

Bioaugmentation For Recovery of Anaerobic Digesters Subjected to Organic Overload

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BIOAUGMENTATION FOR RECOVERY OF ANAEROBIC DIGESTERS
SUBJECTED TO ORGANIC OVERLOAD

by

Vaibhav P. Tale

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ABSTRACT

BIOAUGMENTATION FOR RECOVERY OF ANAEROBIC DIGESTERS SUBJECTED TO ORGANIC OVERLOAD

Vaibhav P. Tale

Marquette University, 2010

Anaerobic digester upset due to organic overload is common and methods to reduce recovery time would be beneficial. One potential method is bioaugmentation, the addition of an external culture for performance improvement. Methanogenic community structure differs from digester to digester and there may exist a relation between specific methanogenic activity (SMA) and microbial community composition. The research presented herein tested whether there is a relationship between SMA and community structure. Also, the effectiveness of bioaugmentation was tested by hypothesizing that bioaugmenting with a methanogenic, propionate-degrading culture acclimated to small oxygen doses will help rapid recovery of organically overloaded digesters.

Fourteen different anaerobic cultures were tested for SMA and microbial community using the *mcrA* gene and DGGE to establish a relationship between SMA and community structure. The culture with the highest SMA was enriched by feeding 0.17g propionate/L-day and different oxygen doses. The enrichment cultures were used to bioaugment organically overloaded anaerobic digesters. Microbial communities present in bioaugmented, non-bioaugmented and undisturbed control digesters as well as the bioaugmentation culture were analyzed using 16S rDNA.

A statistically significant relationship between SMA and community structure could not be established, highlighting the difficulty in establishing activity/community structure relationships. However, the results indicated that there was a relation between SMA and methanogenic community compositions studied. Enriching a culture for 25mgO₂/L-day increased its SMA by 29.7%, but higher oxygen doses yielded lower SMA values. Bioaugmentation with this enrichment culture reduced the time required for upset digester effluent to decrease below 1000mgSCOD/L by 114 days (11.4 SRTs) and the time required to reach 25mLCH₄/day by 37 days (3.7 SRTs) respectively. Bioaugmented digesters consistently produced lower effluent SCOD and more methane than non-bioaugmented digesters. Bioaugmentation is a promising approach for speeding up recovery of organically overloaded digesters. Bacterial and archaeal communities of the bioaugmented and undisturbed control digesters had similar phylogenetic tree structures ($p > 0.3$), whereas the tree structures of non-bioaugmented and undisturbed control digesters differed significantly ($p < 0.01$). Bioaugmentation helped restore the microbial communities of overloaded digesters to their original undisturbed state. Higher relative abundance of clones related to *Methanospirillum hungatei* may have caused better performance of bioaugmented digesters.

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Table of Contents

Acknowledgements	i
Table of Contents	ii
List of Tables	v
List of Figures	vi
CHAPTER 1: Community Structure and Specific Methanogenic Activity of Anaerobic Biomass	1
1.1: Introduction	1
1.1.1: Anaerobic Wastewater Treatment and Propionate	1
1.1.2: Methanogens and Methanogenesis	4
1.2: Methods and Materials	6
1.2.1: Sample Collection and Locations	6
1.2.2: SMA Tests of Anaerobic Cultures Against Calcium Propionate	8
1.2.3: SMA Before and After Enrichment	10
1.2.4: <i>mcrA</i> Gene as a Tool For Phylogenetic Analysis	11
1.2.5: Molecular Techniques	13
1.2.5.1: DNA Extraction	13
1.2.5.2: Agarose Gel Electrophoresis	13
1.2.5.3: Polymerase Chain Reaction (PCR)	14
1.2.5.4: Denaturing Gradient Gel Electrophoresis (DGGE)	15
1.2.6 Image Analysis	17
1.2.6.1: Cluster Analysis	17
1.2.6.2: Range Weighted Richness (Rr) and Functional Organization (Fo)	19
1.2.6.3: Regression Analysis	23
1.3: Results and Discussion	24
1.3.1: SMA Results	24
1.3.2: Effect of Initial SMA on SMA After Enrichment	26
1.3.3: <i>mcrA</i> – DGGE Analysis	28
1.3.3.1: Cluster Analysis	31
1.3.3.2: Range Weighted Richness (Rr)	35
1.3.3.3: Functional Organization (Fo)	36
1.3.3.4: Regression Analysis	39
1.4: Conclusions and Recommendations for Future Work	47
1.5: References	50
CHAPTER 2: Bioaugmentation for Recovery of Organically Overloaded Digesters	56
2.1: Anaerobic Wastewater Treatment and Propionate	56
2.1.1: Importance of Hydrogen in Propionate Degradation	56
2.1.2: Propionic Acid Toxicity	56
2.1.3: Bioaugmentation for Enhancing Microbial Community Function	59
2.1.3.1: Removal of Contaminants from Environmental Systems	60
2.1.3.1.1: Chlorinated Volatile Organic Compounds (CVOCs)	60
2.1.3.1.1.2: Benzene, Toluene, Ethylene and Xylene (BTEX)	62
2.1.3.1.1.3: Phenols	63

2.1.3.1.4: Methyl Tertiary-Butyl Ether (MTBE)	64
2.1.3.1.5: Polychlorinated Biphenyls (PCBs)	64
2.1.3.2: Wastewater Treatment	65
2.1.3.3: Bioaugmentation and Nitrification	68
2.1.3.4: Anaerobic Bioaugmentation	71
2.1.3.4.1: Specific Organics Removal	71
2.1.3.4.2: Fats Oil and Grease Removal	75
2.1.3.4.3: Cellulose Degradation	75
2.1.3.4.4: Increased Biogas Production	77
2.1.3.4.5: Odor Reduction	80
2.1.3.4.6: Stressed Reactor Recovery	80
2.1.3.4.7: Anaerobic Augmentation	82
2.1.4: Oxygen and Methanogenic Systems	84
2.2: Methods and Materials	86
2.2.1: Acclimation of Anaerobic Cultures to Propionate and Oxygen	86
2.2.2: Bioaugmentation Experiment	88
2.2.3: Analytical Methods	90
2.2.4: Statistical Analysis	91
2.3: Results and Discussion	92
2.3.1: Effect of Oxygen on Activity of an Enrichment Culture	92
2.3.2: Bioaugmentation Experiments	93
2.3.2.1: Effluent SCOD	93
2.3.2.2: Effluent VFA Concentrations	101
2.3.2.3: Methane Production	105
2.3.2.4: Glass Floor Effluent SCOD Concentrations	114
2.4: Conclusions	116
2.5: References	118
CHAPTER 3: Microbial Community Analysis	124
3.1: Introduction	124
3.1.1: Anaerobic Digesters Community Analysis	125
3.1.2: Microbial Survey of Anaerobic Digesters	128
3.1.3: Microbial Communities in the Granules of UASB Digesters	131
3.1.4: Microbial Communities During Start-up of Anaerobic Digesters	133
3.1.5: Microbial Communities for Substrate Degradation/Conversion	134
3.1.5.1: Fatty Acids	135
3.1.5.2: Volatile Organic Sulfur Compounds (VOSCs)	137
3.1.6: Methanogenic Population Shift Due to Change in Operating Conditions	138
3.2: Methods and Materials	141
3.2.1: Molecular Techniques for Community Analysis	142
3.2.1.1: DNA Extraction	142
3.2.1.2: Agarose Gel Electrophoresis	143
3.2.1.3: Polymerase Chain Reaction (PCR)	143
3.2.1.4: Cloning	144
3.2.1.5: PCR Purification	145
3.2.1.6: DNA Sequencing	146

3.2.1.7: Analysis of Sequences	146
3.3: Results and Discussion	152
3.3.1: Archaea	152
3.3.1.1: Archaeal Diversity, Richness and Functional Organization	152
3.3.1.2: Archaeal Phylogenetics	159
3.3.1.2.1: Physiological Characters of the Orders of Methanogens Found	164
3.3.1.2.2: Composition of Archaeal Clone Libraries	165
3.3.1.2.3: Comparison of Archaeal Phylogenetic Trees	169
3.3.2: Bacteria	174
3.3.2.1: Bacterial Diversity, Richness and Functional Organization	174
3.3.2.2: Bacterial Phylogenetics	178
3.3.2.2.1: Physiological Characters of the Phyla Found	184
3.3.2.2.2: Composition of Bacterial Clone Libraries	189
3.3.2.2.3: Comparison of Bacterial Phylogenetic Trees	193
3.4: Conclusions	197
3.5: References	202
APPENDIX A : Additional Data	210
APPENDIX B : Signature Page	216

List of Tables

Table 1.1: Conversion of Propionate to Methane	2
Table 1.2: Substrates Converted to Methane by Methanogenic <i>Archaea</i>	5
Table 1.3: Anaerobic Biomass Samples Collected for Testing SMA Against Propionate	7
Table 1.4: Basal Media Constituents	10
Table 1.5: Primers Used for <i>mcrA</i> Gene Amplification	12
Table 1.6: Clusters of Biomass Samples Based on SMA Values	26
Table 1.7: Biomass Samples Selected for Enrichment	27
Table 1.8: Range Weighted Richness (Rr) of the Biomass Samples	35
Table 2.1: Overview of Bioaugmentation Literature	67
Table 2.2: Overview of Bioaugmentation Experiments Using <i>Pseudomonas</i> Strains	68
Table 2.3: Enrichment Cultures	88
Table 2.4: Bioaugmentation Digesters	90
Table 2.5: Probabilities Associated with Student's t Statistic for Effluent VFAs	105
Table 3.1: Bacteria Found in Anaerobic Digesters Degrading Long-Chain Fatty Acids	136
Table 3.2: Number of Good Archaeal Sequences Obtained	153
Table 3.3: S_{CHAO1} , S_{ACE} and Good's C Estimates for Archaeal Clone Libraries	156
Table 3.4: Shannon-Weaver and Simpson's Indices for Archaeal Clone Libraries	157
Table 3.5: Pair-Wise Comparison of Archaeal Tree Structures	170
Table 3.6: Number of Good Bacterial Sequences Obtained	174
Table 3.7: S_{CHAO1} , S_{ACE} and Good's C Estimates for Bacterial Clone Libraries	176
Table 3.8: Shannon-Weaver and Simpson's Indices for Bacterial Clone Libraries	176
Table 3.9: Pair-Wise Comparison of Bacterial Tree Structures	194
Table A.1: Parameters Used for Bands Detection	211
Table A.2: The Probability Associated With the Student's t Test For Pair-Wise Comparisons of SMA Means	212
Table A.3: Interpretation of Results of the Student's t Test	213
Table A.4: Rank Correlation Coefficient Between Initial and Enriched SMA	214

List of Figures

Figure 1.1: Effect of Organic Overload on Effluent Composition of a CSTR Reactor	4
Figure 1.2: Thermocycler Programs Used	
a) First Amplification for <i>mcrA</i> Gene	15
b) Nested Amplification of <i>mcrA</i> Gene With GC-Clamp	15
Figure 1.3: Possible Cases of P-L Curves	21
Figure 1.4: Results of SMA Testing	24
Figure 1.5: Effect of Initial SMA on SMA After Enrichment	28
Figure 1.6: DGGE Gel Image	
a) Denaturant Concentration Along the Length	30
b) Different Types of Bands Present in the Banding Patterns	30
Figure 1.7: Cluster Analysis of the Biomass Samples Based on DGGE Band Densitometric Data	32
Figure 1.8: Principle Component Analysis Results	34
Figure 1.9: Correlation Between R_r and SMA	36
Figure 1.10: Pareto-Lorenz Curves for the Anaerobic Biomass Samples	37
Figure 1.11: Correlation Between F_o and SMA	39
Figure 1.12: First-Order Degradation Kinetics for Hypothetical Microbial Phylotype 1 and 2	40
Figure 1.13: Plot of Measured SMA Values Versus SMA Values Estimated From DGGE Banding Pattern Data Using Multiple Linear Regression	43
Figure 1.14: Predicted and Estimated SMA Values For the Training and Testing Data	44
Figure 2.1: Effect of Oxygen on SMA Against Propionate After Enrichment	93
Figure 2.2: Effluent SCOD of Digesters Bioaugmented and Augmented With C1a, C1b and C1c	96
Figure 2.3: Effluent SCOD of Digesters Bioaugmented and Augmented With C2a, C2b and C2c	96
Figure 2.4: Effluent SCOD of Digesters Bioaugmented and Augmented With C3	97
Figure 2.5: Effluent SCOD of Digesters Bioaugmented and Augmented With C4	97
Figure 2.6: Time Required by Bioaugmented and Non-Bioaugmented Digesters to attain 1000mg/L Effluent SCOD Concentration Following the Organic Overload	98
Figure 2.7: Effect of Oxygen Acclimation of Bioaugmentation Culture on Percent SCOD Decrease of Bioaugmented Digesters	101
Figure 2.8: Effluent Acetic Acid Concentration of UCD, TD-0% and CD-0%	102
Figure 2.9: Effluent Propionic Acid Concentration of UCD, TD-0% and CD-0%	102
Figure 2.10: Effluent Butyric Acid Concentration of UCD, TD-0% and CD-0%	103
Figure 2.11: Effluent Iso-Valeric Acid Concentration of UCD, TD-0% and CD-0%	103
Figure 2.12: Daily Methane Production from Digesters Bioaugmented and Augmented With C1a, C1b and C1c	106
Figure 2.13: Daily Methane Production from Digesters Bioaugmented and Augmented With C2a, C2b and C2c	107
Figure 2.14: Daily Methane Production from Digesters Bioaugmented and Augmented With C3	108
Figure 2.15: Daily Methane Production from Digesters Bioaugmented and Augmented With C4	109

Figure 2.16: Time Required by Bioaugmented and Non-Bioaugmented Digesters to attain 25 mLCH ₄ /day Following the Organic Overload	111
Figure 2.17: Effect of Oxygen Acclimation of Bioaugmentation Cultures on Percent Methane Increase from Bioaugmented Digesters	113
Figure 2.18: Effluent SCOD Concentration of TD-90% and CD-90%	116
Figure 3.1: Thermocycler Programs for PCR Amplification	
a) Bacterial 16S rDNA	144
b) Archaeal 16S rDNA	144
Figure 3.2: Thermocycler Program for PCR Amplification for Puc Primers	145
Figure 3.3: Rarefaction Curves for the Archaeal Clone Libraries	154
Figure 3.4: Pareto-Lonerz Evenness Curves of Archaeal Clone Libraries	158
Figure 3.5: Phylogenetic Analysis of Culture UCD-1 Archaeal Clone Library	160
Figure 3.6: Phylogenetic Analysis of Culture TD-10%-1 Archaeal Clone Library	161
Figure 3.7: Phylogenetic Analysis of Culture CD-10%-1 Archaeal Clone Library	162
Figure 3.8: Phylogenetic Analysis of Culture C2a Archaeal Clone Library	163
Figure 3.9: Archaeal Diversity of Cultures UCD-1, TD-10%-1, CD-10%-1 and C2a	168
Figure 3.10: Graph of First Two Principle Components for Archaeal Communities	
a) PCoA Performed on Tree Lineages Using UniFrac	173
b) PCA Performed on Distribution of Clones in the Clone Libraries Using MATLAB(v.7.6(R2008a)).	173
Figure 3.11: Rarefaction Curves for the Bacterial Clone Libraries	175
Figure 3.12: Pareto-Lorenz Evenness Curves of Bacterial Clone Libraries	177
Figure 3.13: Phylogenetic Analysis of Culture UCD-1 Bacterial Clone Library	179
Figure 3.14: Phylogenetic Analysis of Culture TD-10%-1 Bacterial Clone Library	180
Figure 3.15: Phylogenetic Analysis of Culture CD-10%-1 Bacterial Clone Library	181
Figure 3.16-A: Phylogenetic Analysis of Culture C2a Bacterial Clone Library	182
Figure 3.16-B: Phylogenetic Analysis of Culture C2a Bacterial Clone Library	183
Figure 3.16-C: Phylogenetic Analysis of Culture C2a Bacterial Clone Library	184
Figure 3.17: Bacterial Diversity of Cultures UCD-1, TD-10%-1, CD-10%-1 and C2a	192
Figure 3.18: Graph of First Two Principle Components for Bacterial Communities	
(a) PCoA Performed on Tree Lineages Using UniFrac (Lozupone et. al, 2006)	195
(b) PCA Performed on Distribution of Clones In The Clone Libraries Using MATLAB(v.7.6(R2008a)).	195

CHAPTER 1: METHANOGENIC COMMUNITY STRUCTURE AND SPECIFIC METHANOGENIC ACTIVITY OF ANAEROBIC BIOMASS

1.1 Introduction

1.1.1 Anaerobic Wastewater Treatment and Propionate

Anaerobic biological treatment converts chemical oxygen demand (COD) exerted by organic substances into methane gas as the means of COD removal from wastewater, sludge or other organic feedstocks. Essentially there are four steps involved in anaerobic digestion of a complex substrate. In the first step, complex organic matter is hydrolyzed to simple organics. In the second step, the simple organics are further broken down into volatile fatty acids such as propionate, by a group of organisms called acidogens. Then the volatile fatty acids are converted to acetate and hydrogen gas by the acetogens. The fourth and final step involves the conversion of acetate as well as the hydrogen and carbon dioxide to methane by a microbial group called the methanogens. During the degradation process, many odd-numbered carbon chains pass through propionate. Efficient flow of electrons through this metabolic intermediate requires efficient methanogenic metabolism of hydrogen and acetate and efficient metabolism of propionate (Speece, 2008).

Gracia et al. (2006) applied anaerobic digestion model -1 for evaluating the COD flow for anaerobic digestion of a simulated organic compound exerting 100 g COD. Out of the 100 g total COD, 20 g COD was exerted by carbohydrates, 20 g COD was exerted by proteins and 30 g COD was exerted by lipids. The remaining 30 g COD was considered to be exerted by non-biodegradable material present in the substrate. Out of

70 g of biodegradable COD, 6.14 g COD (8.7%) passed through propionate. This may seem to be relatively small, but this mass of propionate or propionic acid can lower pH, causing process upset or failure.

Under standard conditions, metabolism of propionate to acetate and hydrogen gas is energetically unfavorable as demonstrated by the first reaction given in Table 1.1, but conversion of acetate to methane and carbon dioxide and conversion of hydrogen and carbon dioxide to methane drives the overall reaction in the forward direction (see the last reaction Table 1.1). Degradation of propionate is theoretically favorable only when the hydrogen concentration in an anaerobic digester lies between 10^{-4} to 10^{-6} atm (McCarty and Smith, 1986). If the dissolved hydrogen gas concentration goes above this range, then propionate degradation becomes energetically unfavorable and stops. This may result in build-up of propionic acid and other reduced compounds such as *n*-propanol and four-through seven-carbon *n*-carboxylic acids in the digester, such as butyric acid (McCarty and Smith, 1986). This acid build-up can cause the pH to decrease which inhibits or stops methane production.

Table 1.1: Conversion of Propionate to Methane (McCarty and Smith, 1986).

	$\Delta G^{0'}$ kJ/mole propionate
$\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} = \text{CH}_3\text{COO}^- + 3\text{H}_2 + \text{CO}_2$	+71.67
$3\text{H}_2 + (3/4) \text{CO}_2 = (3/4) \text{CH}_4 + (3/2) \text{H}_2\text{O}$	-98.06
$\text{CH}_3\text{COO}^- + \text{H}^+ = \text{CH}_4 + \text{CO}_2$	-35.83
$\text{CH}_3\text{CH}_2\text{COO}^- + \text{H}^+ + (1/2) \text{H}_2\text{O} = (7/4) \text{CH}_4 + (5/4) \text{CO}_2$	-62.22

Propionate accumulation has typically been observed as an indicator of anaerobic digester process imbalance. Therefore, propionate-utilizing microbial consortia play an important role when anaerobic digesters are subjected to organic overload (Smith and McCarty, 1990).

Smith and McCarty (1990) studied the effect of substrate overloading on a continuous-stirred tank reactor (CSTR) operated at a 9.1-day solids retention time (SRT) and a 5-day hydraulic retention time (HRT). The reactor was fed daily with 0.1M ethanol and 0.1M propionate until a quasi steady state was achieved in terms of effluent ethanol, propanol, acetate and propionate concentrations. After the steady state, the reactor was shock overloaded with 0.3M ethanol while the same daily dose of 0.1M propionate was also fed. Figure 1.1 shows the effluent characteristics. Although the reactor was shock overloaded with ethanol, the effluent propionate concentration remained elevated even after 18 days (3.6 HRTs), whereas the ethanol concentration decreased to its normal low value after 4 days. This experiment affirmed that propionate concentrations can remain chronically elevated for a significant time after a process upset.

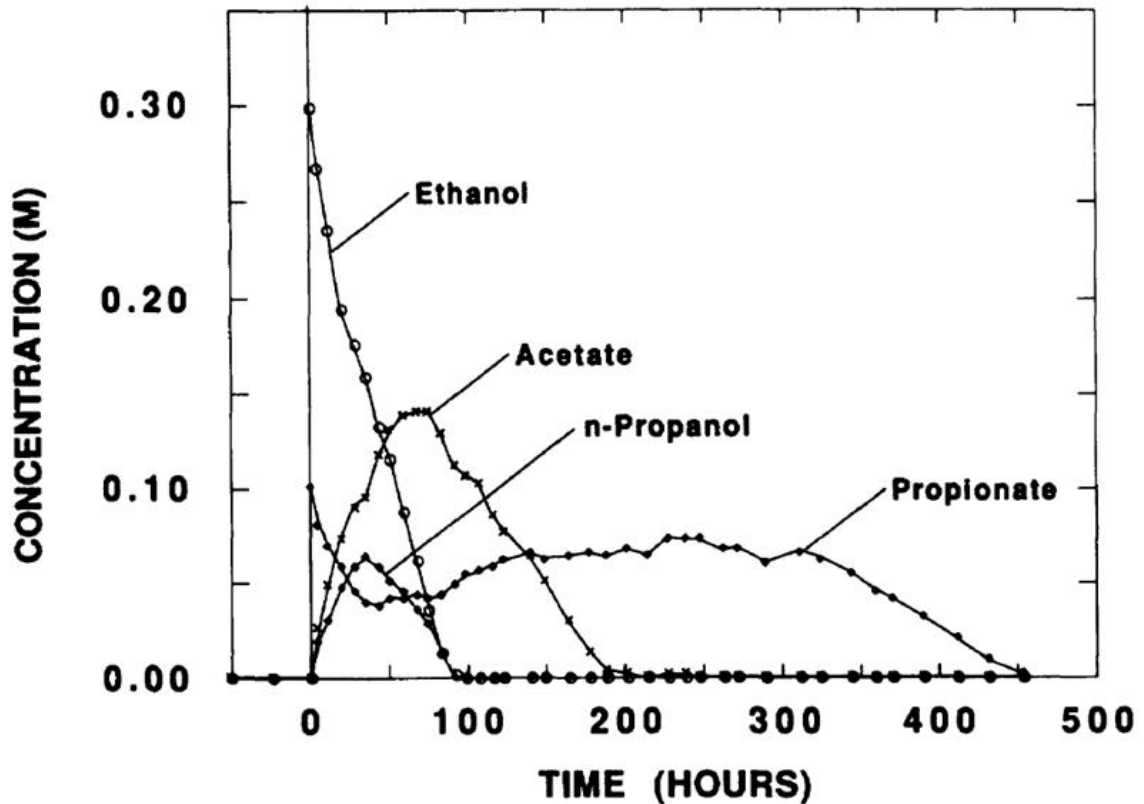


Figure 1.1: Effect of Organic Overload on Effluent Composition of A CSTR Reactor. Adopted from Smith and McCarty (1996)

1.1.2 Methanogens and Methanogenesis

Methanogens have been isolated from virtually every habitat where anaerobic biodegradation occurs (Jones et al., 1987; Stams, 1994; Zheng and Raskin, 2000; Madigan and Martinko, 2006). Methanogens are among the last links in the anaerobic biodegradation process and convert end products of previous degradation steps to methane and carbon dioxide. They also provide a hydrogen sink for degradation of organic acids like propionic and butyric acids which are otherwise difficult to degrade. Previous studies have revealed that methanogens are often the most important fraction of

biomass in anaerobic digesters (Zheng and Raskin, 2000; Diaz et al., 2006; Keyser et al., 2006; Hulshoff Pol et al., 2004; Zheng and Raskin, 2000; Hobson and Wheatley, 1993; Griffin et al., 1998; Chen et al., 2005). Various methanogens are known to consume one or more of the eleven different substrates given in Table 1.2 and convert them to methane (Madigan and Martinko, 2006). This process involves unique methanogenic coenzymes and the process is called methanogenesis (Woese and Fox, 1977; White, 2000).

Table 1.2: Substrates Converted to Methane by Methanogenic *Archaea* (Madigan and Martinko, 2006)

Carbon dioxide, CO_2 (with electrons derived from H_2 , certain alcohols or pyruvates)

Formate, HCOO^-

Carbon monoxide, CO

Methanol, CH_3OH

Methylamine, CH_3NH_3^+

Dimethylamine, $(\text{CH}_3)_2\text{NH}_2^+$

Trimethylamine, $(\text{CH}_3)_3\text{NH}^+$

Methylmercaptan, CH_3SH

Dimethylsulfide, $(\text{CH}_3)_2\text{SH}$

Acetate, CH_3COO^-

Pyruvate, $\text{CH}_3\text{COCOO}^-$

Anaerobic biomass samples contain a microbial community with syntrophic relationships. Closely co-existing microbial groups utilize metabolic products of other groups, finally leading to the production of methane. Characteristics of the microbial communities present may significantly differ from one anaerobic culture to another (Leclerc et al., 2004) and hence may affect the overall digester function.

Since hydrogenotrophic methanogens are responsible for the conversion of hydrogen and carbon dioxide to methane, it was hypothesized that the methanogenic community structure of an anaerobic culture may significantly influence the overall propionate degradation function of the community. There may exist a relationship between the propionate degradation rate and the methanogenic community structure of an anaerobic biomass sample. To test the hypothesis, a study was undertaken with the aim of establishing a relationship between specific methanogenic activity (SMA) value against propionic acid and methanogenic community organization.

1.2 Methods and Materials

1.2.1 Sample Collection and Locations

Anaerobic biomass samples were collected from wastewater treatment plants serving different industries and municipalities. All the biomass samples were maintained at 4 °C following their collection until SMA tests were conducted. Table 1.3 summarizes the anaerobic biomass samples collected.

Table 1.3: Anaerobic Biomass Samples Collected for Testing SMA Against Propionate

Biomass Sample Source	Naming Used	Type of Reactor
City Brewery Wastewater Treatment Plant	Brewery WWTP-1	UASB*
New Belgium Brewery Wastewater Treatment Plant	Brewery WWTP-2	Anaerobic plug flow
Anheuser Busch Brewery Wastewater Treatment Plant	Brewery WWTP-3	UASB
City of Des Moines Municipal Wastewater Treatment Plant	Municipal WWTP-1	CSTR**
Ocean County, New Jersey Municipal Wastewater Treatment Plant	Municipal WWTP-2	CSTR
City of Akron Municipal Wastewater Treatment Plant	Municipal WWTP-3	High solids plug flow digester
Philadelphia Water Department Municipal Wastewater Treatment Plant	Municipal WWTP-4	CSTR
Kerry Ingredients Wastewater Treatment Plant	Food Industry WWTP-1	UASB
Axiom Foods Wastewater Treatment Plant	Food Industry WWTP-2	CSTR
Smuckers Wastewater Treatment Plant	Food Industry WWTP-3	UASB
Hilmar Cheese Wastewater Treatment Plant	Food Industry WWTP-4	EGSB***
Wis-Pak Wastewater Treatment Plant	Soft Drink Bottling Industry WWTP	UASB
American Crystal Sugar Wastewater Treatment Plant	Sugar Industry WWTP	Anaerobic contact process
F & A Dairy Products Wastewater Treatment Plant	Dairy Industry WWTP	CSTR

*UASB – Upflow anaerobic sludge blanket, **CSTR- Completely stirred tank reactor, ***EGSB- Expanded granular sludge blanket

1.2.2 SMA Tests of Anaerobic Cultures Against Calcium Propionate

Maximum propionate utilization rates can be determined by conducting specific methanogenic activity (SMA) testing (Sorensen and Ahring, 1993; Speece, 2008; Zitomer et al., 2008). SMA of the biomass samples was analyzed using propionate as the substrate by a standard protocol (Owen et al., 1979).

The assays were conducted in 160-mL glass serum bottles. All test and control assays were run in triplicate. All the test and control assays were supplied with 25mL of diluted biomass samples having less than 2g volatile suspended solids (VSS)/L. Basal media given in Table 1.4 was used for diluting biomass samples. All test assays received 3 g/L of propionate in the form of calcium propionate, whereas control assays contained no substrate. This substrate concentration is typically employed for propionate utilization testing (Speece, 2008; Zitomer et al., 2008), is not toxic to anaerobic biomass and is significantly higher than the Monod half-saturation constant values. Therefore, the systems were not substrate limited during the initial testing period. Biogas generated by control assays accounted for endogenous methane production during the testing period. Headspace for all test and control assays was flushed by a nitrogen and carbon dioxide gas mixture (7:3 volumetric proportion, respectively) to ensure anaerobic conditions. Further, all assays were incubated at 35 ± 2 °C and were continuously shaken at 150 rpm using a gyratory shaker-incubator. Gas production from each assay was monitored every 12 hours using a syringe displacement method over a period of 30 days. Graphs of cumulative gas production versus time were plotted. At the end of the testing period, methane content of the biogas was measured by gas chromatography (Series 600,

GLOW-MAC Instrument Co., Bethlehem, MA) using a thermal conductivity detector (TCD) and a CTR I column (Agilent Associates, Inc., Deerfield, IL). Helium was used as the carrier gas at a flow of 30 ± 2 mL/min with the temperature of the injector and detector set at 120° C and the temperature of the oven set at 38° C. VSS concentration of the diluted biomass was measured before and after the test using standard methods (APHA, 1998) and the average of initial and final VSS concentration was used for calculating SMA. Cumulative methane production was calculated by subtracting the average methane produced by the control assays. A portion of the resulting curve having the steepest slope was used to calculate SMA using linear regression. The activity of each test assay was expressed as mL CH_4 /hr-gVSS. Finally the average and standard deviation of the SMA values were calculated. Statistical analysis of the SMA values was done by pair-wise comparison of the SMA using Student's t statistic for unequal population variances. A probability threshold of 0.05 (95% confidence interval) was used as a criterion for clustering samples based on similarity in their SMA values. The DOTUR program (v. 1.53), was used for assigning samples to different clusters by the farthest neighbor algorithm (Schloss et al., 2005).

Table 1.4: Basal Media Constituents

	Concentration (mg/L)
NH ₄ Cl	400
MgSO ₄	250
KCl	400
CaCl ₂	120
(NH ₄) ₂ HPO ₄	80
FeCl ₃ .6H ₂ O	55
CoCl ₂ .6H ₂ O	10
KI	10
Metals*	0.5
Alkalinity	5000
Resazurin	1

*Metals include MnCl₂.4H₂O, NH₄VO₃, CuCl₂.2H₂O, Zn(C₂H₃O₂)₂.2H₂O, AlCl₃.6H₂O, NaMoO₄.2H₂O, H₃BO₃, NiCl₂.6H₂O, NaWO₄.2H₂O and Na₂SeO₃ added together to make a 0.5mg/L of each metal in solution.

1.2.3 SMA Before and After Enrichment

The effect of the initial (before enrichment) SMA value of an anaerobic culture on the SMA value after enrichment was evaluated for selected samples.

Three cultures with the highest SMA and three cultures with the lowest SMA were selected for enrichment against calcium propionate. When selecting the cultures for enrichment, cultures having no initial SMA against propionic acid were excluded. The selected cultures were enriched in 750-mL serum bottle digesters operated in CSTR mode. The serum bottle digesters were inoculated with 150 mL of the selected culture.

Headspace of all the digesters was sparged with a nitrogen-carbon dioxide gas mixture (mixed in 7:3 ratio v/v) to establish anaerobic conditions. The enrichment digesters were shaken continuously at 150 rpm and maintained at 35 ± 2 °C. All the enrichment digesters were fed 0.17g propionate/L-day (0.25 gCOD/L-day) with basal medium (see Table 1.4) while maintaining a 15-day SRT. After 580 days (38.6 SRTs), the SMA of all the enrichment cultures against propionate was measured by following the procedure described in Section 1.2.1.

The cultures were ranked and listed according to their initial SMA values. Also a second list was prepared which contained cultures ranked according to their enriched SMA values. Spearman's rank correlation coefficient was used to determine if the initial SMA values before enrichment had a significant effect on the SMA values after enrichment based on the ranks of the cultures in the lists.

1.2.4 *mcrA* Gene as a Tool For Phylogenetic Analysis

Methyl coenzyme-M reductase (MCR) is the terminal enzyme complex in the biological methane generation pathway and catalyzes the reduction of the methyl group bound to coenzyme-M, with the concomitant release of methane (Woese and Fox, 1977). This enzyme complex is thought to be unique to and ubiquitous in methanogens (Thauer, 1998), making it a suitable tool for the detection of methanogens. The MCR operon exists in two forms, MCRI and MCRII. The MCRI form is thought to be present in all methanogens, while the MCRII form has been found to be present in the members of the orders *Methanobacteriales* and *Methanococcales*. Researchers have selected one peptide of the MCRI complex, encoded by the *mcrA* gene, as a suitable candidate for the

development of PCR-based detection of methanogens. Suitability of this marker for analysis of the order *Methanosarcinales* has also been demonstrated (Springer et al., 1996). Further studies have highlighted the use of the *mcrA* gene as a target for the detection of methanogens in a wide range of environments including rice paddies (Lueders et al., 2001), peat bogs (Hales et al., 1996; Lloyd et al., 1998; Nercessian et al., 1999; Juottonen et al., 2006), termite gut (Ohkuma et al., 1995), anaerobic digesters (Rastogi et al., 2008), polluted water (Ufnar et al., 2007), hypereutrophic lakes (Earl et al., 2003), hydrothermal sediments (Dhillon et al., 2005), subsurfaces of tidal flats (Wilms et al., 2007) and marine environments (Bidle et al., 1999). Table 1.5 shows the primers developed by Luton et al. (2002) targeting the *mcrA* gene that leads to a 470-base-pair-long amplified DNA product. These primers are commonly used by researchers (Wilms et al., 2007; Juottonen et al., 2006; Ufnar et al., 2007; Rastogi et al., 2008).

Table 1.5: Primers Used for *mcrA* Gene Amplification

Primers	Sequence
mcrA1f	5'- GGTGGTGTMGGA TTCACACARTAYGCWACAGC -3'
GCmcrA1f	5'- *GC-clamp-GGTGGTGTMGGA TTCACACARTAYGCWACAGC -3'
mcrA500r	5' – TTCATTGCRTAGTTWGGRTAGTT – 3'
* GC-clamp = 5' – CGCCCGCCGCGCCCCGCGCCCGTGCCGCCGCCCCCGCCCG – 3'	

1.2.5 Molecular Techniques

1.2.5.1 DNA Extraction

The DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions modified by the inclusion of the alternative lysis method. This alternative lysis method states that "if the cells are difficult to lyse, a 10 min incubation at 70 °C after addition of solution C1 can be performed." (PowerSoil Protocol, 2007). This method replaces the horizontal vortexing of the PowerBead Tubes and is meant to reduce DNA shearing. The presence of extracted DNA was confirmed using agarose gel electrophoresis.

1.2.5.2 Agarose Gel Electrophoresis

A 1% agarose gel (w/v) was prepared by mixing agarose with tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer (Sambrook and Russell, 2001). The resulting mixture was heated in a microwave until all the solid agarose was melted and dissolved in the TAE buffer. Afterwards, 0.8mL/L ethidium bromide was added to the gel mixture for staining nucleotides. A mixture of 2 uL 6X blue-orange loading dye and a 5-uL DNA sample were placed into the wells (Hartwell et al., 2004). A $\lambda\phi$ ladder with 40 ng/ μ L Lambda (λ) DNA, HindIII cut and 30 ng/ μ L phi X 174 (ϕ) DNA, HaeIII cut was used for comparison. A 100 volt of current was passed across the gel which caused migration of the DNA. The DNA bands were illuminated and photographed under ultraviolet light using GDS-8000 Bioimaging System (UVP Inc. Upland, CA).

1.2.5.3 Polymerase Chain Reaction (PCR)

PCR was performed on DNA samples using EconoTaq® PLUS 2X Master Mix, which includes the Taq polymerase (Lucigen Corporation, Middleton, WI). Forward and reverse primers were added to the PCR tube with nuclease-free H₂O to make a 100- μ L reaction volume. The extracted DNA was first amplified for *mcrA1f* and *mcrA500r* primers and then the amplified product was re-amplified for *GCmcrA1f* and *mcrA500r* primers which yielded a gene product that was approximately 500 nucleotides long (Luton et al., 2002). The primers used for nested PCR amplification of the *mcrA* gene are given in Table 1.5. The PCR was carried out using a thermo-cycler (Biometra T-Personal). Figure 1.2 shows the thermocycler programs used for the nested PCR amplification.

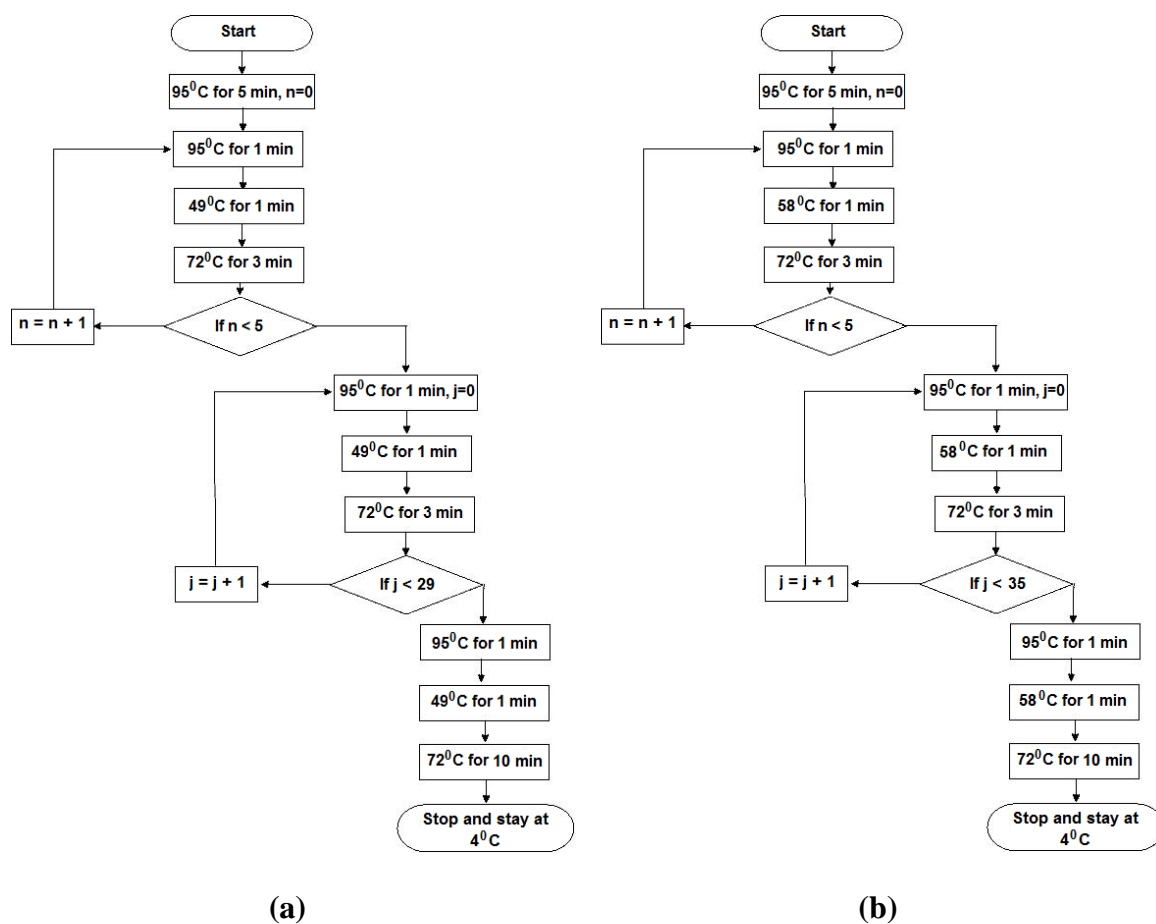


Figure 1.2: Thermocycler Programs Used for (a) First Amplification for *mcrA* Gene (b) Nested Amplification of *mcrA* Gene With GC-Clamp

1.2.5.4 Denaturing Gradient Gel Electrophoresis (DGGE)

DNA is a two stranded molecule made of four types of nucleotide bases i.e. adenine, cytosine, guanine and thymine. A series of these bases form a deoxyribose sugar backbone of each DNA strand and both the strands of the molecule are held together with hydrogen bonds shared by the nucleotides present on opposite strands. Adenine and thymine share two hydrogen bonds, whereas guanine and cytosine share three hydrogen

bonds, so separation of guanine and cytosine takes more energy or denaturant concentration than separation of adenine and thymine (Muyzer et al., 1998).

Genetic fingerprinting techniques like denaturing gradient gel electrophoresis (DGGE) can provide a community diversity profile on the basis of physical separation of unique nucleic acid sequences (Muyzer et al., 1999). The DGGE technique is based on decreased electrophoretic mobility of partially melted, double-stranded DNA molecules in polyacrylamide gels containing a linear concentration gradient of DNA denaturants (a mixture of urea and formamide). Molecules with different nucleotide compositions have different melting behavior, and, therefore, may stop migrating at different positions in the gel (Muyzer et al., 1998). The DGGE technique has been extensively used in the field of microbial ecology to compare microbial communities and use of DGGE with *mcrA* as a target gene have been reported (Wilms et al., 2007; Galand et al., 2002).

Urea and formamide were used as denaturing reagents in acrylamide gels. Gels with a linear gradient of 40% at the top of the gel to 70% denaturant concentration at the bottom of the gel (expressed as v/v of the total gel volume) were used for electrophoresis. The highest and the lowest concentration of the denaturant was 75 mm apart. A BioRad Universal DCode Mutation Detection System device was used to run the DGGE gels. DGGE was performed on 1 mm thick 8 % polyacrylamide gel prepared as per the manufacturer's protocol. Forty μ L of the amplified DNA product (equivalent to approximately 75 ng of DNA) was added to each lane of the polyacrylamide gel with 2X loading dye. An electric current of 100 V was run across the gel for 12 hours. A 1 % solution of SYBR[®] Green (Invitrogen, CA USA) dye was used for gel staining purpose.

The gel was immersed in the staining solution and rotated on a gyratory shaker table at 1 rpm for 30 min before observing it under ultra violet light using GDS-8000 Bioimaging System (UVP Inc. Upland, CA).

1.2.6 Image Analysis

The obtained DGGE gel was visualized under ultraviolet light and its image was taken using a digital camera. The Lab Works software (v. 4.6.00.0) was used for digitally analyzing the number of bands present in each lane and their respective intensities in terms of optical density (OD). Parameters used for bands detection are presented in Appendix A, Table A.1. For the purpose of comparing densitometric data from two gel images, a common DNA sample extracted from a non-fat-dry-milk-fed, lab-scale anaerobic reactor and amplified for primers given in Table 1.5 was used as a marker (ladder). The marker was run in the first lane on every gel (Boon et al., 2002). The ratio between the densitometric data from the marker lanes of the first and the second gel images was used to normalize the densitometric data from all the other lanes of the second DGGE gel image.

1.2.6.1 Cluster Analysis

Dice and Jaccard coefficients as well as Pearson's correlation coefficients have been used in the past to calculate similarity coefficients between banding patterns obtained from DGGE gels to make dendrograms showing differences between different banding patterns (Jackson et al., 1989; Zhang and Fang, 2000; Boon et al., 2002; Kosman and Leonard, 2005; Griffiths et al., 2000). Among all the similarity coefficients,

Pearson's correlation coefficient seems to be the best suited for the application because, if used for the overall digitized lane data, then it takes into account the brightness of an individual band along with the presence or absence of the band, whereas other coefficients do not consider band intensities but only presence-absence of a band.

Pearson's correlation coefficients were calculated for each pair of densitometric data. The obtained values represented the similarities between the banding patterns of each lane. For calculating dissimilarities, one minus Pearson's correlation coefficient was calculated and the values were used to make a distance matrix. The obtained distance matrix was used to make a dendrogram using the Phylogeny Inference Package (PHYLIP, v 3.68) and the unweighted pair group method with arithmetic mean (UPGMA) algorithm was used for clustering. Further, all the biomass samples were listed in ascending order of their pair-wise distances from the sample having the highest SMA (i.e. Brewery-1) and the obtained list of samples was ranked from 1 to 14. Another list of the biomass samples was prepared by arranging them in descending order of their SMA values and the list was ranked from 1 to 14. If there was a strong correlation between the SMA data and the densitometric data, then a strong correlation between the ranks of two lists was expected, so Spearman's rank correlation coefficient (Zar, 1972 and Spearman, 1904) was used to compare the two lists.

Principle component analysis (PCA) was performed on the densitometric data using the MATLAB(v.7.6(R2008a)) software package. Optical densities of the bands were used as dimensional values and each biomass sample represented a data point. Finally a graph of the first versus the second principle component was plotted. The

samples were clustered into three groups using MATLAB(v.7.6(R2008a)) based on their first two principle components by the farthest neighbor algorithm. The SMA values were represented by a third dimension (diameter of the points) on the graph. If there was a strong correlation between the densitometric data and the SMA values, then samples with relatively similar SMA values were expected to cluster together.

1.2.6.2 Range Weighted Richness (Rr) and Functional Organization (Fo)

Marzorati et al (2008) defined two parameters, range weighted richness (Rr) and functional organization (Fo), for comparing DGGE banding patterns for the *16S rRNA* gene.

Range weighted richness (Rr) is a product of the square of the number of bands present in a particular banding pattern and the denaturant concentration difference between the first and last band locations (Marzorati et al., 2008). For DGGE of the *16S rRNA* gene, Marzorati et al. (2008) hypothesized that $Rr < 10$ is indicative of an environment particularly adverse or restricted to colonization, leading to low range-weighted richness. Rr values between 10 and 30 are hypothesized to be indicative of medium range-weighted richness, and samples having $Rr > 30$ are considered to have high range-weighted richness (Marzorati et al., 2008).

To calculate Rr, the gel image was divided into sections, each representing a 5 % increase in the denaturant concentration along the gel length. From the image, the number of bands present and the number of sections of gel image comprising all the bands in a

lane were obtained. Finally Rr was calculated using Equation 1.1. In this equation, N is the total number of bands present in a particular lane and D_g is the denaturant concentration (expressed as a v/v fraction) difference between the first and last band of a particular lane. Rr was calculated as,

$$Rr = (N^2 \times D_g) . \quad (1.1)$$

The Rr values obtained for each banding pattern were categorized as low, medium or high richness as proposed by others for *16S rRNA* genes (Marzorati et al., 2008).

Pareto-Lorenz (PL) evenness curves can be constructed from DGGE banding patterns to graphically represent the diversity of identified phylotypes (Mertens et al., 2005; Wittebolle et al., 2008). To draw a PL curve using a banding pattern, respective bands present in a pattern are ranked from high to low abundance based on their band intensities (measured in terms of optical densities). The individual band intensity is divided by the total band intensity within a lane to normalize the data. The normalized band intensity represents the cumulative proportion of the phylotype relative abundance and is used as the y-coordinate for plotting PL curves as shown in Figure 1.3. Similarly, the number of bands is normalized by dividing by the total number of bands and the cumulative normalized number of bands is used as the x-coordinate for plotting PL curve as shown in Figure 1.3. A line with slope of 45° through the origin represents perfect evenness. As the un-even distribution among the community increases, the PL curve deviates from the line of perfect evenness as shown in Figure 1.3.

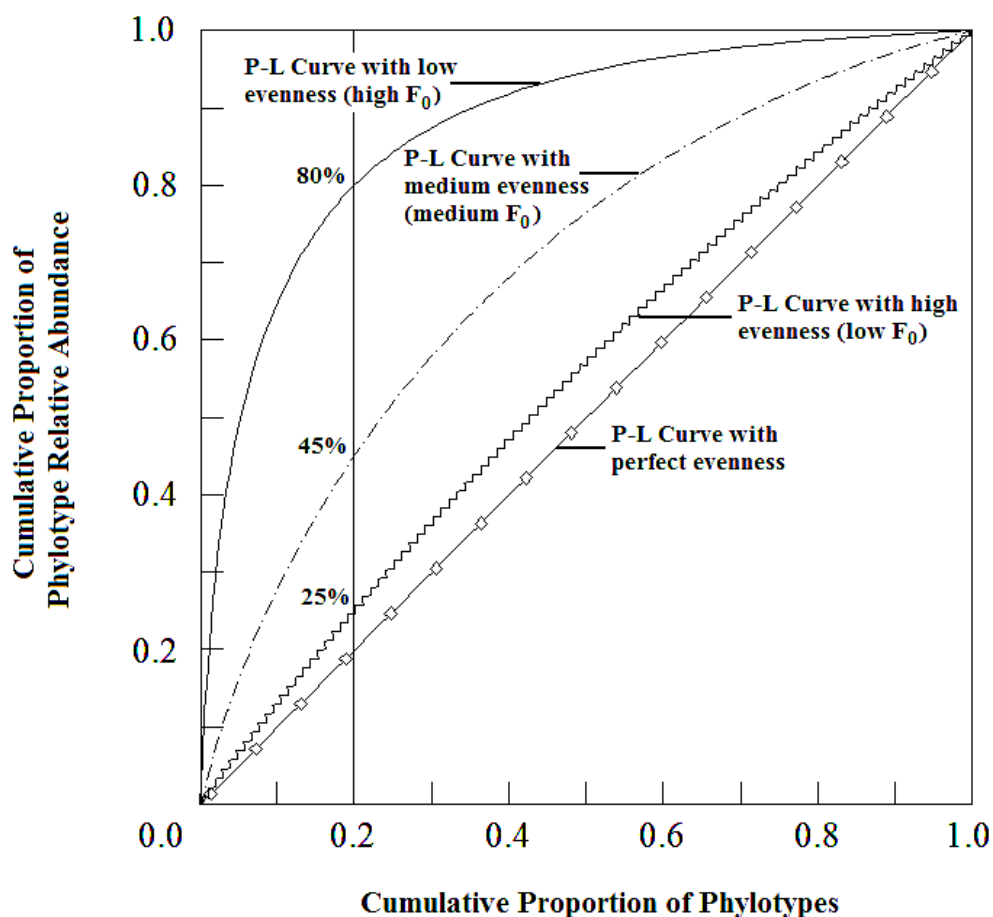


Figure 1.3: Possible Cases of P-L Curves. Adopted from Marzorati et al. (2008)

For numerically interpreting a PL curve, it is suggested to focus on the cumulative number of bands that describe 20% of the normalized total population (Wittebolle et al., 2008). For the *16S rRNA* gene in ecological terms, it was concluded that the PL curve representing 25% cumulative band intensity (y-coordinate projection) for 20% of the total number of bands (x-coordinate) represents a community with high evenness. A PL Curve with high evenness is shown in Figure 1.3. The microbial community represented by a PL curve with high evenness has a low F_0 value and it may result from a lack of selective

pressure. Such a microbial community doesn't demonstrate a well defined structure since there are no phylotype present in a high relative concentration. A relatively long lag phase could be needed to counter a sudden stress experienced by this type of microbial community. The PL Curve representing 45% cumulative band intensity for 20% of the total number the bands represents a community with medium evenness as shown in Figure 1.3. In this case, the most fitting phylotypes are dominant and hence are present in high relative numbers while the other phylotypes are present in lower numbers. Such a community has a medium F_o value and may be able to preserve its functionality under changing environments. Finally the PL curve representing 80% cumulative band intensity for 20% of the total number of bands represents a specialized community having a small number of members dominating the overall number of microbes present. Such a distribution is categorized as high F_o and may be fragile when external changes are made because disruption may require long recovery times (Marzorati et al., 2008).

Functional organization (F_o) of the banding patterns was evaluated using PL curves (Marzorati et al., 2008). For the purpose of analysis, it was assumed that the band intensity was a function of phylotype richness. For each lane, the respective bands were ranked from high to low based on their optical densities. The fraction of the total number of bands represented by an individual band was calculated by taking the reciprocal of the total number of bands present in a banding pattern as shown by Equation 1.2. In this equation, σ_{xi} is the fraction of the total number of bands represented by i^{th} band and N is the total number of bands. Similarly, the fraction of band intensity represented by every individual band in a banding pattern was calculated by taking the ratio of band intensity of that particular band and the sum of intensities of all the bands present in the banding

pattern as shown by Equation 1.3. In Equation 1.3, σ_{yi} is the fraction of the total intensity represented by i^{th} band and X_i is the intensity of i^{th} band. σ_{xi} and σ_{yi} were calculated as,

$$- , \quad (1.2)$$

$$\text{---} . \quad (1.3)$$

Subsequently, the cumulative fraction of the total number of bands (σ_{xi}) was used as the x -coordinate, and the respective cumulative fraction of the intensity of the bands (σ_{yi}) was used as the y -coordinate. Furthermore, a vertical line was drawn to evaluate the fraction of microbial community represented by 20 % of the dominant bands. Functional organization for each biomass sample was categorized as low, medium or high as proposed by others (Marzorati et al., 2008).

1.2.6.3 Regression Analysis

The densitometric data obtained by the image analysis was also used to fit a multiple regression equation expressing SMA (dependent variable) as a function of band intensities (independent variables). Microsoft Excel® (v.2007) software was used for regression analysis by the least squares method and the results obtained were used to test the significance of the regression equation.

1.3 Results and Discussion

1.3.1 SMA Results

Figure 1.4 shows the SMA results for the anaerobic biomass samples arranged in descending order. The bar graph represents average SMA values, whereas the error bars represent standard deviation among the replicates.

