# DETERMINATION OF VINYL-CHLORIDE UTILIZING KINETIC PARAMETERS PARAMETERS FOR A *MYCOBACTERIUM* STRAIN AND AN ENRICHMENT CULTURE

A Thesis

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Mak, Kin Teng

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# ABSTRACT

A recent study in our lab had suggested that aerobic oxidation of vinyl chloride (VC) was responsible for VC loss at extremely low oxygen concentrations that would be practically classified as anaerobic conditions. However, no aerobic VC-assimilating bacterium had been isolated from the enrichment cultures involved, and therefore no further characterization had been conducted. This study investigated the VC-utilizing kinetics of a *Mycobacterium* isolate from one of the enrichment cultures (from Cecil Field, FL), and another highly-enriched culture (from Industrial Site 4, CA). By limiting VC and $O_2$ concentrations, thus separately limiting the utilization rates of respective substrates, various kinetic parameters of both cultures were determined. The maximum VC utilization rate ($k_{VC}$) and half velocity constant for VC ($K_{s(VC)}$) of the *Mycobacterium* isolate were 11.5 nmol/min/mg of protein and 0.16 µM, respectively, while those of the S4 culture were 4.7 nmol/min/mg of protein and 0.51 µM, respectively. The half velocity constants for $O_2$ ($K_{s(O2)}$) of the *Mycobacterium* isolate and S4 culture were 0.06 mg/L and 0.03 mg/L, respectively. There was no measureable $O_2$ threshold identified for either culture. Overall, this study further supported the hypothesis that aerobic VC oxidation could account for loss of VC under commonly defined "anaerobic" conditions, and provided data for kinetic behavior of related aerobic VC-assimilating bacteria.

# BIOGRAPHICAL SKETCH

Born in Macau S.A.R., China on 11/26/1987, when the 34th Formula 3 Macau Grand Prix was taken place outside the hospital, Mak, Kin Teng has been experiencing his 24th year of life. He finished his high school at Pui Ching Middle School (Macao) at the age of 18. In the following four years, he spent his time in Huazhong University of Science and Technology, China, to pursue his study and earned his Bachelor degree in environmental engineering in July, 2009. After graduation, he continues his master study in environmental processes in the department of civil and environmental engineering at Cornell University and is expected to receive his master degree in January, 2012.

*To my family who continually supports me.*
*To those I love and those who loves me.*
*Most importantly, to the only GOD who has provided me all these.*

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# CHAPTER 1

# INTRODUCTION

Vinyl chloride (VC), a known human carcinogen (USEPA, 2000), is one of the major contaminants presented in groundwater at many chloroethene-contaminated sites. VC monomer is produced in large quantity each year, almost all of which is used for production of polymers, primarily polyvinylchloride (PVC) (Smits et al., 2011). Although direct discharge of VC happens, evidence clearly suggests that incomplete reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), which are widely used and disposed as solvents, results in accumulation of their daughter products – *cis*-dichloroethene (cDCE), VC, and ethene (ETH) (Habeck, 1996-2010; He et al., 2003; Distefano et al., 1991; Verce et al., 2002; Smits et al., 2011). The accumulation of such products can be attributed to the absence of certain microbial species, e.g. *Dehalococcoides ethenogenes*, which can reduce chloroethenes beyond DCE, or unfavorable site conditions, e.g. limited electron donors, which limit reductive dechlorination (Mattes, 2011). Even though ETH has its own deleterious effects on plants and environments, it is benign compared to other chloroethenes, and can only be generated when microbes that are capable of reducing VC to ETH exist (Mattes, 2011; Coleman et al., 2002). Therefore, incomplete reductive dechlorination of PCE and TCE not only doesn't attenuate the environmental hazards, but instead turns out to be more hazardous to human health, given the higher toxicity and carcinogenicity of VC, compared to PCE and TCE. This makes the remediation of VC at these contaminated sites more important and urgent.

Not many microorganisms and enrichments have been reported to be able to completely reductively dechlorinate PCE and TCE to ETH. A few examples, however, have been identified, such as *Dehalococcoides ethenogenes* strain 195 isolated by

Maymó-Gatell et al. (Maymó-Gatell et al., 1997), some other strains of *Dehalococcoides ethenogenes*, and some enrichment cultures (Nielsen and Keasling, 1999; Major et al., 2002; Cupples et al., 2003; Bunge et al., 2003). These processes are commonly carried out but may be constrained by environmental conditions or factors, such as limited substrate concentration (Bradley et al., 1998a).

Alternatively, the aerobic oxidation of VC is apparently rapid and commonly occurs in many environmental niches (Coleman et al., 2002; Singh et al., 2004; Smits et al., 2011; Bradley et al., 1998a). Both assimilatory (growth-coupled) and co-metabolic (fortuitous oxidation accompanying oxidation of substrates other than VC, such as toluene and phenol) degradation of VC can be observed under aerobic conditions (Coleman et al., 2002; Hopkins et al., 1995). Additionally, the anaerobic oxidation of VC has been reported, allegedly involving electron acceptors such as Fe(III), Mn(IV) or humic acid (Bradley et al., 1998b; Bradley et al., 1998a; Bradley et al., 1998c).

Although many studies relate the disappearance of VC under anaerobic conditions to anaerobic VC oxidation, not even one anaerobic VC-oxidizer has been isolated, which leads us to think that the "anaerobic" disappearance of VC claimed in previous studies maybe due to other existing mechanisms, namely aerobic oxidation. It is actually possible for this to happen because firstly, it is difficult to exclude all oxygen from samples. The cited oxygen concentrations were <3µM (~0.01 mg/L) and <5µM (~0.016 mg/L) for samples, in the studies related to Mn(IV) and humic acid, respectively, conducted by Bradley et al. (Bradley et al., 1998a; Bradley et al., 1998c). On the other hand, half-velocity constants and oxygen thresholds (0.02-0.1 mg/L) of various VC-degraders estimated by Coleman et al. from their isolates suggest that aerobic microbes could effectively degrade VC at low oxygen concentration (0.03-0.30 mg/L) (Coleman et al., 2002). Environments described as "anaerobic"

might, in reality, be environments with low but positive oxygen concentrations, albeit close to the oxygen thresholds where VC oxidations might proceed aerobically.

A recent study by Gossett concerning VC oxidation by two enrichments derived from chloroethene-contaminated sites under low-oxygen concentration provides strong evidence for the sustained, aerobic oxidation of VC at extremely low oxygen concentrations (Gossett, 2010). A considerable amount of VC was remediated through aerobic VC oxidation at oxygen concentrations <0.02 mg/l, maintained by permeation tubes. This study illuminates one of the causes, probably the most important one, of apparent loss of VC in the claimed anaerobic regions of subsurface sites, enabling a more in-depth understanding of processes undergone at contaminated sites and providing us with more remediation strategies. However, the study was performed with enrichment cultures, and no specific microorganism was isolated from them. Isolation of specific microorganisms from the enrichments is of great importance, because studies of the isolates will add to our growing database of aerobic VC-degraders. Specifically, VC- and oxygen-utilization kinetics of these microorganisms are of interest. Such information is useful for us to more accurately apply bioremediation strategies to chloroethene-contaminated sites, resulting in improved removal efficiency and cost savings compared to other existing alternatives.

Thus, there are two main objectives for this present study:

1. Isolation of microorganisms from the two enrichments responsible for the oxidation of VC under low-oxygen concentrations;
2. Evaluation of the kinetic parameters for VC- and oxygen-utilization by the isolated strains. Note: if isolation is not achieved, kinetics will be studied in highly enriched cultures derived from the previous study by Gossett.

# CHAPTER 2

# BACKGROUND

## 2.1 Chlorinated Ethenes

### 2.1.1 Occurrence, Properties, and Health Effects

Chlorinated ethenes are a group of compounds that possess the same structure of ethene, but with one or more hydrogen atoms substituted by chlorine(s). This group of compounds includes tetrachloroethene (PCE), trichloroethene (TCE), dichloroethenes (DCEs), and vinyl chloride (VC). There are three different isomers of DCE, namely 1,1-DCE, *trans*-1,2-DCE (tDCE), and *cis*-1,2-DCE (cDCE). PCE and TCE are manufactured and widely used as dry cleaning solvents and degreasing agents, while DCE is manufactured as solvent and intermediate for production of other chlorinated solvents (Olaniran et al., 2004). VC has been produced in bulk quantity since its discovery, and most of it has gone into the production of polyvinyl chloride (PVC) products (Rossberg et al., 2006). PVC production in 2009 achieved the level of approximately 30 million tons/year worldwide (Deloitte & Touche Regional Consulting Services Limited, 2010).

Every member of the group of chlorinated ethenes enters into and remains in the environment through different scenarios. Widespread use of PCE and TCE in commercial, industrial, and military applications, as well as improper disposal practices, have contributed to the majority of PCE and TCE contaminations of ambient groundwater of the United States (Squillace et al., 1999; Nielsen et al., 1999). On the other hand, DCE and VC contamination of groundwater can be more ascribed to incomplete reductive dechlorination of higher-chlorinated ethenes (e.g. PCE and TCE) than from direct disposal of these compounds (Habeck, 1996-2010; Distefano et

al., 1991). All chlorinated ethenes are toxic to human beings, but at different levels. VC has long been categorized as a known carcinogen, proven by ample evidence that it does trigger cancer, especially hepatic cancer (USEPA, 2001a). PCE and TCE were previously categorized as suspected carcinogens. Nonetheless, more and more evidence proved their intimate relationships with various cancers (USEPA, 2008, 2010). TCE is listed as a carcinogen by USEPA recently because significant evidence proves that TCE can prompt various cancers and can result in increased risk of many other cancers and Parkinsons disease (USEPA, 2010; Mattes et al., 2010; Goldman et al., 2011). Epidemiologic evidences have related PCE exposure with excess risks for a number of cancers, but a causal association has yet to be definitively established, which means PCE is still listed as suspected carcinogen thus far (USEPA 2008). The new maximum concentration levels (MCL) of PCE and TCE have not yet been identified and published. Their current MCL are both 0.005 mg/L while MCL of VC is 0.002 mg/L (USEPA, 2006).

