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Exploring the diversity of vinyl chloride assimilating bacteria in enriched groundwater cultures

Carly Faye Lintner
University of Iowa

EXPLORING THE DIVERSITY OF VINYL CHLORIDE ASSIMILATING BACTERIA IN ENRICHED GROUNDWATER CULTURES

by

Carly Faye Lintner

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Thesis Supervisor: Associate Professor Timothy E. Mattes

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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MASTER'S THESIS

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This is to certify that the Master's thesis of

Carly Faye Lintner

has been approved by the Examining Committee
for the thesis requirement for the Master of Science
degree in Civil and Environmental Engineering at the May 2014 graduation.

Thesis Committee: _____
Timothy E. Mattes, Thesis Supervisor

_____
Gene F. Parkin

_____
Richard L. Valentine

To T.S.B.

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CHAPTER 1

INTRODUCTION

Groundwater contamination by chloroethenes is prevalent worldwide due poor use and disposal practices of these compounds. Chlorinated ethenes, and particularly vinyl chloride (VC), are found in groundwater primarily through use as solvents in commercial, industrial, and military operations (Chuang et al., 2010). Under anaerobic conditions, certain bacteria can reduce higher chlorinated ethenes, such as tetrachloroethene (PCE) and to trichloroethene (TCE), to lesser chlorinated ethenes. The reductive dechlorination biodegradation process converts tetrachloroethene (PCE) to trichloroethene (TCE), TCE to *cis*-1,2-dichloroethene (*cis*-1,2-DCE), *cis*-1,2-DCE to VC, and VC to ethene.

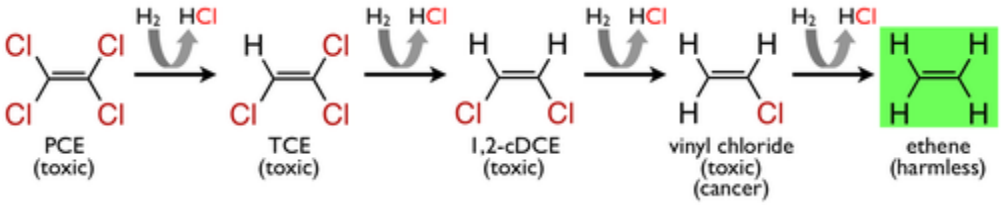


Figure 1: Process of reductive dechlorination of chloroethenes.

When partial reductive dechlorination occurs, with the reduction stopping at *cis*-1,2-DCE or VC, VC can quickly accumulate in groundwater plumes as a secondary pollutant. VC is a known human carcinogen and has the lowest regulatory limit in drinking water of 2 ppm (Chuang et al., 2010). The high mobility of VC in groundwater coupled with the slow degradation of VC to ethene pose a threat to drinking water supplies near VC contaminated plumes.

Anaerobic reductive dechlorinating bacteria are able to use the highly-chlorinated ethenes as electron acceptors, thus degrading the substrate to a lesser-chlorinated compound, and ideally to ethene. Many bacteria can partially reduce these higher-chlorinated ethenes through anaerobic dechlorination, but only *Dehalococcoides* has been shown to reduce PCE and TCE to the lesser chlorinated ethenes fully to VC and cometabolically to ethene (MaymoGatell et al., 1997).

In some situations, the groundwater returns to aerobic conditions down gradient of the source with methane, ethene, VC, and possibly cis-DCE still present. Natural attenuation of VC can occur via oxidation by aerobic microorganisms as a VC plume intersects with groundwater containing molecular oxygen. Certain bacteria that use ethene as a growth substrate can fortuitously metabolize VC. Some co-metabolizing bacteria can adapt, or assimilate, to using VC as a carbon and energy source. These bacteria can be isolated from aerobic environments as well as anaerobic environments, indicating that they are participating in the aerobic natural degradation of VC within the groundwater (Mattes et al., 2010).

Studies have revealed that the initial reaction of ethene-oxidizing bacteria to degrade ethene and VC is catalyzed by an alkene monooxygenase. A VC-assimilating bacterium, *Mycobacterium aurum* L1, was found to convert VC to chlorooxirane; a similar degradation pattern was observed from an ethene-grown L1 culture. This suggested that AkMO is responsible for the initial attack on both VC and ethene (Hartmans and Debont, 1992). *Mycobacterium* strain JS60 and *Nocardioides sp.* strain JS614, both VC-assimilating bacteria, were also found to be capable of oxidizing ethene to epoxyethane (Mattes et al., 2005).This evidence indicates that AkMO is the initial enzyme in the pathway that converts VC and ethene to VC epoxide and epoxyethane, respectively (Mattes et al., 2007; Mattes et al., 2005).

For co-metabolism to occur, enough primary substrates and cofactors must be provided to sustain an active population of microorganisms that possess the co-

metabolizing enzymes. These variables have added great complexity and cost to co-metabolic biotransformations, leading to extensive research of the microbes found within contaminated sites and how to better utilize their functions for bioremediation (Mattes et al., 2010).

Research Objectives

The primary research objective of this study is to explore the diversity of isolated VC assimilators within contaminated groundwater. The secondary research objective is to create a method for the enrichment and isolation of microorganisms using PCR that allows for sequencing of the isolate. Specific research objectives of this study include:

1. Enrich and maintain growth of microbes within contaminated groundwater through VC supplementation.
2. Isolate VC assimilators using a method of media plating and microbial analysis.
3. Assess the purity and bias associated with isolation methods and analyze suitability for work with in-situ and stable isotope probing applications.

CHAPTER 2

LITERATURE REVIEW

VC and Ethene Contamination

VC contamination in groundwater can be remediated through both anaerobic and aerobic degradation. Studies have found there are not only bacteria that will co-metabolically degrade VC while using a different substrate as a carbon and energy source, but also bacteria that will assimilate and begin to use VC as a growth substrate itself. Bacteria that co-metabolically degrade VC will use additional substrates for growth, including ethene, BTEX, methane, and others. Bacteria that directly metabolize VC as a growth substrate under aerobic conditions are commonly called VC-assimilators (Begley et al., 2012).

The catalyst used by ethene-oxidizing bacteria to degrade VC has been found through experimentation to be alkene monooxygenase (AkMO). It is the initial enzyme required in the oxidation pathway, converting ethene and VC into epoxides (Jin and Mattes, 2010). Sequencing and analysis of the AkMO genes (*etnABCD*) in studies has suggested involvement in VC oxidation. Studies have indicated through microbial analysis techniques that *etnABCD* is the VC monooxygenase, and have found the increased co-expression of an epoxyalkane coenzyme M transferase (EaCoMT) (Mattes et al., 2010).

EaCoMT catalyzes the second step in the pathways of oxidation of VC and ethene assimilators (Jin and Mattes, 2010). Early studies indicated that CoA, along with another cofactor, was required for metabolism. Later research showed that EaCoMT was present in ethene and VC metabolizing bacterial cells, suggesting that EaCoMT catalyzes the second step in the pathways (Coleman and Spain, 2003a). Further analysis through PCR and microbial assays support the enzymes AkMO and EaCoMT as initializing the beginning steps of VC and ethene oxidation (Mattes et al., 2005).

Downstream reactions in these pathways are largely unknown, although predictions include conversion of the two pathways after an alcohol dehydrogenase-mediated oxidation. Reactions further downstream have been suggested in the literature via experimental evidence indicating possible enzymes for oxidation. (Coleman and Spain, 2003b) Studies have indicated that the genes required for VC and ethene degradation pathways are plasmid-borne, and conclusive evidence for this theory was provided with the sequencing of the *JS614* genome consisting of a chromosome and plasmid, as the plasmid carried the gene for epoxidation (Coleman et al., 2011). Figure 2 is a simplified flowchart of the proposed pathway for assimilation of ethene and VC, although the reactions further along in the pathway are unknown (Begley et al., 2009).

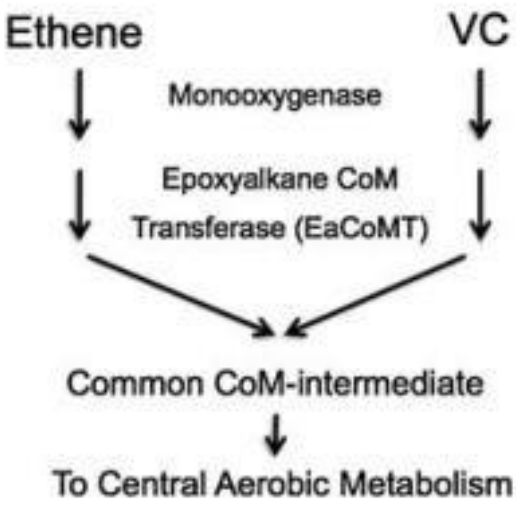


Figure 2: Simplified flowchart of the proposed pathway for assimilation of ethene and VC (Begley et al., 2009).

Biodegradation

Anaerobic dechloriating bacteria are promising agents for the bioremediation of chloroethene-contaminated sites because they are both active under the anoxic conditions

typically encountered in the subsurface and have the ability catalyze the complete reduction of PCE and TCE to ethene, which is a preferred end product. Many anaerobic dechlorinating organisms can reduce PCE and TCE to cDCE, including *Dehalobater restrictus* (Holliger et al., 1998), *Dehalospirillum multivorans* (Neumann et al., 1994), *and Geobacter lovleyi* (Sung et al., 2006). Only members of the genus *Dehalococcoides* are known to reduce PCE or TCE beyond DCE. *Dehalococcoides ethenogenes* strain 195 is the only known bacteria that can fully reduce PCE to ethene, using PCE, TCE, and cDCE as electron acceptors and the reduction of VC to ethene as a co-metabolic substrate (MaymoGatell et al., 1997). *Dehalococcoides* strain BAV1 is notable for its ability to reduce VC to ethene for growth (He et al., 2003), strain FL2 reduces TCE to VC for growth (He et al., 2005), and strain GT can reduce TCE to ethene for growth (Sung et al., 2006). These dechlorinating bacteria are an option for remediation of contaminated groundwater with anaerobic environments.

Despite this, incomplete dechlorination of chlorinated ethenes leads to the accumulation of cDCE and VC at many sites. With the use of alkene monooxygenases (AkMOs) as a catalyst, aerobic bacteria can oxidize VC and ethene by converting substrates through cometabolism, using ethene as a carbon and energy source (Mattes et al., 2010). Although using other substrates, such as ammonia and a variety of aromatics, alkanes, and alkenes for growth, these aerobic bacteria will co-metabolize the chlorinated ethenes, including VC. Some of these bacteria will assimilate or evolve to use the VC as a growth substrate itself (Jin and Mattes, 2010). These VC-cometabolizing bacteria that use ethene as an energy source, known as etheneotrophs, and VC assimilating bacteria are a relevant topic for study, as they can combat the accumulation of undesirable chlorinated ethenes in groundwater.

Both gram-negative and gram-positive bacteria are known to assimilate to VC while originally oxidizing ethene. The conversion to assimilation of VC seems to depend upon chemical and microbial conditions, as well as the length of exposure to the gas.

(Mattes et al., 2010) Four ethene-utilizing Mycobacterium strains (JS622, JS623, JS624, and JS625) were found to initially lag and eventually utilize VC as a growth substrate after an extensive period of incubation (Jin and Mattes, 2008). Studies have since shown that many VC and ethene assimilators have the ability to degrade chloroethenes at very low dissolved oxygen levels, further suggesting that the bacteria are active in groundwater environments with very little oxygen present (Gossett, 2010).

A common thread in studying the evolution of VC-assimilating bacteria is the presence of a higher EaCoMT activity and etnE presence. Higher EaCoMT activity is typically seen in cell extracts of VC-assimilating *Mycobacterium* strains compared with strains isolated on ethene (Coleman and Spain, 2003a). Later studies confirmed the presence of etnE genes with high EaCoMT activities in VC assimilators (Mattes et al., 2005).

Isolation of VC-assimilating Microorganisms

Isolation of VC assimilators has been approached in different ways. Isolation attempts begin with an enrichment culture using environmental groundwater samples being suspended in a mixed salts and metal solution. Samples of groundwater were mixed with mineral salts medium (MSM) agar in 160 ml serum bottles, 72 ml liquid volume, and sealed with Teflon-faced rubber stoppers and aluminum crimp caps. VC was added at 20-40 µmol/bottle in the initial microcosms. The microcosms and enrichments were incubated with the bottles in an inverted position and with shaking at the ambient temperature (Coleman et al., 2002).

Previous studies performed isolations using standard spread plating of prepared enrichment cultures on MSM plates and incubation in desiccators in a 1% (vol/vol) VC-air atmosphere. The plates were incubated at ambient temperature for 1 to 3 months, and representative colonies were re-streaked onto two MSM plates. The plates were incubated, one in 1% VC and the other in air; the colonies showing significant growth in

the VC incubator was further investigated with the use of 16S rRNA sequencing and resuspended into liquid MSM with VC to confirm VC degradation (Coleman et al., 2002).

Additional studies utilized an approach of plating onto 1/10 Tryptic Soy Agar plus 1% glucose (TSAG) media and incubating at 30 °C until colonies appeared and utilizing 16S rRNA to identify the bacterium, then resuspending into liquid MSM with VC to confirm VC degradation (Jin and Mattes, 2008). A more recent study combines an etheneotrophic activity assay with a PCR assay to evaluate the aerobic VC bioremediation potential of a groundwater. Serial dilutions of a groundwater microcosm were plated onto nutrient rich R2A media and incubated until colonies were present. Representative colonies were isolated and PCR was performed to amplify the EaCoMT gene in the DNA providing an idea of the ethenotrophic potential of the groundwater (Begley et al., 2009).

These studies served as a beginning method model for this thesis study. By incorporating the 16S rRNA gene analysis into the Begley method of culturing and plating, the method allows for isolation identification and further characterization of the groundwater, fully utilizing molecular tools for analysis.

Even with repeated streak plating and 16S rRNA gene sequencing, absolute purity can be difficult to obtain. Extremely rare, difficult to detect variants within the culture can exist. A recent study of *G. sulfurrenducens* strain DL1 found contamination, *G. sulfurrenducens* strain KN400, within a bioelectrical system biofilm after purity plate streaking and 16S rRNA gene sequencing and analysis. Attempts to remove the KN400 contaminant through repeated re-streaking of isolated colonies proved futile, as the KN400 strain continued to be present at a low frequency in the isolated colonies (Shrestha et al., 2013). This research suggests that only true way to ensure that a culture is pure is to cultivate it from an initial pure cell, which can be near impossible for many environmentally significant microbes.

Additionally, enrichment and isolation biases have been studied and should be considered when working with an environmental sample. The use of cultured instead of uncultured bacteria for exploring diversity of environmental samples is a well-known source of potential bias. Batch culture enrichment is widely used for isolation of bacteria, and has been recognized to limit the number and relative growth rates of organisms obtained from the environment (Harder and Dijkhuizen, 1982). Using R2A plates, the bias of enrichment cultures was studied through direct plating and plating of enriched cultures from environmental soil samples. The diversity of isolates found from direct plating was greater than that found from enrichment. Seventy-four isolates were taken from both the direct plating and the enrichment; the direct plating produced 25 different populations, while the enrichment plating produced only 7 (Dunbar et al., 1997).

Isolation biases have also been studied. In a study on the influence of isolation medium on the biodiversity of *Burkhoderia cepacia* strains, the genetic diversity of isolations obtained through serial dilution plating on two different selective medias was analyzed. Fifty random colonies were isolated from the both of the selective medias, TB-T (2 g glucose, 1 g L-asparagine, 1 g $NaHCO_3$, 0.5 g KH_2PO_4, 0.1 g $MgSO_4.7H_2O$, 0.05 g trypan blue, 0.02 g tetracycline, 15 g agar, in 1 L of distilled water, pH 5.5) and PCAT (2 g azelaic acid, 0.2 g tryptamine, 0.1 g $MgSO_4.7H_2O$, 4 g KH_2PO_4, 4g K_2HPO_4, 0.02 g yeast extract, 15 g agar, in 1 L of distilled water, pH 5.5), and analyzed for partial 16S rRNA genes amplified with PCR. All strains isolated from TB-T medium were assigned to the *B. cepacia* species, where only 74% were assigned to the *B. cepacia* species from the PCAT media. Genetic diversity was also evaluated by random amplified polymorphic DNA technique. The results indicated that there was a higher degree of genetic diversity observed among strains isolated from PCAT medium than among those isolated for TB-T medium (Tabacchioni et al., 2000).

