetate/Ethylene	0.021	0.043	--
Acetate/VC	0.019	--	--

* Growth likely occurred at temperature above $20^{\circ}C$.

Table. 3.7. Some kinetic parameters calculated based on the data sets provided by this experiment.

Substrate	Yield (g protein/ e- equiv)	Yield (g protein/ g substrate)	Gross Utilization Rate (nmol/hr/ mg protein)
Acetate	0.10	0.69	12.1
Ethylene	0.01	0.87	6.5
Acetate/Ethylene	0.11	0.87	9.2
VC	0.08	0.31	5.2
Acetate/VC	0.09	0.47	9.9

Table. 3.8. The growth rate comparisons for duplicate trials of the same experiment.

Substrate	Duplicate Trial 1 Growth Rate (hour^{-1})	Duplicate Trial 2 Growth Rate (hour^{-1})
Ethylene	0.015	0.015
VC	0.007	0.006
Acetate	0.019	0.018
Acetate/Ethylene	0.022	0.021
Acetate/VC	0.019	0.019

Table. 3.9. A summary of kinetic parameters calculated based on the data sets provided by a duplicate trial of the same experiment. The duplicate trial appears to be similar.

Substrate	Duplicate Trial 1 Gross Utilization Rate (nmol/hour/mg protein)	Duplicate Trial 2 Gross Utilization Rate (nmol/hour/mg protein)
Acetate	12.1	12.6
Ethylene	6.5	6.7
Acetate/Ethylene	9.2	9.4
VC	5.2	5.1
Acetate/VC	9.9	9.4

Growth on Vinyl Chloride in the Presence of Variable Amounts of Acetate

This experiment was designed to determine the effects of different amounts of acetate on the rate of VC utilization. Each batch reactor contained 0.1 mg cells, and 62.5 μmol of VC, and a different amount of acetate added ranging from 0 mg/L to 120 mg/L. At 95 hours into the experiment, just before the VC was fully consumed, all of the batch bottles were re-fed with VC only. See Table 3.10 for substrate additions during the experiment.

Table. 3.10. VC and acetate additions to the batch reactors.

Growth Reactor	VC Addition at 0 Hours	Acetate Addition at 0 Hours	e- Equiv Added at 0 Hours	VC Addition at 95 Hours
VC Control	62.5 μmol	0 mg/L	0.63	62.5 μmol
1	62.5 μmol	20 mg/L	0.70	62.5 μmol
2	62.5 μmol	40 mg/L	0.77	62.5 μmol
3	62.5 μmol	80 mg/L	1.05	62.5 μmol
4	62.5 μmol	120 mg/L	1.20	62.5 μmol

Duplicate sets of reactors indicate that the growth rates, yield, and utilization rates were very reproducible. Results of duplicate experimental set are shown in Figure 3.10 and 3.11, and Table 3.10 and 3.11.

The growth curves show that the OD peak is highest in the reactor provided the most substrate, and lowest with the VC control. The OD peak for the reactor containing 120 mg/L acetate is almost two times higher than the reactor containing 20 mg/L acetate. The growth curves accurately reflect the amount of substrate provided to each reactor.

The VC consumption curves of all the reactors containing acetate were equally enhanced, even with the lowest amount of acetate added (20 mg/L). After the reactors were fed VC at 95 hours, the VC consumption rate was still much higher in the reactors that had initially contained acetate, compared to the VC control reactor. It was apparent that the enhanced rates were not due to greater cell mass (Figure 3.10 and 3.11). Reactors fed 20 mg/L acetate produced half the OD_{600} of reactors fed 120 mg/L of acetate, yet all reactors containing acetate exhibited similar VC utilization rates. Zero-order rates were initially a factor of three times greater than the VC control reactor. After the VC addition at 95 hours, the zero-order rates remained a factor of two greater than the VC control reactor.

As indicated by the growth curves, the calculated growth rates were highest in the reactors with the most acetate, and lowest on the VC control. The VC growth rate was determined to be 0.007 hour^{-1}. This rate was reproducible in three out of four VC degradation experiments. The growth rates for the reactors containing acetate increased by 1% between reactors 1 and 2, and 14% between the other reactors, as the amount of acetate substrate provided increases (Table 3.10). In the duplicate experiment the rates increase by 3%, 6%, and 16% between each reactor (Table 3.11).

The growth yield was lowest in the reactor given VC alone and highest in the reactor with the most amount of acetate. It is interesting to notice that the only yield that increases after the addition of VC at 95 hours was that of the VC control reactor. All of the other reactors do not experience the same amount of protein increase during the second phase of the experiment as they did during the initial phase.

The utilization rates calculated from the biomass measured before and after the

VC addition at 95 hours are provided in Table 3.10 and 3.11. Substrate utilization rates were highest in the reactor with only 20 mg/L of acetate. The utilization rate prior to the VC addition at 95 hours was three times higher than the utilization rate in the VC control reactor. In the reactor containing the 120 mg/L of acetate, the utilization rate was similar to the VC control. After the VC addition, the utilization rates in all the reactors increased significantly. The largest increase occurred in the VC control reactor. However, the utilization rate for VC control was much lower than those calculated for VC in previous experiments. This could have been due to the lower concentrations of VC used in this experiment.

Acetate consumption data was not taken for this experiment. In all other experiments acetate was fully consumed by hour 80, so therefore it is unlikely that there was any acetate present at hour 95 when the bottles were fed VC. Additionally, the yield calculation before the reactors were fed at 95 hours with VC supports the assumption that all acetate had been consumed, since the yields are similar to those found in other experiments containing the same amount of substrate (i.e. reactor 4 is identical to the Acetate/VC reactor in the previous experiment.) (Table 3.11).
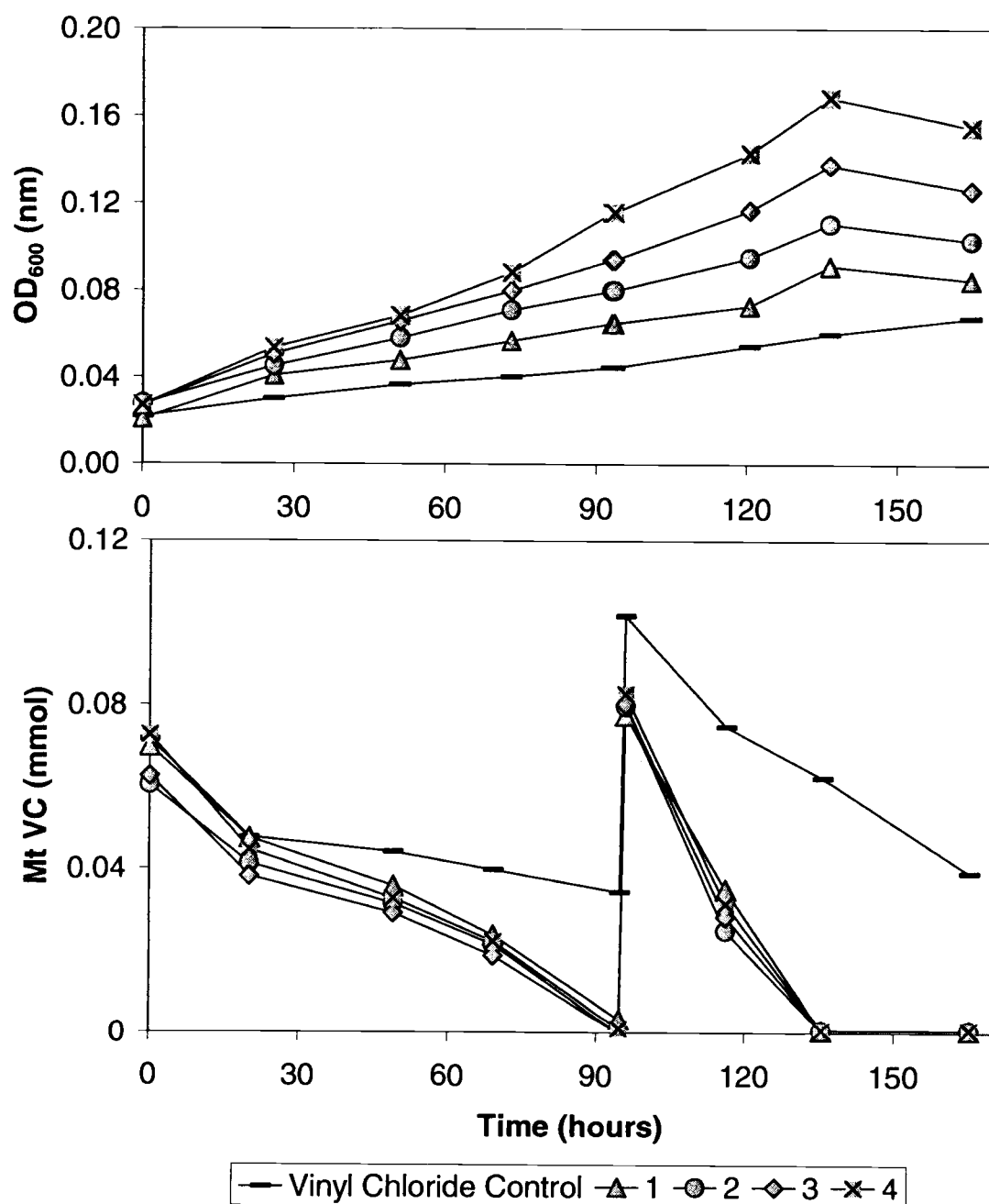
Figure. 3.10. Experimental results for Set 1 of the degradation of VC in the presence of different acetate concentrations; (a) growth curves, (b) VC consumption. ▲-20 mg/L of acetate, ●-40 mg/L of acetate, ♦- 80 mg/L of acetate, and ■-120 mg/L.
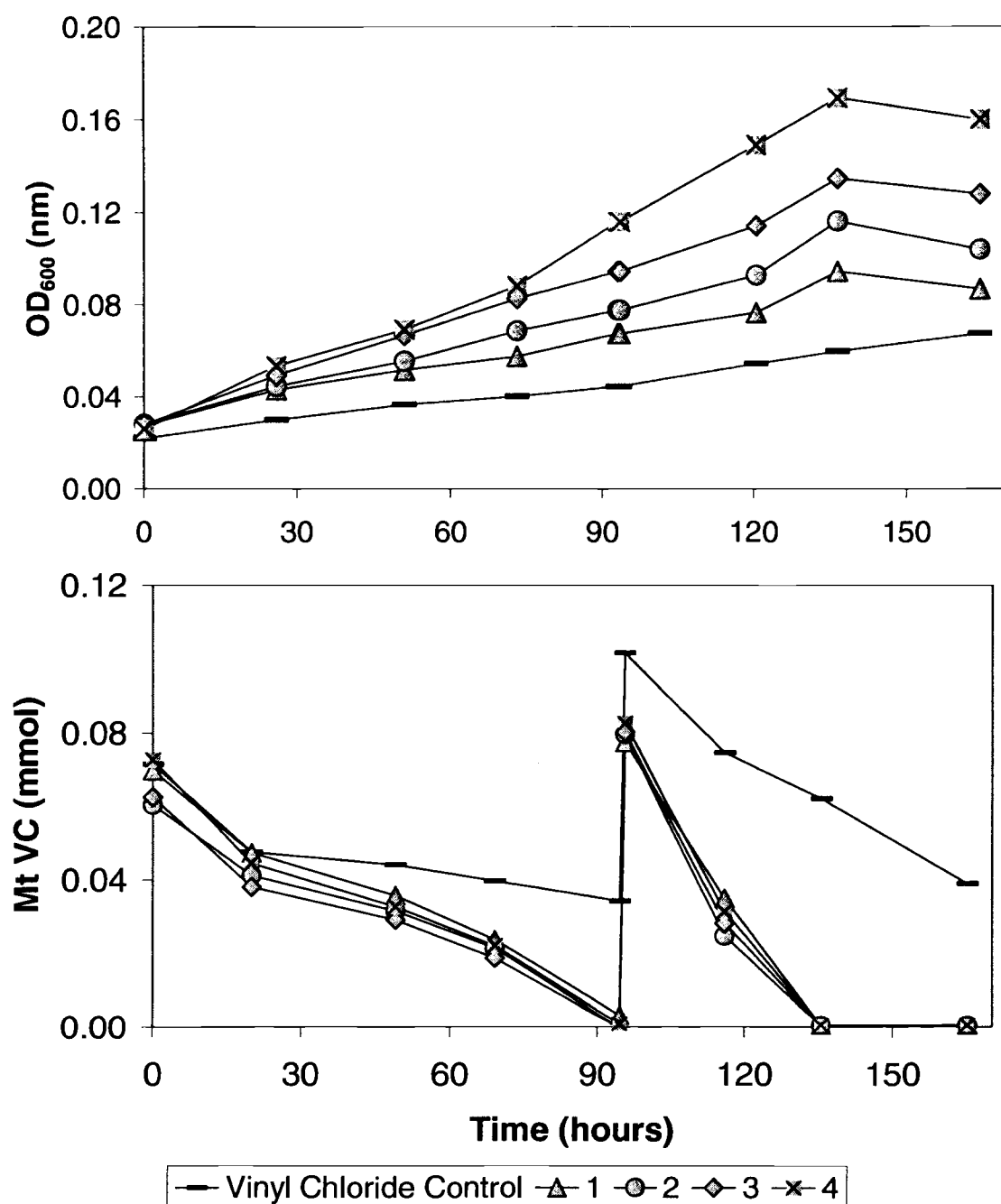
Figure. 3.11. Experimental results for Set 2 of the degradation of VC in the presence of different acetate concentrations; (a) growth curves, (b) VC consumption. ▲-20 mg/L of acetate, ●-40 mg/L of acetate, ♦- 80 mg/L of acetate, and ■-120 mg/L.

Table. 3.11. Kinetic results for Set 1 of the VC degradation test with different concentrations of acetate in each batch reactor.

Substrate	Growth Rates (hour^{-1})	Prior to VC Addition Yield (g protein/ e- equiv)	Yield for Entire Experiment (g protein/ e- equiv)	Prior to VC Addition Utilization Rate (nmol/hour/ mg protein)	After VC Addition Utilization Rate (nmol/hour/ mg protein)
VC Control	0.007	0.07	0.05	5.9	21.8
1	0.009	0.09	0.06	16.4	34.5
2	0.009	0.10	0.07	11.5	30.6
3	0.011	0.09	0.08	8.5	23.6
4	0.013	0.09	0.08	5.5	18.6

Table. 3.12. Kinetic results for Set 2 of the VC degradation test with different concentrations of acetate in each batch reactor.

Substrate	Growth Rates (hour^{-1})	Prior to VC Addition Yield (g protein/ e- equiv)	Yield for Entire Experiment (g protein/ e-equiv)	Prior to VC Addition VC Utilization Rate (nmol/hour/ mg protein)	After VC Addition VC Utilization Rate (nmol/hour/ mg protein)
VC Control	0.007	0.07	0.05	5.5	21.5
1	0.009	0.01	0.07	16.0	34.9
2	0.009	0.10	0.08	11.2	30.3
3	0.010	0.09	0.08	8.7	23.2
4	0.012	0.09	0.09	5.6	18.7

CONCLUSIONS

The purpose of these experiments was to determine how ethylene and VC

utilization by *Mycobacterium* strain JS60 was effected by the presence of organic acids

that could serve as potential growth substrates in contaminated aquifers.

- Coleman et al. (2002) provided results showing that strain JS60 is capable of

    growth on ethylene and VC as the sole carbon source. We confirmed these results

    in our initial growth tests. The growth rates calculated in our experiments of 0.33

    day^{-1} on ethylene, and 0.15 day^{-1} on VC were similar to the rates reported by

    Coleman et al. (2002) of 0.58 day^{-1} on ethylene, and 0.22 day^{-1} on VC.

- Culture purity was maintained throughout the tests, based on plating and

    molecular assays. Except for the one experiment where growth rates were

    approximately doubled, the behavior of the cells during growth was consistent

    throughout the experiments. The reproducibility of the growth rates, yields, and

    OD peak times, except for where already noted, attested to the consistent behavior

    of the batch reactors. JS60 when grown on plates was light yellow colored,

    opaque and mucoid colony in about 1-2 weeks, consistent with the observations

    of Coleman et al. (2002). The culture showed no clumping and only limited wall

    climbing in liquid media, which was also consistent with observation of Coleman

    et al. (2002).