Over the years, chlorinated ethenes have become one of the most common contaminants among all other contaminants. Out of 1662 National Priorities List (NPL) sites identified by US Environmental Protection Agency (USEPA), 616 sites were found contaminated by VC, and out of 1430 NPL sites, 771 and 852 were found with TCE and PCE, respectively (USEPA, 1997, 2003, and 2006). One thing to note is that the percentage of NPL sites affected by VC has risen compared to the same data reported in 1996 (He et al., 2003). This could indicate an increasing threat posed by VC, and could serve as an urgent call for remediating these sites.

### 2.1.2 Environmental Fate and Transport

Anaerobic reductive dechlorination, or halorespiration, of chlorinated ethenes is the primary biological action observed that describes biotransformation between

different chlorinated ethenes (Distefano et al., 1991). In this anaerobic respiration process, chlorinated ethenes serve as electron acceptors and hydrogen as electron donor, which is usually made available by degradation of other existing organic compounds (Tiehm et al., 2011). In general, at the initial point of the chloroethenes plume, where PCE and TCE are being disposed, the environment is usually anaerobic and reducing, probably because of the high COD level associated with co-contaminants. Halorespiration of higher-chlorinated ethenes, in other words more oxidized compounds, such as PCE and TCE, are favorable. Starting with PCE (as an example), through anaerobic reductive dechlorination, hydrogen consecutively replaces chlorine on the ethene molecule, if the environmental conditions are favorable, and thus PCE is transformed stepwise to TCE, DCE, VC and finally ethene which is a benign end product. It is, however, not assured that complete dechlorination to ethene is achieved. Research in the past reveals that only some microorganisms within a specific specie are able to reduce higher-chlorinated ethenes (PCE, TCE or DCE) completely to ethene, namely *Dehalococcoides ethenogenes* (He et al., 2003; Maymó-Gatell et al., 1997; Cupples et al., 2003). *Dehalococcoides ethenogenes* strain 195 is yet the only microorganism isolated which can completely reduce PCE to ethene. Other isolated dehalorespirers, such as *Dehalobacter restrictus* (Holliger et al., 1999) or *Desulfitobacterium* (Gerritse et al., 1996), cannot dechlorinate PCE and TCE beyond DCE. The lack of *Dehalococcoides ethenogenes* at contaminated sites, which is rather common, results in the accumulation of DCE and VC in the plume. At this point, oxygen may have slowly fluxed into the plume through diffusion and/or recharge from external environments, thus creating some aerobic niches in the plume.

Many studies in the past have focused on biological transformation of lower-chlorinated ethenes apart from reductive dechlorination. These include:

1. Anaerobic oxidation to carbon dioxide with co-substrates serving as electron acceptors, e.g., Mn(IV) and humic acid (Bradley et al., 1998a; Bradley et al., 1998c), or oxidation coupled to methane formation under methanogenic conditions (Bradley et al., 2000).
2. Aerobic oxidation to carbon dioxide, with DCE or VC being assimilated into biomass as primary substrate (Coleman et al., 2002a; Bradley et al., 2000; Hartmas et al., 1985; Singh et al., 2004).
3. Aerobic oxidation to carbon dioxide but is rather a co-metabolic reaction. Co-substrates such as methane, ammonium, ethene, and toluene can serve as primary substrates which sustain growth of microorganisms, which simultaneously co-metabolize VC to carbon dioxide (Broholm et al., 2005; Coleman et al., 2002a).

As the plume of chlorinated ethenes flows through the subsurface, more than one of the above biological activities could occur attenuating the contamination. One of the key steps to achieve effective attenuation is the removal of VC because reductive dechlorination of other higher-chlorinated ethenes would generate more VC. Although VC is a gas under ambient pressure and temperature, groundwater systems are gas-phase limited and unmixed, resulting in limited volatilization; in other words, most newly generated VC would remain in the system which would increase the importance for VC attenuation downstream. In the next section, we focus on the biodegradation of VC.

## 2.2 Remediation of Chlorinated Ethenes

### 2.2.1 General Remediation Methods

Over the years, many remedial technologies for chlorinated-ethene contaminated plumes have emerged but in this section, three of the most common

technologies – pump and treat, permeable reactive barrier, and in-situ bioremediation – are briefly discussed. These technologies have both advantages and disadvantages. Sometimes, application of one technology for treating contaminated plumes is infeasible or ineffective due to its limitations and the complexity of environmental conditions. So, they are often used together to achieve better remediation of contaminated plumes.

Pump and treat is the most conventional method; it relies on pumping the contaminated groundwater to the surface through extraction wells, followed by either offsite disposal or re-injected into the subsurface after treatment at the surface to remove contaminants. It is an ex-situ treatment method. Though it is widely adopted at contaminated sites, it has several drawbacks that hinder its application to chloroethene remediation. One of the biggest problems is that the effectiveness of removal by the pump and treat method is highly related to characteristics of the target contaminant and the subsurface matrix, especially the contaminant's solubility in water and tendency to adsorb onto soil particles, which can be inferred by the contaminant's octanol-water partition coefficient ($K_{ow}$) and adsorption coefficient ($K_{oc}$). The pump and treat method is only able to treat the contaminants which exist in aqueous phase. Research indicates that PCE, TCE, and DCE are rather hard to desorb from soil particles, thus making them hard to be extracted from subsurface and enter treatment facilities (Yong et al., 2005). This could greatly influence the effectiveness of removing these contaminants from plumes. Moreover, other drawbacks include the high cost of extended operation of pump and treat systems, geological restriction, and potential contamination by other by-products generated from on-site treatments (De Wildeman et al., 2003). Finally, the migration of contaminants into low-transmissive zones impedes the ability of pump and treat to extract them.

A permeable reactive barrier (PRB) is a barrier, typically constructed of highly permeable emplacements of materials, used to minimize the potential migration of contaminants downgradient. These barriers are filled with electron donors, e.g., vegoil, bark, compost, chitin or zero-valent iron which can help reduce chlorinated ethenes through reductive dechlorination as the plume flows through (USEPA, 2005). Problems of this method in remediation of chlorinated ethenes include geological restriction of installation of barriers, and ineffective removal of lesser-chlorinated ethenes, like DCE and VC (USEPA, 1998, 2002). However, compared to a conventional pump and treat method, PRB is less expensive to install and operate (USEPA, 2001b). PRBs are able to simultaneously treat multiple contaminants, if the conditions are appropriate, and without the risk of cross contamination which can happen in pump and treat applications (ITRC, 2005). On the other hand, PRBs are more of a containment technology than a remediation technology. However, coupled with other remedial technologies, they can play an important role in site remediation.

Bioremediation is the use of microbial metabolism to remove contaminants. It has been greatly studied and regarded as an important strategy for cleanup of chlorinated-ethene contaminated sites, because of its comparative advantages over other current methods. These advantages include its lower cost for treatment, its potential for complete destruction of contaminants, its ability to be conducted in situ, and that it represents a natural initiative that is more widely accepted by the public (Vidali, 2001).

There are many bioremediation technologies used in different pollution-treatment situations, but three typical technologies are discussed here: Monitored natural attenuation (MNA), enhanced natural attenuation, and bioaugmentation. MNA utilizes microorganisms which already exist at the contaminated sites without disturbing the system but simply monitoring the

disappearance of contaminants and concentrations of subsequent products resulting from natural attenuation. Compared to other bioremediation technologies, MNA is a relatively passive method. Although MNA can be cost-effective, it is slow and may not be applicable to every site because of the absence of suitable microorganisms and/or the time-length to achieve regulatory limits for site closure. Enhanced natural attenuation is an upgraded version of MNA, which attempts to accelerate degradation processes by addition of amendments that stimulate a naturally occurring consortium of bacteria to increase the rate of degradation. The application of enhanced natural attenuation utilizes knowledge about the indigenous consortia of bacteria but still relies upon (and therefore is limited by) the types of indigenous microorganisms at the site. Lastly, bioaugmentation is a type of enhanced attenuation which involves amending the system with additional species or higher levels of indigenous species that can confer a degradation ability (or enhanced rate) the system does not otherwise possess. Bioaugmentation usually involves the addition of enhancing chemicals (nutrients, substrates, buffers, etc.), along with microbes. It is the bioremediation approach that is the most interventionist. However, there is always the concern about whether introduced species can survive and be active in the environments to which they are bioaugmented (Early et al., 2006). Application of bioaugmentation usually requires detailed information about the site, the degradation pathways, the introduced species, as well as their genetic characteristics. Because of these added complexities, various field studies are often required before implementation.

### 2.2.2 Biodegradation of VC

As mentioned before, possible VC biodegradation pathways include reductive dechlorination, anaerobic oxidation and aerobic oxidation. Many research studies have focused on these degradation pathways, specific strains which are involved in

them, as well as molecular genetic analyses of them, influences of external factors to the microorganisms, and the effectiveness of degradation processes.

Only some strains of *Dehalococcoides* are able to biotransform chlorinated ethenes to ethene, indicating their special importance to bioremediation (He et al., 2003; Maymó-Gatell et al., 1997; Cupples et al., 2003). However, presence of these strains at chlorinated-ethene contaminated sites is not assured, limiting the applicability of MNA or enhanced MNA based on reductive dechlorination in many instances.