Stable Isotope Probing and Fractionation

Stable Isotope Probing (SIP) uses a labeled substrate to identify and characterize the behaviors of a bacterial community, allowing a general determination of the metabolic pathways that are being utilized. By using a substrate enriched with a stable isotope, microorganisms can be labeled following consumption of the substrate. The bacteria will become enriched with the substrate, and through molecular and analytical techniques, can be identified as having incorporated the substrate into their system (Neufeld et al., 2007).

The VC isotope used for SIP has an extra neutron, becoming a ^{13}C isotope. Cells that metabolize the substrate with the ^{13}C will become "heavier" and a distinction between the light and heavy DNA will be clear. This distinction is more obvious in a pure culture than a mixed groundwater sample, so additional analysis is often utilized.

Further research is being performed to optimize the use of stable isotope probing regarding bioremediation. A new NSF project aims to provide a procedure to differentiate between VC assimilators and VC co-metabolizers through the use of stable isotope probing. The hypothesis of the project is "stable isotope probing (SIP) techniques when used in conjunction with existing molecular tools will differentiate between etheneotrophs and VC-assimilators in both laboratory and field-based applications." (Mattes and Cupples, 2012).

The NSF project involves using both soil and groundwater samples to reveal the presence, identity, and activity of VC assimilating bacteria through DNA SIP. Using laboratory cultures, a SIP assay that differentiates between VC assimilating and co-metabolizing bacteria can be developed for field application (Mattes and Cupples, 2012).

A sampling approach using a passive sampling device allows biofilms to grow, consuming the enriched substrate and effectively labeling VC assimilators. In combination with qPCR, identification and characterization of the microbial groundwater communities will be obtained for analysis (Mattes and Cupples, 2012).

CHAPTER 3

MATERIALS AND METHODS

Chemicals and Media

VC (99.5%) was from Sigma-Aldrich, and ethene (99%) was from Airgas. All other chemicals were of reagent grade. Minimal Salts Medium (MSM) was prepared as follows: 0.95 g KH_2PO_4, 2.27 g K_2HPO_4, 0.67 g $(NH_4)SO_4$ per liter of deionized water. One liter of MSM was autoclave sterilized at 121 degrees Celsius (°C) for 30 minutes.

One liter of trace metals solution (TMS) was prepared as follows: 60 g $MgSO_4 \cdot 7H_2O$, 6.37 g EDTA ($Na_2(H_2O)_2$),1 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $CaCl_2 \cdot 2H_2O$, 2.5 g $FeSO_4 \cdot 7H_2O$, 0.1 g $NaMoO_4 \cdot 2H_2O$, 0.1 g $CuSO_4 \cdot 6H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.52 g $MnSO_4 \cdot H_2O$ per liter of deionized water. TMS was filter sterilized by 0.22µm, GP Express membrane from Steritop and stored at 4°C in a foil wrapped container to prevent photo degradation. TMS was added to sterilize MSM in a dose of 2 ml per liter of solution.

One liter of R2A agar (R2A) was prepared as follows: 18.2 grams per liter of deionized water. One liter of R2A was autoclave sterilized at 121°C for 30 minutes. Broth was poured into 2 sleeves of sterile petri dish in the laminar flow hood and stored sealed at 4°C.

Development of Enrichment Cultures

Contaminated groundwater collected from monitoring wells at a contaminated groundwater site in Carver, MA was used to prepare ethene- and VC-fed microcosms. The contamination on the site is a large PCE plume (300 x 400 x 30 ft) originating from a leaky landfill, which has moved N-NW of the source area. The plume, although a PCE release is primarily stalled at VC, with anoxic conditions, low VC concentrations and high iron and sulfate concentrations. Through 2006, in-situ bioremem diaton occurred at multiple biobarriers, stimulating VC degradation with oxygen and ethene delivery.

Groundwater taken from wells at the Carver site was added to sterile 160 ml serum bottles (Wheaton) containing sterile mineral salts media (MSM) (Coleman, 2002) to a total liquid volume of 72 ml. Cultures were created in multiple ratios of groundwater to MSM for ethene and VC. Ethene (400 µmoles) or VC (20-60 µmoles) was added using sterile syringe and needles, with filter, as appropriate. The bottles were stored covered and inverted on a shaker. Headspace samples (100 µl) were analyzed 2-3 times per week by gas chromatography with flame ionization detection. Compounds were identified by retention time. Ethene showed a retention time of 1.7 minutes, while VC showed a retention time of 4.8 minutes.

Table 1 gives a summary of all enrichment cultures that were created and monitored. Two groundwater samples were used in enrichments, groundwater taken from well 63-I on the Carver site and a composite sample of groundwater taken from multiple wells on the Carver site (composite Carver). Multiple dilutions of the two different groundwaters with MSM were created and monitored for enrichment via both VC and ethene. Additional enrichments that were monitored were created prior to this study, including the cultures labeled SERDP 63-I and BCI 63-I (a) for both VC and ethene (all previously created cultures with Carver 63-I groundwater). BCI 63-I (b) enrichments for both VC and ethene were dilutions created with groundwater form the BCI 63-I (a) cultures.

Table 1: Summary of enrichment cultures created for growth and monitoring using groundwater from multiple wells at the Carver, MA field site and sterile mineral salts media.

Name	Date Created	Composition	Growth Substrate
SERDP 63-I	9/20/2012	68 ml MSM, 4 ml Culture	VC
63-I Groundwater	9/20/2012	36 ml MSM, 36 ml GW	VC
63-I Groundwater	9/20/2012	54 ml MSM, 18 ml GW	VC
63-I Groundwater	9/20/2012	68 ml MSM, 4 ml GW	VC
Composite Carver	9/27/2012	36 ml MSM, 36 ml GW	VC
Composite Carver	9/27/2012	54 ml MSM, 18 ml GW	VC
Composite Carver	9/27/2012	68 ml MSM, 4 ml GW	VC
63-I Groundwater	10/4/2012	36 ml MSM, 36 ml GW	Ethene
63-I Groundwater	10/4/2012	54 ml MSM, 18 ml GW	Ethene
63-I Groundwater	10/4/2012	68 ml MSM, 4 ml GW	Ethene
Composite Carver	10/4/2012	36 ml MSM, 36 ml GW	Ethene
Composite Carver	10/4/2012	54 ml MSM, 18 ml GW	Ethene
Composite Carver	10/4/2012	68 ml MSM, 4 ml GW	Ethene
MSU Composite Carver (unfiltered)	10/10/2012	70 ml MSM, 2 ml Culture	VC
MSU Composite Carver (filtered)	10/10/2012	68 ml MSM, 4 ml Culture	VC
BCI 63-I (a)	4/15/2011	100 ml MSM, 1 ml GW	VC
BCI 63-I (b)	11/1/2012	70 ml MSM, 2 ml BCI 63-I VC (a)	VC
BCI 63-I (a)	4/15/2011	100 ml MSM, 1 ml GW	Ethene
BCI 63-I (b)	11/1/2012	70 ml MSM, 2 ml BCI 63-I Eth (a)	Ethene

Note: Cultures used for 63-I isolations and composite Carver groundwater isolations are highlighted.

Bacterial Isolation Procedures

Serial dilutions (10^{-2} to 10^{-7}) of samples (100 µl) from the 1:1 groundwater: MSM enrichment culture were plated on R2A medium and incubated for 10 days at 30 °C. Individual colonies from the 10^{-4} dilution spread plate were streaked onto R2A plates and allowed to grow for 10 days.

A representative clump of cells were taken from streak plates with a sterile pipette tip and suspended for PCR analysis. DNA templates for PCR were prepared by suspension in 40 µL of sterile water and boiling cell suspensions at 95° C for 10 minutes, or by suspension in 600 µL STE buffer (100 mM NaCl, 10 mM Tris-Cl, 50 mM EDTA,

pH 8.0) and a beadbeating DNA extraction method (Jin and Mattes, 2008). Cell lysis was achieved by bead-beating at high speed for 2 min with a Bio-Spec MiniBeadbeater-8. DNA concentrations were measured on a Qubit fluorometer with high sensitivity buffers; one ng of the boiled DNA was used as the DNA template for PCR without purification.

PCR mixtures of 25 µl contained 12.5 µl Qiagen Master Mix, 1.5 µl of each primer, the equivalent of 1 ng of DNA template and completed with sterile DI water. Volumes were doubled for PCR mixtures of 50 µl. Primers for 16S rRNA amplification were 27F and 1492R, with an expected base pair size of approximately 1500 bp. Primers for etnE gene amplification used were CoM- F1L and CoM-R2E, with an expected base pair size of 891 (Coleman and Spain, 2003a). Thermocycling protocol was 94°C for 10 min, then 30 cycles of 94°C (1 minute), 55°C (2 minutes), 72°C (1 minute), followed by a final extension cycle (72°C for 10 min). The PCR products were run on a 1.0% agarose gel with Invitrogen SYBR Safe DNA Gel Stain Dye to confirm presence of bands of expected size.

PCR products were purified with Qiagen MinElute PCR Purification Kit. DNA concentrations were measured on a Qubit fluorometer with high sensitivity buffers and dyes and prepared for sequencing. Sequencing solutions consisted of 10 pmol primer, 500 ng DNA, and sterile DI water to 11 µl and were sequenced by the Iowa Institute of Human Genetics Genomics Division.

Representative colonies from the each of the analyzed streak plates were suspended in 1 ml sterile MSM, and injected into a 160 ml serum bottle containing 71 ml sterile MSM. Each bottle was fed 40 µmol VC and incubated at room temperature (22° C) inverted with shaking (180 revolutions per minute) in the dark. Headspace samples (100 µl) were analyzed 2-3 times per week by gas chromatography with flame ionization detection.

Periodically, frozen stocks were made of bacterial strains for cell line maintenance. Liquid cultures were centrifuged until a cell pellet was formed. After the

removal of supernatant, the cultures were suspended in 10 ml of MSM and stored in 1 ml increments at -80° C.

Experimental Design

Experiments were targeted at isolating a pure microorganism culture and confirming presence of 16S and etnE genes within these organisms. Cultures were plated onto sterile R2A plates and incubated at 30° C. Once colonies had developed, plates were re-streaked until an apparently pure culture was observed on the plate. Representative colonies were removed from the plate and prepared for use as a DNA template. This DNA template was used for 16S and etnE PCR.

DNA concentrations were measured, and 1 ng of DNA was used as template in PCR. Gel electrophoresis was run with the PCR product. PCR product was purified and sent for sequencing. Purity check methods for the isolations included streak plates and 16S rRNA gene sequencing and analysis, and verified culture purity.

Analytical Methods

Ethene and VC degradation was monitored by subjecting headspace samples (0.1 ml) to gas chromatography with flame ionization detection. A 0.25-ml Pressure-Lok gas-tight syringe with a sterile side-ported needle was used to take 0.1 ml headspace samples during each measurement. Gases were analyzed on a Hewlett-Packard 5890 series II gas chromatograph using a stainless steel column (8.0 ft. x 1/8 in. x 2.1mm) packed with 1% SP-1000 phase on 60/80 Carbopack B. A flame ionization detector was used with a nitrogen carrier gas flow rate of 30 ml/min and an oven temperature of 90 °C. Standard curves created for ethene and VC ranged from 0 µmol to 600 µmol and from 0 µmol to 60 µmol, respectively. These gas standards are included in Appendix A. Retention times for the gases were found to be 1.7 minutes and 4.3 minutes, respectively.

Growth was indirectly estimated by measuring the optical density at 600 nm (OD_{600}) with a Cary 50 Bio UV-Visible spectrophotometer. The liquid culture was

inverted and shaken, and less than 250 µL of liquid sample was extracted using a 1-ml plastic syringe with a sterile needle (22G 1.5) and added into a disposable cuvette.

Statistical Methods

To analyze the sequences, alignments and phylogenetic trees were created using MEGA5 software. Sequences for the alignment and tree were found using NCBI Basic Local Alignment Search Tool (BLAST) and Ribosomal Database Project (RDP) Seqmatch. Sequences were found on NCBI Genbank. Additionally, RDP Classifier program was used to characterize the 16S rRNA sequences from the isolates.

CHAPTER 4

RESULTS AND DISCUSSION

Results of this research are presented and discussed in this chapter. The data collected for this research was collected from August 2012 through April 2013. In partnership with MSU, this research is being conducted to further the knowledge of contaminated groundwater enrichment, isolation, and characterization.

Composite Carver Groundwater Isolations

Enrichment cultures were created using a composite contaminated groundwater sample collected from several wells at a site in Carver, MA, discussed in chapter 3. Cultures were created using stock culture had previously been growing on VC. The cultures from which the isolates were obtained were created from the initial culture in March and July of 2013.

Culturing and Isolation

The archived microcosm samples were suspended into two microcosms. The microcosms were grown on VC and monitored for gas degradation and growth through headspace readings and optical density measurements. Figures 3 and 4 show the degradation of VC gas for the filtered and unfiltered microcosms, respectively.

Archived microcosm was filtered before suspension in sterile MSM to potentially remove glycerol. The lag in degradation in the initial enrichment culture, shown by the black diamonds on Figure 3, indicates that glycerol was present in the suspension and used for growth by the microorganisms. To remove glycerol, 2 ml of culture was taken from the microcosm and filtered using a 0.22 µm Sterivex filter unit. The filtered culture was suspended in 70 ml of MSM and fed 40 µmol/bottle of VC. The degradation of the filtered culture is indicated by the blue crosses on Figure 3.

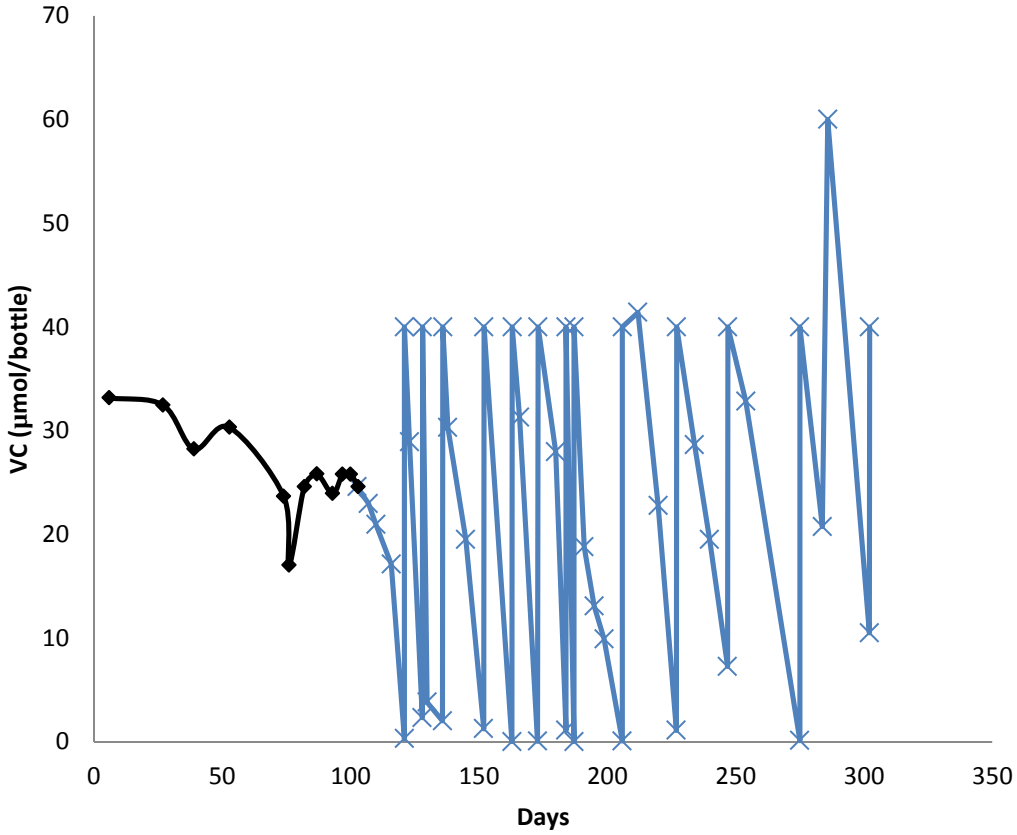


Figure 3: Degradation of VC in filtered composite Carver enrichment microcosm over time. Archived microcosm was suspended in sterile MSM for growth without filtration, then 2 ml was filtered using a Sterivex filter and resuspended in sterile MSM. Microcosms were fed 40-60 µmol/bottle of VC when VC was found to be close to depletion.