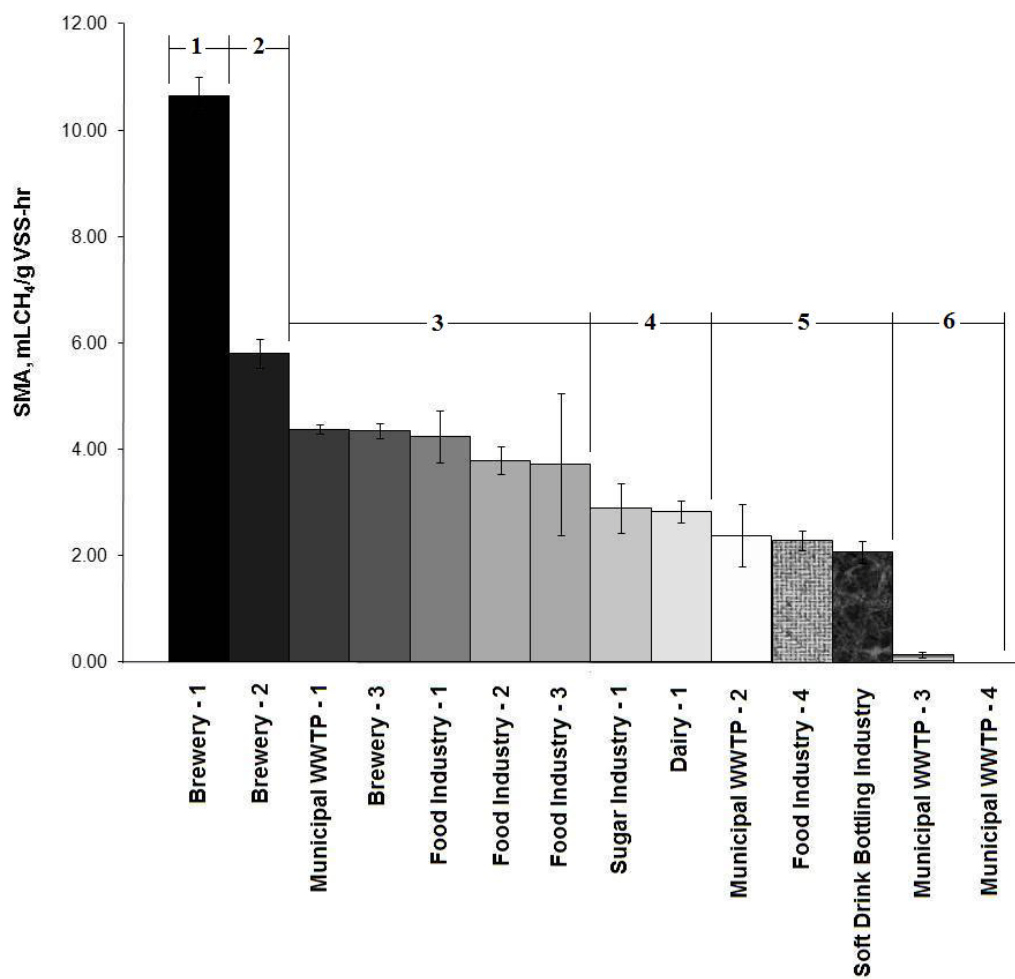


Figure 1.4: Results of SMA Testing.

Groups 1 to 6 represent biomass samples having statistically distinct SMA values.

Interestingly all the brewery biomass samples had higher SMA values as compared to other biomass samples and 75 % of the municipal biomass samples had lower SMA values except the Municipal WWTP-1 biomass. The brewery biomass samples may have contained more suitable microbial communities for faster degradation of propionate as compared to the municipal biomass samples leading to higher SMA values. A reason for municipal biomass having a lower SMA may be the fact that the municipal biomass was used to digest sludge and some of the undigested sludge solids may have contributed to errors in estimating active biomass quantity measured in terms of VSS. This error using VSS to estimate active biomass may have caused lower SMA of municipal biomass samples. The probabilities (p) associated with the Student's t statistic for pair-wise comparisons of SMA means are presented in Appendix A, Table A.2 in a 14×14 sized, lower triangular matrix. The null hypothesis for the Student's t test was that the population means (SMA values) of the two biomass samples compared were equal. Table A.3 in Appendix A, shows interpretation of the results for a 5% level of significance. In Table A.3, every gray colored cell, containing 'True' represents biomass samples with statistically equal SMA values whereas a cell containing signal 'False' represents biomass samples whose SMA values are statistically unequal.

Figure 1.4 and Table 1.6 represent the clusters of the biomass samples formed by the farthest neighbor algorithm using $(1-p)$ as the distance between two samples and a 95% level of significance as the cutoff for cluster definition. The data were found to be clustered in six groups based on similarity in SMA values. Brewery-1 and Brewery-2 had unique SMA values among the samples tested.

Table 1.6: Clusters of Biomass Samples Based on SMA Values

Cluster No.	Members	Average SMA, mLCH₄/gVSS-hr	Standard Deviation, mLCH₄/gVSS-hr
Cluster 1	Brewery-1	10.65	±0.36
Cluster 2	Brewery-2	5.80	±0.26
	Municipal WWTP-1		
	Brewery-3		
Cluster 3	Food Industry-1	4.09	±0.61
	Food Industry-2		
	Food Industry-3		
Cluster 4	Sugar Industry-1	2.86	±0.32
	Dairy-1		
	Municipal WWTP-2		
Cluster 5	Food Industry-4	2.25	±0.35
	Soft Drink Bottling		
Cluster 6	Municipal WWTP-3	0.07	±0.08
	Municipal WWTP-4		

1.3.2 Effect of Initial SMA on SMA After Enrichment

Based on the results of initial SMA screening (Figure 1.4), biomass samples with higher and lower SMA values were selected for enrichment. Since the Municipal WWTP-4 biomass sample was found to have zero initial SMA against propionate, it was

not selected for enrichment. Table 1.7 shows the list of biomass samples selected for enrichment and their initial and final SMA values after 580 days (38.6 SRTs).

Table 1.7: Biomass Samples Selected for Enrichment

Sample	Initial SMA, mLCH₄/gVSS-hr	Final SMA after enrichment, mLCH₄/gVSS-hr
Brewery-1	10.65 ± 0.36	10.65 ± 3.27
Brewery-2	5.80 ± 0.26	8.89 ± 3.20
Municipal WWTP-1	4.38 ± 0.09	4.18 ± 0.50
Food Industry-4	2.29 ± 0.18	2.21 ± 0.46
Soft Drink Bottling	2.08 ± 0.21	2.14 ± 0.41
Municipal WWTP-3	0.14 ± 0.06	1.94 ± 1.37

Figure 1.5 shows the effect of initial SMA on the SMA after 580 days (38.6 SRTs) of enrichment for different methanogenic cultures. The SMA values after enrichment tend to follow the pattern of initial SMA values of the samples which was verified statistically by ranks correlation coefficient. The ranks correlation coefficient used for proving the hypothesis that the SMA values after enrichment followed the trend of initial SMA values of the biomass samples was found to be 0.94, for which the critical value at 5% level of significance was only 0.886. This means there was a strong influence of the initial SMA and the microbial community on the SMA after enrichment. This

further highlights the need for analysis of the methanogenic communities present in the samples with higher or lower initial SMA. This indicates that the microbial communities present in the seed culture used for starting an anaerobic digester can dictate the long-term digester behavior.

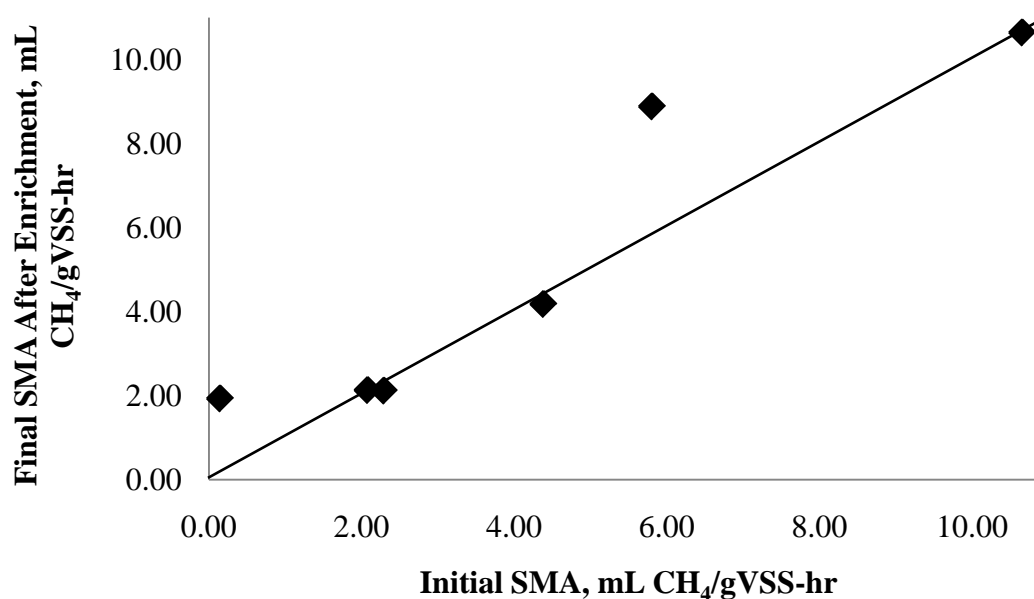
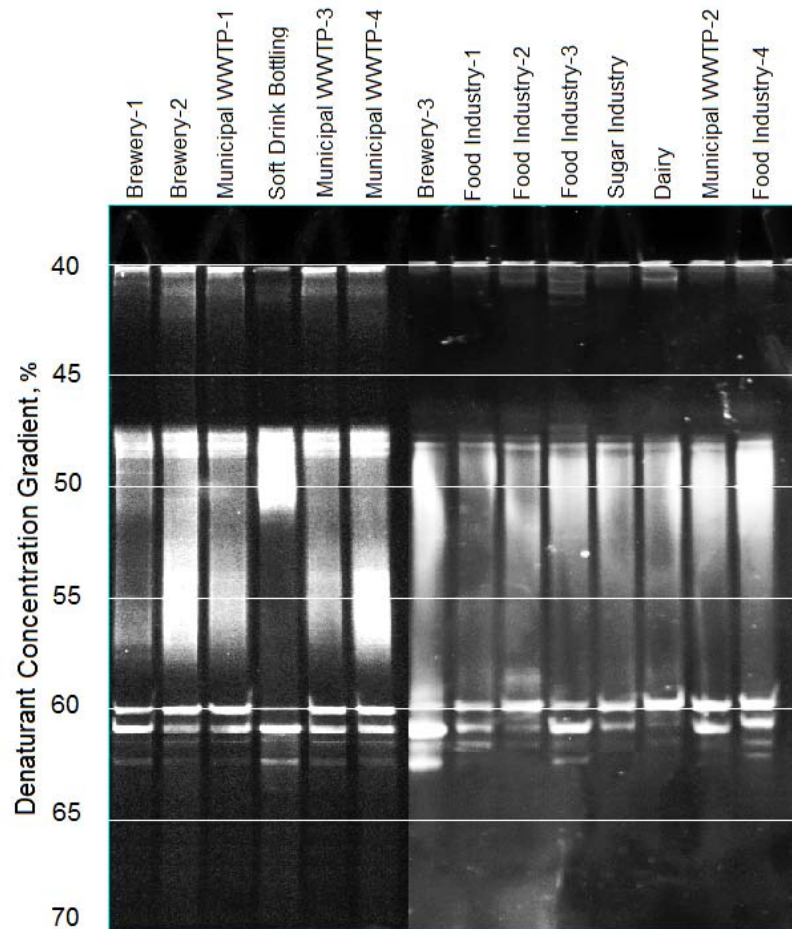


Figure 1.5: Effect of Initial SMA on SMA After Enrichment

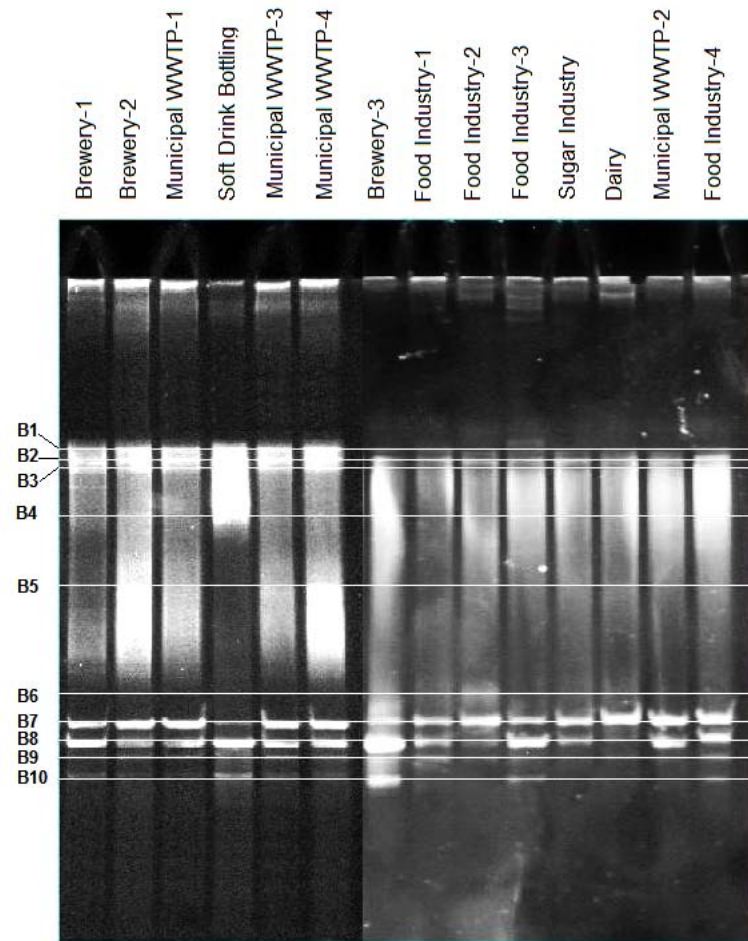
1.3.3 *mcrA* – DGGE Analysis

Two DGGE gel images were compared by lining up the bands present in the common marker (ladder). Figure 1.6 shows the combined DGGE image. Densitometric data from the two images were combined as described previously (see section 1.2.2.5) and used to calculate R_r and F_o . The densitometric data were also used to perform cluster

and multiple linear regression analysis. Significant bands were detected for the minimum optical density threshold of 0.15 and visual inspection. Each significant band in Figure 1.6 b (i.e. B1, B2, B3.....B10) was assumed to represent one phylotype of methanogen. The presence of the same band in different sample lanes indicated the presence of that particular phylotype in both samples. The brightness of a band in terms of optical density (OD) was considered to be an indicator of the abundance of that methanogenic phylotype as has been assumed by other researchers (Marzorati et al., 2008). Although helpful, these assumptions may not be accurate because of biases involved in molecular techniques such as DNA extraction, PCR amplification and DGGE (Head et al., 1998; Spiegelmann et al., 2005; Potens et al., 2007; Marzorati et al., 2008).



(a)



(b)

Figure 1.6: DGGE Gel Image Indicating (a) Denaturant Concentration Along the Length (b) Different Types of Bands Present in the Banding Patterns

1.3.3.1 Cluster Analysis

Figure 1.7 presents the dendrogram obtained for banding patterns constructed using UPGMA algorithm and densitometric data. The distance between each pair of samples was calculated as one minus the correlation coefficient between densitometric data of two samples. If there was a strong relation between the *mcrA* DGGE banding patterns and the SMA values, then the clustering of the densitometric data was expected to follow results presented in Table 1.6. For example, Municipal WWTP - 1 was expected to be clustered with Brewery – 3, Food Industry -1, Food Industry -2 and Food Industry -3 according to results in Table 1.6. However these four biomass samples were present in different dendrogram clades. Also, Brewery – 1 biomass was found to cluster with Municipal WWTP -3 biomass in the dendrogram, whereas these biomass samples demonstrated significantly different SMA values.

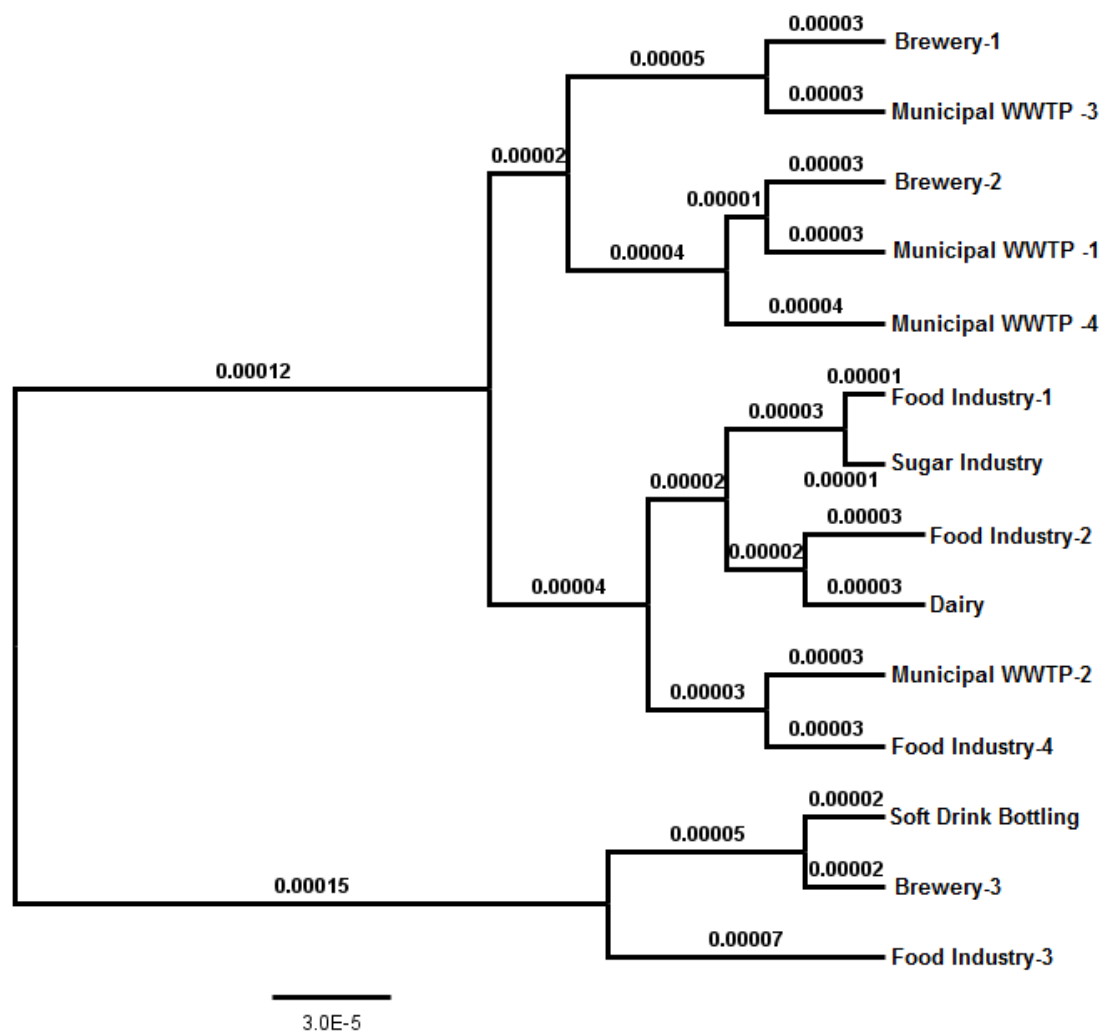


Figure 1.7: Cluster Analysis of the Biomass Samples Based on DGGE Band Densitometric Data. The scale bar represents one minus correlation coefficient between the densitometric data of the two samples.

Further, the rank correlation coefficient between the SMA data and the densitometric data was only 0.015, which was less than the critical value of 0.538 for a 95% confidence level (Zar, 1972). The rank data used to calculate the rank correlation coefficient are presented in Appendix A, Table A.4. An insignificant rank correlation coefficient indicates that clustering based on densitometric data did not follow the clustering trend based on SMA values.

Figure 1.8 shows the three clusters obtained for principle component (PCA) performed using densitometric data. The first two principle components were employed as x and y-coordinate respectively and explain 81.6% of the total variation for densitometric data. On the right hand side of the figure, the biomass samples are listed in descending order of their SMA values and the samples are ranked from 1 to 14 according to their respective SMA values. In Figure 1.8, the circle diameter is used as the third dimension indicating SMA values (i.e., samples with higher SMA values are represented by circles with larger diameters). This means SMA values were not used to determine the principle components, but they were super-imposed on the plot as the third dimension (dot diameter). In Figure 1.8, Municipal WWTP-3 (Rank 13) and Municipal WWTP (Rank 14) were represented by '×' symbols because of their negligible SMA values which led to non visible circle diameters. Projections of the densitometric data of the five bands having the highest contribution to the principle components (B₁, B₂, B₃, B₇ and B₈) were also shown in Figure 1.8. The biomass samples clustered in three groups based on their principle component coordinates (see Figure 1.8).

Cluster 1 represented biomass samples having high SMA ranks, whereas Cluster 2 represented those with lower ranks. Cluster 3 represented biomass samples having variable SMA ranks. The banding pattern correlates to a high degree with the SMA ranks. However, Brewery-3 (Rank 4) and Municipal WWTP-2 (Rank 10) were outliers in Cluster 3 since ideally they would be contained in Cluster 1 and Cluster 2 respectively.

Projections of the five dimensions causing the highest variation in data showed that higher OD values for bands B₁ and B₂ contributed significantly to Cluster 1. This

indicates that these may have a significant metabolic function leading to higher SMA values. This may be an indication of an underlying relationship between banding pattern and the SMA value, but as mentioned above, Figure 1.8 explains only 81.6% of the total variation in the data.

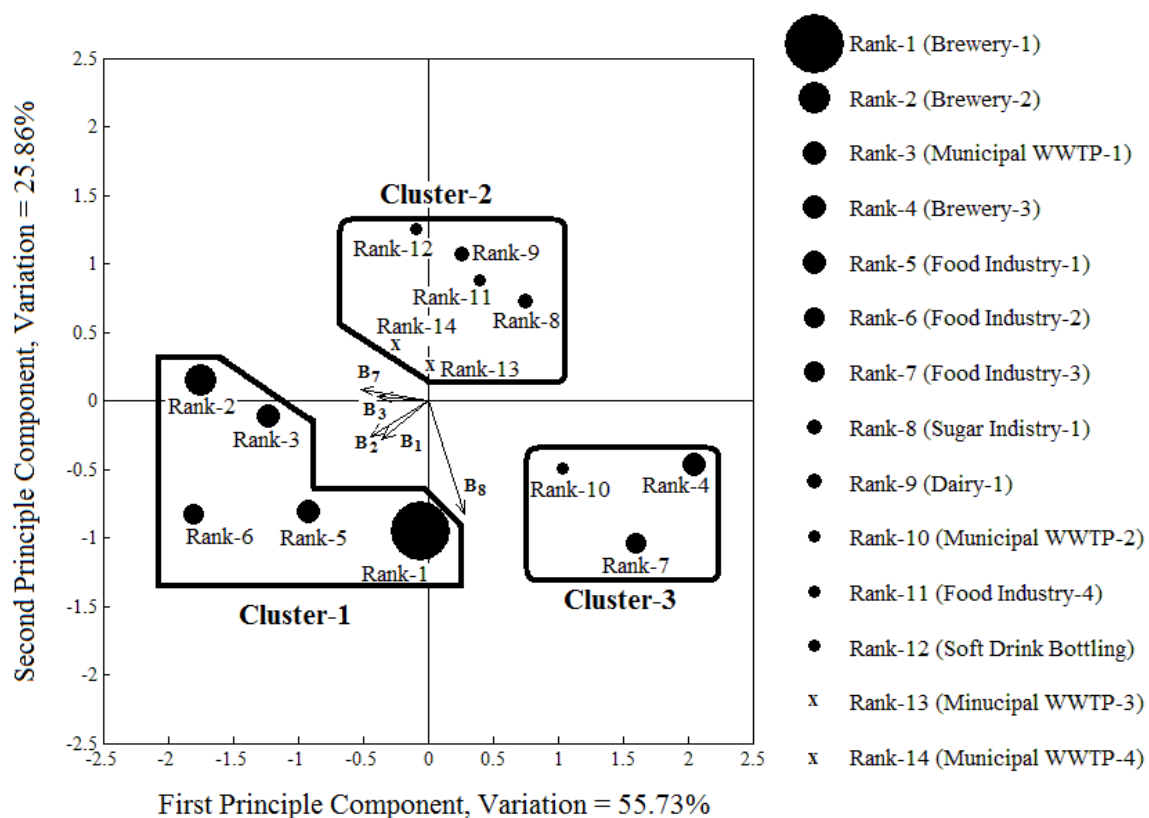


Figure 1.8: Principle Component Analysis Results

First principle component = $-0.4655(X_1) - 0.5228(X_2) - 0.3663(X_3) - 0.0875(X_4) + 0.0044(X_5) + 0.035(X_6) - 0.5116(X_7) + 0.2959(X_8) + 0.0478(X_9) + 0.1237(X_{10})$

Second principle component = $-0.3467(X_1) - 0.3190(X_2) + 0.0285(X_3) + 0.0070(X_4) - 0.0662(X_5) + 0.0129(X_6) + 0.0786(X_7) - 0.8571(X_8) - 0.0402(X_9) - 0.1740(X_{10})$

Where, $X_1, X_2, X_3, \dots, X_{10}$ are the normalized optical densities for band B1, B2, B3, ..., B10 of a banding pattern as shown in Figure 1.6 b)

1.3.3.2 Range Weighted Richness (Rr)

Figure 1.6 a and b show the gel image used to calculate Rr and Table 1.8 shows Rr values of biomass samples listed in descending order of SMA values. The Rr values for the biomass samples varied between 3.2 to 12.8 and 50% of the biomass samples had an Rr values of 12.8. Table 1.8 also shows classification of Rr values in low, medium and high categories as proposed by others for *16S rRNA* gene (Marzorati et al., 2008).

Table 1.8: Range Weighted Richness (Rr) of The Biomass Samples

	Brewery-1	Brewery-2	Municipal WWTP-1	Brewery-3	Food Industry-1	Food Industry-2	Food Industry-3	Sugar Industry	Dairy	Municipal WWTP-2	Food Industry-4	Soft Drink Bottling	Municipal WWTP-3	Municipal WWTP-4
Rr	128	128	128	98	98	128	128	98	72	98	98	32	128	128
Classification	M*	M	M	L**	L	M	M	M	L	L	L	L	M	M

* M – Medium Rr

** L – Low Rr

Figure 1.9 Presents SMA versus Rr values. The correlation coefficient for the data presented in Figure 1.9 was found to be 0.18 and Student's t test for testing significance of the correlation coefficient showed that the obtained correlation coefficient was not significant at a 5% level of significance ($p=0.53$). This means that the Rr values of the samples did not correlate with the SMA values.

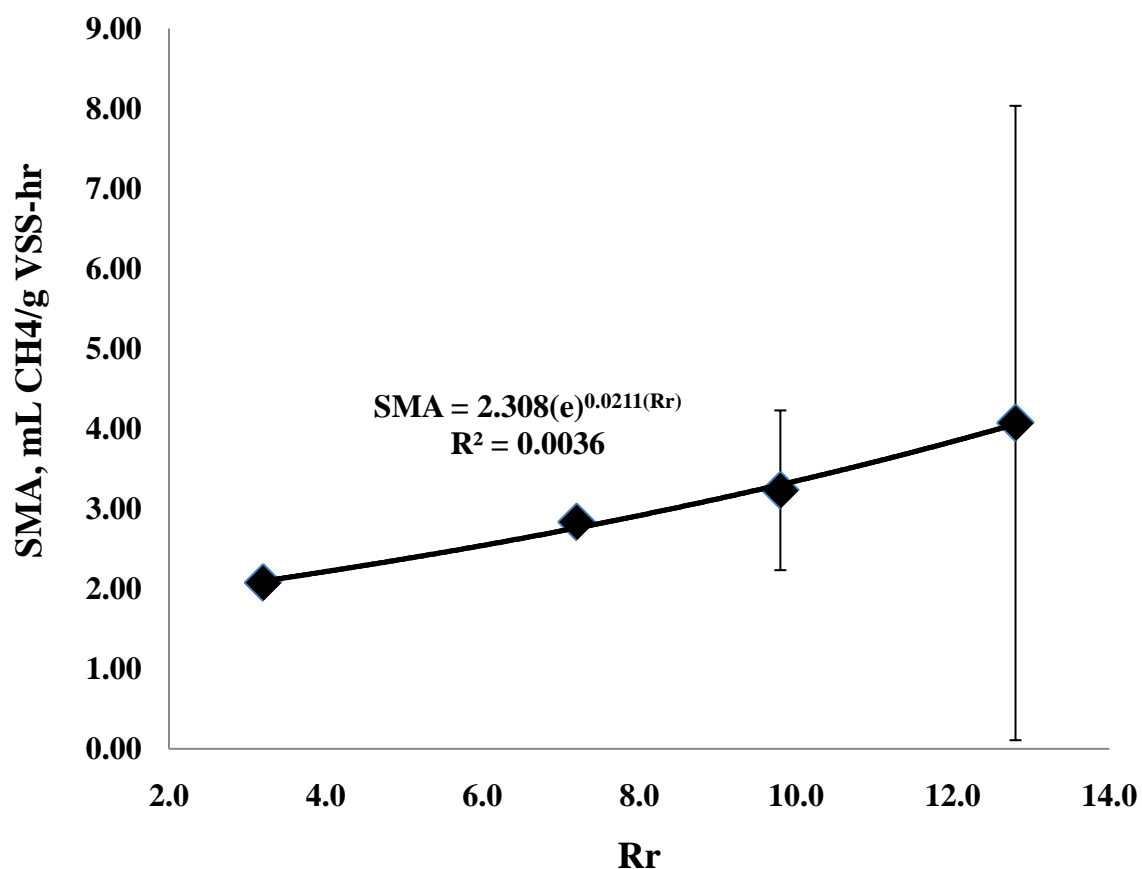


Figure 1.9: Correlation Between Rr and SMA.

Error bars represent the standard deviation among the replicates of the biomass samples. Absence of error bar represents that only one sample had the Rr value represented by the point.

1.3.3.3 Functional Organization (Fo)

Figure 1.10 shows PL curves used to describe functional organization values (Fo) of biomass microbial communities existing in the biomass samples. PL curves for all the samples except Municipal WWTP - 3 and Food Industry - 4 were in the medium functional organization range (45 to 80% cumulative phylotype abundance for 20% cumulative population) proposed by others for various environmental niches (Marzorati

et al., 2008). The Municipal WWTP - 3 and the Food Industry - 4 biomass samples were categorized as having low functional organization, and their 20 % cumulative population values represented 44% and 43% cumulative phylotype abundance respectively. In contrast, 80% of the cumulative phenotype abundance for dairy biomass was observed at the 20%-cumulative-population value; meaning the dairy biomass had lower diversity.

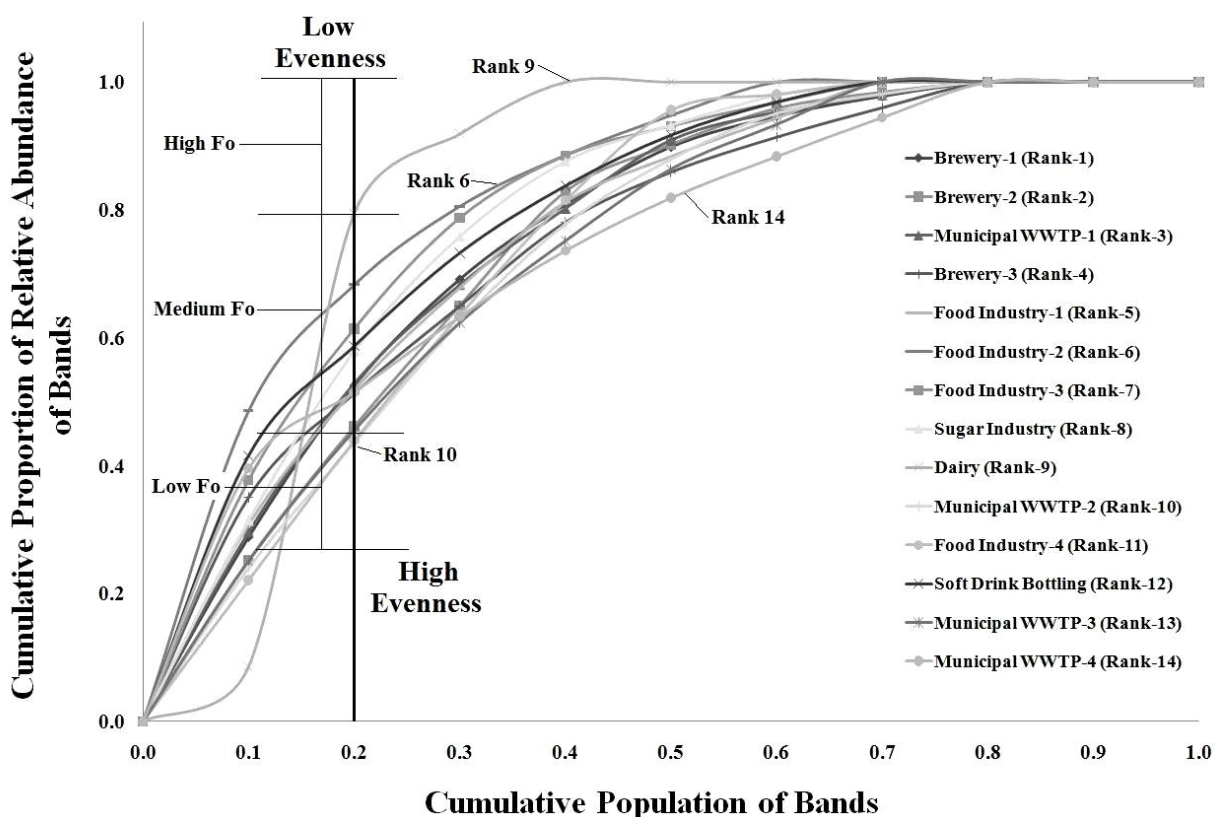


Figure 1.10: Pareto-Lorenz Curves for the Anaerobic Biomass Samples

The correlation coefficient between Fo and SMA values was 0.061 and Student's t test for testing significance of the correlation coefficient showed that the correlation was

not significant at a 5% level of significance ($p=0.83$). When two samples with the lowest SMA values were dropped from the correlation analysis (Municipal WWTP-3 and Municipal WWTP-4), the correlation coefficient was found to be 0.594 which was statistically significant ($p=0.013$). Figure 1.11 shows the graph of F_o and five groups of SMA values (see Figure 1.4 and table 1.6 for groups of SMA). The group of SMA values with the two low-SMA outliers was removed. Horizontal error bars represent the standard deviation among F_o values of the samples having the same SMA. Vertical error bars represent standard deviation among the replicates. The Brewery-1 biomass sample was represented by a point that is far away from the curve because of its significantly higher SMA value. Interestingly, F_o seems to have a negative correlation with the SMA value which indicates that high evenness in the methanogenic community may be important for higher propionate degradation rates.

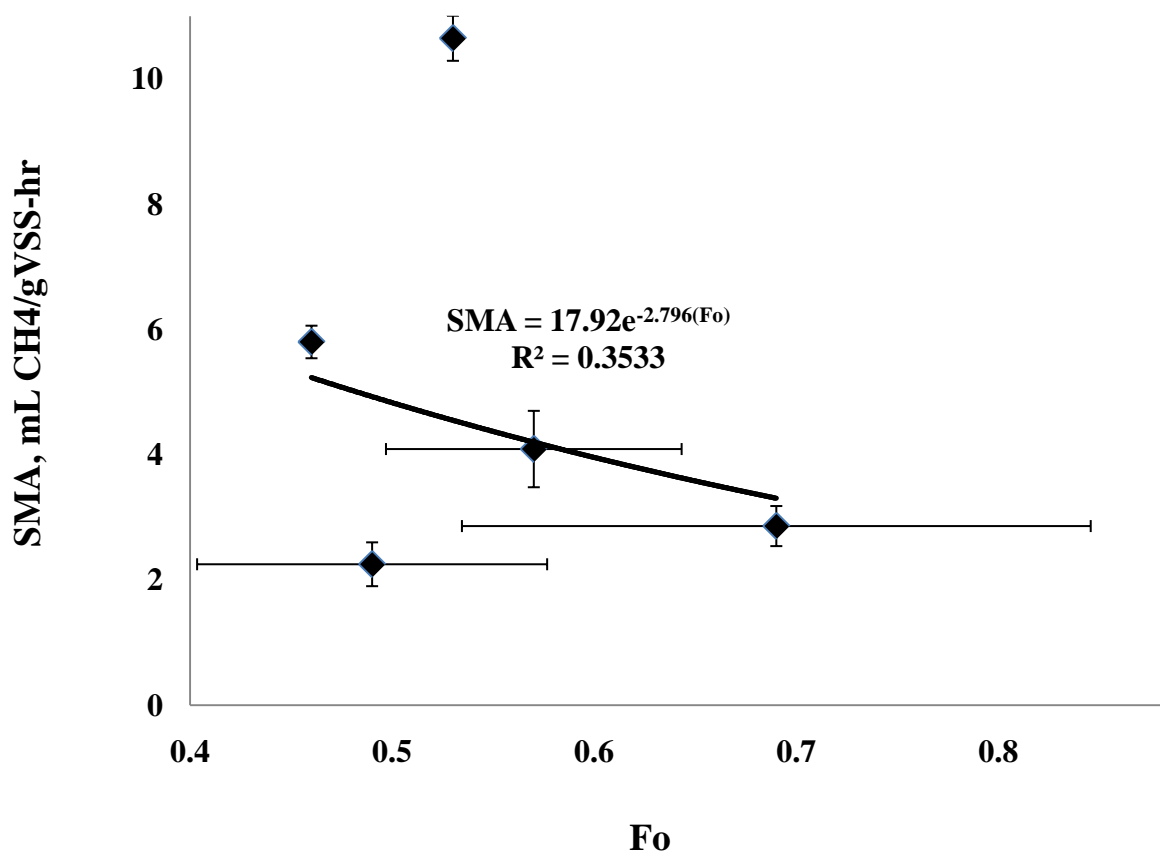


Figure 1.11: Correlation Between Fo and SMA.

Error bars represent the standard deviation among the replicates of biomass samples. Vertical error bars represent standard deviation among the SMA replicates. Horizontal error bars represent the standard deviation among Fo values of the samples.

1.3.3.4 Regression Analysis

The following theory was considered to attempt to establish a relationship between SMA values against propionate and the DGGE bands. It was assumed that the SMA test systems operated at low H_2 concentrations (10^{-4} to 10^{-6} atm partial pressure), which was necessary for propionate conversion to methane (McCarty and Smith, 1986). Also, it was assumed that the H_2 concentration was constant when methane production

rate was constant during the SMA test utilized, and the CH_4 production rate was proportional to the H_2 utilization rate.

Figure 1.12 shows the first-order degradation kinetic relationship with respect to H_2 concentration for two hypothetical microbial phylotypes 1 and 2. Previous researchers have stated that many hydrogenotrophic methanogenic archaea require a minimum threshold hydrogen concentration to be metabolically active (Karadagli and Rittmann, 2007a; Karadagli and Rittmann, 2007b).

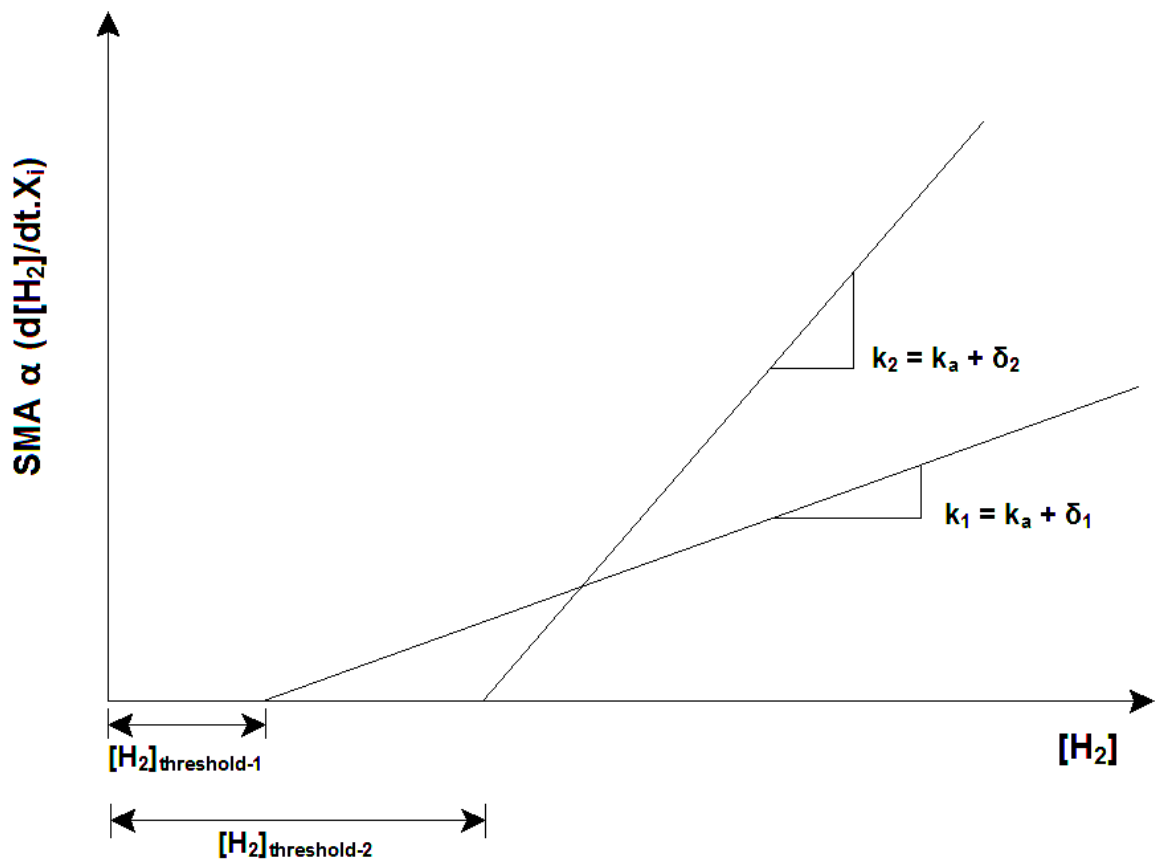


Figure 1.12: First-Order Degradation Kinetics for Hypothetical Microbial Phylotype 1 and 2

Equation 1.4 shows the expression for specific methanogenic activity (r_1 , moles- $\text{CH}_4/\text{gVSS}_1\text{-hr}$) due to methanogenic phylotype 1 against hydrogen, k_1 is the first order kinetic rate constant for phylotype1 and has units (hr^{-1}), $[\text{H}_2]$ is the hydrogen concentration and has units (moles/L), $[\text{H}_2]_{\text{threshold}}$ is the threshold hydrogen concentration for methanogenic phylotype1 and has units (moles/L), X is concentration of all the methanogenic phylotypes and has units (gVSS) and X_1 is the concentration of methanogenic phylotype 1 and has units (gVSS₁). Specific methanogenic activity due to phylotype 1 was assumed to be given as,

$$\text{SMA}_1 = r_1 = k_1 ([\text{H}_2] - [\text{H}_2]_{\text{threshold-1}}) (X_1/X). \quad (1.4)$$

Average first order degradation rate constant for all the phylotypes (k_a , hr^{-1}) can be given by Equation 1.5. In Equation 1.5, δ_1 is the difference between the average first-order rate constant and first-order rate constant for microbial phylotype 1 and has units (hr^{-1}). Average first-order degradation rate constant was assumed to be given as,

$$k_1 = k_a + \delta_1 . \quad (1.5)$$

Substituting the value of k_1 in Equation 1.4 leads to,

$$\begin{aligned} r_1 &= (k_a + \delta_1) ([\text{H}_2] - [\text{H}_2]_{\text{threshold-1}}) (X_1/X) , \\ r_1 &= (k_a [\text{H}_2] + \delta_1 [\text{H}_2] - k_a [\text{H}_2]_{\text{threshold-1}} - \delta_1 [\text{H}_2]_{\text{threshold}}) (X_1/X) , \\ r_1 &= k_a [\text{H}_2] (X_1/X) + (\delta_1 [\text{H}_2] - [\text{H}_2]_{\text{threshold-1}} (k_a + \delta_1)) (X_1/X) . \end{aligned} \quad (1.6)$$

If the overall SMA (SMA_T , moles- $CH_4/gVSS_1$ -hr) of a biomass sample is assumed to be the sum of individual SMA values of the different methanogenic phylotypes present in a biomass sample, then,

$$SMA_T = r_1 + r_2 + r_3 \dots r_n , \quad (1.7)$$

where, n is the total number of methanogenic phylotypes present in the biomass.

From Equations 1.6 and 1.7, the following theoretical linear equations can be generated,

$$\begin{aligned} SMA_T = & k_a[H_2]((X_1+X_2+X_3\dots X_n)/X) + (\delta_1[H_2]-[H_2]_{\text{threshold-1}}(k_a+\delta_1))(X_1/X) \\ & + (\delta_2 [H_2]-[H_2]_{\text{threshold-2}}(k_a+\delta_2))(X_2/X) + (\delta_3[H_2]-[H_2]_{\text{threshold-3}}(k_a+\delta_3))(X_3/X) \\ & + \dots + (\delta_n[H_2]-[H_2]_{\text{threshold-n}}(k_a+\delta_n))(X_n/X) \end{aligned} \quad (1.8)$$

$$SMA_T = a \quad n/X) + m_1(X_1/X) + m_2(X_2/X) + m_3(X_3/X) + \dots + m_n(X_n/X) \quad (1.9)$$

Let $a=k_a[H_2]$, the y-intercept of the linear equation, and $m_i=(\delta_i[H_2]-[H_2]_{\text{threshold}-i}(k_a+\delta_i))$, the slope of the linear equation, then Equation 1.9 is valid for the following conditions:

1. if $\delta_i < 0$, then $|\delta_i| < k_a$;
2. $[H_2] > [H_2]_{\text{threshold}}$;
3. $m_i < 0$ if $\delta_i [H_2] < [H_2]_{\text{threshold}-i} k_i$.

A multiple linear regression equation was generated by the least sum of squared residual method using the OD of DGGE bands shown in Figure 1.6 b). The obtained regression equation was,

$$\begin{aligned}
 \text{SMA} = & 0.0942(X_1/X) + (-6.4968(X_1/X)) + 5.2921(X_2/X) + 0.6974(X_3/X) \\
 & + 5.1697(X_4/X) + 1.1422(X_5/X) + (-0.0549(X_6/X)) + (-1.3621(X_7/X)) \\
 & + (-1.8603(X_8/X)) + (-25.6866(X_9/X)) + 17.1986(X_{10}/X) .
 \end{aligned} \tag{1.10}$$

In Equation 1.10, (X_1/X) and (X_2/X) are the standardized ODs of band B1 and B2 shown in Figure 1.6 b). For the above equation, the coefficient of determination (r^2) was found to be 0.864. Figure 1.13 shows a plot of predicted SMA values versus actual SMA values. The diagonal line in Figure 1.13 represents the locus of perfect correlation.

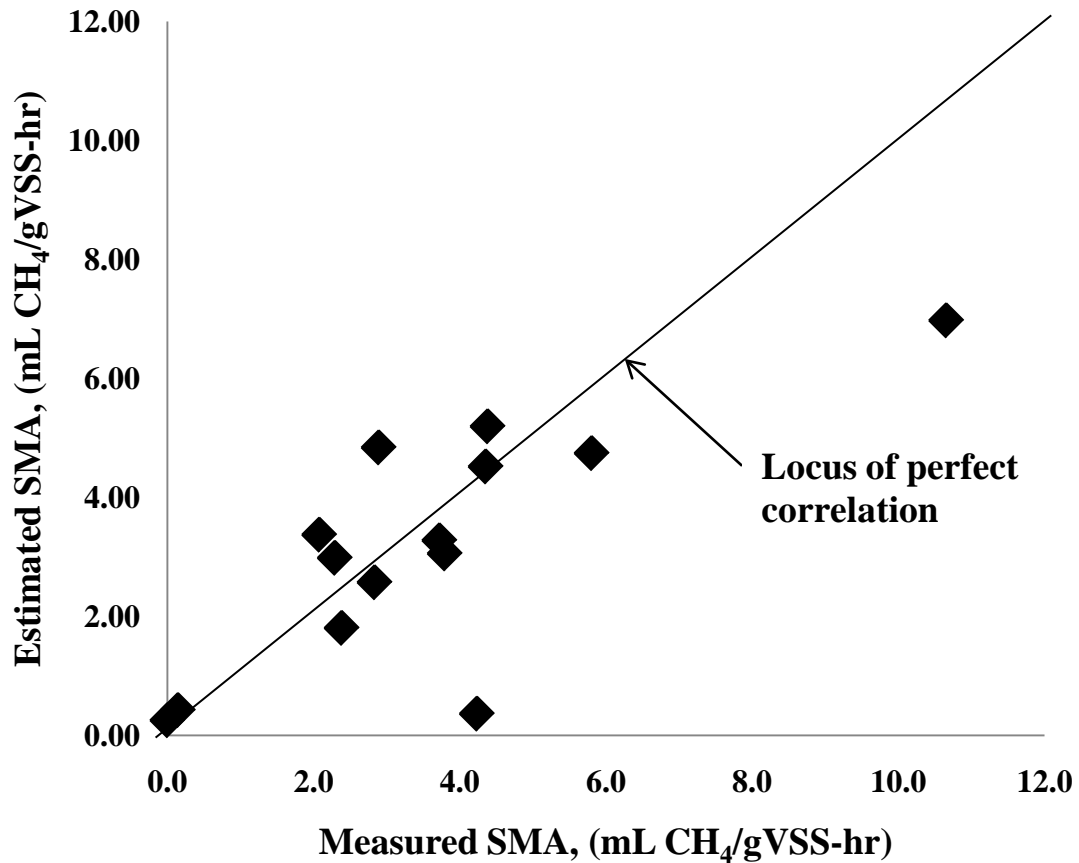


Figure 1.13: Plot of Measured SMA Values Versus SMA Values Estimated From DGGE Banding Pattern Data Using Multiple Linear Regression

Although the equation seems to fit relatively well to the test data, the ‘F-statistic’ for the regression equation revealed that there was a 35.6% chance of obtaining a better ‘F-statistic’ value by random occurrence. Hence the obtained equation does not represent a strong relationship between banding patterns and SMA values for the data evaluated. To demonstrate this, the data were randomly divided into two groups. One group was used as the training set to obtain a regression equation and the second group was used to test the resulting correlation equation. Figure 1.14 shows the graph of estimated versus measured values. The equation predicted the training data SMA values well, but test data were found to be scattered. This highlights the finding that there was no predictive correlation between the SMA values and the densitometric data from the DGGE image for the tested dataset.

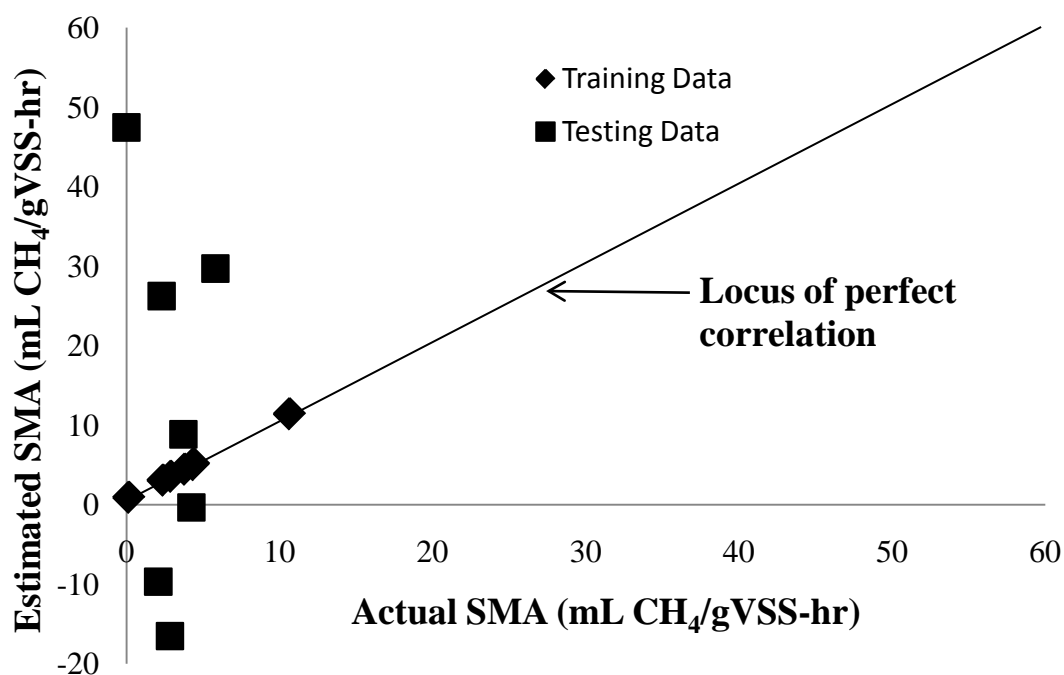


Figure 1.14: Predicted and Estimated SMA Values For the Training and Testing Data

There may be several reasons behind the multiple regression equation being statistically insignificant, including an insufficient amount of data, the amplified *mcrA* gene not being a true estimator of the active MCR enzyme in anaerobic cultures, the possibility of the MCR enzyme not being the rate-limiting enzyme during degradation of propionate, and the difficulties in measuring the actual quantity of biomass present in a sample using VSS values. These factors are discussed below.

- 1) When estimating SMA values, volatile suspended solids (VSS) concentration was used as a measure of the amount of active biomass present in the samples. It was assumed that all the VSS present in the samples was active biomass. This assumption may not be true since biomass samples digesting insoluble wastes (particulate material) such as waste activated sludge contain undigested particulate material that is not active biomass. This may have led to erroneous SMA values. These erroneous SMA values may also have led to a statistically insignificant regression relationship between the DGGE banding patterns and SMA values.
- 2) The number of samples used to perform the regression analysis was insufficient for the number of variables tested. Topliss and Costello (1972) recommended that for quantitative structure-activity relationship (QSAR) models, if $r^2 = 0.4$ is considered to be an acceptable level of correlation, then ≥ 30 samples are required to test 5 variables and ≥ 50 samples are required to test 10 variables. In the current study, only fourteen samples were used to fit an equation having ten variables, whereas ≥ 50 samples were required.

- 3) The *mcrA* gene copy number may not be representative of the active biomass involved in methane production. It is known that gene copies don't necessarily correlate to the number of translated enzymes (Freitag et al., 2009; Radl et al., 2007; Steinberg et al., 2009). An approach based on mRNA quantification may yield better data to establish such a relationship because the presence of mRNA indicates that the gene is not only present but also actively transcribed.
- 4) Also there are a number of biases involved in the molecular techniques such as DNA extraction, PCR amplification, DNA purification and DGGE (Head et al., 1998; Spiegelmann et al., 2005; Potens et al., 2007; Marzorati et al., 2008). These biases may have led to DGGE results that were not necessarily true representations of the methanogenic communities.
- 5) Another reason behind the poor regression could be that there was actually very little or no correlation between the methanogenic community structure and SMA values against propionate because the rate of propionate degradation by syntrophic bacteria may actually be the rate-limiting step. Although consumption of H₂ by hydrogenotrophic methanogens is a very important step in the degradation of propionate, the consumption rate of H₂ may not be representative of the overall propionate degradation rate.
- 6) To avoid complications during analysis, it was assumed that the methanogenic phlotypes worked independently and any interdependence among two or more methanogenic phlotypes was ignored. This led to the assumption that the total SMA value was an additive function of individual SMA values. The validity of this assumption is not known and if two or more methanogenic phlotypes do

share interdependence, then the additive relationship assumed would not be accounted for during regression analysis.