- Strain JS60 was able to grow on acetate, propionate, and butyrate, but not on

    lactate or formate. The cells grew most rapidly on acetate (0.0182 hour^{-1}),

followed by propionate (0.0128 hour^{-1}), and butyrate (0.0066 hour^{-1}). Rates were therefore fastest the lower the carbon number, however growth was not observed on formate, which contains a single carbon.

- When batch reactors were provided acetate or propionate in addition to either ethylene or VC the growth rate increases, compared to growth on ethylene or VC alone.

- The zero-order degradation rate of VC is three times greater in the presence of at least 0.012 mmol of acetate, when compared to the degradation rate of VC alone. The degradation rate was found to be the same regardless of the amount of acetate added between 0.012 mmol to 0.072 mmol. However, due to the increase in cell density in the reactors containing more acetate substrate, the utilization rate of VC was found to be the greatest in low concentrations of acetate.

Our experimental results therefore show that degradation rates of ethylene and VC are increased in the presence of acetate. These experimental results indicate that there may be a benefit of having organic acids present for the aerobic natural attenuation of VC in contaminated groundwater sites.

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CHAPTER 4

Ethylene and Vinyl Chloride Kinetics, and
Cometabolic Utilization of c-DCE, t-DCE, and 1,1-DCE

SUMMARY

Better information is needed to understand the degradation kinetics of aerobic VC natural attenuation in contaminated groundwater. Coleman et al. (2002) determined t K_S and k_{max} values for strain JS60. We performed similar tests as part of our study on the effects of acetate on VC and ethylene degradation kinetics. Batch kinetic tests were conducted on both ethylene and VC utilization at three different concentrations. The ethylene tests produced concentration versus time depletion curves that follow zero-order kinetics at high concentrations and first-order kinetics at lower concentrations. To estimate half-saturation constant (K_S) and k values, these data were fit using a nonlinear least squared regression (NLSR) model described by Smith et al. (1998). The fit produced a K_S for ethylene of 0.93 µM. This value is in good agreement with the K_S reported by Coleman et. al. (2002) of 0.9 µM. The k value of 0.23 day^{-1}, was lower than the value reported by Coleman et al. (2002) of 0.53 day^{-1}. Due to analytical detection limits we were unable to determine a K_S or k for VC. However, we estimated a pseudo 1st order reaction rate for VC of 0.12 day^{-1}.

Another important process is the ability of JS60 when grown on acetate, ethylene,

or VC to cometabolize more chlorinated ethenes. Coleman et. al. (2002) found that strain JS60 could not grow on c-DCE as the sole carbon source, however further tests with c-DCE were not conducted. We conducted cometabolic transformation tests with c-DCE using cells grown on acetate, ethylene, and VC. Strain JS60 tested positively for cometabolic transformation of c-DCE with acetate, ethylene, and VC as growth substrates. The presence of c-DCE appeared to partially inhibit VC degradation. The cometabolic transformation of t-DCE and 1,1-DCE was also evaluated with acetate as the growth substrate. Some transformation of t-DCE was observed, but no significant transformation activity was obtained for 1,1-DCE. c-DCE was the most rapidly transformed of the three isomers.

Another important process is inhibition. Kinetic inhibition tests were conducted to more fully understand the effects that each substrate has on strain JS60 degradation capabilities and substrate preferences. Strain JS60 was found to preferentially utilize VC over ethylene. Ethylene degradation was not significantly effected by the presence of c-DCE, and c-DCE was slowly transformed after ethylene concentrations fell below about $6\text{-}\mu M$. After all the ethylene was consumed, the transformation rate of c-DCE increased briefly, and then ceased. VC degradation rates were significantly effected by c-DCE when both VC and c-DCE were added simultaneously to resting cells. However, when VC was added before c-DCE the VC degradation rates showed no inhibition.

INTRODUCTION

Kinetic parameters of K_S and k for ethylene and VC for strain JS60 have been previously determined by Coleman et. al. (2002). The K_S for ethylene and for VC are 0.9 μM and 0.5 μM, respectively. The k values for ethylene and VC were 0.58 day^{-1} and 0.22 day^{-1}, respectively. The K_S values indicate that substrate growth on VC will be preferential to ethylene as a substrate. Since both ethylene and VC are often found together in contaminated groundwater plumes, it is beneficial that this bacterial stain will consume VC prior to ethylene. VC is the only chlorinated ethylene to be listed as a carcinogen, and is therefore of the utmost human health concern when designing a treatment strategy.

The DCE isomers at this point are suspected carcinogens. c-DCE is also a common groundwater contaminant, resulting from the anaerobic transformations of PCE and TCE. Disappearance of c-DCE at the aerobic fringe due to natural attenuation has been documented in the field (Cox et al., 1995; Edwards and Cox, 1997). One possible explanation of this disappearance is that c-DCE is cometabolized by aerobic bacteria capable of growth on VC or ethylene. *Mycobacterium aurum* L1 was the first VC-grown organism reported to cometabolize DCEs (Hartmans and de Bont, 1992). Also, aerobic cometabolism of chlorinated ethenes has been demonstrated using microbes stimulated by methane, phenol, toluene, and other regulated organic compounds (US EPA, 2000). Acetate, a non-regulated organic compound is common in contaminated groundwater plumes containing chlorinated ethenes (Figure. 2.4), and would therefore be an ideal substrate for driving aerobic cometabolism.

The DCE isomers, t-DCE and 1,1-DCE, occur in much smaller percentages in contaminated chlorinated ethene plumes. *B. cepacia* G4 expressing a toluene *ortho-monooxygenase* is one microbe capable of cometabolizing t-DCE (Rui, 2004). Unfortunately, due to high transformation product toxicity, 1,1-DCE is a problematic compound to transform via aerobic cometabolism (Dolan and McCarty, 1995; Hopkins and McCarty, 1995). Through batch growth studies, we determined that *Mycobacterium* strain JS60 is capable of cometabolism of c-DCE and t-DCE with acetate and ethylene as the growth substrates.

MATERIALS AND METHODS

Chemicals

Ethylene gas (99.5%), and vinyl chloride gas (99.5+%) were purchased from Aldrich (St. Louis, MO); sodium acetate anhydrous (99.3%) and lactic acid (85%) were purchased from Mallinckrodt Chemical (Paris, KT); sodium propionate (99%), sodium butyrate (99%) and sodium formate (99+%) were purchased from Aldrich (St. Louis, MO).

VC gas was transferred directly from the stock bottle to the batch reactors used for the kinetic, inhibition, or cometabolic experiments. Ethylene gas was transferred into a double-flask container. Measured quantities of ethylene gas were then filtered with a

Pall 0.2 μm PTFE Acrodisc prior to injection into the batch reactors. Aqueous stock solutions of c-DCE, t-DCE, and 1,1-DCE were prepared at room temperature by adding specific amounts of the liquid to a 27 mL crimp-capped bottle containing 15 mL of autoclaved deionized water to achieve the desired aqueous concentration. All experimental growth reactors contained 50 mL of 1/5 MSM growth media and deionized water in 125 mL batch bottles. All kinetic and inhibition experiment reactors contained 4 mL of MSM growth media in 27 mL batch vials. See Appendix A for MSM mixture.

Analysis

All of the batch growth reactors were continuously shaken at 200 rpm on a rotary shaker to achieve effective mass transfer between the gas and liquid phases. Consumption of ethylene and vinyl chloride, and the transformation of the DCE isomers were monitored by gas chromatograph (GC) analysis of headspace samples (Kampbell at al., 1989). 100 μl samples were injected into a HP6890 series GC using a DB 624 column, 30 m long, I.D. 0.25 mm narrowbore, 1.4 μm film (Agilent Technologies) operated at 50°C. The gaseous concentrations were determined by headspace analysis, as previously described in Chapter 3.

Consumption of acetate was monitored by high pressure liquid chromatograph (HPLC). A 1 mL liquid sample was taken daily from the batch growth reactors for acid analysis. The concentrations of the acetate was measured using a Dionex 500 series

high-pressure liquid chromatograph (HPLC) with a Prevail Organic Acid 5-µm column

(Altech), operated at 25°C, with a AD 20 series Absorbance Detector. The HPLC was

calibrated as previously described in Chapter 3.

Optical density (OD) was measured at 600 nm using a HP8453 UV-Visible

spectrophotometer. The biomass concentration was determined by measuring TSS

(American Public Health Association, 1985). A 1 mL liquid sample was filtered using a

0.22 µm membrane filter (Osmonics Inc., Minnetonka, MN), then placed in a drying

oven at 105°C for at least 24 hours.

The kinetic data was modeled using a nonlinear least squared regression

(NLSR) model described by Smith et al. (1998), to estimate half-saturation constant (K_S)

and k values. The Monod equation for the susbstrate reaction rate is a bacterial culture

is;

$$-(dC_L/dt) = kX_aC_L/(K_S+C_L) \qquad \text{Equation 4.1}$$

where C_L is the liquid-phase concentration (mg/L), t is time (day), k is the maximum

specific rate of substrate utilization (mg substrate/mg cells/day), X_a is the concentration

of active cells (mg/L), and K_S is the half-velocity coefficient (mg susbtrate/L).

The active cell concentration with growth susbstrate utilization can be described

by;

$$X_a = X_{a0} + Y(C_{L0} - C_L) \qquad \text{Equation 4.2}$$

where Y is the cell yield (g cells/g substrate), and the subscript 0 denotes the time zero.

By combining these equations, the kinetic parameters of K_S and k can be

determined by NLSR analysis by fitting measured substrate concentrations to the

integrated form of the Monod equation (Equation 4.2.) (Smith et al., 1998);

$$t = \frac{1}{k} \left\{ \left(\frac{K_S}{X_{a0} + YC_{L0}} + \frac{1}{Y} \right) \ln[X_{a0} + Y(C_{L0} - C_L)] \right.$$

$$\left. + \left(\frac{K_S}{X_{a0} + YC_{L0}} \right) \ln\left(\frac{C_{L0}}{X_{a0}C_L} \right) - \frac{1}{Y} \ln(X_{a0}) \right\}$$

Equation 4.3

The fitting parameters of this NLSR model were the half velocity constant K_S, the initial substrate concentration C_L, and the substrate utilization constant k. NLSR requires an initial estimate for each unknown parameter as input. Kinetic data for strain JS60 degradation of ethylene reported by Coleman et al. (2002) we used as the initial estimates.

The NLSR method used has some inherent uncertainties. When the liquid concentration values in Equation 4.1 are less than the K_S calculated, then the Excel Solver function begins to encounter numerical errors during the iterations.

Culture Maintenance

Strain JS60 was grown in batch culture as previously described in Chapter 3. For growth experiments the initial amount of cells was low, at 0.025-0.04 OD_{600} (approximately 0.1 mg cells), for kinetic experiments the initial amount of cells was high, at 1.1-1.6 OD_{600} (3-5 mg cells) for kinetic and inhibition experiments. In order to prepare enough cells for the kinetic tests, a 20-mL aliquot of the cell suspension grown in the 125 mL mother reactor was transferred to each of eight 500 mL capped-bottles containing 30

mL of ethylene gas, 300 mL 1/5 MSM growth media, and 450 mL of headspace containing air. The bottles were shaken at 200 rpm at 20°C. The cells were fed on a weekly basis, and allowed to incubate for one to two months prior to usage. Cells were harvested by centrifuge (7,500 x g for 20 minutes), washed and suspended in 10 mL of MSM growth media, to give a final cell density of 1.6 OD_{600} or 5.2 mg cells. All kinetic experiments were conducted within a 48 hour time period after the cells were harvested.

Batch Kinetic and Inhibition Reactors

Ethylene, VC, or c-DCE were added to 27 mL batch kinetic vials. The initial concentrations of substrates were determined after 30 minutes of rotary shaking at 300 rpm. The concentrations were determined by GC analysis using a 100 µL sample. All kinetic experiments were conducted in duplicate or triplicate, and also included an abiotic control, which contained substrate but no cells. The control bottle was used to ensure that there were negligible losses of substrates through the cap for the duration of the experiment.

After an initial substrate concentration was determined, a 1 mL aliquot of the cell suspension (5-7 mg cells) was then added to the 4 mL of MSM growth media and 23 mL of headspace containing air. The vials were vigorously shaken at 300 rpm and 20°C to minimize mass transfer limitations. 100 µL samples for GC head-space analysis were periodically taken throughout the duration of the experiment. These kinetic experiments lasted until the substrate was completely consumed, anywhere between 2-13 hours.

RESULTS AND DISCUSSION

Ethylene Kinetics

The ethylene kinetic experiments were conducted at three different starting concentrations, 9 μmol, 4.5 μmol, and 2.2 μmol (Figure. 4.1). The amount of biomass added was 5.2 mg of cells. The experiment was repeated again with the addition of only 2.2 μmol of ethylene, and increased sampling frequency to obtain a higher number of data points (Figure 4.2). The non-linear least squared regression (NLSR) method (Smith et. al., 1998) was applied to these data sets (Figures 4.3 and 4.4). The initial kinetic guesses for strain JS60 degradation of ethylene were found in Coleman et. al. (2002). The NLSR method produced a K_S of 0.93 μM and 0.89 μM, and k of 0.29 day^{-1} and 0.23 day^{-1}, Coleman et. al. (2002) reported a K_S of 0.9 and a k of 0.58 day^{-1} (Figure 4.3 and Table 4.1).

The equation used in the model (Equation 4.3) calculates the k for a non-volatile substrate that is completely in the liquid phase, such as acetate. However, ethylene is a volatile growth substrate with a high Henry's constant of 7.64. Therefore, the k value produced by the model is lower than the expected k for the substrate because the substrate is present in two phases, as follows;

$$M = C_L V_L + C_G V_G \qquad \text{Equation 4.4}$$

where M is total mass (mg), C_G is the gas-phase substrate concentration (mg/L), V_L is the liquid volume (L), and V_G is the gas volume (L).

Henry's Law is then used to account for the distribution of the substrate between the liquid and gas phases in equilibrium;

$$H_C = C_G/C_L \qquad\qquad \text{Equation 4.5}$$

where H_C is the Henry's constant of the substrate (7.64 for ethylene).

Where the equalities can be written as;

