ANAEROBIC DIGESTION OF BIOFUEL PRODUCTION RESIDUES

By

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To my parents who have always been supportive of my education

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ANAEROBIC DIGESTION OF BIOFUEL PRODUCTION RESIDUES

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The production of renewable fuels is expanding globally as the supply of petroleum reserves diminish and concerns regarding environmental impacts rise. Fuels produced from biomass have the potential to reduce reliance on petroleum resources and reduce greenhouse gas emissions. Commercially produced liquid biofuels are biodiesel and ethanol. Obtaining from coproduced residues remains one of the major challenges in full scale operations. This study investigated the potential of using anaerobic digestion to convert three waste residues generated from biodiesel production and cellulosic and non-cellulosic ethanol production. The wastes were glycerol byproduct from biodiesel production, distillery wastewater (stillage) from sugarcane bagasse and hardwood-based cellulosic ethanol production and tailings from sugarbeet-based noncelluloisc ethanol production. Anaerobic digestion is an engineered biochemical process that converts organic matter to biogas (a mixture of methane and carbon dioxide) by the concerted action of syntrophic microbial populations under oxygen free conditions. Biogas can be used as a fuel at the biorefinery displacing fossil fuel use.

Biochemical methane potential assays were performed on glycerol byproduct at mesophilic temperature (37 ^{o}C). The methane-producing potential was determined to be 450 ml CH_4 (ml sample)$^{-1}$. The produced methane is able to generate 1058 KJ (kg biodiesel)$^{-1}$, which is sufficient to offset the fuel energy consumption of 495 KJ (kg biodiesel)$^{-1}$ in a typical small scale biodiesel production, leaving in excess of energy that can be sold to make profit. A semi-continuous anaerobic digestion of glycerol byproduct was also carried out at laboratory scale. It was found that if glycerol byproduct was fed by itself, the degradation rate was only 0.67 ml (day)$^{-1}$(L) $^{-1}$.

Ethanol production using fermentation process produces a stillage waste from distillation of fermentation broth. Stillage is generated from ethanol distillation that is usually conducted at 60-70^{o}C. To take advantage of these warm temperatures, anaerobic digestion was carried out at thermophilc temperature (55 ^{o}C). Stillage obtained from cellulosic ethanol production process using sugarcane bagasse as well as hardwoods, as feedstock was used in these studies. Along with an aqueous fraction, the stillage contained of unconverted ligno-cellulosic material. The biochemical methane producing potential of these two different fractions of the stillage was first determined. It was observed that 70% of the methane-producing potential was in the aqueous filtrate. This meant that only the filtered fraction needs to be digested. The lignin-rich fibrous residue can be used for other applications like making bioproducts. Biochemical methane potential studies were followed by long term digestion of stillage in a continuously fed laboratory scale (13L) anaerobic digester. The digester was operated at hydraulic retention times (HRT) of 21 and 14 days for 90 days. The methane yield averaged 10 ml at STP (ml stillage)$^{-1}$. The organic matter removal efficiency was about

80%. An energy balance developed for integrating anaerobic digestion with the ethanol production process showed that up to 70% of the energy consumed in steam generation can be displaced using biogas from anaerobic digestion of stillage.

Sugar beet is a widely used feedstock for sugar-based non-cellulosic ethanol production in Europe. Tailing waste is generated when raw sugar beets are washed and mainly consists of sugar beet pieces, weeds, sugar beet tops, and other debris and soils. Anaerobic digestion of sugar beet tailings was implemented in a single-stage, batch system. Digestion performance was investigated and compared under mixed and non-mixed conditions. A higher methane yield of 0.35 L CH_4 $(g\ VS)^{-1}$ was obtained under non-mixed conditions, whereas only 0.25 ml CH_4 at STP $(g\ VS)^{-1}$ was obtained at mixed condition. The rate of methane production was also higher in the unmixed digester. The microbial community structure was investigated using 16s rDNA analysis for both mixed and non-mixed digesters, revealing marked differences. For example, an abundance of hydrogen-producing bacteria was detected in the mixed digester.