1.4 Conclusions and Recommendations for Future Work

The anaerobic biomass samples demonstrated SMA values that varied over two orders of magnitude and the samples were statistically categorized in 6 different groups based on their SMA values (for 5% level of significance). After enrichment of six different biomass samples it was observed that the initial SMA value had a strong influence on the final enriched SMA values even after enrichment over 38 SRTs (580 days). The samples with higher initial SMAs demonstrated higher enriched SMAs. This means that the initial microbial community present in an anaerobic culture significantly affects the enriched SMA value. This is particularly important since the biomass used as a seed culture to start an anaerobic culture may significantly influence the operating characteristics of a reactor over a long period of time.

Cluster analysis of DGGE banding pattern densitometric data using UPGMA algorithm showed that the grouping pattern based on DGGE pattern did not correlate to the clustering based on SMA values and samples with significantly different SMA values were found to be clustered together (see Figure 1.7). This indicates that there was no correlation between the densitometric data and the SMA values. The principle component analysis of the densitometric data indicated that the biomass samples were clustered in three groups according to their SMA values on a plot of the first two principle components. This may indicate an underlying relation since the principle components

were found without using the SMA values and the SMA values were imposed as a third dimension on the plot using DGGE banding pattern principle components. Furthermore, band B1 and B2 were found to have the highest positive influence on the SMA values of the biomass samples analyzed, which means the presence of phylotypes represented by bands B1 and B2 had a positive effect on biomass SMA values.

Range weighted richness (Rr) and functional organization (Fo) showed statistically insignificant correlation with SMA values. This indicates that the distribution of phylotypes among samples was independent of their SMA values. Also, multiple regression analysis done using SMA values as the dependent variable and band intensities as independent variables showed a 0.864 r^2 value but the associated F statistic value indicated that there was a 35.6% chance of obtaining a better correlation by random occurrence. Hence, the obtained relationship between DGGE banding patterns and SMA values was not statistically significant.

In conclusion, there are several factors that may have caused poor correlation between molecular and the SMA analysis such as an insufficient amount of data, the amplified *mcrA* gene not being the true estimator of the active MCR enzyme in anaerobic cultures, the possibility of MCR enzyme not being the rate limiting enzyme for degradation of propionate, and complications in measuring the actual quantity of biomass present in the samples using VSS data.

In lieu of the current study, the following recommendations for future work are made. At least 50 samples representing different SMA values should be considered to attempt to obtain a statistically significant QSAR model.

Instead of genes (DNA), mRNA should be extracted from the biomass samples and reverse transcriptase polymerase chain reaction (RT-PCR) should be performed on the extracted mRNA to convert them to stable DNA sequences. The stable DNA can then be quantified using real-time PCR techniques and then finally a fingerprinting technique like DGGE could be used for analysis of the amplified DNA. This approach may be more reliable since mRNA is a better estimator of the quantity of active enzyme involved in methane production and active biomass. Furthermore, the amount of target mRNA found in each sample can be used instead of VSS to calculate SMA values to eliminate any bias involved in VSS measurement to determine active biomass i.e. SMA values may be expressed in units of mLCH₄/(g target mRNA-hr). Importantly, mRNA coding for the enzyme that determines the overall rate of degradation should be selected for the study since it may show better correlation with the methane production rate.

In conclusion, SMA values against propionate for biomass from different anaerobic digesters varied over two orders of magnitude. SMA values after enrichment showed high correlation with the initial SMA values of cultures after 580 days (38.6 SRTs) of enrichment. Therefore, seed biomass for new reactors should be chosen carefully, and activity testing is recommended when selecting seed biomass. Comparison of DGGE banding patterns for the *mcrA* gene with SMA values for 14 biomass samples indicate an underlying relation between methanogenic community structure and activity. However, more research is required to establish a quantitative structure-activity relationship (QSAR). In the future, QSARs may be developed, and more highly-defined microbial communities may be employed to improve specific aspects of anaerobic digester performance.

1.5 References

- American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF) (1998) *Standard Methods for the Examination of Water and Wastewater* (20th ed.). APHA et al.
- Bidle, K.A., Kastner, M., and Bartlett, D.H. (1999) "A Phylogenetic Analysis of Microbial Communities Associated With Methane Hydrate Containing Marine Fluids and Sediments in the Cascadia Margin (ODP site 829B)." *FMES Microbiology Letters*. 177.1:101-108.
- Boon, N., Windt, W.D., Verstraete, W., and Top, E.M. (2002) "Evaluation of Nested PCR-DGGE (Denaturing Gradient Gel Electrophoresis) With Group-Specific *16S rRNA* Primers for the Analysis of Bacterial Communities from Different Wastewater Treatment Plants." *FMES Microbiology Ecology*. 39:101-112.
- Chen, Y., Higgins, M.J., Maas, N.A., Murthy, S.N., Toffey, W.E., and Foster, D.J. (2005) "Roles of Methanogens on Volatile Organic Sulfur Compound Production in Anaerobically Digested Wastewater Biosolids." *Water Science and Technology*. 52.1-2: 67-72.
- Dhillon, A., Lever, M., Lloyd, K.G., Albert, D.B., Sogin, M.L., and Teske, A (2005) "Methanogen Diversity Evidenced by Molecular Characterization of Methyl Coenzyme M Reductase A (*mcrA*) Genes in Hydrothermal Sediments of the Guaymas Basin." *Applied Environmental Microbiology*. 71.8:4592-4601.
- Diaz, E.E., Stams, A.J.M., Amils, R., and Sanz, J.L. (2006) "Phenotypic Properties and Microbial Diversity of Methanogenic Granules from a Full-Scale Upflow Anaerobic Sludge Bed Reactor Treating Brewery Wastewater." *Applied and Environmental Microbiology*. 72.7: 4942-4949.
- Earl, J., Hall, G., Pickup, R.W., Ritchie, D.A., and Edwards, C (2003) "Analysis of Methanogen Diversity in a Hypereutrophic Lake Using PCR-RFLP Analysis of *mcr* Sequences." *Microbial Ecology*. 46.2:270-278.
- Freitag, T.E., and Prosser, J.I. (2009) "Correlation of Methane Production and Functional Gene Transcriptional Activity in a Peat Soil." *Applied and Environmental Microbiology*. 75.21:6679-6686.

- Galand, P.E., Saarnio, S., Fritze, H., Yrjälä, K. (2002) "Depth Related Diversity of Methanogen *Archaea* in Finnish Oligotrophic Fen". *FEMS Microbiol Ecol.* 42.3: 441-449.
- Gracia M. de, Sancho L, Garcia-Heras J. L., Vanrolleghem P., and Ayesa E. (2006) "Mass and Change Conservation Check in Dynamic Models: Application to the New ADM 1 Model." *Water Science and Technology.* 53.1:225-240.
- Griffin, M.E., McMahon, K.D., Mackie, R.I., and Raskin, L. (1998) "Methanogenic Population Dynamics During Start-Up of Anaerobic Digesters Treating Municipal Solid Waste and Biosolids." *Biotechnology and Bioengineering.* 57.3: 342-355.
- Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., and Bailey, M.J. (2000) "Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA- Based Microbial Community Composition." *Applied and Environmental Microbiology,* 66.12:5488-5491.
- Hales, B.A., Edwards, C., Ritchie, D.A., Hall, G., Pickup, R.W. and Saunders, J.R. (1996) "Isolation and Identification of Methanogen-Specific DNA from Blanket Bog Peat by PCR Amplification and Sequence Analysis." *Applied Environmental Microbiology.* 62.2:668-675.
- Hartwell, L.H., Hood L., Goldberg, M.L., Reynolds, A.E., Silver, L.M., and Veres, R.C. (2004) *Genetics: From Genes to Genomes.* 2nd ed. McGraw-Hill Companies, Inc. New York, NY.
- Head, I.M., Sauders, J.R., and Pickup, R.W. (1998) "Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms." *Microbial Ecology.* 35:1-21.
- Hobson, P.N. and Wheatley, A.D. (1993) *Anaerobic Digestion, Modern Theory and Practice.* Elsevier Science Publishers, LTD. Essex, United Kingdom.
- Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., and Lens, P.N.L. (2004) "Anaerobic Sludge Granulation." *Water Research.* 38: 1376-1389.
- Jackson, A.A., Somers, K.M., and Harvey, H.H. (1989). "Similarity Coefficients: Measures for Co-Occurrence and Association or Simply Measures of Occurrence?" *The American Naturalist.* 133.3: 436-453.
- Jones, W.J., Nagle, D.P., and Whitman, W.B. (1987) "Methanogens and the Diversity of Archaeobacteria." *Microbiological Reviews.* 51.1: 135-177.

- Juottonen, H., Galand, P.E., and Yrjala, K. (2006) "Detection of Methenogenic *Archaea* in Peat: Comparison of PCR Primers Targeting the *mcrA* Gene." *Research in Microbiology*. 157.10:914-921.
- Karadagli, F. and B. E. Rittmann (2007) (b). "Mathematical Model for the Kinetics of H₂ Utilization by *Methanobacter bryantii* M.o.H. Considering Hydrogen Thresholds." *Biodegradation*. 18:453-464.
- Karadagli, F., and B. E. Rittmann (2007) (a). "Thermodynamic and Kinetic Analysis of H₂ Threshold for *Methanobacter bryantii* M.o.H.." *Biodegradation*. 18:439-452.
- Keyser, M., Witthuhn, R.C., Lamprecht, C., Coetzee, M.P.A., and Britz, T.J. (2006) "PCR- Based DGGE Fingerprinting and Identification of Methanogens Detected in Three Different Types of UASB Granules." *Systematic and Applied Microbiology*. 29.1: 77-84.
- Kosman, E. and Leonard, K.J. (2005) "Similarity Coefficients for Molecular Markers in Studies of Genetic Relationships Between Individuals for Haploid, Diploid, and Polyploid Species." *Molecular Ecology*. 14:415-424.
- Leclerc, M., Delgenes, J., and Godon, J. (2004) "Diversity of the Archaeal Community in 44 Anaerobic Digesters as Determined by Single Strand Conformation Polymorphism Analysis and 16S rDNA Sequencing." *Environmental Microbiology*. 6.8: 809-819.
- Lloyd, D., Thomas, K.L., Hayes, A., Hill, B., Hales, B.A., Edwards, C., Saunders, J.R., Ritchie, D.A., and Upton, M. (1998) "Micro-Ecology of Peat: Minimally Invasive Analysis Using Confocal Laser Scanning Microscopy, Membrane Inlet Mass Spectrometry and PCR Amplification of Methanogen-Specific Gene Sequences." *FMES Microbiology Ecology*. 25.2:179-188.
- Lueders, T., Chin, K.J., Conrad, R., and Friedrich, M (2001) "Molecular Analysis of Methyl-Coenzyme M Reductase α -Subunit (*mcrA*) Gene in Rice Field Soil and Enrichment Cultures Reveal the Methanogenic Phenotype of a Novel Archaeal Lineage." *Environmental Microbiology*. 3.3:194-204.
- Luton, P.E., Wayne, J.M., Sharp, R.J., and Riley, P.W. (2002) "The *mcrA* Gene as an Alternative to *16S rRNA* in the Phylogenetic Analysis of Methanogen Populations in Landfill." *Microbiology*. 148: 3521-3530.
- Madigan, M.T. and Martinko, J.M. (2006) *Brock Biology of Microorganisms*. 11th ed. Pearson Prentice Hall, Pearson Education, Inc. Upper Saddle River, NJ.

- Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., and Verstraete, W. (2008) "How to Get More Out of Molecular Fingerprints: Practical Tools for Microbial Ecology." *Environmental Microbiology*. 10.6:1571-1581.
- McCarty, P.L. and Smith, D.P. (1986) "Anaerobic Wastewater Treatment." *Environmental Science and Technology*. 20.12: 1200-1206.
- Mertens, Y., Boon, N., and Verstraete, W. (2005) "Stereospecific Effect of Hexachlorocyclohexane on Activity and Structure of Soil Methanotrophic Communities." *Environmental Microbiology*. 7.5:660-669.
- Muyzer, G. (1999) "DGGE/TGGE a Method for Identifying Genes from Natural Ecosystems." *Current Opinion in Microbiology*. 2: 317-322.
- Nercessian, D., Upton, M., Lloyd, D., and Edwards, C. (1999) "Phylogenetic Analysis of Peat Bog Methanogen Population." *FMES Microbiology Letters*. 173.2:425-429.
- Ohkuma, M., Noda, S., Horikoshi, K. and Kudo, T., (1995) "Phylogeny of Symbiotic Methanogens in the Gut of the Termite *Reticulitermes speratur*." *FMES Microbiology Letters*. 134.1:45-50.
- Owen, W.F., Stuckey, D.C., Healy, Jr., J.B., Young, L.Y., and McCarty P.L. (1979) "Bioassay for Monitoring Biochemical Methane Potential and Anaerobic Toxicity." *Water Research*. 13: 485-492.
- Pontes, D.S., Lima-Bittencourt, C.I., Chartone-Souza, E., and Nascimento, A.M.A. (2007) "Molecular Approaches: Advantages and Artifacts in Assessing Bacterial Diversity." *Journal of Industrial Microbiology and Biotechnology*. 34: 463-473.
- Radl, V., Gattinger, A., Chronakova, A., Nemcova, A., Cuhel, J., Simek, M., Munch, J.C., Schloter, M., and Elhottova, D. (2007) "Effects of Cattle Husbandary on Abundance and Activity of Methanogenic Archaea in Upland Soils." *The ISME Journal*. 1:443-452.
- Rastogi, G., Ranade, D.R., Yeole, T.Y., Patole, M.S. and Shouche, Y.S. (2008) "Investigation of Methanogen Population Structure in Biogas Reactor by Molecular Characterization of Methyl-Coenzyme M Reductase A (*mcrA*) Genes". *Bioresource Technology*. 99.13:5317-5326.
- Schloss, P. D. and Handelsman, J. (2005) "Introducing DOTUR, a Computer Program for Defining Operational Taxonomic Units and Estimating Species Richness." *Applied and Environmental Microbiology*. 71.3:1501-1506.

- Smith, D.P., and McCarty, P.L. (1990) "Factors Governing Methane Fluctuations Following Shock Loading of Digesters." *Research Journal of the Water Pollution Control Federation*. 62.1: 58-64.
- Smith, L.H. (1996) *Performance of a Two-Stage Process for Cometabolic Treatment of Trichloroethylene-Contaminated Water by Methane-Oxidizing Mixed Cultures*, Ph.D. Dissertation, Department of Civil Engineering, Stanford University, Stanford, CA.
- Sorensen, A. H. and Ahring B. K. (1993) "Measurements of the Specific Methanogenic Activity of Anaerobic Biomass." *Applied Microbiology and Biotechnology*. 40:427-431.
- Spearman, C. (1904) "The Proof and Measurement of Association Between Two Things." *The American Journal of Psychology*. 15.1:72-101.
- Speece, R. (2008) *Anaerobic Biotechnology and Odor/Corrosion Control for Municipalities and Industries*. Archae Press. Nashville, TN.
- Spiegelman, D., Whissell, G., and Greer, C. (2005) "A Survey of the Methods for the Characterization of Microbial Consortia and Communities." *Canadian Journal of Microbiology*. 51.5: 355-386.
- Springer, E, Sachs, M.S., Woese, C.R. and Boone, D.R. (1996) "Partial Gene Sequence for the A Subunit of Methyl-Coenzyme M Reductase (*mcrI*) as a Phylogenetic Tool for the Family *Methanosarcinaceae*." *International Journal of Systematic Bacteriology*. 45.3:554-559.
- Stams, A.J.M. (1994) "Metabolic Interactions Between Anaerobic Bacteria in Methanogenic Environments." *Antonie van Leeuwenhoek*. 66: 271-294.
- Steinberg, L.M., and Regan, J.M. (2009) "*mcrA*-Targeted Real-Time Quantitative PCR Method to Examine Methanogen Communities." *Applied and Environmental Microbiology*. 75.13:4435-4442.
- Thauer, R.K. (1998) "Biochemistry of Methanogenesis : A Tribute to Marjory Stephenson." *Microbiology*. 144:2377-2406.
- Topliss, J.G., and Costello, R.J. (1972) "Chance Correlations in Structure-Activity Studies Using Multiple Regression Analysis." *Journal of Medicinal Chemistry*. 15.10:1166-1168.

- Ufnar, J.A., Ufnar, D.F., Wang, S.Y., and Ellender, R.D. (2007) "Development of a Swine-Specific Fecal Pollution Marker Based on Host Differences in Methanogen *mcrA* Genes." *Applied and Environmental Microbiology*, 73.6:5209-5217.
- White, D. (2000) *The Physiology and Biochemistry of Prokaryotes*. Oxford University Press, Inc. New York, NY.
- Wilms, R., Sass, H., Köpke, B., Cypionka, H., and Engelen, B. (2007). "Methane and Sulfate Profiles Within the Subsurface of a Tidal Flat are Reflected by the Distribution of Sulfate-Reducing Bacteria and Methanogenic Archaea." *FMES Microbiology Ecology*. 59.3:611-621.
- Wittebolle, L., Vervaeren, H., Verstraete, W., and Boon, N. (2008) "Quantifying Community Dynamics of Nitrifiers in Functionally Stable Reactors." *Applied Environmental Microbiology*. 74.1:286-293.
- Woese, C.R. and Fox, G.E. (1977) "Phylogenetic Structure of the Prokaryotic Domain: the Primary Kingdoms." *Proceedings of the National Academy of Sciences of the United States of America*. 74.11: 5088-5090.
- Zar, J.H. (1972) "Significance Testing of the Spearman Rank Correlation Coefficient." *Journal of the American Statistical Association*. 67.339:578-580.
- Zhang, T., Fang, H.H.P. (2000) "Digitization of DGGE (Denaturing Gradient Gel Electrophoresis) Profile and Cluster Analysis of Microbial Communities." *Biotechnology Letters*. 22.5:399-405.
- Zheng, D. and Raskin, L. (2000) "Quantification of *Methanosaeta* Species in Anaerobic Bioreactors Using Genus- and Species-Specific Hybridization Probes." *Microbial Ecology*. 39.3: 246-262.
- Zitomer, D.H, Johnson, C.C. and Speece R.E. (2008) "Metal Stimulation and Municipal Digester Thermophilic/Mesophilic Activity." *Journal of Environmental Engineering*. 134.1:42-47.

CHAPTER 2: BIOAUGMENTATION FOR RECOVERY OF ORGANICALLY OVERLOADED DIGESTERS

2.1 Anaerobic Wastewater Treatment and Propionate

2.1.1 Importance of Hydrogen in Propionate Degradation

Traditionally, propionate accumulation has been seen as an indicator of process imbalance in an anaerobic digester and propionate-utilizing microbes play an important role when anaerobic digesters are subjected to organic overload due to complex substrate. Under standard conditions, metabolism of propionate to acetate and hydrogen gas is energetically unfavorable (see Table 1.1), but conversion of hydrogen and carbon dioxide to methane drives the overall reaction in the forward direction. Degradation of propionate is theoretically favorable only when the hydrogen concentration in an anaerobic digester lies between 10^{-4} to 10^{-6} atm (McCarty and Smith, 1986). If the dissolved hydrogen gas concentration goes above this range, then propionate degradation becomes energetically unfavorable and stops. (see section 1.1.1 for the review).

2.1.2 Propionic Acid Toxicity

Besides being an energetically challenging substrate, propionic acid can be inhibitory to methanogens at high concentrations. Barredo and Evison (1991) studied the effect of propionate toxicity at pH values of 6.5, 7.0 and 8.0 on methanogen-enriched sewage sludge and pure cultures of the hydrogen-utilizing methanogens *Methanobrevibacter smithii* and *Methanospirillum hungatii*. Test assays fitted with screw caps and butyl rubber septa were fed with different doses of the sodium salt of propionate

and equal amounts of biomass to determine propionate toxicity. Toxicity of propionate was characterized by a decrease in the amount of methane produced and the microbial count. Results of the study indicated that even 20 to 30 mM (1.48 g/L to 2.22 g/L) of propionic acid decreased the methanogen count from the enriched sludge at all pH ranges. At a propionic acid concentration of 80 mM (5.92 g/L), the methanogen count in enriched sludge decreased by two orders of magnitude. Also, comparison of pure cultures with the enriched culture showed that they were equally sensitive to propionic acid. There was a clear tendency for cumulative methane production to decrease as the propionate concentration increased.

Ahring et al. (1995) studied the effect of volatile fatty acids (VFAs) on methane production rates during digestion of manure in batch experiments. Different doses of sodium salts of acetate, propionate, butyrate and valerate were added to the mixed culture to evaluate their toxicity. Methane production rate increased with increasing concentrations of VFA up to 50 mM (3.7 g/L of propionic acid) for all VFA tested. A slight decrease was observed in the methane production rate at 200 mM (1200 mg/L of acetic acid) acetate and butyrate, while methane production rate from propionate decreased by 50% when the propionate concentration was increased from 50 mM (3.7 g/L) to 100 mM (7.4 g/L).

Dhakad et al. (2003) studied the effect of propionic acid toxicity on a mixed culture degrading night soil at psychrophilic (10 °C) and mesophilic (30 °C) temperatures at different pH conditions. The decrease in biogas production of 60.4% at 10 °C and 77% at 30 °C was reported at the propionate dose of 200 mM (14.8 g/L equivalent to 30.8 g propionate/g VS) at pH 7. Also, a 2 log decrease in methanogenic cell counts was

observed for the pH values 6, 7 and 8 at 10 °C for a propionic acid concentration of 200 mM (14.8 g/L), whereas a similar decrease in methanogenic count at 30 °C was only observed at a pH 7.

Savant and Ranade (2004) studied toxicity of propionate on *Methanibrevibacter acididurans* (MCM B 613) and *Methanobacterium bryantii* (MCM B 608) separately at mesophilic temperatures. Autoclaved growth medium suitable for growing *Methanibrevibacter acididurans* and *Methanobacterium bryantii* was added to 38-mL glass vials and the medium pH was adjusted to 5.0, 6.0 or 7.0 respectively using NaHCO₃ or HCl. Furthermore, the sodium salt of propionate was added to provide 0 and 1.0 g/L propionate concentration in the vials maintained at pH 5. Vials maintained at pH 6 were supplied with 0, 1.0, 5.0 and 10.0 g propionate/L, and the vials maintained at pH 7 were supplied with 0, 1.0, 5.0, 10.0 and 20.0 g/L propionate. Sodium salt of propionate was used for the experiments. Pure cultures of *M. acididurans* and *M. bryantii* having optical density of 0.4 were mixed in a 1:9 (w/w) proportion in the vials containing propionate and the growth medium. H₂:CO₂ gas (80:20 v/v) was used as the substrates for the methanogenic strains. The headspace of each vial was flushed by H₂:CO₂ gas once a day and a headspace pressure of 2 atm (absolute) was maintained. All the vials were maintained anaerobically at 35 °C on a shaker table for 8 days. Samples of the headspace gas were analyzed for methane content every day using gas chromatography and the cumulative methane produced after 8 days was determined to evaluate propionate toxicity. Results of the study revealed that at pH 7.0, cumulative methane production from *M. acididurans* was reduced to half of its maximum value at 14 g/L propionate concentration, whereas there was a 30% decrease in cumulative methane production by

M. bryantii at 20g/L propionate concentration. At pH 6.0, cumulative methane production from *M. acididurans* and *M. bryantii* was reduced by 50% of its maximum value at 9 g/L and 4.6 g/L propionate concentration respectively, whereas at pH 5.0, cumulative methane production from *M. acididurans* and *M. bryantii* was reduced by 25% and 32% of their maximum values at a 1 g/L propionate concentration.

2.1.3 Bioaugmentation for Enhancing Microbial Community Function

Bioaugmentation is defined as adding specialized microorganisms or enzymes to biological systems to improve process performance (Rittmann and Whittemann, 1994; Maier et al., 2000; Deflaun and Steffan, 2002; Mulligan, 2002; Evans and Furlong, 2003). Bioaugmentation can also be viewed as the use of preselected specialized, mutant or adapted bacterial blends that can help enhance overall system performance and stability and reduce costs (Nyer and Bourgeois, 1980). Bioaugmentation has been traditionally considered for remediation of hazardous waste sites, but more recently has been studied for use in other applications, such as improving anaerobic digesters. Applications for wastewater treatment include more reliable nitrification, improved sludge settling, enhanced grease degradation and accelerated transformation of xenobiotic organic contaminants (Rittmann and Whittemann, 1994). A review of bioaugmentation research relating to environmental remediation, wastewater treatment and finally, anaerobic digestion is presented below.

2.1.3.1 Removal of Contaminants from Environmental Systems

Bioaugmentation has been used for remediation of sites contaminated with specific chemicals such as chlorinated volatile organic compounds (CVOCs) (Deflaun and Steffan, 2002; Streger et al., 2002; McCarty, 1997); benzene, toluene, ethylene and xylene (BTEX); phenols; methyl tertiary butyl ether (MTBE) and polychlorinated biphenyls (PCBs). The added organisms may mineralize the pollutant in the environment and may remediate the site at a faster rate than the indigenous microorganisms (Singer et al., 2005).

2.1.3.1.1 Chlorinated Volatile Organic Compounds (CVOCs)

For a review of various types of bioaugmentation projects to remediate CVOC contaminated sites, refer to Deflaun and Steffan (2002) and Cupples (2008).

Cupples (2008) provides an overview of the applications for investigating the importance of *Dehalococcoides sp.* for bioremediation purposes. *Dehalococcoides sp.* is known to reductively dechlorinate a wide variety of environmental contaminants including CVOC's. It was therefore concluded that these organisms can be used to bioaugment various sites that may contain these pollutants (Ellis et al., 2000; Major et al., 2002; He et al., 2002; Lendvay et al., 2003; Rahm et al., 2006; Sleep et al., 2006).

Ellis et al., (2000) studied bioaugmentation for dechlorinating trichloroethylene (TCE) to ethene in a contaminated aquifer. Before implementation of bioaugmentation, there was no dechlorination beyond cis-dichloroethylene (DCE). An ethene-forming microbial mixed culture that contained *Dehalococcoides ethenogenes* was introduced on

days 269 and 284 of the study. Vinyl Chloride (VC) was observed in the aquifer by day 360, which was followed by ethene on day 367. By day 509, TCE and cDCE were completely degraded to ethene, suggesting that bioaugmentation can act as a supplement to natural attenuation and make bioremediation more effective.

Major et al. (2002) studied bioaugmentation of groundwater contaminated with perchloroethylene (PCE), TCE and cDCE for reductive dechlorination. The initial biostimulation of the site using methanol and acetate effected dechlorination of PCE to cDCE. A natural dechlorinating culture containing organisms related to *Dehalococcoides ethenogenes* was then added to the groundwater. Results of the study indicated that the dechlorination proceeded until ethene became the prominent constituent (88% of the total concentration) in the groundwater 142 days after bioaugmentation.

He et al. (2002) determined the reductive dechlorination process end points in microcosms established with aquifer material contaminated with chloroethene using a variety of electron donors including H₂ and acetate. The microorganisms in the aquifer dechlorinated PCE, TCE, cis-DCE, and VC completely to ethene within 14 weeks. These microcosms were bioaugmented with *Dehalococcoides multivorans* or a defined *Dehalococcoides sp.* containing a strict hydrogenotrophic, PCE-dechlorinating mixed culture.

Lendvay et al. (2003) compared bioaugmentation and biostimulation of chloroethene-contaminated aquifers. Biostimulation was achieved by continuous lactate and nutrient injection which resulted in complete dechlorination after a three-month lag period. The bioaugmentation experiment involved addition of a *Dehalococcoides-*

containing consortium that degraded PCE to ethene and resulted in complete dechlorination to ethene within six weeks.

Rahm et al. (2006) studied the microbial communities of two different sites that experienced biostimulation and bioaugmentation. At the first site, biostimulation with lactate led to complete dechlorination of TCE to ethane, whereas at the second site, biostimulation resulted in incomplete dechlorination. The bioaugmentation strategy was then explored by using a mixed culture that contained *Dehalococcoides sp.*. The bioaugmentation resulted in complete reduction to ethene.

Sleep et al. (2006) observed enhanced PCE reduction from a dense nonaqueous phase liquid (DNAPL) source. Using contaminated soil and groundwater from Dover Air Force Base in Delaware, dechlorination was studied using biostimulation alone and biostimulation along with bioaugmentation. The biostimulation using methanol, acetate and ethanol addition produced no dechlorination, but when one system was bioaugmented with a dechlorinating culture containing *Dehalococcoides sp.*, dechlorination to ethene was effectively achieved.

2.1.3.1.2 Benzene, Toluene, Ethylene and Xylene (BTEX)

Da Silva and Alvarez (2004) studied whether anaerobic bioaugmentation enhances the natural attenuation of BTEX in groundwater contaminated with ethanol-blended gasoline. They studied bioaugmentation using two different microbial consortia: one enriched with toluene and o-xylene and another enriched with benzene. High BTEX removal efficiency (88% removal of benzene and 99% removal of o-xylene) was

sustainable for over one year in soil bioaugmented with both consortia. When investigating soil that was not bioaugmented, toluene was the only contaminant that could be biodegraded and this took a period of two years.

2.1.3.1.3 Phenols

Zou et al. (2000) investigated the removal of pentachlorophenol (PCP) through a comparison of biostimulation and bioaugmentation. The culture used for bioaugmentation was a PCP-degrading methanogenic consortium from a fluidized bed reactor and was immobilized on a porous silica/alumina support material that was approximately 0.5 mm in diameter. Biostimulation was accomplished using 2 g glucose/kg soil and was almost as effective as bioaugmentation with 0.14 g VSS/kg soil (90% removal in 20 days by biostimulation as compared to 90% removal in 15 days by bioaugmentation). A combination of both treatments removed PCP at a faster rate than the treatments by themselves with the combination reaching 90% removal of PCP in less than 14 days.

Ramadan et al. (1990) investigated the mineralization of p-nitrophenol (PNP) in lake water using *Burkholderia cepacia*. *Burkholderia cepacia* was known to grow on and mineralize PNP in a salt solution. This species did not mineralize 1.0 µg of PNP per mL and the cell population declined in 13 hours until no cells were detected. The authors suggested that the decline in the population of the bioaugmented culture may have occurred because they were eliminated by protozoan grazing or nutrient deficiencies. Results of Ramadan et al. (1990) validated earlier findings of Goldstein et al., (1985) who also arrived at similar conclusions when studying bioaugmentation for remediation of

PNP in various environmental systems. It was concluded that it is important to introduce a bioaugmentation culture that can establish itself in the environment and the environment must have the required nutrients for the survival of the bioaugmentation culture.

2.1.3.1.4 Methyl Tertiary-Butyl Ether (MTBE)

Streger (2002) studied removal of MTBE using *Hydrogenophaga flava* ENV735 and the adhesion-deficient strain ENV735:24 using lab soil columns and serum bottles. The authors concluded that even though the rate of MTBE degradation was slightly lower in the adhesion-deficient strain, the improved transport through the soil of that strain outweighed the slight decrease in the removal rate. This adhesion-deficient strain can disperse throughout the column and cause a significant reduction of contaminant levels throughout a contaminated aquifer.

2.1.3.1.5 Polychlorinated Biphenyls (PCBs)

Luepromchai et al. (2002) studied bioaugmentation of PCB-contaminated soil with a combination of PCB-degrading bacteria (*Ralstonia eutrophus* and *Rhodococcus sp.* strain ACS) and earthworms. Results of the study showed that 50% PCB removal occurred in the top 9 cm of the soil when bioaugmentation and earthworms were used in combination. When the bioaugmentation or earthworms were implemented separately, 50% removal could only occur within the top 3 cm.

Singer et al. (2000) studied the use of two bacterial species (*Arthrobacter sp.* strain BIB and *Ralstonia eutrophus* H850) along with a surfactant to remove PCBs from

contaminated soil. After bioaugmentation for 34 weeks, PCB removal in the bioaugmented soil was found to be 55-59% as compared to only 30-36% removal in the soil that was introduced to only the surfactant. Even though complete removal of PCBs did not occur, introducing the bacteria did provide partial decontamination and could be used with other remediation strategies.

Specific microorganisms able to degrade specific compounds can be used for bioaugmentation if the environment is right for the establishment and growth of these microorganisms. Without the proper environmental conditions, however, the microorganisms may not survive and therefore the degradation of the pollutant may not take place (Goldstein et al., 1985; Ramadan et al., 1990).

2.1.3.2 Wastewater Treatment

Bioaugmentation for wastewater treatment may have started in the 1960s in order “to address problems of slow biomass recovery and to supplement lost bacterial populations.” (Rittmann and Whiteman, 1994). Bioaugmentation can theoretically help prevent problems from BOD/COD overload, improve the degradation of pollutants, induce or stabilize nitrification, speed recovery from plant upsets, and prevent odor, foaming, and algae growth (Gaiek, 1988).

Stephenson and Stephenson (1992) provided a review of the bioaugmentation literature completed by independent investigators. The review consisted of five full-scale cases and 14 lab-scale cases. There was varied performance when bioaugmentation was applied as seen in Table 2.1. A full explanation of each of these studies with references

can be found in Stephenson and Stephenson (1992). The authors concluded that bioaugmentation can sometimes be successful for the improvement of various wastewater treatment systems but failures can also occur when adding cultures to wastewater treatment systems. A number of reasons for failure have been postulated including inhibitory substances or conditions in the system, competition with other microorganisms, low microorganism count and improper environment for the specific bioaugmentation culture. In order to clearly understand these successes and failures, more bioaugmentation studies are needed.

Van Limbergen et al. (1998) compiled a review of wastewater treatment bioaugmentation. They suggested that bioaugmentation can be used to improve flocculation and degradation of recalcitrant compounds. They reviewed four activated sludge bioaugmentation projects using different *Pseudomonas* organisms, as shown in Table 2.2. Since survival of the bioaugmented microorganisms is very important for bioaugmentation in wastewater treatment applications to succeed, the authors proposed that pre-adaptation of the strains to the activated sludge conditions before bioaugmentation could help prevent problems with lack of survival caused by a sudden change in environmental conditions.

Table 2.1: Overview of Bioaugmentation Literature (Stephenson and Stephenson, 1992)

Waste Type	Process	Scale	Result
Bearing factory	Trickling filter	Full	Improved BOD removal in cold weather
Dairy	Extended aeration system	Full	Improved BOD removal and prevented bulking
Municipal	Aerated lagoon	Full	Reduced sludge blanket
Municipal	Activated sludge	Lab	Little effect on performance
Dairy	Fill and draw	Lab	Failed to prevent bulking
PNP	Activated sludge	Lab	Product gave same result as adding fresh organisms
Phenol	Batch	Lab	Municipal sludge better than product
Municipal	Activated sludge	Lab	Little effect
Hazardous	Batch	Lab	Feasibility uncertain
Phenol	SBR steady state	Lab	Improved start-up
Phenol	SBR non-steady state	Lab	Improved performance
Potato	Activated sludge	Lab	Improved COD removal
Pineapple waste	Activated sludge	Lab	Increased total organic carbon (TOC) removal and nitrification
3-CB	SBR	Lab	Improved start-up but not performance
3-CB	SBR	Lab	Enhanced degradation rates
Shock organic load	Anaerobic filters	Lab	No significant influence on recovery

Table 2.2: Overview of Bioaugmentation Experiments Using Pseudomonas Strains (Van Limbergen et al., 1998)

Strain	Process	Scale	Result
<i>Pseudomonas putida</i> ATCC11172	Sequencing Batch Reactor (SBR)	Lab	Increased phenol degradation
<i>Pseudomonas sp.</i> FR120	CSTR	Lab	Increased degradation of 3- Chlorobenzoate and 4-methylbenzoate
<i>Pseudomonas putida</i> EB62	CSTR	Lab	Slight increase in degradation of 4- ethylbenzoate
<i>Pseudomonas putida</i> UWC1	Activated sludge	Lab	No increased degradation of 3- Chlorobenzoate

2.1.3.3 Bioaugmentation and Nitrification

Addition of nitrifying bacteria has been studied for wastewater treatment bioaugmentation (Rittmann and Whitman, 1994). Using bioaugmentation for nitrification is especially promising because the nitrifying bacteria have slow growth rates and therefore recover very slowly when encountering issues such as uncontrolled biomass loss, pH swings, toxic shocks, or temperature decrease (Rittmann and Whitman, 1994; Abeysinghe et al., 2002; Satoh et al., 2003; Head and Oleszkiewicz, 2005). Also, the nitrifying activity can be easily monitored in a wastewater treatment system using measures such as loss of Kjeldahl-N, formation of NO₃-N, and consumption

of alkalinity (Rittmann and Whiteman, 1994). Finally, molecular tools can be used to track nitrifying bacteria throughout the system (Rittmann and Whiteman, 1994).

Rittmann and Whiteman (1994) presented two case studies that demonstrate successful bioaugmentation related to nitrification. The first case study involved a municipal wastewater treatment system that was unable to nitrify during the cold temperatures of winter. The 5,000 m³/day facility was bioaugmented with an initial dose and maintenance dose that were not fully described in the paper (Rittmann and Whiteman, 1994). The effluent ammonia levels were reduced from approximately 35 mg/L at the beginning of the experiment to 10-15 mg/L after 15 days. It was determined that the maintenance dose would have to continue for the bioaugmentation to be successful because conditions were not sufficient to build up the *Nitrosomonas* biomass to a stable level (Rittmann and Whiteman, 1994).

The second case study was performed at a chemical-manufacturing plant using *Nitrosomonas* bioaugmentation cultures (Rittmann and Whiteman, 1994). An initial dose was introduced into the system, followed by a maintenance dose. The initial dose and the maintenance dose were not fully described in the paper (Rittmann and Whiteman, 1994). Full nitrification was seen after approximately 15 days of bioaugmentation with ammonia removal of 100%, whereas other oxidized forms of nitrogen (NO₂⁻ and NO₃⁻) increased. This bioaugmentation program was successful and the chemical facility continued to add the microorganisms for five years in order to improve process performance without any additional plant modifications.

Satoh et al. (2003) conducted a laboratory study to observe the effect of bioaugmentation and biostimulation on the start-up of nitrification process in three rotating disk biofilm reactors. One reactor was bioaugmented with an enrichment culture of nitrifying bacteria, another was fed with synthetic medium that contained NH_4^+ and NO_2^- for biostimulation, and the third was used as a control. NH_4^+ and NO_2^- oxidation occurred 10 to 20 days faster in the bioaugmented reactor as compared with the control, whereas the biostimulated reactor achieved NH_4^+ and NO_2^- oxidation 10 to 20 days after the control (Satoh et al., 2003).

Operating a nitrifying system at cold temperatures can cause nitrifiers to wash out of a system and, therefore, Abeysinghe et al. (2002) studied the effect of bioaugmentation during this stressed condition. Laboratory-scale CSTRs were operated by decreasing the temperature to 4°C with an SRT of 5 days. When the nitrifying organisms were added to this low temperature system, the effluent NH_4^+ -N concentration decreased to approximately 25 mgN/L, whereas the control reactor had an effluent concentration of approximately 100 mgN/L. Following the one-time bioaugmentation event, the NH_4^+ -N concentration increased again to levels similar to the control reactor which was attributed to the washout of the organisms. These results demonstrate the possible need of continual bioaugmentation in order to ensure low effluent NH_4^+ -N concentrations at cold temperature and low SRT.

Head and Oleszkiewicz (2005) studied the effect of the bioaugmentation culture temperature (10, 20, 25, and 30°C) on the success of bioaugmentation of a sequencing batch reactor (SBR) operating below 10°C . The authors concluded that partial NH_3 -N

removal could be achieved using the bioaugmentation cultures that were acclimated to 20, 25 and 30°C when the HRT was 24 hours but continual and consistent bioaugmentation was necessary for sustained NH₃-N removal.

2.1.3.4 Anaerobic Bioaugmentation

The effect of bioaugmentation on the anaerobic removal of specific organic chemicals, removal of fats oil and grease, degradation of cellulose, odor reduction and recovery of stressed reactors has been studied to a limited extent.

2.1.3.4.1 Specific Organics Removal

Guiot et al. (2000) studied degradation of phenol, ortho- and para-cresol in upflow anaerobic sludge blanket reactors bioaugmented with different amounts of an enriched methanogenic mixed culture that was able to degrade these specific chemicals. Addition of 2 to 5% enrichment culture (expressed as mass/mass with respect to the non-acclimated granular inoculum biomass) decreased the start-up period of the reactor to 55 days to achieve 80% phenol degradation, whereas the control reactor fed with no enrichment culture took 100 days to achieve the same level of phenol degradation. During continuous operation of the reactors, the bioaugmented reactors showed at least two-fold more specific activity to degrade the targeted compounds as compared to the non-bioaugmented reactor.

Guiot et al. (2002) studied enrichment of anaerobic sludge for the degradation of pentachlorophenol (PCP) by an on-line control-based selective stress strategy (controlling the feeding rate of PCP by feedback control from methane percentage in the biogas

produced) and bioaugmentation of anaerobic sludge using the PCP-degrading microbe *Desulfotobacterium frappieri* (PCP-1). Both the selective stress strategy and the PCP-1-augmented culture resulted in a specific degradation rate of $4 \text{ mg PCP g}^{-1} \text{ VSS day}^{-1}$, but the selective stress control system culture took 120 days to attain complete degradation capacity while the bioaugmented culture took only 56 days. Furthermore, fluorescent in situ hybridization (FISH) of granule cross sections showed no fluorescence signal for PCP-1 specific probes in the selective stress strategy enrichment culture, whereas a strong fluorescence signal for PCP-1 was present in the culture bioaugmented with PCP-1 after 5 and 9 weeks.

Ahring et al. (1992) bioaugmented anaerobic granules using a pure culture of a 3-chlorobenzene (3-CB) degrading microbe (*Desulfomonile tiedjei*) to impart 3-CB dechlorinating ability to UASB reactors. Also a three-member consortium containing *D. tiedjei*, a benzoate degrading coculture and a hydrogen-utilizing methanogen was used to bioaugment a separate UASB reactor. A third control UASB reactor with no bioaugmentation was also operated. All the reactors were fed with basal medium, formate, acetate and 3-CB. Results of the study indicated that 3-CB did not degrade in the control reactor, whereas the reactors bioaugmented with *D. tiedjei* and the three-member consortium transformed 3-CB at a rate of $54 \mu\text{mol/day/g}$ granule biomass. Even after reducing the HRT of the bioaugmented reactors to 0.5 days (which is much shorter than the generation time of *D. tiedjei*), the reactors still dechlorinated 3-CB, indicating immobilization of microbes in the granules which was further confirmed by immunological studies.

Saravanane et al. (2001) bioaugmented fluidized bed reactors with a cephalixin-enriched anaerobic culture to evaluate cephalixin-degrading behavior of the reactor. Results of the study revealed an initial COD removal of 88% for the first 2 to 8 days after which the removal efficiency rapidly declined suggesting cell biomass washout. Further study revealed that periodic inoculation of the enrichment culture every 2 days yielded COD removal efficiency of 88% for the entire duration of the experiment (32 days).

Tartakovsky et al. (1999) inoculated anaerobic sludge granules obtained from a UASB treating food processing wastewater with a strain of pentachlorophenol (PCP) degrader, *Desulfitobacterium frappieri* PCP-1, and used competitive polymerase chain reaction (cPCR) to observe the adaptability of PCP-1 strains in the granules. Also, the PCP degrading ability of the resulting consortium was tested using a lab-scale UASB reactor. The PCP-1 strain succeeded in competing within the microbial community present in the granule and it increased from 10^6 to 10^{10} cells/g volatile suspended solids within 70 days resulting in PCP removal efficiency of 99%.

Tawfiki et al. (2000) studied the effect of mixing two different anaerobic consortia, one capable of removing phenol and ortho-cresol and the other capable of removing para-cresol, in a fixed film anaerobic reactor. For continuous flow, phenolic compounds removal with the mixed consortia was as good as that achieved by each of the two individual consortia against their respective substrates. Further batch studies revealed that, for the mixture of cultures, phenol removal was complete after 11 days while the phenol degrading consortium alone took 35 days for the same amount of phenol degradation. Also, the mixed consortia totally removed o-cresol after 22 days while no removal of o-cresol was observed even after 35 days in the phenol degrading consortium

alone. On the other hand, the mixed consortium took 17 days more for degradation of p-cresol as compared with the time taken for degradation of p-cresol by the p-cresol degrading consortium alone.

Horber et al. (1998) studied dechlorination of PCE in UASB reactors. A strictly anaerobic, reductively dechlorinating bacterium, *Dehalospirillum multivorans*, was incorporated into granular sludge used for the test assay. Also a control reactor (R1) containing pre-autoclaved granular sludge was supplied with *D. multivorans* and a third UASB reactor (R2) was seeded with the same amount of active granular biomass but no bioaugmentation. All the reactors were fed PCE, formate and acetate. Both the test reactor and reactor R1 converted 93% of the PCE to DCE, whereas the non-bioaugmented reactor (R2) converted only 43% of the PCE to trichloroethane. Interestingly, the test reactor and reactor R1 showed conversion of PCE to DCE at hydraulic retention times (HRTs) much lower than the reciprocal maximum growth rate of *D. multivorans*, indicating immobilization of the microbe in the living and autoclaved granules which was further confirmed by immunological studies conducted on the granules.

Lenz et al. (2009) studied the effect of bioaugmentation of a UASB reactor with immobilized selenate-accumulating *Sulfurospirillum barnesii* cells on selenate removal. Initially, *S. barnesii* cells were immobilized in acrylamide gels and the gel cubes were used for bioaugmentation of a mesophilic anaerobic digester fed with lactate (electron donor) at an organic loading rate (OLR) of 5 gCOD/L-day. The reactor was also fed 2mM sulfate and 10 μ M selenate and 15mM nitrate (electron acceptor). Selenate was reduced efficiently (more than 97%) in the reactor and the scanning electron micrograph

revealed that the selenate was reduced by the immobilized *S. barnesii* cells. Furthermore, to validate these findings under a microbial competitive environment, *S. barnesii* immobilized cells were added to a granular UASB biomass and the reactor was operated on the same synthetic waste described above. Operation of the reactor showed that the bioaugmented reactor took 24 HRTs to attain 97% selenate removal as compared with 44 HRTs required by the non-bioaugmented reactor. Microbial community analysis of the reactor biomass revealed that the *S. barnesii* cells were effectively immobilized in the bioaugmented reactor even after 58 days of operation.

2.1.3.4.2 Fats Oil and Grease Removal

Cirne et al. (2006) studied the effect of bioaugmentation of anaerobic reactors degrading lipid rich waste (containing 10% lipids triolein) with a lipolytic bacterial strain, *Clostridium lundense* (DSM 17049^T). Results of the study indicated that bioaugmentation increased the methane production rate and hence reduced the time required for 80% of the total methane recovery by approximately 30 %.

2.1.3.4.3 Cellulose Degradation

Many organic substrates contain cellulosic material which degrades relatively slowly under anaerobic conditions. Bioaugmentation strategies were examined to improve the overall degradation rate of cellulose-containing substrates under anaerobic conditions.

Weiß et al. (2010) studied degradation of xylan (heteropolysaccharides with a homopolymetric backbone chain of β -1,2-linked _D-xylopyranose units) using enriched

hemicellulolytic bacteria immobilized on activated zeolite. Second stage sludge containing hemicellulolytic bacteria from a two stage biogas plant was enriched for 0.1% (w/v) xylan in a nutrient medium. After 5 days of cultivation, 10% (v/v) of the enriched culture again was transferred in fresh nutrient medium containing 0.1% (w/v) xylan. After 13 enrichment cycles, 10% of dry cell mass was immobilized on 5g zeolite and the immobilized bacterial cells and the zeolite was air dried for two days. About 0.2% (w/v) of this dry material was introduced in batch anaerobic digesters containing xylan. Control digesters were augmented with 0.2% (w/v) zeolite material without bacterial cells. All the digesters were operated for 34 days. The effect of bioaugmentation was visible in terms of methane production from day 9. On the 26th day of the experiment, bioaugmentation with the immobilized bacteria increased methane recovery by 73.2 LCH₄/kgVS equivalent to 121% more methane production than control digesters. The authors concluded that bioaugmentation with hemicellulolytic bacteria immobilized on activated zeolite increased xylan degradation rate of anaerobic digesters.

Nielsen et al. (2007) studied the effect of bioaugmentation of two thermophilic strains *Caldicellulosiruptor lactoaceticus* (strain 6A) and *Dictyoglomus* (strain B4a) on the degradation of manure, liquid fraction of manure, fibers from the manure and anaerobically digested fibers. Results of the batch studies revealed that both strains increased methane recovery from all the substrates tested and strain 6A showed better potential for improving the methane yield as compared to strain B4a. Strain 6A was further used to bioaugment a two-stage thermophilic (68°C/55°C) digester. Results of the study indicated a 93% increase in the methane yield of the pretreatment reactor, whereas the overall methane yield of both the reactors was only slightly improved. The

improvement was attributed to degradation of cellulose material in the pretreatment reactor due to bioaugmentation. Also it was observed that the increased methane yield lasted till day 47 of the experiment and at day 50, the methane production suddenly dropped which was attributed to washout of strain 6A from the reactor.

Mladenovska et al. (2001) carried out batch studies on anaerobic Avicel- and xylan-degrading bacteria *Ruminococcus albus* DSM 20455, *Acetivibrio cellulolyticus* DSM 1870, *Clostridium cellulovorans* DSM 3052, *Clostridium aerotolerans* DSM 5434, and two new isolates (SA14 and KMX1). It was found that under methanogenic conditions, DSM 3052 and SA14 showed the maximum potential for cellulosic biomass degradation, so they were further used for bioaugmentation studies of a mesophilic continuously stirred tank reactor (CSTR). Bioaugmentation with isolate SA14 resulted in increased methane production by 62 % for the first five days and a decrease to background values on day 7 which was attributed to washout of the cell biomass of SA14 from the reactor.

Angelidaki and Ahring (2000) carried out experiments on the improvement of biogas yield from manure by the treatment of recalcitrant matter present in the manure by hemicellulose degrading bacterium B4. The treatment resulted in a 30% increase in methane production compared to the control reactors.

2.1.3.4.4 Increased Biogas Production

Savant and Ranade (2004) studied the effect of bioaugmenting with a *Methanobrevibacter acididurans* strain on the cumulative methane production from a distillery wastewater. Anaerobic cultures from lab-scale acidogenic and methanogenic

digesters treating distillery wastewater were used as inocula and were added to 125-mL serum bottles with 10 mL distillery waste. Headspace of the bottles was flushed with N₂ gas and sealed to maintain anaerobic conditions. One liter of the freshly grown *M. acididurans* culture was centrifuged to concentrate *M. acididurans* biomass. Further, one gram of the concentrated biomass was resuspended in 10 mL basal media and the resuspension liquid was used for bioaugmentation of test assays. Respective control assays were supplied with 10 mL of basal media. All the test and control assays were maintained anaerobically at 35⁰C under shaking conditions for 8 days. The test assays containing acidogenic culture showed 12% more cumulative methane than their respective control assays. The increase in cumulative methane production from the test assays supplied with methanogenic culture was found to be 7.5% as compared with respective control assays. Also headspace of the test assays supplied with acidogenic cultures showed 23.5% less hydrogen accumulation as compared to their control assays. The authors concluded that bioaugmenting with *M. acididurans* increased the methane production rate of methanogenic systems.

Bagi et al. (2007) carried out laboratory-scale experiments on bioaugmentation of batch fed anaerobic digesters operating on sewage sludge, pig manure and Jerusalem choke with two different hydrogen producing bacteria (HPB) *Caldicellulosyruptor saccharolyticus* (DSM 8903) and *Enterobactor cloacae* (DSM 16657) at thermophilic (55⁰C) and mesophelic (35⁰C) temperatures. An anaerobic culture obtained from an anaerobic, thermophilic wastewater sludge digester was used as an innoculum for carrying out the experiment. An equal amount of the innoculum was added to 12 serum bottles simulating anaerobic digesters. The serum bottles were divided in three groups

and each group was supplied with either sewage sludge, pig manure or Jerusalem choke so that the substrate:biomass ratio of 10:90 (v/v) was maintained in each reactor. The pH value of all the assays was adjusted to 7.0 using phosphate buffer. Furthermore, two test assays from each group were bioaugmented with pure cultures of *C. saccharolyticus* and *E. cloacae*. The pure cultures represented 5% of the active volume of the test assays. The associated control assays from the same group were supplied with equivalent amounts of the growth medium of the respective organism. Headspace of all the assays was flushed with nitrogen gas to maintain anaerobic conditions. The test assays bioaugmented with *C. saccharolyticus* and respective control assays having growth medium of *C. saccharolyticus* were incubated at 55⁰C and the remaining assays were incubated at 35⁰C to provide optimal growth temperatures for the bioaugmented cultures. Biogas from all the assays was collected by a water displacement method and all the assays were operated for 30 days. The biogas produced by the test assays per unit mass of substrate dry matter was higher by 42 to 57% as compared to that of their respective control assays. Further field studies carried out on the effect of bioaugmentation by a pure culture of *C. saccharolyticus* on a 5 m³, anaerobic CSTR digester operated at 55⁰C confirmed the earlier findings. Molecular techniques used during the field study revealed that the *C. saccharolyticus* strain was absent in the field reactor before bioaugmentation, but *C. saccharolyticus* was detectable in the reactor even after 12.6 SRTs following bioaugmentation, which proved its survival in the reactor.

2.1.3.4.5 Odor Reduction

Duran et al. (2006) studied the effects of bioaugmenting laboratory-scale anaerobic biosolids digestion with a commercial product containing microbes from the genera *Bacillus*, *Pseudomonas* and *Actinomyces* marketed by Organica Biotech, (Norristown, PA). The bioaugmented digester generated 29% more net methane during the 8 weeks of operation. In addition, the average residual propionic acid concentration in the bioaugmented digester was 54% of that in control and the biosolids digested in the bioaugmented digester generated a negligible amount of odorous methyl mercaptan (CH_3SH) during 10 days of post-digestion storage, while CH_3SH concentration in the control reached nearly 300 ppm_v during the same period. Similarly, peak dimethyl sulfide (CH_3SCH_3) generated by the stored biosolids from the bioaugmented digester was only 37% of that from the control. Thus lower concentrations of CH_3SH and CH_3SCH_3 in the bioaugmented reactor result in lesser odor and may have been due to improved methanogenesis during storage of the digested and dewatered biosolids. Although laboratory-scale results were promising, full scale pilot-testing did not result in process improvement (Toffey et al., 2007).