$$dM/dt = (V_L + H_C V_G)\, dC_L/dt = (kX_{a0}C_L/(K_S + C_L))V_L \qquad\qquad \text{Equation 4.6}$$
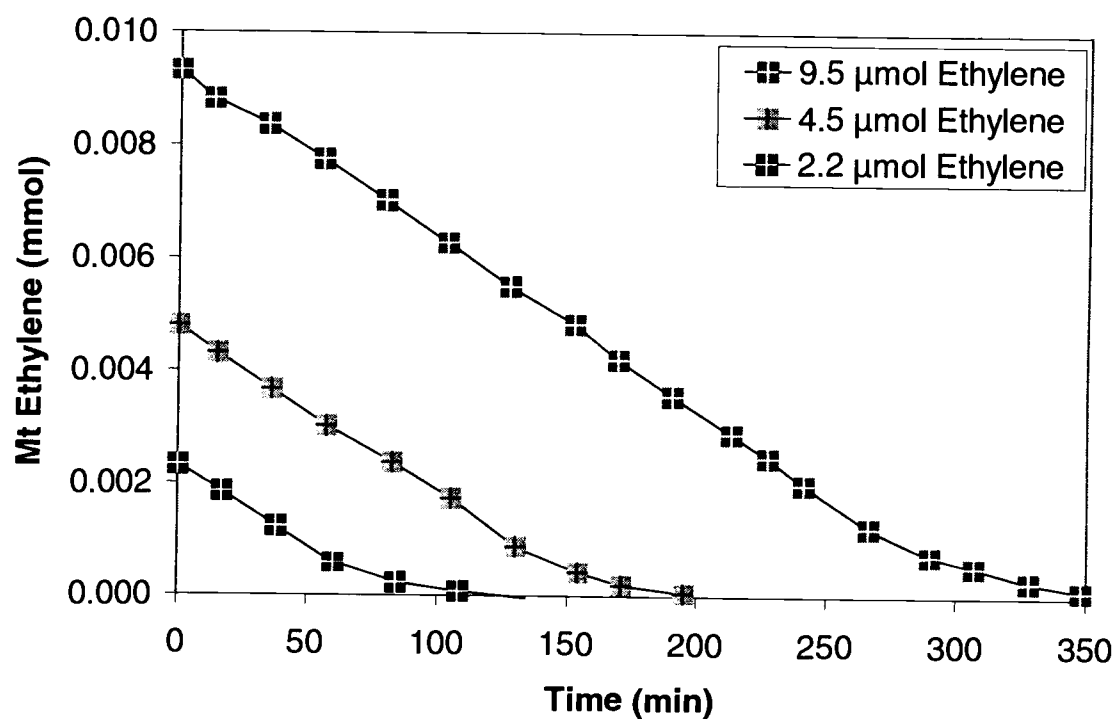
Figure. 4.1. Ethylene kinetics at three different starting concentrations, substrate additions made were 2.2 μmol, 4.5 μmol, 9 μmol.
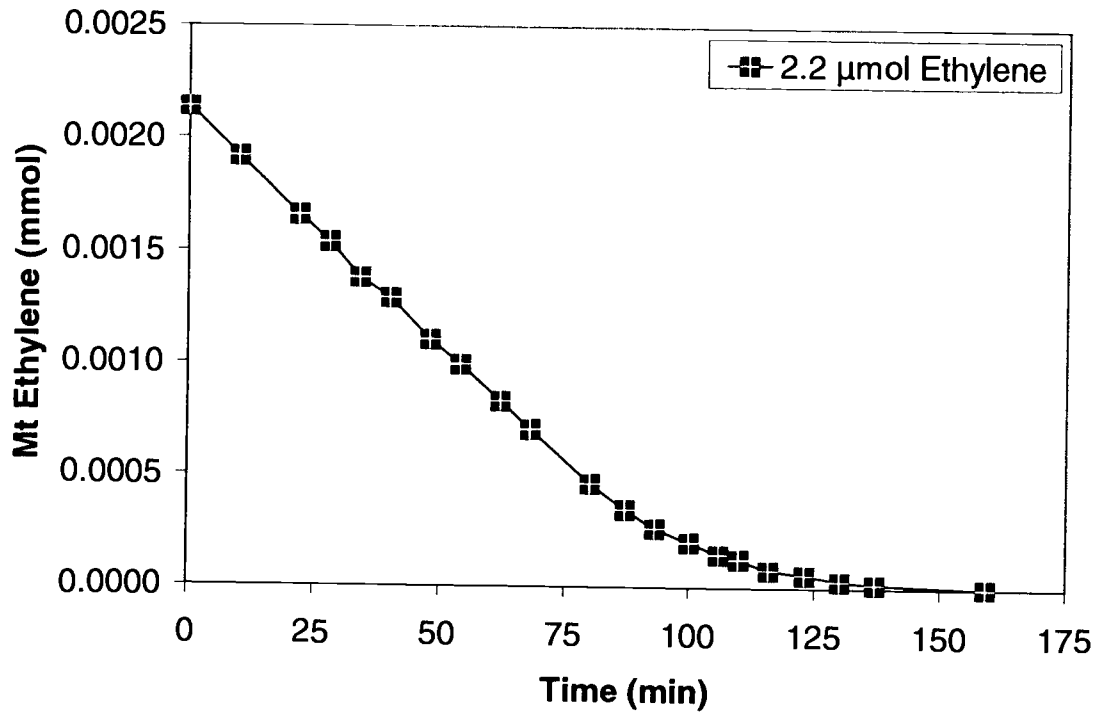
Figure. 4.2. Ethylene kinetics at 2.2 μmol.
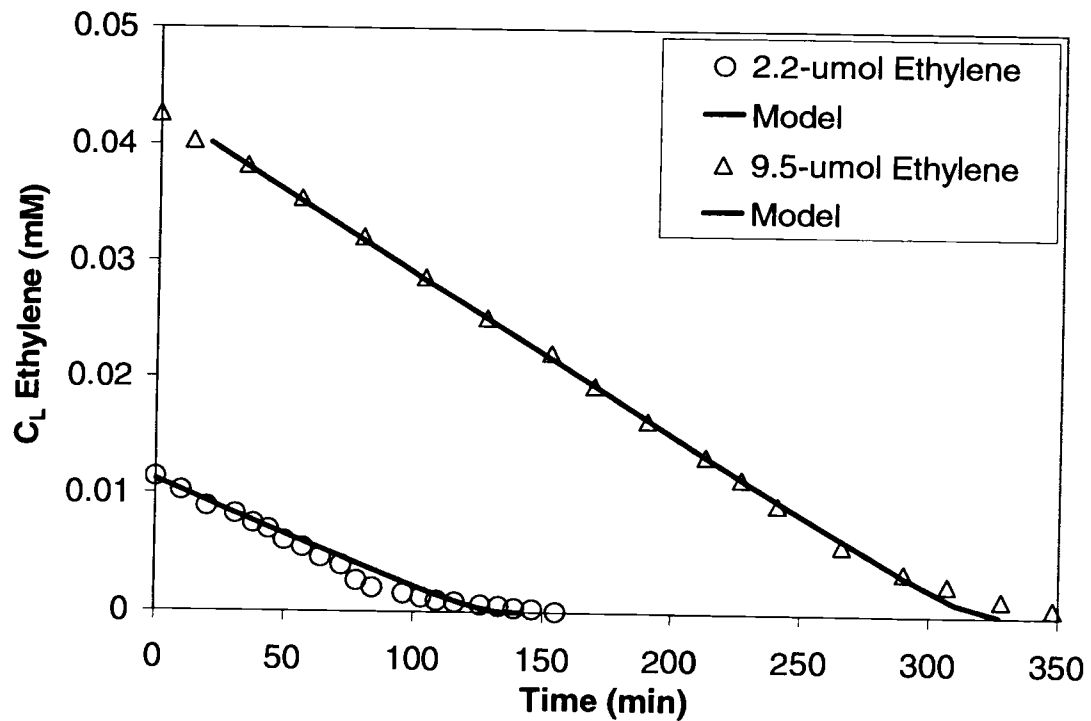
Figure. 4.3. Degradation kinetics fit by NLSR model.

Table. 4.1. The NSLR results compared to the kinetic parameters reported in Coleman et al. (2002).

Model Fit	K_S (μM)	k (day^{-1})
9 μmol Ethylene Trial	0.93	0.29
4.5 μmol Ethylene Trial	--	--
2.2 μmol Ethylene Trial	0.89	0.25
0.7 mmol Ethylene (Coleman et al. (2002))	0.9	0.58

Based on this kinetic analysis, as well as the growth behavior of the cell cultures, the culture remained pure throughout the studies. The colony appearance when grown on 1/10 TCA plates were light yellow in color, round, opaque, and mucoid. This was consistent with the observations of strain JS60 made in Coleman et al. (2002).

Vinyl Chloride Kinetics

The ethylene kinetics tests described previously were repeated with VC as a substrate. Due to analytical detection limits and sampling time constraints the initial concentration of cells added was decreased from OD of 1.6 (5.2 mg cells) to OD of 1.1 (3.6 mg cells). The substrate additions were also decreased to; 4.5 μmol, 2.2 μmol, and 1.1 μmol of VC. The degradation curve remained linear until the detection limit was reached around 0.065 μmol total mass of VC, representing an aqueous concentration of

0.0025 μmol/L (Figure 4.4). The experimental results were too limited to model K_S with

the NLSR method previously used. However, based on our observations in the kinetic

inhibition tests discussed later, K_S was lower for VC than ethylene. The K_S as reported

by Coleman et al. (2002) was 0.5 μM for VC, and 0.9 μM for ethylene. The pseudo 1st

order reaction rates for the VC kinetic trials were calculated by fitting a linear trendline to

the degradation data (Figure 4.5). The results are shown in Table 4.2. The pseudo 1st

order reaction rate was approximately 0.12 day^{-1} for all three trials.
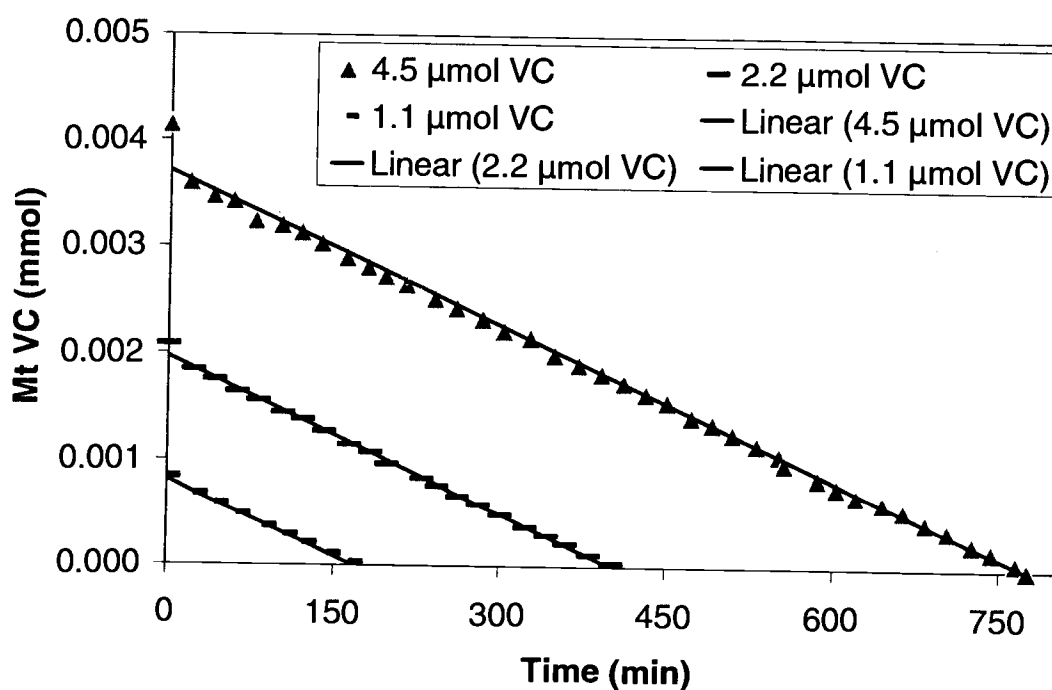
Figure. 4.5. VC kinetic tests at three different starting concentrations, substrate additions made 1.1 μmol, 2.2 μmol, and 4.5 μmol. The linear fit trendlines producing the pseudo 1st order reaction rate values.

Cometabolism of c-DCE, t-DCE, and 1,1-DCE with Acetate, Ethylene and VC as Growth Substrates.

The cometabolic transformation experiments were conducted with ethylene, VC, and acetate as the growth substrates. c-DCE was tested with all three substrates, while t-DCE and 1,1-DCE were tested with only acetate as a growth substrate. Each DCE isomer was tested separately by adding 3 μmol to a reactor, similar to the studies conducted by Coleman et al. (2002). The experiment lasted until the growth substrate was completely consumed, anywhere between 70-230 hours. The experiments were conducted similarly to the growth experiments described previously in Chapter 3.

The DCE isomers were tested for cometabolism with cells growing on acetate. Approximately 3 μmol of either c-DCE, t-DCE, or 1,1-DCE along with 0.144 mmol acetate was added into each reactor. The transformation curves of c-DCE, t-DCE, and 1,1-DCE are shown in Figure 4.6a. A significant amount of c-DCE was transformed, t-DCE showed some transformation, while 1,1-DCE showed no clear transformation activity (Figure 4.6a). All of the acetate in each reactor was consumed by 100 hours (Figure 4.6b). The acetate is the 1,1-DCE reactor was completely consumed by 75 hours. The majority of the CAH transformation activity occurred after acetate was consumed. This might indicate catabolic repression, where the induction of enzymes to metabolize a less desirable substrate also requires the absence of the preferable substrate. The preferred substrate, when present, represses the metabolism of alternative substrates (Brock, 2000).
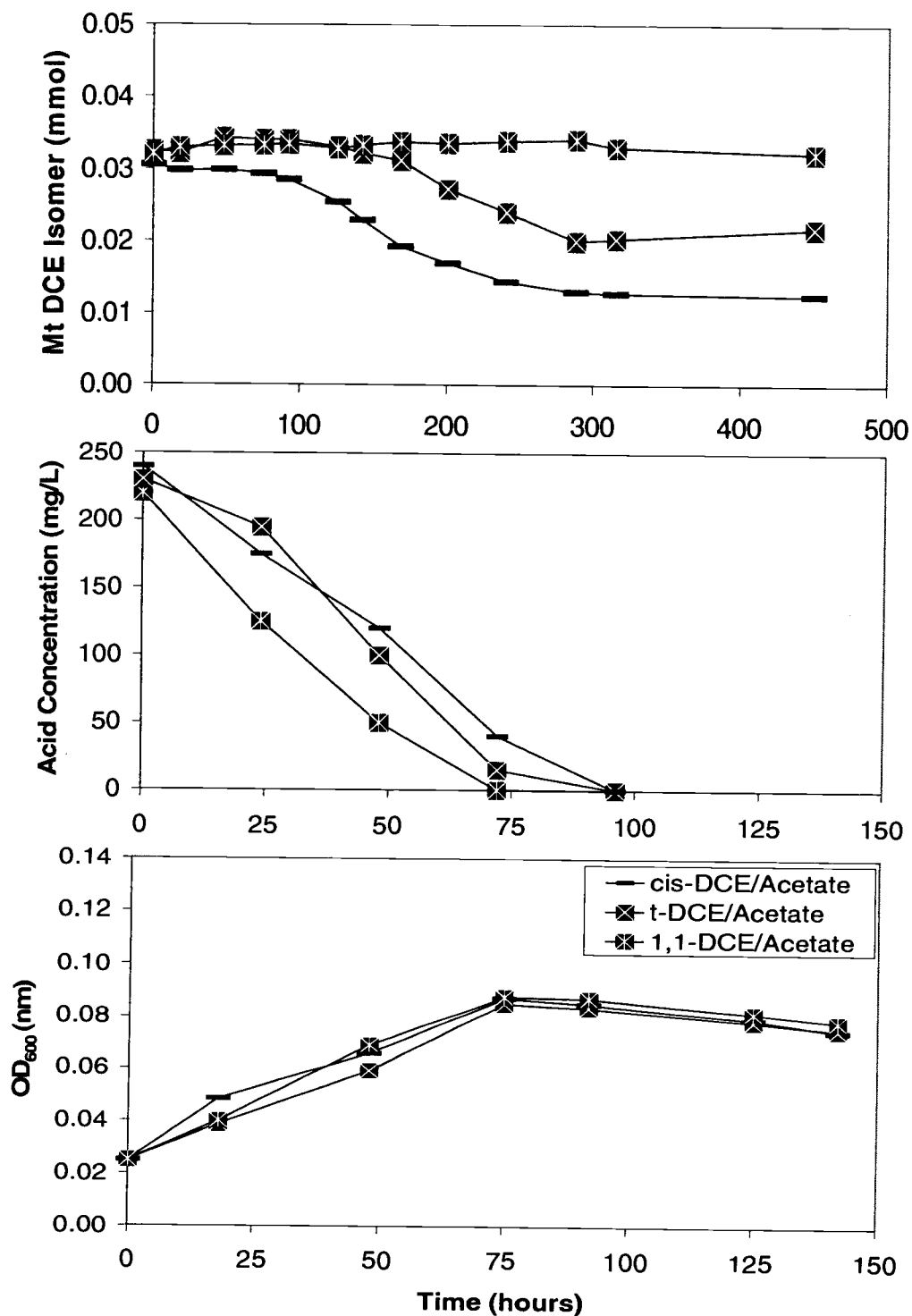
Figure. 4.6. The (a) transformation curves of DCE isomers by cells grown on acetate, (b) acetate consumption curves, (c) growth curves.