Figure 4 shows the degradation of VC in the unfiltered composite Carver groundwater microcosms. This culture was not filtered, as it was not stored in glycerol. As the figure shows, the lack of glycerol allowed the bacteria to degrade the VC immediately.

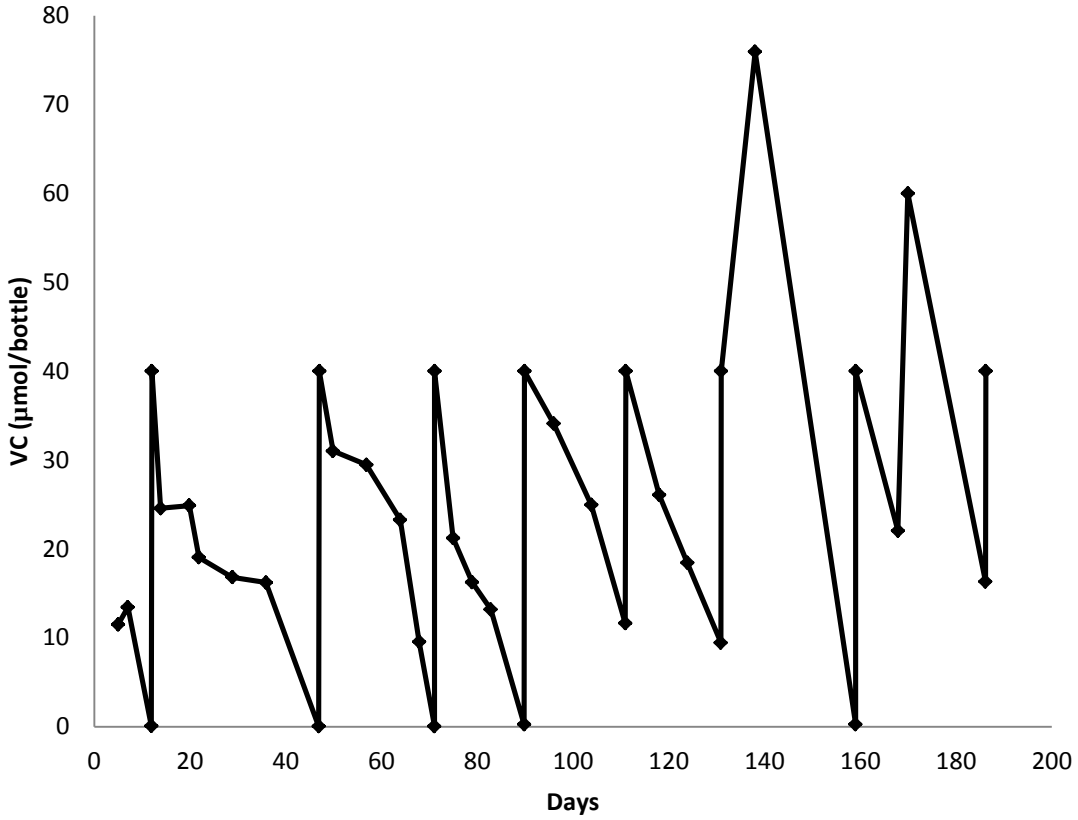


Figure 4: Degradation of VC in unfiltered composite Carver groundwater microcosm over time. Archived microcosm was suspended in sterile MSM for growth without filtration. Microcosms were fed 40-60 µmol/bottle of VC when VC was found to be close to depletion.

Figure 5 shows the observed optical density at 600 nm for the filtered and unfiltered microcosms.

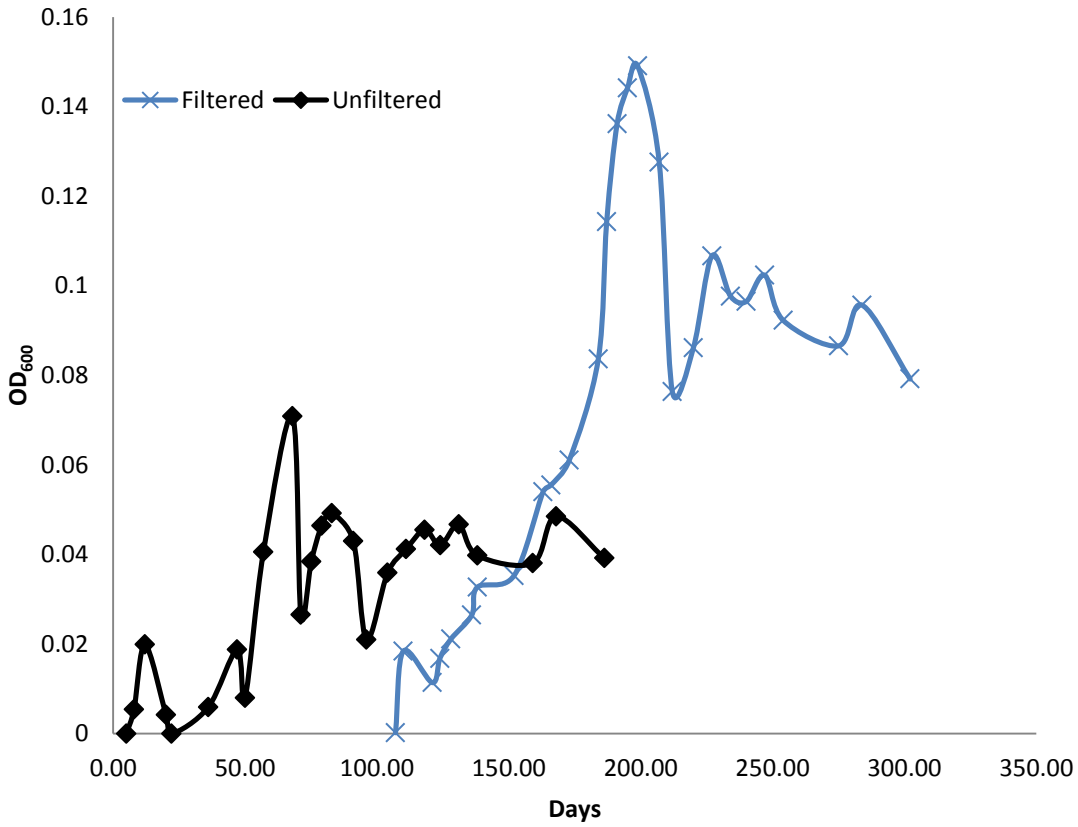


Figure 5: OD600 measurements for the composite carver site cultures. Filtered microcosm is represented in black and unfiltered microcosm is represented in blue.

During the growth period, clumps of cells of increasing size could be seen in the cultures. While steps were taken to distribute the cellular material throughout the medium, the clumps of cells impaired the reliability of the OD600 measurements. The uneven distribution of the cells in the suspension could have caused the fluctuations in the measurements over time.

After degradation and growth had been confirmed in VC enrichments, serial dilutions of the cultures were plated onto R2A media for isolations. Five representative colonies from each plated culture were streaked onto new plates for isolation growth. The streaking process was repeated to ensure pure colonies were present on each individual

plate. The assumed pure colonies were taken from the plate, suspended in sterile MSM, and used as DNA in PCR as outlined in chapter 3 for both 16S and etnE presence confirmation.

Four isolates showing positive gel bands for 16S and etnE were sequenced and analyzed, 2 from the filtered culture and 2 from the unfiltered culture. Figures 6 and 7 show the gel electrophoresis results with positive 16S and etnE bands.

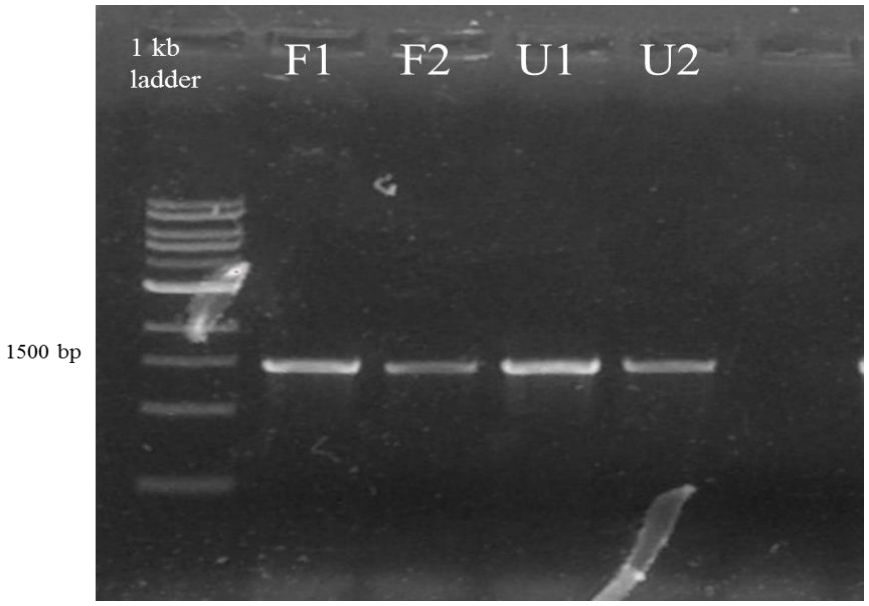


Figure 6: Gel electrophoresis of the amplified partial 16S rRNA gene for 4 isolates from the composite Carver well enrichment cultures. The ladder is a 1 kilo-basepair ladder; the positive bands are located at approximately 1500 bps. The first 2 isolates are from the filtered microcosm, and the second 2 isolates are from the unfiltered microcosm.

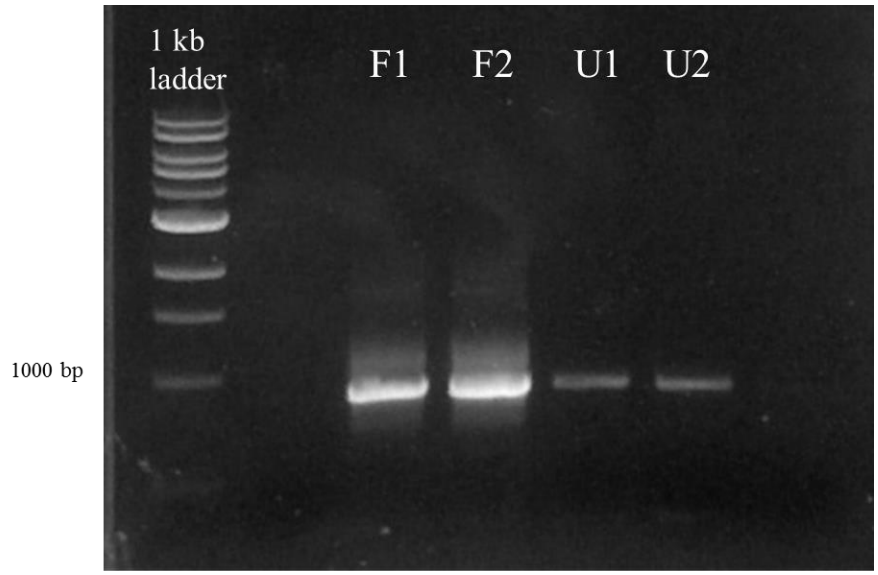


Figure 7: Gel electrophoresis of the amplified etnE gene for 4 isolates from the composite Carver well enrichment cultures. The ladder is a 1 kilo-basepair ladder; the positive bands are located at approximately 800 bps. The first 2 positive isolates are from the filtered microcosm, and the second 2 positive isolates are from the unfiltered microcosm.

Sequence data

DNA was prepared and sent to the Iowa Institute of Human Genetics Genomics Division, where it underwent Sanger sequencing. The 16S rRNA sequences relative to each other and other known VC assimilators are shown in Figure 8.

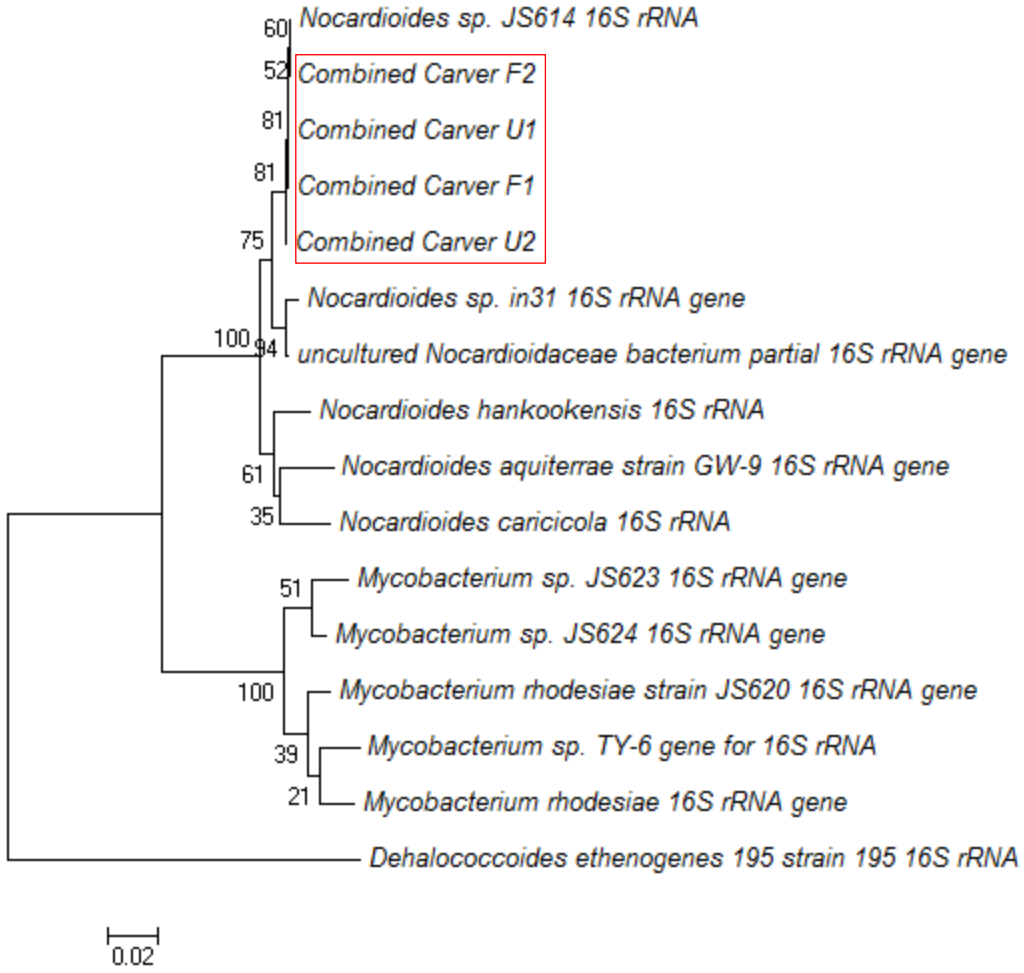


Figure 8: Phylogenetic analysis of the relationships between the partial 16S rRNA gene from the 4 isolates from the composite Carver enrichment cultures and other known VC assimilators. The optimal tree was created using Neighbor-Joining method, and evolutionary distances were computed using the Jukes-Cantor method in MEGA5. Bootstraping with 500 replicates was completed and 839 base pairs (bp) were included in the alignment. Isolate F1 16S rRNA sequence showed a 8% bp difference from the 16S rRNA sequence, and sequences from isolates F2, U1, and U2 showed less than 2% bp difference.

Sequences for this phylogenetic analysis were chosen through NCBI BLAST analysis and RDP Seqmatch results. Sequences with the highest identities to the isolates

were used for the phylogenetic comparison. The 16S sequences are most closely related to previously reported VC assimilator *JS614.* The sequences, Filtered 1 and 2 (F1, F2) and Unfiltered 1 and 2 (U1, U2), all show strong identity and coverage to the 16S and etnE sequences of *Nocardioides sp. JS614*. Figure 9 shows the etnE sequences relative to each other and the sequences of other known VC assimilators as a nucleotide alignment.