2.1.3.4.6 Stressed Reactor Recovery

Lynch et al. (1987) studied the effect of bioaugmentation using two anaerobic cultures enriched for (i) propionic acid and butyric acid and (ii) lactic acid and ethanol on organically overloaded anaerobic filters. Following a shock overload, the filter receiving propionate-butyrate enriched culture was inoculated on day 95, whereas the other shock-overloaded filter receiving lactic acid-ethanol enriched culture was inoculated on days 95

and 105. All the shocked filters (including controls which didn't receive any bioaugmentation) recovered from the shock and apparently bioaugmentation didn't play a role in recovery. To confirm the findings, one more filter which was not subjected to shock overload was bioaugmented with propionate-butyrate enrichment culture on day 98 and lactic acid-ethanol enrichment culture on day 105. In both the cases, a temporary decrease in effluent volatile fatty acid concentration and improvement in TCOD removal was observed which was attributed to normal variations. The authors further attributed failure of the bioaugmentation experiment to the following :

(1) low number of enrichment organisms introduced during bioaugmentation, (2) introduced organisms exposed to inhibitory environment and (3) possible antagonistic relationship between the introduced organisms and the indigenous community. The authors concluded that bioaugmentation with this type of enrichment may be more successful in a suspended growth systems where original biofilm biomass is not dominant.

O'Flaherty et al. (1999) and O'Flaherty and Colleran (1999) performed bioaugmentation studies on 17-L hybrid reactors treating potentially toxic, high sulphate-containing waste. One hybrid reactor received 4 g/L sulphate in the influent which resulted in less than 60% COD removal efficiency in the reactor, whereas a control reactor received no sulphate and maintained a COD removal efficiency of more than 95%. Successful bioaugmentation was achieved when 25g VSS/L of sulphate-adapted sludge from a full-scale digester was inoculated in the reactor. Improvement in the COD removal efficiency was observed after one retention time (48 hrs) and the COD removal efficiency then exceeded 95%. Inoculation resulted in the establishment of propionate-

and hydrogen-utilizing, sulphate-reducing bacteria in the biofilm section, but not in the granular sludge bed section of the anaerobic hybrid reactor.

Schauer (2008) studied the effect of bioaugmenting with cultures acclimated to (1) hydrogen, carbon dioxide gases and glucose (C1) and (2) hydrogen, air, carbon dioxide and glucose (C2) on anaerobic reactors subjected to a toxicant (oxygen). A series of anaerobic digesters was operated and subjected to oxygen in air. Following the air exposure, half of the digesters were bioaugmented with either C1 or C2 and the remaining digesters were supplied with autoclaved C1 or C2 cultures which served as controls. The augmentation rate was maintained at 1.2 mgVSS/L-day over the entire period of bioaugmentation. Results of the experiment showed 47% more methane production from digesters that received active cultures as compared to their respective controls. Also, the effluent soluble COD concentrations from the bioaugmented digesters decreased below 2,000 mg/L 83 days before that of the control digesters.

2.1.3.4.7 Anaerobic Augmentation

Augmentation of anaerobic digesters means the addition of non-active organisms, enzymes, or components of organisms that can aid in the digestion process.

Rigo et al. (2008) studied the effect of lipases for pre-treatment of oil- and grease-rich dissolved air floatation (DAF) float from the swine and bovine meat industry. Two different lipases were tested. The first lipase (Lipolase 100T) was obtained from a commercial firm and the second lipase (Lipase-SEP) was produced by solid-state fermentation of babassu cake by *Penicillium restrictum*. Meat industry wastewater mixed

with different concentrations of DAF float (250 to 3000 mg/L) was used. Experiments were carried out in batch anaerobic digesters using 0.1 and 5.0% (w/v) lipase. A control digester fed with wastewater without DAF float was also operated. The high concentration of lipase (5.0 %) decreased biodegradation due to production of excess amounts of volatile fatty acids, whereas benefits of lipase treatment were visible at the low lipase concentration (0.1%). Lipase-SEP yielded better digester performance than Lipolase 100T in terms of effluent COD. At a 1200 mg/L FOG float concentration, Lipolase 100T pre-treatment caused only a 3 % increase in effluent COD removal than the control digester, whereas Lipase-SEP pre-treatment showed a 22 % increase in effluent COD removal. The authors concluded that augmenting with lipases may improve performance of biological treatment systems treating oil- and grease-rich wastes.

Noyola and Tinajero (2005) studied the effect of augmentation on anaerobic digestion of primary sludge from enhanced primary treatment (EPT) of municipal wastewater. Two additives, (a) lyophilized *bacilli* and enzymes and (b) a solution of micronutrients (iron, cobalt, nickel, and molybdenum) were studied separately and in combination to determine their effect. The lyophilized *bacilli* alone was found to yield higher methane production than the control (95% higher on day 17) and the combination of lyophilized microorganisms and micronutrients resulted in methane production that was 167% higher than the control on day 17. The combination also achieved lower VFA concentrations and greater volatile solids reduction than the control and digestion with the *bacilli* alone. The authors concluded that the use of biological additives and micronutrients had a positive effect on sludge stabilization.

Davidsson et al. (2007) studied the effect of augmenting with polysaccharide-degrading enzymes, lipase, and protease in the pre-treatment of pilot-scale anaerobic digestion of primary sludge and waste activated sludge mixed in a 1:1 proportion. It was hypothesized that the enzymes would hydrolyze biopolymers resulting in increased release of proteins and carbohydrates from the substrate. A 71% increase in methane yield (compared to non-augmented sludge) was observed when the enzymes were added together with the fresh sludge as compared to a 57% increase in the methane yield (compared to non augmented sludge) observed when the enzymes were added through the recirculation pipe together with the digested sludge.

2.1.4 Oxygen and Methanogenic Systems

Oxygen toxicity tolerance of anaerobic cultures used for bioaugmentation is of particular importance due to difficulties involved in handling large volumes of anaerobic cultures. Contact with oxygen is almost inevitable if cultures have to be transported from one location to another during handling. In the past, researchers have studied the effect of oxygen addition on methanogenic cultures as presented below.

Zitomer and Shrout (1997) studied the effect of oxygen addition on anaerobic digestion in batch reactors fed ethanol and propionate as the primary carbon source. Three assay cultures for each substrate were prepared and 0, 10 and 30 mL air (20 °C) was added to each assay, respectively. The cultures supplied with air showed 10- to 18-hr lag periods, whereas the cultures supplied with no air showed no lag.

Zitomer and Shrout (1998) reported the effect of oxygen addition on anaerobic digestion systems in batch and continuously stirred tank reactors (CSTRs). In the batch studies, a factorial approach was used in which two oxygenation conditions and three oxygen doses corresponding to 10, 30 and 125% of the added COD were supplied. Cultures maintained under the first oxygenation condition (30% oxygen) received oxygen at the time of medium addition whereas the cultures maintained under the second condition (125% oxygen) received oxygen 1 day in a two- day feeding cycle. It was observed that cultures receiving an oxygen dose of 125% were able to produce methane and they showed a 20% higher specific methanogenic activity (mL biogas/g-VSS-day) than the cultures maintained under strictly anaerobic conditions. Also the cultures maintained under oxygen-limited conditions showed a yield of 0.13 to 0.07 gVSS/g COD which is more typical for strictly methanogenic processes. In the CSTR studies, two bench scale anaerobic reactors were operated and fed 1 and 0.1 g O₂/L_(reactor)-day and additionally conventional anaerobic and aerobic reactors were operated in parallel. Organic loading rates of 0.25 to 4 g COD/L_(reactor)-day was fed to all the reactors. Methane was detected in the headspace gas of both CSTRs operated under oxygen-limited conditions. Furthermore, the reactor fed with 1 g O₂/L-day showed a residual COD concentration of 1400 mg/L, whereas the aerobic CSTR showed a much higher residual COD concentration of 2400 mg/L. Also it was observed that reactor pH after a pH drop caused by a shock overload of COD returned to >7 in the 1 g O₂/L-day and 0.1 gO₂/L-day reactors after 34 and 28 days respectively, whereas the strictly anaerobic reactor showed no pH recovery even after 52 days.

As described above, propionate plays a major role in contributing to elevated effluent COD of some organically overloaded anaerobic digesters. It was hypothesized that the function of upset digesters can possibly be restored at a faster rate if the elevated effluent SCOD concentrations are lowered by using a bioaugmentation strategy. Using bioaugmentation cultures especially enriched for the degradation of propionate may prove beneficial. Exposure of bioaugmentation cultures to small amounts of oxygen may help retain its activity during its transfer from enrichment digester to stressed digester. The study presented herein involved enrichment of an anaerobic culture for propionate and different doses of oxygen and evaluation of bioaugmentation effectiveness following the organic overload of an anaerobic digester.

2.2 Methods and Materials

2.2.1 Acclimation of Anaerobic Cultures to Propionate and Oxygen

Results of the SMA test conducted on different anaerobic cultures were used to select cultures with the highest and lowest SMA against propionate. Figure 1.4 shows the results of the SMA testing for cultures arranged in descending order. The bar graph represents average SMA of the cultures against propionate, whereas the error bars represent the standard deviations among the replicates.

The selected cultures presented in Table 2.3 were enriched in 750-mL serum bottle digesters operated in CSTR mode. The serum bottle digesters were supplied with 150 mL of the selected culture. Digester headspace was sparged with a nitrogen-carbon dioxide gas mixture (mixed in 7:3 ratio v/v) so that anaerobic conditions were

maintained. The enrichment cultures were shaken continuously at 150 rpm and maintained at 35 ± 3 °C. All the enrichment digesters were fed 0.17g propionate/L-day (0.25 gCOD/L-day) with basal medium (see Table 1.4). All enrichment cultures were operated at a 15-day SRT. The feeding schedule followed for enrichment cultures is presented in Table 2.3. After 580 days of operation, the SMA of all the enrichment cultures against propionate was tested by following the procedure described in Section 1.2.2.

The effect of oxygen on bioaugmenting ability of the culture and its SMA against calcium propionate were observed for the culture with the highest initial SMA for propionate (Brewery WWTP-1). The selected culture was enriched for propionate in the same manner as explained above and additionally oxygen equivalent to 0%, 10%, 50% and 90% of the COD exerted by the daily propionate dose was supplied in the form of atmospheric air. The equivalent doses of air were 14, 68 and 124 mL air @35°C/ L-day respectively. The resulting oxygen doses were 0, 0.025, 0.125 and 0.225 gO₂/L-day respectively. The schedule of feeding for enrichment cultures acclimated to different doses of atmospheric oxygen is given in Table 2.3.

Table 2.3: Enrichment Cultures

Biomass	Designation
1. Culture with best activity against propionate	
(a) Replicate -1	C1a
(b) Replicate -2	C1b
(c) Replicate -3	C1c
2. Culture with best activity against propionate and O ₂ – dose 1 (10% of the COD fed daily)	
(a) Replicate -1	C2a
(b) Replicate -2	C2b
(c) Replicate -3	C2c
3. Culture with best activity against propionate and O ₂ – dose 2 (50% of the COD fed daily)	
	C3
4. Culture with best activity against propionate and O ₂ – dose 3 (90% of the COD fed daily)	
	C4

2.2.2 Bioaugmentation Experiment

The effectiveness of bioaugmentation of anaerobic digesters was evaluated by organically overloading small-scale, non-fat-dry-milk-fed anaerobic digesters and bioaugmenting them with cultures enriched for propionate and different oxygen doses. Enrichment cultures selected for the bioaugmentation study are described in Table 2.4.

For the bioaugmentation study, anaerobic seed sludge was from a laboratory-scale digester fed with non-fat-dry milk. The obtained sludge was used to seed different 160-

mL serum bottle digesters and each digester received 50 mL of seed sludge. Headspace of all the digesters was sparged with a nitrogen-carbon dioxide gas mixture (mixed in 7:3 ratio v/v) so that anaerobic conditions were maintained. The digesters were operated at a 10-day SRT by wasting sludge volumetrically equivalent to the daily feed volume from the digesters. The digesters were incubated and maintained at 35 ± 3 °C. All the digesters were fed daily with basal medium (composition given in Table 1.4) and non-fat-dry milk (2.7 g COD/L-day) for a period of 3 SRTs. Biogas production from the digesters and pH of the waste biomass was recorded daily and effluent soluble COD (SCOD) concentration and methane percentage in the biogas was measured twice a week. After attaining a quasi-steady state condition (i.e. after 3 SRTs), a shock overload of non-fat-dry milk substrate (32g COD/L digester volume) was given for one day to all the digesters except the undisturbed control digesters. This shock dose of COD was expected to cause an increase in VFAs leading to reduced methane production and increased effluent COD.

Following the organic overload, bioaugmented digesters were provided with a daily dose (1.7 mL/day equivalent to 70 mgVSS/L-day) of an enrichment culture. Non-bioaugmented digesters were fed with 1.7 mL/day of the autoclaved version of the appropriate acclimated enrichment culture. Table 2.4 shows the number of bioaugmented and non-bioaugmented digesters maintained for the study.

Table 2.4: Bioaugmentation Digesters

Name of the digester	Enrichment culture used	Number of Replicates
Undisturbed Control Digester (UCD)	-	4
Bioaugmented Digester (TD) – 0% - 1	C1a	1
TD – 0% - 2	C1b	1
TD – 0% - 3	C1c	1
TD – 0% - 4	C1c	1
Non-bioaugmented Digester (CD) – 0% - 1	Autoclaved C1a	1
CD – 0% - 2	Autoclaved C1b	1
CD – 0% - 3	Autoclaved C1c	1
CD – 0% - 4	Autoclaved C1c	1
TD – 10% - 1	C2a	1
TD – 10% - 2	C2b	1
TD – 10% - 3	C2c	1
CD – 10% - 1	Autoclaved C2a	1
CD – 10% - 2	Autoclaved C2b	1
CD – 10% - 3	Autoclaved C2c	1
TD – 50%	C3	1
CD – 50%	Autoclaved C3	1
TD – 90%	C4	1
CD – 90%	Autoclaved C4	1

2.2.3 Analytical Methods

Effluent samples from the digesters were analyzed for SCOD and pH and also the digesters were tested for daily biogas production quantity and headspace gas

composition. The SCOD concentration was determined using procedure 5220-D in Standard Methods (APHA et al., 1998). For SCOD analysis, solids were removed at 13,000 rpm for 10 minutes using a centrifuge (Galaxy 14D, VWR International, West Chester, PA) and further prepared by filtering the supernatant through a 0.45- μ m filter (Whatman International Ltd., Maidstone, England). The filtrate was then tested for COD using the above method.

The pH was measured using a bench-top pH meter (Orion Model 720A, Thermo Fisher Scientific, Inc., Waltham, MA) and a general-purpose pH electrode (Orion, Thermo Fisher Scientific, Inc., Waltham, MA).

The digester biogas quantity was measured daily using the plunger displacement method of a water lubricated glass syringe. The headspace gas composition was determined using gas chromatography using either a GOW-MAC Series 600 (GOW-MAC Instrument Co., Bethlehem, MA) equipped with a thermal conductivity detector (TCD) or an Agilent 7890A GC system, equipped with a packed CTR I column Agilent Associates, Inc., Deerfield, IL and Helium as the carrier gas at a flow of 30 ± 2 mL/min with the temperature of the injector and detector set at 120°C and the temperature of the oven set at 38 ± 2 $^{\circ}\text{C}$.

2.2.4 Statistical Analysis

Two sample Student's *t* test with unequal variances was used for comparison of results of (1) SMA against propionate before and after enrichment for the enrichment

cultures given in Table 2.3 and (2) bioaugmented and the non-bioaugmented digesters effluent SCOD and daily biogas production.

2.3 Results and Discussion

2.3.1 Effect of Oxygen on Activity of an Enrichment Culture

Figure 2.1 shows the effect of oxygen addition to an enrichment culture on its SMA against propionate. The error bars in Figure 2.1 represent the standard deviations among the replicates. The horizontal line represents the initial SMA before enrichment.

Surprisingly, there was no increase in SMA after 580 days (38.6 SRTs) of enrichment for 0 mgO₂/L-day dose, but addition of 25 mgO₂/L-day increased the average SMA by 29.7%; however, this increase in the activity was found to be statistically insignificant (p=0.23). Further increase in the oxygen dose had a negative effect on SMA. Addition of 225mgO₂/L-day decreased the SMA by 90.3%; this decrease was found to be statistically significant for 5% level of significance (p=0.03). Addition of 25 mgO₂/L-day to the enrichment culture may have increased its oxygen-tolerating capacity so that, when it was being transferred from enrichment digester to SMA assay digesters, it may have suffered minimal oxygen toxicity due to exposure to atmospheric air and, therefore retained its activity. The culture fed with no oxygen may have less capability to withstand any toxic effects due to air exposure. Excess doses of oxygen (>25 mgO₂/L-day) may have decreased SMA of the enrichment culture since higher doses of oxygen may have been toxic to anaerobic microbes.

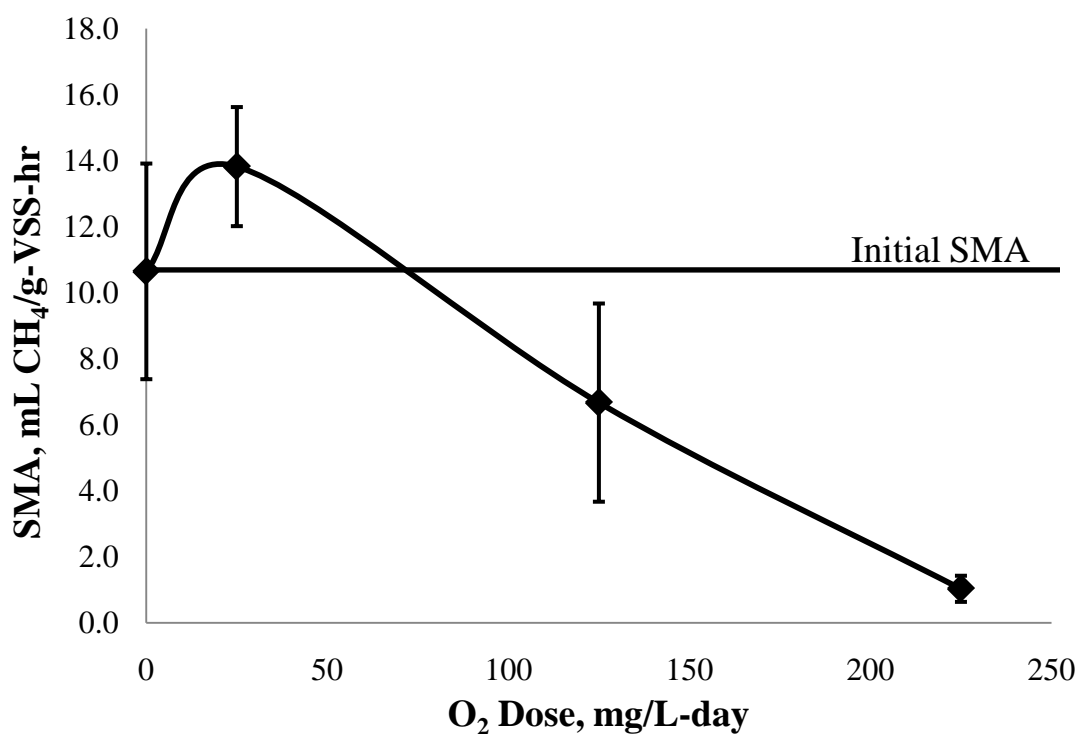


Figure 2.1: Effect of Oxygen on SMA Against Propionate After Enrichment.
Error Bars Represent Standard Deviations Among Replicates.

2.3.2 Bioaugmentation Experiments

The purpose of the bioaugmentation investigation was to determine if faster recovery occurred with addition of a bioaugmentation culture after a digester was subjected to a shock organic overload. Also the aim was to evaluate if addition of oxygen to the bioaugmentation culture have any effect on its bioaugmenting ability.

2.3.2.1 Effluent SCOD

Figures 2.2 through 2.5 show the effluent SCOD concentrations of the experimental digesters (see Table 2.4 for the designations). In Figures 2.3 through 2.5,

the series of solid diamonds represent the average effluent SCOD of digesters which did not receive organic overload, designated as the undisturbed control digesters (UCD), the series of hollow circles represent average effluent SCOD of the bioaugmented (test) digesters and the series of solid squares represent average effluent SCOD of non-bioaugmented digesters. Since all the digesters were operated with daily feeding-wasting, the peak SCOD caused by the organic overload was expected to be diluted due to loss of SCOD in the effluent. Therefore, a dilution curve was plotted by using Equation 2.1 to account for the SCOD lost in the effluent which is shown by the series of hollow triangles in Figures 2.2 through 2.5. In Equation 2.1, B (mg/L) is the effluent SCOD concentration resulting due to dilution on day θ , A (mg/L) is the average of highest SCOD concentration of the bioaugmented and the non-bioaugmented digesters following the organic overload, θ (days) represents the time after the organic overload, and SRT (days) is the solid retention time. The dilution curve was plotted using the equation,

$$B = A \times e^{-(\theta/SRT)} . \quad (2.1)$$

For simplicity in calculations, daily COD added to the digesters in the form of feed following the shock overload was not considered for plotting the dilution curve. The error bars in Figures 2.2 through 2.5 represent standard deviations among the replicates.

As seen in the Figures 2.2 through 2.5, all the digesters initially required about 40 days (4 SRT's) to attain an average quasi steady-state effluent SCOD concentration of 290 ± 150 mg/L before shock organic overload. The shock organic overload was given on day 57 as explained in section 2.2 and shown in Figures 2.2 through 2.5. The organic overload resulted in higher effluent SCOD concentrations for all the bioaugmented (test)

and non-bioaugmented digesters and the resulting effluent SCOD was 5000 ± 750 mg/L as shown in Figures 2.2 through 2.5. Following the organic overload, the effluent SCOD of the digesters started to decrease due to the dilution effect biological reactions and bioaugmentation. After about 3 to 6 SRTs following the overload event, the effect of bioaugmentation started to become visible in terms of lower effluent SCOD concentration of the bioaugmented digesters as compared with their respective non-bioaugmented digesters.

One of the four non-bioaugmented digesters fed with autoclaved bioaugmentation culture enriched for $0 \text{ mgO}_2/\text{L-day}$ oxygen dose (CD-0%-4), never recovered after receiving the organic overload and its effluent SCOD concentration never became less than 4000 mg/L throughout the study. Data from this digester were not considered in further analysis and not shown in Figure 2.2.

Before organic overload, the average quasi-steady state pH of all the digesters was found to be 6.9 ± 0.2 . After the organic overload, the average pH of the overloaded digesters dropped to 6.5 ± 0.2 . Moreover, the average pH of the overloaded digesters 6 SRTs following the organic overload was found to be 7.1 ± 0.3 .

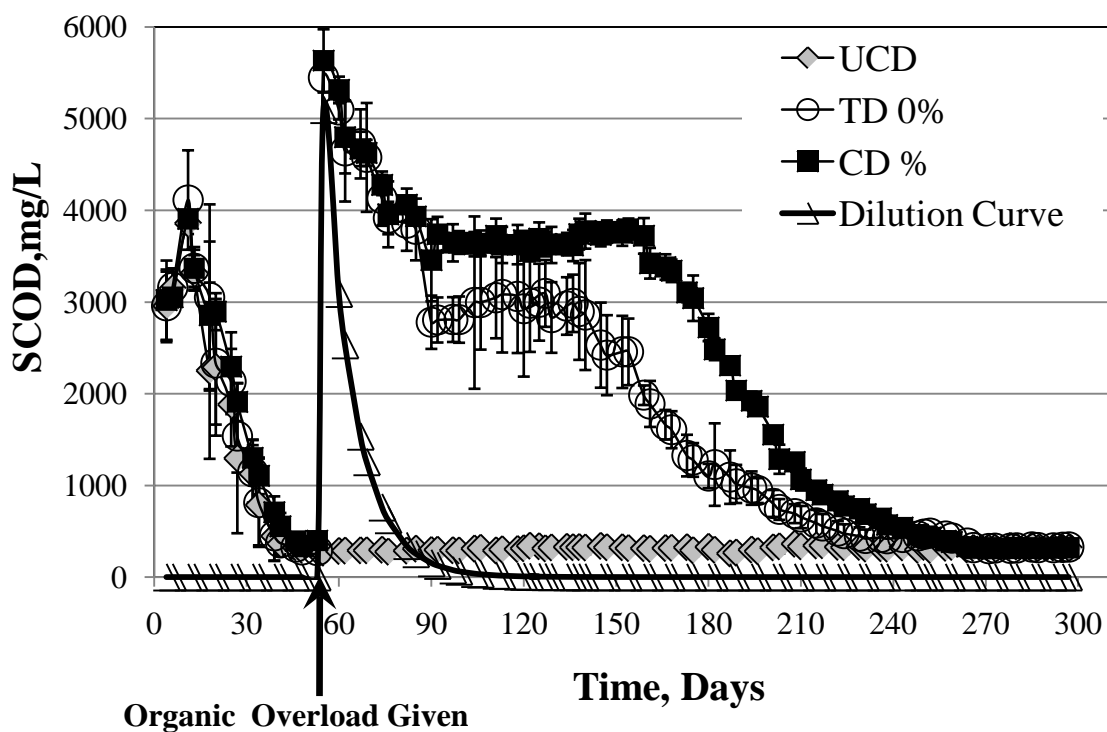


Figure 2.2: Effluent SCOD of Digesters Bioaugmented and Augmented With C1a, C1b and C1c. Error Bars Represent Standard Deviations Among Replicates.

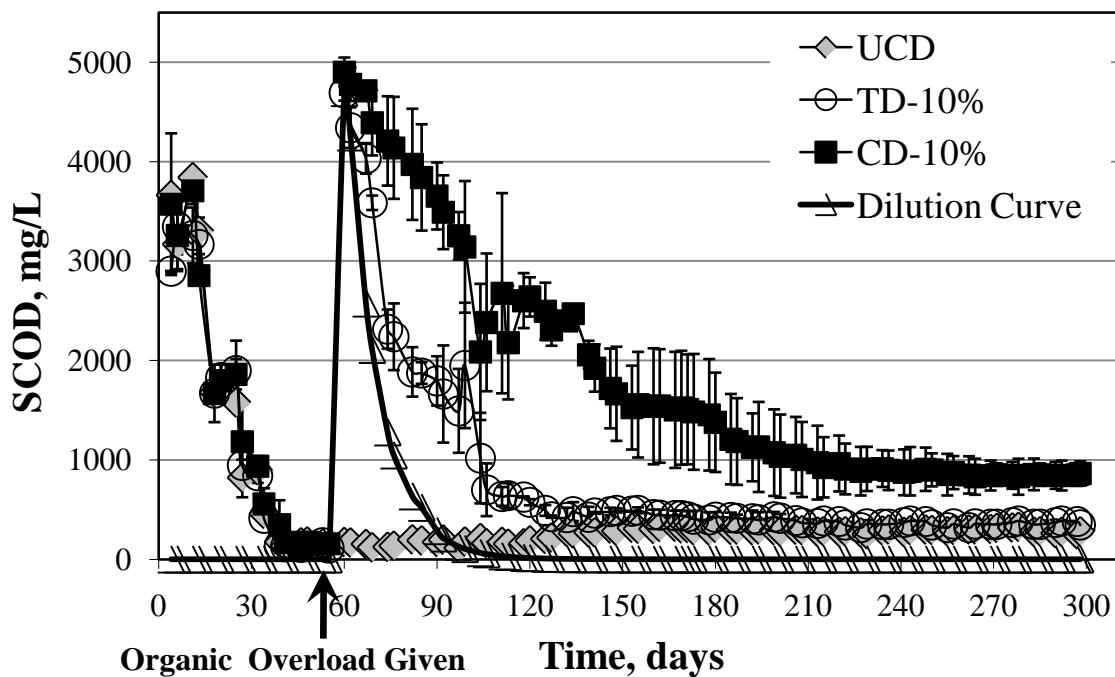


Figure 2.3: Effluent SCOD of Digesters Bioaugmented and Augmented With C2a, C2b and C2c. Error Bars Represent Standard Deviations Among replicates.

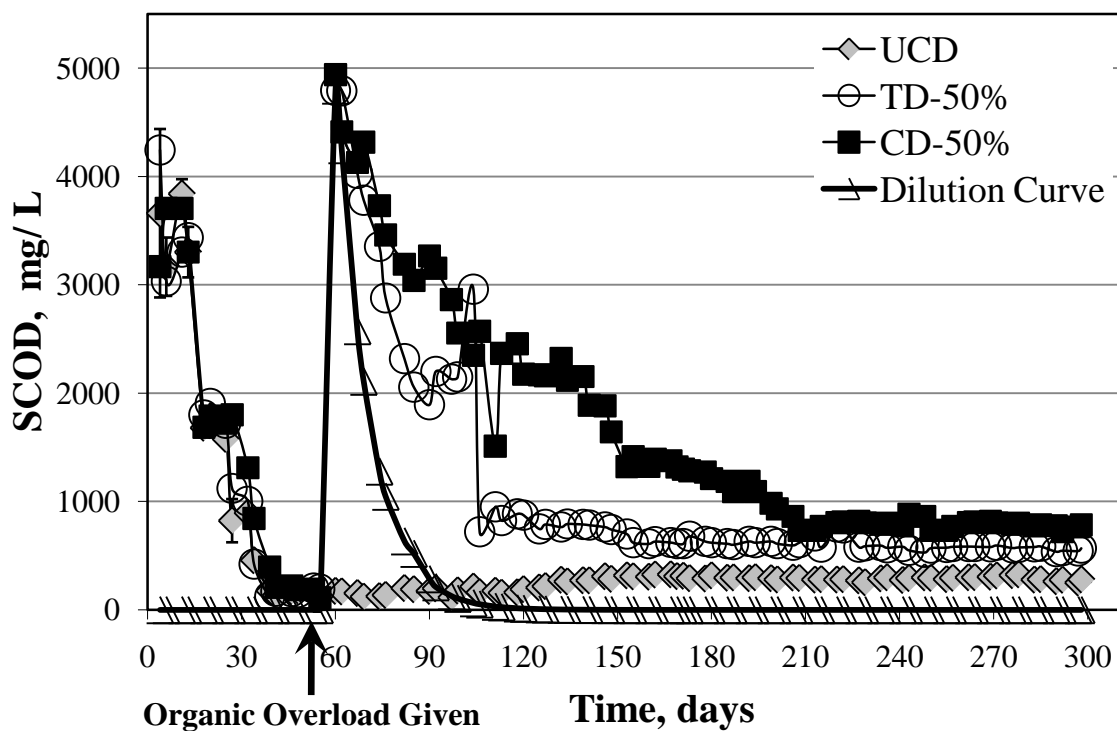


Figure 2.4: Effluent SCOD of Digesters Bioaugmented and Augmented With C3.
Error Bars Represent Standard Deviations Among Replicates.

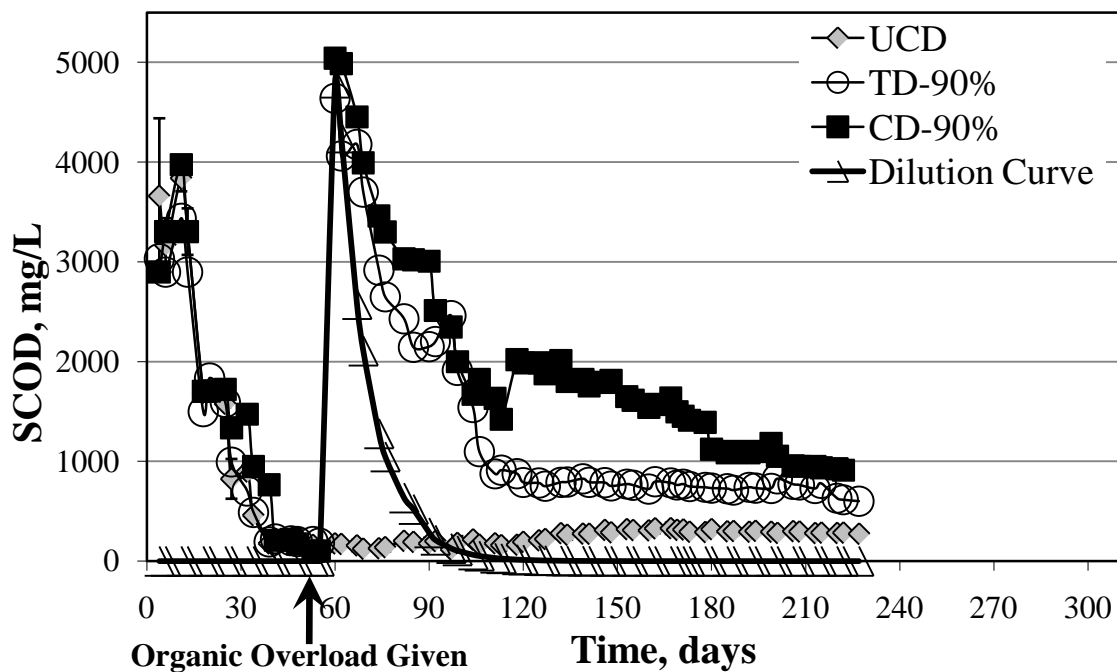


Figure 2.5: Effluent SCOD of Digesters Bioaugmented and Augmented With C4.
Error Bars Represent Standard Deviations Among Replicates.

The time required after organic overload to reach an effluent SCOD concentration of 1000mg/L was used as a measure of recovery time. Figure 2.6 shows the time required by bioaugmented and non-bioaugmented digesters to attain a 1000mg/L effluent SCOD concentration.

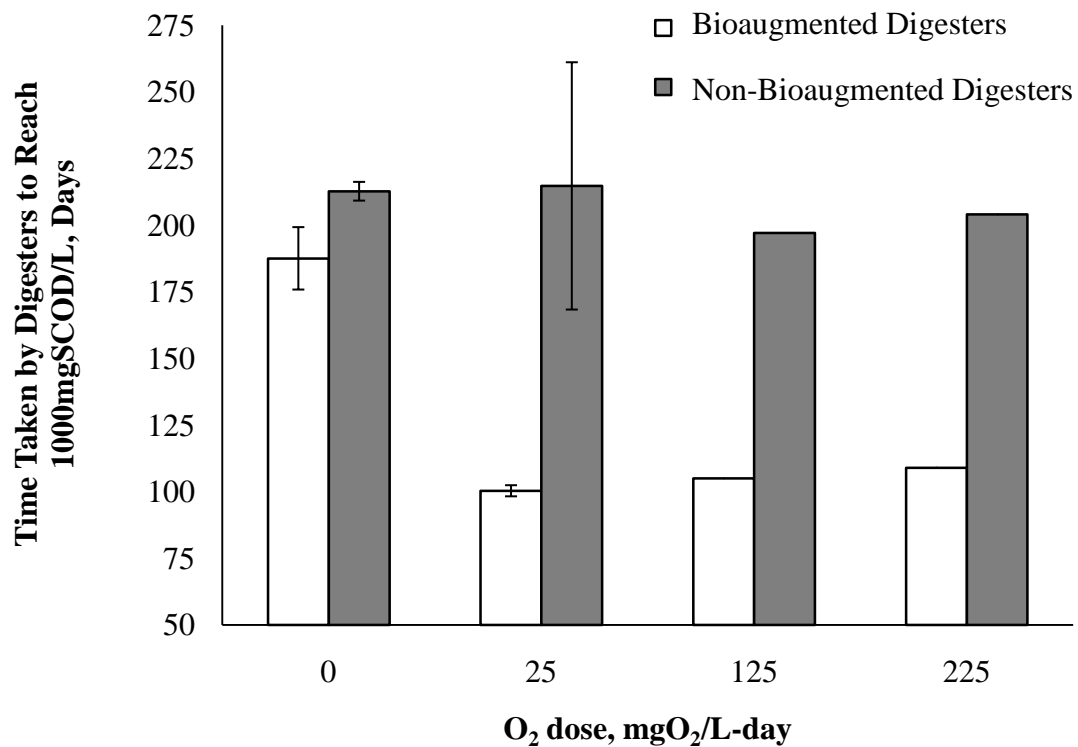


Figure 2.6: Time Required by Bioaugmented and Non-Bioaugmented Digesters to attain 1000mg/L Effluent SCOD Concentration Following the Organic Overload. Error bars represent standard deviation among replicates. Absence of error bars indicates that no replicates were operated.

Analysis of the time required to cross the effluent 1000mg/L SCOD concentration limit revealed that the bioaugmented digesters took less time (25 and 114 days less by TD-0% and TD-10% respectively) to attain 1000mgSCOD/L as compared to their

respective non-bioaugmented digesters ($p < 0.03$). Statistical analysis of bioaugmented and non-bioaugmented digesters fed with enrichment cultures enriched for 125 and 225 mgO₂/L-day was not possible because no replicates were operated for these systems. Although statistical analysis of effluent SCOD data of these digesters was not possible, it was observed that there was considerable difference between the days required to reach 1000 mg/L effluent SCOD (92 and 95 days less by TD-50% and TD-90%, respectively) by the bioaugmented digesters (see Figures 2.4 through 2.6).

It is interesting to note that all the bioaugmented digesters consistently produced lower average effluent SCOD as compared to their respective non-bioaugmented digesters after 6 SRTs following the shock overload. To demonstrate the same, the percent difference between the SCOD concentrations of the bioaugmented and the non-bioaugmented digesters after 6, 9 and 12 SRTs following the organic overload were evaluated. Figure 2.7 shows the comparison of the percent decrease in effluent SCOD concentration of the bioaugmentation digesters for each oxygen dose used for bioaugmentation after 6, 9 and 12 SRTs following the overload. The average percent difference in the effluent SCOD of the bioaugmented and the non-bioaugmented digesters for each oxygen dose is shown as the percent decrease in effluent SCOD in Figure 2.7. All curves in Figure 2.7 followed a similar pattern which was tested by calculating the ranks correlation coefficient for each pair of curves. The rank correlation for each pair of curves was found to be 1.0, which means that the percent decrease in effluent SCOD of the bioaugmented digesters followed a similar pattern after 6, 9 and 12 SRTs. This shows that the beneficial effect of bioaugmentation was observed for a prolonged period of time following the shock overload. This is in contrast to the findings

of other researchers (Lynch et al., 1987) who found that bioaugmenting with cultures enriched for propionate and butyrate following an organic overload did not speed up recovery (see section 2.1.3.4.6) for prolonged periods. The reason behind successful and prolonged recovery of shock overloaded digesters may be because, in the current study, the bioaugmentation cultures were supplied on a daily basis, whereas the previous researchers (Lynch et al., 1987) supplied bioaugmentation culture only once leading to washout from the bioaugmented digester.

Moreover, as shown in the Figure 2.7, the set of digesters receiving bioaugmentation culture enriched for $25\text{mgO}_2/\text{L-day}$ (C2a, C2b and C2c) produced a higher percent decrease in the effluent SCOD as compared to the other enrichment cultures. The reason behind the better performance may be the higher SMA associated with the bioaugmentation culture enriched for $25\text{mgO}_2/\text{L-day}$ (see Section 2.3.2) which may have led to more rapid metabolism of propionate in the bioaugmented digesters following the organic overload.

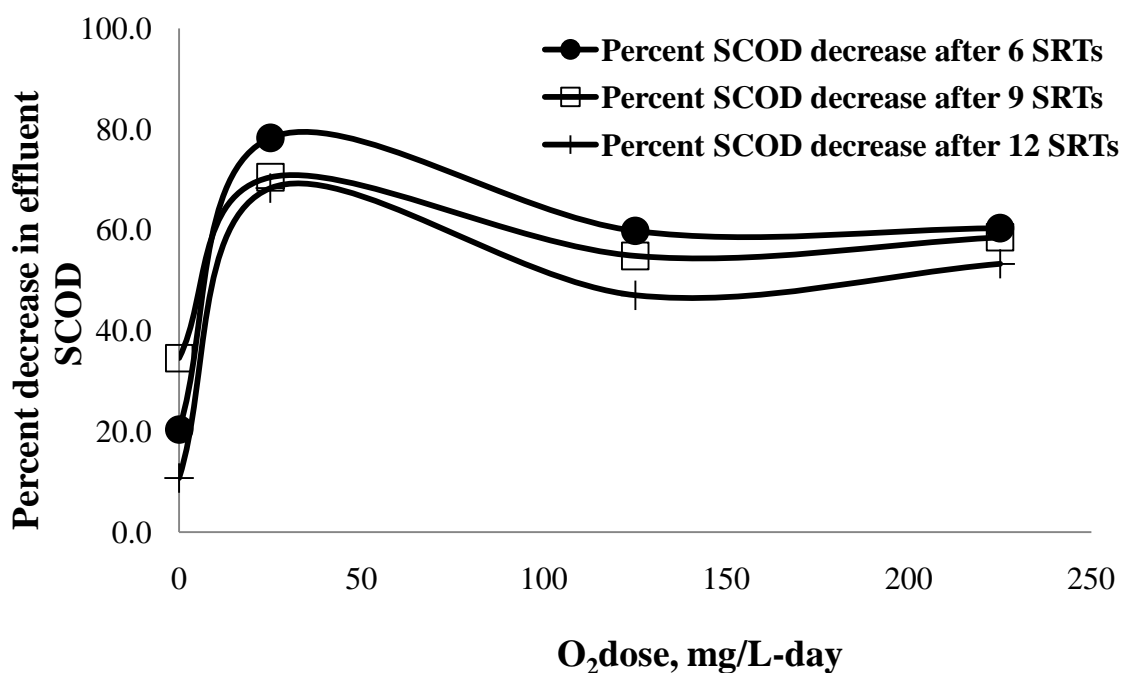


Figure 2.7: Effect of Oxygen Acclimation of Bioaugmentation Culture on Percent SCOD Decrease of Bioaugmented Digesters

2.3.2.2 Effluent VFA Concentrations

Concentrations of acetic, propionic, butyric and iso-valeric acid in the effluent from digesters UCD, TD-0% and CD-0% (see Table 2.4 for designation) were analyzed after day 170. The VFA analysis showed that initially when there was higher effluent SCOD difference between TD-0% and CD-0%, the effluent VFA concentration difference between TD-0% and CD-0% was also higher, and as the difference between SCOD decreased with time, the effluent VFA concentration difference also decreased.

Figures 2.8 through 2.11 show the effluent acetic, propionic, butyric and iso-valeric acid concentration of UCD, TD-0% and CD-0% on day 170. Error bars in Figures 2.8 through 2.11 represent the standard deviations among the replicates.

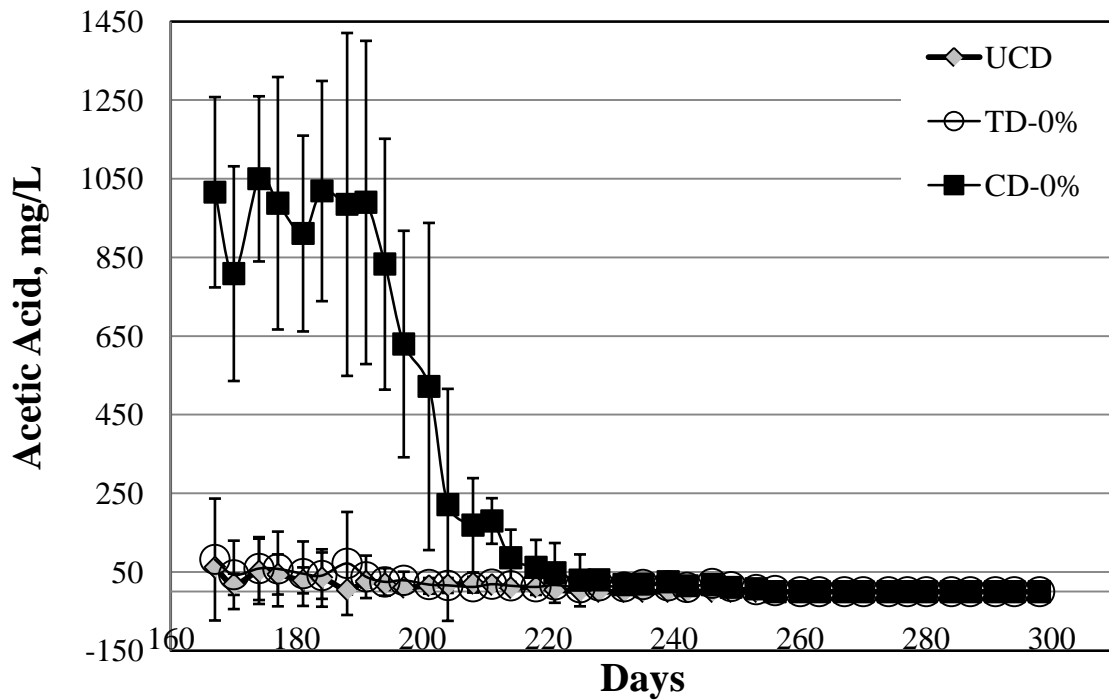


Figure 2.8: Effluent Acetic Acid Concentration of UCD, TD-0% and CD-0%.
Error Bars Represent Standard Deviations Among Replicates.

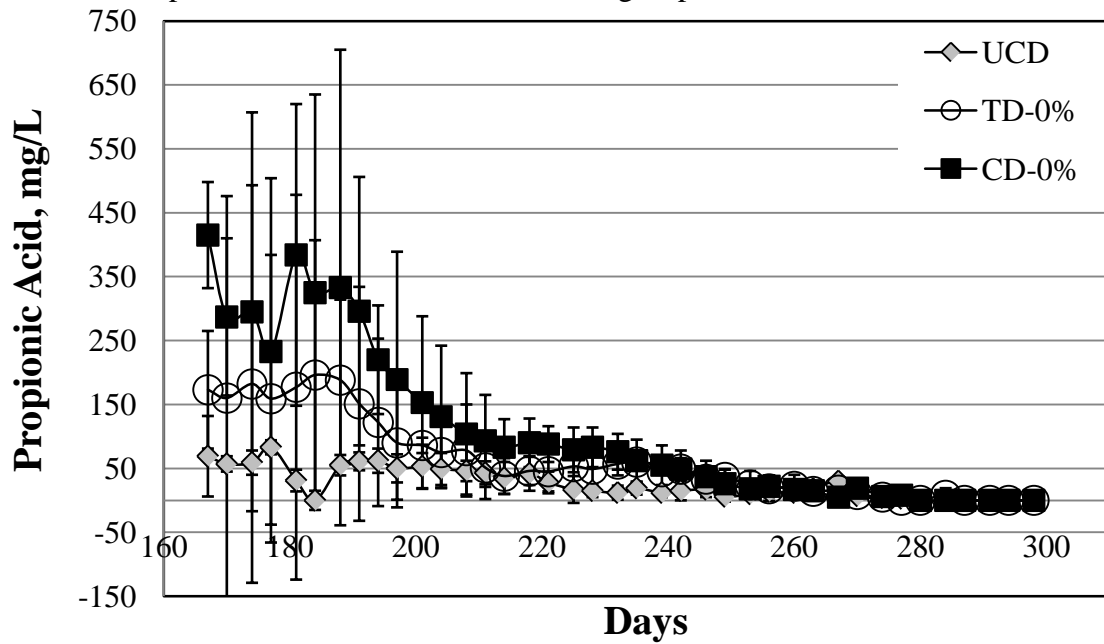


Figure 2.9: Effluent Propionic Acid Concentration of UCD, TD-0% and CD-0%.
Error Bars Represent Standard Deviations Among Replicates.

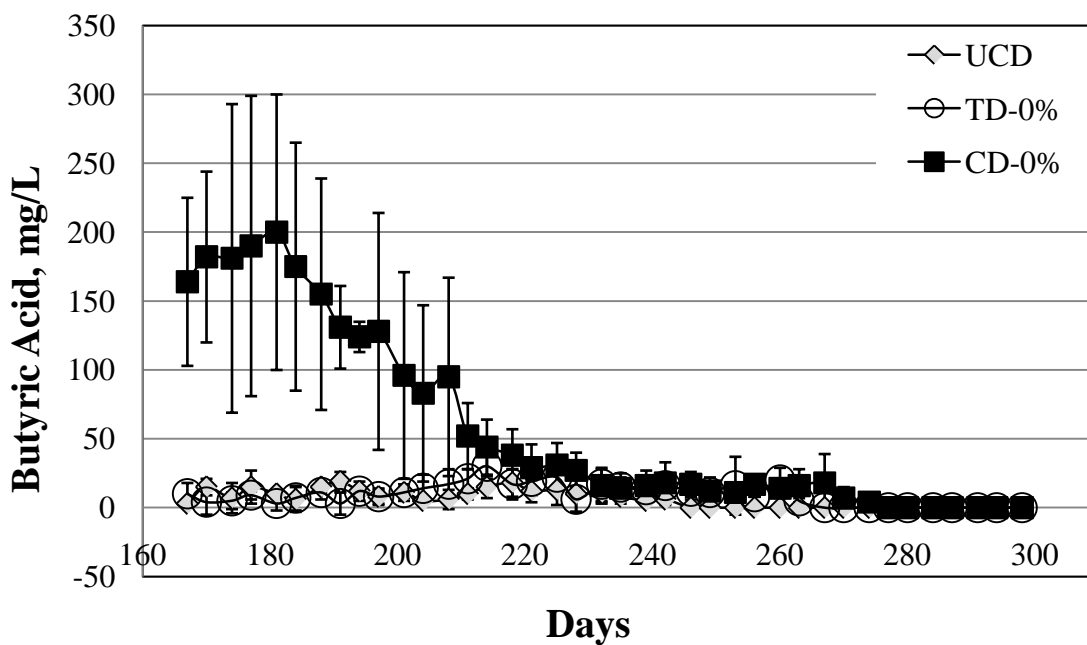


Figure 2.10: Effluent Butyric Acid Concentration of UCD, TD-0% and CD-0%.
Error Bars Represent Standard Deviations Among Replicates.

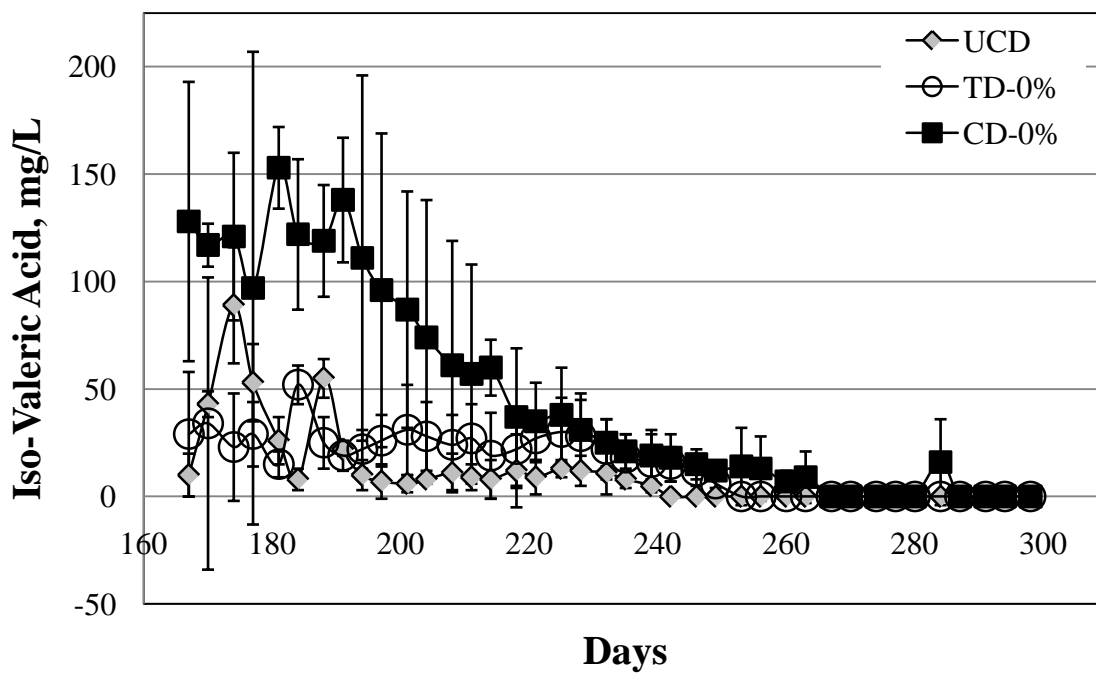


Figure 2.11: Effluent Iso-Valeric Acid Concentration of UCD, TD-0% and CD-0%.
Error Bars Represent Standard Deviations Among Replicates.

VFA concentration data on day 170 was selected for statistical analysis because on day 170, there were statistically significant differences between effluent SCOD of TD-0% and CD-0% ($p=0.0001$) and between SCOD of TD-0% and UCD ($p=0.0006$). These differences between SCOD concentrations were reduced as the digesters recovered from the shock. Table 2.5 shows probabilities associated with the null hypothesis that the average effluent VFA concentrations of UCD, TD-0% and CD-0% were equal on day 170. In Table 2.5, the gray cells have invalid null hypothesis at a 5% level of significance.

It was observed that there were significant differences between effluent acetic acid, propionic acid and butyric acid concentrations of CD-0% and TD-0%, whereas there was non-significant differences between effluent iso-valeric acid concentrations on day 170 (see last column of Table 2.5). Also there were non-significant differences between average effluent VFA concentrations between UCD and TD-0% (see first column of Table 2.5) on day 170. It is important to note that, not only TD-0% received shock overload on day 120, but also it received more daily COD exerted by the bioaugmentation culture, whereas UCD did not receive any organic overload or daily excess COD like TD-0%. Moreover there were significant differences between effluent propionic acid concentration and iso-valeric acid concentration of UCD digesters and CD-0% digesters.

The differences between the effluent VFA concentrations during the recovery of bioaugmented and non-bioaugmented digesters showed that the digesters receiving active bioaugmentation culture had effluent VFA characters much similar to the UCD digesters.

Moreover, the non-bioaugmented digesters had higher effluent acetic acid, propionic acid and butyric acid concentrations than their respective bioaugmented digesters.

Table 2.5: Probabilities Associated with Student's t Statistic for Effluent VFAs

	Null Hypothesis		
	Average concentration in UCD= Average concentration in TD-0%	Average concentration in UCD= Average concentration in CD-0%	Average concentration in TD-0%= Average concentration in CD-0%
Acetic Acid	0.84	0.02*	0.01*
Propionic Acid	0.11	0.16	0.02*
Butyric Acid	0.29	0.04*	0.05*
Iso-Valeric Acid	0.39	0.09	0.13

*cells for which null hypothesis is invalid at 5% level of significance

2.3.2.3 Methane Production

Figures 2.12 through 2.15 show the methane production from bioaugmentation digesters. In Figures 2.12 through 2.15, the series of solid diamonds represents daily methane produced by the control digesters which didn't receive an organic overload (UCD), the series of hollow circles represents daily methane produced by the bioaugmented test digesters and the series of solid squares represents daily methane produced by the non-bioaugmented digesters. The error bars represent standard deviations among the replicates.