Anaerobic oxidation of VC has been claimed to occur in several different enrichments under Fe(III)-reducing (Bradley et al., 1998b; Bradley & Chapelle, 1998d), Mn(IV)-reducing (Bradley et al., 1998c), methanogenic (Bradley & Chapelle 1998d; Bradley et al., 2000), humic-acid (Bradley et al., 1998a), and $SO_4^{=}$-reducing conditions (Bradley et al., 1998a), indicating the possible widespread importance of these biological activities in environmental niches. Oxidation of VC amended by co-substrates (Fe(III), Mn(IV)) was compared with aerobic oxidation (Bradley et al., 1998b,c). Higher percentage mineralization of VC to $CO_2$ was observed to occur under aerobic than anaerobic conditions, with only anaerobic oxidation amended by Mn(IV) demonstrating similar effectiveness in VC mineralization (Bradley & Chapelle, 1998d; Bradley et al., 1998a,b,c). Nevertheless, there has been not even one anaerobic VC-oxidizer isolated thus far, a situation which inhibits in-depth studies on their phylogeny and physiology. Furthermore, in a recent publication of Bradley (Bradley, 2011), he states that some previous studied environmental conditions (Fe(III)-, Mn(IV)-, and $NO_3^-$-reducing conditions) were not reducing enough to preclude the concurrent contribution of aerobic oxidation. This suggests that the effectiveness of VC mineralization achieved in previous studies might have been the result of aerobic oxidation, rather than anaerobic oxidation.

Compared to the other two biological activities mentioned above, aerobic oxidation of VC is more studied and well-documented. Discovery of aerobic VC oxidizers can be traced back to 1985, when de Bont and van der Linden found a *Mycobacterium* capable of growing on vinyl chloride, named as *Mycobacterium* L1. Hartmans and his colleagues further investigated its application to remove vinyl chloride from industrial-waste gases (Hartmans et al., 1985). Since then, various strains from different genera have been identified with ability to carry out aerobic VC oxidation. Typically, they are divided into two categories: (1) co-metabolic VC degradation in the presence of co-substrates like phenol, toluene, ethene, methane, etc. (Hopkins et al., 1995; Fox et al., 1990; Koziollek et al., 1999); and (2) VC-assimilation, or growth-coupled VC oxidation, meaning that certain strains are able to utilize VC as primary substrate and assimilate it into their own biomass.

Co-metabolic VC degradations are, however, comparatively slow compared to growth-coupled VC oxidation, mainly because of substrate competition, enzyme inactivation and accumulation of toxic metabolites (Newman and Wackett, 1997; Ensign et al., 1992). In addition, the need for substrate other than VC could be problematic for treating contaminated sites where such substrates are absent. All these factors make it hard to implement co-metabolic oxidation to treat VC contamination.

On the other hand, many genera that are capable of assimilating VC have been isolated, such as *Mycobacterium* (Hartmans et al., 1985; Coleman et al., 2002; Hartmans et al., 1992), *Pseudomonas sp*. (Verce et al., 2001), *Ralstonia* sp. (Alango et al., 2006) and *Nocardioides sp*. (Coleman et al., 2002). The isolation of VC-assimilating strains provides us opportunities to investigate not only the VC-utilizing kinetics but also the physiology and molecular genetics of this group of microorganisms. Through kinetic studies, relationships between substrate utilization rate and substrate concentration of these microorganisms are known, which can assist

prediction of environmental behavior at contaminated sites. One of the most remarkable studies was done by Coleman and his colleagues. They isolated 12 aerobic VC-assimilators (11 *Mycobacterium* and 1 *Nocardioides* strains) from various chlorinated-ethene contaminated sites and reported kinetic parameters for 5 of the 12 VC-assimilators. This study elucidates the diversity and distribution of some aerobic VC-assimilating bacteria, and thus their biological behaviors at VC contaminated sites (Coleman et al., 2002a). The kinetic parameters found in the study are shown in Table 2.1.

**Table 2.1 Kinetic parameters of VC-assimilating bacteria (Coleman et al., 2002)**

| **Strain** | $k_{VC}$ **(nmol/min/mg of protein)**[a] | $K_{s(VC)}$ **(μM)** | $K_{s(O2)}$ **(μM)** | $O_2$ **threshold (mg/L)** |
|---|---|---|---|---|
| *Mycobacterium* | | | | |
| JS60 | 9.7 ± 0.2 | 0.5 ± 0.1 | 0.17 ± 0.06 | 0.02 ± 0.01 |
| JS61 | 9.0 ± 0.2 | 0.8 ± 0.2 | 0.03 ± 0.04 | 0.07 ± 0.01 |
| JS616 | 15.4 ± 0.6 | 3.2 ± 0.3 | 0.30 ± 0.24 | 0.10 ± 0.02 |
| JS617 | 16.0 ± 0.4 | 0.8 ± 0.1 | 0.07 ± 0.06 | 0.06 ± 0.02 |
| *Nocardioides* sp. strain JS614 | 43.1 ± 4.2 | 1.2 ± 0.2 | 0.11 ± 0.04 | 0.06 ± 0.01 |

[a] All data from the study were averages of three replicates; error values represent 95% confidence intervals.

Moreover, physiology and molecular genetic analyses enables us to identify functional genes which are related to degradation pathway. To date, the enzymes involved in the first two steps of aerobic VC assimilation have been identified, namely Alkene monooxygenase (AkMO) and EaComT (Mattes et al., 2010; Mattes et al., 2005). These findings enable us to more easily monitor degradation activities at contaminated sites, through biosensors for example, or create transgenic strains for better VC bioremediation. Interestingly, the enzymes found for VC-assimilation are

the same enzymes involved in ethene degradation. Several researchers suggest that some aerobic VC-assimilating bacteria could have evolved from aerobic ethene-assimilating bacteria through prolonged exposure to VC (Mattes et al., 2010; Yang and Mattes, 2008).

A recent study by Gossett suggests that aerobic VC oxidation can be sustained at low oxygen concentrations, which offers an alternative explanation for VC degradation under ill-defined “anaerobic” conditions (Gossett, 2010). After this paper was published, several other papers re-defined the role of aerobic VC oxidation as the major contributor to natural VC attenuation, which accounts for 85%-65% of VC degradation at contaminated sites (Bradley and Chapelle, 2011; Bradley, 2011). These studies suggest that aerobic VC oxidation is worthy to be developed and researched as an effective tool for VC remediation in the years ahead.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Experimental Strategy

In this study, vinyl chloride degradation by microorganisms under aerobic conditions was investigated. A modified, Michaelis-Menten equation (Equation 3.1) was the basis for describing VC-utilization kinetics:

$$\frac{dS_{VC}}{dt} = -\frac{V_{\max(VC)}(S_{VC}-S_{th(VC)})}{K_{s(VC)}+(S_{VC}-S_{th(VC)})} \cdot \frac{(S_{O2}-S_{th(O2)})}{K_{s(O2)}+(S_{O2}-S_{th(O2)})} \quad (3.1)$$

where

$V_{max(VC)}$: maximum rate of VC depletion in moles per liter per hour.

$S_{VC}$: VC aqueous concentration in moles per liter.

$S_{O2}$: dissolved oxygen concentration in milligrams per liter.

$S_{th(VC)}$: threshold VC concentration, if there is any, in moles per liter.

$S_{th(O2)}$: threshold dissolved oxygen concentration, if there is any, in milligrams per liter.

$K_{s(VC)}$: half-velocity constant with respect to VC in moles per liter.

$K_{s(O2)}$: half-velocity constant with respect to oxygen in milligrams per liter.

This model describes the rate of VC oxidation as potentially limited by aqueous concentrations of either VC or oxygen, if either is at concentration that is not much greater than its respective $K_s$. On the other hand, when both $S_{VC}$ and $S_{O2}$ are >> than $K_{s(VC)}$ and $K_{s(O2)}$, respectively, VC depletion will proceed at some maximum rate ($V_{max(VC)}$) limited only by biomass concentration and environmental conditions

(temperature, pH, etc.).

$V_{max(VC)}$, $K_{s(VC)}$, $K_{s(O2)}$, $S_{th(VC)}$ and $S_{th(O2)}$, of two cultures were determined by observing VC degradation under a VC-limiting condition, and $O_2$ consumption under an $O_2$-limiting condition. During each observation, a set of triplicate samples was used. Excess VC and $O_2$ were purged from the samples with oxygen-free nitrogen gas. For establishing the VC-limiting condition, samples were fed with enough oxygen so that VC concentration would limit kinetics. Conversely, enough VC was added to the samples so as to make $O_2$ the kinetically limiting factor in establishing the $O_2$-limiting condition. Headspace samples were analyzed using gas chromatography methods. The data were then fit to the Michaelis-Menten kinetics by the Matlab® curve-fitting toolbox to estimate the values of the parameters. A complicating factor is that both VC and oxygen are volatile and therefore partition between headspace and aqueous phases in our experimental bottles. This was taken into account, as described later in "Data Analysis".

3.2. Chemicals and Media

Vinyl chloride, 99.5% purity, was from Sigma Aldrich. All other reagents were reagent grade. Both broth enrichments and pure cultures were maintained in minimal salts medium (MSM), buffered at pH 7.1-7.2 with 20 mM phosphate and supplemented with trace metals as described by Coleman et al. (Coleman et al., 2002). One-quarter-strength Trypticase soy agar (1/4-TSA; pH 7.0; Difco) was used as nonselective medium and noble agar (NA; pH 7.0; Difco) dissolved into MSM was used as selective medium with VC for isolation. Bovine serum albumin (BSA), 1 mg/mL protein standard, was from Sigma-Aldrich.

Measurement of oxygen concentration at low level is difficult and easily

impacted by carry-over of oxygen remaining in a syringe used for sampling. A "zero oxygen" serum bottle was created for syringe-flushing prior to acquiring headspace samples from culture bottles. Zero-oxygen bottles were prepared by adding excess $Na_2SO_3$ to water with 100 mg/L $CoCl_2$ catalyst in a 160-ml serum bottle sealed with Teflon® rubber stopper and an aluminum crimp cap. The bottle was purged 20 minutes and over-pressurized with $O_2$-free nitrogen gas (scrubbed through titanium (III)-citrate solution (Zehner et al., 1976)). From stoichiometry, oxygen which might enter the zero-oxygen bottle oxidizes sulfite to sulfate and is thus removed. Each time before sampling a culture bottle or low-oxygen standard bottle, the syringe was flushed with headspace from the zero-oxygen bottle to minimize any oxygen carry-over from a previous sampling.