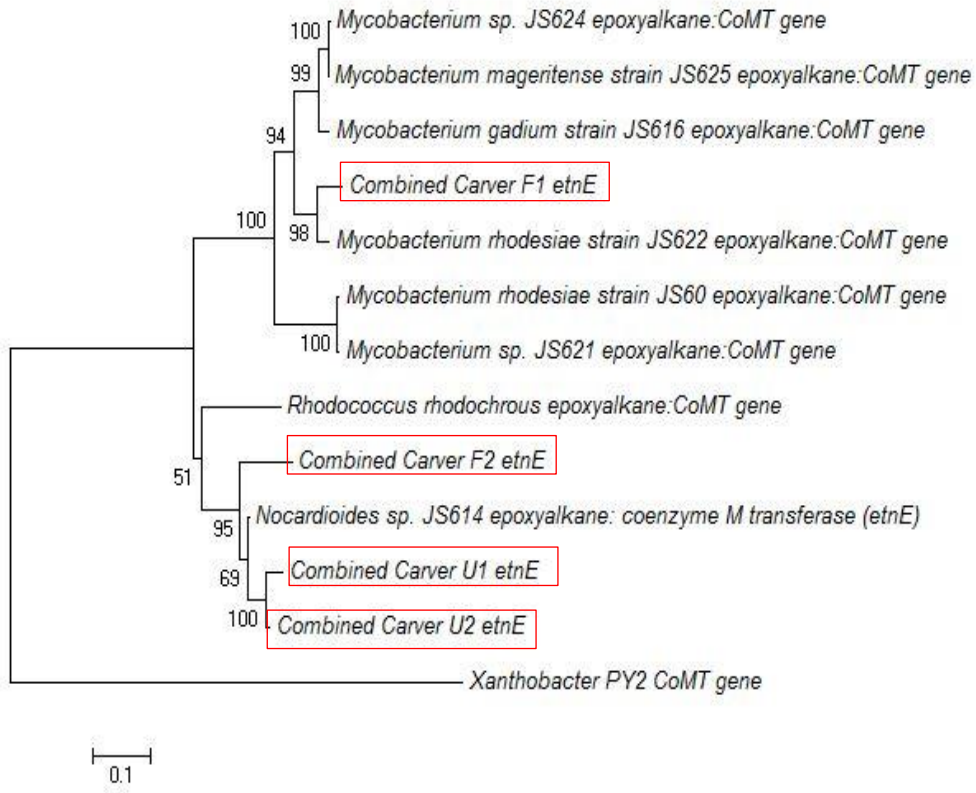


Figure 9: Phylogenetic analysis of the etnE gene from the 4 isolates from the composite Carver enrichment cultures and other known VC assimilators. The optimal tree was created using Neighbor-Joining method, and evolutionary distances were computed using the Jukes-Cantor method in MEGA5. Bootstraping with 500 replicates was completed and 798 base pairs were included in the alignment.

Figure 10 shows the etnE sequences relative to each other and the sequence of other known VC assimilators as an amino acid alignment. The trees display similar evolutionary distances and relationships for the bacteria and isolates, although they do have slightly differing bootstrap values.

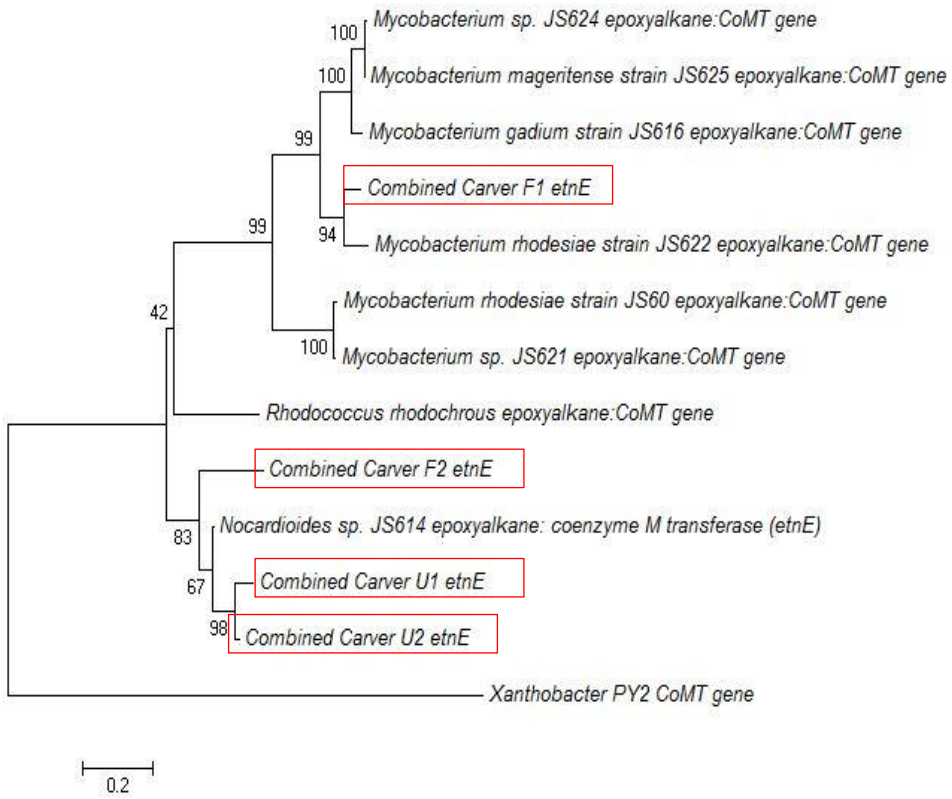


Figure 10: Phylogenetic amino acid analysis of the etnE gene from the 4 isolates from the composite Carver enrichment cultures and other known VC assimilators. The optimal tree was created using Neighbor-Joining method, and evolutionary distances were computed using the Poisson correction method in MEGA5. Bootstraping with 500 replicates was completed and 798 base pairs were included in the alignment.

Most of the etnE sequences are more closely related to the previously reported VC assimilator JS614, with the exception of F1, which aligns most clearly with

Mycobacterium sp. JS622. Isolate F1 shows a strong identity with *Mycobacterium rhodesiae* strain JS622, which contrasts with the results found from the 16S analysis. The other composite carver isolates, F2, U1, and U2, are more closely related to JS622. Partners at Michigan State University completed a high-throughput Illumina sequencing run with the microcosm created previous to the filtered and unfiltered cultures, finding a variety of microorganisms within the culture. Table 2 shows the variety of microorganisms obtained through their culturing and sequencing efforts, organized by operational taxonomic unit (OTU).

Table 2: Results of SIP experiment with enriched composite Carver well microcosm.

Phylum	Family	OTU	Present in fractions
Actinobacteria	*Nocardioidaceae*	Nocardioides	5
Proteobacteria	*Caulobacteraceae*	Brevundimonas	5
Acidobacteria	*Acidobacteria Gp6 family incertae sedis*	Gp6	4
Firmicutes	*Clostridiales Incertae Sedis XI*	Tissierella	4
Firmicutes	*unclassified Clostridiales*	Clostridiales	4
Firmicutes	*Clostridiales Incertae Sedis XI*	Sedimentibacter	3
Firmicutes	*unclassified Bacillales*	Bacillales	3
Proteobacteria	*Sphingomonadaceae*	Sphingomonadaceae	3
Verrumicrobia	*Opitutaceae*	Opitutus	3
Firmicutes	*Erysipelotrichaceae*	Erysipelotrichaceae	2
Proteobacteria	*Rhodospirillaceae*	Azospirillum	2
Proteobacteria	*Burkholderiaceae*	Burkholderia	2
Proteobacteria	*Rhizobiaceae*	Rhizobium	2

Note: Total number of microcosms present displays how prevalent the family was throughout fractions taken during the study.

The sequences found through the SIP study by Michigan State University display a much larger variety of species than what was found through isolation techniques.

Bacteria from the family *Nocardioidacae* were most prevalent in the sequencing results, aligning with the isolation findings in this study. Laboratory biases could be at play; rich media was used that could facilitate a bias during plating and culture growth.

Isolate regrowth on VC

Isolates were archived in sterile MSM and stored in -80℃ freezer. The cultures were revived in serum bottles as previously described in chapter 3 and fed 40 µmol of VC. The degradation of the VC was recorded through GC measurements. Figure 11 shows the degradation of the VC after cell suspension in MSM for isolates F1, F2, U1, and U2, respectively.

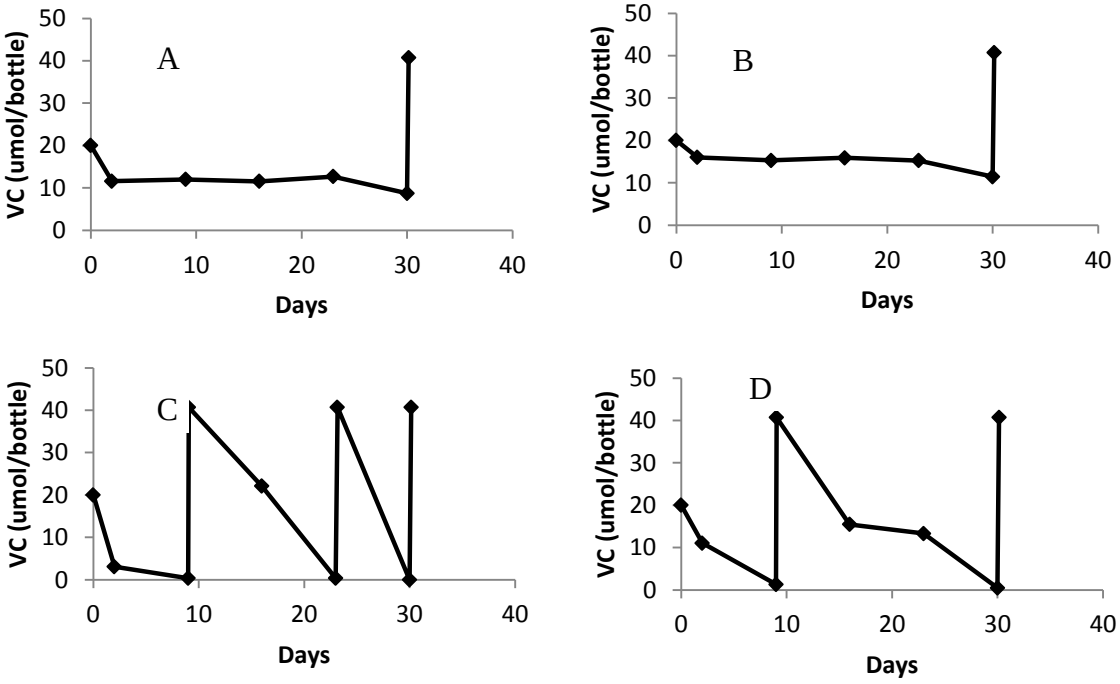


Figure 11: Degradation of VC in composite Carver groundwater isolates A) F1, B) F2 C) U1 D) U2. Isolates were suspended in sterile MSM for growth. Microcosm was fed 20 µmol/bottle and headspace measurements were reported over 30 days.

The degradation shows that the unfiltered isolates, U1 and U2, were able to degrade VC after being re-introduced to the gas. Isolates F1 and F2 have degraded a small amount of VC, but are displaying signs of lagging. Further monitoring of the re-suspended isolates should continue to display VC degradation. Figure 12 shows the OD600 measurements for the isolates F1, F2, U1, and U2. The isolates show a general cellular growth, and will continue to be monitored for growth.

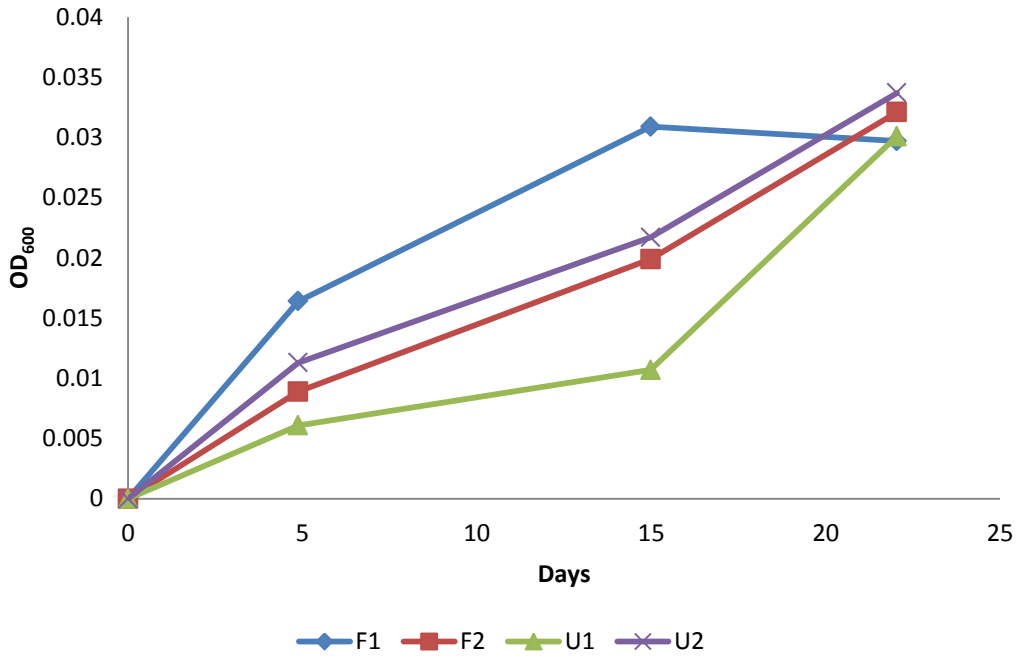


Figure 12: OD_{600} measurements for isolates F1, F2, U1, and U2. The isolates are showing a general cellular growth.

63-I Groundwater Isolations

Enrichment cultures were created using contaminated groundwater collected from well 63-I at a site in Carver, MA. Cultures were created at the University of Iowa in September 2012.

Culturing and Isolation

Groundwater and sterile MSM were added to 160 ml serum bottles (Wheaton) in ratios of 1:1, 1:3, 1:17. VC was added to the cultures in 20-40 µmol increments, replenished as needed. The bottles were stored covered and inverted at room temperature on a shaker. Headspace samples (100 µl) were analyzed 2-3 times per week by gas chromatography with flame ionized detection. Groundwater cultures were transferred to new MSM microcosms and archived over time. Figure 13 shows the degradation of VC for the 63-I groundwater microcosm from which the isolates were extracted.

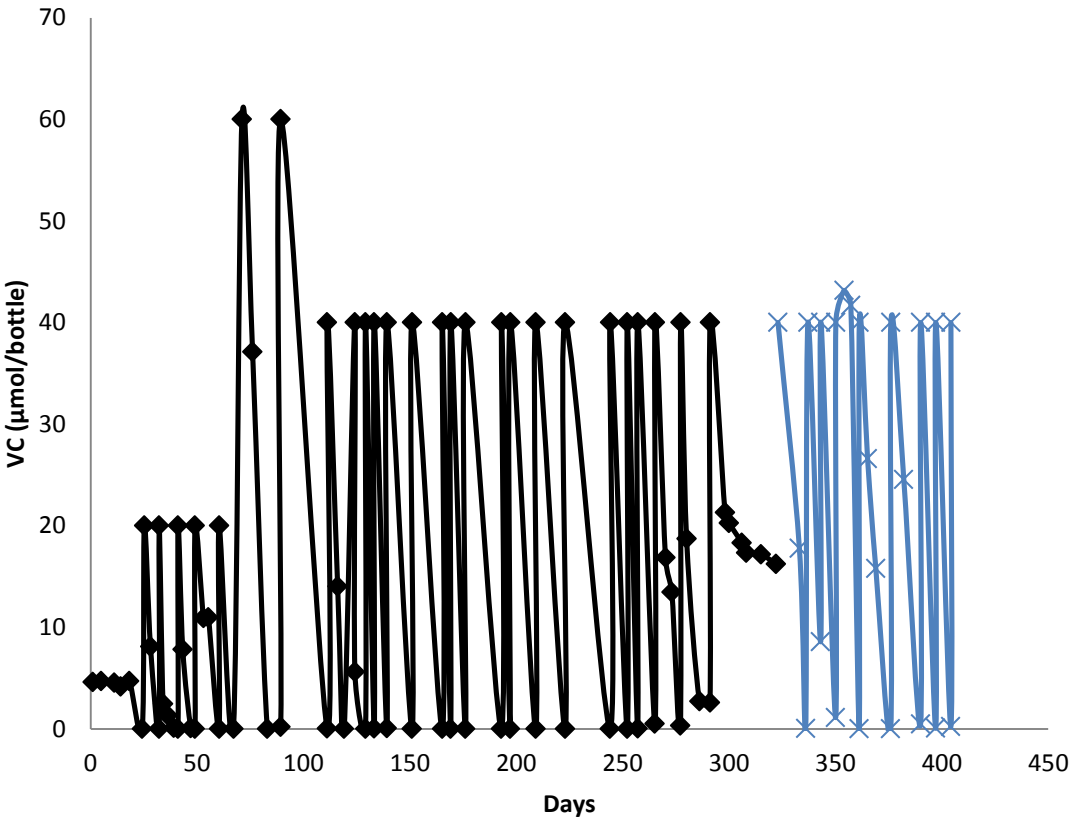


Figure 13: Degradation of VC in 63-I groundwater microcosm over time. Groundwater was suspended in sterile MSM for growth. Microcosm was fed 40-60 µmol/bottle of VC when VC was found to be close to depletion.