CHAPTER 1
INTRODUCTION

Project Focus

The rapid increase in demand for petroleum, its finite reserves, and concerns regarding its environmental impact are driving the search for new energy sources and alternative ways to power the world's economy. An alternative fuel is expected to be technically feasible, economically competitive, environmentally acceptable, and readily available (Meher, Sagar et al. 2006). Numerous alternative fuels have been proposed, including bioethanol, biodiesel, methanol, hydrogen, natural gas, liquefied petroleum gas (LPG), Fischer–Tropsch fuel, electricity, solar fuels and algae based fuels (Balat 2011). Among these, biomass-based fuels or biofuels have several advantages over petroleum with a major one being easy availability from locally grown biomass (Demirbas 2008). The most commonly produced biofuels include ethanol and biodiesel because these can be used as a substitute fuel for motor vehicles (Demirbas 2011). Biofuel production has increased in recent years. This, however, has also resulted in increases in the amount of associated wastes. For example, biodiesel production generates crude glycerol byproduct and ethanol production generates distillery wastewater (stillage) and fermentation residues as wastes, which present significant disposal or treatment problems. The studies presented in this dissertation investigated anaerobic digestion as a viable and sustainable approach for simultaneous waste treatment and energy production. The biogas produced from anaerobic digestion can be used as a fuel on site in the biorefinery to displace fossil fuels.

Anaerobic digestions of three different residues from biofuel production process are considered in this dissertation: 1) glycerol byproduct (GBP) from biodiesel

production, 2) stillage from sugarcane-bagasse and hardwood-based cellulosic ethanol production and 3) tailings from sugarbeet based noncelluloisc ethanol production.

Glycerol Byproduct from Biodiesel Production Process

Biodiesel is typically produced by reacting vegetable oil or animal fat (triglycerides) with methanol or ethanol in the presence of an alkali catalyst. Glycerol is generated during the transesterification process, as shown in the stoichiometric reaction in Figure 1-1 (Parawira 2009).

Transesterification is the process of exchanging the alk-oxy group of an ester compound with another alcohol. The overall process is a sequence of three consecutive and reversible reactions, in which diglycerides and monoglycerides are formed as intermediate compounds (Ma and Hanna 1999). In general, transesterification process produces about 1 kg glycerol per 10 kg biodiesel. Glycerol byproduct stream may contain impurities like unreacted oil, excess alcohol, catalyst and soap. Since the transesterfication reaction is reversible, alcohol is usually added in excess to shift the equilibrium in favor of production of biodiesel (Meher, Sagar et al. 2006). Excess methanol is distributed between the ester and glycerol phases. Methanol is typically recovered even by small-scale biodiesel processors from these phases for reuse as reactant. Purified glycerol, or glycerin, is a fairly high value chemical that is primarily used in the manufacture of various foods and beverages, pharmaceuticals, cosmetics, and other personal care products. Thus, a possible option to add value to GBP would be to refine it to pure glycerol or small scale biodiesel manufactures can directly sell it. However, as the biodiesel industry has expanded at a global scale, greatly increased GBP production has impacted the market, causing a 10-fold slump in crude glycerol price in recent years and recession among glycerol refineries (Sabourin-Provost and

Hallenbeck 2009). As a result, biodiesel refiners are faced with limited options for managing GBP, which has essentially gone from being a potentially valueable product to a waste stream in the biodiesel industry (Johnson and Taconi 2007). Currently utilized options for glycerol management include selling it as boiler fuel or as a supplement for animal feed. However, when burned to produce thermal energy, the impurities contained in GBP may create a significant amount of ash with potential to cause environmental and health problems, and the presence of water decreases its heating value. When sold as animal feed, the market value of GBP is too low to be economically attractive (Hu and Wood 2010). Consequently, it is crucial to develop an alternative process to convert GBP to a value-added product to increase the competiveness of biodiesel industry.