## 3.3 Enrichment Cultures and Isolation

### 3.3.1 Aseptic Technique

Several aseptic techniques were adopted to prevent contamination during culture operations. All bottles were autoclaved before use with liquid media in them at 121℃ for 40 minutes. Sterile syringes and needles were used when delivering vinyl chloride, oxygen, transferring cultures, and sampling liquids. For all operations, the tops of bottles and connecting parts were swabbed with ethanol and flamed to sterilize. For GC analysis, sterile needles (but not sterile syringes) were used; the sterility of the sample needle was maintained by inserting it (connected to its syringe) for 1 minute into a 250˚C GC injector port just prior to inserting through a culture-bottle septum for sampling. Headspace samples were thus acquired via sterile needles into non-sterile syringes, but the syringe plungers were only withdrawn, never pushed to expel, while needles were inserted into bottles. Furthermore, all plating was

conducted under a laminar-flow hood. Flame-sterilized loops were used for picking colonies and streaking, and were flamed between each action of use.

3.3.2 Enrichment Cultures

Two aerobic, VC-degrading enrichment cultures were obtained from a previous study conducted by Gossett (Gossett, 2010). They were derived from subsurface materials acquired from different chloroethene-contaminated sites. Industrial Site 4 (S4) enrichments were derived from shallow subsurface sediment and groundwater at an electronics manufacturing site contaminated mainly by TCE and 1,1-DCE and slightly by VC. Cecil Field (CF) enrichments were derived from stream-bed sediments and groundwater at the former NAS Cecil Field, Jacksonville, FL, which was primarily impacted by VC (Gossett, 2010).

The dilution-to-extinction method was used in an attempt to purify enrichment cultures and isolate specific VC-utilizing microorganisms. Serial dilutions from $10^{-5}$ to $10^{-8}$ of the enrichments were made in MSM, supplemented with VC. Remaining VC and turbidity were monitored over time. In the end, the $10^{-6}$ dilution was chosen for further study in each case because they were the greatest degree of dilution showing positive activity. However, cultures created from these dilutions were later shown to be highly enriched, but not pure. Nonetheless, they were used as the starting point for further purification attempts in this study.

All cultures were maintained with MSM in 160-mL serum bottles (100 mL MSM and 60 mL headspace) sealed with Teflon® -faced rubber stoppers and aluminum crimp caps, and periodically supplemented with VC (ca. 42 µmol). Oxygen was provided by air trapped in the headspace of the bottles during preparation. Enrichment cultures were transferred to fresh medium (0.5% v/v) every 1-2 months and were incubated aerobically at 22℃ at an inverted angle, agitated at 160 rpm on a

rotary-shaker.

3.3.3 Isolation

After confirming that the dilution-to-extinction method was not successful, we adopted an alternative of using selective media to isolate aerobic, VC-utilizing bacteria. Enrichment cultures were streaked onto noble agar-MSM plates and incubated at ambient temperature in a desiccator with VC (1% v/v) in the headspace. Noble agar is the highest purity agar available and is believed to be free from other carbon sources. Under this circumstance, any colony growing in this condition is likely to be an aerobic, VC-utilizing bacterium. After incubation for 1-2 months, several colonies were sampled via sterile loop and inoculated into 25-mL serum bottles with 7 mL MSM and 0.2 mL gaseous VC (ca. 8.4 µmol/bottle, depending upon barometric pressure and temperature at the time VC was added). Subsequent VC-utilization activity was routinely monitored by checking turbidity and VC depletion over time. Aseptic techniques described in section 3.3.1 were used for GC analysis and maintaining cultures to prevent contamination. Once growth or disappearance of VC was confirmed, cultures were re-streaked onto ¼ -TSA agar plates and submitted for partial sequencing (500 bp) of the amplified 16S rRNA gene (MIDILab, 2010).

Only one pure culture was obtained from the CF enrichment; and none from S4 cultures. Our kinetic study therefore utilized a pure CF culture and a highly enriched S4 culture.

3.3.4 Identification and Characterization

The partial 16S rRNA gene sequence was uploaded to a database – Basic Local Alignment Search Tool (BLAST) – to compare with microorganisms reported therein. Phylogenetic relationship between our isolate and microorganisms with

similar 16S rRNA gene sequence were provided by the report from MIDI Lab (MIDILab, 2010).

## 3.5 Kinetic Experiment Procedure

### 3.5.1 Experiment Preparation

For every experiment, triplicate culture samples were produced using the following procedure. An aliquot of 0.5 mL from the source culture was transferred into three 160-mL serum bottles containing 100 mL MSM, supplemented with 1 mL of VC (ca. 42 µmol, depending upon the barometric pressure and temperature at the time of VC addition). All culture samples were prepared, monitored and maintained using aseptic techniques described in section 3.3.1 for about 45 days at 22˚C, inverted at an angle on a rotary-shaker at 160 rpm, from the day of transfer to the day when kinetic experiments took place. Six to eight spikes of VC (ca. 42 µmol for each spike) were consumed during this period. This allowed the cultures to produce a sufficient amount of active biomass for the experiments. One design objective was that growth of new biomass should be insignificant during the period of usable-kinetic-data acquisition. It was also desired that depletion rates be neither too fast nor too slow during kinetic monitoring. Before every experiment, bottles were purged with oxygen-free nitrogen gas for 20 minutes to evacuate excess $O_2$ and VC. Then 5 mL of nitrogen gas was injected to over-pressurize each culture before initiating kinetic experiments.

For bottles to be monitored under VC-limiting condition, 0.05 mL of VC and 3 mL of $O_2$ (ca. 2.1 µmol/bottle of VC and 1.97 mg/L of $O_2$, depending upon barometric pressure and temperature at the time of addition) were added to each culture at start-up. For the $O_2$-limiting condition, 1 mL of VC and 0.7mL of $O_2$ (ca. 42 µmol/bottle of VC and 0.46 mg/L of $O_2$, depending upon barometric pressure and

temperature at the time of addition) were added. The bottles were allowed to equilibrate to 22℃ on a rotary shaker at 160rpm. A headspace sample (0.25-mL) was withdrawn using a locking, 0.5-mL gas-tight syringe (Pressure-Lok series A, glass with Teflon® -tipped plunger), previously made "oxygen-free" by several flushes in the "zero oxygen" bottle just before sampling; and this headspace sample was then injected into gas chromatograph 1 (GC1) for $O_2$ detection. Another 0.25-mL sample was then similarly acquired and injected to gas chromatograph 2 (GC2) for VC quantification. Samples were collected by this procedure at intervals as the substrates depleted. One thing to note: For kinetic experiments, in either condition, the difficulty of maintaining the sterility of sample was high when purging bottles with nitrogen gas. During this operation, samples were opened to air for about 20 minutes and contaminants might have gotten into them. Remedial methods adopted included completing respective experiments within 2 days and using aseptic techniques mentioned before for GC analysis. In any event, it was judged that the important thing was that the samples were handled aseptically leading up to the kinetic experiments themselves. If any microbial contamination were to have occurred thereafter, it would be of little consequence, since the kinetic experiments were designed to result in insignificant new growth. In other words, there was insufficient new substrate added to have resulted in appreciable growth of contaminants, in comparison to the starting culture.

At the end of the experiments, 5 mL of liquid media with active biomass was withdrawn from each replicate bottle for protein measurement. Since only the lower part of the depletion curves was of interest, increases in biomass over this part of the depletion curve should have been negligible.

### 3.5.2 Analytical Methods

#### 3.5.2.1 Vinyl Chloride Measurement

A Hewlett-Packard 5890 Series II gas chromatograph (GC1), equipped with a 16-ft × 1/8-in. column packed with 1% SP 1000 on 60/80 Carbopack B (Supelco, Inc.) and a flame-ionization detector was used to measure VC concentration. The oven temperature was set isothermally at 150℃. Headspace samples (0.25-mL) were injected into GC1. The correlation factor between peak height and total mass of VC per bottle was determined by measuring the headspace samples (0.25-mL) of triplicate standards. The standards were prepared with identical procedures: 100 mL of MSM was added to each 160-mL serum bottle which was sealed with Teflon® rubber stopper and an aluminum crimp cap. 1 mL of vinyl chloride was added and the total mass of VC in the bottle was calculated by the Ideal Gas Law (Equation 4.3):

$$n = \frac{PV}{RT} \tag{3.2}$$

where

n: Total moles of VC in the bottle (moles).

P: the barometric pressure in atm.

V: the volume of VC added in liter.

R: the ideal gas constant which has the value 0.08206 $L \cdot atm \cdot K^{-1} \cdot mol^{-1}$.

T: the temperature in Kelvin.

The triplicate standards were then allowed to equilibrate for 2 hours before VC analysis by GC. A correlation between total μmoles of VC and peak height was established and thus the factor was determined as 0.00384 μmol/peak height for GC1.

During kinetic experiments, VC was measured by another gas chromatograph of the same model (GC2) but with a 30-m × 0.53-mm, 1-μm film Super Cowax-10

column. The oven temperature was set isothermally at 40℃. Calibration procedure for GC2 was identical to that described above to identify the relationship between peak height and VC content. The correlation factor for GC2 was 0.00505 μmol/peak height.

3.5.2.2. Oxygen Measurement

Oxygen was measured in replicates during kinetic experiments by GC1 with a 3-ft × 1/8-in. column packed with 60/80 Molecular Sieve 5A (Supelco, Inc.) with a thermal-conductivity detector and helium for carrier gas. Oven temperature was set isothermally at 30℃. Before sampling, a 0.5-mL, locking, gas tight syringe with needle was flushed several times in the "zero oxygen" serum bottle. Headspace samples (0.25-mL) were withdrawn and injected into GC1. Although we only measured the oxygen concentration of the headspace ($C_g$, mg/L), aqueous oxygen concentration ($C_w$, mg/L) can be calculated through Henry Law's where $C_g=H_cC_w$. $H_c$ is the pseudo-dimensionless Henry's constant which is 31.676 [mg/L gas per mg/L water] at 22℃, based on an oxygen solubility in pure water (1 atm) of 8.743mg/L (American Public Health Association, 1998).