The culture showed a short lag and then began degrading VC. After approximately 300 days, a lag in degradation was noticed, likely due to oxygen depletion in the microcosm. This initial culture is represented by black diamonds. Culture was transferred into fresh sterile MSM and the new culture was spiked with 40 µmol/bottle. The new culture is indicated by the blue crosses on Figure 11.

After degradation and growth had been confirmed, serial dilutions of the cultures were plated onto R2A media for growth. Varying volumes (1, 10, 50, 100 µl) of the enrichment culture sample containing a groundwater and MSM ratio of 1:1 (36 ml sterile MSM and 36 ml 63-I groundwater) and fed VC were plated on R2A medium and incubated at 30 °C. After 3 days, representative colonies were chosen and analyzed via etnE PCR to confirm presence of etnE gene. The analysis showed no evidence of etnE, as expected due to the short growth time. Experiment growth time for previous etnE positive isolations has been 1 to 3 months.

The groundwater culture was again plated in serial dilutions onto R2A medium and incubated at 30 degree Celsius for 10 days. Individual colonies from the 10^{-4} dilution spread plate were streaked onto R2A plates and allowed to grow for 10 days. The streaking process was repeated to ensure pure colonies were present on each individual plate. If an isolate appeared to have multiple colony types by looking at plates, streaking was repeated until pure. A total of 9 isolates were found to have positive gel bands for 16S and etnE during gel electrophoresis analysis. Figure 14 shows the gel electrophoresis results with positive 16S and etnE bands.

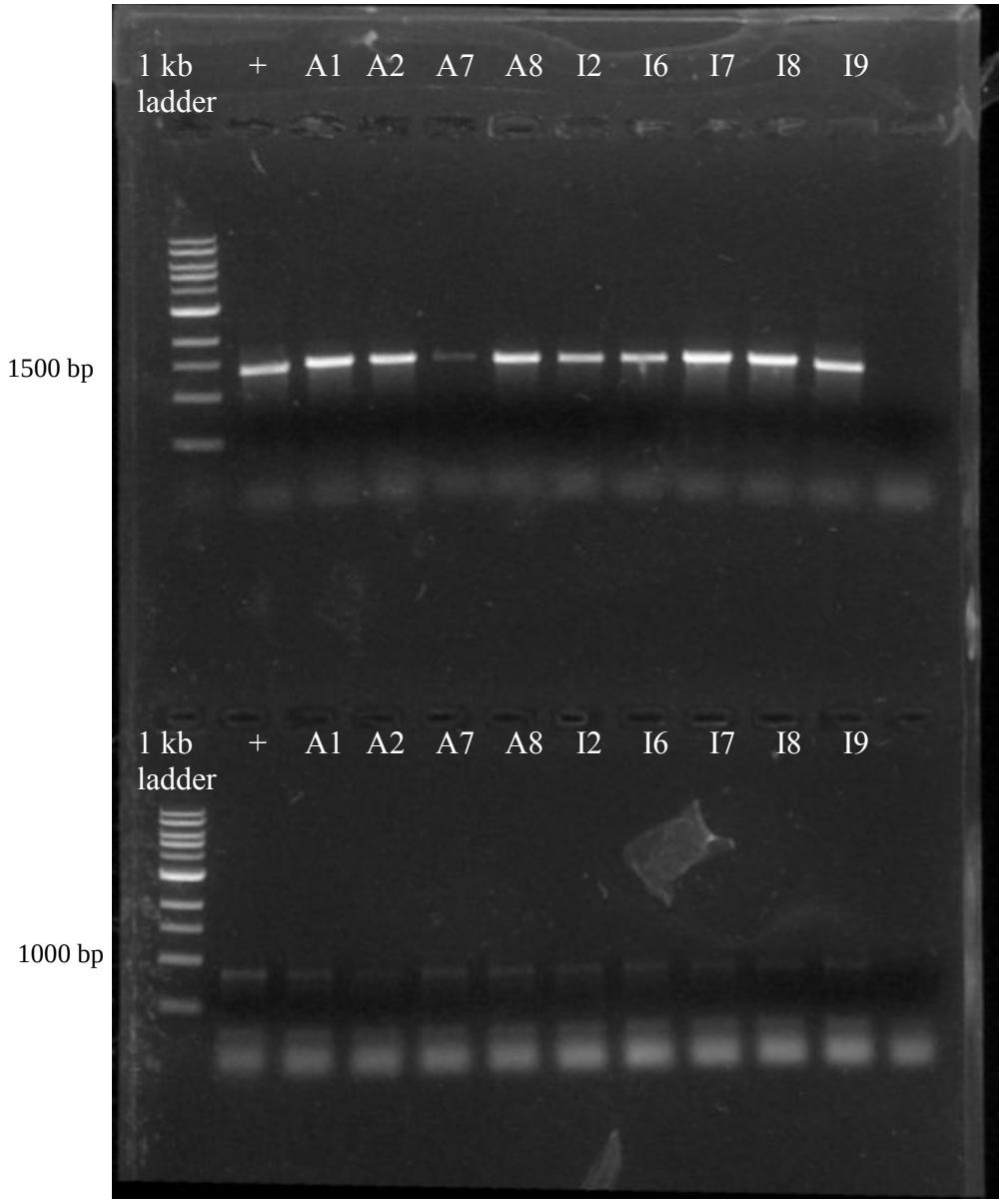


Figure 14: Gel electrophoresis of the amplified partial 16S rRNA gene (top row) and etnE gene (bottom row) for 9 isolates from the 63-I groundwater enrichment culture. The ladder is a 1 kilo-basepair ladder. The positive bands are located at approximately 1500 bps and 800 bps in the top and bottom rows, respectively. The first positive band in each row is *Nocardioides sp. JS622*, acting as a positive control. The isolates in both rows are ordered as follows: A1, A2, A7, A8, I2, I6, I7, I8, and I9. Sterile DI water was used as a negative control and follows isolate I9 in both rows.

The isolates were taken from the plates and suspended in 160 ml serum bottles for growth. VC was added to the cultures in 20-60 µmol increments, replenished and transferred as needed. The bottles were stored covered and inverted at room temperature on a shaker. Headspace samples (100 µl) were analyzed 2-3 times per week by gas chromatography with flame ionized detection. Degradation data and OD600 measurements for the isolates can be found in Appendix B.

Sequence data

DNA was prepared and sent to the Iowa Institute of Human Genetics Genomics Division, where it underwent Sanger sequencing. The 16S rRNA sequences relative to each other and other known VC assimilators are shown in Figure 15.

Sequences for this phylogenetic analysis were chosen through NCBI BLAST analysis and RDP Seqmatch results. Sequences with the highest identities to the isolates were used for the phylogenetic comparison. The sequences align with various bacteria, including *Nocardioides sp. JS614*, *Microbacterium sp. DmB 4*, and uncultured *Microbacterium*, *Stephomonas*, and *Aminobacter* clones. Isolates A1, A2, and A8 most clearly aligned with *Microbacterium sp. DmB 4.* Isolates I2 and A7 most clearly aligned with previously reported VC assimilator *JS614I.* Isolates I6, I7, I8, and I9 aligned with a variety of uncultured bacteria clones, including clones of *Microbacterium*, *Stephomonas*, and *Aminobacter* phylums. This variety of bacterial sequences differs from the results found in the enriched cultures from the composite Carver site wells.

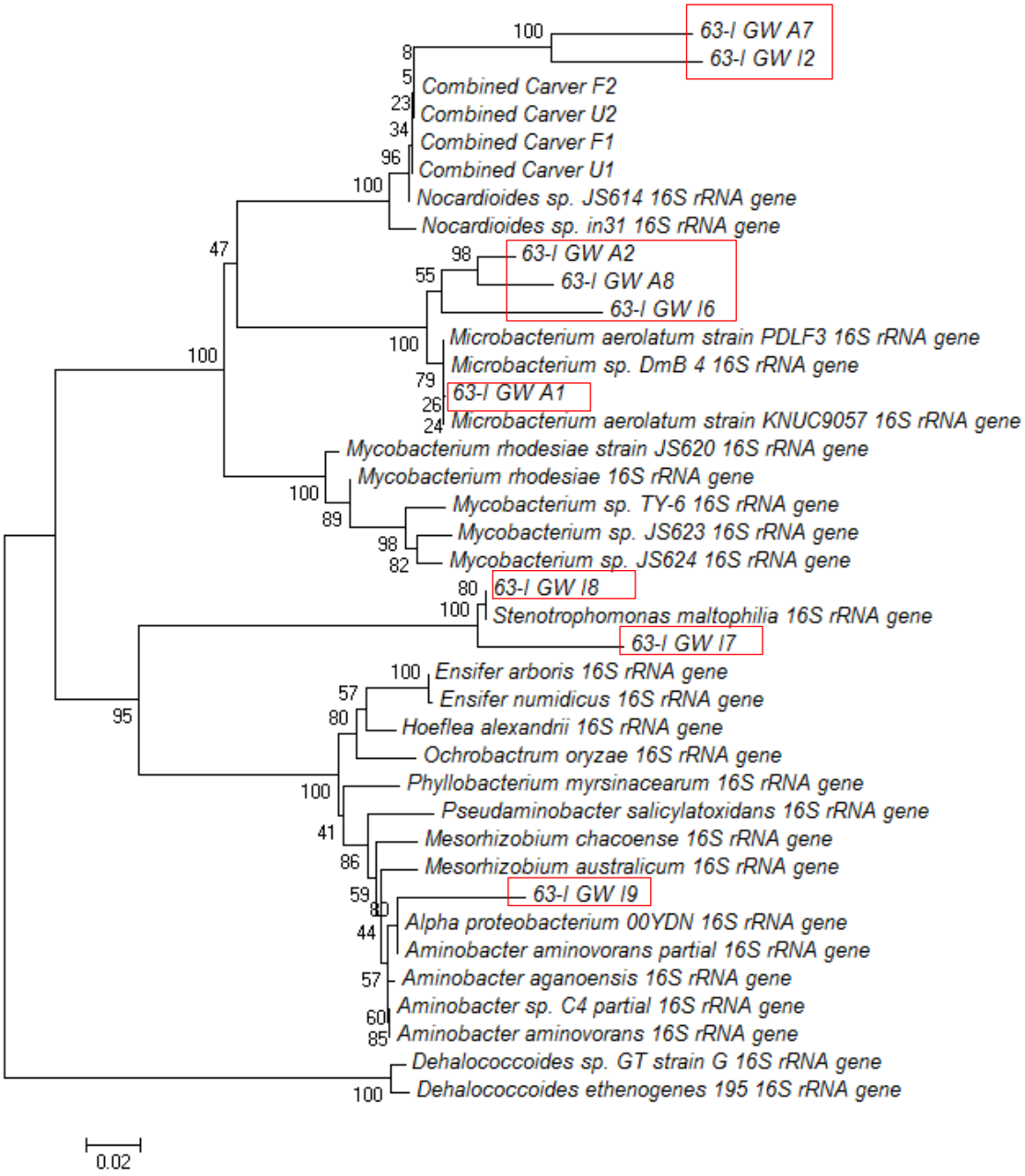


Figure 15: Phylogenetic analysis of the partial 16S rRNA gene from the 9 isolates from the 63-I groundwater enrichment cultures and other known VC assimilators. The optimal tree was created using Neighbor-Joining method, and evolutionary distances were computed using the Jukes-Cantor method in MEGA5. Bootstraping with 500 replicates was completed and 759 base pairs were included in the alignment.

The sequences for the CoM genes have been harder to obtain. The PCR product that has shown to have positive gel bands for CoM has extremely low concentrations, and are not high enough to be sequenced. Multiple method adjustments have been explored, including increasing the DNA template concentration, adjusting the thermocycling program to increase the chance of targeting the CoM gene, and nesting the PCR by using the PCR product as a DNA template in a second round of thermocycling. While these method adjustments have shown positive bands in a gel electrophoresis test, concentrations high enough for sequencing still have not been found.

Using RDP Classifier, the isolate 16S rRNA sequences were used to characterize the isolates, shown in Table 3. The isolates were classified as bacteria from the *Microbacterium* and *Nocardioidaceae* families, as well as *Xanthomonadaceae* and *Aminobacter* strains, and were found to have varying degrees of confidence in the classifications.

Table 3: Isolate characterization through RDP Classifier analysis.

Isolate	Family	Genus	Confidence
A1	*Microbacteriaceae*	*Microbacterium*	100%
A2	*Microbacteriaceae*	*Microbacterium*	95%
A7	*Nocardioidaceae*	*Pimelobacter*	90%
A8	*Microbacteriaceae*	*Okibacterium*	57%
I2	*Nocardioidaceae*	*Pimelobacter*	76%
I6	*Microbacteriaceae*	*Okibacterium*	56%
I7	*Xanthomonadaceae*	*Stenotrophomonas*	97%
I8	*Xanthomonadaceae*	*Stenotrophomonas*	100%
I9	*Phyllobacteriaceae*	*Aminobacter*	99%

Isolate regrowth on VC

Isolates were archived in sterile MSM and stored in -80̊ C freezer. The cultures were revived in serum bottles as previously described in the methods section and fed 20 µmol of VC. The degradation of the VC was recorded through GC measurements. Figures 16 and 17 shows the degradation of the VC after cell suspension in MSM.

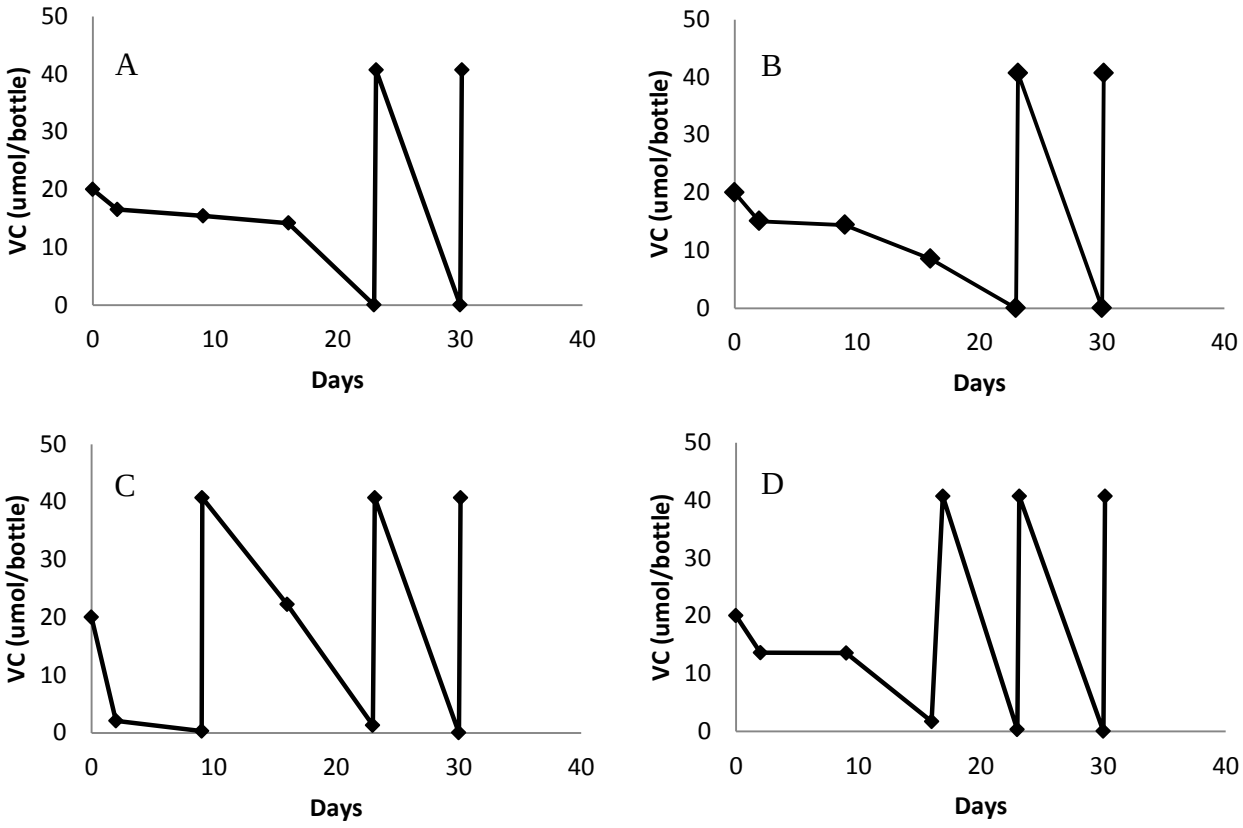


Figure 16: Degradation of VC in 63-I groundwater isolates A) A1 B) A2 C) A7 D) A8. Isolates were suspended in sterile MSM for growth. Microcosm was fed 20 µmol/bottle and headspace measurements were reported over 15 days.