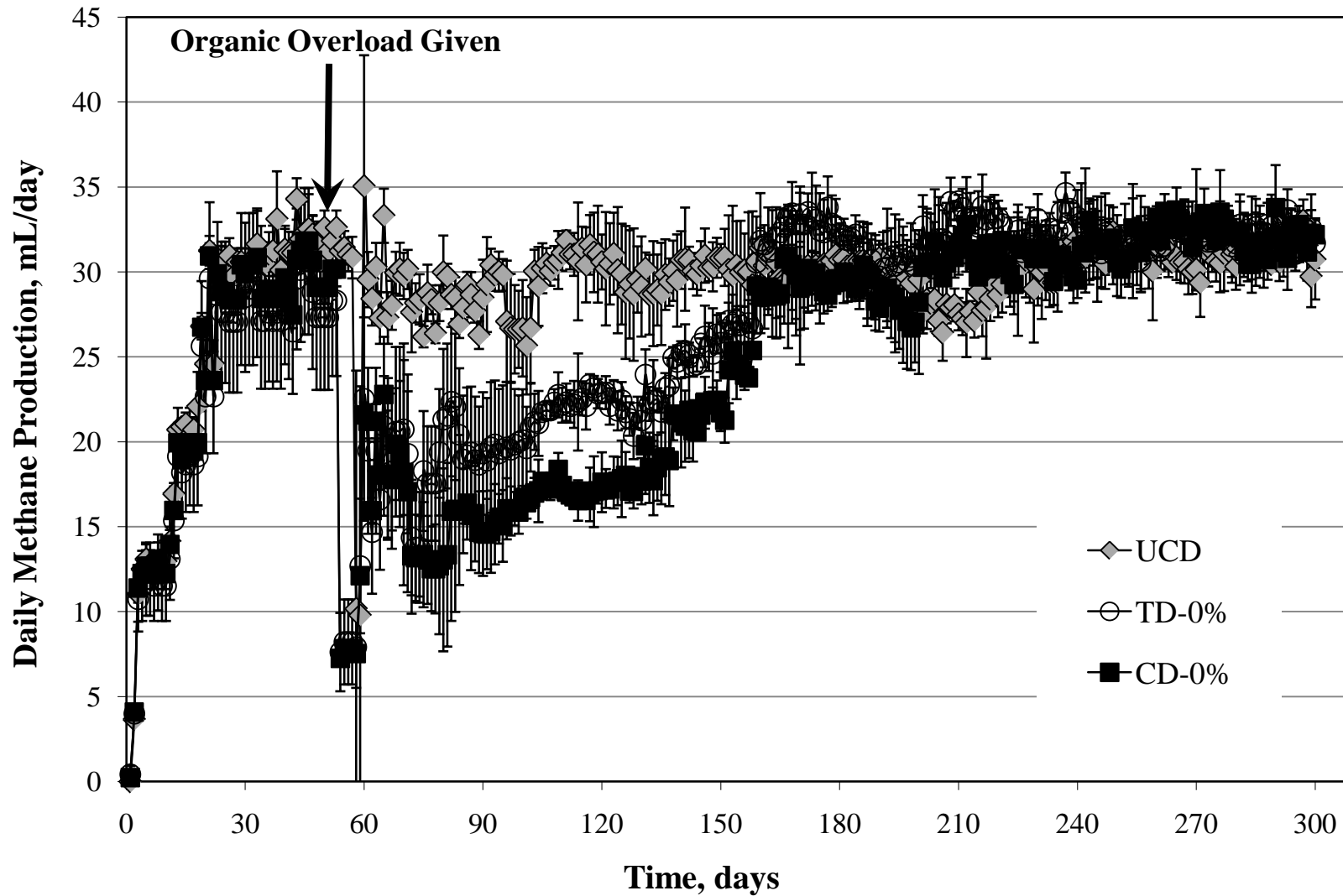


Figure 2.12: Daily Methane Production from Digesters Bioaugmented and Augmented With C1a, C1b and C1c.
 Error Bars Represent Standard Deviations Among Replicates.

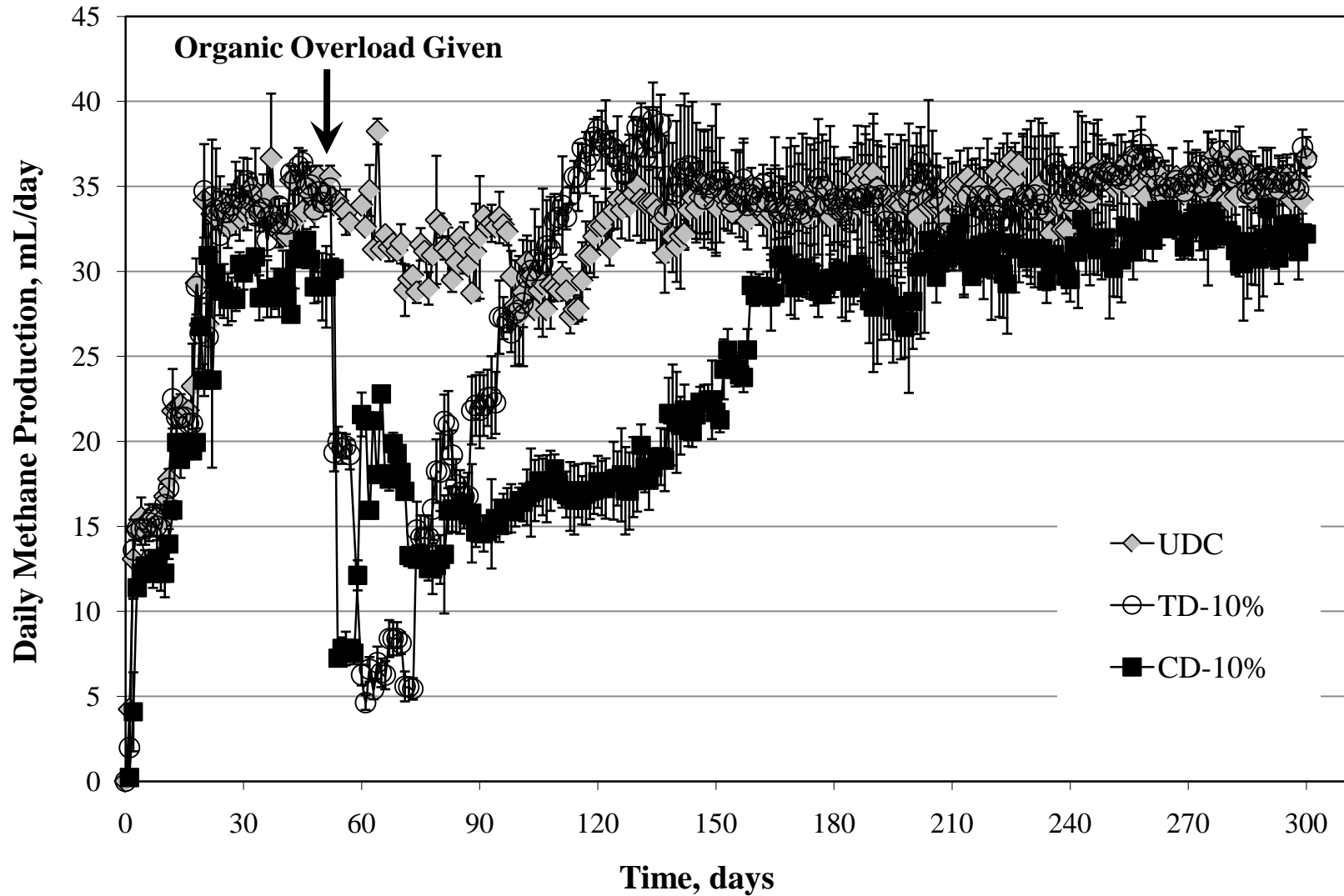


Figure 2.13: Daily Methane Production from Digesters Bioaugmented and Augmented With C2a, C2b and C2c.
 Error Bars Represent Standard Deviations Among Replicates.

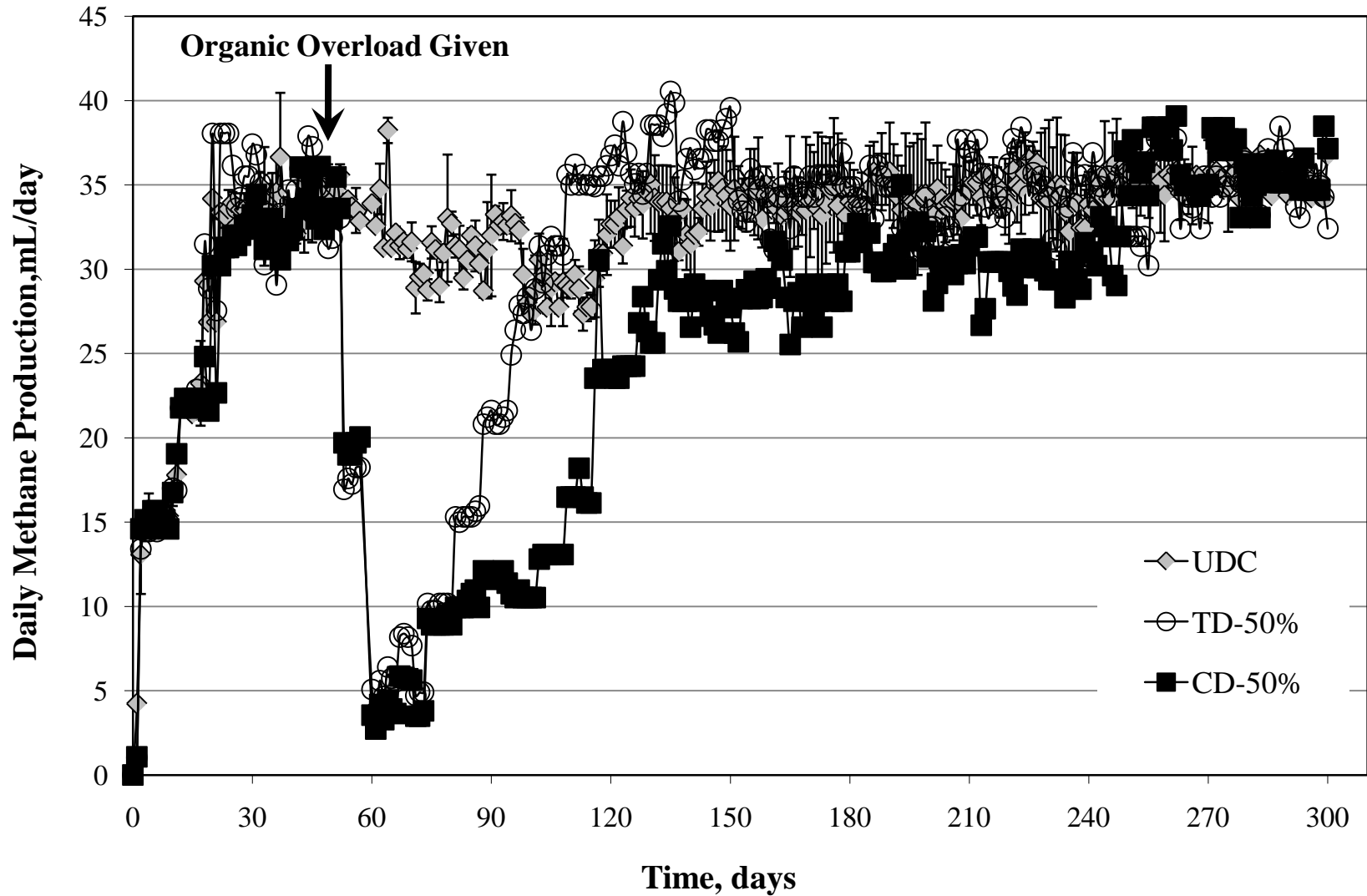


Figure 2.14: Daily Methane Production from Digesters Bioaugmented and Augmented With C3.
 Error Bars Represent Standard Deviations Among Replicates.

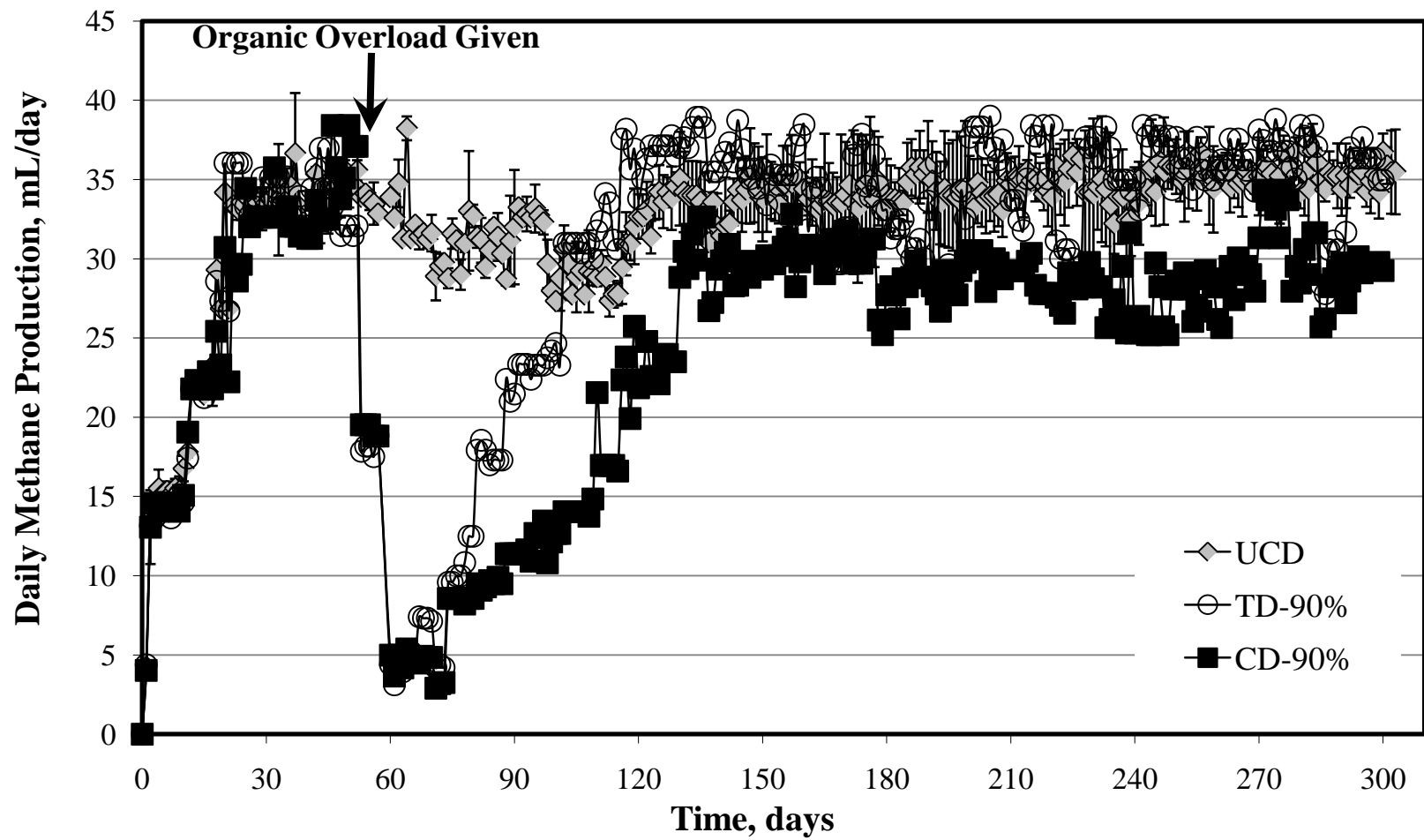


Figure 2.15: Daily Methane Production from Digesters Bioaugmented and Augmented With C4.
 Error Bars Represent Standard Deviations Among Replicates.

Like effluent SCOD concentration, all the digesters required about 40 days (4 SRTs) to reach an average quasi-steady-state 32 ± 4 mLCH₄/day methane production. During this period, average methane content in the biogas produced by all the digesters was $59\pm 4\%$ (v/v). The organic overload was given on day 57 and, following the organic overload, methane production from the digesters decreased to 18 ± 13 mLCH₄/day and the average methane content in biogas produced by the all the digesters was $16\pm 11\%$ (v/v). The daily methane production started to recover and after about 180 days of operation (i.e. 12 SRTs after the organic overload), all the digesters attained their quasi-steady state average gas production. For statistical analysis, the time required to attain 25 mLCH₄/day following the organic overload was considered. Figure 2.16 shows the time required by bioaugmented and non-bioaugmented digesters to attain 25 mLCH₄/day methane production following the organic overload.

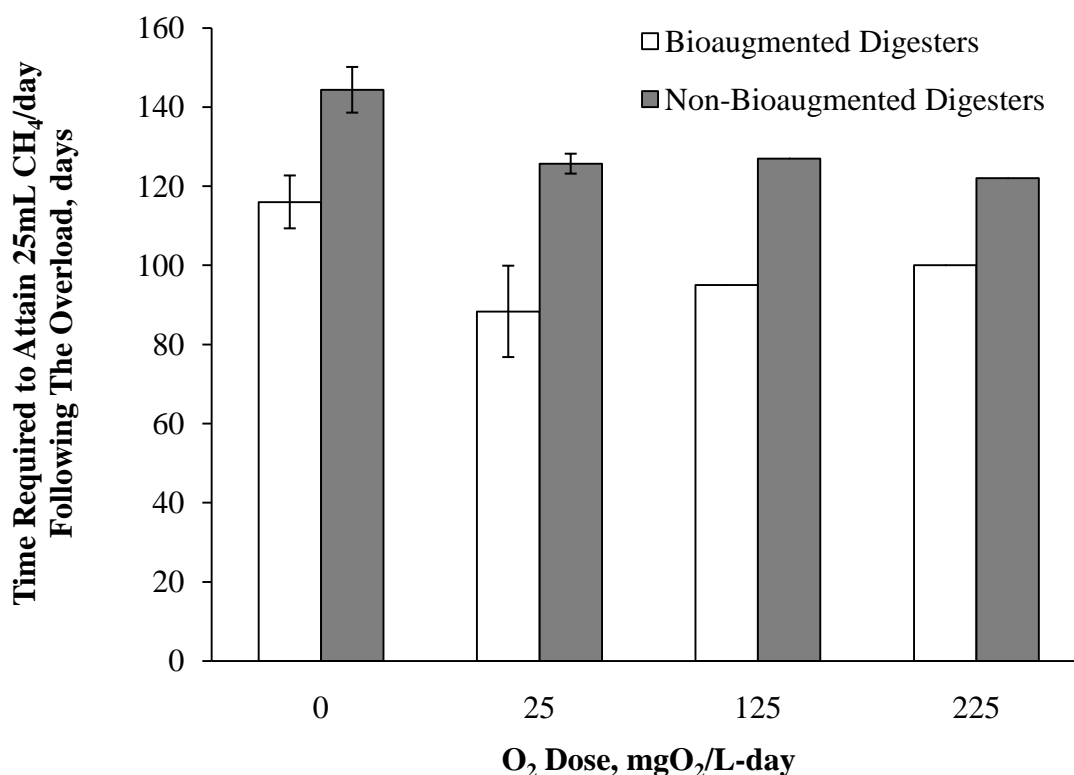


Figure 2.16: Time Required by Bioaugmented and Non-Bioaugmented Digesters to attain 25 mLCH₄/day Following the Organic Overload.

Error bars represent standard deviation among replicates. Absence of error bars indicates that no replicates were operated.

Statistical analysis for the 25-mLCH₄/day methane production limit was possible only for digester sets bioaugmented or augmented with cultures enriched for 0 mgO₂/L-day and 25 mgO₂/L-day since cultures enriched for 125 and 225mgO₂/L-day had no replicates. Student's t test revealed that there was significant difference between the time taken by the digesters bioaugmented with cultures enriched for 0 mgO₂/L-day and 25 mgO₂/L-day and the respective non-bioaugmented digesters to reach 25mLCH₄/day (28 and 37 days lesser respectively) ($p < 0.01$). Also it was observed that the bioaugmented digester receiving the culture enriched against 125mgO₂/L-day took 32 days less to reach

25mLCH₄/day than its non-bioaugmented digester, whereas the bioaugmented digester receiving culture enriched against 225mgO₂/L-day oxygen took 22 days less to reach 25mLCH₄/day than its non-bioaugmented digester.

To evaluate the consistency of bioaugmentation on methane production during the recovery period, the percent increase in methane production after 6, 9 and 12 STRs following the organic overload was evaluated. Figure 2.17 shows the percent increase in methane production after 6, 9 and 12 SRTs which was calculated as the percent difference between daily methane production from bioaugmented digesters and non-bioaugmented digesters. The ranks correlation coefficient between the percent methane increase after 6 and 9 SRTs was found to be 1.0. This means the methane production from digesters bioaugmented with cultures acclimated to different doses of oxygen followed a similar trend during recovery (i.e. after 6 and 9 SRTs). The bioaugmentation culture acclimated to 25 mgO₂/L-day showed the highest increase in the methane production after 6 and 9 SRTs following the organic overload. After 12 SRT's following the organic overload, the bioaugmentation culture acclimated to 225 mgO₂/L-day showed the highest increase in methane production. The rank correlation coefficient for percent increase in methane production after 9 and 12 SRTs was 0.83. This rank correlation coefficient was found to be statistically insignificant for 5% level of significance (p=0.08). This means the percent increase in the methane production after 12 SRTs didn't follow the 6- and 9-SRT trend. This may be because, after 12 SRTs, all the digesters nearly recovered to their pre-overloaded quasi steady-state methane production values.

Moreover, as shown in the Figure 2.17, the set of digesters receiving bioaugmentation culture enriched for 25mgO₂/L-day (C2a, C2b and C2c) produced a higher percent increase in methane production up to 6 SRTs after upset. The reason behind the better performance may be the higher SMA associated with the bioaugmentation culture enriched for 25mgO₂/L-day (see Section 2.3.1) which must have led to quicker metabolism of propionate.

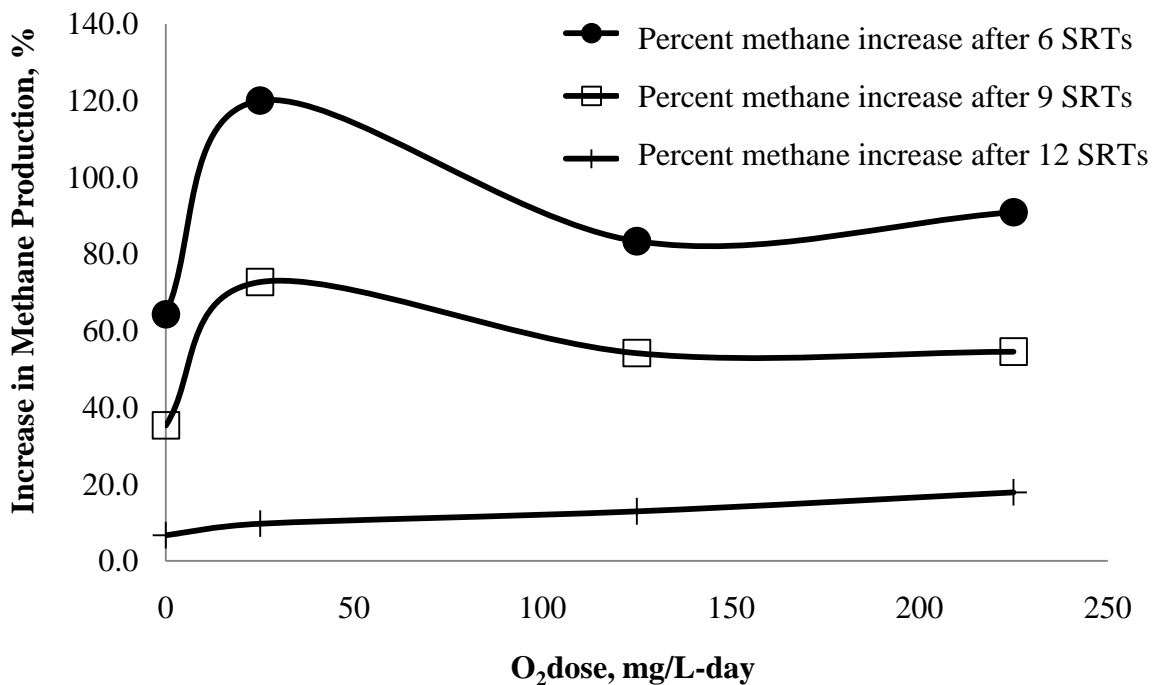


Figure 2.17: Effect of Oxygen Acclimation of Bioaugmentation Cultures on Percent Methane Increase from Bioaugmented Digesters

2.3.2.4 Glass Floor Effluent SCOD Concentrations

Interestingly, the “glass floor”, that is, the quasi steady-state effluent SCOD concentration, for the digesters augmented with the inactivated version of bioaugmentation cultures were greater than the glass floor effluent SCOD concentration of the respective bioaugmented digesters (see Figures 2.3 through 2.5). However, the glass floor effluent SCOD concentrations of the digesters augmented with inactivated bioaugmentation culture fed with 0 mgO₂/L-day equaled the glass floor effluent SCOD concentrations of the respective bioaugmented digesters (see Figure 2.2). It was hypothesized that a fraction of bioavailable trace nutrients present in the bioaugmentation cultures was oxidized due to the addition of oxygen to the bioaugmentation cultures and the higher effluent SCOD was due to unavailability of oxidized nutrients in the autoclaved cultures.

To test this hypothesis, digesters fed with active bioaugmentation culture C13 and its inactivated version (i.e. TD-90% and CD-90%) were operated after day 227 without bioaugmentation and augmentation. Three times higher concentration of basal medium containing trace nutrients (see Table 1.4) was provided to CD-90% with the daily feed. Figure 2.18 shows the effluent SCOD of TD-90% and CD-90% after day 227. The effluent SCOD of CD-90% remained higher than TD-90% until day 325. This may have been caused by the oxidized nature of the nutrients present in the basal medium. From day 325 onwards, the nutrients were first acidified to pH 2.0 by using 0.1 N HCl solution in stock solution before addition to CD-90%. Since the salts of metals present in the basal medium remain in dissolved form at the lower pH, acidifying the nutrients prior to their

addition may have increased their bioavailability. Addition of the acidified nutrients resulted in an immediate decrease in the effluent SCOD of CD-90% as shown in Figure 2.18. This decrease highlights the importance of the form of trace nutrients added to anaerobic digesters which is often ignored in actual practice. It is important to note that addition of acidified nutrients had negligible effect on the digester pH of both test and control digesters which stayed in the range of 7.01 ± 0.1 .

From day 405 onwards, TD-90% was also supplied with three times more concentrated acidified basal medium containing trace nutrients, while the SRT was kept constant. The effluent SCOD concentration of TD-90% after day 405 remained constant for 3 SRTs indicating that the digester was not trace-nutrient limited. During day 227 and day 435, effluent volatile acid concentrations from TD-90% and CD-90% were evaluated by gas chromatography as described in Section 2.2.3. Acetic, propionic, butyric and valeric acid concentrations in the effluent from TD-90% and CD-90% were below the detection limits.

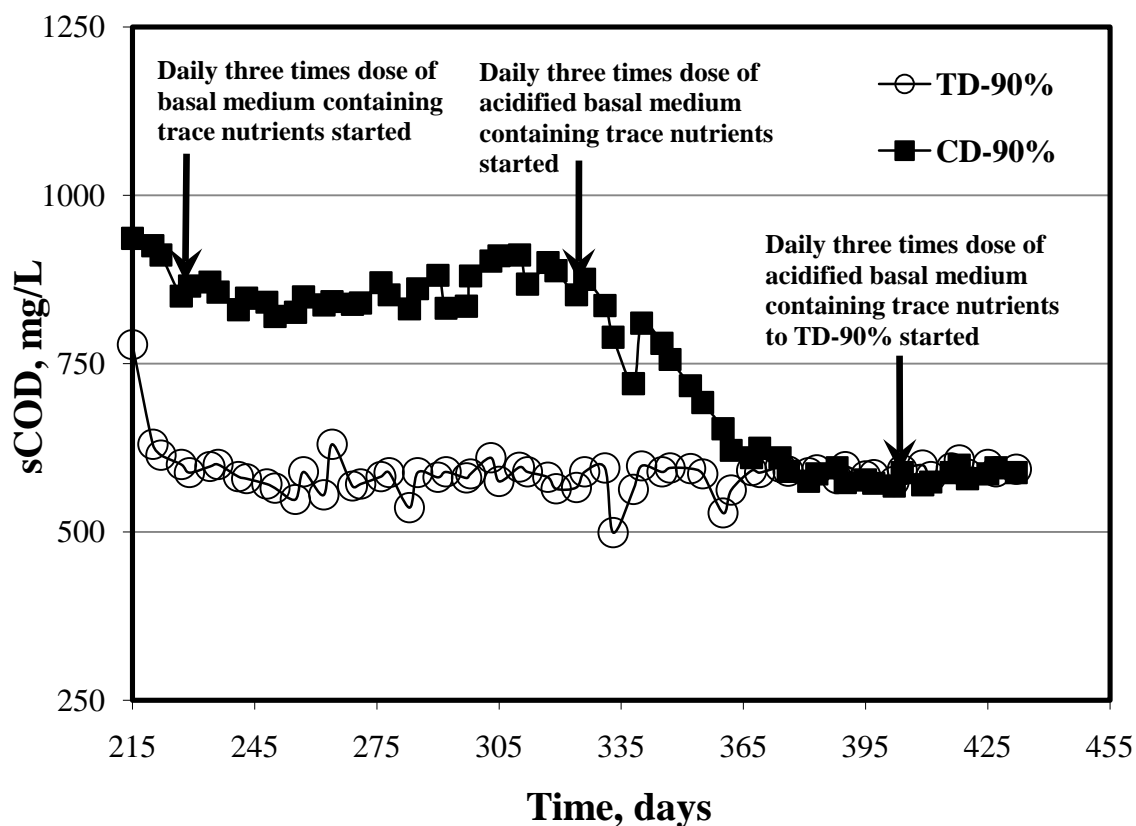


Figure 2.18: Effluent SCOD Concentration of TD-90% and CD-90%

2.4 Conclusions

Enrichment of an anaerobic culture for propionate degradation did not result in increase in SMA even after 580 days of enrichment (38.6 SRTs) for 0 mgO₂/L-day oxygen dose, whereas addition of 25 mgO₂/L-day oxygen resulted in increased SMA by 29.7%. Additional increase in oxygen dose resulted in lower SMA values.

Shock organic overloaded digesters were bioaugmented with cultures enriched to degrade propionate. Digesters bioaugmented with cultures enriched for propionate resulted in lower effluent SCOD than non-bioaugmented digesters. The difference

between effluent SCOD of the bioaugmented and non-bioaugmented digesters was apparent even after 12 SRTs following the shock overload. Digesters bioaugmented with cultures acclimated to $25\text{mgO}_2/\text{L-day}$ consistently showed higher reduction in effluent SCOD as compared to other bioaugmented digesters. These digesters took 114 days less to reach 1000 mgSCOD/L effluent concentration. Higher SCOD reduction from the digesters bioaugmented with $25\text{mgO}_2/\text{L-day}$ may have been due to the higher SMA value associated with this bioaugmentation culture, which may have led to more rapid metabolism of propionic acid.

On day 170 of the experiment (11.3 SRTs following the organic overload), Effluent VFA concentrations of the bioaugmented digesters were found to be statistically equal to the effluent VFA concentrations of the undisturbed control digesters, whereas there was more acetic and butyric acid concentration in the non-bioaugmented digesters as compared with the undisturbed control digesters.

Bioaugmentation with cultures enriched for propionate and oxygen resulted in higher methane production from shock overloaded digesters and the effect of bioaugmentation was apparent for 6 SRTs following the shock overload, after which digester methane production reached quasi steady-state. Digesters bioaugmented with $25\text{mgO}_2/\text{L-day}$ showed a higher increase in methane production for 9 SRTs and required 37 days less than non-bioaugmented digesters to reach $25\text{ mLCH}_4/\text{day}$. Higher methane production from the digesters bioaugmented with $25\text{mgO}_2/\text{L-day}$ may have been due to the higher SMA value associated with this bioaugmentation culture, which may have led to more rapid metabolism of propionic acid.

Addition of acidified nutrients reduces the final quasi-steady-state effluent SCOD concentration in some digesters.

In conclusion, recovery time of some digesters subjected to organic overload can be reduced by bioaugmentation with cultures enriched to degrade propionate. Also, the effect of bioaugmentation was apparent for a long period of time after recovery. The enrichment culture acclimated to 25mgO₂/L-day showed a 29.7% increase in SMA value, but further addition of oxygen resulted in decreased SMA values.

2.5 References

- Abeysinghe, D.H., De Silva, D.G.V., Stahl, D.A., and Rittmann, B.E. (2002) "The Effectiveness of Bioaugmentation in Nitrifying Systems Stressed by a Washout Condition and Cold Temperatures." *Water Environment Research*. 74.2: 187-199.
- Ahring B. K., Sandberg M., and Angelidaki I. (1995) "Volatile Fatty Acids as Indicators of Process Imbalance in Anaerobic Digesters." *App. Microbiol. Biotechnol.*, 43:559-565.
- Ahring, B.K., Christiansen, N., Mathrani, I., Hendriksen, H.V., Macario, A.J.L., and Conway de Macario, E. (1992) "Introduction of a De Novo Bioremediation Ability, Aryl Reductive Dechlorination, into Anaerobic Granular Sludge by Inoculation of Sludge with *Desulfomonile tiedjei*." *Applied and Environmental Microbiology*. 58.11: 3677-3682.
- American Public Health Association (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF) (1998) *Standard Methods for the Examination of Water and Wastewater* (20th ed.). McGraw-Hill Companies, Inc. New York, NY.
- Angelidaki, I. and Ahring, B.K. (2000) "Methods for Increasing the Biogas Potential from the Recalcitrant Organic Matter Contained in Manure." *Water Science and Technology*. 41.3: 189-194.
- Bagi, Z., Acs, N., Balint, B., Horvath, L., Dobo, K., Perei, K.R., Rakhely, G., and Kovas, K.L. (2007) "Biological Intensification of Biogas Production." *Appl. Microbiol. Biotechnol.*, 76:473-482.

- Barredo, M. S., and Evison, L. M. (1991) "Effect of Propionate Toxicity on Methanogen-Enriched Sludge, *Methanobrevibacter smithii*, and *Methanospirillum hungatii* at different pH values." *Applied Environmental Microbiology*. 57.6: 1764-1769.
- Cirne, D.G., Bjornsson, L., Alves, M., and Mattiasson, B. (2006) "Effects of Bioaugmentation by an Anaerobic Lipolytic Bacterium on Anaerobic Digestion of Lipid-Rich Waste." *Journal of Chemical Technology and Biotechnology*. 81: 1745-1752.
- Cupples, A.M. (2008) "Real-Time PCR Quantification of *Dehalococcoides* Populations: Methods and Applications." *Journal of Microbiological Methods*. 72: 1-11.
- Da Silva, M.L. and Alvarez, P.J.J. (2004) "Enhanced Anaerobic Biodegradation of Benzene-Toluene-Ethylbenzene-Xylene-Ethanol Mixture in Bioaugmented Aquifer Columns." *Applied and Environmental Microbiology*. 70.8: 4720-4726.
- Davidsson, A., Wawrzynczyk, J., Norrflow, O., and La Cour Jansen, J. (2007) "Strategies for Enzyme Dosing to Enhance Anaerobic Digestion of Sewage Sludge." *Journal of Residuals Science and Technology*. 4.1: 1-7.
- Deflaun, M.F. and Steffan, R.J. (2002) "Bioaugmentation." In: Bitton, G. (ed) *Encyclopedia of Environmental Microbiology*. (Vol. 1, p. 434-442). Wiley Interscience. New York, NY.
- Dhakad R. K., Waghmare C. K., Alam S. I., Kamboj D. V. and Singh L. (2003) "Effect of Propionate on Methanogenesis of Night Soil at Psychrophilic Temperature." *Bioresource Technology*, 87:299-303.
- Duran, M., Tepe, N., Yurtsever, D., Punzi, V.L., Bruno, C., and Mehta, R.J. (2006) "Bioaugmenting Anaerobic Digestion of Biosolids With Selected Strains of *Bacillus*, *Pseudomonas*, and *Actinomycetes* Species for Increased Methanogenesis and Odor Control." *Applied Microbiology and Biotechnology*. 73: 960-966.
- Ellis, D.E., Lutz, E.J., Odom, J.M., Buchanan, Jr., R.J., Bartlett, C.L., Lee, M.D., Harkness, M.R., and Deweed, K.A. (2000) "Bioaugmentation for Accelerated In-Situ Anaerobic Bioremediation." *Environmental Science and Technology*. 34.11: 2254-2260.
- Evans, G.M. and Furlong, J.C. (2003) *Environmental Biotechnology: Theory and Application*. John Wiley and Sons, Ltd. West Sussex, England.
- Gaiek, R.L. (1988) "The Effect of Bioaugmentation Under Steady and Non-Steady State Influent Conditions." *Environmental Engineering Proceedings of the 1988 Joint CSCE-ASCE National Conference*. 130-136.

- Goldstein, R.M., Mallory, L.M., and Alexander, M. (1985) "Reasons for Possible Failure of Inoculation to Enhance Biodegradation." *Applied and Environmental Microbiology*. 50.4: 977-983.
- Guiot, S.R., Tartakovsky, B., Lanthier, M., Levesque, M.J., Manuel, M.F., Beaudet, R., Greer, C.W., and Villemur, R. (2002) "Strategies for Augmenting the Pentachlorophenol Degradation Potential of UASB Anaerobic Granules." *Water Science and Technology*. 45.10: 35-41.
- Guiot, S.R., Tawfiki-Hajji, K., and Lepine, F. (2000) "Immobilization Strategies for Bioaugmentation of Anaerobic Reactors Treating Phenolic Compounds." *Water Science and Technology*. 42.5-6: 245-250.
- He, J., Sung, Y., Dollhopf, M.E., Fathepure, B.Z., Tiedje, J.M., and Loffler, F.E. (2002) "Acetate Versus Hydrogen as Direct Electron Donors to Stimulate the Microbial Reductive Dechlorination Process at Chloroethene-Contaminated Sites." *Environmental Science and Technology*. 36: 3945-3952.
- Head, M.A. and Oleszkiewicz, J.A. (2005) "Bioaugmentation with Nitrifying Bacteria Acclimated to Different Temperatures." *Journal of Environmental Engineering*. 131.7: 1046-1051.
- Horber, C., Christiansen, N., Arvin, E., and Ahring, B.K. (1998) "Improved Dechlorinating Performance of Upflow Anaerobic Sludge Blanket Reactors by Incorporation of *Dehalospirillum multivorans* into Granular Sludge." *Applied and Environmental Microbiology*. 64.5: 1860-1863.
- Lendvay, J.M., Loffler, F.E., Dollhopf, M., Aiello, M.R., Daniels, G., Fathepure, B.Z., Gebhard, M., Heine, R., Helton, R., Shi, J., Krajmalnik-Brown, R., Major, Jr., C.L., Barcelona, M.J., Petrovskis, E., Hickey, R., Tiedje, J.M., and Adriaens, P. (2003) "Bioreactive Barriers: A Comparison of Bioaugmentation and Biostimulation for Chlorinated Solvent Remediation." *Environmental Science and Technology*. 37: 1422-1431.
- Lenz, M., Enright, A.M., O'Flaherty, V., van Aest, A.C., and Lens P.N.L. (2009) "Bioaugmentation of UASB Reactors with Immobilized *Sulfurospirillum barnesii* for Simultaneous Selenate and Nitrate Removal." *Applied Microbiology and Biotechnology*, 83.2: 377-388.
- Luepromchai, E., Singer, A.C., Yang, C., and Crowley, D.E. (2002) "Interactions of Earthworms with Indigenous and Bioaugmented PCB-Degrading Bacteria." *FEMS Microbiology Ecology*. 41: 191-197.
- Lynch, N., Daniels L., and Parkin, G.F. (1987) "Bioaugmentation of Stressed Anaerobic Filters with Methanogenic Enrichment Cultures." *Proceedings of the 42nd*

- Industrial Waste Conference*. (pp. 285-296). West Lafayette, Indiana: Purdue University.
- Maier, R.M., Pepper, I.L., and Gerba, C.P. (2000) *Environmental Microbiology*. Academic Press. San Diego, CA.
- Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J., and Buonamici, L.W. (2002) "Field Demonstration of Successful Bioaugmentation to Achieve Dechlorination of Tetrachloroethene to Ethene." *Environmental Science and Technology*. 36.23: 5106-5116.
- McCarty, P.L. and Smith, D.P. (1986) "Anaerobic Wastewater Treatment." *Environmental Science and Technology*. 20.12: 1200-1206.
- McCarty, P.L. (1997) "Microbiology: Breathing with Chlorinated Solvents." *Science*. 276.5318: 1521-1522.
- Mladenovska, Z., Ishoy, T., Mandiralioglu, A., Westermann, P., and Ahring, B. (2001) "Bioaugmentation of a Mesophilic Biogas Reactor by Anaerobic Xylanolytic- and Cellulolytic Bacteria." *Proceedings of the 9th World Congress on Anaerobic Digestion*. Antwerpen, (pp. 183-188). Belgium.
- Mulligan, C.N. (2002) "Environmental Biotreatment: Technologies for Air, Water, Soil, and Waste." *Journal of Hazardous Materials*. 93.2:253-255.
- Nielsen, H.B., Mladenovska, Z., and Ahring, B.K. (2007) "Bioaugmentation of a Two-Stage Thermophilic (68°C/55°C) Anaerobic Digestion Concept for Improvement of the Methane Yield from Cattle Manure." *Biotechnology and Bioengineering*. 97.6: 1638-1643.
- Noyola, A. and Tinajero, A. (2005) "Effect of Biological Additives and Micronutrients on the Anaerobic Digestion of Physicochemical Sludge." *Water Science and Technology*. 52.1-2: 275-281.
- Nyer, E. K. and Bourgeois, H. J., Jr. (1980) "Operational Troubleshooting in Industrial Biological Treatment Systems." *Proceedings of the 35th Industrial Waste Conference*. (pp. 849-854). West Lafayette, Indiana: Purdue University.
- O'Flaherty, V. and Colleran, E. (1999) "Effect of Sulphate Addition on Volatile Fatty Acid and Ethanol Degradation in an Anaerobic Hybrid Reactor. I: Process Disturbance and Remediation." *Bioresource Technology*. 68: 101-107.
- O'Flaherty, V., Colohan, S., Mulkerrins, D., and Colleran, E. (1999) "Effect of Sulphate Addition on Volatile Fatty Acid and Ethanol Degradation in an Anaerobic Hybrid

- Reactor. II: Microbial Interactions and Toxic Effects.” *Bioresource Technology*. 68:109-120.
- Rahm, B.G., Chauhan, S., Holmes, V.F., Macbeth, T.W., Sorenson, Jr., K.S., Alvarez-Cohen, L. (2006) “Molecular Characterization of Microbial Populations at Two Sites With Differing Reductive Dechlorination Abilities.” *Biodegradation*. 17.6: 523-34.
- Ramadan, M.A., El-Tayeb, O.M., and Alexander, M. (1990) “Inoculum Size as a Factor Limiting Success of Inoculation for Biodegradation.” *Applied and Environmental Microbiology*. 56.5: 1392-1396.
- Rigo, E., Rigoni, R.E., Lodea, P., de Oliveira, D., Freire, D.M.G., and Luccio, M.D. (2008) “Application of Different Lipases as Pretreatment in Anaerobic Treatment of Wastewater.” *Environmental Engineering Science*, 25.9:1243-1248.
- Rittmann, B.E. and Whiteman, R. (1994) “Bioaugmentation: A Coming of Age.” *Water Quality International*. 1: 12-16.
- Saravanane, R., Murthy, D.V.S., and Krishnaiah, K. (2001) “Bioaugmentation and Anaerobic Treatment of Pharmaceutical Effluent in Fluidized Bed Reactor.” *Journal of Environmental Science and Health*. 36.5: 779-791.
- Satoh, H., Okabe, S., Yamaguchi, Y., and Watanabe, Y. (2003) “Evaluation of the Impact of Bioaugmentation and Biostimulation by In Situ Hybridization and Microelectrode.” *Water Research*. 37: 2206-2216.
- Savant, D.V., and Ranade, D.R. (2004) “Application of *Methanobrevibacter acididurans* in Anaerobic Digestion.” *Water Science and Technology*, 50.6:109-114.
- Schauer, A. (2008) *Bioaugmentation for Recovery of Anaerobic Digesters Subjected to a Toxicant*. Ph.D. Dissertation, Marquette University, Milwaukee, WI.
- Singer, A.C., van der Gast, C.J., and Thompson, I.P. (2005) “Perspectives and Vision for Strain Selection in Bioaugmentation.” *TRENDS in Biotechnology*. 23.2: 74-77.
- Sleep, B.E., Seepersad, D.J., Mo, K., Heidorn, C.M., Hrapovic, L., Morrill, P.L., McMaster, M.L., Hood, E.D., Lebron, C., Sherwood Lollar, B., Major, D.W., and Edwards, E.A. (2006) “Biological Enhancement of Tetrachloroethene Dissolution and Associated Microbial Community Changes.” *Environmental Science and Technology*. 40.11: 3623-3633.
- Stephenson, D. and Stephenson, T. (1992) “Bioaugmentation for Enhancing Biological Wastewater Treatment.” *Biotechnology Advances*. 10: 549-559.
- Streger, S.H., Vainberg, S., Dong, H., and Hatzinger, P.B. (2002) “Enhancing Transport of *Hydrogenophaga flava* ENV735 for Bioaugmentation of Aquifers

- Contaminated with Methyl *Tert*-Butyl Ether.” *Applied and Environmental Microbiology*. 68.11: 5571-5579.
- Tartakovsky, B., Levesque, M.-J., Dumortier, R., Beaudet, R. and Guiot, S.R. (1999) “Biodegradation of Pentachlorophenol in a Continuous Anaerobic Reactor Augmented with *Desulfitobacterium frappieri* PCP-1.” *Applied and Environmental Microbiology*. 65.10: 4357-4362.
- Tawfiki Hajji, K., Lepine, F., Bisailon, J.G., Beaudet, R., Hawari, J., and Guiot, S.R. (2000) “Effects of Bioaugmentation Strategies in UASB Reactors with a Methanogenic Consortium for Removal of Phenolic Compounds.” *Biotechnology and Bioengineering*. 67.4: 417-423.
- Toffey, W.E. and Higgins, M.J. (2007) “An Odor Odyssey : Review of Scientific Progress toward Eliminating Odorant Emissions from Philadelphia’s Biosolids.” *Proceedings of Moving Forward Wastewater Biosolids Sustainability : Technical, Managerial and Public Synergy, International water Association, Moncton, New Brunswick Canada*.
- Van Limbergen, H., Top, E.M., and Verstraete, W. (1998) “Bioaugmentation in Activated Sludge: Current Features and Future Perspectives.” *Applied Microbiology and Biotechnology*. 50: 16-23.
- Weiß, S., Tauber, M., Somitsch, W., Meincke, R., Muller, H., Berg, G., Guebitz, G.M. (2010) “Enhancement of Biogas Production by Addition of Hemicellulolytic Bacteria Immobilised on Activated Zeolite.” *Water research*, 44.6:1970-1980.
- Zitomer, D.H. and Shrout, J.D. (1997) “Kinetics of COD Biotransformation Under Methanogenic, Limited-Aeration Conditions.” *WEFTEC '97 the 70th Annual Conference and Exposition of the Water Environment Federation*. Chicago, Illinois. 18-22
- Zitomer, D.H. and Shrout, J.D. (1998) “Feasibility and Benefits of Methanogenesis Under Oxygen-Limited Conditions.” *Waste Management*. 18: 107-116.
- Zou, S., Anders, K.M., and Ferguson, J.F. (2000) “Biostimulation and Bioaugmentation of Anaerobic Pentachlorophenol Degradation in Contaminated Soils.” *Bioremediation Journal*. 4.1: 19-25.

CHAPTER 3: MICROBIAL COMMUNITY ANALYSIS

3.1 Introduction

Anaerobic digesters contain a complex microbial community. Members of this community carry out diverse metabolic functions leading to the overall digestion process. These microorganisms include members of the domains *Bacteria* and *Archaea* which work together to convert many organic substrates into methane and carbon dioxide. Syntrophic relations make the understanding of the microbial community important for the successful application of anaerobic biotechnology, but practitioners in the past haven't paid significant attention to the delicate balance of microorganisms doing the work in anaerobic digesters (Amann et al., 1998; Scully et al., 2007).

It was believed that a smoothly operating digester possessed a stable microbial community. But recent insight in the composition of these communities reveals that there may be continuous change within the microbial consortia although the overall function of the community appears to be at steady state (Fernandez et al., 1999; Fernandez et al., 2000; Zumstein et al., 2000). It is difficult to obtain pure cultures of many members of these complex communities and, for a number of years, the underlying microbiology of anaerobic digesters remained undiscovered and was often thought of as a 'black box' (Godon et al., 1997; Riviere et al., 2009). With the advent of new molecular tools, efforts have been made to view these consortia in their habitats and to observe their compositional changes following environmental perturbation (Fernandez et al., 1999; Verstraete, 2007; Riviere et al., 2009; Leclerc et al., 2004).

An overall review of the microbiology associated with anaerobic digestion is presented below, followed by a discussion of the methanogens and their syntrophic role with bacteria in a phenomenon called interspecies hydrogen transfer.

3.1.1 Anaerobic Digesters Community Analysis

In the past, it was difficult to analyze the microbial community of anaerobic digesters because classical techniques (presence/absence of indicator organisms, isolation of pure cultures) and direct monitoring approaches (profiles of respiratory quinones, polyamine, phospholipid, fatty acid patterns) proved to have limitations (Raskin et al., 1994; Amann et al., 1998; Godon et al., 1997; Riviere et al., 2009). But recently, molecular techniques like direct rRNA gene sequence analysis followed by hybridization, direct cloning-sequencing (metagenomic approaches), randomly amplified polymorphic DNA (RAPD), fluorescence in situ hybridization (FISH) as well as polymerase chain reaction (PCR) followed by clone library or amplified ribosomal DNA restriction analysis (ARDRA), denaturing gradient gel electrophoresis (DGGE), single strand conformation polymorphism (SSCP), and ribosomal intergenic spacer analysis (RISA) have been developed to explore microbial diversity. Use of these molecular techniques has been initiated to explore the community profile of anaerobic digesters (Raskin et al., 1994; Raskin et al., 1995; Griffin et al., 1998; McHugh et al., 2003; Leclerc et al., 2004; McHugh et al., 2004; McMahon et al., 2004; Calli et al., 2005; Chen et al., 2005; Diaz et al., 2006; Hori et al., 2006; Keyser et al., 2006; Mladenovska et al., 2006; McGarvey et al., 2007; Hatamoto et al., 2007). Moreover, a few other techniques like metagenomics and pyrosequencing can be used without any need to culture microbes (Ansorge, 2009).

Although these techniques have opened up an entirely new world for the understanding of microbial communities and diversity in anaerobic digesters, it is important to realize that there are potential problems associated with them which can lead to misleading conclusions (von Wintzingerode et al., 1997; Head et al., 1998; Spiegelman et al., 2005; Pontes et al., 2007; Janda and Abbott et al., 2007; Marzorati et al., 2008). There are biases involved in the DNA extraction step due to the difference in the cell wall structures of microbes which may lead to preferential extraction of DNA from a few species (von Wintzingerode et al., 1997). Many techniques require PCR amplification of the target DNA gene and PCR involves many potential problems such as chimera formation, deletion or insertion mutation, preferential amplification of the desired gene from a few species due to primer specificity, and inhibition due to the presence of humic or other inhibitory substances (von Wintzingerode et al., 1997). Subsequent steps used for the separation of amplified DNA from a mixture of DNA may lead to preferential cloning of particular DNA types leading to erroneous data (von Wintzingerode et al., 1997). Finally, the obtained sequence data is compared to data present in databanks which are not conclusive, which may ultimately lead to identifying microbes as being 'uncultured or unnamed' which doesn't give much information about phenotypic properties (von Wintzingerode et al., 1997).

Community profiles of anaerobic digesters have been studied for different purposes. For example, comparing microorganism surveys for a variety of digesters can help to understand the organisms living in the digesters (Raskin et al., 1994; Raskin et al., 1995; McHugh et al., 2003; Leclerc et al., 2004). Another type of community profile survey has been completed specific to UASB digesters (Zheng and Raskin, 2000; Diaz et

al., 2006; Keyser et al., 2006). These digesters use granules that serve as a habitat for the microorganisms. Studying the underlying complex microbiology of these granules can be beneficial for understanding how microorganisms interact in these reactors and the knowledge gained can be used to shorten start-up time or overcoming upset events.

The effect of specific conditions on microbial community structure has also been explored with the objective to analyze the microorganisms responsible for various process functions and aid in digestion optimization. One of the process functions that has been studied is the start-up of anaerobic digesters (Griffin et al, 1998; McMahon et al., 2004). Establishment of desirable microbiota is crucial for anaerobic digester start-up and without the proper microorganisms the digester may fail (Hobson and Wheatley, 1993; Griffin et al., 1998). Therefore, knowing the identity of the microorganisms that aid in the start-up of anaerobic digesters is important.

More complete treatment of waste is another important process function (McGarvey et al., 2007; Mladenovska et al., 2006). Studies have aimed to identify the microbial communities that were capable of carrying out additional enhanced treatment so that reactor size could be reduced and may help to uncover organisms that are unique to a specific treatment goal (McGarvey et al., 2007; Mladenovska et al., 2006).

Also, the microbial communities within digesters treating specific substrates have been studied (Chen et al., 2005; McHugh et al., 2004; McMahon et al., 2004; Hatamoto et al., 2007). Acclimation of these microbes to substrates they were used to treat, showed potential for better degradation in an anaerobic digester (McMahon et al., 2004). Acclimation was found to be important if the reactor contained an excess of the substrate

or if the substrate was toxic to members of the microbial community (Hobson and Wheatley, 1993).

3.1.2 Microbial Survey of Anaerobic Digesters

Molecular techniques have been used to survey microbial communities in various environmental habitats such as soils, groundwater, aquifers, tidal flats, marine and lake waters as well as anaerobic digesters.

Riviere et al. (2009) surveyed seven different municipal anaerobic digesters operated at mesophilic temperatures. All the digesters had a cylindrical shape and were used for the digestion of municipal sludge with or without industrial waste. The industrial effluents represented less than 30% (v/v) of the total influent volume. A clone library approach based on the *16S rRNA* gene for comparison of microbial communities was followed. A core group of microbes was ubiquitously found in all the samples tested. The core group consisted of microbes related to *Methanosarcinales*, *Meathanomicrobiales* and Arc I from the domain *Archaea* and *Chloroflexi*, *Betaproteobacteria*, *Bacteroidetes* and *Firmicutes* from the domain *Bacteria*. The researchers concluded that the role of this core group appears to be critical for overall digester function and needs to be investigated further. A non-core group of microbes was identified and members were related to *Crenarchaeota* from the domain *Archaea* and *Aminanaerobia*, *WWE-1*, *Actinobacteria*, *Synergistetes*, *Coprothermobacteria* and *Spirochaete* from the domain *Bacteria*.