The cometabolic tests on c-DCE using VC and ethylene as a substrate were set up in a similar manner to those described above. Approximately 3 μmol of c-DCE was added each batch bottle, along with either 0.097 mmol of ethylene or 0.125 mmol of VC. The growth curves of ethylene and VC in the presence of c-DCE show a similar shape as previous growth tests on ethylene and VC alone. However, the OD_{600} peak is approximately 0.02 OD_{600} lower (Figure 4.7). The results of the c-DCE transformation with ethylene as the growth substrate shows the majority of the c-DCE was transformed after ethylene was consumed (Figure. 4.8). However, there is a limited amount of data available, so it is difficult to draw definite conclusions. In the VC cometabolism experiment, the degradation of VC appears to be slightly inhibited by the presence of c-DCE (Figure 4.9). For example, Figure. 3.9 shows an uninhibited VC degradation curve under the same initial conditions, where VC was removed at a faster rate.
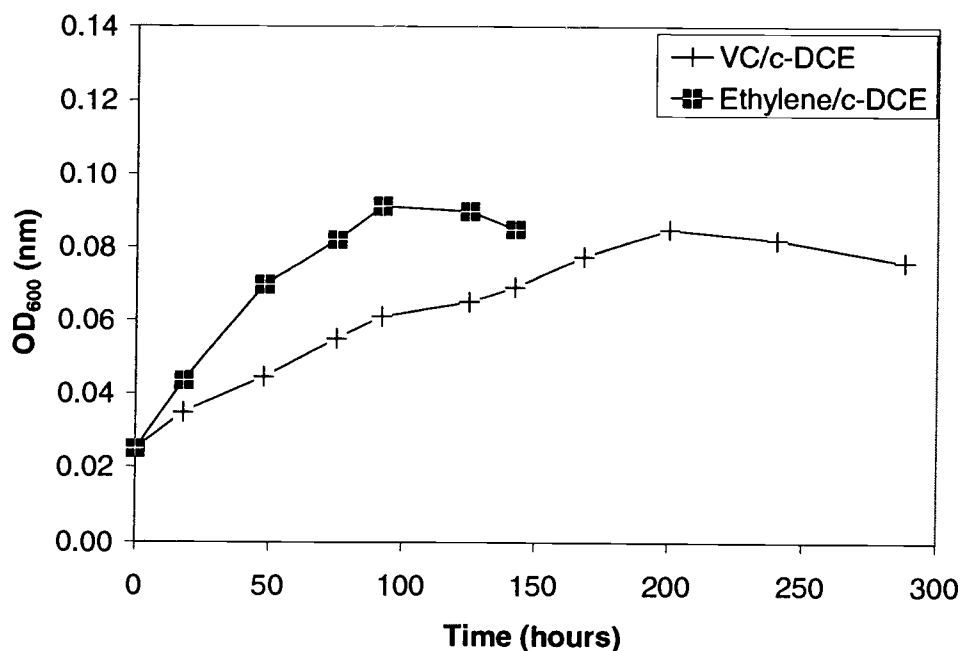
Figure. 4.7. Growth curves of strain JS60 cometabolizing c-DCE with VC and ethylene as growth substrates.

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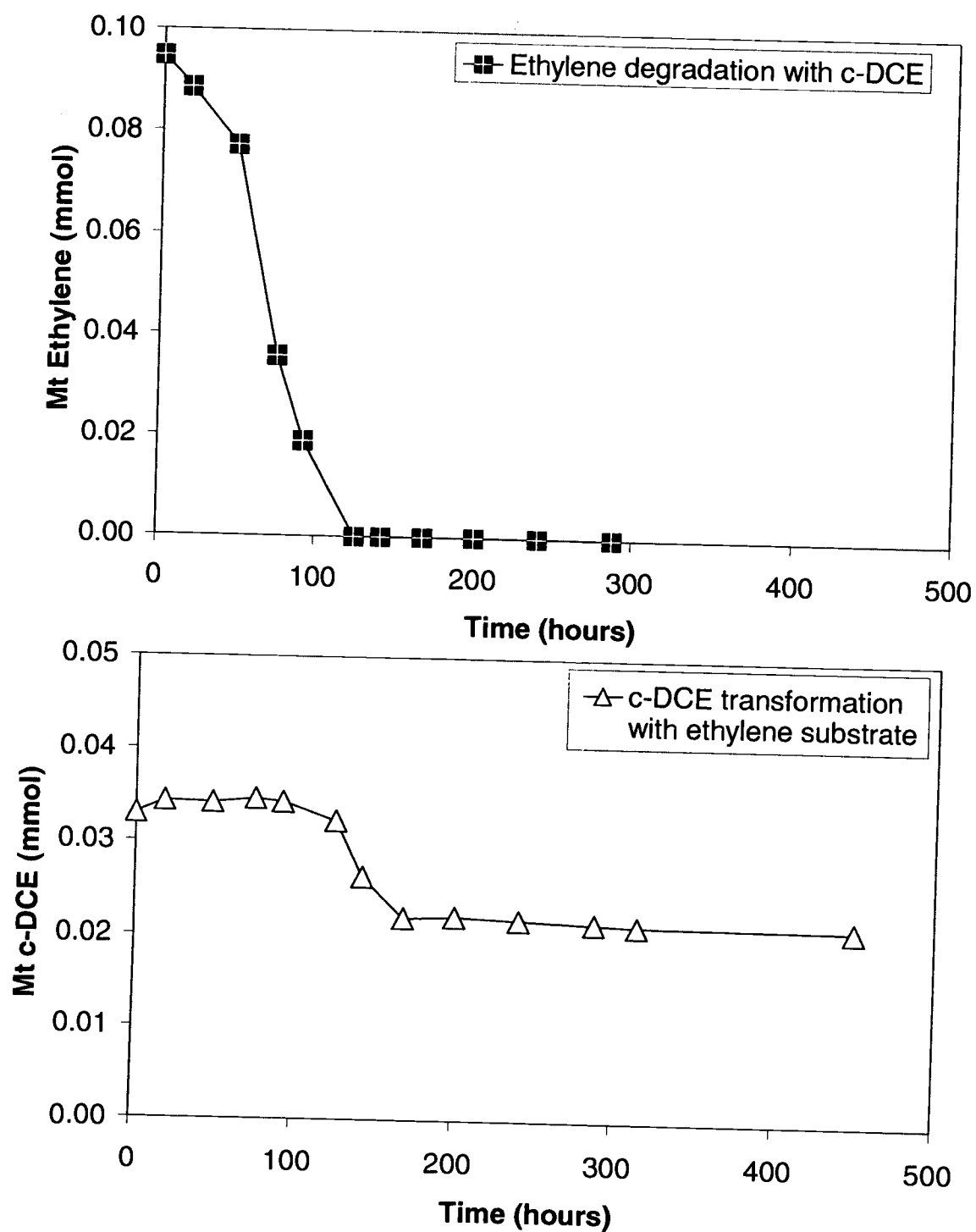
Figure. 4.8. Cometabolism of c-DCE by cells growing on ethylene.
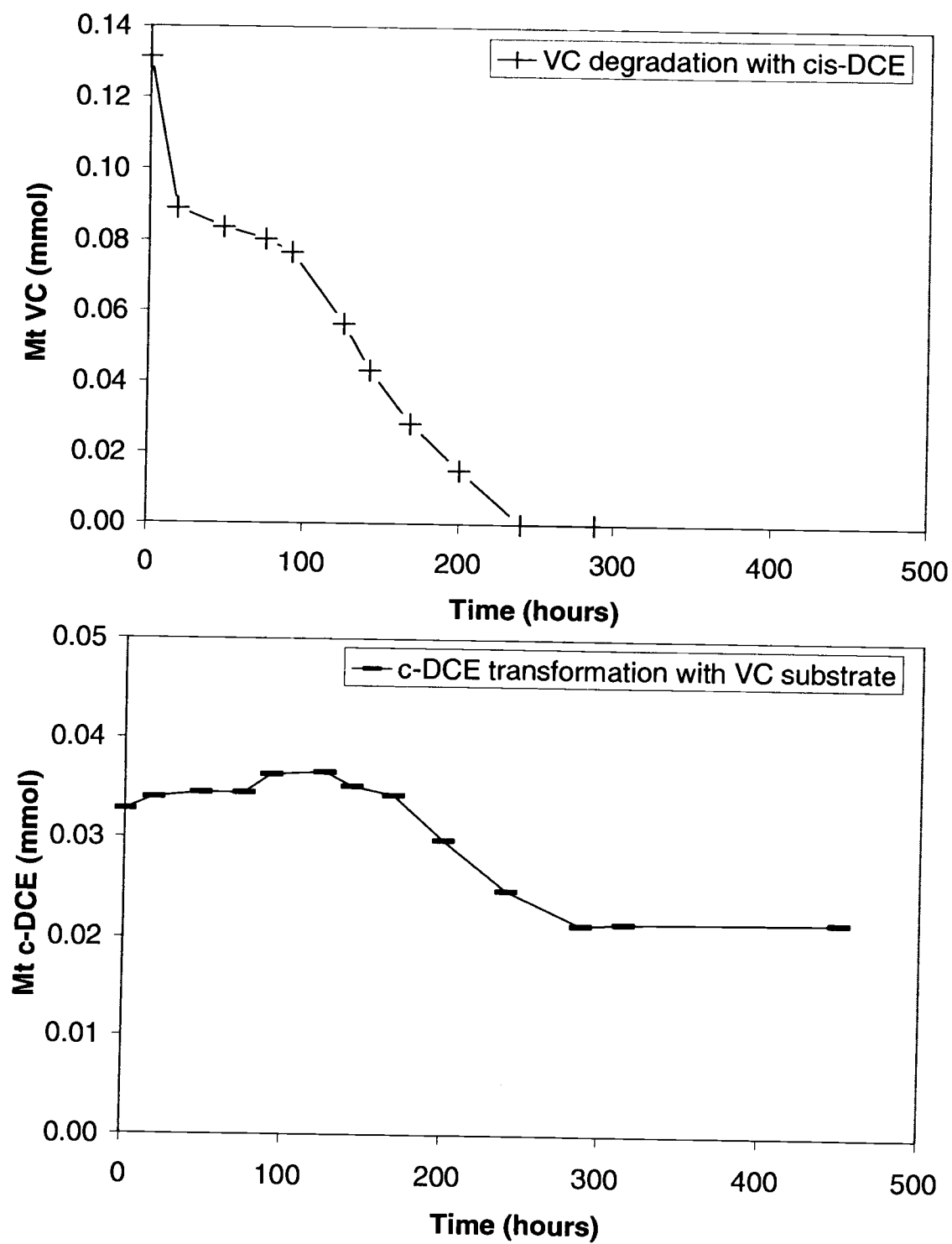
Figure. 4.9. Cometabolism of c-DCE by cells grown on VC.

The transformation yields in mg CAH/mg substrate are presented in Table 4.3 and Table 4.4. The transformation yield is much higher with acetate as a substrate than with either ethylene or VC when calculated based in electron equivalents. Acetate as a growth substrate produces a transformation yield approximately 51% higher than when ethylene was used as a growth substrate. The transformation yield for c-DCE with VC is only 46% of that achieved with acetate. In future studies it would be interesting to see if using ethylene and VC as a growth substrates would show similar effects on the transformation yield of t-DCE.

The growth rates of strain JS60 here were approximately 50% of those observed in the absence of c-DCE in earlier experiments (Chapter 3). The growth rate on acetate, ethylene, and VC were decreased by 45%, 41%, and 44%, respectively (Table 4.5). This may indicate some toxicity of the DCE isomers for strain JS60 that inhibited growth. Additionally, cell yields were also decreased. The yields on acetate, ethylene, and VC were decreased approximately 36%, 17%, and 21%, respectively (Table 4.6). It is interesting to note that the yield on acetate decreased by almost two times more than the yields on both of the other growth substrates. These observations support that the transformation of the DCE isomers require energy to support the reactions, and that the presence of the DCE isomers is toxic to the cells.

Table. 4.2. Transformation yield comparisons.

Transformation Yields	Acetate	Ethylene	VC
c-DCE	0.23 mg c-DCE/ mg acetate	0.36 mg c-DCE/ mg ethylene	0.12 mg c-DCE/ mg VC
t-DCE	0.11 mg t-DCE/ mg acetate	--	--
1,1-DCE	0 mg 1,1-DCE/ mg acetate	--	--

Table. 4.3. Transformation yields based on electron equivalents.

Transformation Yields	Acetate	Ethylene	VC
c-DCE	1.68 mg c-DCE/ e- equiv	0.85 mg c-DCE/ e- equiv	0.77 mg c-DCE/ e- equiv
t-DCE	0.84 mg t-DCE/ e- equiv	--	--
1,1-DCE	0 mg 1,1-DCE/ e- equiv	--	--

Table. 4.4. Growth rate comparisons.

Growth Rate (hour^{-1})	Acetate	Ethylene	VC
c-DCE	0.001	0.009	0.004
t-DCE	0.010	--	--
1,1-DCE	0.011	--	--
Previous Experiments	0.018	0.015	0.007

Table. 4.5. Yield comparisons.

Yield (mg protein/ mg substrate)	Acetate	Ethylene	VC
c-DCE	0.42	0.72	0.26
t-DCE	0.41	--	--
1,1-DCE	0.43	--	--
Previous Experiments	0.66	0.87	0.33

Table. 4.6. Yield comparisons based on electron equivalents.

Yield (mg protein/ e- equiv)	Acetate	Ethylene	VC
c-DCE	0.076	0.084	0.068
t-DCE	0.076	--	--
1,1-DCE	0.077	--	--
Previous Experiments	0.104	0.097	0.076

Inhibition Kinetic Tests

Inhibition kinetic experiments were conducted similarly to the previous kinetic tests, with a high starting amount of cells at 1.6 OD_{600}. However, at 50 minutes into the experiment, some bottles were dosed with an additional substrate. See Table 4.6 for all ethylene, VC, and c-DCE additions. The monitoring continued until all the substrate was consumed, or until transformation activity ceased.

Table. 4.6. A summary of the experimental set-up of each kinetic inhibition tests.

Inhibition Test One	Initial Additions	Aqueous Concentrations	Addition at Time 50 Minutes
Reactor 1	2.2 μmol of ethylene	85 μM of ethylene	none
Reactor 2	2.2 μmol of ethylene	85 μM of ethylene 64 μM of VC	2.2 μmol of VC
Reactor 3	none	64 μM of VC	2.2 μmol of VC

Inhibition Test Two	Initial Additions	Aqueous Concentrations	Addition at Time 50 Minutes
Reactor 1	2.2 μmol of ethylene	85 μM of VC	none
Reactor 2	2.2 μmol of ethylene, 0.1 μmol of c-DCE	85 μM of VC 2.3 μM of c-DCE	none
Reactor 3	2.2 μmol of ethylene	85 μM of VC 2.3 μM of c-DCE	0.1 μmol of c-DCE
Reactor 4	none	2.3 μM of c-DCE	0.1 μmol of c-DCE

Inhibition Test Three	Initial Additions	Aqueous Concentrations	Addition at Time 50 Minutes
Reactor 1	2.2 μmol of VC	64 μM of VC	none
Reactor 2	2.2 μmol of VC, 0.1 μmol of c-DCE	64 μM of VC 2.3 μM of c-DCE	none
Reactor 3	2.2 μmol of VC	64 μM of VC 2.3 μM of c-DCE	0.1 μmol of c-DCE
Reactor 4	none	2.3 μM of c-DCE	0.1 μmol of c-DCE

INHIBITION TEST ONE

The inhibition tests were performed to identify which substrates were preferable to strain JS60 preferred (VC or ethylene). We also determined whether there were any inhibition effects of c-DCE on the degradation of ethylene or VC. As reported by Coleman et. al. (2002), the K_S for ethylene was 0.9 μM and the K_S for VC was 0.5 μM. The lower K_S for VC indicates that VC is a more preferential substrate than ethylene. We would therefore expect ethylene degradation to be inhibited in the presence of VC.