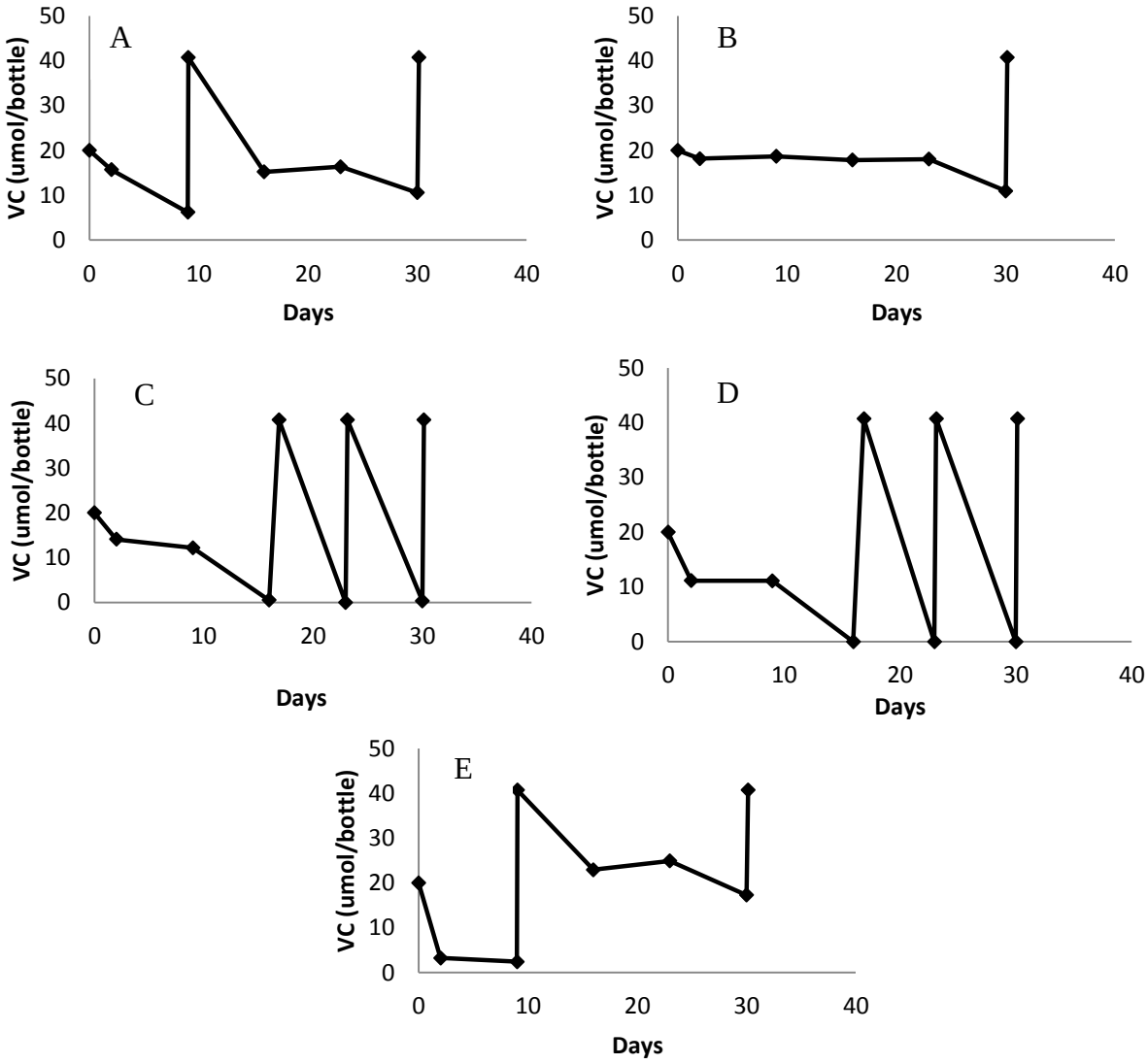


Figure 17: Degradation of VC in 63-I groundwater isolates A) I2 B) I6 C) I7 D) I8 E) I9. Isolates were suspended in sterile MSM for growth. Microcosm was fed 20 µmol/bottle and headspace measurements were reported over 30 days.

The degradation shows that some of the isolates were able to degrade VC after being re-introduced to the gas. Isolates A1, A2, A7, A8, I2, I7, I8, I9 show complete

degradation of 20 µmol of VC over a 30 day period. Isolates A1, A2, and I6 showed a slower degradation of the VC over a 30 day period.

OD600 measurements were taken for the isolates to monitor cell growth. Figure 18 shows the OD600 measurements for the 63-I isolates.

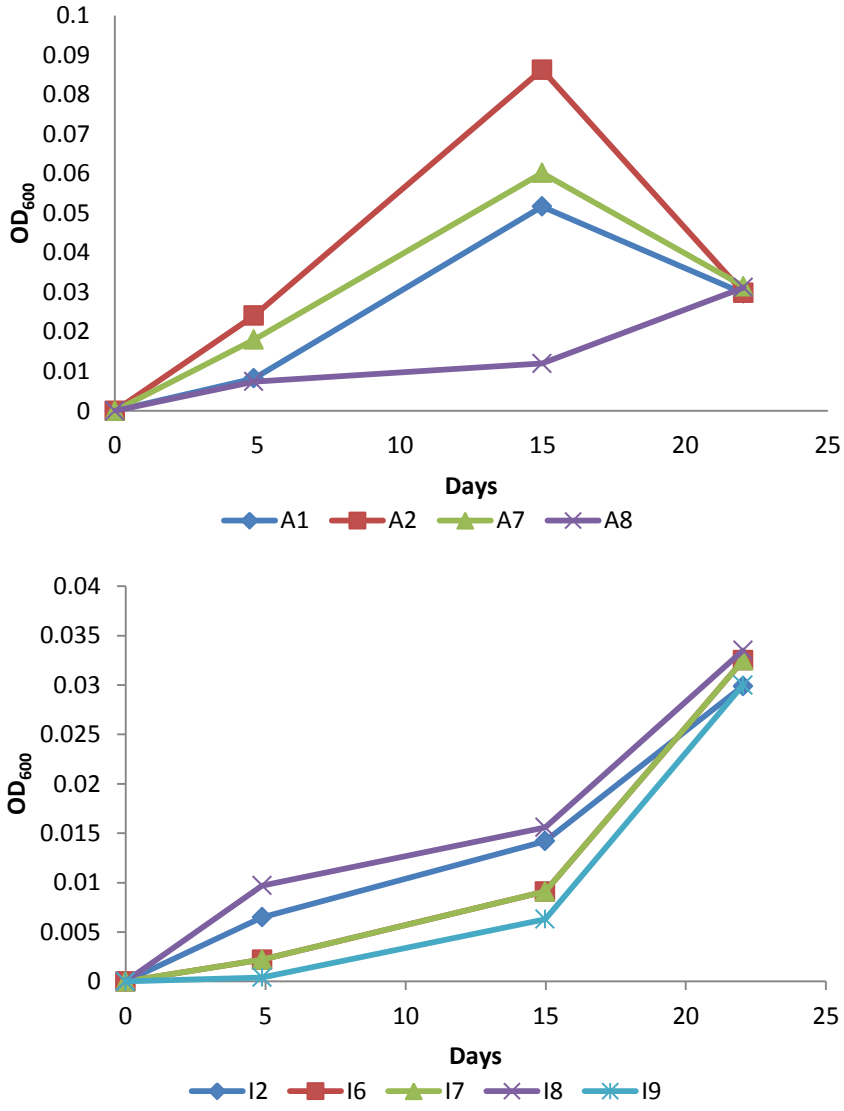


Figure 18: OD_{600} measurements for isolates A1, A2, A7, A8 and I2, I6, I7, I8, and I9. The isolates are showing a general cellular growth.

Purity and Bias

Purity of the isolates was considered a central research objective and was observed in multiple methods.

Plating Colonies

The isolates were checked for purity during growth through plating onto R2A agar and incubating at 30 degrees. The plates were checked for single colony types; if contamination was suspected, isolates were re-streaked onto new R2A agar plates and isolation cultures were recreated. After sequencing analysis, isolates were confirmed pure through plating. Figure 19 shows streak plates of isolates showing a single colony type and multiple colony types.

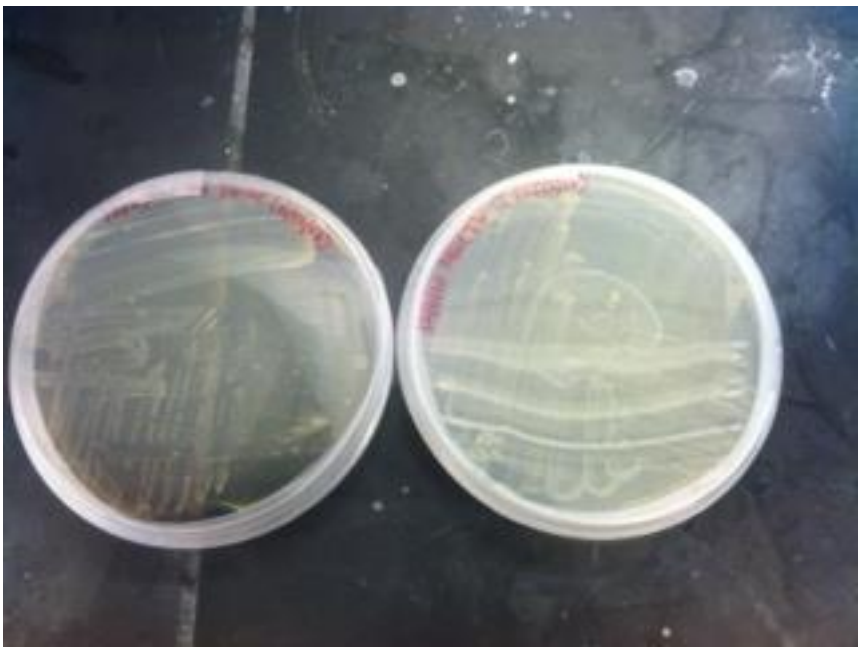
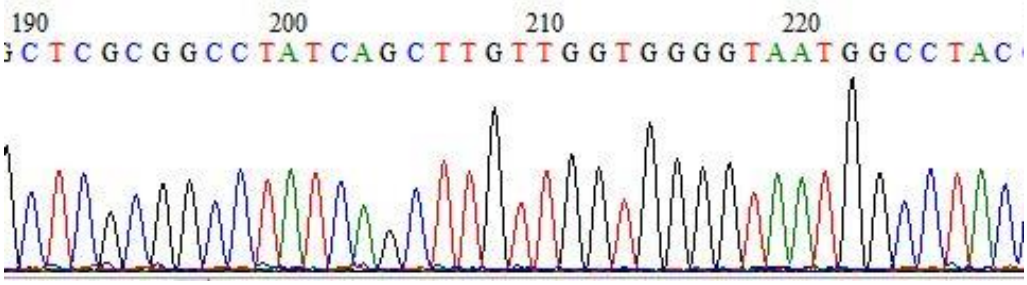


Figure 19: Streak plates of isolates showing a) a single colony type and b) multiple colony types. The plates were analyzed to ensure a single colony type was represented on an isolate plate.

Sequence Identity

Isolates sequences were checked for purity through the sequencing chromatograph and BLAST sequence identity. Chromatographs of the pure isolates displayed strong, individual peaks throughout the sequence, while impure sequences contained multiple peaks for an individual base pair. The isolates all showed strong, individual peaks throughout the center of the sequence. The partial 16S rRNA and etnE gene sequences also showed strong identity with previously reported bacterium, indicating a single sequence was present. Figure 20 shows electropherogram samples with strong single peaks, indicative of a pure sequence, and with small multiple peaks, indicative of a contaminated sequence.

a)



b)

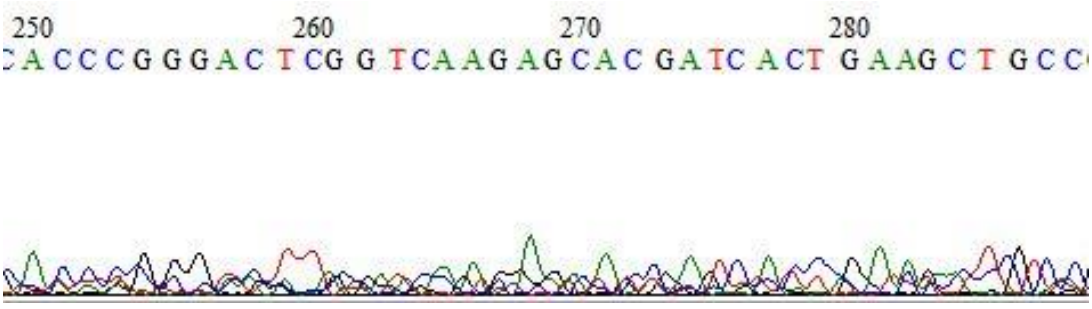


Figure 20: Electropherogram samples showing a) a single strong sequence for an isolate and b) a poor sequence with multiple peaks.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The purpose of this research was to explore the diversity of isolated VC assimilators within contaminated groundwater, and attempt to enrich and isolate these microorganisms using molecular tools. As indicated in this study, a plating technique for isolation, in combination with PCR and DNA sequences, can be effective in isolating bacteria from an enriched groundwater microcosm.

Through plating isolations and microbial analysis, VC assimilating bacteria were found within enriched groundwater microcosms made from groundwater taken from wells at a contaminated site in Carver, MA. Streak plating was performed for cell growth, and PCR amplification was used to sequence the 16S rRNA gene and EaCoMT gene for analysis. From the composite Carver site groundwater, isolates were found to be closely similar to 16S rRNA gene sequence of *Nocardioides sp. JS614.* The data found at Michigan State University with the same groundwater shows a larger variety of bacteria within the enriched groundwater culture than was found through isolation methods. From the Carver 63-I groundwater, isolates were found to be closely similar to 16S rRNA gene sequences of *Nocardioides sp. JS614, Microbacterium sp. DmB 4,* and uncultured *Microbacterium, Stephomonas,* and *Aminobacter* clones. Isolates were also confirmed to have coordinating etnE genes with these sequences.

There should, however, be an understanding of potential isolation bias through this method. Cell growth was performed on rich R2A media and in controlled laboratory microcosms, which unintentionally provides a biased growth environment for certain microbes. The variety of bacteria found through high-throughput sequencing of the composite carver groundwater culture by Michigan State University provides an indication of isolation bias in spread and streak plating.

Purity was an important factor in the isolation method development. The isolates were confirmed pure through repeated streak plating analysis and 16S and etnE sequence analysis.

CHAPTER 6
ENGINEERING SIGNIFICANCE AND RECOMMENDATIONS FOR FUTURE RESEARCH

VC contamination within groundwater is a prevalent environmental groundwater problem that could be combated with further studies into bioremediation and the production of tools that utilize biodegradation for remediation.

The application of stable isotope probing and molecular tools to capture the characteristics of a groundwater in-situ is a novel idea, and would allow a microbial environment to be analyzed without laboratory bias. This research and application of molecular tools and microbial analyses will open new pathway to find effective and efficient bioremediation for groundwater.