Residues from Bioethanol Production

Bioethanol can be produced from different kinds of feedstocks. The materials can be classified into three categories: sucrose-containing feedstocks (e.g. sugar cane, sugar beet, sweet sorghum and fruits), starch-based materials (e.g. corn, wheat, rice, potatoes, cassava and sweet potatoes) and lignocellulosic materials (e.g. wood, straw and grasses) (Balat 2011). Sugar beet provides an abundant source of sucrose which can be easily converted to ethanol by yeast fermentation, whereas producing ethanol from maize and wheat requires enzymes to convert starch to sugar first (Antoni, Zverlov et al. 2007). On a per hectare basis, sugar beet is one of the most productive sources of ethanol and was the second most common feedstock used in European Union for ethanol production in 2009 (Panella 2010; Takara, Nitayavardahana et al. 2010). However, commercial ethanol production from sugar beet has not been implemented in United States to date (Outlaw, Ribera et al. 2007).

Sugarbeet processing generates significant quantities of both solid and liquid wastes. Raw sugar beets, when brought into the processing plant from storage in outdoor stockpiles, are first washed and separated from “tailings”. The washed beets proceed for further processing and juice extraction generating another solid waste stream, the spent beet pulp. The tailings mainly consist of 10% to 30% sugar beet chips and 70 to 90% weeds, sugar beet tops, debris, and soil (Kumar, Rosen et al. 2002). Sugar beet tailings have been anaerobically digested to produce biogas ((Weiland 2003; Klocke, Mähnert et al. 2007; Felde 2008).

The alternate option to produce ethanol from nonfood biomass resources like forestry and agricultural residues and urban wastes is currently gaining ground. Biofuel produced this way is termed second generation biofuel. Lignocellulosic materials serve as a cheap and abundant feedstock and have the potential to produce up to 442 billion liters of ethanol that could help meet the future demand (Kim and Dale 2004). Ninety percent of dry matter in lignocelluloses consists of cellulose, hemicellulose and lignin with the rest consisting of extractables and ash. Ethanol is produced from cellulose, hemicellulose and other sugar components in the feedstock. (Balat 2011). The difficulties in bioconversion of lignocellulosic materials to ethanol are (1) the resistant nature of biomass to breakdown; and (2) inefficient fermentation of sugars released from degradation of cellulose and hemicellulose by naturally occurring organisms. At the Biofuels Pilot Plant in University of Florida, a recombinant E. Coli KO101 strain developed in the Microbiology and Cell Science Department is employed to ferment both the hexose and pentose sugars to ethanol (Yomano, York et al. 1998; Yomano, York et al. 2008; Yomano, York et al. 2009). Figure 1-2 shows the flow diagram of

bioethanol production from sugarcane bagasse conducted in the Biofuel Pilot Plant. The flow quantities were calculated by assuming the plant being able to produce 1 million gallon of ethanol annually. As shown in Figure 1-2, the process generates 12 liters of stillage per 1 liter of ethanol produced. Stillage organic content can exceed 100 g COD/L and exhibits a considerable pollution potential (Sheehan and Greenfield 1980; Lele, Rajadhyaksha et al. 1989; Yeoh 1997). Wilkie et al. (Wilkie, Riedesel et al. 2000) conducted an extensive literature review to investigate methods to process and utilize the stillage associated with ethanol production from conventional and cellulosic feedstocks and revealed a consensus toward anaerobic digestion as an economical technology.

Research Objectives

The overall goal of this research was to investigate the feasibility and ascertain environmental benefits of deploying anaerobic digestion for wastes, byproducts and residues generated in biofuel production process. Three different types of feedstocks were separately investigated. 1) mostly defined liquid feedstock (GBP), 2) fiber containing slurry (stillage), and 3) solid feedstock (tailings)

Specific objectives were to

1) determine the biochemical methane potential of the feedstocks
2) investigate the long term operability of the anaerobic digestion process to operate on these feedstocks by employing appropriate digester designs at the laboratory scale. Operational parameters monitored included
    a. quality of the effluent
    b. biogas production rate
    c. biogas composition

d. microbial population and dynamics

3) estimate fuel savings when anaerobic digestion is integrated with the biofuel production process.