The inhibition experiment was preformed with three batch reactors containing 5.2 mg cells; an ethylene reactor (Reactor 1), a VC reactor (Reactor 3), and a reactor that started initially with ethylene, and then had VC added after 50 minutes (Reactor 2). At 50 minutes into the experiment, 2.2 μmol of VC was added to the VC reactor and to the ethylene inhibition reactor. The substrate additions are shown in Table 4.6. Results presented in Figure 4.10 showed the ethylene utilization rate decreased after VC was added. The decrease in rate can be compared to the ethylene reactor (Figure 4.10a). The VC degradation occurring in the VC reactor and the VC degradation occurring in the ethylene inhibition reactor are similar. This indicates that the VC degradation curve is unaffected by the presence of ethylene.
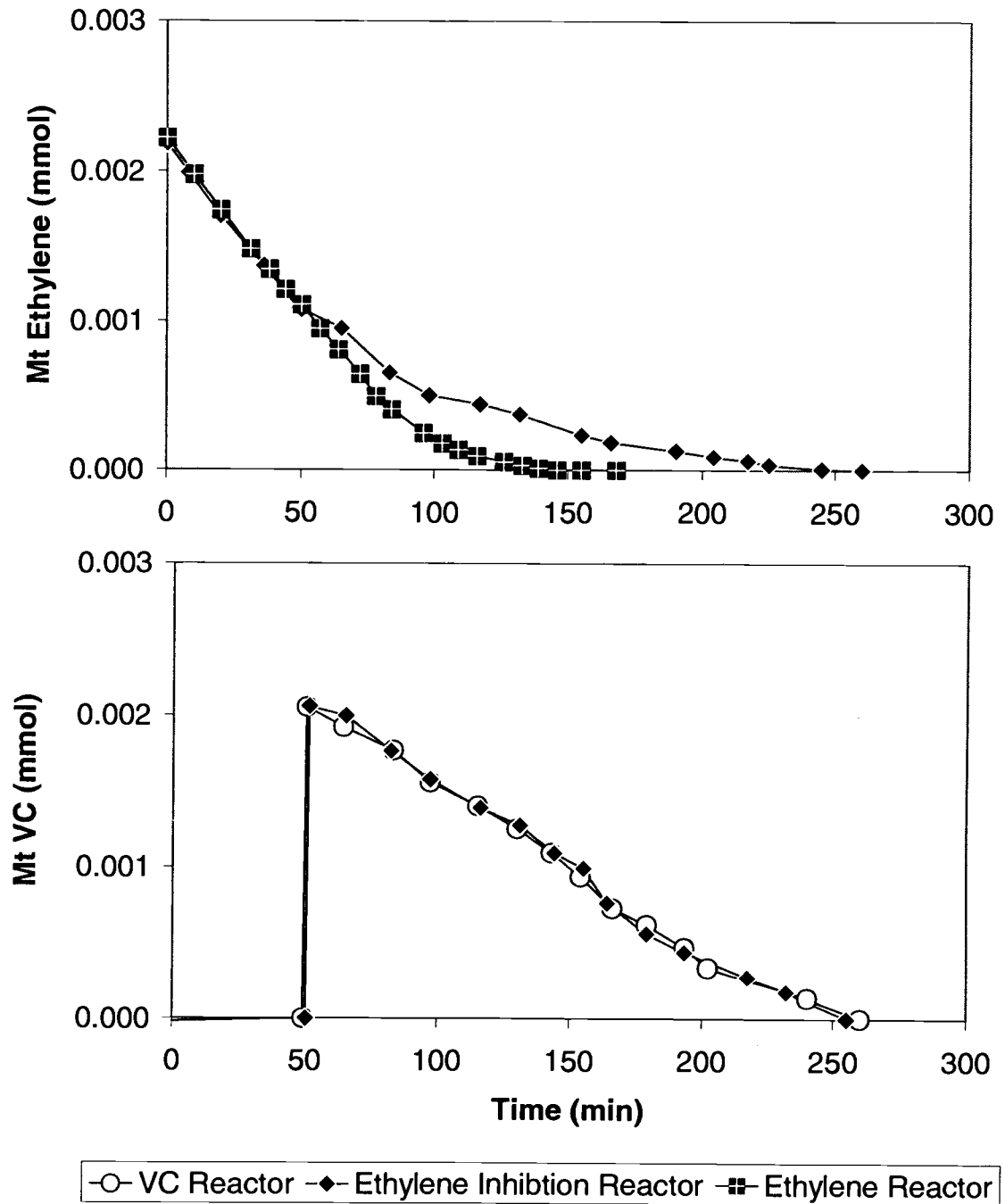
Figure. 4.10. Results of VC inhibition on ethylene utilization. O-VC only, ■-ethylene only, ♦-ethylene added at time zero, VC was added at time 50 minutes.

INHIBITION TEST TWO

This inhibition test was designed to identify how strain JS60 ethylene degradation would be effected by the presence of c-DCE. The experiment was performed with four batch reactors containing 5.2 mg cells each; a reactor containing ethylene (Reactor 1), a reactor containing c-DCE and ethylene at time zero (Reactor 2), a reactor containing ethylene at time zero, with an addition of c-DCE at 50 minutes (Reactor 3), and a reactor containing c-DCE only (Reactor 4). The substrate additions are shown in Table 4.6. Figure 4.11a shows similar ethylene degradation curves for Reactor 1, Reactor 2, and Reactor 3. This would indicate that the presence of c-DCE had little effect on the degradation of ethylene.

There appears to be a lag period prior to c-DCE transformation (Figure 4.11b). This lag period might be associated with the time required for the enzyme induction for the cometabolism of c-DCE. The lag could also be associated with ethylene inhibition of c-DCE transformation. It is interesting to note that c-DCE was less effectively transformed after being added 50 minutes into the experiment (Reactor 3). This could indicate that the time for enzyme induction is important.
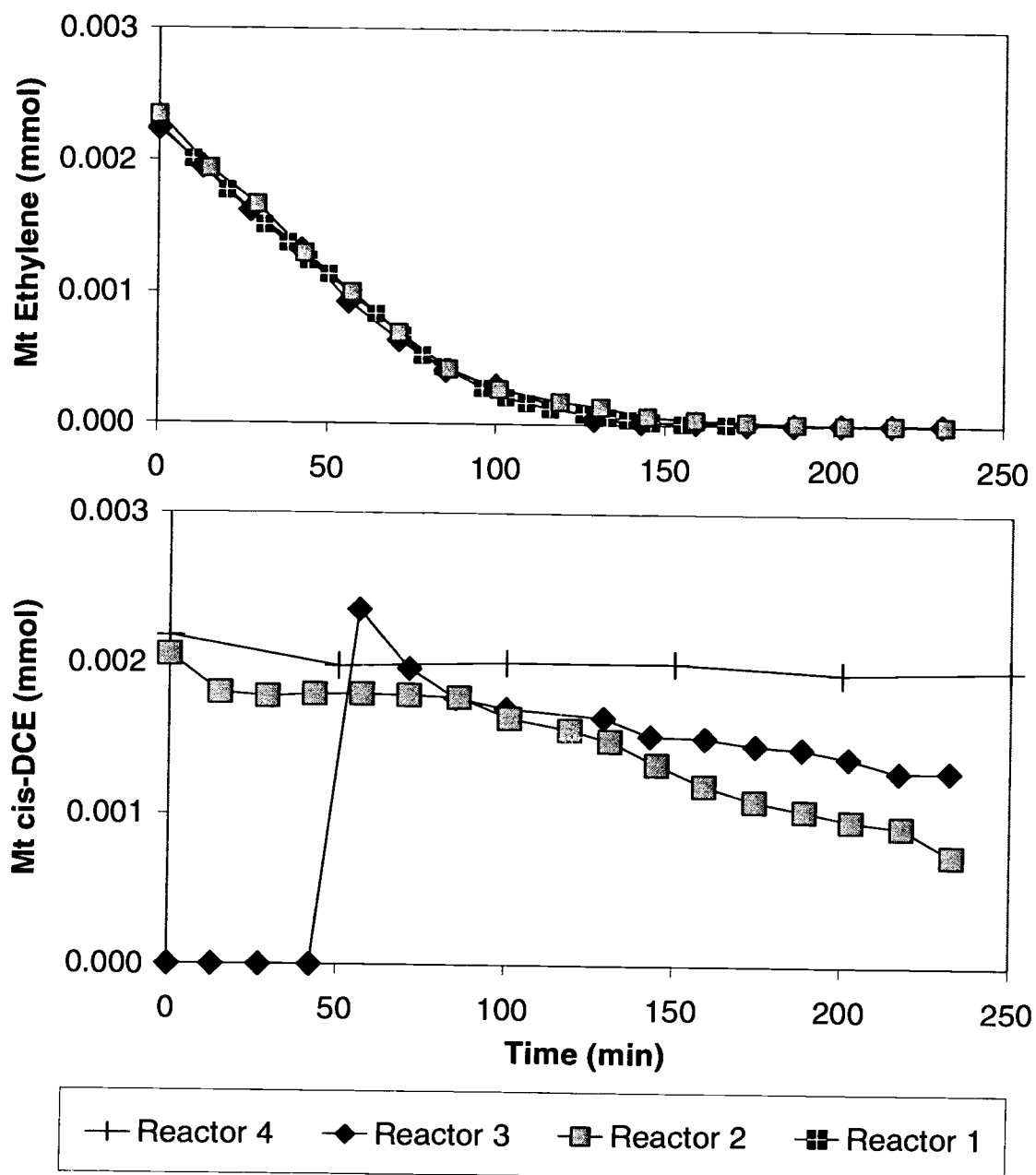
Figure. 4.11. Results of tests of c-DCE inhibition on ethylene utilization. ■-ethylene and c-DCE added together at time zero (Reactor 2), ◆-ethylene added at time zero, c-DCE added at time 50 minutes (Reactor 3), + -c-DCE only (Reactor 4), ■-ethylene only (Reactor 1).

INHIBITION TEST THREE

This test evaluated c-DCE inhibition of VC utilization. The experiment was preformed with four batch reactors containing 5.2 mg cells; a reactor containing VC (Reactor 1), a reactor containing c-DCE and VC at time zero (Reactor 2), a reactor containing VC at time zero, with an addition of c-DCE at 50 minutes (Reactor 3), and a reactor containing c-DCE only (Reactor 4). The substrate additions can be seen in Table 4.6. This kinetic inhibition test was similar to inhibition test two; however VC was substituted for ethylene as the substrate. c-DCE was not observed to be transformed to any significant extent (Figure 4.12b). However, in the previous experiment, the majority of the c-DCE is transformed after most of the ethylene is consumed (Figure 4.11). Therefore, since data was not collected for a sufficient amount of time following the disappearance of VC from the reactors, no conclusion about c-DCE transformation can be made.

The reactor 2, containing c-DCE and VC together at time zero, appears to be considerably inhibited, when compared to the reactor containing only VC. Reactor 3, where c-DCE was added at a time of 50 minutes, transformed VC as the same rate as the Reactor 4, which contained only VC (Figure 4.12a). The pseudo 1st order reaction rate decreased significantly from 0.12 day^{-1} to 0.09 day^{-1} in the reactor containing c-DCE.
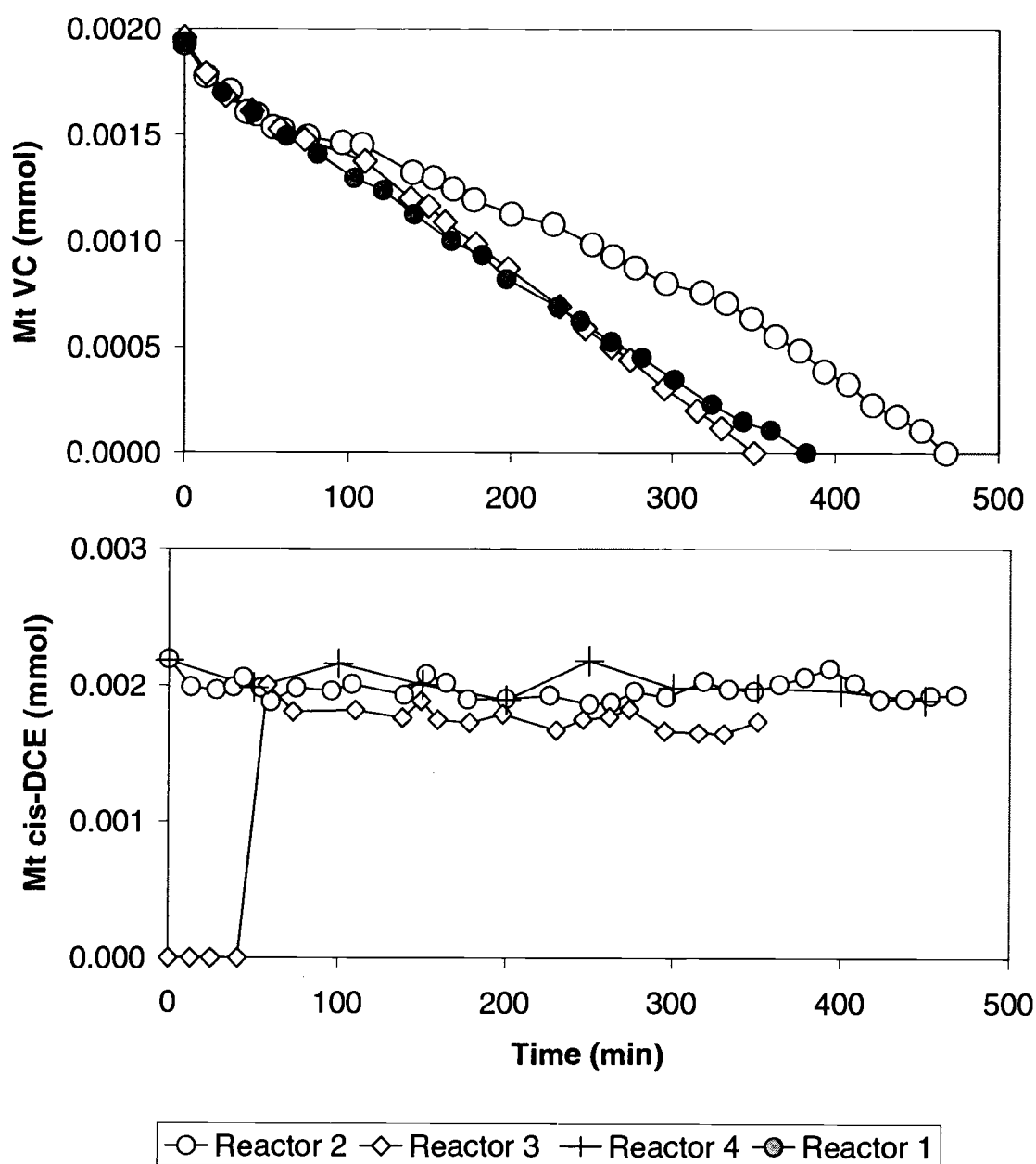
Figure. 4.12. Results of c-DCE inhibition on VC utilization. o-VC and c-DCE added together at time zero (Reactor 2), ◊-VC added at time zero, c-DCE added at time 50 minutes (Reactor 3), + -c-DCE only (Reactor 4), ●-VC only (Reactor 1).

CONCLUSIONS

The results presented provide kinetic, cometabolic, and inhibition information on strain JS60. Prior to this study, limited kinetic data had been collected on strain JS60 by Coleman et al. (2002), and Coleman and Spain (2003). Our results indicated that strain JS60 showed good degradation kinetics for VC and cometabolic activity on c-DCE with only slight inhibition.

- The NSLR fit of the ethylene data produced a K_S of 0.93 µM and a k of 0.23 day^{-1}. This compares to the K_S and k Coleman et. al. (2002) reported of 0.9 µM and 0.58 day^{-1}, respectively.

- A pseudo 1st order reaction rate was estimated for VC at 0.12 day^{-1}.

- JS60 cells when grown on acetate was capable of cometabolizing c-DCE, and t-DCE, but not 1,1-DCE. c-DCE was also cometabolically transformed with strain JS60 cells growing on ethylene and VC.

- In the c-DCE transformation test with ethylene as the growth substrate, significant transformation did not occur until after most of the ethylene was consumed, indicating inhibition by ethylene. The same trend appeared in the kinetic tests, performed with higher cell densities. In this case, most of the c-DCE was transformed over a short period (30 minutes) after ethylene was consumed.

- In the VC cometabolic transformation tests, VC utilization was slightly inhibited by the presence of c-DCE in both the growth test and the kinetic tests. c-DCE transformation in the growth test occurred only after the concentration of VC

reaches a level of approximately 0.03-mmol of mass. In the kinetic tests, no significant c-DCE transformation occurred, however these tests were not monitored for a long enough period after VC was consumed.

- The transformation yields calculated for c-DCE when strain JS60 is grown on acetate, ethylene, and VC showed that ethylene was the best substrate for the cometabolism of c-DCE. The yields were; 0.23 mg c-DCE/mg acetate, 0.36 mg c-DCE/mg ethylene, and 0.12 mg c-DCE/mg VC. The transformation yield of t-DCE with acetate as a growth substrate at 0.11 mg t-DCE/mg acetate was one half the rate of c-DCE transformation on acetate at 0.23 mg c-DCE/mg acetate.

- The pseudo 1st order rate estimates from the c-DCE inhibition tests on VC showed a 26% decrease when c-DCE and VC were added together at time zero. The pseudo 1st order rates calculated for the VC reactor and the reactor where c-DCE was introduced 50 minutes into the experiment are very close to the same pseudo 1st order rate for VC estimated earlier in the report. This shows that VC degradation is inhibited by c-DCE.

The results from these studies have implications on the intrinsic aerobic transformation of VC and DCE isomers. The degradation of VC is preferential to ethylene. This is important since both substrates would be present in a chlorinated ethylene contaminated groundwater plume. In addition, c-DCE and t-DCE can be cometabolized, however the cell yields are lower in the presence of the DCE isomers, indicating a toxic effect.