Oxygen measurement was calibrated through a linear relationship between peak height and $O_2$ aqueous concentration. A series of concentrations from 0 mg/l to 3.3 mg/L was made and assayed using the same method described before. Every peak height reading was modified by subtraction of the peak height at the concentration of 0 mg/l added oxygen. The linear calibration curve is shown below in Figure 3.1.

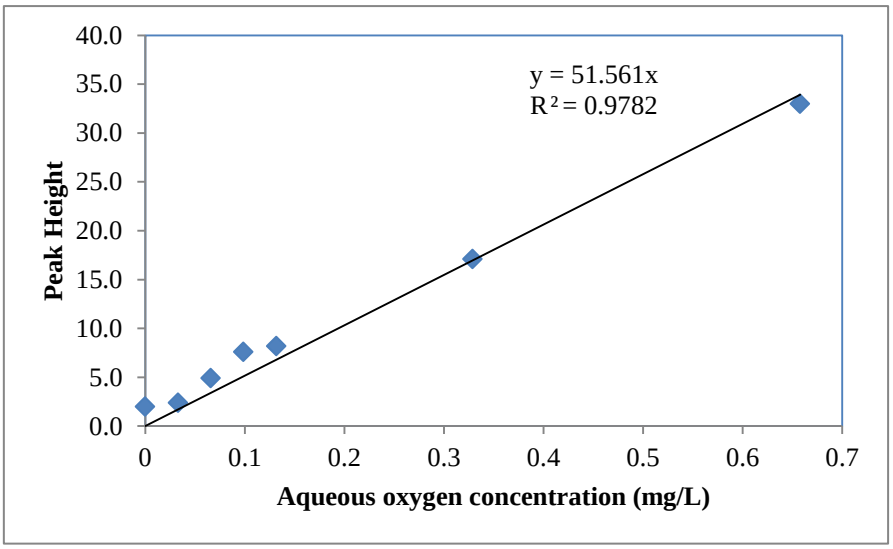


**Figure 3.1 Oxygen calibration curve. The trend line was being forced to go through zero.**

3.5.2.3 Protein Measurement

Protein contents of samples were quantified by a UV absorbance assay, based on that described by Coleman et al. (Coleman et al., 2002a). Sample fluid (0.45-mL) was mixed with 10M NaOH (0.15 mL). Then the samples were heated at 95℃ for 10 minutes using a block heater. After the samples cooled down, 400 µL (10M HCl and MSM in 5:3 ratio) was added to each sample. All the samples were centrifuged at 13000x*g* for 10 minutes. Absorbances at 230 and 260 nm of the supernatant were measured by Hewlett-Packard 8452A Diode Array Spectrophotometer and the protein content was calculated in mg/L as $(183\times A_{230}) - (75.8\times A_{260})$ (Coleman et al., 2002a). A correction factor was determined by repeatedly comparing the concentrations calculated from absorbance at 230 and 260 nm versus the concentrations diluted from 1000-mg/L Bovine Serum Albumin (BSA) protein standards to several concentrations over the range from 0 mg/L to 200 mg/L. A correction factor of 0.9125 was thus determined, and the raw, $(183\times A_{230}) - (75.8\times A_{260})$ value was divided by this correction factor to arrive at reported protein concentrations.

## 3.6 Data analysis

### 3.6.1 VC-Limiting Condition

The Matlab® curve-fitting toolbox was used to fit VC depletion curves to a modified Michaelis-Menten equation to determine $V_{max(VC)}$, $K_{s(VC)}$ and $S_{th(VC)}$ values. A complicating factor in measuring VC depletion rates is that VC partitions between headspace and liquid phases in the bottles. Therefore, for a constant molar VC degradation rate, the rate of change in its aqueous concentration will be a function of headspace/liquid ratio, among other things. Thus, it is more useful to monitor rate of VC depletion by monitoring total mass (or moles) of VC in a bottle over time. If we assume that agitation is sufficiently rapid so that equilibrium can be assumed between gas and liquid phases, then the relationship between VC's aqueous concentration ($S_{VC}$) and its total moles in a bottle ($M_{t(VC)}$) is given by

$$S_{VC} = \frac{M_{t(VC)}}{(V_g H_{c(VC)} + V_l)} \tag{3.3}$$

$V_g$: gas-phase volume in liter.

$V_l$: liquid-phase volume in liter.

$H_{c(VC)}$: pseudo-dimensionless Henry's constant of VC at 22°C which is 0.965 [mol/L gas per mol/L water] (USEPA).

Substituting into equation 3.1 then gives

$$\frac{dM_{t(VC)}}{dt} = -\frac{V_{\max(VC)}(M_{t(VC)} - S_{th(VC)} \times (V_g H_{c(VC)} + V_l))}{(V_g H_{c(VC)} + V_l) K_{s(VC)} + (M_{t(VC)} - S_{th(VC)} \times (V_g H_{c(VC)} + V_l)} \cdot \frac{(S_{O2} - S_{th(O2)})}{K_{s(O2)} + (S_{O2} - S_{th(O2)})}$$

$V_{max(VC)}$ was redefined as the maximum rate of VC depletion in moles per

hour — i.e., a mass-based maximum rate, rather than a concentration-based one.

In the VC-limiting condition, oxygen was provided excessively so that $S_{O2} >> K_{S(O2)}$ and thus $K_{S(O2)}$ could be neglected. Therefore, the latter portion of the equation becomes essentially 1 and the equation can be expressed as:

$$\frac{dM_{t(VC)}}{dt} = -\frac{V_{\max(VC)}(M_{t(VC)} - S_{th(VC)} \times (V_g H_{c(VC)} + V_l))}{(V_g H_{c(VC)} + V_l) K_{s(VC)} + (M_{t(VC)} - S_{th(VC)} \times (V_g H_{c(VC)} + V_l)}$$

And

$$V_{max(VC)} = k_{VC} X V_l \qquad (3.4)$$

Where $k_{VC}$ is the specific substrate utilization rate of VC [moles per day per mg protein], and X is the protein concentration of the media [mg protein/L]. The equation can be integrated and becomes equation 3.5:

$$t = \frac{1}{k_{VC} X V_w}\left[(V_g H_{c(VC)} + V_l) K_{s(VC)} \ln \frac{M_i - S_{th(VC)} \times (V_g H_{c(VC)} + V_l)}{M_{t(VC)} - S_{th(VC)} \times (V_g H_{c(VC)} + V_l)} + (M_i - M_{t(VC)})\right] \qquad (3.5)$$

$M_i$ is the VC content of the sample at time = 0, which has a unit of moles. $S_{th(VC)}$ is the threshold of VC expressed in concentration (mole/L). If the VC content goes below detection limit (about 0.03μmol/bottle for VC as determined by Gossett (Gossett, 2010)), the threshold term will be omitted because such a value is too small to be measured.

Parameters t and $M_t$ were the two parameters we obtain directly from the experiments. For every single replicate bottle, the two parameters were fitted to equation 3.5 with Matlab® . Corresponding $K_{s(VC)}$, $S_{th(VC)}$ and $k_{VC}$ were determined by the software where a mean for each parameter is given with 95% confidence interval.

Therefore, for every experiment and every parameter, three sets of values were generated from the three biological replicate bottles. In order to generate a mean of means, t-tests were applied to determine if there were significant differences among biological replicates to prevent their being pooled. The outlier, if there was any, would be discarded. A mean of means for every parameter was calculated after running the t-tests with a 95% confidence interval.

### 3.6.2 $O_2$-Limiting Condition

For the $O_2$-limiting condition, a similar procedure was used to determine $K_{s(O2)}$ and $S_{th(O2)}$. However, since oxygen level was measured in milligrams/liter, it would be easier to fit the data with equation 3.5 in concentration instead of in total mass content, assuming the equilibrium of aqueous/gaseous oxygen concentration was reached. Equation 3.6 was used for curve fitting in this section:

$$t = \frac{1}{V_{\max(O2)}}\left[K_{s(O2)}\ln\frac{S_i - S_{th(O2)}}{S_{t(O2)} - S_{th(O2)}} + (S_i - S_{t(O2)})\right] \quad (3.6)$$

$S_i$ is the oxygen concentration of the sample at time = 0, which has a unit of milligrams/liter. $S_{th(O2)}$ is the threshold of $O_2$ expressed in concentration (mg/L), which will be omitted during curve fitting if final concentration of oxygen was immeasurable, in other words, below 0.01mg/L, the detection limit reported before (Gossett, 2010). $V_{max(O2)}$ was not of interest as a variable because its relationship to $V_{max(VC)}$ can be derived from a stoichiometric relationship between VC and $O_2$ consumption, given as 2.205 mol $O_2$/mol VC in previous literature (Gossett, 2010). Note, however, that the specific value of the stoichiometric proportion between VC and $O_2$ consumptions is unimportant to subsequent estimation of $K_{s(O2)}$ and $S_{th(O2)}$; all that matters is that a proportion exists. That is, we assumed that maximum oxygen

uptake rate corresponded to maximum VC degradation rate, and that when the oxygen uptake rate was one-half its maximum, the oxygen concentration equaled the $K_{s(O2)}$ value.

# CHAPTER 4

# RESULT AND DISCUSSION

Although purification was attempted with both enrichments, only one pure strain was obtained – that from the CF enrichment. Colonies of that isolate are round, flat, watery and yellow-pigmented when cultured on Noble agar and ¼ TSA (with VC in the headspace of desiccators). According to the 16S rRNA gene sequence, its closest relative is *Mycobacterium gadium* strain JS616 (98% sequence identity), which was isolated and characterized by Coleman et al. (Coleman et al., 2002). Figure 4.1 illustrates the relationship between our CF isolate and other closely related strains.