Anaerobic Digestion Technique

Anaerobic digestion is an engineered biochemical process that converts organic matter to biogas (a gas mixture of methane and carbon dioxide) by the concerted action of microbial populations under oxygen-free conditions. The bioconversion of organic matter into biogas is accomplished by a series of interdependent metabolic reactions in which different classes of microorganisms take part (Alkaya and Demirer 2011). Hydrolysis and acidogenesis reactions are conducted by bacterial populations that produce hydrogen and organic acids, and the methanogenesis reaction is performed by archaeal group that produce methane using the products of acidogenesis reactions. Organic matter is converted into biogas as well as new bacterial biomass (Romano and Zhang 2008).

The Microbiology of Anaerobic Digestion

The microbiological nature of anaerobic digestion was discovered more than a century ago (Koster 1988). While a diverse array of microorganisms is implicated, anaerobic digestion is mostly driven by bacteria and methanogens. Although the microbiology and chemistry of anaerobic decomposition are complicated, it can be conceptualized into three transformation steps: 1) hydrolysis of complex organic molecules to soluble monomer molecules such as amino acids, fatty acids, glucose and glycerol; 2) fermentation of those soluble substrates to produce carbon dioxide, hydrogen and organic acids with acetic acid being the main product, with smaller

amounts of propionic and butyric acids; 3) conversion of the acetic acid, hydrogen and a portion of the carbon dioxide to methane.

Four categories of microorganisms: 1) hydrolytic bacteria, 2) fermentative acidogenic bacteria, 3) acetogenic bacteria and 4) methanogens are involved in step 1, 2 and 3 respectively. A large number of obligate and facultative anaerobic bacteria carry out the hydrolysis and fermentation steps. These include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactrobacillus* and *Streptococcus* (Bitton 2005). The *Syntrobacter* and *Syntrophomonas* genera are known to include acetogenic species that convert propionic and butyric acid to acetate, hydrogen and carbon dioxide (McInerney, Bryant et al. 1981). This group requires low H_2 partial pressure for acetate production. There is a synergistic relationship between acetogenic bacteria and methanogens, in which acetogenic bacteria provide substrates for methanogens and methanogens help to achieve low H_2 tension by converting it to CH_4. Distinct from the other 3 categories, methanogens belong to a separate domain, *Archaea*, and are only able to use a limited number of substrates that include acetate, H_2, CO_2, formate and methanol. The bioconversion to CH_4 by methanogens can be divided into 2 pathways: hydrogenotrophic and aceticlastic methanogenesis. Hydrogenotrophic methanogens, which belongs to the orders of *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales* and *Methanococcales*, reduce CO_2 to methane by using H_2. Aceticlastic methanogens produce CH_4 and CO_2 from acetic acid. These are considered as the dominating methanogens in many anaerobic reactors treating waste water (Leclerc, Delgenes et al. 2004; Karakashev, Batstone et al. 2005). An estimated 65-70 % of CH_4 produced in an anaerobic digester is believed to originate from acetate

(Mackie and Bryant 1981; Liu.Y. and Whitman 2008). Unlike the diverse hydrogenotrophic methanogens, acetotrophic methanogens were only identified among the order of *Methanosarcinales* (Liu.Y. and Whitman 2008).