Despite the insight that this research provides, there are still methods to improve upon and applications for this work that have not yet been explored. Specific recommendations for future work regarding VC assimilating and co-metabolizing bacteria include:

1. Further assess the bias associated with methods of isolation, including but not limited to, microcosm suspension (headspace, gas feed rate, etc.), plating media type, and laboratory scaled experiments.
2. Perform additional experiments to investigate the applicability of alternative media for isolations of VC assimilating bacteria within laboratory environments and in-situ collection.

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APPENDIX A

STANDARD CURVE SUPPORTING DOCUMENTS

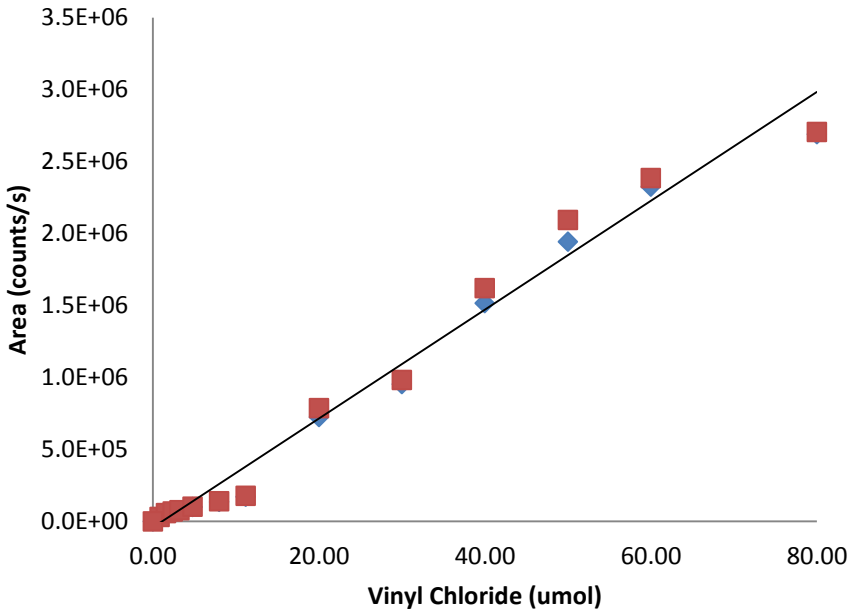

Figure A1. Standard curve for VC

Table A1. Raw data for VC mass conversion

VC (umol)	Area (counts/s)	Area (counts/s)
0	0	0
20	94059	94317
30	115264	113166
40	1515510	1618870
50	1940960	2093810
60	2325180	2384880
80	2689240	2705010