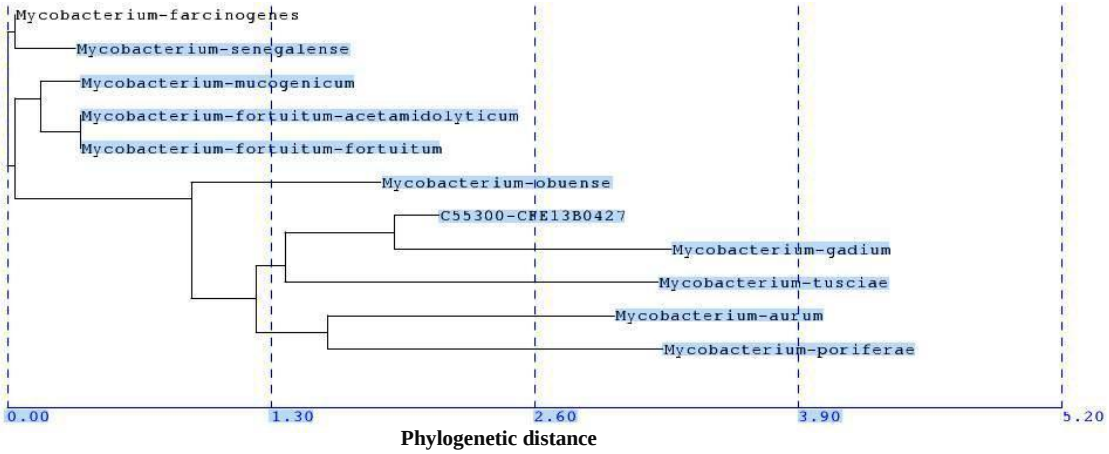


**Figure 4.1 Phylogeny of VC-assimilating bacterium (C55300-CFE13B0427) from CF enrichment based on partial 16S rRNA gene sequences (500 b.p.) (MIDILab, 2010).**

Purification of the S4 enrichment, however, was not successful. An assumption was made that the S4 enrichment was highly enriched in terms of VC-degraders due to multiple consecutive transfers and culturing over a period of 6 months – with VC as the only significant carbon source provided. The two cultures (i.e., the pure culture derived from CF and the highly enriched culture derived from

S4) were selected for more detailed analysis of kinetic parameters with respect to VC degradation and oxygen consumption.

## 4.1 VC-Limiting Condition

Figures 4.2 and 4.3, respectively, depict the data points of the three VC depletion curves of the CF isolate and S4 enrichment. Dashed curves (or dotted curves) show the curve-fitting results based on Matlab's simulations. From Equation 3.5, we know that in order to calculate $k_{VC}$ values, two variables, $V_{max(VC)}$ and $X \cdot V_l$, are employed. As explained in Methods, $V_{max(VC)}$ is the maximum rate of VC depletion which can be inferred from curve-fitting the depletion-curve data. From the Figures below, the slopes of curves suggest that the $V_{max(VC)}$ values among the three biological replicates are similar, which concurs with estimates by Matlab (~10% differences). If we assume that biological replicates are identical to each other, which we, of course, intended, similarity in $V_{max(VC)}$ values could represent similar maximum-specific VC utilizing. However, in this study, for both CF and S4 cultures, there was quite a large variance in protein measurements among biological replicates, resulting in large variance in their respective $k_{VC}$ values if the X-value for each biological replicate is used in calculation of its $k_{VC}$ value from its $V_{max(VC)}$ value.

So, it is necessary to discuss how protein measurements were dealt with, before we report the $k_{VC}$ values of both cultures. Were the true protein concentrations in biological replicates nearly the same, and there was merely a large variance in protein measurement? Or were the true protein concentrations actually different? The answers to these questions determine whether we should use the grand mean of all X measurements (i.e., 9 measurements — 3 samples from each of 3 biological replicates of a culture-type) in calculating $k_{VC}$ for each biological replicate; or,

alternatively, whether we should use a separate, mean X-value for each biological replicate in calculating its $k_{VC}$.

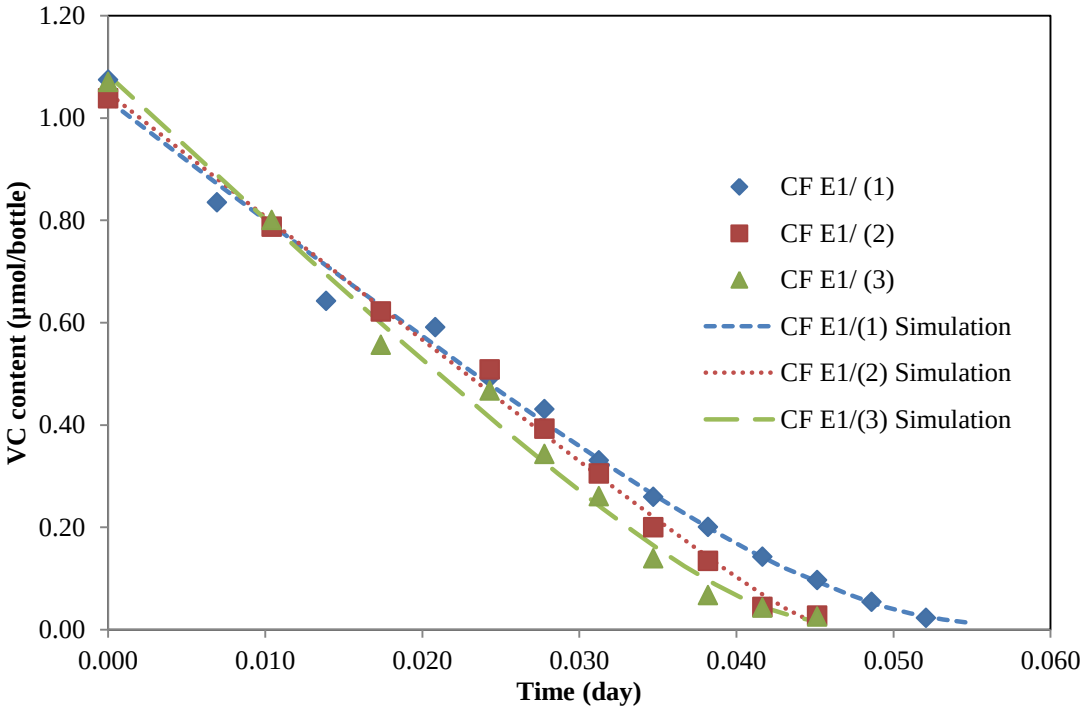


**Figure 4.2 VC depletion curves of three CF replicates with curve fitting by Matlab.**

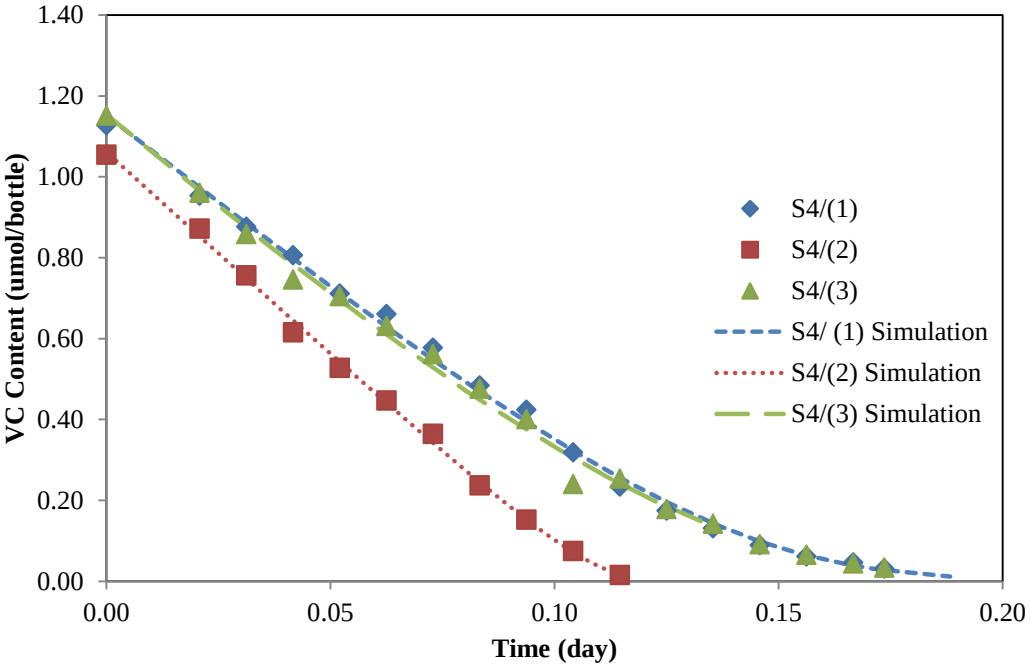


**Figure 4.3 VC depletion curves of three S4 enrichment replicates with curve fitting by Matlab.**

Table 4.1 presents the mean and standard deviation (SD) of protein measurements for all six culture replicates studied, three for CF isolate and the other three for S4. For each culture type (CF or S4), t-tests were run between every combination of pairs among the three biological replicates to determine if any mean is significantly different from the others. T-test results indicated that replicate 3 of CF isolate and replicate 1 of S4 were significantly different among their respective culture-types, so that using a global mean of protein concentrations in the calculation of $k_{VC}$ values would be inappropriate. Given the preparation of replicate samples, which involved extended culturing for 45 days with different amounts of VC fed (6-8 spikes with 42 μmol/bottle of VC in each spike), different protein levels among biological replicates is understandable.