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CHAPTER 5

Engineering Significance and Conclusions

ENGINEERING SIGNIFICANCE

Chlorinated solvents and their natural transformation products are frequently observed groundwater contaminants in the United States (Semprini, 1998). Methods commonly used for remediation of groundwater, such as pump-and-treat, are fairly effective, but costly, and result in the transfer of contaminants into other environmental compartments, or require surface treatment, and still often do not reach final acceptable treatment levels (Semprini, 1998). However, under favorable conditions, natural attenuation, and even enhanced bioremediation will result in significant savings in cost over a more intrusive remedial alternative. Natural attenuation is mainly comprised of aerobic and anaerobic degradation processes (Azadpour-Keeley, 1999). Enhanced bioremediation involves supplying limiting compounds to achieve effective biological treatment. For example, for anaerobic reductive dehalogenation, an electron donor addition might be required.

The lesser chlorinated ethenes, cis-1,2-dichloroethylene (c-DCE) and vinyl chloride (VC) often accumulate in anaerobic contaminated groundwater plumes due to incomplete reductive dechlorination of higher chlorinated compounds. Aerobic

degradation of c-DCE and VC is possible on the fringes of contaminant plumes. In fact, VC can be directly utilized as a growth substrate by some microorganisms, including *Mycobacterium*, *Nocardiodes*, and *Pseudomonas* (Coleman et al., 2002; Hartmans et al., 1992; Verce et al., 2000). Currently there is not much information on the degradation abilities of these microbes.

The *Mycobacterium* strain JS60 is capable of degrading VC, and based on the low K_s of VC the enzymes will likely degrade VC to low concentrations over other compounds, such as ethylene, under appropriate conditions. Our experimental results indicated that VC was preferentially degraded over ethylene.

Strain JS60 has also been shown to grow on several organic acids, which are often found in contaminated groundwater plumes. JS60 was observed to grow on acetate, propionate, and butyrate, but not formate or lactate. The growth rate of strain JS60 on acetate was 0.018 hour^{-1}, which was greater than ethylene and VC, at 0.015 hour^{-1} and 0.007 hour^{-1}, respectively. When acetate was in the presence of VC in equal parts, the growth rate was increased by 65%, and the yield was increased by 30% from VC alone. When acetate was present as 16% of the substrate mass, the utilization rate of VC was three times higher than when acetate was 50% of the growth substrate mass. Therefore, although not as many cells where produced, strain JS60's specific utilization rate of VC is much higher when acetate is present in lower amounts. Because acetate is a product of microbial metabolism, low concentrations of acetate are much more likely to be present in a contaminated aquifer. These findings about the increase in degradation rates of VC in the presence of organic acids, may lead to improved site assessment, better remedial-action decision making, and more reliable enhanced bioremediation technologies.

CONCLUSIONS

Based on the original objectives, the following conclusions can be drawn from this work;

- The *Mycobacterium* strain JS60 was capable of growth on both ethylene and VC as the sole carbon and energy sources.

- Strain JS60 was capable of growth on acetate, propionate, and butyrate, but not on lactate or formate. Strain JS60 showed the fastest growth rate on acetate, 0.018 hour^{-1}, followed by propionate, 0.013 hour^{-1}, and finally butyrate, 0.007 hour^{-1}. The general trend appeared to be that the growth rate decreased as the number of carbons in the chain increased.

- The presence of acetate increased the degradation of rate of ethylene. The utilization rate of ethylene alone was 6.5 nmol/hr/mg protein and the yield was 0.87 mg protein/mg substrate. When acetate was present as 50% of the substrate provided, the utilization rate increased by 30% to 9.2 nmol/hr/mg protein, and the yield increased by 8%. The presence of acetate significantly increased the degradation rate of VC. The utilization rate of VC alone was 5.2 nmol/hr/mg protein and the yield is 0.3 mg protein/mg substrate. In the presence of acetate these increased to 9.9 nmol hr/mg protein and 0.47 mg protein/mg substrate. This resulted in a 48% increase in utilization rate, and 33% increase in yield.

- The ethylene K_S of 0.93 µM and k of 0.23 day^{-1} determined through kinetic testing and NSLR fitting was fairly close to the K_S of 0.9 µM and k of 0.58 day^{-1} reported by Coleman et al. (2002). The VC kinetic tests encountered analytical

detection limits and therefore the K_S could not be determined by NLSR. The pseudo 1st order rate was estimated to be 0.12 day^{-1}.

- When strain JS60 was in the presence of both VC and ethylene, VC was degraded preferentially. This was to be expected based on the value of K_S of VC at 0.5 μM compared to ethylene at 0.9 μM.

- Cometabolism of c-DCE was promoted by strain JS60 cells growing on acetate, ethylene, or VC. The transformation yields were determined to be 0.23 mg c-DCE/mg acetate, 0.36 mg c-DCE/mg ethylene, and 0.12 mg c-DCE/mg VC. In addition, strain JS60 was capable of cometabolizing t-DCE with acetate as the growth substrate with a yield of 0.11 mg t-DCE/mg acetate, but transformation of 1,1-DCE was not observed. The transformation of t-DCE with acetate is approximately 50% of the transformation yield of c-DCE with acetate.

- When both ethylene and c-DCE are present together, the ethylene degradation rate was unaffected by c-DCE. However, c-DCE transformation did not begin until ethylene was reduced to very low concentrations (2 μM). This possibly indicates catabolic repression, or substrate inhibition.

- The presence of c-DCE interfered with the degradation of VC. The uninhibited pseudo 1st order rate of VC was observed to be 0.12 day^{-1} in multiple experiments. In the presence of c-DCE, the pseudo 1st order rate decreased to 0.09 day^{-1}. Therefore, when VC and c-DCE were present together, VC degradation was slightly inhibited. Also, c-DCE transformation was not observed in the growth test until the aqueous concentration of VC was reduced to 350 μM.

FUTURE WORK

In order to more fully understand the *Mycobacterium* strain JS60 degradation of chlorinated ethenes, more experiments are needed;

- Further kinetic modeling work would be an ideal next-step in this project. Utilizing the modeling software AQUASIM to model the results of the inhibition experiments would be of interest.

- In the kinetic inhibition test containing c-DCE and VC, it appeared that the presence of c-DCE inhibited the degradation of VC. This effect should be more fully explored, because the presence of c-DCE and VC together in a contaminated aquifer is a common occurrence, and in order to rely on natural attenuation as a reliable remediation option, inhibitory effects are important to consider.

- The cometabolism of c-DCE with VC grown cells should be investigated in the future. The short-term kinetic test showed no transformation of c-DCE, but the growth test showed a slow transformation of c-DCE. This test needs to be repeated since the short-term test was not conducted for a long enough period.

- The cometabolism of t-DCE and 1,1-DCE will need to be explored more thoroughly. Only one growth test was conducted with acetate, so the repeatability of the experiment was not analyzed. Tests also need to be performed with VC and ethylene as the substrate.

A different experimental set-up should be explored to more closely simulate aquifer conditions. In our experiments, batch growth reactors containing 125-mL of air and 50-mL of 1/5 MSM media were used. This set-up likely produced the best conditions for degradation and transformation tests. In a groundwater aquifer the growth conditions for microorganisms are likely nutrient limited. It is therefore important to determine degradation rates in situations more like similar to those present in contaminated aquifers. Some possible studies are;

- Conduct growth experiments on acetate in the presence of VC and ethylene, and the cometabolic growth tests of c-DCE and t-DCE, in groundwater instead of dilute growth media.

- Change from batch reactors to reactors that contain aquifer solids, groundwater, and dissolved oxygen instead of a headspace. Then compare these results to determine how the different conditions effected to degradation and transformation rates of strain JS60.

- A column study would be one of the closest ways to simulate groundwater conditions in the laboratory. Acetate and VC could be continuously circulated through the column at a flow rate similar to that found in the environment and the degradation rate could be monitored by samples taken at injection/extraction ports. Also, c-DCE transformation experiments could be conducted in a similar manner.

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APPENDICES

APPENDIX A

MSM Growth Media Constituents

A1. MSM GROWTH MEDIA CONSTITUENTS

Part A:

15-mL 1-mM KNO_3 stock

5-mL KH_2PO_4 stock (12.4-g/100-mL)

1.1-mL 1.0-M $MgSO_4$ stock (24.5-g/100-mL)

0.02-mL 50-mM $CuSO_4 \cdot 5H_2O$ (125-mg/10-mL)

0.30-mL 1.0-M $CaNO_3 \cdot 4H_2O$ (2.36-g/100-mL)

0.50-mL $FeSO_4$ (30-mM $FeSO_4$ in 50-mM EDTA)

7.5-mL Trace Elements (In 100-mL of dH_2O; 0.01-g $Na2MoO_4 \cdot 2H_2O$, 0.02-g $MnCl_2$,

0.2-mg $CoCl_2 \cdot 6H_2O$, 0.01-g $ZnSO_4 \cdot 7H_2O$, 0.002-g $CuSO_4 \cdot 5H_2O$)

1439.75-mL dH_2O

Autoclave Part A for 20 minutes. If ppt. forms during sterilization it may be necessary to add 100-μL of 10-M H_2SO_4 to Part A before autoclaving.

Part B:

3.75-mL 0.4-M PO_4^{2-} stock (53-g K_2HPO_4, 2.75-g NaH_2PO_4 in 750-mL H_2O)

12.5-mL K_2CO_3 (6.5% w/v)

Adjust the pH of the mixture to 7.2 by making slight additions of 10M NaOH or 10M H_2SO_4.

APPENDIX B

Standard Curves

B1. STANDARD CURVE OF ETHYLENE

Ethylene Added (mL)	Ethylene Peak Area 1	Ethylene Peak Area 2	Ethylene Average	Cg (mol/sample)
2	2.67E+05	2.63E+05	2.65E+05	1.30E-06
1	1.39E+05	1.34E+05	1.36E+05	6.70E-07
0.5	7.30E+04	7.06E+04	7.18E+04	3.52E-07
0.25	1.52E+04	1.44E+04	1.48E+04	7.07E-08
0.1	3.94E+03	3.82E+03	3.88E+03	1.68E-08
0.05	2.33E+03	2.40E+03	2.37E+03	9.30E-09
0.025	1.49E+03	1.44E+03	1.46E+03	4.84E-09
0	6.69E+00	0.00E+00	3.34E+00	-2.35E-09

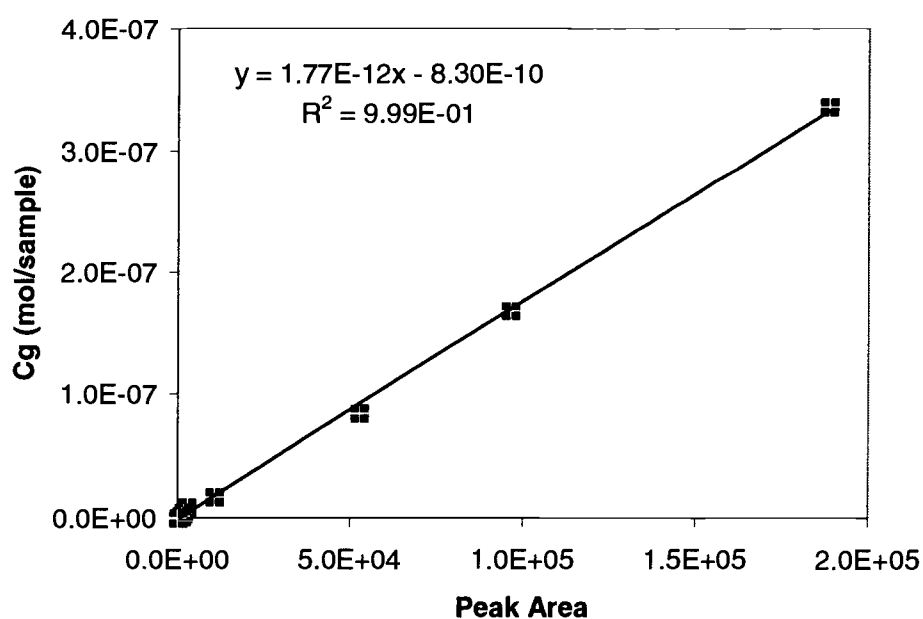Table. B1.1. Ethylene calibration data of the GC series HP6890.

Figure. B1.1. Ethylene calibration curve.

B2. STANDARD CURVE OF VINYL CHLORIDE

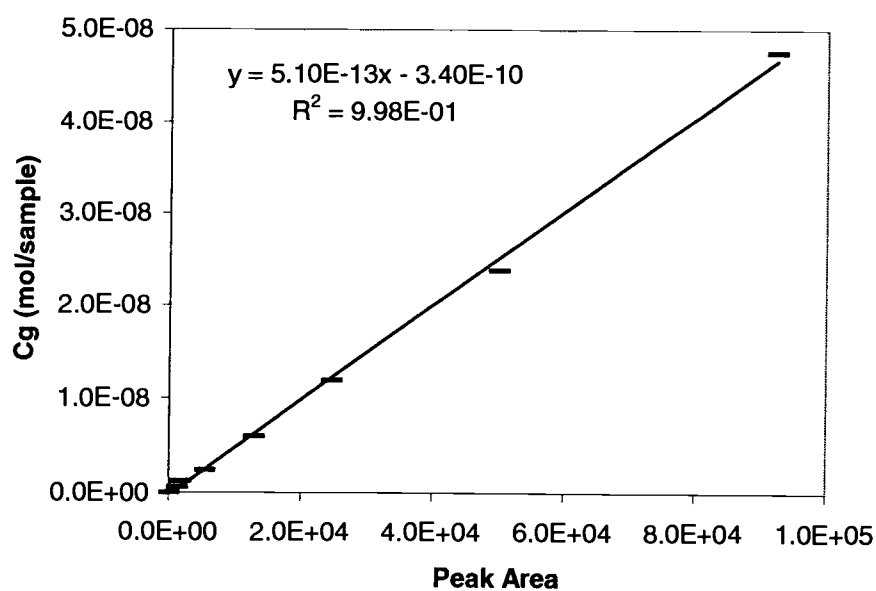| VC Added (mL)	VC Peak Area 1	VC Peak Area 2	VC Average	Cg (mol/sample)
2	9.17E+04	9.29E+04	9.23E+04	1.29E-06
1	4.96E+04	5.05E+04	5.01E+04	6.96E-07
0.5	2.43E+04	2.49E+04	2.46E+04	3.38E-07
0.25	1.29E+04	1.29E+04	1.29E+04	1.73E-07
0.1	5.64E+03	5.32E+03	5.48E+03	6.79E-08
0.05	1.76E+03	1.84E+03	1.80E+03	1.59E-08
0.025	1.27E+03	1.36E+03	1.31E+03	9.14E-09
0	9.15E+00	8.75E+00	8.95E+00	-9.27E-09

Table. B2.1. VC calibration data.

Figure. B2.1. VC calibration curve.

B3. STANDARD CURVE OF ACETATE

Acetate Concentration (mg/L)	Acetate Peak Area 1	Acetate Peak Area 2	Acetate Average	Response Factor
22	156603	176673	166638	1.30E-04
43	357398	359390	358394	1.21E-04
72	552663	604854	578759	1.25E-04
108	903485	904316	903901	1.20E-04

Average Response Factor = 1.23692E-04

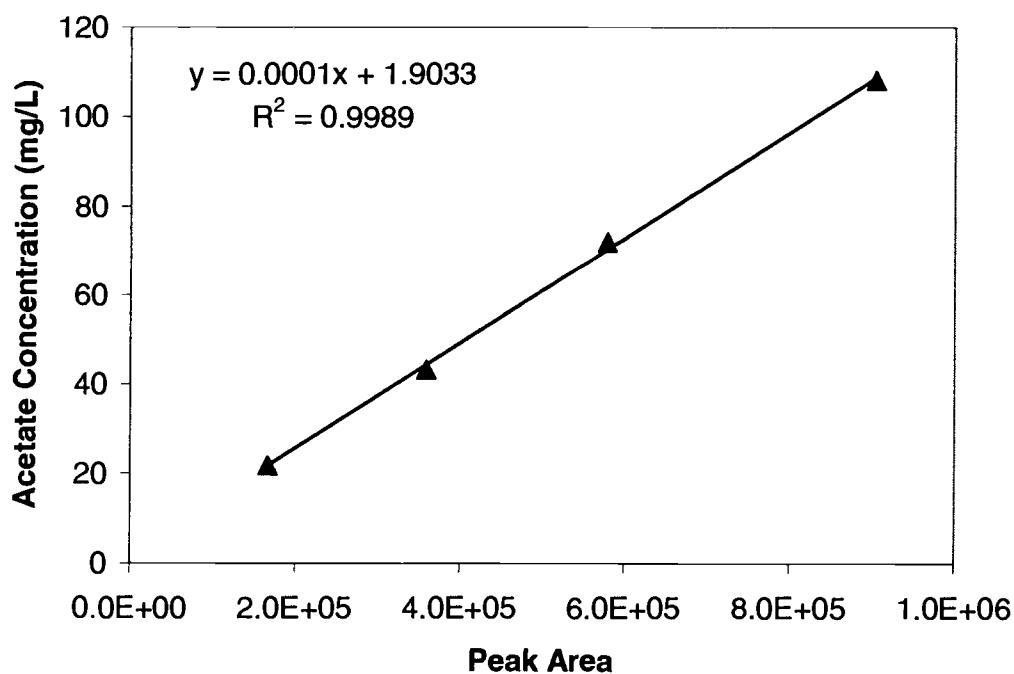Table. B3.1. Data from the calibration of the HPLC for acetate.