Tang et al. (2005) studied two mesophilic anaerobic chemostats fed the protein bovine serum albumin as the substrate. Chemostat-1 was supplied with $0.1 \text{ mgNi}^{2+}/\text{L}$ and Chemostat-2 was supplied with $0.12 \text{ mgCo}^{2+}/\text{L}$. Chemostat-1 produced higher effluent

VFA and NH_3 as well as negligible CH_4 , whereas CH_4 , CO_2 and NH_3 were the main products of Chemostat-2 at steady state. Microbial community analysis based on *16S rRNA* gene analysis showed that the genera *Methanosaeta* and *Methanoculleus* were abundantly present in chemostat-2, whereas there were very limited total archaeal cells present in chemostat-1. In the bacterial clone library, clones related to the phyla *Firmicutes* (32%), *Bacteroidetes* (11%) and *Proteobacteria* (13%) were found in Chemostat-2 and the remaining clones were found to be uncultured organisms (44%). Clones related to phyla *Firmicutes* (43%), *Bacteroidetes* (50%) and *Proteobacteria* (7%) were found in Chemostat-1 and no uncultured bacterial clones were detected.

Leclerc et al. (2004) surveyed the archaeal communities of 44 anaerobic digesters using the SSCP technique. The V3 region of the *16S rRNA* gene was selected for the analysis. These various-sized digesters treated different waste types, including agricultural, food processing, petro-chemical, pulp and paper, brewery, slaughterhouse, and municipal wastes. The organisms found most frequently among the different digesters included an acetoclastic methanogen *Methanosaeta concilii* (84% of the digesters) and clone vadinDC06, which was located within the hydrogenotrophic genus *Methanobacterium* (73% of the digesters).

McHugh et al. (2003) surveyed three full-scale anaerobic digesters treating industrial wastes and three lab-scale anaerobic hybrid reactors operated at different temperatures (mesophilic, thermophilic and psychrophilic). Microbial diversity was quantified based on a clone library and the ARDRA patterns. *Methanosaeta sp.*, was present in all digesters. Other methanogens found included *Methanococcus*,

Methanosarcina and members of the methanogen orders *Methanobacteriales* and *Methanomicrobiales*.

Raskin et al. (1995) studied the community profiles of 21 single-phase, full-scale, anaerobic digesters digesting municipal sewage sludge using oligonucleotide probes. It was found that all of these digesters had similar community profiles, with the majority of the Archaea belonging to the genera *Methanosaeta* and order *Methanomicrobiales*. Methanogenic members of the order *Methanococcales* were also found. The digester which was determined to have “poor” performance due to increased concentrations of acetate and propionate had low levels of *Methanosaeta* and *Methanomicrobiales* species.

Raskin et al. (1994) analyzed the communities of various types of digesters: lab-scale solid waste digesters and full-scale sewage sludge digesters using oligonucleotide probes complementary to conserved regions of the *16S rRNAs* of groups of methanogens. In the solid-waste lab digester, members of the order *Methanobacteriales* were the major hydrogenotrophic methanogens (Raskin et al., 1994). The full-scale digesters had similar methanogenic community profiles, which consisted of *Methanogenium* and *Methanosaeta* (Raskin et al., 1994).

The data presented above suggests that *Methanosaeta sp.* was found as a common component of digesters (Raskin et al., 1994; Raskin et al., 1995; McHugh et al., 2003; Leclerc et al., 2004) and also at least one hydrogenotrophic group of methanogens existed in each digester.

3.1.3 Microbial Communities in the Granules of UASB Digesters

UASB digesters typically form a blanket of anaerobic granules that have a higher settling velocity than flocculent biomass. These granules also provide a location for the microorganisms to attach and grow. Because of a low HRT, the UASB is one of the most preferred anaerobic biotechnologies for treating high strength organic wastes. Each granule from the anaerobic blanket of a UASB consists of a complex microbial colony structure containing members that can share syntrophic relationships.

Zheng and Raskin (2000) investigated the microbial community profile of UASB granules from a full-scale UASB reactor treating corn milling waste and two lab-scale UASB reactors treating synthetic wastewaters containing glucose or glucose and propionate by using genus- and species-specific hybridization probes. The authors concluded that the outer layer of granules from all the reactors was thin and contained mostly bacterial cells with a small number of archaea, whereas the inner layer was denser and contained almost only archaea. *Methanosaeta concilii* was found to be present throughout the granule structure, and it was most abundant in the cores. It was concluded that the spatial orientation of the granule microbes may be critical for optimal reactor function (Keyser et al., 2006).

Diaz et al. (2006) studied UASB granules from a full-scale facility treating brewery wastewater that were sorted by color (black, brown, and grey) using a combination of molecular techniques (FISH, DGGE, and cloning). They compared the granule structure, microbial diversity, and age-specific development of each type of methanogenic granule. The black granules were characterized as “young” and contained

the archaea *Methanospirillum* and *Methanosarcina*. The black granules were the only type that contained active microorganisms within the entire interior of the granule. The grey granules were classified as “middle-aged” and contained *Methanosaeta*, which was similar to the brown, or “old” granules, the only difference being they were more filamentous, soft and fluffy (Diaz et al., 2006).

Keyser et al. (2006) identified the methanogens present in UASB granules treating different wastewaters. Using DGGE and further sequencing of DGGE bands, the microorganisms from waste treatment for three different industries, winery, brewery, and canning, were studied. The brewery and winery UASB granules contained *Methanosaeta concilii*. This was important because studies by Hulshoff Pol et al. (2004) indicated that *Methanosaeta* played a key role in UASB granulation. These organisms were considered to be responsible for the formation of the nucleus of the granules and they provided a structure for the bacteria and other methanogens to grow syntrophically, forming the entire granule (Hulshoff Pol et al., 2004). The brewery granules also contained *Methanosaeta thermophila* and *Methanobacterium formicicum*, whereas the winery granules contained *Methanosarcina mazeii* (Keyser et al., 2006). The canning industry granules contained three uncultured archaea named APL1, APL2, and APL3. These uncultured archaea showed the closest similarity to previously uncultured archaeons with a sequence similarity of only 88%, 85% and 88%, respectively (Keyser et al., 2006).

Diaz et al (2006) and Keyser et al (2006) suggested that certain microorganisms seem to favor specific conditions when it comes to granule habitat. But, similar to Zheng and Raskin (2000), these studies concluded that *Methanosaeta* was an important

organism in nearly all UASB granules. Also *Methanosaeta* was found to form the core of the granule so its presence appeared to be extremely important for starting the granulation process (Zheng and Raskin, 2000; Diaz et al., 2006).

Therefore, knowing the organisms present in optimally performing UASB reactors may help digester operators choose supplemental organisms to help recover poorly performing reactors. Also, in order to develop the proper granules for a reactor, bioaugmentation with a proper methanogenic culture may be an option for reducing start-up time.

3.1.4 Microbial Communities During Start-up of Anaerobic Digesters

Start-up of anaerobic digesters is a very crucial step; it can be time-consuming and failures are possible (Hobson and Wheatley, 1993; Griffin et al., 1998). Community profile analysis of microorganisms during successful start-up can be used to design subsequent reactors favoring these particular organisms allowing for a shorter start-up time.

Griffin et al. (1998) analyzed a methanogenic community during start-up of two anaerobic lab scale CSTRs. One CSTR was maintained at a mesophilic temperature (37°C) and the other at a thermophilic temperature (55°C). Using rRNA-targeted oligonucleotide probes, large variations in the digester methanogenic community structures were observed when start-up was not performing well as indicated by the accumulation of VFA. The thermophilic digester had a shorter start-up period and the levels of hydrogenotrophic methanogens (*Methanobacteriaceae*) were found to be higher in the thermophilic digester than the mesophilic digester. The reason behind the quicker

start by the thermophilic digester may have been the presence of a higher amount of hydrogenotrophic methanogens which might have led to more efficient metabolism of certain VFAs (like propionate and butyrate), but further research is needed to confirm this assumption.

McMahon et al., (2004) studied the microbial communities during start-up of lab-scale anaerobic co-digesters treating municipal solid waste and sewage sludge using oligonucleotide probes. The authors concluded that *Methanosaeta concilii* was an important methanogen for successful start-up and digesters with poor start-up performance contained a higher proportion of *Methanosarcina spp.* than *Methanosaeta*. The study also found that the presence of syntrophic propionate-oxidizing bacterium *Syntrophobacter wolinii* helped in degrading propionate during successful start-up.

This research suggests that the acetotrophic methanogen, *Methanosaeta sp.*, is an important component for successful start-up of anaerobic digesters. Also, since the start-up may be process specific, the presence of other hydrogenotrophic methanogens (like *Methanobacteriaceae*) may also be critical (Griffin et al., 1998).

3.1.5 Microbial Communities for Substrate Degradation/Conversion

In the past, the microorganisms that specifically degrade or convert substrates like fatty acids and volatile sulfur compounds in anaerobic environments have been studied.

3.1.5.1 Fatty Acids

Hatamoto et al. (2007) investigated long-chain fatty acids used as a substrate in a digester by comparing the microbial communities of lab-scale and full-scale reactors, along with the effect of different temperatures (mesophilic and thermophilic). They used RNA-based stable isotope probing, terminal restriction fragment length polymorphism (T-RFLP), cloning and sequencing. It was found that a diverse group of organisms were present to degrade long-chain fatty acids in anaerobic digesters, as shown in Table 3.1. Syntrophic associations were found to be important for the degradation of long-chain fatty acids because complete oxidation of the long-chain fatty acids is thermodynamically unfavorable in the anaerobic environment unless the consumption of hydrogen and/or formate is coupled with the oxidation of the long-chain fatty acids (Hatamoto et al, 2007).

Table 3.1: Bacteria Found in Anaerobic Digesters Degrading Long-Chain Fatty Acids

	<i>Acidobacteria</i>
Phylum	<i>Spirochaetes</i>
	<i>Chlorobi</i>
Class	<i>Clostridia</i>
	<i>Geobacteraceae</i>
Family	<i>Deferribacteraceae</i>
	<i>Syntrophaceae</i>
	<i>Anaerobaculum</i>
	<i>Synergistes</i>
	<i>Coprothermobacter</i>
Genus	<i>Syntrophomonas</i>
	<i>Syntrophothermus</i>
	<i>Tepidanaerobacter</i>
	<i>Thermotoga</i>

McHugh et al. (2004) conducted a microbial community comparison for anaerobic digesters operating at psychrophilic temperatures that were fed different substrates and they used various molecular techniques (clone library, ARDRA, and sequencing). One reactor was fed sucrose and the other was fed a mixture of fatty acids (acetate, butyrate and propionate) along with ethanol. The VFA-fed reactor maintained successful performance levels at low temperatures (95% COD removal at 18°C), but

declined in performance when a shock (increase in upflow velocity) was introduced. The sucrose-fed reactor did not perform particularly well (50-60% COD removal at 16°C), but when the shock was introduced, the performance was actually enhanced (COD removal increased to greater than 80%). This showed that the sucrose-fed microorganisms may have been able to handle a shock to the reactor more easily than the VFA-fed microorganisms, which appeared to perform better under stable feeding conditions. The stressed condition caused an increase in *Methanomicrobales sp.* (hydrogenotrophic methanogens) along with a decrease in *Methanosarcina sp.* and *Methanosaeta sp.* (acetotrophic methanogens) in the microbial community profile of both the reactors (McHugh et al., 2004).

McMahon et al. (2004) studied the microbial community in anaerobic digesters during organic overload. It was shown that the overload caused a rapid increase in VFAs, which inhibited the methanogens. But those reactors that had VFA accumulation in the past were shown to perform better during subsequent overload periods. This implied that there could be acclimation to high VFAs by microorganisms, or there was a community shift to those organisms that used the VFAs which may help when a future overload occurs.

3.1.5.2 Volatile Organic Sulfur Compounds (VOSCs)

The presence of hydrogen sulfide (H₂S) and VOSCs, including dimethyl sulfide cause odor problems during anaerobic digestion. Methanogens have been found to degrade dimethyl sulfide and, therefore, can play a role in VOSC reduction. Chen et al.

(2005) analyzed the microbial communities from 12 anaerobically digested biosolid cakes using PCR-DGGE techniques. The profiles were found to be the same for all of the mesophilic digesters and the identified microorganisms were in the family *Methanosarcinaceae*. The Archaea (including methanogens) demonstrated a simplified community profile compared to the Bacterial community, which was important because any stress imposed on the former community could result in a decrease in the degradation of VOSCs and a subsequent release of odors. The authors also suggested that the abundance of the overall methanogenic community was more important than the existence of a specific methanogen on VOSC degradation.

3.1.6 Methanogenic Population Shift Due to Change in Operating Conditions

Molecular techniques were also used to monitor community profiles when a set of operating conditions changed. When an environment is perturbed, the community may change in order to adapt to the change. The analysis of the microbial community shifts can lead to a determination of the function of certain microorganisms within a community and ultimately an ecosystem.

Schauer (2008) Studied the archaeal community profiles of four lab-scale anaerobic enrichments (C1, C2, C3 and C4) using *16S rRNA* gene and molecular techniques (PCR, cloning, restriction digests, and sequencing). The enrichment conditions differed based upon whether or not glucose and/or oxygen was fed to the enrichment. The substrates used for enriching the cultures were as follows: C1 – H₂:CO₂, C2 – H₂:CO₂ + glucose, C3 – H₂:CO₂ + air, and C4 – H₂:CO₂ + glucose and air. When

comparing the enrichment cultures that received air versus those that did not, a shift in the methanogen community occurred. In C1 and C2, *Methanosaeta* was the dominant genus. On the other hand, *Methanosaeta* was not dominant in C4 and was not detected in C3. *Methanospirillum* was the dominant methanogen genus in C3. Organisms related to *Methanospirillum* were also detected in C4 but *Methanosaeta* was the dominant genus. It was concluded that addition of air in absence of glucose significantly increased the *Methanospirillum* population in the hydrogen-enriched cultures.

Hori et al. (2006) used a combination of molecular techniques (SSCP, quantitative PCR (qPCR), FISH) and concluded that the hydrogenotrophic methanogen, *Methanoculleus sp.* was the predominant methanogen during stable reactor performance with no VFAs in the reactor. Once the VFA concentrations increased, the community shifted and *Methanothermobacter sp.* became dominant with an approximately 10,000-fold increase. Once the VFA levels returned to normal (after 60 days), *Methanoculleus sp.* started to increase again. This means that methanogens can potentially recover from stressed conditions once the conditions return to favorable and the presence of certain hydrogenotrophic methanogens may correspond with lower effluent VFA concentration (propionate in particular).

Delbes et al. (2001) analyzed a community profile shift of an anaerobic digester during an increase in acetate concentration by using SSCP analysis of 16S rDNA and rRNA PCR products. During the stressed period of 80 days, the effluent acetate concentration exceeded 1500 mg/L and small amounts of propionate started to accumulate. A shift in Archaea occurred during this period from *Methanobacterium-*

related to *Methanosaeta concilii*-related and then back to *Methanobacterium*-related once the acetate levels decreased. This demonstrated a shift from hydrogenotrophic to acetotrophic methanogens in order to potentially degrade the excess acetate in the system. The Bacterial community also changed during this period with an increase in the *Spirochetes spp.* and then a return to the *Synergistes spp.* once the acetate levels decreased.

Calli et al. (2005) investigated the effect of increased ammonia levels on various lab-scale digesters using a combination of molecular techniques (DGGE, FISH, cloning and sequencing). The free ammonia nitrogen (FAN) increase in these digesters caused a stressed condition. As FAN increased, the *Methanosaeta*-related species decreased and *Methanosarcina*-related methanogens were detected. One reactor was able to resist FAN levels as high as 750 mg/L. This reactor was seeded with sludge taken from a laboratory-scale UASB reactor treating high ammonia acidogenic landfill leachate. The authors suggested that the inorganic particles from the seed sludge provided a good surface support for *Methanosarcina* species which enabled the reactor to withstand high concentration of FAN.

The analyses of the microbial communities in the studies mentioned above are important for understanding the beneficial and critical microbes involved in the process of anaerobic digestion. As the above review suggests, stressed conditions may cause microbial community shifts in anaerobic digesters (Hori et al., 2006; Delbes et al., 2001; Calli et al., 2005). In light of the experimentation explained in the previous sections (see Section 2), it is important to know the composition of the microbial community in

organically overloaded conditions. It is also important to know which individual microbes are present in the recovering digesters following an organic overload. Knowledge of such microbial consortia may provide clues for improved recovery. Also, microbial analysis of the bioaugmentation culture may provide the underlying reasons behind the findings of earlier authors (Lynch et al., 1987) who concluded that the effect of bioaugmentation on the organically overloaded anaerobic packed bed reactors by the enrichment cultures acclimated to propionate was short-lived.

In this study the microbial community analysis of an undisturbed control digester, a bioaugmented digester, a non-bioaugmented digester and the enrichment culture was carried out to test the hypothesis that bioaugmentation helps restore the microbial community of an anaerobic digester following an organic overload.

3.2 Methods and Materials

To study the community structure in digesters during recovery from an organic overload, microbial community analysis was carried out. Microbial community structures were evaluated for the bioaugmentation culture enriched for both propionate and $25\text{mgO}_2/\text{L-day}$ (culture C2a), the digester bioaugmented with culture C2a (i.e. TD-10%-1), the non-bioaugmented digester augmented with an autoclaved version of the culture C2a (i.e. CD-10%-1) and the undisturbed control digester (i.e. UCD-1) (see Table 2.3 and 2.4 for designations). Effluent samples from digesters were collected on day 120 (6 SRTs following the organic overload). Day 120 was selected for collecting samples because

there was a statistically significant difference between the effluent SCOD concentrations of TD-10% and CD-10% on day 120 (see Section 2.2). Also DNA from culture C2a was extracted on the same day.

3.2.1 Molecular Techniques for Community Analysis

Clone libraries for the *16S rRNA* gene of bacteria and archaea were constructed. Molecular techniques used for microbial community analysis and the analytical procedures followed are explained in this section.

3.2.1.1 DNA Extraction

Microbial DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions modified by the inclusion of the alternative lysis method. This alternative lysis method is recommended if the cells are difficult to lyse and entails a 10 min incubation at 70 °C to aid in cell lysis (PowerSoil Protocol, 2007). This method replaced the horizontal vortexing of the PowerBead Tubes and was meant to reduce shearing of DNA. The presence of extracted DNA was confirmed using agarose gel electrophoresis.

3.2.1.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed by the procedure explained in section 1.2.5.2.

3.2.1.3 Polymerase Chain Reaction (PCR)

PCR was performed on DNA samples using 50 μL of EconoTaq® PLUS 2X Master Mix (Lucigen Corporation, Middleton, WI), which included 0.1 units/ μL of EconoTaq DNA Polymerase, Reaction Buffer (pH 9.0), 400 μM each of dATP, dGTP, dCTP, and dTTP, 3mM MgCl_2 , and a proprietary PCREnhancer/Stabilizer (EconoTaq® PLUS 2X Master Mix Protocol). One μL per reaction of forward and reverse primer solution (concentration of 0.1 μM) were added to the PCR tubes with nuclease-free H_2O to make a 100- μL reaction volume. The *Bacteria* primers used for amplification of the *16S rRNA* gene were 27F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane, 1991). The *Archaea* primers were A571 (5'-GCY TAA AGS RIC CGT AGC -3') and UA1406R (5'-ACG GGC GGT GWG TRC AA -3') specific to *Archaea* (Baker et al., 2003). The PCR reactions were completed by using a Bio-Rad PTC-200 DNA Engine Cycler thermal cycler. Figures 3.1 a) and b) show the thermocycler programs used for bacterial and archaeal amplification.

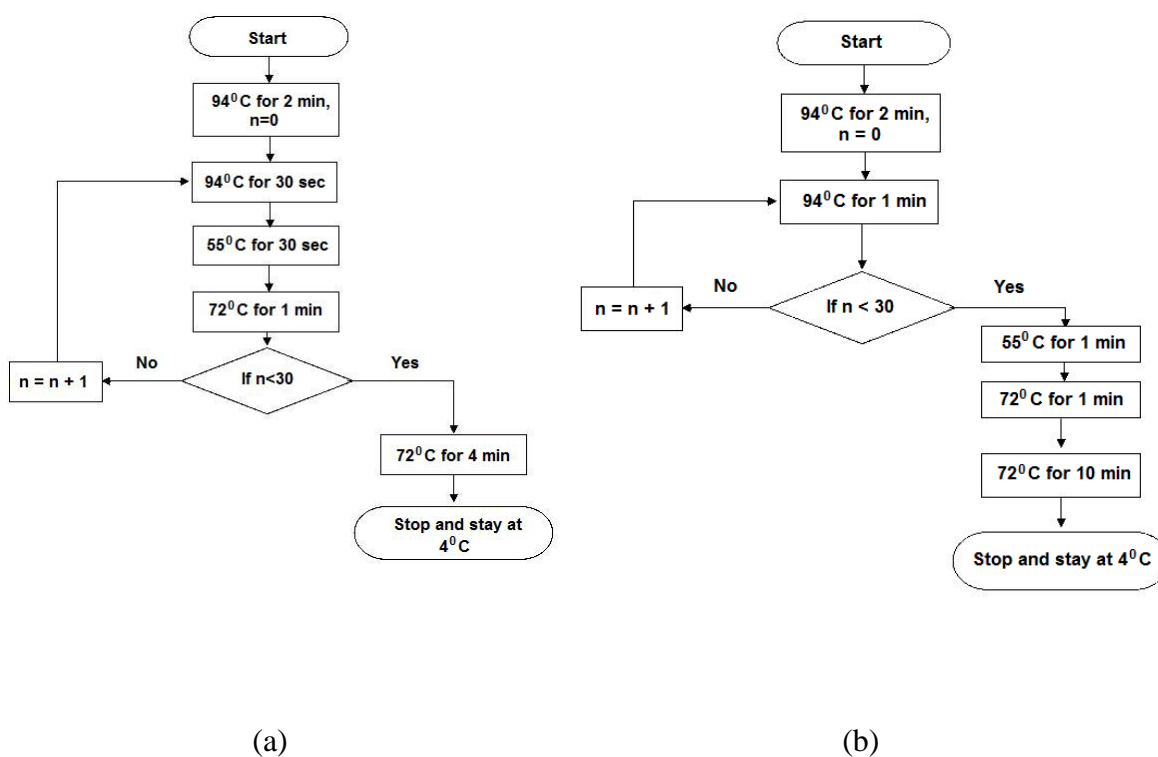


Figure 3.1: Thermocycler Programs for PCR Amplification of (a) Bacterial 16S rDNA (b) Archaeal 16S rDNA (Fode-Vaughan et al., 2001 and Baker et al., 2003)

3.2.1.4 Cloning

Cloning of the PCR product was done utilizing the TOPO TA Cloning® Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Transformants containing plasmids with amplified product were screened via blue/white selection (Sambrook and Russell, 2001). The light-colored colonies were picked and a PCR, with PucF (5'-GGA ATT GTG AGC GGA TAA CA-3') and PucR (5'-GGC GAT TAA GTT GGG TAA CG-3') primers was run on each colony to amplify the DNA. Figure 3.2

shows the PCR amplification programme that was used for Puc amplification. The PCR products obtained were run on an agarose gel to confirm the presence of amplified DNA.

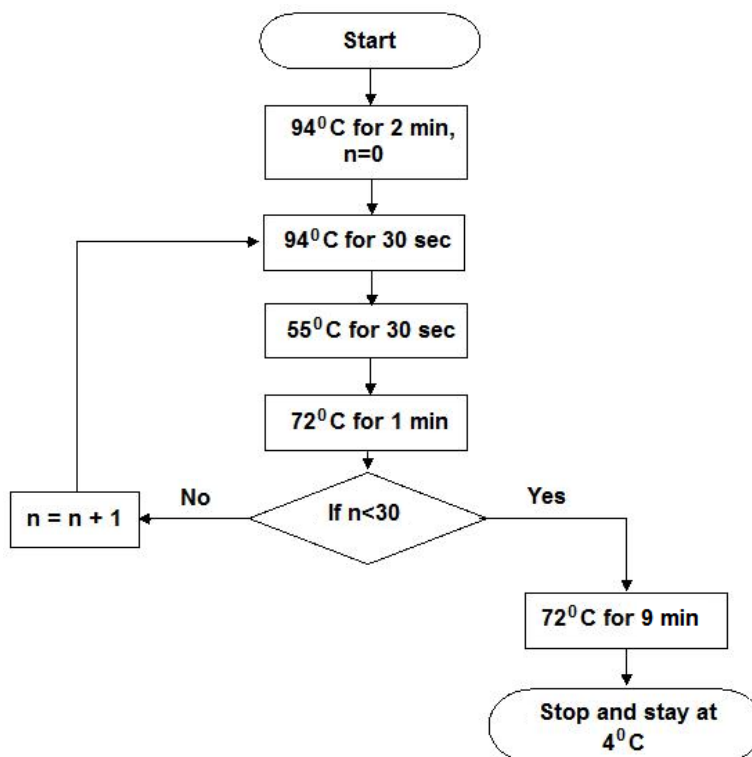


Figure 3.2: Thermocycler Program for PCR Amplification for Puc Primers

3.2.1.5 PCR Purification

To prepare the samples for sequencing, samples were cleaned using the UltraClean™ PCR Clean-up™ Kit (MoBio Laboratories, Carlsbad, CA). Clean-up was done in an effort to remove unwanted reaction components and purify the DNA for sequencing. The protocol included with the kit was used without modification.

3.2.1.6 DNA Sequencing

The amplified products were sequenced at the University of Chicago Cancer Research Center's DNA sequencing facility which sequenced individual clones for universal forward and reverse primers using a capillary automated DNA sequencer (Applied Biosystems, Foster City, CA).

3.2.1.7 Analysis of sequences

The forward and reverse sequences obtained from the sequencing facility were analyzed using the FinchTV, v 1.4.0 (Geospira Inc., Seattle, WA) and Vector NTI, v 11.0 (Invitrogen Corporation, Carlsbad, CA) software. Contiguous sequences were assembled for each clone using forward and reverse sequences. Vector segments from the contiguous sequences were removed using Vecscreen, a tailor-made computer program written by Dr. Craig Struble, Department of Mathematics, Statistics and Computer Science, Marquette University. Vecscreen searches the UniVec database of the National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html>) using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997) for cloning vectors. If there is a match in the UniVec database sequences and the submitted query sequence then the program removes vector segments of the submitted sequence.

Chimera detection analysis was performed using Chimera Check, v 2.7 (Cole et al., 2003; Maidak et al., 2000) and Bellerophon (Huber et al., 2004). Unaligned

sequences were submitted to online versions of Chimera Check and Bellerophon tools. While using Bellerophon tool, a window size of 200 was selected and no distance correction method was applied. The identified chimera sequences were removed from the data.

After checking for chimeras, the sequences were submitted to Ribosomal Database Project (RDP) (Cole et al., 2003; Cole et al., 2007; Cole et al., 2009; Maidak et al., 2000; Wang et al., 2007) which uses the program Infernal, v 1.0 (Nawrocki et al., 2009) that aligns *16S rRNA* gene sequences based on their secondary RNA structure. The resulting alignment was downloaded from the RDP server (<http://rdp.cme.msu.edu/>).

Distance matrices were calculated from the alignment by using 'DNADIST' algorithm of the Phylogeny Inference Package (PHYLIP, v 3.68) (Felsenstein, 2005). All the distances were calculated by the Kimura "2-parameter" model which assigns different probabilities for the transitions and transversions for a nucleotide base change.

Operational taxonomic units (OTUs) were formed from the distance matrices using the DOTUR program, v 1.53 which assigned sequences to OTUs by the farthest neighbor algorithm (Schloss et al., 2005). A criterion of 99% sequence similarity was used to form OTUs. DOTUR was also used to plot rarefaction curves and calculate coverage, richness and diversity indices.

The S_{CHA01} estimator was calculated from the clustering pattern obtained for the clones in each clone library. The S_{CHA01} estimator is based on mark-release-recapture

techniques that yield an unbiased estimate of the probable number of phylotypes present in the source assemblage (Chao, 1984; Chao, 1987). S_{CHAO1} estimator was calculated as

$$\text{---} - \text{---} \quad (3.1)$$

In Equation 3.1, S_{OBS} is the number of OTUs observed in the library, F_1 is the number of OTUs containing one sequence only and F_2 is the number of OTUs containing two sequences only.

The S_{ACE} estimator was calculated for the clustering pattern of clones in each clone library. S_{ACE} estimator is another estimate for the probable number of phylotypes present in the source assemblage (Chao et al., 1993). The S_{ACE} estimator was calculated as,

$$\text{---} - \text{---} \quad (3.2)$$

The Y_{ACE}^2 in Equation 3.2 can be calculated as,

$$\text{---} - \text{---} ,$$

,

and $\text{---} - \text{---}$.

In the above equations, n_i is the number of OTUs with i individuals, S_{rare} is the number of OTUs with 10 or fewer individuals, and S_{abund} is the number of OTUs with more than 10 individuals.

OTU data from each clone library were used to calculate Good's C estimate (Good, 1953). Good's C estimate determines the number of OTUs that would actually be found in a library of infinite size based on the OTU data. Good's C was calculated as,

$$C = 1 - \frac{n_1}{N}, \quad (3.3)$$

where, n_1 is the number of OTUs containing one sequence only and N is the library size.

OTU heterogeneity in a sample was calculated by the Shannon-Weaver Index (Shannon and Weaver, 1963). Shannon-Weaver Index was calculated as,

$$H_{\text{Shannon}} = -\sum_{i=1}^n p_i \log_2 p_i \quad (3.4)$$

In Equation 3.4, H_{Shannon} represents Shannon-Weaver Index, n is the total number of OTUs present in a library, and p_i is the ratio of number of sequences present in an OTU with the total number of sequences.

Diversity of OTUs in a clone library was also found by Simpson's index. The Simpson's index value gives the probability of two individual clones randomly selected from a sample belonging to the same OTU. Simpson's diversity index was calculated as,

$$D_{\text{Simpson}} = \frac{1}{\sum_{i=1}^n p_i^2} \quad (3.5)$$

In Equation 3.5, H_{Simpson} is Simpson's Index, and S_i is number of sequences in the i^{th} OTU.

All the unique sequences were submitted to the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for nucleotide (BLASTN) search to find the closest related microorganisms in the database and subsequently DNA sequences of the closest related organisms from the database were collected to use as reference organisms for plotting phylogenetic trees. The selected reference sequences and the sample sequences were re-aligned using the Ribosomal Database Project server (Cole et al., 2009; Maidak et al., 2000). Neighbor-joining, maximum parsimony and maximum likelihood trees were created using the Phylogeny Inference Package (PHYLIP, v 3.68) (Felsenstein, 2005) and bootstrap analysis was done for 100 replications. Consensus trees generated by bootstrap analysis were visualized using the software FigTree v1.2.2 (Rambaut, 2008). Comparisons among different tree structures were done using the Tree Climber software package (Schloss et al., 2006). The online version of UniFrac tool (Lozupone et. al, 2006) was used to perform principle coordinate analysis (PCoA) on the phylogenetic trees. A combined tree containing all the bacterial or archaeal clone libraries was submitted to UniFrac software. UniFrac calculated pair-wise distances between the samples using ratio of sum of lineage lengths between two samples and total lineage length. While calculating pair-wise distances, the lineages that lead to descendants from either of the two samples were considered, whereas the lineages that contained clones from both the samples were not considered. The resulting distance matrix was used to perform PCoA. While performing PCoA, phylotype abundance weights were considered. Finally the first two principle components were used for plotting the data.

To perform PCA on the archaeal clone libraries, all the archaeal clone libraries were combined together and the distance matrix for the combined library was calculated using the RDP aligner and the DNADIST algorithm of the PHYLIP Package (PHYLIP, v 3.68) (Felsenstein, 2005). All the distances were calculated by the Kimura "2-parameter" model which assigns different probabilities for the transitions and transversions for a nucleotide base change. The resulting distance matrix was submitted to the DOTUR program (v. 1.53) and OTUs were formed using 99% similarity criterion. Contribution of each sample to individual OTU was determined by calculating ratio of the number of clones present in that OTU from that particular samples and the total number of clones in that OTU. These fractions were used as dimensions for performing PCA. PCA was performed using MATLAB (v.7.6(R2008a)) software package which calculated a covariance matrix for the given data. The first two coordinates that represented the highest amount of variation among the data were determined. These two coordinates, called principle components, were used for plotting the data. The samples were clustered on the two dimensional space in three groups using farthest neighbor algorithm. Projections of the three dimensions that had highest contribution to the principle components were also projected on the plot of the first two principle components. Same procedure was followed for performing PCA on bacterial clone libraries and projections of the six dimensions that had highest contribution to the principle components were also projected on the plot of the first two principle components. The resulting PCA plots were expected to follow the same pattern as that of PCoA plots obtained from UniFrac software.

3.3 Results and Discussion

3.3.1 Archaea

3.3.1.1 Archaeal Diversity, Richness and Functional Organization

PCR of *16S rRNA* gene fragments followed by clone library construction yielded DNA sequence data. The data were analyzed and cleaned by removing chimera sequences as mentioned in the methods section (see Section 3.2.1.6). Following the chimera check, the data were submitted to RDP seqmatch query (Cole et al., 2003; Cole et al., 2007; Cole et al., 2009; Maidak et al., 2000). Interestingly it was found that many of the sequences obtained were classified under the domain *Bacteria*. The reason behind obtaining bacterial clone for the primers specific to the domain *Archaea* may be that the archaeal primers might have annealed to the bacterial genes present in the PCR product due to the lower annealing temperature used. This suggested that the PCR program used needs further fine-tuning. The clones classified under the *Bacteria* domain were not considered for further analysis. Table 3.2 shows the summary of the chimera-and bacteria-free archaeal clones obtained for each sample.

Table 3.2: Number of Good Archaeal Sequences Obtained

DNA Sample	Number of Clones Sequenced	Number of Good Sequences Obtained, Classified Under Domain <i>Archaea</i>
UCD-1	96	66
TD-10%-1	96	68
CD-10%-1	96	57
C2a	96	55

Rarefaction curves were constructed using the DOTUR program (Schloss et al., 2005). Rarefaction curves are based on empirical calculations that help to estimate if the number of samples sequenced provides an accurate estimate of the diversity present in the population. The rarefaction value increases with each new OTU obtained in the sample (Kemp and Aller, 2004).

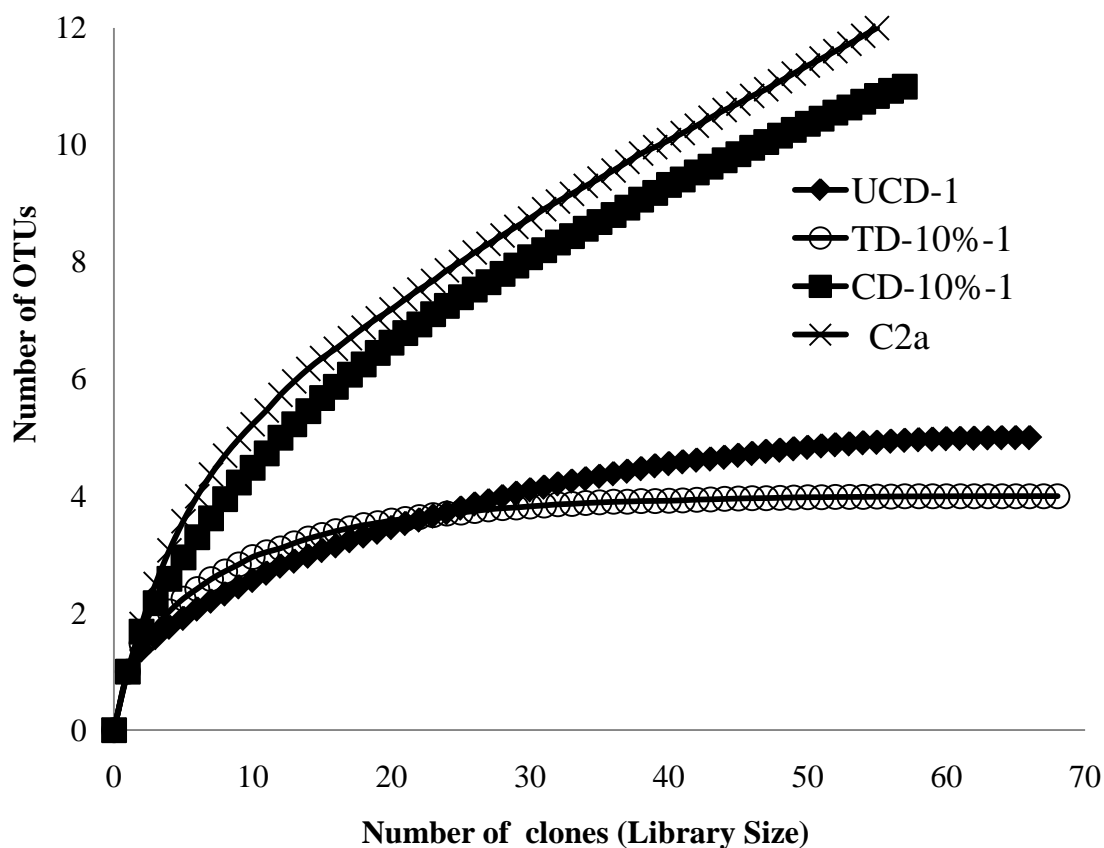


Figure 3.3: Rarefaction Curves for The Archaeal Clone Libraries

Figure 3.3 shows that for the given library sizes, TD-10%-1 and UCD-1 clone libraries attained nearly complete coverage, whereas C2a and CD-10%-1 clone libraries did not attain complete coverage for a library size of 57 and 55, respectively. This indicates that the archaeal communities of C2a and CD-10%-1 were more diverse than the archaeal communities of UCD-1 and TD-10%-1.

S_{CHAO1} and S_{ACE} provide estimates of the number of clones expected in a clone library (Kemp and Aller, 2004) and Good's C provides another estimate of the amount of coverage obtained using the number of unique clones and the total library size (Good,

1953). Richness indices like S_{CHAO1} , S_{ACE} and Good's C are sensitive to the number of rare OTUs and so must be applied with caution but the confidence intervals associated with them give a better clue of accuracy of the richness estimation (Riviere et al., 2009).

Table 3.3 shows S_{CHAO1} , S_{ACE} and Good's C estimates for the archaeal clone libraries. Again, all the estimates indicated that archaeal diversity of UCD and TD-10%-1 was covered by the library size of 66 and 68, respectively, whereas there were still archaeal OTUs to be detected in samples CD-10%-1 and C2a. According to the S_{CHAO1} estimates, approximately 78% of the archaeal diversity of sample CD-10%-1 was covered, whereas only 36% of the archaeal diversity of sample C2a was covered. According to S_{ACE} estimates, 64% of the archaeal diversity of sample CD-10%-1 was covered whereas 36% of the archaeal diversity of sample C2a was covered. Good's C estimates indicated 91 and 87% coverage for samples CD-10%-1 and C2a respectively.

It is known that the S_{ACE} estimator yields smaller coverage values than S_{CHAO1} and Good's C estimates since "it is derived from a larger subset of the complete frequency distribution than S_{CHAO1} estimator" (Kemp and Aller, 2004). Therefore, the S_{ACE} estimator is particularly appropriate for data sets in which some phylotypes (OTUs) occur more frequently. On the other hand, the S_{CHAO1} estimator is derived from the number of phylotypes appearing either one or two times in a given library so it is particularly appropriate for data sets in which most phylotypes are relatively rare in the library (Kemp and Aller, 2004). In the case presented herewith, the S_{ACE} estimator was found to be an appropriate choice due to skewed distribution of clones among the OTUs and because more than two clones were often associated with an OTU (explained in following paragraphs and Figure 3.4).

Table 3.3: S_{CHAO1} , S_{ACE} and Good's C Estimates for Archaeal Clone Libraries

	Observed	S_{CHAO1}	S_{ACE}	Good's C
		Predicted	Predicted	
	Number of	Number of	Number of	
	OTUs	OTUs*	OTUs	
UCD-1	5	5 (5-5)	5 (5-5)	1.00
TD-10%-1	4	4 (4-4)	4 (4-4)	1.00
CD-10%-1	11	14 (12-33)	17 (12-43)	0.91
C2a	12	33 (17-98)	33 (16-127)	0.87

* values in the parenthesis indicate 95% confidence interval

The Shannon-Weaver index is a measure of the diversity within a sample (Shannon and Weaver, 1963). Higher Shannon-Weaver index values indicate a higher diversity of microbes in a clone library and vice versa.

Simpson's Index gives another estimate of the diversity in a sample. The index value is the probability of two individual clones randomly selected from a sample belonging to the same OTU. A higher Simpson's Index value indicates a less diverse sample and vice versa (Schloss et al., 2005).

Table 3.4 shows Shannon-Weaver and Simpson's diversity indices for the archaeal clone libraries obtained by using DOTUR program (Schloss et al., 2005).

Culture C2a had the highest archaeal diversity among the samples tested, followed by

CD-10%-1, whereas the DNA sample from TD-10%-1 and UCD-1 had lower archaeal diversity.

Table 3.4: Shannon-Weaver and Simpson's Indices for Archaeal Clone Libraries

	H_{Shannon}	H_{Simpson}
UCD-1	0.76	0.63
TD-10%-1	0.93	0.51
CD-10%-1	1.57	0.34
C2a	1.94	0.17

Both digesters UCD-1 and TD-10%-1 had lower archaeal diversity than CD-10%-1 and C2a. Moreover, both UCD-1 and TD-10%-1 performed better than CD-10%-1 in terms of methane production and effluent SCOD removal. Interestingly since C2a was used as the bioaugmentation culture and was introduced in TD-10%-1 on a daily basis, TD-10%-1 was also expected to have a higher diversity of archaea similar to culture C2a, but this was not the case.

All the diversity and coverage estimated indicated that both UCD-1 and TD-10%-1 had lower diversity than CD-10%-1 which indicates that the better function of anaerobic digesters digesting milk waste may require less diverse, but a dedicated, archaeal community.

Functional organization (f_o) of microbes in a sample can be represented by Pareto-Lorenz (P-L) evenness curves (Marzorati et al., 2008) (see Section 1.2.2.4). Figure 3.4 represents P-L curves for the archaeal clone libraries. Interestingly, functional organization of all the archaeal clone libraries fell into the medium category (Marzorati et al., 2008). Moreover, the f_o values of C2a and TD-10%-1 were about 0.6, whereas the f_o values of UCD-1 and CD-10%-1 were 0.8 and 0.7 respectively. This means archaeal community distributions in the libraries C2a and TD-10%-1 were more even as compared to the distribution of the clones in UCD-1 and CD-10%-1.

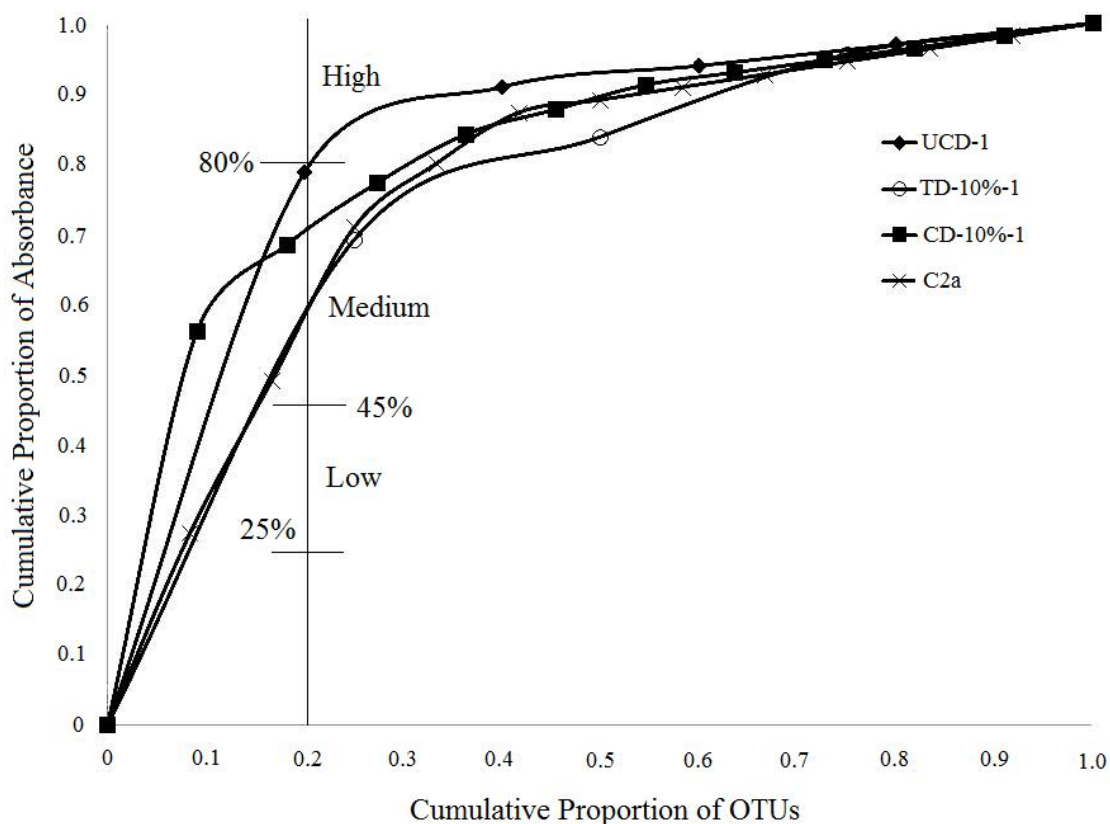


Figure 3.4: Pareto- Lorenz Evenness Curves of Archaeal Clone Libraries

3.3.1.2 Archaeal Phylogenetics

A nucleotide BLAST query (BLASTN) conducted for the archaeal sequences using the GenBank database revealed either uncultured or named organisms based on similarity in *16S rRNA* genes of query and database sequences. Almost all the archaeal sequences obtained were related to either methanogens or other uncultured archaea. The closely related sequences were used to construct phylogenetic trees as mentioned in the methods section (see Section 3.2.1.6). Phylogenetic trees were constructed by neighbor-joining, maximum likelihood and maximum parsimony algorithms. It was confirmed that different algorithms had no effect on the distribution of clones among clades of the different trees. Hence, the same organisms were grouped together in trees formed by different algorithms. Figures 3.5 through 3.8 show the bootstrapped phylogenetic trees constructed by using the neighbor joining algorithm. The numbers at the nodes represent the bootstrap values. The non-bold taxa identify the reference sequences (both cultured and uncultured), followed by their GenBank accession number. The sample sequences are shown in bold-type font and are designated by the name of the culture following the letter “A” for archaea and the OTU number. Only one sequence per OTU is shown and the number of sequences represented by that particular sequence is mentioned in a bracket at the end of sequence designation. The scale at the bottom of the tree indicates the number of nucleotide changes per sequence position. All the archaeal trees were rooted to the organism *Sulfolobus solfataricus* (X03235). Classification of all the OTUs based on RDP classifier (Wang et al., 2007; Cole et al., 2007) is presented at the right side of the trees.

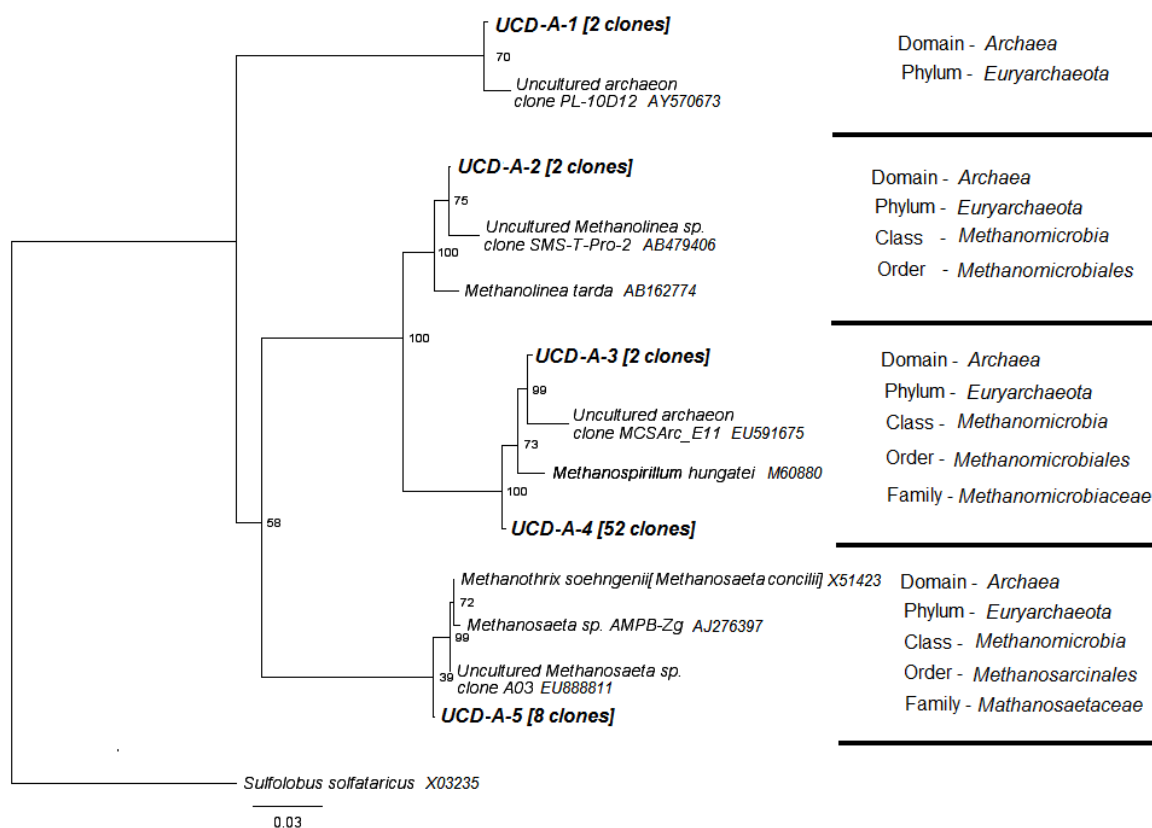


Figure 3.5: Phylogenetic Analysis of Culture UCD-1 Archaeal Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

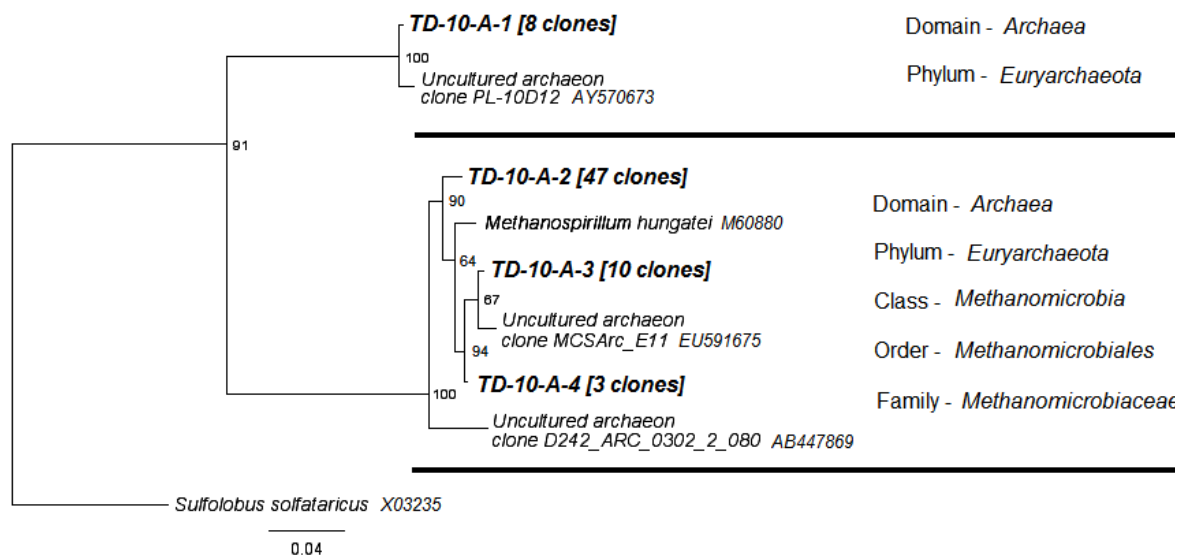


Figure 3.6: Phylogenetic Analysis of Culture TD-10%-1 Archaeal Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

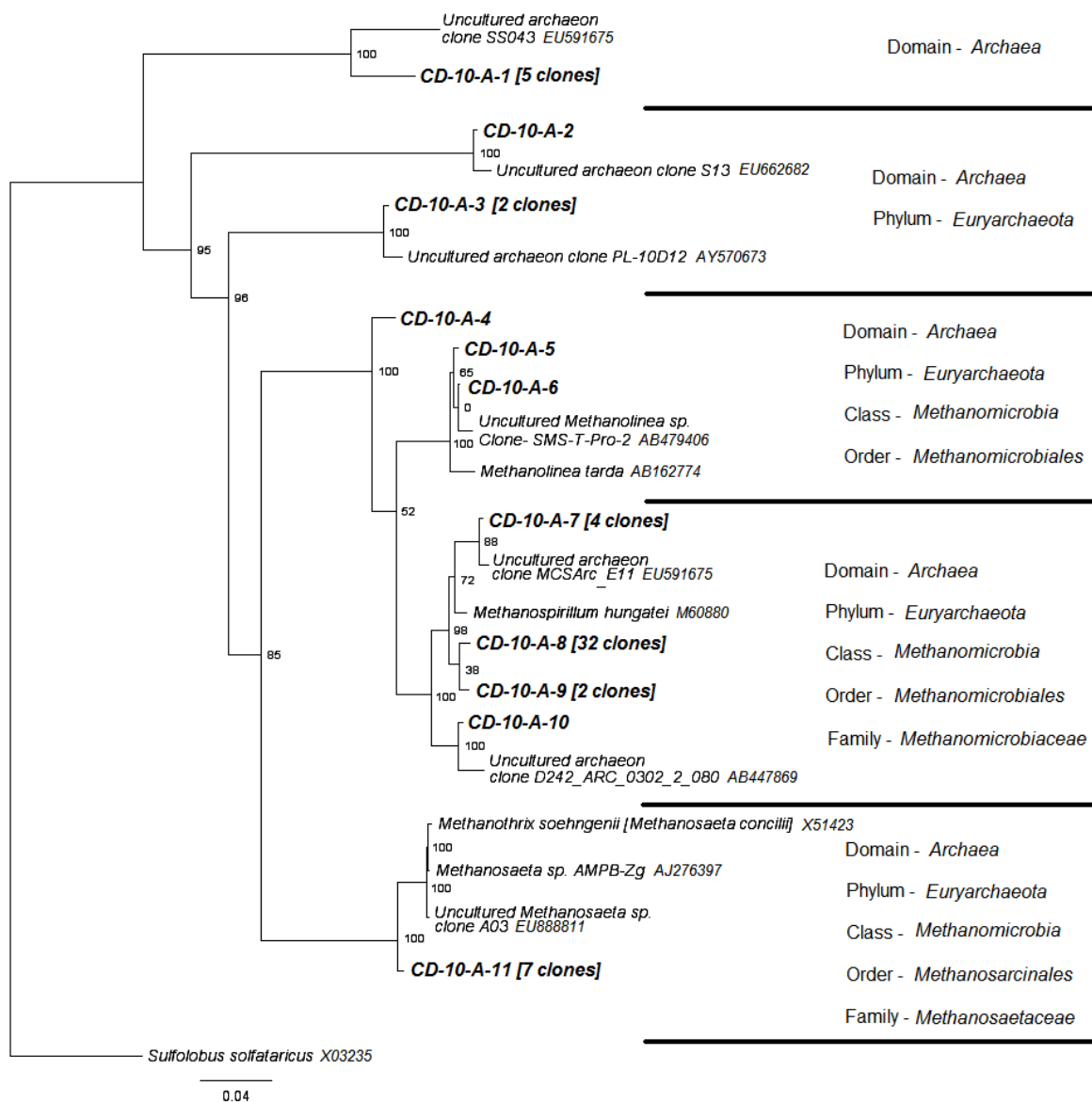


Figure 3.7: Phylogenetic Analysis of Culture CD-10%-1 Archaeal Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

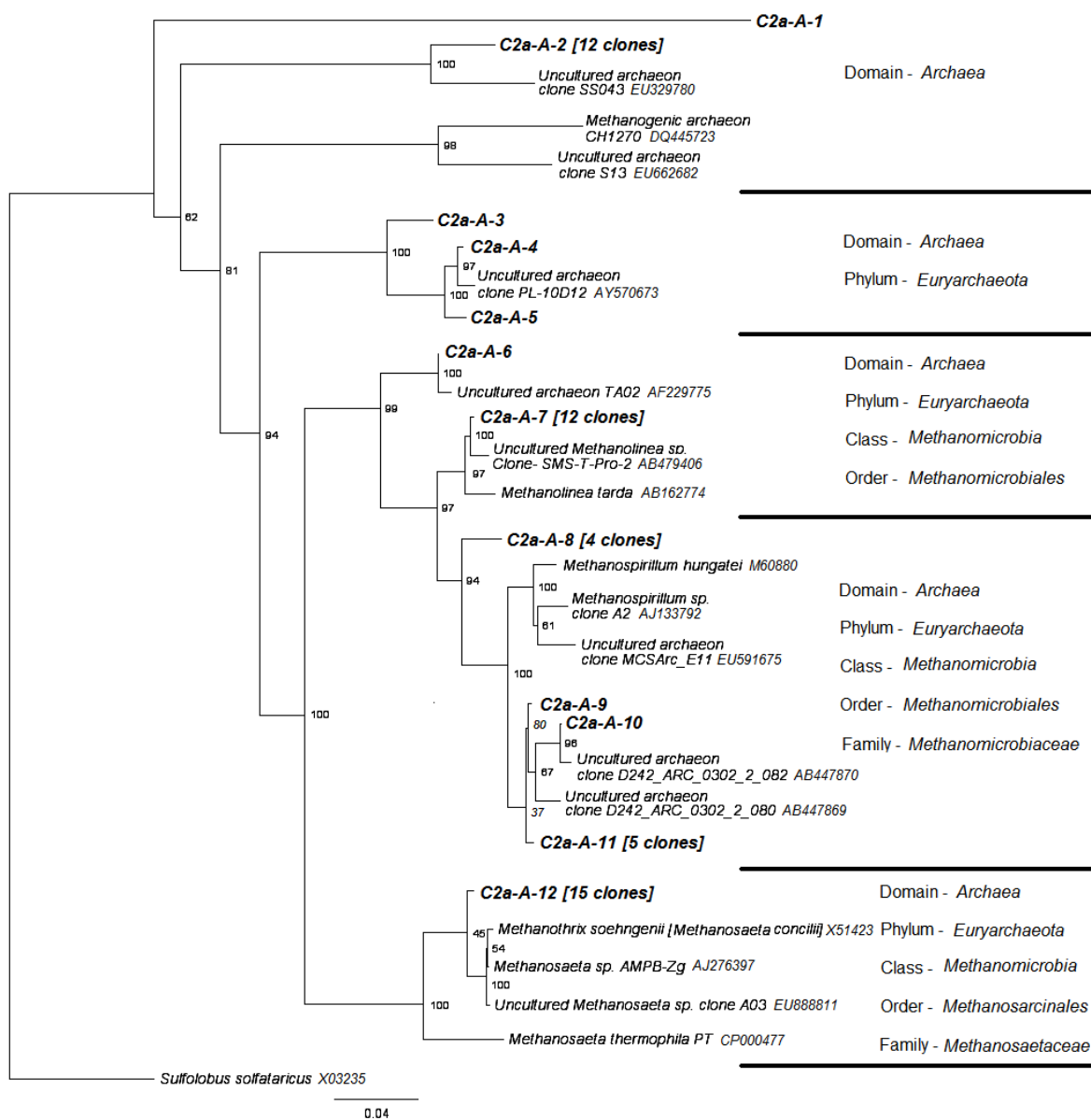


Figure 3.8: Phylogenetic Analysis of Culture C2a Archaeal Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Sulfolobus solfataricus* (X03235).