**Table 4.1 Protein measurements of all six cultures studied**

| Strain | Replicate 1 | | Replicate 2 | | Replicate 3 | |
|---|---|---|---|---|---|---|
| | Mean (mg/L) | SD | Mean (mg/L) | SD | Mean (mg/L) | SD |
| CF isolate | 17.05 | 1.25 | 13.85 | 1.37 | 33.14 | 1.59 |
| S4 enrichment | 33.21 | 0.51 | 14.68 | 2.47 | 10.52 | 0.52 |

Table 4.2 presents the VC-degradation kinetic parameters of the two strains studied under a VC-limiting condition. Percent changes of oxygen concentration in both experiments were small (~10%) and the final oxygen concentrations were much higher than their respective $K_{s(O2)}$ values reported later in this chapter. This means that oxygen was not kinetically limiting, enabling focus upon intrinsic VC-degradation kinetics alone.

T-tests were run between every combination of pairs among the three replicates in order to determine if outliers of $k_{VC}$ existed (which would have been

ruled out from further calculation of reported means). Values of the parameters are reported in Table 4.2 as the calculated means of the replicates ± 95% confidence intervals (mean ± 1.96·standard error).

**Table 4.2 Kinetic parameters of VC-assimilating bacteria**

| **Strain** | $k_{VC}$ **(nmol/mg protein/min)** | $K_{s(VC)}$ **(μM)** |
|---|---|---|
| S4 enrichment (0)[a] | 4.7 ± 2.9 | 0.51 ± 0.14 |
| CF E1 (1) | 11.5 ± 1.9 | 0.16 ± 0.11 |

[a] Number of outliers ruled out from further calculation of reported mean among three replicates (both for $k_{vc}$ and $K_{s(vc)}$ values).

The substrate utilization rate of the *Mycobacterium* isolate was 11.5 nmol/mg protein/min, and that of the S4 enrichment was 4.7 nmol/mg protein/min —only half of the maximum rate of the *Mycobacterium* isolate. This agreed with the experiments by Gossett which showed that VC utilization rate of CF enrichments was more rapid than that of S4 enrichments (Gossett, 2010). The $k_{VC}$ value of the CF isolate fell within the range from 9 to 16 nmol/ mg protein/min of four different *Mycobacterium* strains studied by Coleman et al., as tabulated in Background (Coleman et al., 2002). The value was similar to the $k_{VC}$ value of JS616, the closest relative to our isolate, which was 15.4 nmol/ mg protein/min.

Bear in mind, however, that our protein measurement might not be the best representation of active biomass content of the samples. Most protein measurement techniques, like the method used in this study, cannot distinguish active biomass from other protein content, such as dead cells and other microorganisms, in samples. In general, even though active biomass content might be high, it usually only contributes to a fraction of the total protein content measured. Protein measurement consequently over-estimates active biomass level in samples. This was especially likely to happen

in our kinetic experiments because of the way the cultures were prepared. An extended culturing period of 45 days with different amounts of VC fed to each bottle would have contributed to a significant, inactive fraction of the protein content. Over-estimation of active biomass content was inevitable and thus led to under-estimation of actual VC utilization rates. True active biomass contents of samples were unknown and difficult to assess; the true $k_{VC}$ values of the cultures should be greater than or equal to the values reported in this study.

The maximum VC utilization rate of the S4 enrichment was low compared to our *Mycobacterium* isolate and other *Mycobacterium* strains, as discussed above. Its maximum VC-utilization rate, however, was similar to the $k_{VC}$ value of the *Pseudomonas* sp. strain DL1 (4.6 nmol/ mg protein/min — assuming cellular dry mass is 55% protein) studied by Verce et al. (Verce et al., 2000). In our study, the S4 enrichment differed from the isolated *Mycobacterium* (from CF enrichment) in several respects, including colony morphology on noble agar plate, and rate of VC-utilization. These lead us to believe that the dominant specie of VC-degraders within the S4 enrichment is quite different from *Mycobacterium* strains discussed above.

The large confidence intervals of reported $k_{VC}$ values are worth discussion, especially with respect to the $k_{VC}$ value of the S4 enrichment. As mentioned in a previous paragraph, $V_{max(VC)}$ and protein concentration (X) are the two determinants of $k_{VC}$ values, and the imprecision of these two determinants contributes to the imprecision of calculated $k_{VC}$. Imprecision of $V_{max(VC)}$ comes from the curve fitting of the Michaelis-Menten equation to our data points, probably due to high degree of scattering in them. But it was proven that the protein concentrations between biological replicates are significantly different and the imprecision of them should also be influential in the variance of calculated means of $k_{VC}$ values. The question then

is as follows: Which one is the major contributor to the imprecision of reported $k_{VC}$ values, the degree of scattering in depletion curves or imprecision in protein measurement?

To evaluate the relative influence of the two factors mentioned above, the variance in each can be propagated to arrive at an estimate of total variance in the resulting $k_{VC}$. Culture volume ($V_l$) is assumed to be without significant error (0.1 liter). It is well-known that the square of percent standard deviation of *a quotient* is equal to the sum of the squares of percent standard deviations of its numerator and denominator if we neglect the product term with $\sigma_x\sigma_y$ (Taylor, 1982). Therefore, in our case,

$$\frac{\left(\sigma_{k_{VC}}\right)^2}{(k_{VC})^2} = \frac{(\sigma_y)^2}{y^2} + \frac{(\sigma_x)^2}{x^2} \qquad (4.2)$$

where $y$ is $V_{max(VC)}$ and $x$ is $X{\cdot}V_1$

By comparing the values of respective $\frac{(\sigma_x)^2}{x^2}$ and $\frac{(\sigma_y)^2}{y^2}$, we were able to identify whether the scattered data in curve-fitting or imprecision in protein measurements was more responsible for the large variance in $k_{VC}$ values. Results illustrated that for all the data, $\frac{(\sigma_x)^2}{x^2}$ on average contributed 35%, (for CF) and 30% (for S4) to the total variances in resulting $k_{VC}$ values, while $\frac{(\sigma_y)^2}{y^2}$ contributed the balance. This suggests that the degree of scattering in depletion-curve data is somewhat more responsible for the large confidence interval in reported $k_{VC}$ values, though both factors contribute significantly.

The half-velocity constants of the *Mycobacterium strain* and S4 enrichment were 0.16 and 0.51 μM VC, respectively. The $K_{s(VC)}$ values of both strains were

smaller than other $K_{s(VC)}$ values reported, such as from *Ralstonia* sp. strain TRW-1 (9.09 µM), *Mycobacterium* strains characterized by Coleman et al. (JS60, JS61, JS616, JS617, 0.5 – 3.2 µM), and *Nocardioides* sp. strain JS614 (1.2 µM) (Coleman et al., 2002), and *Pseudomonas* sp. strain DL1 (1.2 µM) (Verce et al., 2000; Alango et al., 2006). The low $K_{s(VC)}$ values and the absence of thresholds indicate the ability of our CF and S4 strains to degrade VC to a very low level without rate slowing appreciably from VC depletion. Thus the CF isolate and S4 enrichment should be environmentally relevant to natural attenuation of VC at contaminated sites. The $K_{s(VC)}$ value of the CF isolate was only one-twentieth that of JS616, its nearest phylogenetic match. Even though the CF isolate resembles *Mycobacterium* sp. strain JS616 phylogenetically, their VC-utilization kinetics are different, based on its $K_{s(VC)}$ and $k_{VC}$ values found in this study. The CF isolate is able to degrade VC faster than JS616 when VC concentration is low.

4.2 Oxygen-Limiting Condition

Two approaches were used when fitting oxygen depletion curves with the data — one considered oxygen threshold and the other did not. Previous studies suggested that the existence of oxygen thresholds was common among *Mycobacterium* species when they assimilate VC (Coleman et al., 2002). All six separate data sets were fit to the Michaelis-Menten equation containing a threshold term. However, results of curve fitting indicated that a measureable oxygen threshold might not exist in our cultures. Threshold values of five curve fittings turned out to be very insignificant (the largest was ~0.0003 mg/l). Only the simulation of S4 replicate 2 gave a significant oxygen threshold — 0.028 mg/L. Nevertheless this simulation was considered invalid because the software estimated the half velocity constant for

oxygen as 0.0048 mg/L, lower than the oxygen threshold it estimated, which is impossible. As a result, the second approach was adopted which assumed that oxygen thresholds did not exist in either culture. The half velocity constants of oxygen were then estimated as $K_{s(O2)}$ = 0.06 ± 0.07 mg/L (only two replicates were taken into account) and 0.03 ± 0.005 mg/L for the CF isolate and the S4 enrichment culture, respectively.

In fact, the assumption that oxygen thresholds did not exist in these cultures was rather arbitrary because data in what might have been the threshold region was absent. As shown on Figures 4.4 and 4.5, all experiments were terminated when oxygen concentration reached < 0.03 mg/L. Without support of lower-oxygen data, it was hard for Matlab to accurately depict what was happening at that low oxygen level. Although simulations of our experimental data suggested that there would not be thresholds, there remains the possibility that such thresholds might exist. However, if such a threshold existed, it would be less than 0.01 – 0.02 mg/L – and therefore likely not significant – according to the studies of VC oxidation at low oxygen concentrations by Gossett on the same enrichments (Gossett, 2010). These earlier studies indicated that the enrichments were able to sustain VC degradation when $O_2$ levels remained ≤ 0.02 mg/L.

Also, there were large variances among the evaluated $K_{s(O2)}$ values of CF replicates, leading to a large confidence interval of $K_{s(O2)}$. Two reasons could explain that. Firstly, oxygen levels were imprecise at low concentration such as 0.05 mg/L. Even though the sampling syringe was flushed with gas from the "zero" bottle before acquiring samples, thus scavenging remnant oxygen from the needle and barrel, oxygen from the lab environment could still have affected sampling. For instance, oxygen in the barrel behind the plunger, a space regularly exposed to air when the plunger is all the way forward, might adhere to the barrel wall, and a small amount

might get into the sample when the plunger is pulled back in sampling. Also oxygen in the air might get into the needle after sampling and before injection. Even a trace amount of extra oxygen could cause large variation in detecting low oxygen concentration. Secondly, inadequate data points at lower oxygen concentration might also lead to significant error in evaluating kinetic parameters.