Figure. B3.1 Acetate calibration curve.

B4. STANDARD CURVE OF PROPIONATE

Propionate Concentration (mg/L)	Propionate Peak Area 1	Propionate Peak Area 2	Propionate Average	Response Factor
23	61130	61653	61392	3.74E-04
46	120936	120418	120677	3.81E-04
77	202472	201521	201997	3.79E-04
115	301850	299848	300849	3.82E-04

Average Response Factor = 3.78912E-04

Table. B4.1. Propionate standards run on the HPLC for calibration.

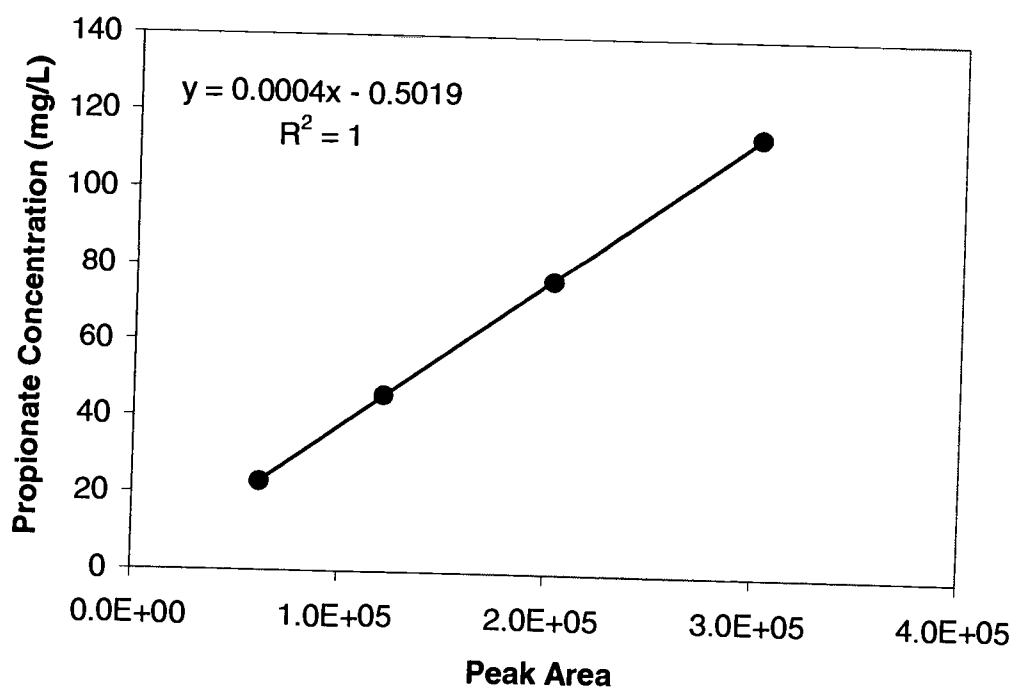
$y = 0.0004x - 0.5019$
$R^2 = 1$

Figure. B4.1. Propionate calibration curve.

B5. STANDARD CURVE OF CIS-1,2-DICHLOROETHYLENE

c-DCE Added (mL)	c-DCE Peak Area 1	c-DCE Peak Area 2	c-DCE Average	Cg (mol/sample)
0.1	3.25 E+04	3.31E+04	3.28E+04	9.53E-07
0.05	1.80E+04	1.86E+04	1.83E+04	5.18E-07
0.04	1.47E+04	1.49E+04	1.48E+04	4.14E-07
0.025	9.51E+03	9.52E+03	9.52E+03	2.56E-07
0.01	4.20E+03	4.22E+03	4.21E+03	9.62E-08
0.005	1.30E+03	1.31E+03	1.30E+03	9.12E-09

Table. B5.1. Standards analyzed for the c-DCE calibration.

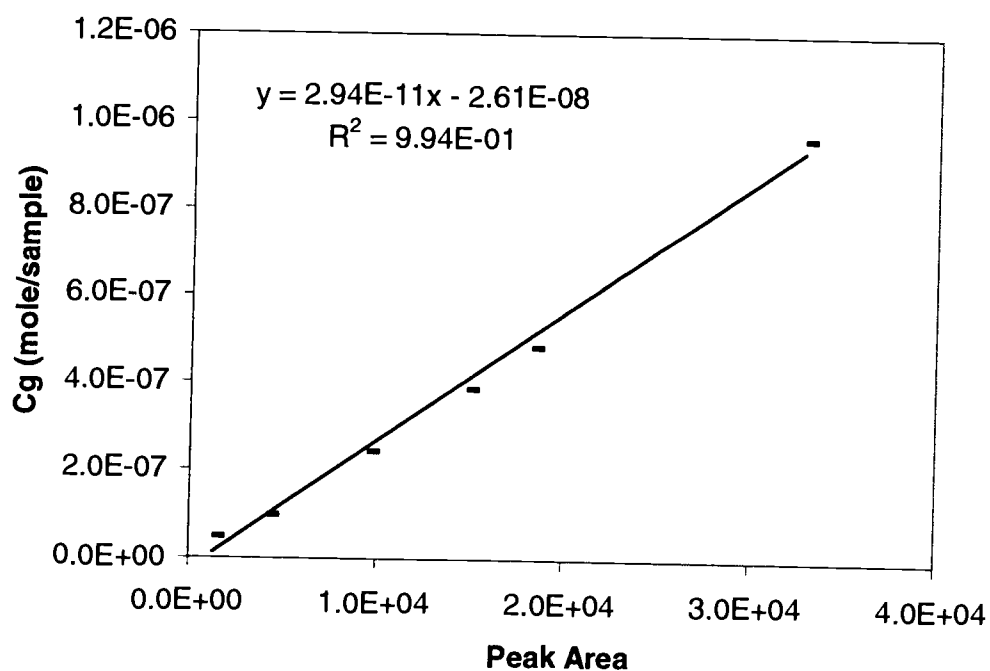$y = 2.94E-11x - 2.61E-08$
$R^2 = 9.94E-01$

Figure. B5.1. c-DCE calibration curve.

B6. STANDARD CURVE OF TRANS-1,2-DICHLOROETHYLENE

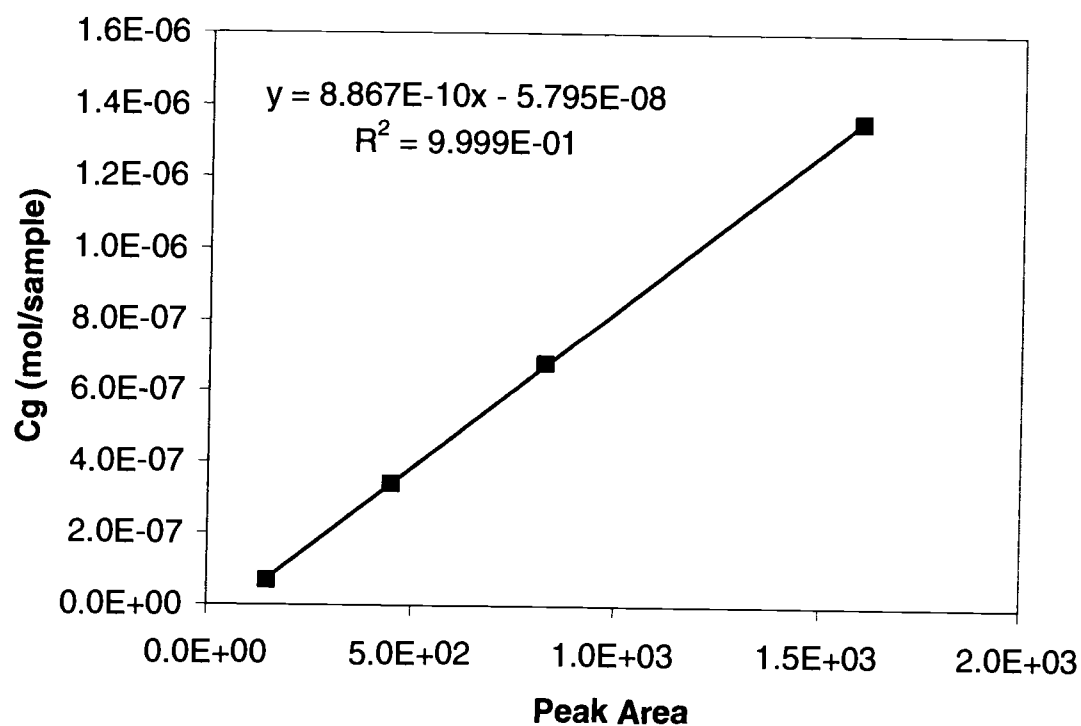| t-DCE Added (mL)	t-DCE Peak Area 1	t-DCE Peak Area 2	t-DCE Average	Cg (mol/sample)
0.1	1.29E+03	1.91E+03	1.60E+03	1.36E-07
0.05	8.22E+02	8.26E+02	8.24E+02	6.78E-07
0.025	4.33E+02	4.63E+02	4.48E+02	3.39E-07
0.005	1.25E+02	1.67E+02	1.46E+02	6.78E-08

Table. B6.1. Data from the t-DCE calibration.

Figure. B6.1. t-DCE calibration curve.

B7. STANDARD CURVE OF 1,1-DICHLOROETHYLENE

1,1-DCE Added (mL)	1,1-DCE Peak Area 1	1,1-DCE Peak Area 2	1,1-DCE Average	Cg (mol/sample)
0.1	9.17E+02	7.87E+02	8.52E+02	4.62E-06
0.05	3.96E+02	4.56E+02	4.26E+02	2.31E-06
0.025	2.43E+02	2.49E+02	2.46E+02	1.16E-06
0.005	3.25E+01	5.51E+01	4.38E+01	2.31E-07

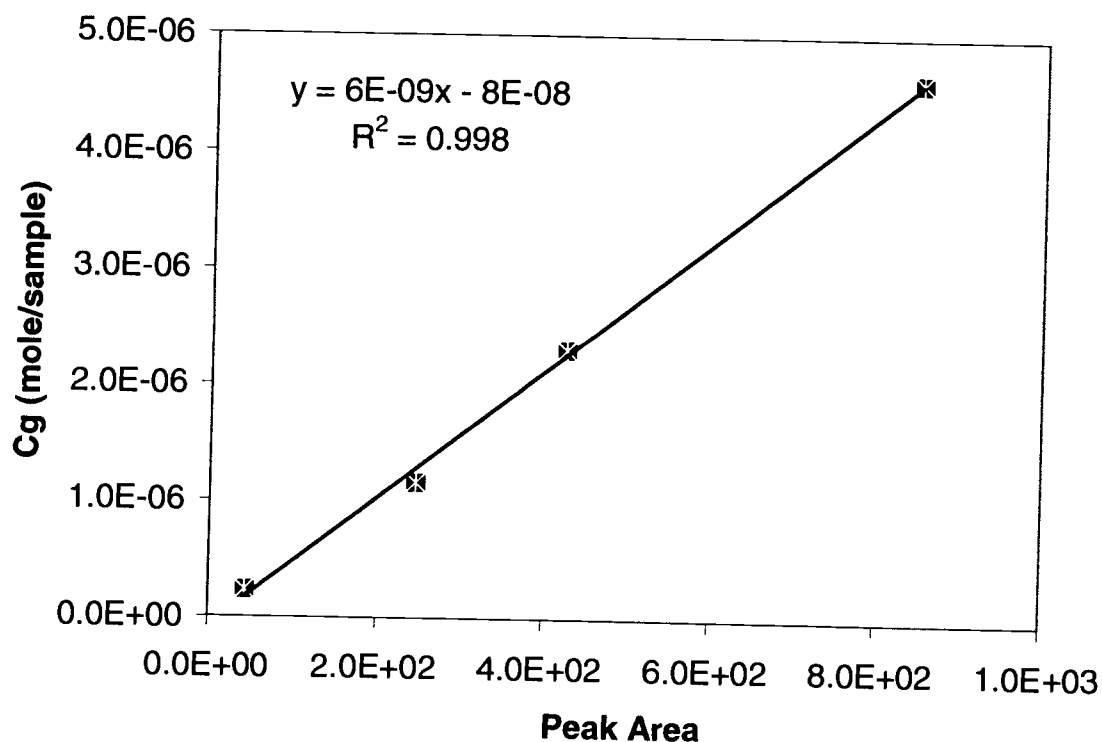Table. B7.1. Data gathered for the 1,1-DCE standard curve.

Figure. B7.1. 1,1-DCE standard curve.

B8. STANDARD CURVE FOR PROTEIN ASSAY

OD_{600}	Protein (g)
0.1299	4.00E-04
0.10108	3.00E-04
7.03E-02	2.00E-04
2.66E-02	5.00E-05
1.98E-02	2.50E-05
1.81E-02	1.00E-05
1.72E-02	6.00E-06
1.54E-02	0.00E+00

Table. B8.1. The protein calibration results.
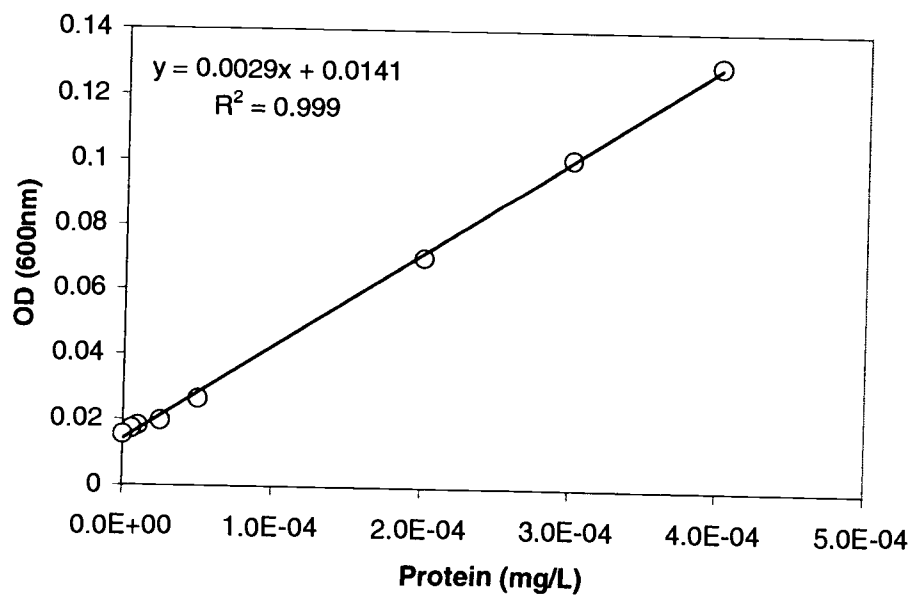
Figure. B8.1. Protein calibration curve.

B9. CORRELATION STANDARD CURVE BETWEEN OD_{600} AND PROTEIN

OD_{600}	Protein (mg)
0.0251	3.1
0.0328	7.5
0.0466	14.2
0.0657	31.8
0.0926	64.4
0.12108	95.7
0.1459	132.4

Table. B9.1. This table contains samples that were analyzed for both OD_{600} and protein content through a protein assay.

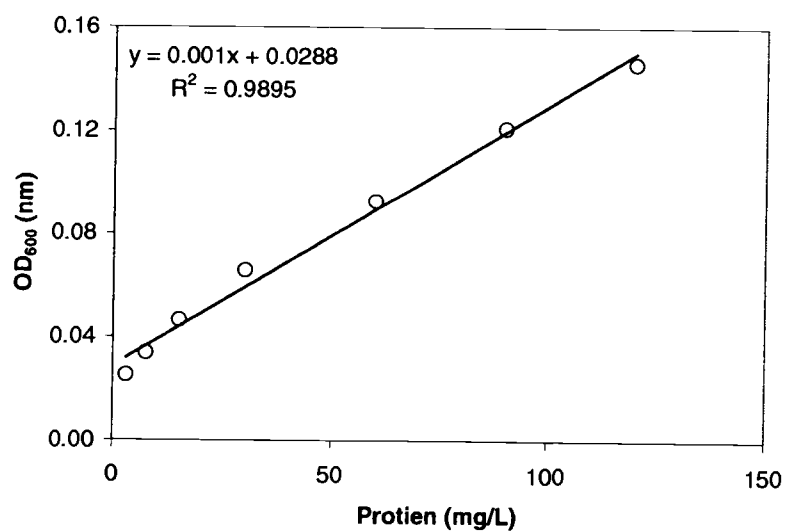Figure. B9.1. This graph shows the correlation between protein concentration and OD. All protein values were calculated using this standard curve.