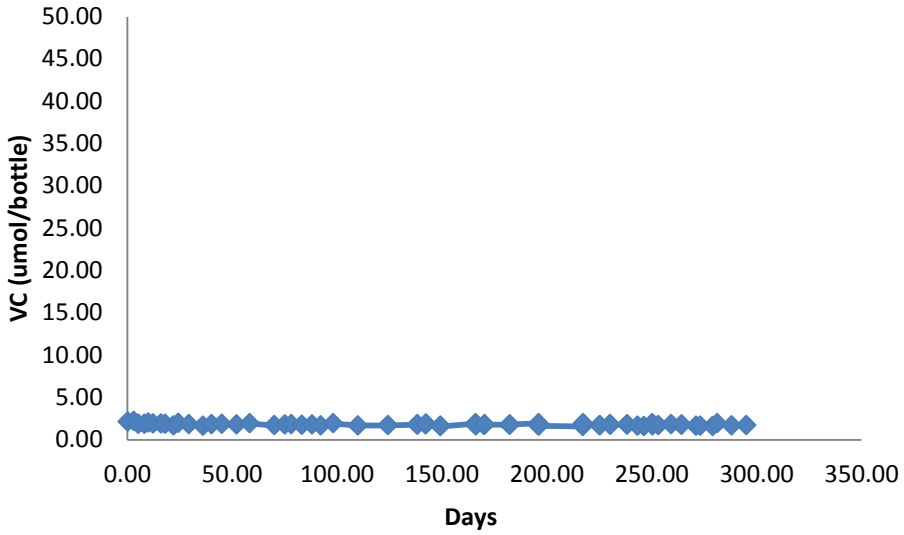

Figure A2. Abiotic Control for VC

APPENDIX B

ISOLATE DEGRADATION AND GROWTH SUPPORTING DOCUMENTS

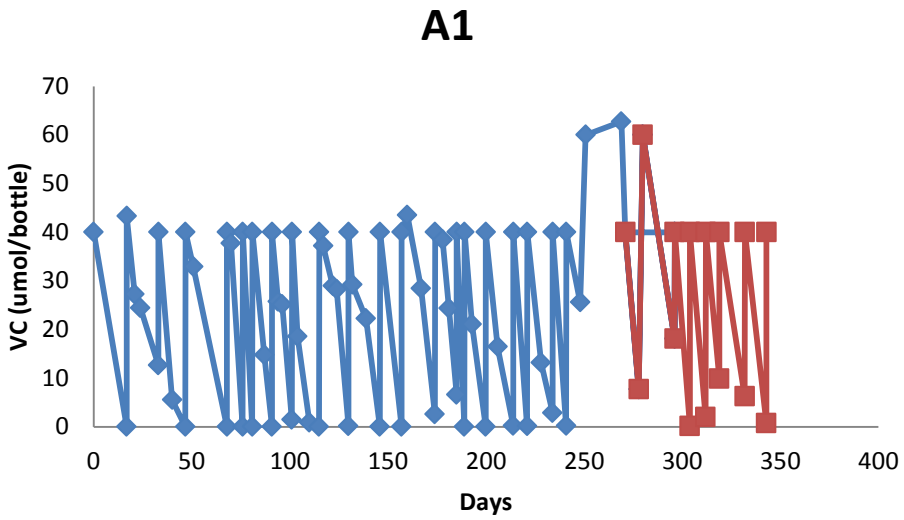

Figure B1. Degradation of VC by isolate A1

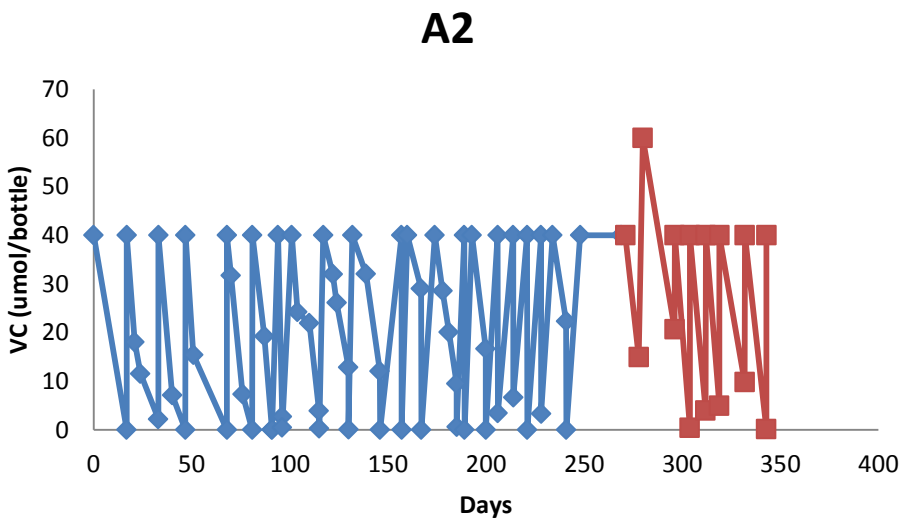

Figure B2. Degradation of VC by isolate A2

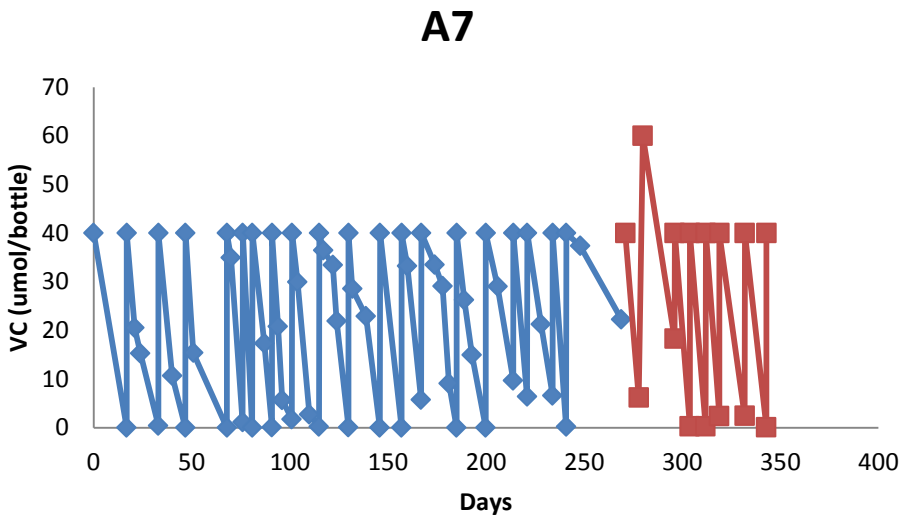

Figure B3. Degradation of VC by isolate A7

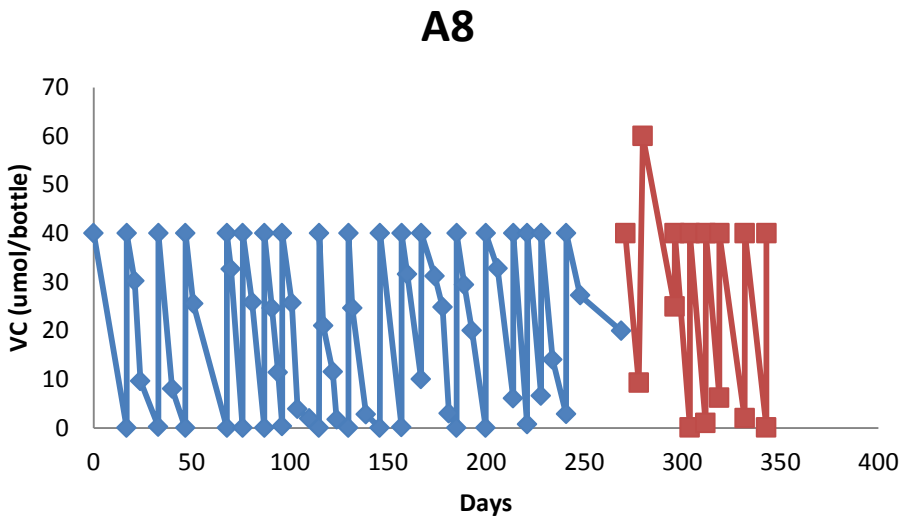

Figure B4. Degradation of VC by isolate A8

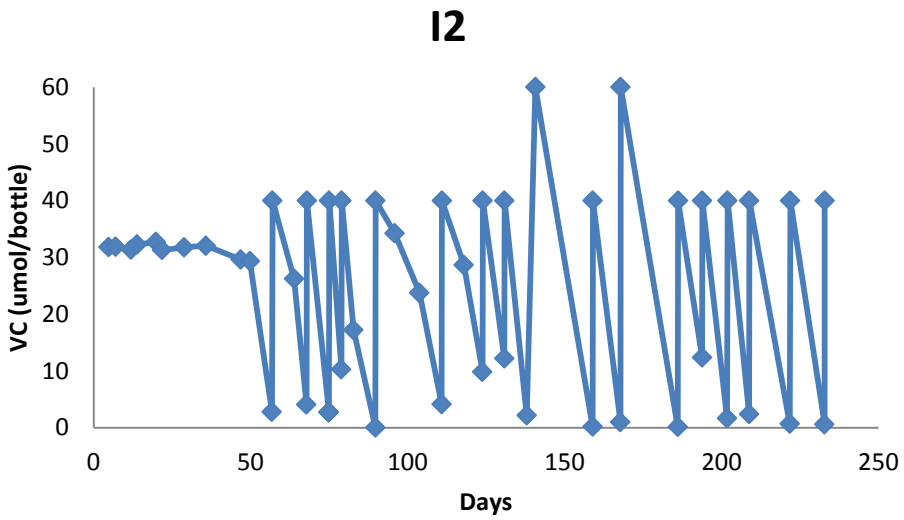

Figure B5. Degradation of VC by isolate I2

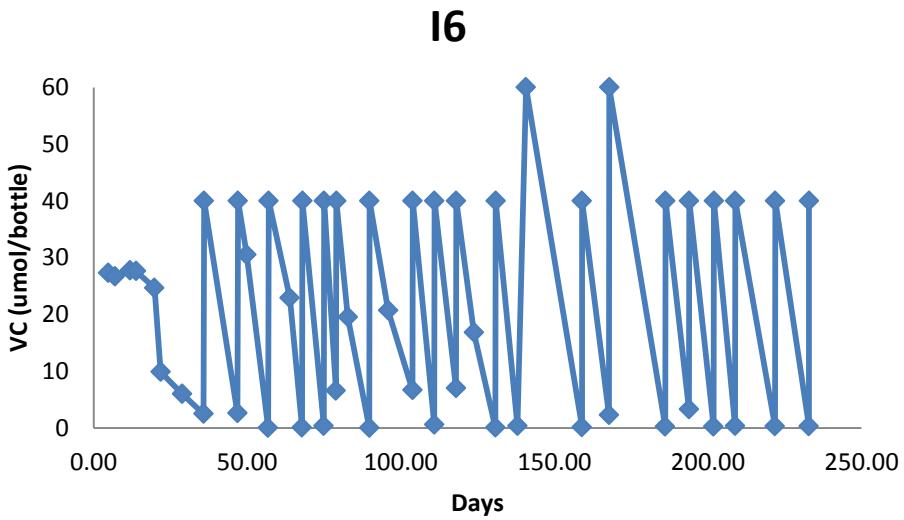

Figure B6. Degradation of VC by isolate I6

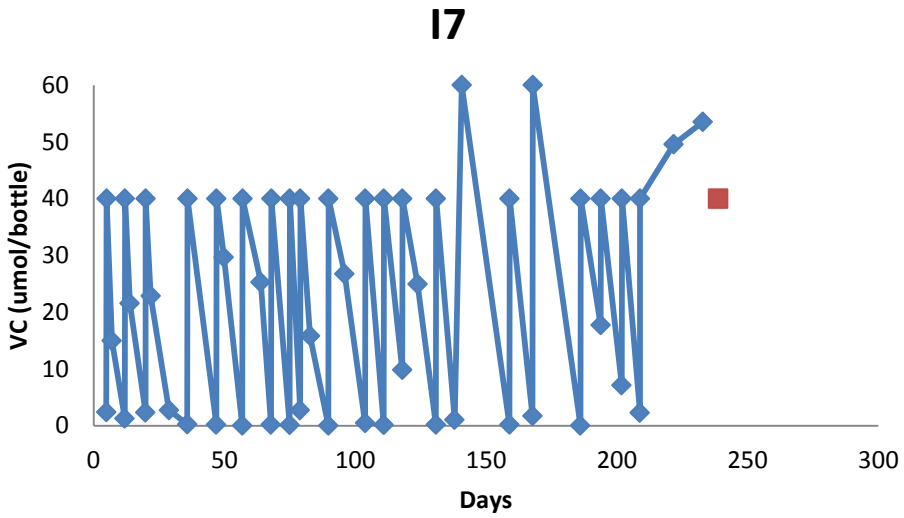

Figure B7. Degradation of VC by isolate I7

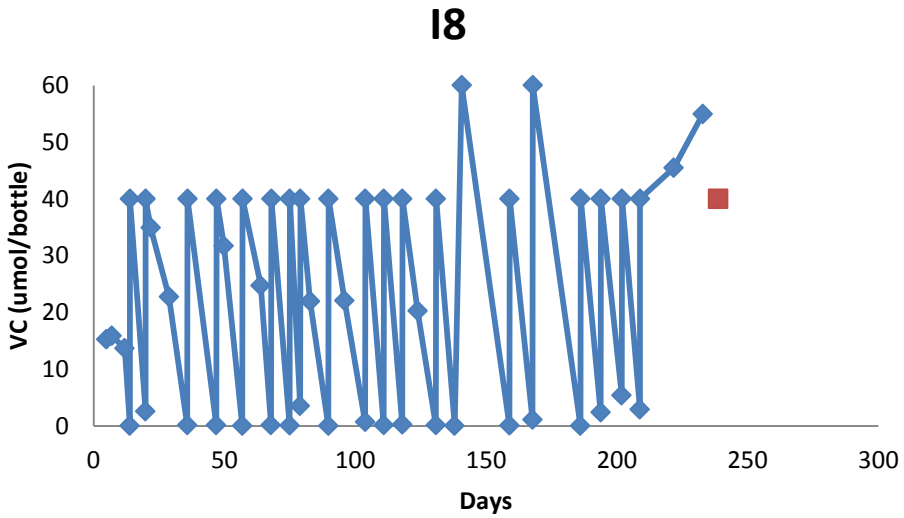

Figure B8. Degradation of VC by isolate I8

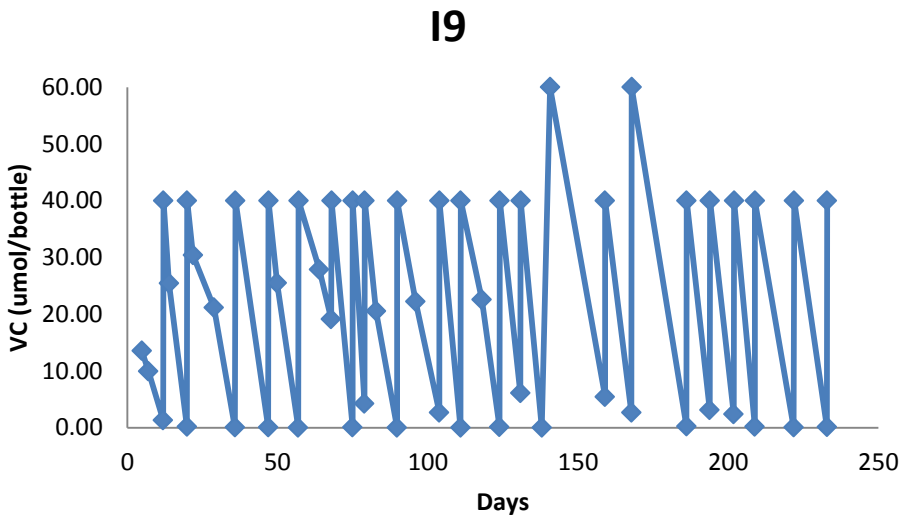

Figure B9. Degradation of VC by isolate I9

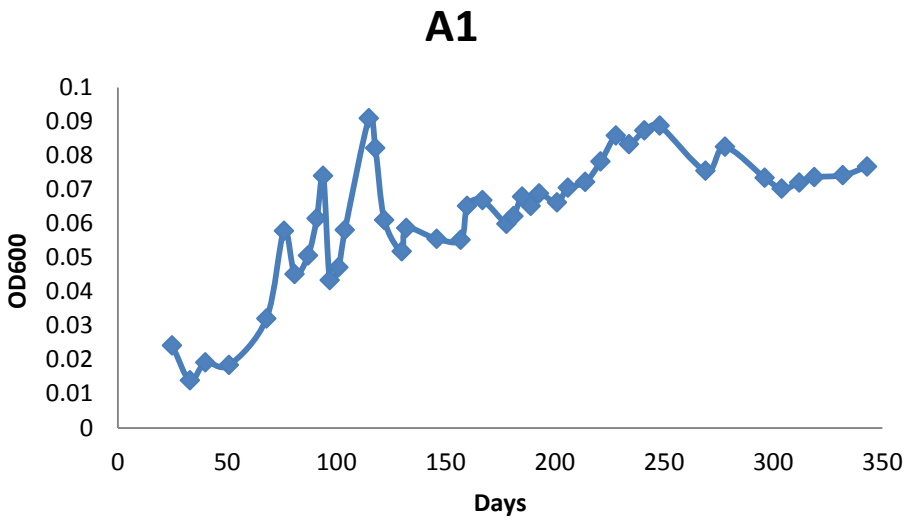

Figure B10. OD_{600} of isolate A1

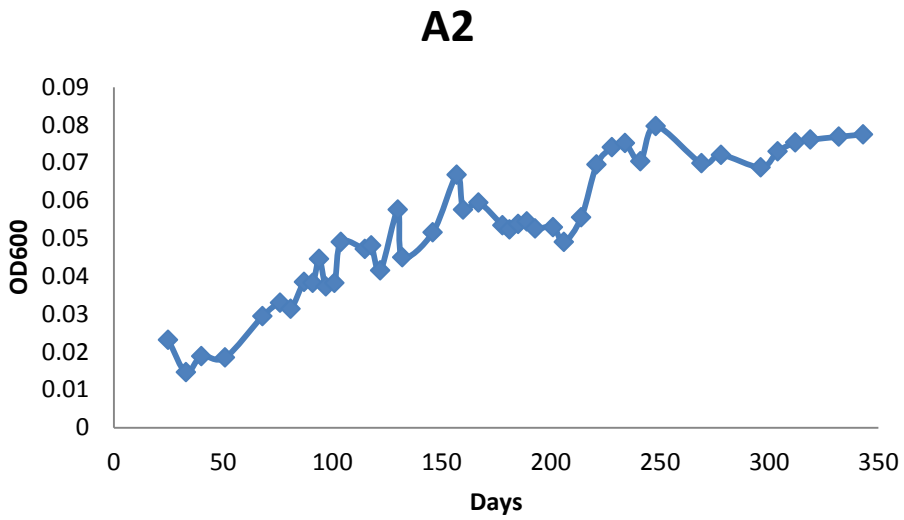

Figure B11. OD_{600} of isolate A2

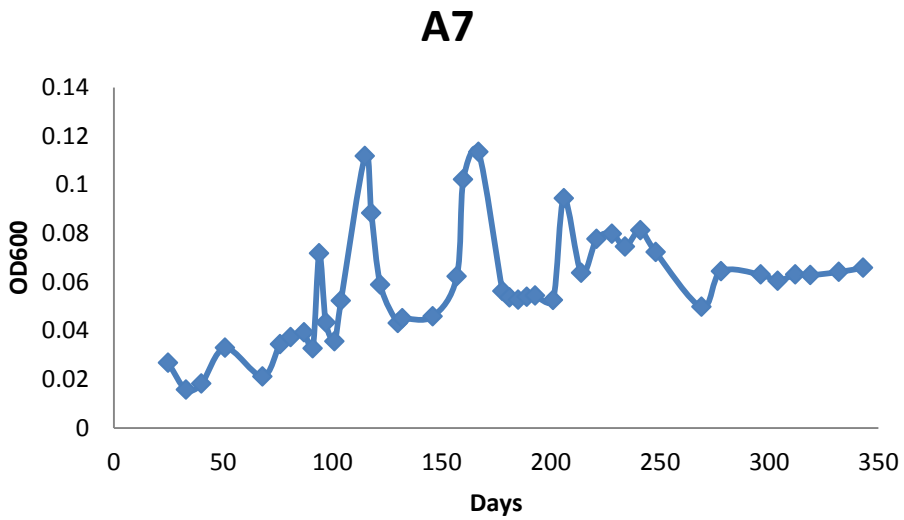

Figure B12. OD_{600} of isolate A7

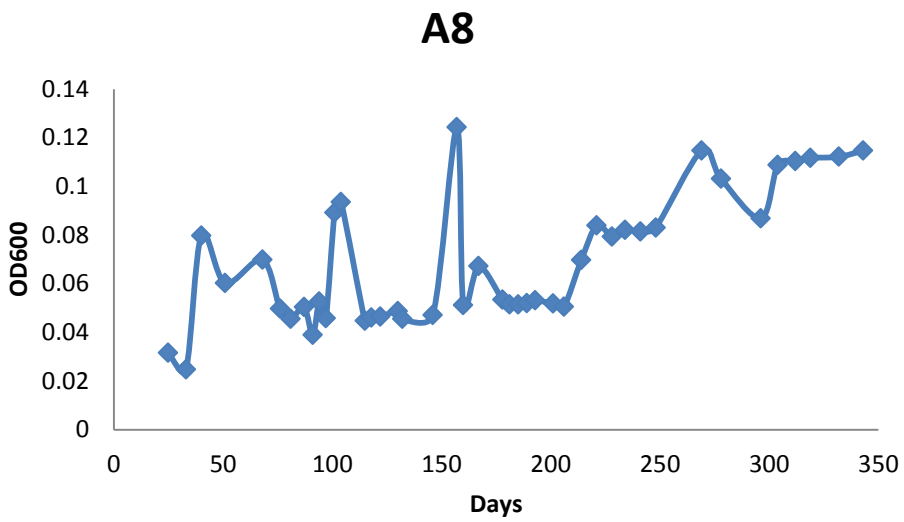

Figure B13. OD_{600} of isolate A8

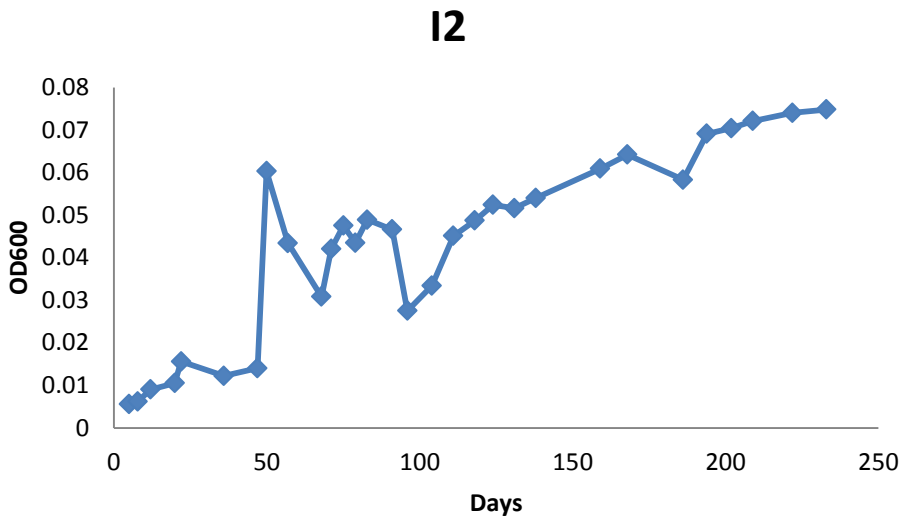

Figure B14. OD_{600} of isolate I2

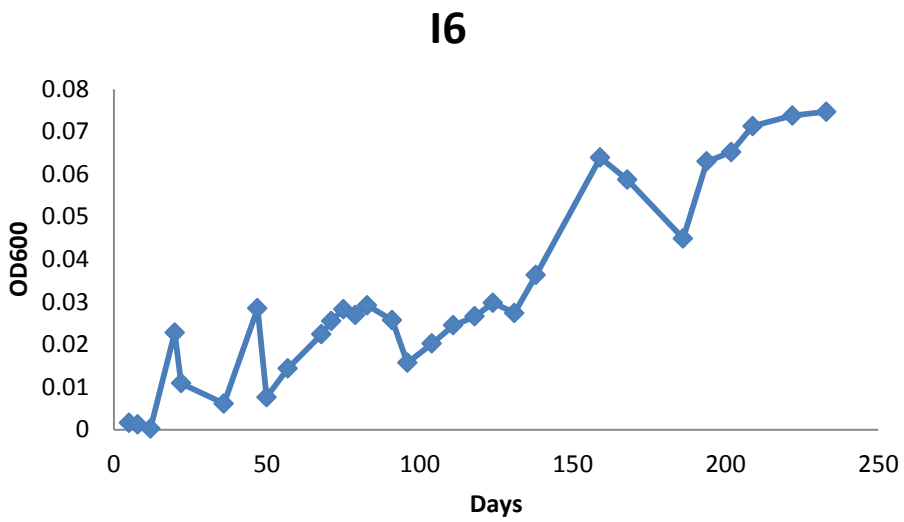

Figure B15. OD_{600} of isolate I6

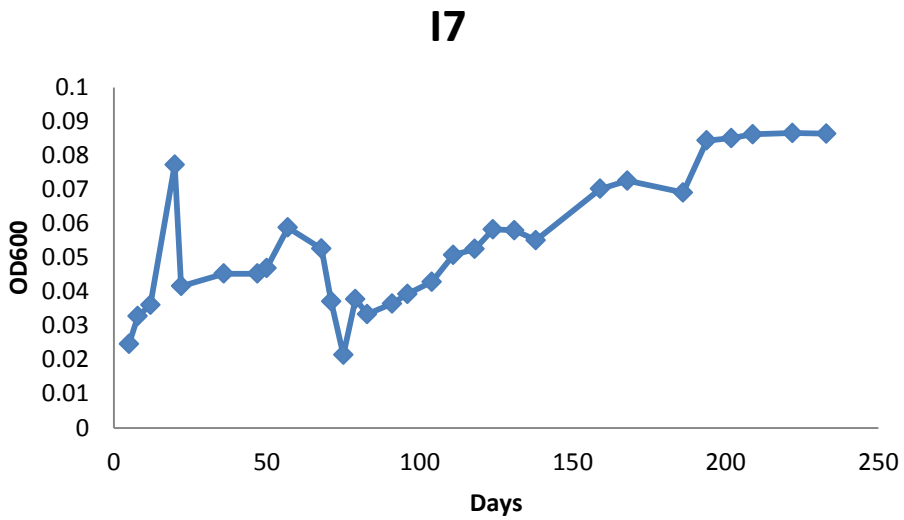

Figure B16. OD_{600} of isolate I7

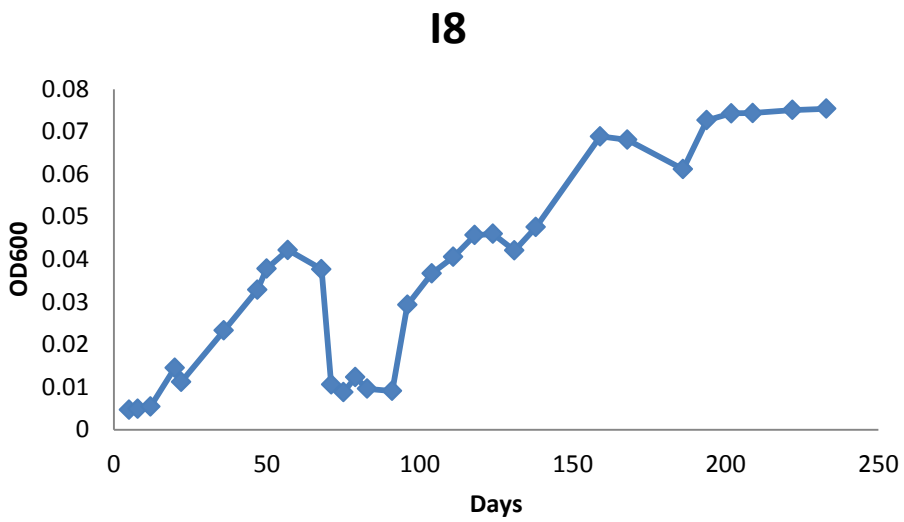

Figure B17. OD_{600} of isolate I8

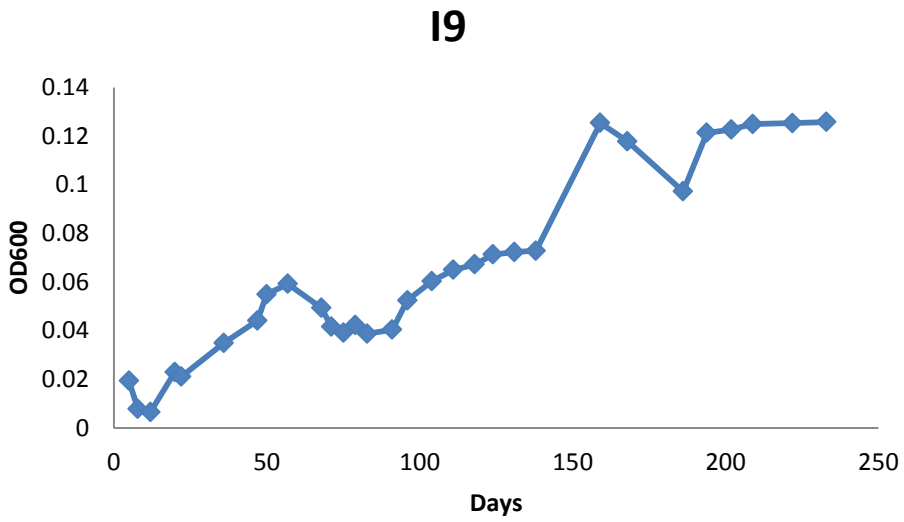

Figure B18. OD_{600} of isolate I9