3.3.1.2.1 Physiological Characters of the Orders of Methanogens Found

Methanomicrobiales

Organisms classified under the order *Methanomicrobiales* utilize H₂ and formate as substrates (Garrity et al., 2001). The clones classified under the order *Methanomicrobiales* were related to methanogens *Methanospirillum hungatei* (M60880) and *Methanolinea tarda* (AB162774). Both these methanogens are classified under the family *Methanomicrobiaceae* and known to grow on H₂ and/or formate as substrates (Madigan and Martinko, 2006; Ferry, 1993). The presence of these hydrogenotrophic methanogens may correspond to a lower effluent VFA concentration (propionate in particular) (Hori et al., 2006).

Methanospirillum hungatei is a mesophilic methanogen (35-40°C optimum) (Yang et al., 1985; Ferry, 1993) and has a relatively short (0.7-day) doubling time (Ferry and Wolfe, 1977) whereas, *Methanolinea tarda* can grow between temperatures varying from 35 to 55°C -(50°C optimum) and has a 4-day doubling time at optimal conditions (Imachi et al., 2008).

Methanosarcinales

Organisms classified under the order *Methanosarcinales* from the clone libraries were also classified under the family *Methanosataceae*. Organisms classified under the family *Methanosataceae* are strict anaerobes and use acetate as the only energy source (Garrity et al., 2001). Also clones classified under family *Methanosataceae* were found to

be related to the organism *Methanosaeta concilii* (X51423) or *Methanosaeta thermophila* (CP000477).

Methanosaeta concilii and *Methanosaeta thermophila* grow optimally at neutral pH. *Methanosaeta concilii* can grow at temperatures varying from 10 to 45°C (35-40°C optimum) and has a doubling time of 2.5 to 2.9-days under optimal growth conditions, whereas *Methanosaeta thermophila* grows at temperatures between 30 to 70°C (55-60°C optimum) and has a doubling time of 1 to 1.5-day under optimal growth conditions (Patel and Sprott, 1990; Garrity et al., 2001).

3.3.1.2.2 Composition of Archaeal Clone Libraries

Figure 3.9 shows the relative abundance of clones from the archaeal libraries. The first column of Figure 3.9 represents the order *Methanomicrobiales* and hence a portion of hydrogenotrophic population of the archaeal community, whereas the second column represents the order *Methanosarcinales*. All the clones classified under the order *Methanosarcinales* were also classified under family *Methanosataceae* which includes the aceticlastic population of the libraries. The third and fourth column in the Figure 3.9 represent the population which could not be further classified based on similarity in *16S rRNA* gene sequences by the RDP classifier (Wang et al., 2007; Cole et al., 2007).

Figure 3.9 shows that both UCD-1 and TD-10%-1 had higher relative abundance of methanogens classified under the order *Methanomicrobiales* ($\geq 85\%$). A higher relative abundance of organisms related to *Methanomicrobiales* may have led to better

metabolism of H₂ and hence more complete degradation of propionate in the digesters UCD-1 and TD-10%-1 leading to better performance than digester CD-10%-1. Out of the total number of clones classified under the order *Methanomicrobiales*, 96, 100 and 92% of the clones from sample UDC-1, TD-10%-1 and CD-10-1 respectively were also classified under the family *Methanomicrobiaceae* and these clones were found to be related to *Methanospirillum hungatei* ($\geq 95\%$ similarity). It is important to note that among the culturable and strict hydrogenotrophic methanogens detected in these samples, *Methanospirillum hungatei* has the lowest doubling time which may be the reason behind the higher relative abundance of clones related to *Methanospirillum hungatei* in UDC-1, TD-10%-1 and CD-10-1.

Archaeal clones from UCD-1, CD-10%-1 and C2a were found to be classified under the order *Methanosarcinales* (family *Methanosataceae*), members of which are known to metabolize acetate (Patel and Sprott, 1990; Fernandez et al., 2000). Since complete metabolism of propionate involves conversion of acetate to methane, finding organisms classified as *Methanosarcinales* (family *Methanosaetaceae*) in C2a was expected. It is known that acetate is a major intermediate in the anaerobic conversion of propionate to methane and during degradation of complex substrates, the majority of electron flow occurs through acetate (Speece, 2008). So the presence of *Methanosarcinales* or other acetate-utilizing methanogens was expected in all the libraries. Surprisingly, however, *Methanosarcinales* was not detected in TD-10%-1 and the rarefaction data show that the archaeal clone library for TD-10%-1 covered most of the novel OTUs. The archaeal clone group from TD-10%-1 classified under the phylum *Euryarchaeota* may contain acetate-utilizing methanogens that are not yet known.

It is important to note that 26.3% of the archaeal clones from C2a were closely related to *Methanolinea tarda* (AB162774) (sequence similarity > 98%) which shows optimum growth at 50⁰C and has a 4-day doubling time (Imachi et al., 2008). Other researchers (Lynch et al., 1987) who tested recovery of organically overloaded anaerobic digesters using bioaugmentation with cultures acclimated to propionate (like C2a) used packed-bed filters. The packed-bed filter used had a 4 day HRT (Lynch et al., 1987) which may have led to washout of the slow-growing hydrogenotrophic methanogenic community members similar to *Methanolinea tarda*. Moreover, the failure of bioaugmentation was attributed to wash-out of the enrichment culture from the overloaded digesters. Also it was recommended that bioaugmentation may produce better recovery for CSTR digesters than for packed-bed filters (Lynch et al., 1987) (see details in Section 2.2). The study described herein presents a successful case of bioaugmentation using cultures acclimated to propionate. One reason for success may be because the CSTR digesters were operated at a 10-day SRT. The higher SRT may have allowed growth of slow-growing methanogens. Also adding the bioaugmentation culture on a daily basis may have helped recovery of the overloaded digesters.

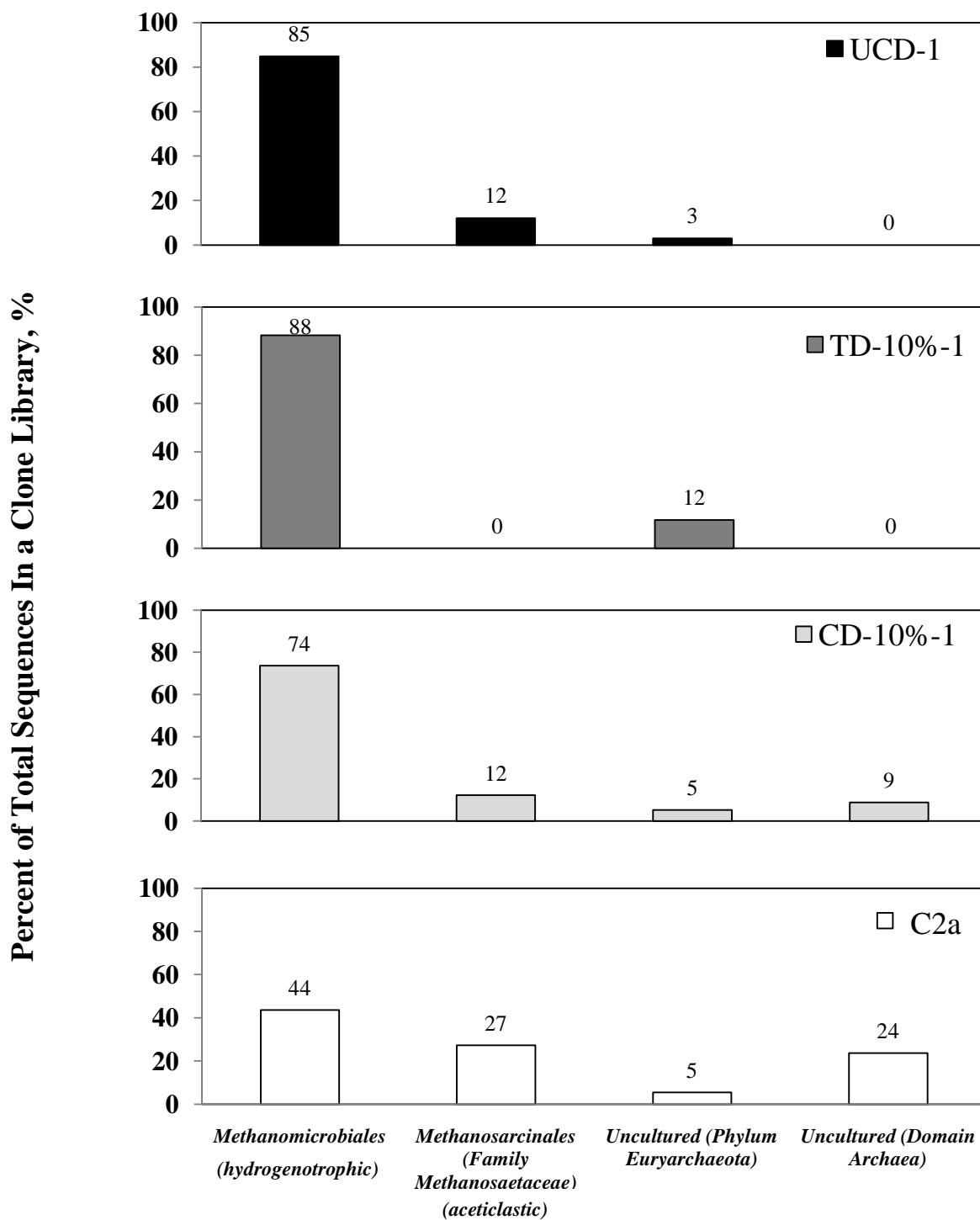


Figure 3.9: Archaeal Diversity of Cultures UCD-1, TD-10%-1, CD-10%-1 and C2a.

3.3.1.2.3 Comparison of Archaeal Phylogenetic Trees

It is important to note that OTUs with the highest archaeal relative abundance in all the digesters, represented by UCD-A-4, TD-10-A-2 and CD-10-A-8, shared $\geq 97\%$ sequence similarity. Also these OTUs shared 95% sequence similarity with OTU represented by C2a-A-8 and 96% sequence similarity with OTU represented by C2a-A-11. It was surprising to find low relative abundance of organisms related to *Methanomicrobiales* in culture C2a but the uncultured *Euryarchaeota* clones from C2a might have a significant proportion of H₂- and formate-utilizing methanogens (hydrogenotrophs).

Pair-wise comparison of phylogenetic tree structures of all the archaeal clone libraries was done using TreeClimber software (Schloss et al., 2006). TreeClimber uses the parsimony test for comparison of tree structures. TreeClimber randomly generates a specified number (in this case 1000) of sub-trees from the combined tree of two samples whose structures have to be compared. All the trees (including the initial combined tree) are given a parsimony score based on substitutions required. In the end, the ratio is calculated by dividing the number of sub-trees that had a better parsimony score than the initial combined tree by the number of sub-trees made (1000). This ratio represents the probability of the two tree structures being similar.

The probabilities obtained for pair-wise comparisons are presented in Table 3.5. Lower scores indicate that the tree structures being compared are relatively different. Samples having similar archaeal tree structure for a 1% level of significance are represented by gray colored cells in Table 3.5. The archaeal tree of culture C2a had a

very unique structure among the archaeal trees ($p=0.001$). Also CD-10%-1 and TD-10%-1 had very different tree structures ($p=0.001$) which means there was a significant difference between the archaeal communities of CD-10%-1 and TD-10%-1 after 6 SRTs. Interestingly TD-10%-1 and UCD-1 had very similar tree structures ($p=0.55$) and the tree structure of UCD-1 and CD-10%-1 had significant differences ($p=0.007$). It is important to note that both UCD-1 and TD-10%-1 had significantly lower effluent SCOD concentrations than CD-10%-1 (see Section 2.3).

This means, that although C2a had a very different archaeal tree structure than all the other samples, addition of C2a to TD-10%-1 restored its archaeal community to its undisturbed state (similar to UCD-1) resulting in more rapid recovery of TD-10%-1 following the shock organic overload.

Table 3.5: Pair-Wise Comparison of Archaeal Tree Structures

	UCD-1	TD-10%-1	CD-10%-1	C2a
UCD-1	-			
TD-10%-1	0.556	-		
CD-10%-1	0.007	0.001	-	
C2a	0.001	0.001	0.001	-

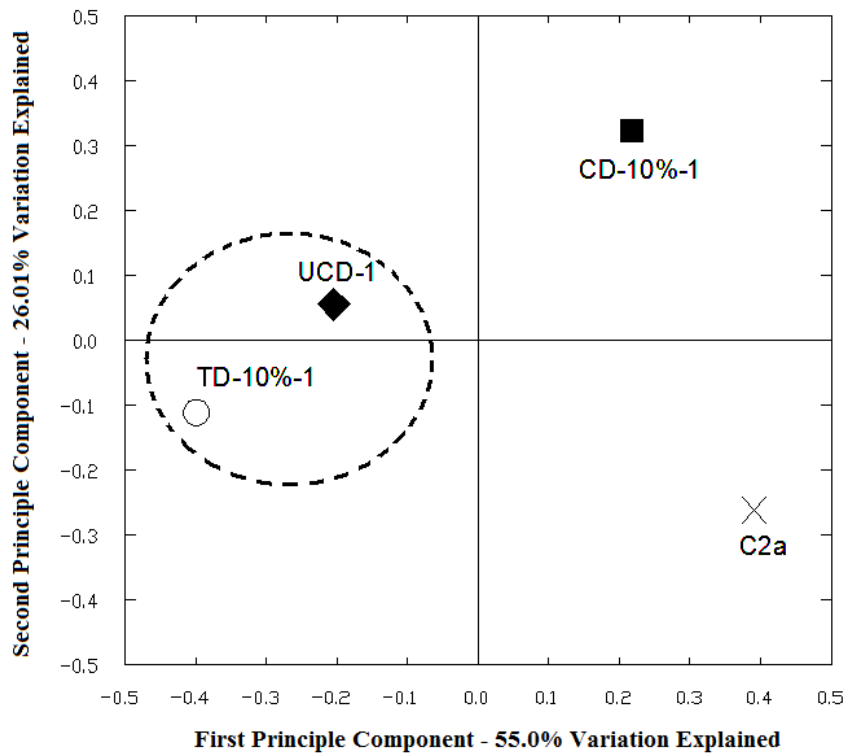
Principle coordinate analysis (PCoA) was performed using online UniFrac software (Lozupone et. al, 2006), using lineages of the phylogenetic trees. Figure 3.10 a)

shows the graph of first two principle components obtained from UniFrac tool which account for 81.01% of the total variation in the data. Grouping of the archaeal clone libraries in Figure 3.10 a) followed the results obtained for the grouping by the parsimony test. In Figure 3.10 a), UDC-1 and TD-10%-1 were found to be closer than UDC-1 and CD-10%-1 which indicates that there were more similarities between the tree structures of UDC-1 and TD-10%-1 than UDC-1 and CD-10%-1. Also the enrichment culture C2a is represented by a point that is far from all the other points indicating that culture C2a possessed a unique archaeal tree structure among all the archaeal clone libraries.

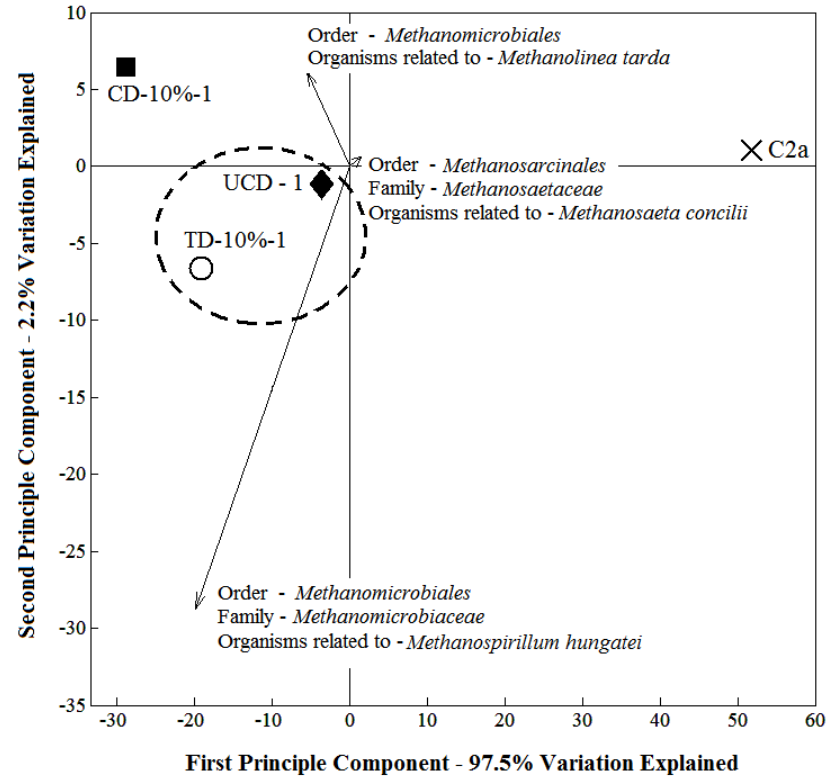
Figure 3.10 b) shows the plot of the first two principle components of the PCA performed using distribution of clones among the archaeal clone libraries using MATLAB(v.7.6(R2008a)) software package. Again grouping of the archaeal clone libraries in Figure 3.10 b) followed the results obtained for the grouping by the parsimony test. In figure 3.10 b), UDC-1 and TD-10%-1 were found to be closer than UDC-1 and CD-10%-1 which indicates that there were more similarities between the tree structures of UDC-1 and TD-10%-1 than UDC-1 and CD-10%-1. Also the enrichment culture C2a is represented by a point that was far from all the other points indicating that culture C2a possessed a unique archaeal tree structure among all the archaeal clone libraries.

Projection of the dimensions indicated that organisms related to *Methanospirillum hungatei* had the highest relative abundance in UDC-1 and TD-10%-1, whereas organisms related to *Methanolinea tarda* had more significant relative abundance in UDC-1 and CD-10%-1. As explained previously, both these organisms are

hydrogenotrophic methanogens, but *Methanolinea tarda* has an optimum temperature of 50°C and at the optimum growth temperature, it takes about 4 days to double its population (Imachi et al., 2008), whereas *Methanospirillum hungatei* is a mesophilic methanogen (35-40°C optimum) (Yang et al., 1985; Ferry, 1993) and has a 0.7-day doubling time (Ferry and Wolfe, 1977). Since the digesters were operated at 35±3°C, the higher relative abundance of organisms related to *Methanospirillum hungatei* may have resulted in better operation of UCD and TD-10% digesters by more rapid metabolism of hydrogen.



(a)



(b)

Figure 3.10: Graph of First Two Principle Components for Archaeal Communities (a) PCoA Performed on Tree Lineages Using UniFrac (Lozupone et. al, 2006) (b) PCA Performed on Distribution of Clones in the Clone Libraries Using MATLAB(v.7.6(R2008a)).

3.3.2 Bacteria

3.3.2.1 Bacterial Diversity, Richness and Functional Organization

Like archaeal clone libraries, bacterial clones were sequenced after PCR cleanup was performed. The obtained sequence data were submitted for chimera detection as mentioned in the methods section. The chimera sequences were detected and subsequently removed from the dataset. Following the chimera check, the data were further submitted to the RDP seqmatch query (Cole et al., 2007). Table 3.6 shows the actual number of good sequences obtained for each bacterial clone library.

Table 3.6: Number of Good Bacterial Sequences Obtained

DNA Sample	Number of Clones Sequenced	Number of Good Sequences Obtained, Classified Under Domain <i>Bacteria</i>
UCD-1	96	85
TD-10%-1	96	88
CD-10%-1	96	86
C2a	96	87

Figure 3.11 shows the rarefaction curves for the bacterial clone libraries. It is evident from the trends of the curves that none of the clone libraries reached complete coverage. Also the S_{CHA01} , S_{ACE} and Good's C indices given in Table 3.7 indicate that all the bacterial clone libraries represented less than 55% of the respective bacterial community in the samples.

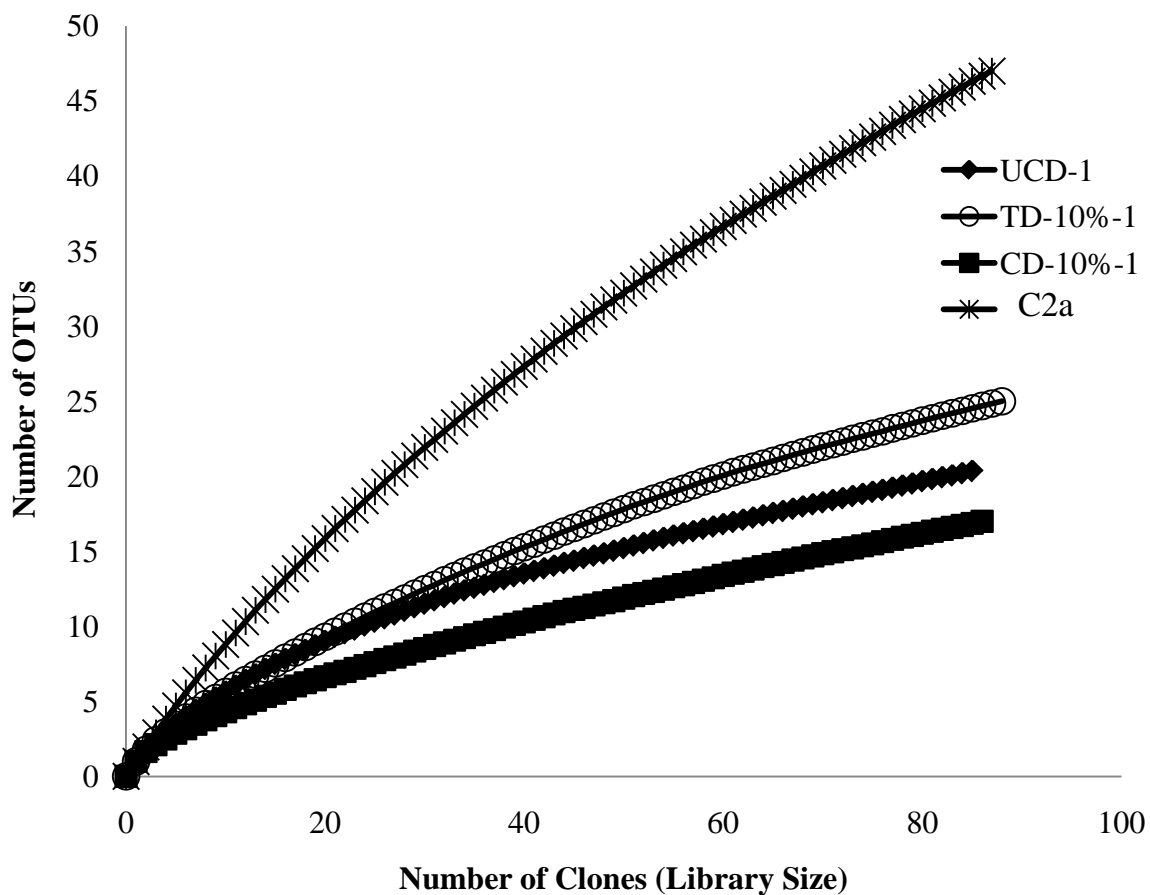


Figure 3.11: Rarefaction Curves for the Bacterial Clone Libraries

Table 3.8 shows the Shannon-Weaver and Simpson's diversity indices obtained for the bacterial clone libraries. Among the samples tested, enrichment culture (C2a) used for bioaugmentation had the highest bacterial diversity and, CD-10%-1 had the lowest bacterial diversity. The diversity indices indicated that UCD-1 and TD-10%-1 had similar bacterial diversity distributed across the OTUs.

Table 3.7: S_{CHA01} , S_{ACE} and Good's C Estimates for Bacterial Clone Libraries

	Observed Number of OTUs	S_{CHA01}	S_{ACE}	Good's C
		Predicted Number of OTUs*	Predicted Number of OTUs*	
UCD-1	21	43 (27-108)	41 (27-89)	0.43
TD-10%-1	25	35 (28-61)	38 (34-43)	0.48
CD-10%-1	17	35 (21-92)	50 (26-141)	0.35
C2a	47	87 (63-146)	104 (71-180)	0.36

* values in the parenthesis indicate 95% confidence interval

Table 3.8: Shannon-Weaver and Simpson's Indices for Bacterial Clone Libraries

	Shannon-Weaver Index	Simpson's Index
UCD-1	2.16	0.22
TD-10%-1	2.24	0.23
CD-10%-1	1.62	0.37
C2a	3.54	0.03

Figure 3.12 shows P-L evenness curves of the bacterial clone libraries. The P-L curves obtained for C2a, UCD-1 and TD-10%-1 were found to be in the medium f_o range, whereas the P-L curve for CD-10%-1 had a higher f_o value. This means that the enrichment culture (C2a) had a relatively more even distribution of clones among OTUs ($f_o = 0.48$) and, on the other hand, the P-L curve for sample CD-10%-1 showed the most uneven distribution of clones among OTUs ($f_o = 0.82$). Interestingly, both UCD-1 and TD-10%-1 had similar f_o values (0.70 and 0.68 respectively).

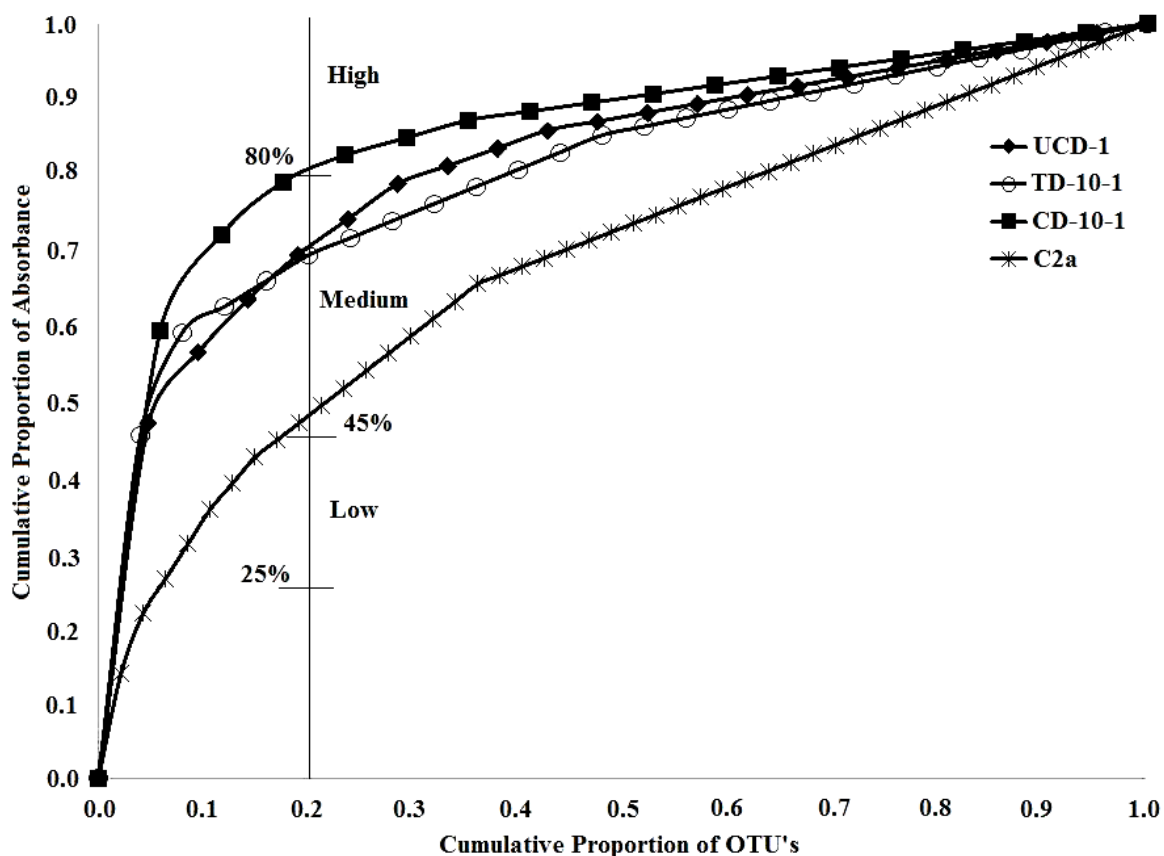


Figure 3.12: Pareto-Lorenz Evenness Curves of Bacterial Clone Libraries

3.3.2.2 Bacterial Phylogenetics

Neighbor joining, maximum parsimony and maximum likelihood algorithms were used to construct phylogenetic trees. Figures 3.13 through 3.16 show phylogenetic trees for the bacterial clone libraries constructed using the neighbor joining algorithm. Since the bacterial phylogenetic tree of culture C2a had the highest diversity, it was broken up into three sections as shown in Figures 3.16-A, B and C. Due to the amount of data involved in the process and limitations on the computational memory, the bootstrapped maximum parsimony tree for culture C2a could not be created. The clades were found to be clustered similarly irrespective of the clustering algorithm employed.

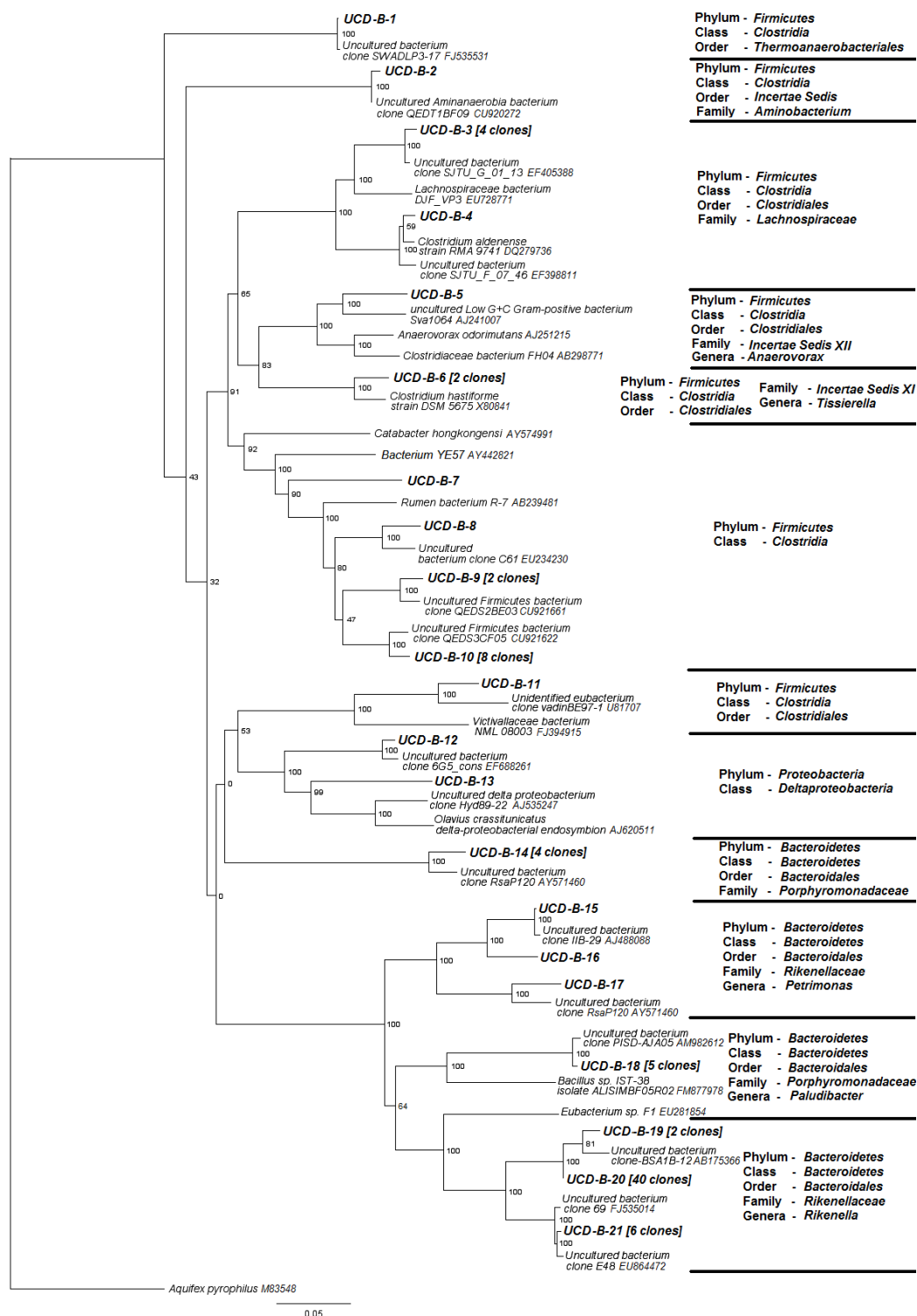


Figure 3.13: Phylogenetic Analysis of Culture UCD-1 Bacterial Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

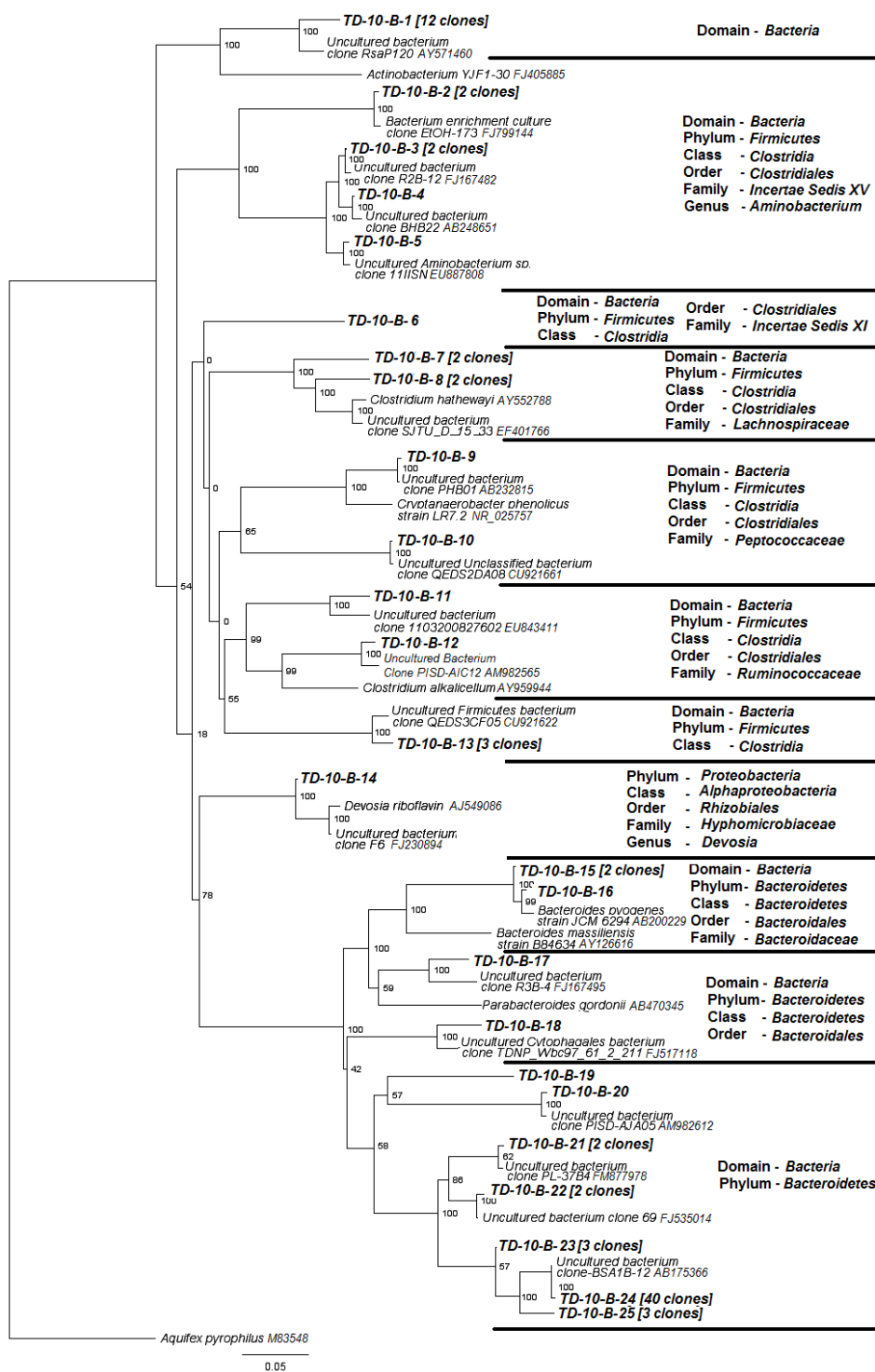


Figure 3.14: Phylogenetic Analysis of Culture TD-10%-1 Bacterial Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

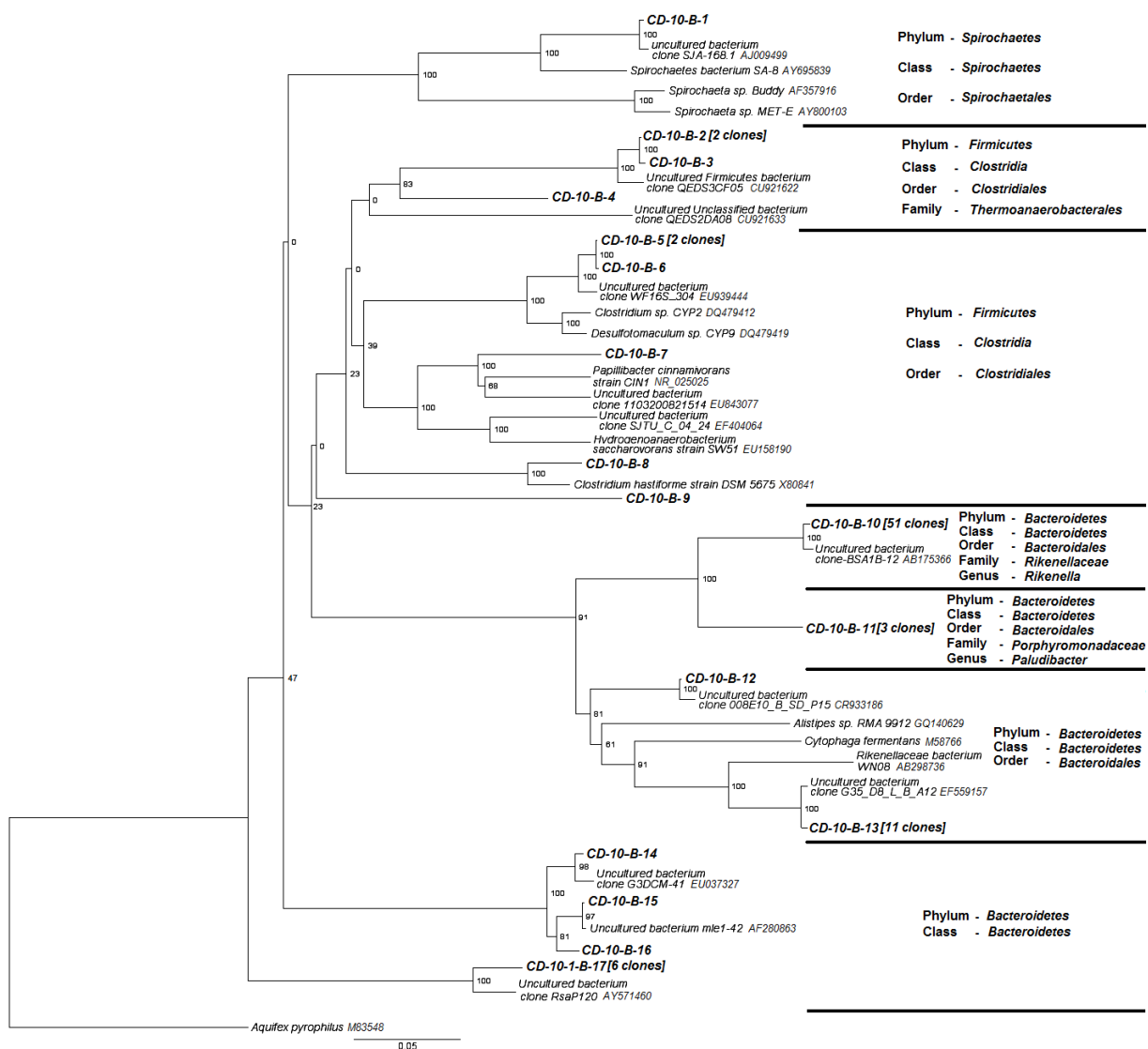


Figure 3.15: Phylogenetic Analysis of Culture CD-10%-1 Bacterial Clone Library.

Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

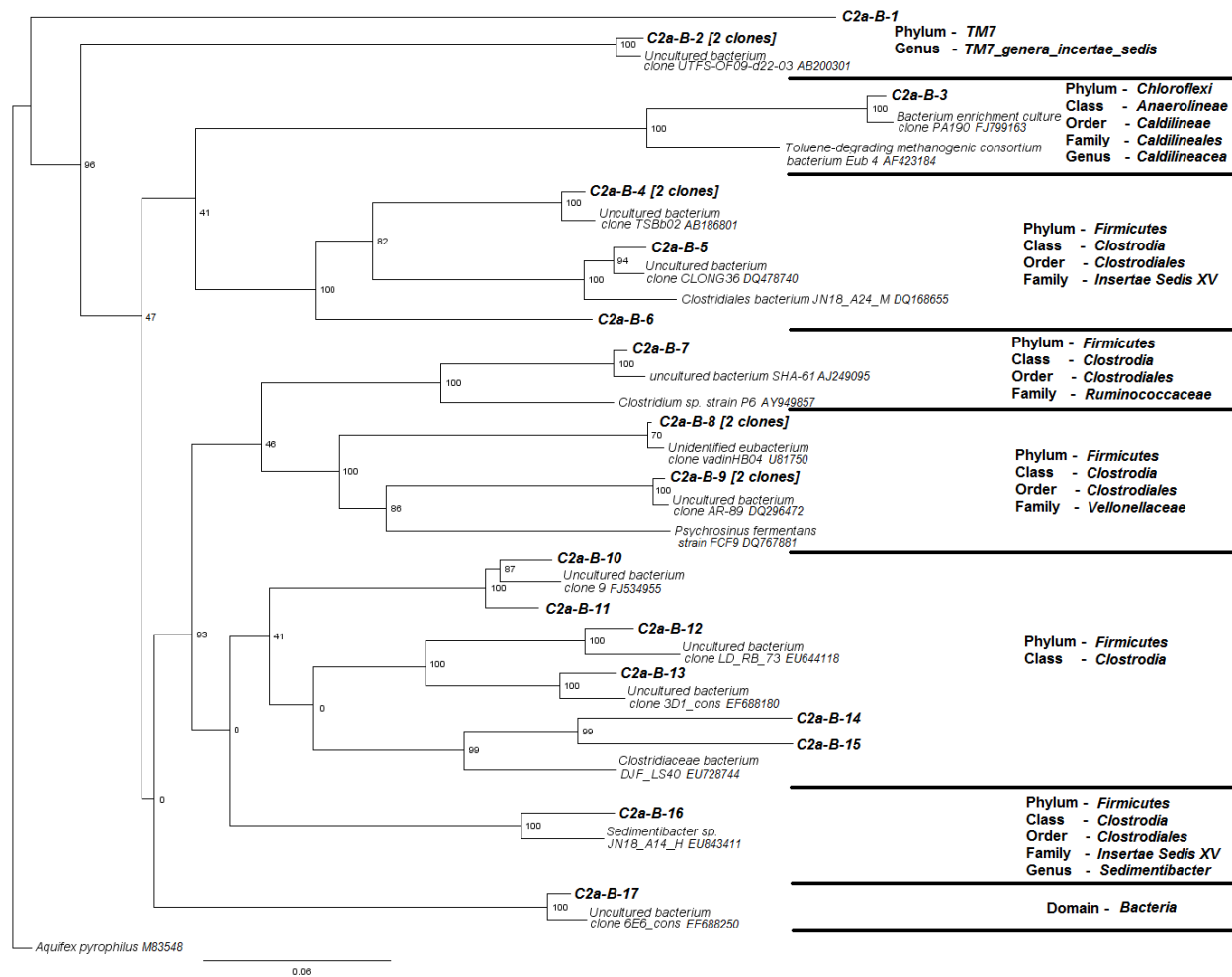


Figure 3.16-A: Phylogenetic Analysis of Culture C2a Bacterial Clone Library.

Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

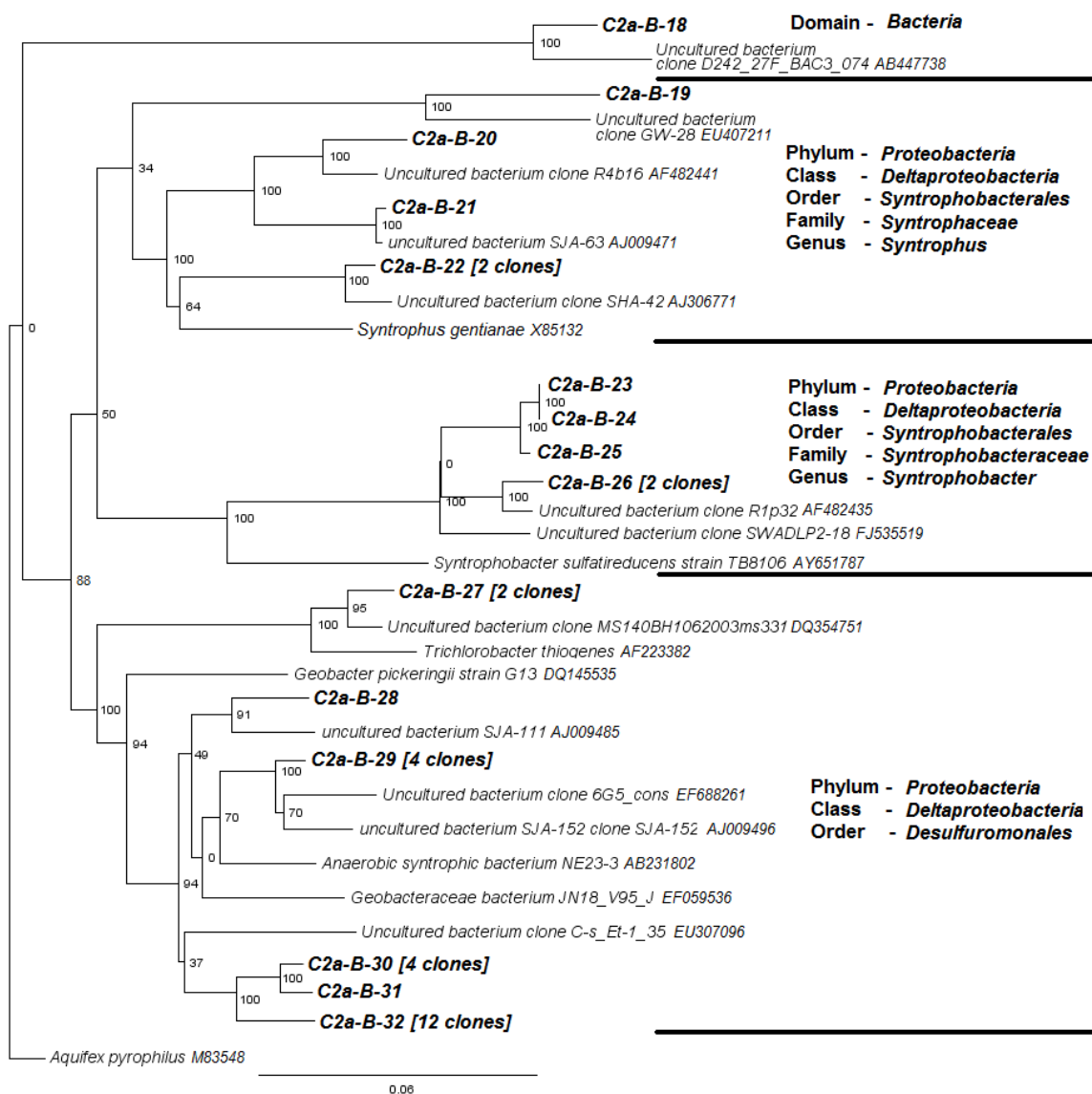


Figure 3.16-B: Phylogenetic Analysis of Culture C2a Bacterial Clone Library.

Calculations were based on neighbor-joining algorithm (bootstrap number = 100).

Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

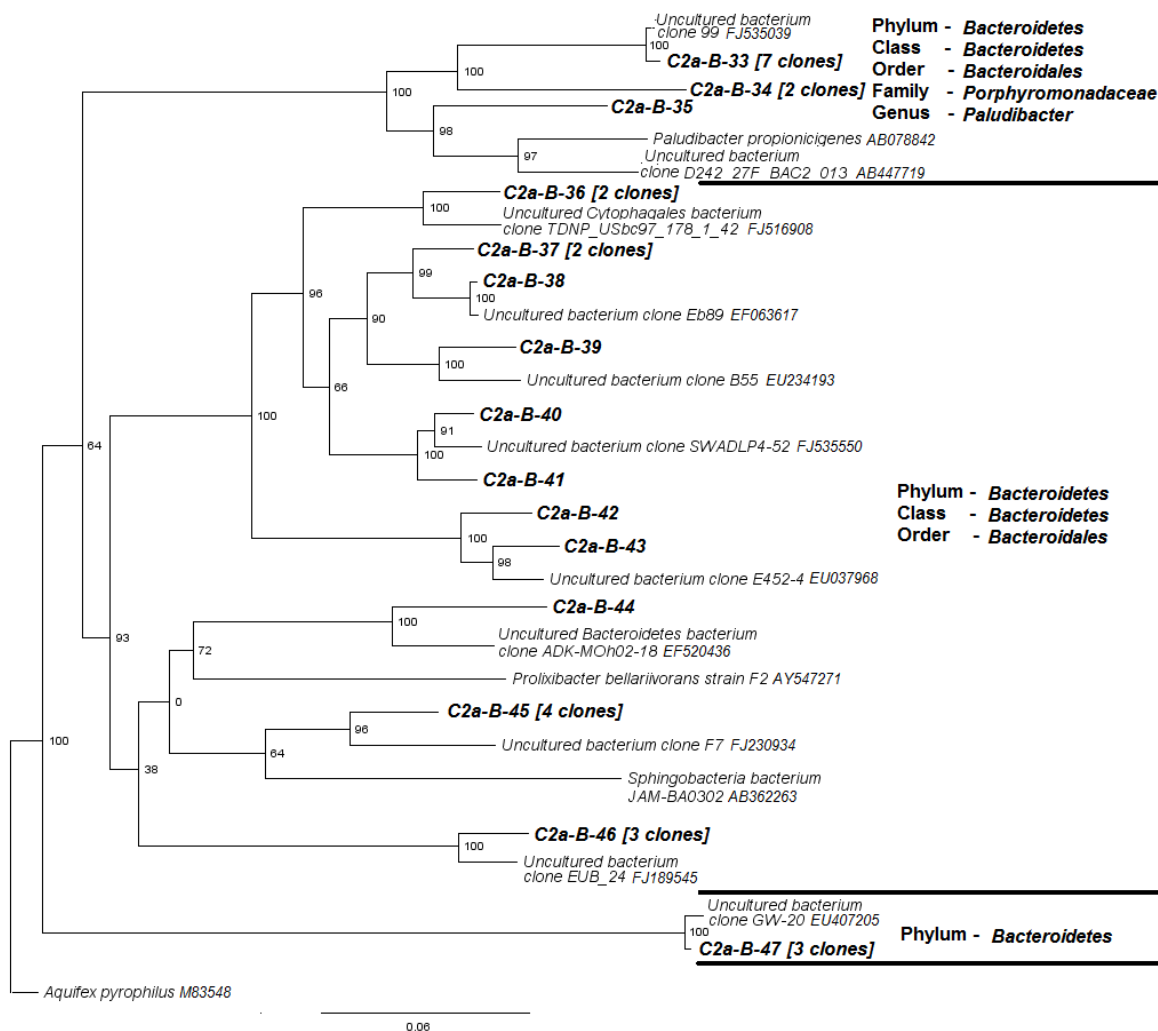


Figure 3.16-C: Phylogenetic Analysis of Culture C2a Bacterial Clone Library. Calculations were based on neighbor-joining algorithm (bootstrap number = 100). Numbers at the node represent bootstrap values. The scale bar represents the number of nucleotide changes per sequence position. The tree was rooted to the organism *Aquifex pyrophilus* (M83548).

3.3.2.2.1 Physiological Characters of the Phyla Found

Like the other studies of bacterial clone libraries of anaerobic digesters, the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were predominant in the clone libraries (Tang et al., 2004; Riviere et al., 2009; Godon et al., 1997; Wilms, et.al, 2006). The

following section gives the classification of the major microbial phyla found in the clone libraries.

Bacteroidetes

The phylum *Bacteroidetes* has three distinct lineages that have been accorded the rank of class: *Bacteroidetes*, *Flavobacteria*, and *Sphingobacteria*; each class contains one order. In all the bacterial clone libraries presented in the current study, only the class *Bacteroidetes* was found. The phylum *Bacteroidetes* is phenotypically diverse and overlaps significantly with members of other phyla because of the *16S rRNA* gene similarity. Members of the phylum *Bacteroidetes* carry out diverse metabolic functions and a few anaerobic species are capable of fermenting sugars or proteins (Madigan and Martinko, 2006). “Member species can be ascribed to the following broad phenotypic categories: Gram-negative aerobic/microaerophilic rods; anaerobic Gram-negative rods; nonphotosynthetic, nonfruiting, gliding bacteria; bacterial symbionts of invertebrate species; sheathed bacteria; nonmotile or rarely motile, curved, Gram-negative bacteria.” (Garrity et al., 2005). In libraries UCD-1, TD-10%-1 and CD-10%-1, microbes related to the genera *Rikenella* were found to be present. Interestingly, although *Rikenella microfuscus* is currently classified under the phylum *Bacteroidetes*, it is considered as an outlier in current phylogenetic classification (Garrity et al., 2005).

Firmicutes

Taxonomic outlines of the phylum *Firmicutes* have been revised recently due to the increase in taxonomic data (Ludwig et al., 2009; Euzéby, 2010). The phylum *Firmicutes* contains three classes, *Bacilli*, *Clostridia* and *Erysipelotrichi* out of which

only the class *Clostridia* was found in all the bacterial clone libraries. It is important to note that some *Clostridium* species are capable of carrying out anoxic digestion of cellulosic material (Madigan and Martinko, 2006) and amino acids (Tang et al., 2005). “Phenotypic groups of the *Firmicutes* include thermophilic and hyperthermophilic bacteria; anaerobic straight, curved, and helical Gram-negative rods; anoxygenic phototrophic bacteria; nonphotosynthetic, nonfruiting, gliding bacteria; aerobic, nonphototrophic, chemolithotrophic bacteria; sulfite- reducing bacteria; symbiotic and parasitic bacteria of vertebrate and invertebrate species; anaerobic Gram-negative cocci; Gram-positive cocci; endospore-forming Gram-positive rods and cocci; regular, nonsporulating Gram-positive rods; irregular, nonsporulating Gram-positive rods; mycoplasmas; and thermoactinomyces.” (Garrity et al., 2005).

Proteobacteria

Proteobacteria is the largest phylum of bacteria known. Many species of this phylum are phototrophic or chemolithotrophic (Madigan and Martinko, 2006).

The phylum *Proteobacteria* has classes *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* (Garrity et al., 2005) out of which, only *Alphaproteobacteria* and *Deltaproteobacteria* were found in the clone libraries. The microbes found in the class *Alphaproteobacteria* were related to the genus *Devosia* which mainly contains soil bacteria. Members of the genus *Devosia* are reportedly aerobic and have oxidase and catalase enzymes (Garrity et al., 2005).

The *Deltaproteobacteria* found in C2a were classified under the genus *Syntrophus* and *Syntrophobacter* and one clade was classified under the order *Desulfuromonales* (see

Figure 3.16-B). “The class *Deltaproteobacta* comprises morphologically diverse, Gram-negative, nonsporeforming bacteria that exhibit either anaerobic or aerobic growth. Most anaerobic members can use inorganic electron acceptors that allow energy conservation by anaerobic respiration. Utilization of inorganic electron acceptors is an important physiological and taxonomic characteristic. However, in a number of isolates the reduction of some electron acceptors (e.g., sulfur, ferric iron) may not be associated with growth (as in the case of oxygen reduction). Some anaerobic members are fermentative and/or exhibit syntrophic growth by proton reduction and interspecies hydrogen transfer. One important feature of the aerobic representatives is the ability to digest other bacteria” (Garrity et al., 2005).

Members of the genus *Syntrophus* are strictly anaerobic and chemoorganotrophic. They possess a fermentative type of metabolism and can ferment crotonate. They can also oxidize substrates, such as benzoate or fatty acids in the presence of H₂ or formate-utilizing methanogenic or sulfate reducing partner bacteria (Garrity et al., 2005).

Members of the genus *Syntrophobacter* are strictly anaerobic chemoorganotrophs. Their growth occurs at neutral pH (6.2-8.0) in low salinity media at mesophilic temperatures by syntrophic metabolism and sulfate reduction. They oxidize propionate to acetate and CO₂ in the presence of either a H₂/formate-using organism (a methanogen or a sulfate reducer) or sulfate as the electron acceptor. Moreover, they cannot oxidize acetate and other fatty acids. Some *Syntrophobacter* species can grow fermentatively with pyruvate, malate, or fumarate. They can also use lactate and propanol as electron donors for syntrophic metabolism or sulfate reduction. *Syntrophobacter* species cannot use other common bacterial substrates such as sugars and aromatic compounds.

Syntrophobacter species can use sulfite or thiosulfate as an electron acceptor and they are known to be found in the sludge from anaerobic waste treatment facilities (Garrity et al., 2005).

The order *Desulfuromonales* contains the families *Desulfuromonaceae* and *Geobacteraceae* having rod-shaped cells. They are strictly anaerobic chemolithoheterotrophs or chemoorganotrophs with respiratory or fermentative metabolism. All the members of the order *Desulfuromonales* are moderate mesophiles with temperature optima for growth around 30°C. Members of order *Desulfuromonales* have been isolated from anoxic freshwater and marine environments (Garrity et al., 2005).

Chloroflexi

Chloroflexi are Gram-negative bacteria with non-motile and filamentous cells. Members of the genus *Caldilineacea* of phylum *Chloroflexi* were found to have grown under both aerobic and anaerobic conditions and were isolated from a UASB reactor operating under thermophilic conditions (Sekiguchi et al., 1998).

Spirochaetes

The phylum *Spirochaetes* is uniform in morphology and all of the validly named species are gram-negative, helically shaped, highly flexible cells motile by periplasmic flagella. They have chemoorganotrophic metabolism and their growth is observed under anaerobic, microaerophilic, facultatively anaerobic, or aerobic conditions. The organisms

are free-living or associated with host animals (arthropods, mollusks, and mammals, including humans) (Garrity et al., 2005).

TM7

Members of candidate phylum TM7 can be PCR amplified by using universal 27F and 1492R bacterial primers (Luo et al., 2009). Members of the candidate phylum TM7 have been referred to as biology's "dark matter" problem (Luo et al., 2009; Marcy et al., 2007), being a focus of study because although they have been identified (via clone sequences) in a wide variety of habitats, researchers have yet to obtain a stable culture of any isolate (Luo et al., 2009).

3.3.2.2.2 Composition of the Bacterial Clone Libraries

Figure 3.17 shows the relative proportion of clones from the bacterial clone libraries. Each column in Figure 3.17 represents a phylum identified in the bacterial clone libraries. The phylum *Bacteroidetes* was the most abundantly-found phylum in the clone libraries of all the digesters (i.e. in UCD-1, TD-10%-1 and CD-10%-1) and it represented more than half of the community from all three digesters, whereas it was the second most abundantly-found phylum in the enrichment culture (C2a). Since the phylum *Bacteroidetes* carries out diverse metabolic functions and is capable of fermenting sugars and proteins under anaerobic conditions (Madigan and Martinko, 2006), its presence was not unexpected in the bacterial clone libraries of these digester fed non-fat-dry milk. The presence of the phylum *Bacteroidetes* in C2a may be because of endogenous decay of the

existing microbial community. The *Bacteroidetes* found in C2a could be degrading the cellular and extracellular materials released by other microbes existing in C2a.

Figure 3.17 shows that the phylum *Firmicutes* was the second most abundantly-found phylum in all the digesters. Moreover, it was the third most abundantly-found phylum in culture C2a. Interestingly UCD-1 and TD-10%-1 had a relatively high proportion of *Firmicutes*. In all the clone libraries, organisms related to the class *Clostridia* were found. Since anoxic members of class *Clostridia* are capable of digesting cellulosic materials and amino acids, their presence in the clone libraries was not surprising. Moreover, it is important to note that some members of this class are capable of reducing sulfate or sulfite or having syntrophic relations in anaerobic environments (Garrity, 2005). Like *Bacteroidetes*, organisms related to the class *Clostridia* found in C2a may be present because of endogenous decay of other microbes.

The phylum *Proteobacteria* includes the organisms that share syntrophic relationships with hydrogenotropic methanogens. As expected, *Proteobacteria* was the most abundantly-found phylum in the bacterial clone library of culture C2a (39%), probably because degradation of propionate requires syntrophs (see Section 1.1). Moreover, about 30% of the *Proteobacteria* community from C2a was related to the order *Syntrophobacterales*. The phylum *Proteobacteria* was also detected in bacterial clone libraries from UCD-1 and TD-10%-1, whereas it was not detected in CD-10%-1. This finding has particular importance because UCD-1 and TD-10%-1 showed better performance in terms of lower effluent SCOD concentrations as compared to CD-10%-1. The presence of the phylum *Proteobacteria* in UCD-1 and TD-10%-1 may be an

indicator of more efficient syntrophic relationships existing among the microbial communities of these digesters leading to better metabolism of propionate, although this assumption needs to be proven by further experimentation.

A clone belonging to the genus *Caldilineacea* of the phylum *Chloroflexi* was detected in culture C2a and a clone belonging to the order *Spirochaetales* of the phylum *Spirochaetes* was detected in digester CD-10%-1. Members of both these phyla are known to survive under anaerobic conditions (Sekiguchi et al., 1998; Garrity et al., 2005) so their presence in the clone library was not surprising.

Figure 3.17 shows that 3% of the bacterial clones from culture C2a were related to phylum TM7 whose function is yet unknown (Luo et al., 2009). These members of the community may be key to some important metabolic functions which are not known.

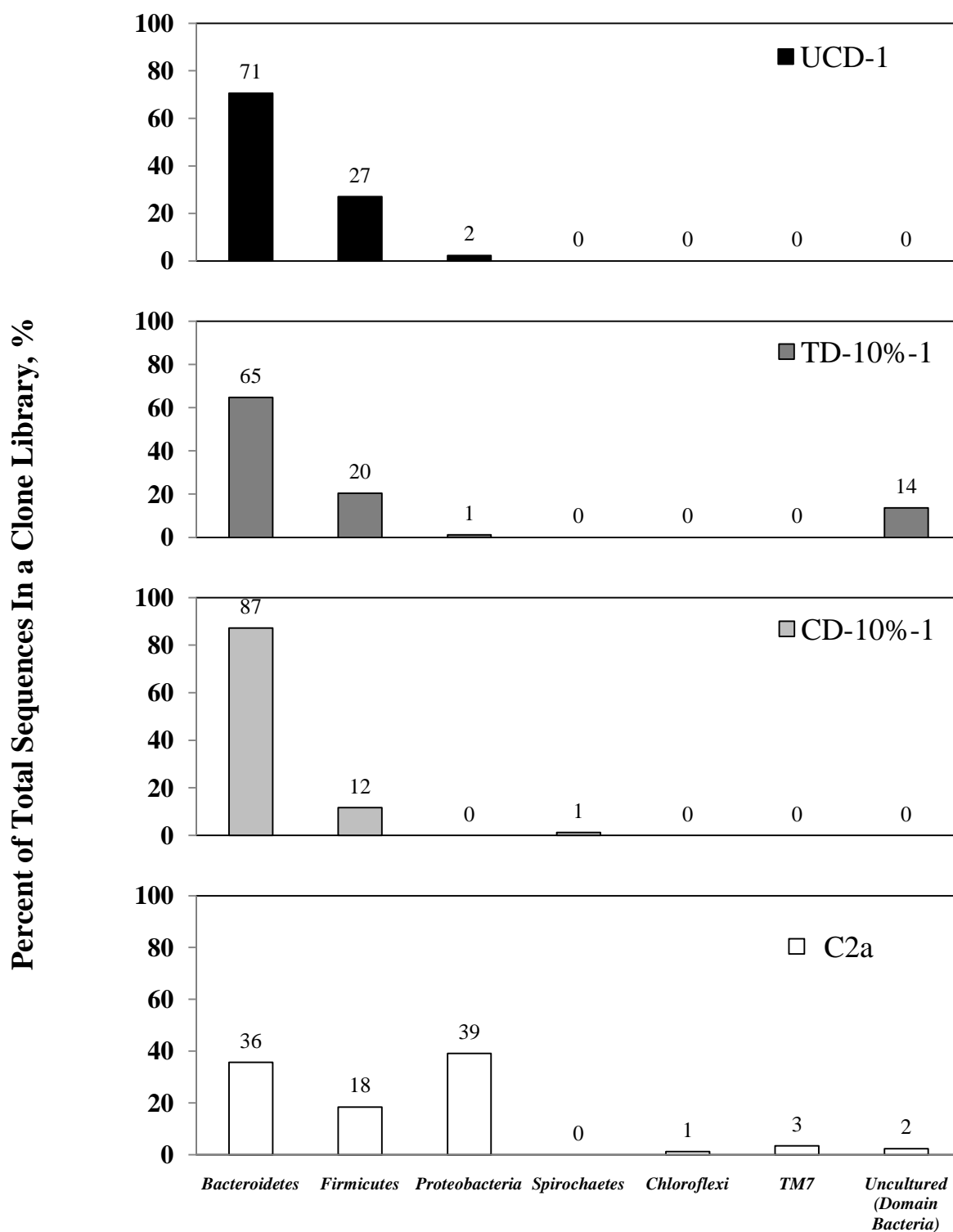


Figure 3.17: Bacterial Diversity of Cultures UCD-1, TD-10%-1, CD-10%-1 and C2a.

3.3.2.2.3 Comparison of Bacterial Phylogenetic Trees

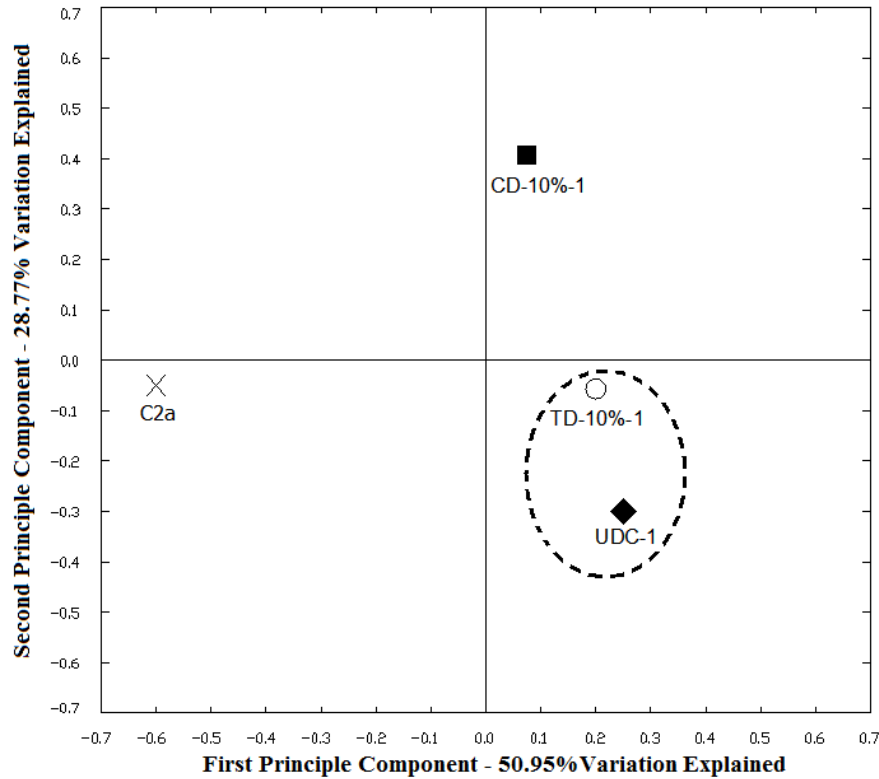
Like archaeal phylogenetic trees, pair-wise comparisons of the bacterial phylogenetic tree structures were performed using TreeClimber (Schloss et al., 2006). Probabilities obtained for the pair-wise comparisons are presented in Table 3.9. Samples having similar archaeal tree structure for 1% level of significance are represented by gray colored cells in Table 3.9. Table 3.9 shows that the bacterial tree of C2a had a very unique structure among all the bacterial trees ($p=0.001$). Also CD-10%-1 and TD-10%-1 had relatively different tree structures ($p=0.008$) which means there was a significant difference between the bacterial communities of CD-10%-1 and TD-10%-1 after 6 SRTs. Interestingly TD-10%-1 and UCD-1 had relatively similar tree structures represented by the highest probability in the table ($p=0.297$) and the tree structure of UCD-1 and CD-10%-1 had significant differences ($p=0.002$). It is important to note that both UCD-1 and TD-10%-1 had significantly lower effluent SCOD concentration than CD-10%-1 (see Section 2.3). In conclusion, although C2a had a very different bacterial tree structure than all the other samples, addition of C2a to TD-10%-1 restored its bacterial community to its undisturbed state (similar to UCD-1) resulting in quicker recovery of TD-10%-1 following the shock organic overload.

Table 3.9: Pair-Wise Comparison of Bacterial Tree Structures

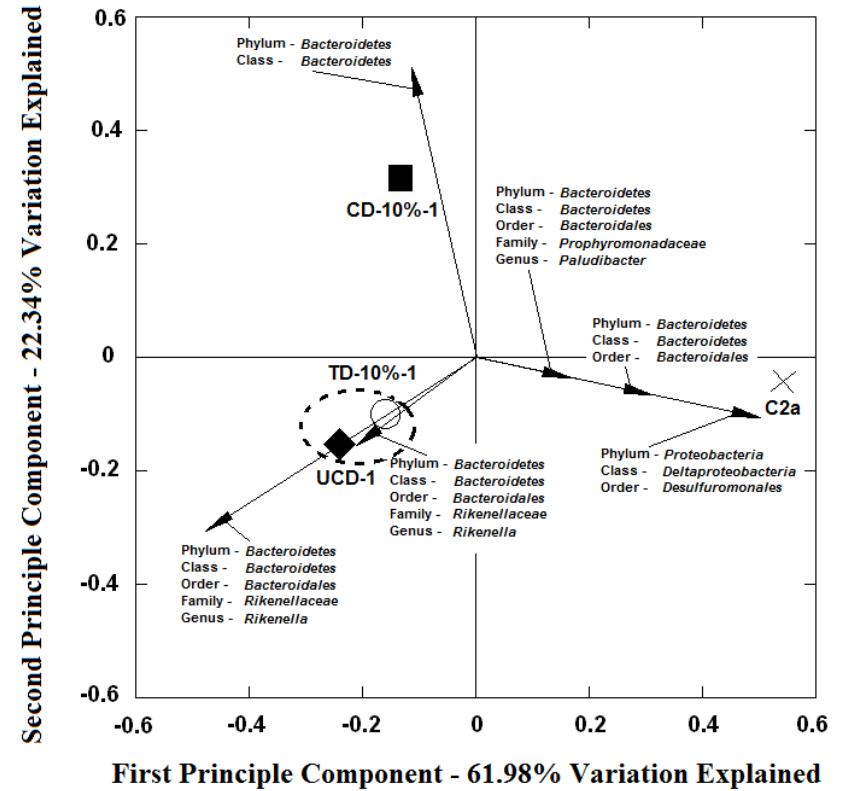
	UCD-1	TD-10%-1	CD-10%-1	C2a
UCD-1	-			
TD-10%-1	0.297	-		
CD-10%-1	0.002	0.008	-	
C2a	0.001	0.001	0.001	-

PCoA was performed using online UniFrac software (Lozupone et. al, 2006). Phylogenetic lineage lengths were used to calculate the distance matrix. The first two principle components accounted for about 80% of the total variation in the data.

The grouping of the bacterial clone libraries in Figure 3.18 a) were similar to the results obtained by the parsimony test. In Figure 3.18, a) UDC-1 and TD-10%-1 were found to be closer than UDC-1 and CD-10%-1 which indicates that there was more similarity between the tree structures of UDC-1 and TD-10%-1 than UDC-1 and CD-10%-1. Also the enrichment culture C2a was represented by a point that was far from all the other points, indicating that culture C2a possessed a unique bacterial tree structure as compared to the other bacterial clone libraries.



(a)



(b)

Figure 3.18: Graph of First Two Principle Components for Bacterial Communities (a) PCoA Performed on Tree Lineages Using UniFrac (Lozupone et. al, 2006) (b) PCA Performed on Distribution of Clones In The Clone Libraries Using MATLAB(v.7.6(R2008a)).

PCA was performed on the bacterial clone libraries as described in the methods section (see section 3.2.1.7). Figure 3.18 b) shows the plot of the first two principle components of the PCA performed using the MATLAB(v.7.6(R2008a)) software package. The first two principle components for PCA analysis of bacterial clone libraries explained about 84% of the total variation among the data. As expected, grouping of the bacterial clone libraries in Figure 3.10 b) was similar to those obtained by the parsimony test (Schloss et al., 2006) and the UniFrac method (Lozupone et. al, 2006). In Figure 3.10 b), UCD-1 and TD-10%-1 clustered together and CD-10%-1 was significantly far from the cluster formed by UCD-1 and TD-10%-1. Also enrichment culture C2a was represented by a point that was far from all the other points indicating that culture C2a possessed a unique bacterial tree structure as compared to all the bacterial clone libraries.

Projection of the six significant dimensions indicated that organisms related to the order *Desulfomononales* had the highest abundance in C2a. As explained above, the order *Desulfuromonales* contains strict anaerobic chemolithoheterotrophs or chemoorganotrophs with respiratory or fermentative metabolism. All the members of the order *Desulfuromonales* are moderate mesophiles with temperature optima for growth around 30°C. Members of the order *Desulfuromonales* have been isolated from anoxic freshwater and marine environments (Garrity et al., 2005). All the other significant vectors represented microbes classified under the phylum *Bacteroidetes* and differences in the clones related to the phylum *Bacteroidetes* resulted in the grouping pattern on the PCA plot. Organisms related to the genera *Rikenella* were found to be abundant in UCD-1 and TD-10%-1. As explained previously, this organism is not accurately classified in the

current phylogenetic classification (Garrity et al., 2005). Also it is important to know that the results obtained for the bacterial clone libraries may be biased due to the low coverage obtained (see Table 3.7).

3.4 Conclusions

Archaeal and bacterial communities from undisturbed control (UCD-1), bioaugmented (TD-10%-1), non-bioaugmented digesters (CD-10%-1) and the bioaugmentation culture (C2a) were analyzed using a clone library approach. DNA samples were extracted when there were significant effluent SCOD differences between TD-10%-1 and CD-10%-1.

Rarefaction curves and the richness indices indicated that archaeal clone libraries for the bioaugmented (TD-10%-1) and undisturbed control (UCD-1) digesters attained complete coverage, whereas the clone libraries for non-bioaugmented digester (CD-10%-1) and bioaugmentation culture (C2a) attained only 91 and 87% coverage according to Good's C estimate. Also both Shannon-Weaver and Simpson's indices showed that TD-10%-1 and UDC-1 had less diverse archaeal communities than archaeal communities from CD-10%-1 and C2a. This indicates that better functioning of anaerobic digesters digesting milk waste may require less diverse but dedicated archaeal communities. Functional organization (*fo*) of the archaeal communities indicated that C2a and TD-10%-1 had relatively more uniform communities than CD-10%-1 and C2a.

Archaeal phylogenetic trees indicated that both UCD-1 and TD-10%-1 had higher relative abundance of methanogens classified under the order *Methanomicrobiales*

($\geq 85\%$) which may have led to better metabolism of H_2 and, hence, more complete degradation of propionate in digesters UCD-1 and TD-10%-1 leading to better performance than digester CD-10%-1. Out of the total number of clones classified under the order *Methanomicrobiales*, 96, 100 and 92% clones from sample UDC-1, TD-10%-1 and CD-10-1 respectively were also classified under the family *Methanomicrobiaceae* and these clones were found to be related to *Methanospirillum hungatei* ($\geq 95\%$ similarity). Archaeal clones from UCD-1, CD-10%-1 and C2a were found to be classified under the order *Methanosarcinales* (family *Methanosataceae*). Since complete metabolism of propionate involves conversion of acetate to methane, finding organisms classified as *Methanosarcinales* (family *Methanosaetaceae*) in C2a was expected.

Importantly, 26.3% of the archaeal clones from C2a were closely related to *Methanolinea tarda* (AB162774) which shows optimum growth at $50^{\circ}C$ and has a 4-day doubling time (Imachi et al., 2008). Other researchers (Lynch et al., 1987) who tested recovery of organically overloaded anaerobic digesters using bioaugmentation with cultures acclimated to propionate (like C2a) used packed-bed filters with a 4 day HRT. This may have led to washout of the slow-growing hydrogenotrophic methanogenic community members similar to *Methanolinea tarda*. The failure of bioaugmentation was attributed to wash-out of the enrichment culture from the overloaded digesters and it was recommended that bioaugmentation may show better recovery for CSTR digesters than for packed-bed filters (Lynch et al., 1987). The reason behind successful bioaugmentation presented herein may be the use of CSTR digesters operated at a longer (10-day) SRT which may have allowed growth of slow-growing methanogens. Also adding the

bioaugmentation culture on a daily basis have helped recovery of the overloaded digesters.

Pair-wise comparison of the tree structures of the archaeal clone libraries was done by parsimony test using TreeClimber software (Schloss et al., 2006). It was found that only UDC-1 and TD-10%-1 had similar archaeal phylogenetic tree structures ($p=0.556$). Surprisingly C2a had a very different community structure than TD-10%-1 ($p=0.001$). Also there was a very low similarity between the tree structure of TD-10%-1 and CD-10%-1 ($p=0.001$). These results were confirmed by PCoA performed on the tree lineages and PCA performed on the clone distribution data. Projection of the impact of each OTU on principle components indicated that organisms related to *Methanospirillum hungatei* had the highest relative abundance in UDC-1 and TD-10%-1, whereas organisms related to *Methanolinea tarda* had significant relative abundance in UDC-1 and CD-10%-1. Among these two organisms, *Methanolinea tarda* has an optimum temperature of 50°C and at its optimum growth temperature it takes about 4 days to double its population (Imachi et al., 2008), whereas *Methanospirillum hungatei* is a mesophilic methanogen (35-40°C optimum) (Yang et al., 1985; Ferry, 1993) and has a 0.7-day doubling time (Ferry and Wolfe, 1977). Since the digesters were operated at 35±3°C, the higher relative abundance of organisms related to *Methanospirillum hungatei* may have resulted in better performance of UCD and TD-10% digesters by more rapid metabolism of hydrogen.

Bacterial clone libraries of all the samples were more diverse than their archaeal clone libraries. Good's C estimate indicated that only 35 to 48% coverage was achieved

in the current study. Among the samples tested, C2a had the highest bacterial diversity, whereas CD-10%-1 had the lowest bacterial diversity. Both UCD-1 and TD-10%-1 had similar bacterial diversities.

The phylum *Proteobacteria* includes the organisms that share syntrophic relationships with hydrogenotropic methanogens, and they were abundantly found in culture C2a (39% of the total community). Moreover, about 30% of the *Proteobacteria* community from C2a was related to the order *Syntrophobacterales*. The phylum *Proteobacteria* was also detected in bacterial clone libraries from UCD-1 and TD-10%-1, whereas it was not detected in CD-10%-1. The detection of the phylum *Proteobacteria* in UCD-1 and TD-10%-1 may be an indicator of efficient syntrophic relationships existing among the microbial communities of these digesters leading to better metabolism of propionate.

The phylum *Bacteroidetes* was the most abundantly found phylum in the clone libraries of all the digesters (i.e. in UCD-1, TD-10%-1 and CD-10%-1) and it represented more than half of the community from all the three digesters, whereas it was the second most abundantly found phylum in the enrichment culture (C2a). Since the phylum *Bacteroidetes* carry out diverse metabolic functions and its members are capable of fermenting sugars and proteins under anaerobic conditions (Madigan and Martinko, 2006), its presence was not unexpected in the bacterial clone libraries of all the milk-fed digesters. The presence of the phylum *Bacteroidetes* in C2a may be because of endogenous decay of the existing microbial community.

The phylum *Firmicutes* was the second most abundantly-found phylum in all the digesters. Moreover, it was the third most abundantly-found phylum in culture C2a. Interestingly UCD-1 and TD-10%-1 had a higher proportion of *Firmicutes*. In all the clone libraries, organisms related to the class *Clostridia* were found. Since anoxic members of class *Clostridia* are capable of digesting amino acids, their presence in the clone libraries was less surprising. Like *Bacteroidetes*, organisms related to the class *Clostridia* found in C2a may be present because of the endogenous decay of the existing microbial community.

Results of pair-wise comparison of the phylogenetic trees indicated that, similar to the archaeal clone libraries, only UCD-1 and TD-10%-1 shared a similar tree structure ($p=0.297$), whereas tree structures for TD-10%-1 and CD-10%-1 had significant differences ($p=0.008$). Also the bacterial tree structure of C2a was unique among the clone libraries ($p=0.001$). These results were also confirmed by PCoA analysis.

In conclusion, community analysis of the clone libraries indicated that the undisturbed control digester and the bioaugmented digester exhibited similar microbial community. In contrast, the non-bioaugmented digester had a very different archaeal and bacterial communities than the undisturbed control and bioaugmented digester. Also, the enrichment culture used for bioaugmentation exhibited a very different community structure than the other cultures. This indicated that addition of the enrichment culture restored the microbial communities to their undisturbed states resulting in better performance of the bioaugmented digesters. Comparison of the archaeal communities indicated that a higher abundance of microorganisms related to *Methanospirillum*

hungatei resulted in similarities in the phylogenetic tree structures of the undisturbed control and bioaugmented digesters. Since *Methanospirillum hungatei* is a fast-growing hydrogenotrophic methanogen, its presence may have resulted in better functioning of the undisturbed control and bioaugmented digesters by causing higher degradation rates of substrates such as H₂ and propionate.

3.5 References

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs." *Nucleic Acids Res.* 25: 3389-3402.
- Amann, R., Lemmer, H., and Wagner, M. (1998) "Monitoring the Community Structure of Wastewater Treatment Plants: A Comparison of Old and New Techniques." *FEMS Microbiology Ecology.* 25: 205-215.
- Ansorge, WJ (2009) "Next-Generation DNA Sequencing Techniques." *New Biotechnology.* 24.4:195-203.
- Baker, G.C., Smith, J.J., and Cowan, D.A. (2003) "Review and Re-Analysis of Domain-Specific 16S Primers." *Journal of Microbiological Methods.* 55:541-555.
- Calli, B., Mertoglu, B., Inanc, B., and Yenigun, O. (2005) "Methanogenic Diversity in Anaerobic Bioreactors Under Extremely High Ammonia Levels." *Enzyme and Microbial Technology.* 37.4: 448-455.
- Chao, A. (1984) "Nonparametric Estimation of the Number of Classes in a Population." *Scandinavian Journal of Statistics.* 11.4:265-270.
- Chao, A. (1987) "Estimating the Population Size for Capture-Recapture Data With Unequal Catchability." *Bioinformatics.* 43.4:783-791.
- Chao, A., and Yang, C.K. (1993) "Stopping Rules and Estimation for Recapture Debugging with Unequal Failure Rates." *Biometrika.* 80.1:193-201.
- Chen, Y., Higgins, M.J., Maas, N.A., Murthy, S.N., Toffey, W.E., and Foster, D.J. (2005) "Roles of Methanogens on Volatile Organic Sulfur Compound Production in

Anaerobically Digested Wastewater Biosolids.” *Water Science and Technology*. 52.1-2: 67-72.

- Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Bandela, A.M., Cardenas, E., Garrity, G.M., and Tiedje, J.M. (2007) “The Ribosomal Database Project (RDP-II): Introducing *myRDP* Space and Quality Controlled Public Data.” *Nucleic Acids Research*. 35 (Database Issue): D169-D172.
- Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., and Tiedje, J.M. (2003) “The Ribosomal Database Project (RDP-II): Previewing a New Autoaligner That Allows Regular Updates and the New Prokaryotic Taxonomy.” *Nucleic Acids Research*. 31.1:442-443.
- Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M., and Tiedje, J.M. (2008) “The Ribosomal Database Project: Improved Alignments and New Tools for rRNA Analysis.” *Nucleic Acids Research*. (Database Issue): D141-D145.
- Delbes, C., Moletta, R., and Godon, J. (2001) “Bacterial and Archaeal 16S rDNA and 16S rRNA Dynamics During an Acetate Crisis in an Anaerobic Digester Ecosystem.” *FEMS Microbiology Ecology*. 35: 19-26.
- Diaz, E.E., Stams, A.J.M., Amils, R., and Sanz, J.L. (2006) “Phenotypic Properties and Microbial Diversity of Methanogenic Granules from a Full-Scale Upflow Anaerobic Sludge Bed Reactor Treating Brewery Wastewater.” *Applied and Environmental Microbiology*. 72.7: 4942-4949.
- Euzeby, J. (2010) “List of New Names and New Combinations Previously Effectively, But Not Validly Published.” *International Journal of Systematic and Evolutionary Microbiology*. 60:469-472.
- Felsenstein, J. (2005) PHYLIP (Phylogeny Inference Package) Version 3.68. Distributed by the Author. Department of Genome Sciences, University of Washington, Seattle.
- Fernandez, A., Huang, S., Seston, S., Xing, J., Hickey, R., Criddle, C., and Tiedje, J. (1999) “How Stable is Stable? Function Versus Community Composition.” *Applied and Environmental Microbiology*. 65.8: 3697-3704.

- Fernandez, A.S., Hashsham, S.A., Dollhopf, S.L., Raskin, L., Glagoleva, O., Dazzo, F.B., Hickey, R.F., Criddle, C.S., and Tiedje, J.M. (2000) "Flexible Community Structure Correlates With Stable Community Function in Methanogenic Bioreactor Communities Perturbed by Glucose." *Applied and Environmental Microbiology*. 66.9: 4058-4067.
- Ferry, J. (ed.). (1993) *Methanogenesis*. Chapman & Hall, Inc., New York, NY.
- Ferry, J.G., and Wolfe, R.S. (1977) "Nutritional and Biochemical Characteristics of *Methanospirillum hungatii*." *Applied and Environmental Microbiology*. 34.4:371-376.
- Fode-Vaughan, K.A., Wimpee, C.F., Remsen, C.C., and Collins, M.L.P. (2001) "Detection of Bacteria in Environmental Samples by Direct PCR Without DNA Extraction." *Biotechniques*. 31: 598-607.
- Garrity, G.M., Boone, D.R., and Castenholz, R.W. (2001) *Bergey's Manual of Systematic Bacteriology*, Vol. 1. New York. Springer.
- Garrity, G.M., Brenner, D.J., Krieg, N.R., and Staley, J.T. (2005) *Bergey's Manual of Systematic Bacteriology*, Vol. 2. New York. Springer.
- Godon, J.J., Zumstein, E., Dabert, P., Habouzit, F., and Moletta, R. (1997) "Microbial 16S rDNA Diversity in an Anaerobic Digester." *Water Science and Technology*. 36.6-7: 49-55.
- Good, I. L. (1953) "The Population Frequency of Species and the Estimation of Sufficient Sample Size." *Ecology*. 56:237-264.
- Griffin, M.E., McMahon, K.D., Mackie, R.I., and Raskin, L. (1998) "Methanogenic Population Dynamics During Start-Up of Anaerobic Digesters Treating Municipal Solid Waste and Biosolids." *Biotechnology and Bioengineering*. 57.3: 342-355.
- Hatamoto, M., Imachi, H., Yashiro, Y., Ohashi, A., and Harada, H. (2007) "Diversity of Anaerobic Microorganisms Involved in Long-Chain Fatty Acid Degradation in Methanogenic Sludges as Revealed by RNA-Based Stable Isotope Probing." *Applied and Environmental Microbiology*. 73.13: 4119-4127.
- Head, I.M., Sauders, J.R., and Pickup, R.W. (1998) "Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms." *Microbial Ecology*. 35:1-21.

- Hobson, P.N. and Wheatley, A.D. (1993) *Anaerobic Digestion, Modern Theory and Practice*. Elsevier Science Publishers, LTD. Essex, United Kingdom.
- Hori, T., Haruta, S., Ueno, Y., Ishii, M., and Igarashi, Y. (2006) "Dynamic Transition of a Methanogenic Population in Response to the Concentration of Volatile Fatty Acids in a Thermophilic Anaerobic Digester." *Applied and Environmental Microbiology*. 72.2: 1623-1630.
- Huber, T., Faulkner, G., and Hugenholtz, P. (2004) "Bellerophon: A Program to Detect Chimeric Sequences in Multiple Sequence Alignments." *Bioinformatics*. 20:2317-2319.
- Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., and Lens, P.N.L. (2004) "Anaerobic Sludge Granulation." *Water Research*. 38: 1376-1389.
- Imachi, H., Sakai, S., Sekiguchi, Y., Hanada, S., Kamagata, Y., Ohashi, A., and Harada, S. (2008) "*Methanolinea tarda* Gen. Nov., Sp. Nov., A Methane-Producing Archaeon Isolated from a Methanogenic Digester Sludge." *International Journal of Systematic and Evolutionary Microbiology*. 58:294-301.
- Janda, J. M. and Abbott, S.L. (2007) "16S rRNA Gene Sequencing for Bacterial Identification in Diagnostic Laboratory: Pluses, Perils, and Pitfalls." *Journal of Clinical Microbiology*. 45.9:2761-2764.
- Kemp, P.F. and Aller, J.Y. (2004) "Bacterial Diversity in Aquatic and Other Environments, What 16S rDNA Libraries Can Tell Us." *FEMS Microbiology Ecology*. 47: 161-177.
- Keyser, M., Witthuhn, R.C., Lamprecht, C., Coetzee, M.P.A., and Britz, T.J. (2006) "PCR- Based DGGE Fingerprinting and Identification of Methanogens Detected in Three Different Types of UASB Granules." *Systematic and Applied Microbiology*. 29.1: 77-84.
- Lane, D.J. (1991) *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, Ltd. Chichester, NY.
- Leclerc, M., Delgenes, J., and Godon, J. (2004) "Diversity of the Archaeal Community in 44 Anaerobic Digesters as Determined by Single Strand Conformation Polymorphism Analysis and 16S rDNA Sequencing." *Environmental Microbiology*. 6.8: 809-819.

- Lozupone, C., Hamady, M., and Knight, R. (2006) "UniFrac - An Online Tool for Comparing Microbial Community Diversity in a Phylogenetic Context." *BMC Bioinformatics*. 7.1:371
- Ludwig, W., Schleifer, K.-H., and Whitman, W.B (2009) *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 3 (The *Firmicutes*), Edited by P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer & W. B. Whitman. New York: Springer.
- Luo, C., Xie, S., Sun, W., Li, X., and Cupples, A.M. (2009) "Identification of a Novel Toluene-Degrading Bacterium from the Candidate Phylum TM7, as Determined by DNA Stable Isotope Probing." *Applied Environmental Microbiology*. 75.13:4644-4647.
- Lynch, N., Daniels L., and Parkin, G.F. (1987) "Bioaugmentation of Stressed Anaerobic Filters with Methanogenic Enrichment Cultures." *Proceedings of the 42nd Industrial Waste Conference*. West Lafayette, Indiana: Purdue University. 285-296.
- Madigan, M.T. and Martinko, J.M. (2006) *Brock Biology of Microorganisms*. 11th ed. Pearson Prentice Hall, Pearson Education, Inc. Upper Saddle River, NJ.
- Maidak, B.L., Cole, J.R., Liburn, T.G., Parker Jr, C.T., Saxman, P.R., Stredwick, J.M., Garrity, G.M., Li, B., Olsen, G.J., Pramanik, S., Schmidt, T.M., and Tiedje, J.M. (2000) "The RDP (Ribosomal Database Project) Continues." *Nucleic Acids Research*, 28.1:173-174.
- Marcy, Y., Ouverney, C., Bik, E.M., Lösekann, T., Ivanova, N., Martin, H.G., Szeto, E., Platt, D., Hugenholtz, P., Relman, D.A., and Quake, S.R. (2007) "Dissecting Biological "Dark Matter" With Single-Cell Genetic Analysis of Rare and Uncultivated TM7 Microbes from the Human Mouth." *Proc. Natl Acad Sci USA*, 104.29:11889-11894.
- Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., and Verstraete, W. (2008) "How to Get More Out of Molecular Fingerprints: Practical Tools for Microbial Ecology." *Environmental Microbiology*. 10.6:1571-1581.
- McGarvey, J.A., Miller, W.G., Zhang, R., Ma, Y., and Mitloehner, F. (2007) "Bacterial Population Dynamics in Dairy Waste During Aerobic and Anaerobic Treatment and Subsequent Storage." *Applied and Environmental Microbiology*. 73.1: 193-202.

- McHugh, S., Carton, M., Collins, G., and O'Flaherty, V. (2004) "Reactor Performance and Microbial Community Dynamics During Anaerobic Biological Treatment of Wastewaters at 16-37°C." *FEMS Microbiology Ecology*. 48: 369-378.
- McHugh, S., Carton, M., Mahony, T., and O'Flaherty, V. (2003) "Methanogenic Population Structure in a Variety of Anaerobic Bioreactors." *FEMS Microbiology Letters*. 219: 297-304.
- McMahon, K.D., Zheng, D., Stams, A.J.M., Mackie, R., and Raskin, L. (2004) "Microbial Population Dynamics During Start-Up and Overload Conditions of Anaerobic Digesters Treating Municipal Solid Waste and Sewage Sludge." *Biotechnology and Bioengineering*. 87.7: 823-834.
- Mladenovska, Z., Hartmann, H., Kvist, T., Sales-Cruz, M., Gani, R., and Ahring, B.K. (2006) "Thermal Pretreatment of the Solid Fraction of Manure: Impact on the Biogas Reactor Performance and Microbial Community." *Water Science and Technology*. 53.8: 59-67.
- Nawrocki, E.P., Kolbe, D.L., and Eddy, S.R. (2009) "Infernal 1.0: Inference of RNA Alignments." *Bioinformatics*, 25:1335-1337.
- Patel, G.B., Sprott, G.D. (1990). "*Methanosaeta concilii* Gen. Nov., Sp. Nov. (*Methanothrix concilii*) and *Methanosaeta thermoacetophila* Nom. Rev., Comb. Nov". *Int. J. Syst. Bacteriol.* 40: 79–82.
- Pontes, D.S., Lima-Bittencourt, C.I., Chartone-Souza, E., and Nascimento, A.M.A. (2007) "Molecular Approaches: Advantages and Artifacts in Assessing Bacterial Diversity." *Journal of Industrial Microbiology and Biotechnology*. 34: 463-473.
- Rambaut, A. (2008) *FigTree: Tree Figure Drawing Tool*. Version 1.1.2. Institute of Evolutionary Biology. University of Edinburgh.
<http://tree.bio.ed.ac.uk/software/figtree>.
- Raskin, L., Amann, R.I., Poulsen, L.K., Rittmann, B.E., and Stahl, D.A. (1995) "Use of Ribosomal RNA-Based Molecular Probes for Characterization of Complex Microbial Communities in Anaerobic Biofilms." *Water Science and Technology*. 31.1: 261-272.
- Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E., and Stahl, D.A. (1994) "Quantification of Methanogenic Groups in Anaerobic Biological Reactors by Oligonucleotide Probe Hybridization." *Applied and Environmental Microbiology*. 60.4: 1241-1248.

- Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., and Sghir, A. (2009) "Towards the Definition of a Core of Microorganisms in Anaerobic Digestion of Sludge." *The ISME journal*. 3:700-714.
- Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. 3rded. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.
- Schauer (2008) *Bioaugmentation for Recovery of Anaerobic Digesters Subjected to a Toxicant*. Ph.D. Dissertation, Marquette University, Milwaukee, WI.
- Schloss, P. D. and Handelsman, J. (2005) "Introducing DOTUR, a Computer Program for Defining Operational Taxonomic Units and Estimating Species Richness." *Applied and Environmental Microbiology*. 71.3:1501-1506.
- Schloss, P. D. and Handelsman, J. (2006) "Introducing TreeClimber, a Test to Compare Microbial Community Structures." *Applied and Environmental Microbiology*. 72.4:2379-2384.
- Scully, C., Collins, G., and O'Flaherty, V. (2007) "Granular Biofilm-Based Anaerobic Digestion: Molecular Biomonitoring and High-Rate Psychrophilic Treatment of Phenolic Wastewater." *Water Science and Technology*. 55.1-2: 43-49.
- Sekiguchi, Y., Kamagata, Y., Syutsubo, K., Ohashi, A., Harada, H., and Nakamura, K. (1998) "Phylogenetic Diversity of Mesophilic and Thermophilic Granular Sludges Determined by 16S rRNA Gene Analysis." *Microbiology*. 144: 2655-2665.
- Shannon, C. E., and Weaver, W. (1963) "The Mathematical Theory of Communication". University of Illinois Press, Urbana.
- Speece, R. (2008) *Anaerobic Biotechnology and Odor/Corrosion Control for Municipalities and Industries*. Archae Press. Nashville, TN.
- Spiegelman, D., Whissell, G., and Greer, C. (2005) "A Survey of the Methods for the Characterization of Microbial Consortia and Communities." *Canadian Journal of Microbiology*. 51.5: 355-386.
- Tang, Y., Shigematsu, T., Morimura, S., and Kida, K. (2005) "Microbial Community Analysis of Mesophilic Anaerobic Protein Degradation Process Using Bovine Serum Albumin (BSA)-Fed Continuous Cultivation." *Journal of Bioscience and Bioengineering*. 99.2:150-164.

- Verstratete, W. (2007) "Microbial Ecology and Environmental Biotechnology." *ISME Journal*. 1: 4-8.
- Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) "Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy." *Applied and Environmental Microbiology*, 73.16:5261-5267.
- Wilms, R., Kopke, B., Sass, H., Chang, T.S., Cypionka, H., and Engelen, B. (2006) "Deep Biosphere-Related Bacteria Within the Subsurface of Tidal Flat Sediments." *Environmental Microbiology*. 8.4:709-719.
- Wintzingerode, F.V., Gobel, U.B., and Stackebrandt, E. (1997) "Determination of Microbial Diversity in Environmental Samples: Pitfalls of PCR-Based rRNA Analysis." *FEMS Microbiology Reviews*. 21: 213-229.
- Yang, D., Kaine, B.P., and Woese, C.R. (1985) "The Phylogeny of *Archaeobacteria*." *Systematic and Applied Microbiology*. 6: 251-256.
- Zheng, D. and Raskin, L. (2000) "Quantification of *Methanosaeta* Species in Anaerobic Bioreactors Using Genus- and Species-Specific Hybridization Probes." *Microbial Ecology*. 39.3: 246-262.
- Zumstein, E., Moletta, R., and Godon, J. (2000) "Examination of Two Years of Community Dynamics in an Anaerobic Bioreactor Using Fluorescence Polymerase Chain Reaction (PCR) Single-Strand Conformation Polymorphism Analysis." *Environmental Microbiology*. 2.1: 69-78.

**APPENDIX A
ADDITIONAL DATA**

Table A.1: Parameters Used for Bands Detection

Minimum band height	0.050
Dark bands and bright background	√
Rows of equal molecular weight	√
Allowed error (%)	±5
Maximum OD level for the image	√
Number of largest bands retained	10
Center peak	√

Table A.2: The Probability Associated With The Student's t Test For Pair-Wise Comparisons of SMA Means

	Brewery-1	Brewery-2	Municipal WWTP-1	Brewery-3	Food Industry-1	Food Industry-2	Food Industry-3	Sugar Industry-1	Dairy-1	Municipal WWTP-2	Food Industry-4	Soft Drink Bottling	Municipal WWTP-3	Municipal WWTP-4
Brewery-1	1.0000													
Brewery-2	0.0001	1.0000												
Municipal WWTP-1	0.0006	0.0062	1.0000											
Brewery-3	0.0003	0.0035	0.7938	1.0000										
Food Industry-1	0.0001	0.0152	0.6565	0.7163	1.0000									
Food Industry-2	0.0000	0.0007	0.0451	0.0431	0.2596	1.0000								
Food Industry-3	0.0084	0.1077	0.4801	0.4959	0.5817	0.9332	1.0000							
Sugar Industry-1	0.0000	0.0021	0.0277	0.0252	0.0264	0.0588	0.3959	1.0000						
Dairy-1	0.0000	0.0002	0.0016	0.0008	0.0252	0.0080	0.3667	0.8608	1.0000					
Municipal WWTP-2	0.0001	0.0036	0.0251	0.0234	0.0145	0.0369	0.2169	0.3095	0.3136	1.0000				
Food Industry-4	0.0000	0.0001	0.0004	0.0002	0.0121	0.0018	0.2012	0.1451	0.0253	0.8129	1.0000			
Soft Drink Bottling	0.0000	0.0001	0.0006	0.0002	0.0083	0.0010	0.1624	0.0781	0.0102	0.4662	0.2510	1.0000		
Municipal WWTP-3	0.0003	0.0004	0.0000	0.0000	0.0042	0.0010	0.0427	0.0088	0.0009	0.0211	0.0010	0.0021	1.0000	
Municipal WWTP-4	0.0004	0.0007	0.0001	0.0003	0.0044	0.0015	0.0399	0.0087	0.0016	0.0195	0.0021	0.0033	0.0514	-

Table A.3: Interpretation of Results of The Student's t Test

	Brewery-1	Brewery-2	Municipal WWTP-1	Brewery-3	Food Industry-1	Food Industry-2	Food Industry-3	Sugar Industry	Dairy	Municipal WWTP-2	Food Industry-4	Soft Drink Bottling	Municipal WWTP-3	Municipal WWTP-4
Brewery-1	True													
Brewery-2	False	True												
Municipal WWTP-1	False	False	True											
Brewery-3	False	False	True	True										
Food Industry-1	False	False	True	True	True									
Food Industry-2	False	False	True	True	True	True								
Food Industry-3	False	True	True	True	True	True	True							
Sugar Industry-1	False	False	False	False	True	True	True	True						
Dairy-1	False	False	False	False	False	False	True	True	True					
Municipal WWTP-2	False	False	False	False	True	True	True	True	True	True				
Food Industry-4	False	False	False	False	False	False	True	True	True	True	True			
Soft Drink Bottling	False	False	False	False	False	False	True	True	False	True	True	True		
Municipal WWTP-3	False	False	False	False	False	False	False	False	False	False	False	False	True	
Municipal WWTP-4	False	False	False	False	False	False	False	False	False	False	False	False	True	-

* True – Null hypothesis valid for 5% significance level (i.e. population means are statistically equal)
 ** False – Null hypothesis is invalid for 5% significance level (i.e. population means are statistically not equal)

Table A.4: Rank Correlation Coefficient Between Initial and Enriched SMA

	SMA Data		Densitometric Data				ρ
	SMA, mLCH ₄ /gVSS- hr	Rank(a)	Distance from Brewery-1	Rank (b)	d_i	$(d_i)^2$	
Brewery-1	10.65	1	0.00	1	0	0	0.015
Brewery-2	5.80	2	0.79	7	-5	25	
Municipal WWTP-1	4.38	3	0.64	3	0	0	
Brewery-3	4.35	4	0.95	13	-9	81	
Food Industry-1	4.23	5	0.87	8	-3	9	
Food Industry-2	3.79	6	0.91	11	-5	25	
Food Industry-3	3.72	7	0.98	14	-7	49	
Sugar Industry-1	2.89	8	0.72	5	3	9	
Dairy-1	2.83	9	0.90	9	0	0	
Municipal WWTP-2	2.38	10	0.91	10	0	0	
Food Industry-4	2.29	11	0.92	12	-1	1	
Soft Drink Bottling	2.08	12	0.67	4	8	64	
Municipal WWTP-3	0.14	13	0.64	2	11	121	
Municipal WWTP-4	0.00	14	0.75	6	8.	64	

$$\text{Spearman's Rank Correlation Coefficient } (\rho) = 1 - \frac{6 \sum d_i^2}{n(n^2 - 1)}.$$

Where,

$$d_i = \text{Rank(a)} - \text{Rank(b)}$$

n = Number of samples