APPENDIX C

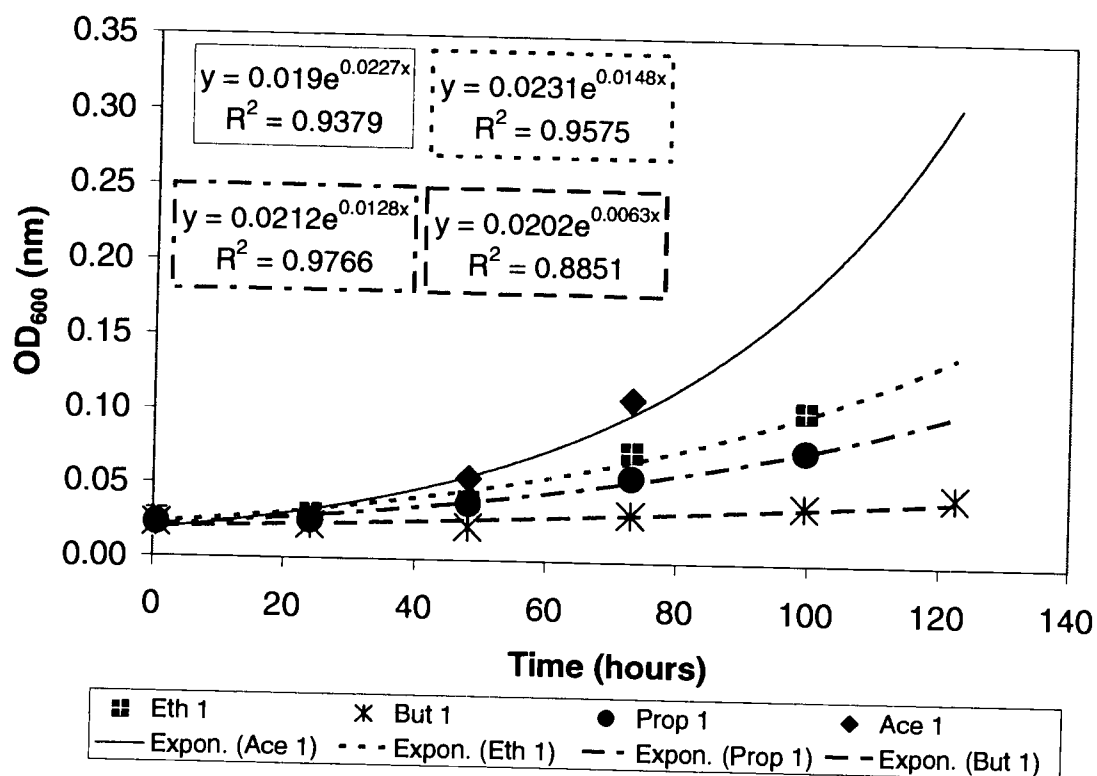# Growth Rate Estimates

C1. GROWTH RATE ESTIMATES

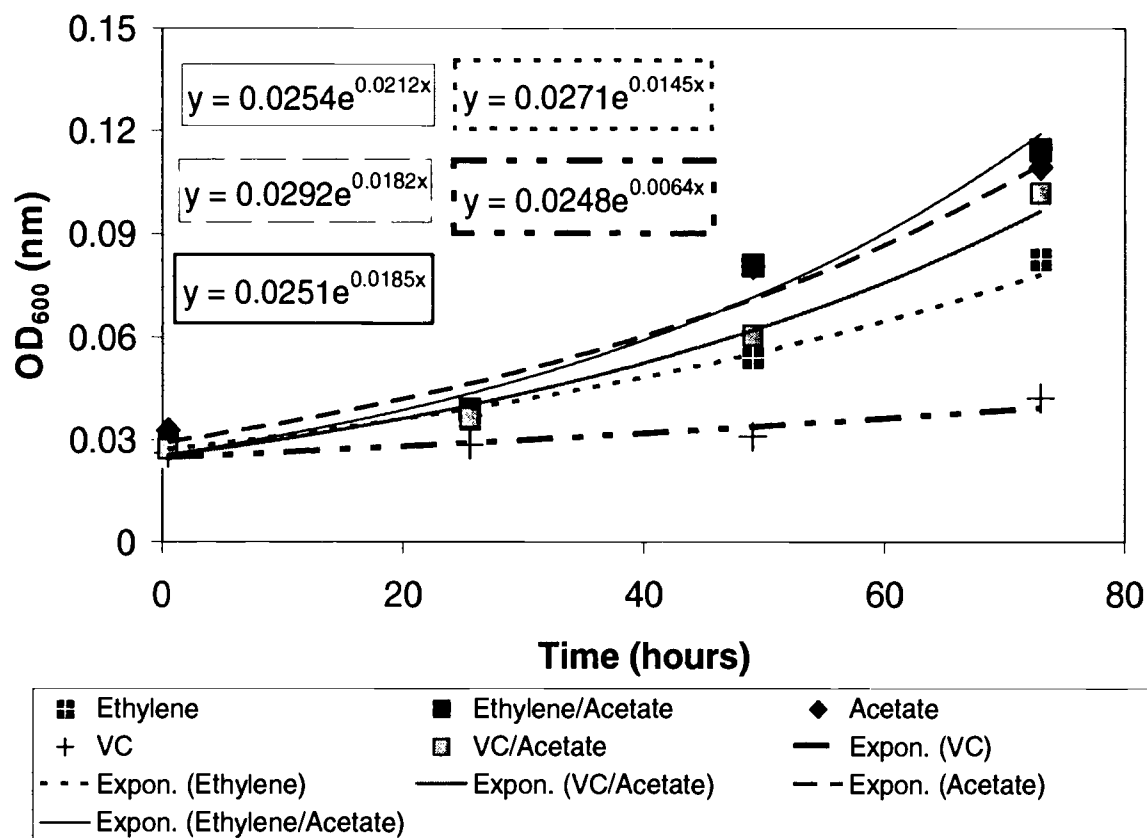Figure. C.1. This data corresponds to the growth rates presented in Table 3.2.

Figure C.2. This data corresponds to the growth rates presented in Table 3.2.
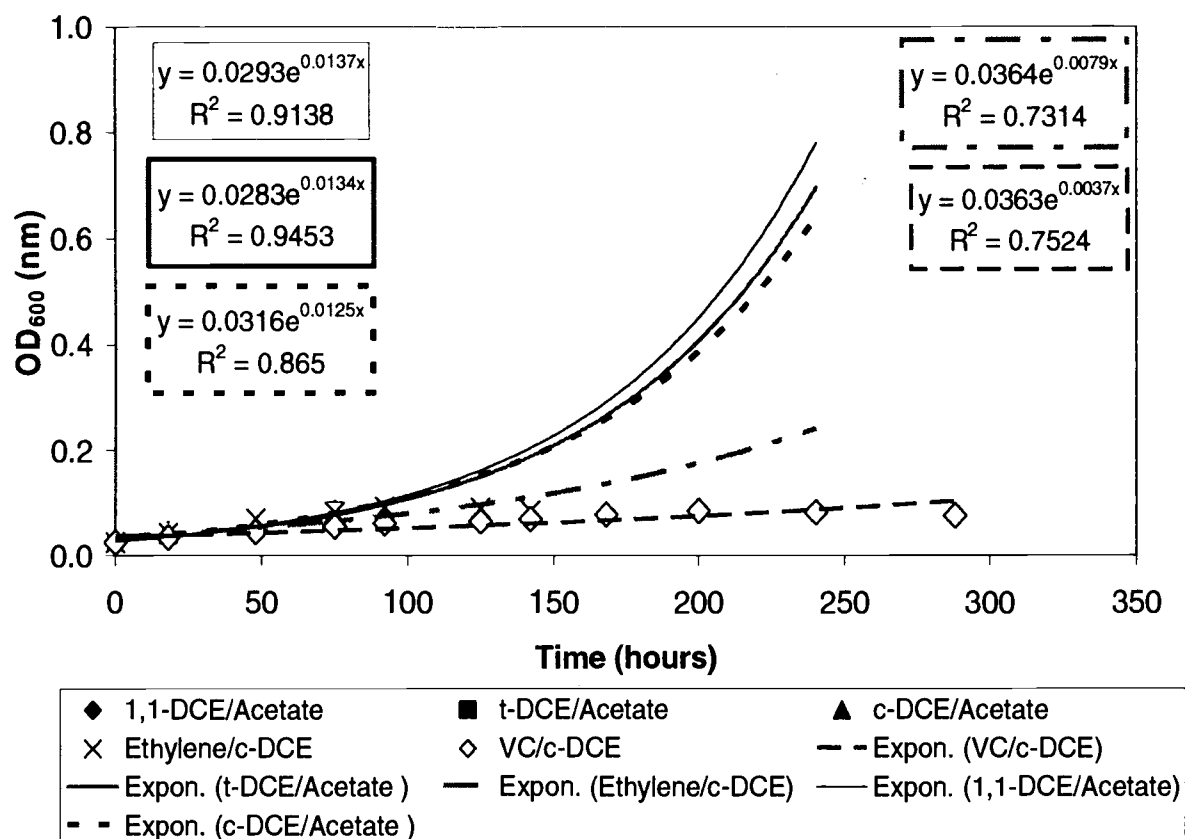
Figure C.3. This corresponds to the data presented in Table 4.4.

APPENDIX D

NLSR Method

D1. NLSR METHOD

Y (g/g) =	0.035	(constant)
k (g/g-d) =	7.460	(fitting parameter - varies)
Ks (mg/L) =	23.681	(fitting parameter - varies)
Xao (mg/L) =	11	(constant)
Clo (mg/L) =	53.646	(fitting parameter - varies)

Observed Data		Predicted (model)			Slope Calculations		Slope	Error t.obs-t.pred	Weighted Error	W. Error Squared
t	CL	t	CL	Xa	t	CL	dCL/dt			
day	(mg/L)	day	(mg/L)	(mg/L)	day	(mg/L)	(mg/L-d)	day	(mg/L)	(mg/L)^2
0	50	0.06	53.55	11	0.00	53.45		-0.06437		
0.08	47.65	0.11	47.65	11.21	0.11	47.55	-55.8534	-0.02625	1.46631	2.15006
0.21	42.17	0.21	42.17	11.4	0.21	42.07	-54.4552	0.00448	-0.24369	0.05939
0.28	39.76	0.25	39.76	11.48	0.25	39.66	-53.6854	0.02992	-1.60643	2.58063
0.41	31.38	0.41	31.38	11.78	0.41	31.28	-50.0545	-0.00121	0.06036	0.00364
0.5	27.66	0.49	27.66	11.91	0.49	27.56	-47.8334	0.01288	-0.61609	0.37957
0.63	21.36	0.63	21.36	12.13	0.63	21.26	-42.8676	0.00439	-0.18800	0.03534
0.71	18.07	0.71	18.07	12.24	0.71	17.97	-39.4805	0.00464	-0.18318	0.03356
0.75	16.16	0.76	16.16	12.31	0.76	16.06	-37.1919	-0.00509	0.18935	0.03585
0.79	14.35	0.81	14.35	12.37	0.81	14.25	-34.7645	-0.01529	0.53167	0.28267
0.85	12.92	0.85	12.92	12.42	0.85	12.82	-32.6436	0.00238	-0.07761	0.00602
0.92	10.51	0.93	10.51	12.51	0.93	10.41	-28.5963	-0.00609	0.17419	0.03034
1	8.16	1.02	8.16	12.59	1.02	8.06	-23.9671	-0.01519	0.36406	0.13254
1.05	6.68	1.08	6.68	12.64	1.09	6.58	-20.6344	-0.03126	0.64495	0.41596
1.08	5.7	1.13	5.7	12.67	1.14	5.6	-18.2211	-0.05141	0.93669	0.87739
1.13	4.76	1.19	4.76	12.71	1.19	4.66	-15.7331	-0.05640	0.88728	0.78726
1.17	4.55	1.20	4.55	12.72	1.21	4.45	-15.1523	-0.02987	0.45266	0.20491
1.22	4.22	1.22	4.22	12.73	1.23	4.12	-14.2203	-0.00213	0.03035	0.00092
1.33	3.5	1.28	3.5	12.75	1.28	3.4	-12.1009	0.05369	-0.64971	0.42212
1.47	2.63	1.36	2.63	12.78	1.37	2.53	-9.3710	0.11353	-1.06385	1.13179
1.58	1.86	1.45	1.86	12.81	1.47	1.76	-6.7862	0.12955	-0.87918	0.77296
1.68	1.26	1.55	1.26	12.83	1.57	1.16	-4.6522	0.12687	-0.59025	0.34839
1.72	1.04	1.60	1.04	12.84	1.63	0.94	-3.8415	0.11713	-0.44995	0.20246
1.79	0.6	1.74	0.6	12.85	1.79	0.5	-2.1715	0.04666	-0.10131	0.01026
1.92	0.55	1.77	0.55	12.85	1.82	0.45	-1.9773	0.15465	-0.30579	0.09351
									Weighted SSE	10.998

D.1. The data set from Smith et al. (1998) reproduced in Excel to get a working model.
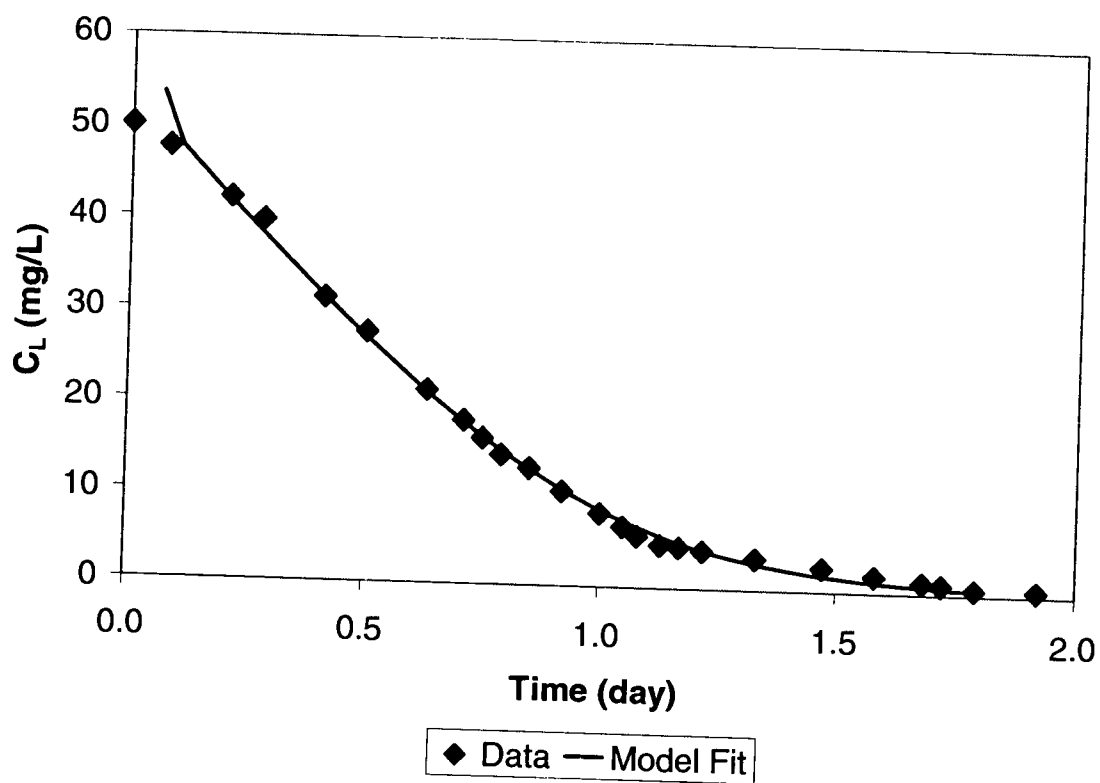
D.1. Model fit to the data set from Smith et al. (1998) reproduced in Excel.