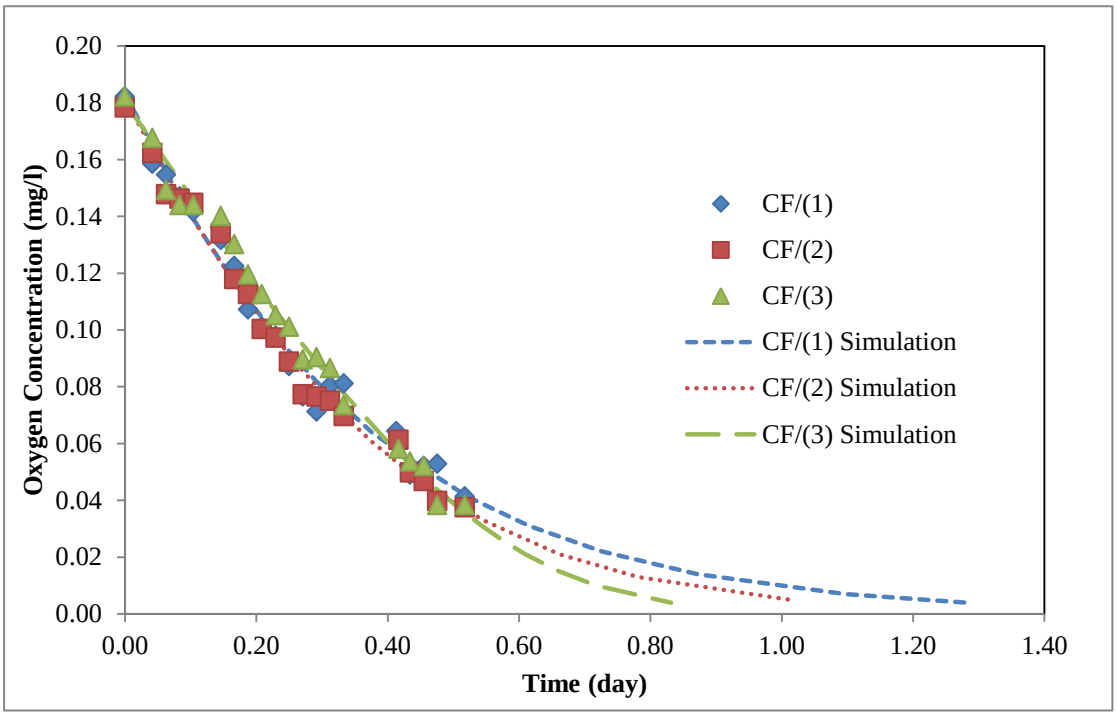


**Figure 4.4 Oxygen depletion curves of three CF replicates with curve fitting by Matlab.**

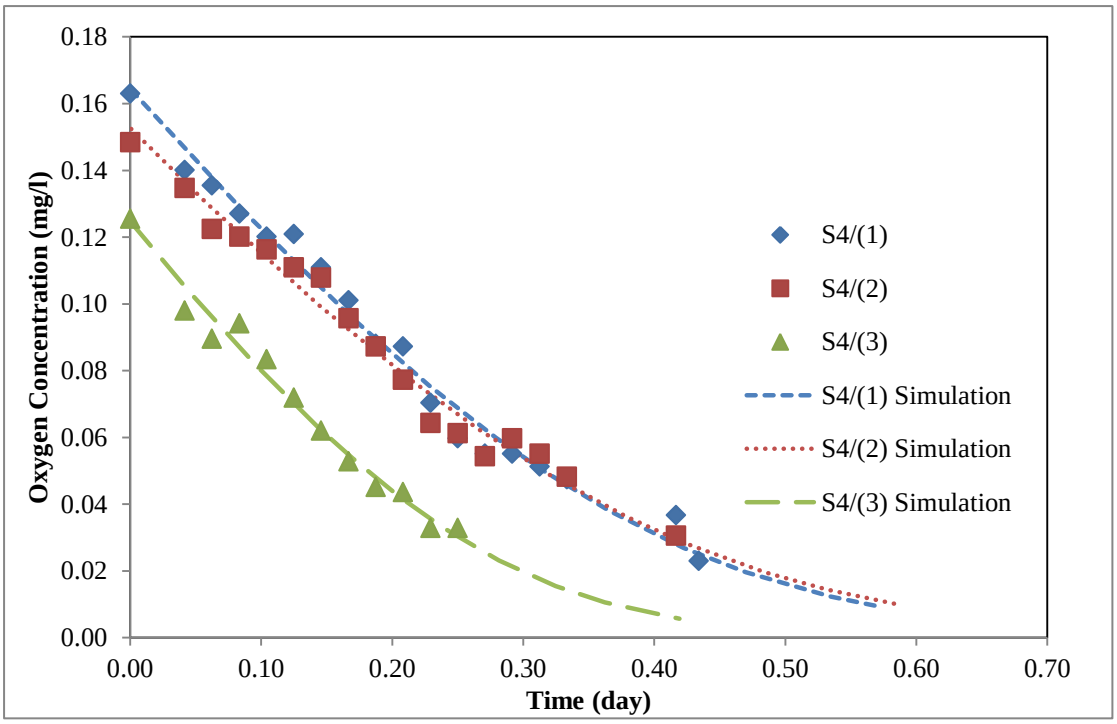


**Figure 4.5 Oxygen depletion curves of three S4 enrichment replicates with curve fitting by Matlab.**

Both low measured $K_{s(O2)}$ values and the lack of a measurable oxygen thresholds suggest that our *Mycobacterium* isolate and S4 enrichment are able to oxidize VC under very low oxygen concentration. Dissolved oxygen (DO) concentration of 0.1 mg/L measured in the field is generally labeled as "anaerobic" in subsurface and groundwater. In the oxygen-limiting experiments, significant VC oxidation rates were observed at such a low oxygen concentration. The result is consistent with the conclusions from other studies that sustained oxidation of VC was observed at extremely low oxygen concentration (0.02 mg/L to 0.1 mg/L) (Coleman et al., 2002; Gossett, 2010). Such small amounts of oxygen could come from any recharge events or diffusion from higher-oxygen zones.

# CHAPTER 5

# CONCLUSION

In this study, an isolated strain was obtained through purification/isolation process of the CF enrichments; its closest 16S rRNA match is to *Mycobacterium gadium* strain JS616 (98% identity). VC- and $O_2$-utilizing kinetic studies of this pure culture showed maximum-specific VC utilization rate ($k_{VC}$) of 11.5 nmol/mg protein/min, and half-velocity constant for VC ($K_{s(VC)}$) and $O_2$ ($K_{s(O2)}$) of 0.16 µM and 0.03 mg/L, respectively. Attempts to isolate microorganisms from S4 enrichments were not successful; therefore, the VC- and $O_2$-utilizing kinetics of an S4 enrichment culture was studied. Its measured $k_{VC}$ value was 4.7 nmol/mg protein/min, while its $K_{s(VC)}$ and $K_{s(O2)}$ were 0.51 µM and 0.06 mg/L, respectively. For both cultures, the low values of $K_{s(VC)}$ suggest their relevance in natural attenuation of VC at contaminated sites.

For both cultures, the oxygen thresholds were so low that they were immeasurable and considered to be absent. Given this situation and the low values of $K_{s(O2)}$ obtained, we believe that aerobic oxidation of VC from supposed anaerobic zones could be appreciable if there is any flux of oxygen into these areas. Such a finding is helpful for understanding natural VC attenuation at many sites and provides an explanation for the poor mass-balances often achieved in site studies. It also enhances the feasibility of bioremediating VC through VC oxidation at many sites which have low DO levels and were once believed to be anaerobic.

# CHAPTER 6

# FUTURE WORK

Several follow-up studies of our two cultures could enable us to dig more into characteristics of them and their possible application in actual bioremediation of chloroethene contaminated sites. Firstly, further purification of the S4 enrichment is of interest because of its kinetic and physiological differences from the *Mycobacterium* isolate, a member of the most common group of VC degraders. However, according to our experiences, it took a rather long period of time for suspected target colonies (yellow-pigmented ones) to show up on noble agar (VC as carbon source), and there was impurity which suggests that perhaps other VC degraders existed in the enrichment, which would have increased the difficulty in obtaining a purified strain. Denaturing Gradient Gel Electrophoresis (DGGE) used in previous studies can be a useful tool to help us obtain partial identity of the dominant members of the S4 culture (Nicholson et al., 2004; Singh et al., 2004). Of course, the most effective way to isolate VC degraders is to use multiple consecutive streaking on noble agar (with VC as the only carbon source), which would take longer time to accomplish.

Another point of interest would be to investigate the capability of our cultures to degrade other, related compounds of interest, such as *cis*-DCE and ethene. Literature suggests that most known VC degraders are also able to utilize ethene for microbial growth (Coleman et al., 2002; Mattes et al., 2005). On the other hand, aerobic degradation of *cis*-DCE was also observed in microcosms from bed and aquifer sediments (Bradley et al., 2000; Verce et al., 2002), and by microbial strain *Pseudomonas aeruginosa* MF1 (Broholm et al., 2005), when different primary substrates were supplemented. These substrates included methane and vinyl chloride.

If our cultures are able to aerobically degrade *cis*-DCE – either by itself or in the presence of other substrates – this could extend their possible use in bioremediation or bioaugmentation at *cis*-DCE, VC contaminated sites.

Finally, experiments are simplified representations of real situations, which might have underestimated the problems at actual sites. Many environmental factors could serve as inhibitors to our targeted degradation pathway. For instance, when VC and *cis*-DCE were present together in *Pseudomonas aeruginosa* MF1, substrate utilization of VC was hampered, and vice versa (Broholm et al., 2005). Such a situation should be very common in real sites with much more complicated systems. Introduction of these elements, such as natural organic matter and intermediates from upstream pathways, into our experiments could better mimic the conditions at actual sites, and thus help us know more and can more accurately predict the biological behaviors and microbial activities in the field.

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