Methane Production and COD Degradation

Anaerobic digestion produces CH_4 which is a sparingly soluble gas, and most is degased from solution and can be recovered easily for subsequent use. Stoichiometry of methane oxidation shows the chemical oxygen demand (COD) equivalent of methane is 4 g COD (g CH_4)$^{-1}$. At standard temperature and pressure (0 ºC and 1 atm), it corresponds to 0. 35 L of CH_4 produced per gram of COD converted to CH_4. This directly relates CH_4 production and COD removal and provides a way to estimate CH_4 production by knowing how much COD has been removed in anaerobic digester. The production of CO_2 does not contribute to COD reduction because the carbon in CO_2 is in the maximum oxidation state. The CH_4 and CO_2 content of biogas varies depending on the nature of the substrate with CH4 content ranging between 50 to 70% (Parkin and Owen 1986).

Anaerobic digesters are typically utilized to stabilize organic matter in wastes with biodegradable COD concentration greater than 1 g/L. Compared to other waste treatment methods, anaerobic processes offer advantages including less solid production, lower nutrient requirement, lower energy consumption and production of a potentially useful product of CH_4 (Sahm 1984; Lettinga, Field et al. 1997).

The first step in developing an anaerobic digestion project involves determining the methane potential of the feedstock using the biochemical methane potential (BMP) assay. BMP assay was conducted by mixing the substrate with inoculum and nutrients, and incubating the mixture in a sealed bottle at predetermined temperature for a period

of time to allow complete degradation of the substrate. In this research the BMP of each of the feedstock was first determined.

Configurations of Anaerobic Digesters

A typical anaerobic digester contains four components, a closed vessel, a heating system, a mixing system and a gas-liquid-solid separation system (Grady, Daigger et al. 1999), but mixing is not always required. A closed vessel is used to exclude dissolved oxygen and ensure the development of anaerobic conditions. Heating is usually required in anaerobic digestion to maintain the optimal temperature for microbial activities, typically ranging from mesophilic (around 37 ºC) to thermophilic (around 55 ºC) temperatures. In many cases, mixing is provided to improve the homogeneity of the digester contents and reduce the resistance to mass and heat transfer. Several methods are used to accomplish mixing, including use of mechanical mixers, slurry recirculation or biogas recirculation (Karim, Hoffmann et al. 2005). Many anaerobic digesters are cylindrical concrete tanks with a cone shaped bottom and steel or concrete covers, though other materials and configurations can be used. In general, there are seven types of anaerobic digesters: 1) continuous stirred tank reactor (CSTR), 2) anaerobic contactor, 3) upflow anaerobic sludge blanket (UASB), 4) anaerobic filter (AF), 5) hybrid UASB and anaerobic filter (UASB/AF), 6) downflow stationary fixed film (DSFF) and 7) fluidized bed (FB) digesters (Grady, Daigger et al. 1999). Among these, a CSTR reactor is well mixed with no solid and liquid separation and its hydraulic retention time (HRT) and solids retention time (SRT) are identical (Malina 1992) . The rest of reactor configurations provide significant retention of active biomass, resulting in large differences between SRT and HRT (Hall 1992; Defour, Derycke et al. 1994). Long SRTs maintain accumulation of active biomass and allows higher organic loading rate.

Therefore, reactor types 2 to 7 are commonly considered as high rate anaerobic processes. Table 1-1 summarizes the primary benefits and drawback of different types of anaerobic digesters. All high rate anaerobic processes share certain properties. High biomass concentration are maintained in the digester and thereby allow long SRTs to be achieved while keep HRT relatively short. High biomass concentrations makes high organic loading rate possible, resulting in small reactor volumes. Choice of digester design is influenced by the solids content of waste. Based on the characteristics of feedstocks studied here, a downflow stationary fixed film digester was used to digest GBP, a continuous mixed digester was used for stillage, and batch high solids mixed and unmixed digesters were used for tailings.

Factors Affecting Anaerobic Digestion

Anaerobic digestion is mainly affected by temperature, pH, retention time, chemical composition of substrates and the presence of toxins (Bitton 2005). Mesophilic anaerobic digestion operates at temperatures from 25 ºC to 40 ºC with an optimum at 37 ºC, while thermophilic anaerobic digestion operates at temperatures ranges of 50 ºC to 60 ºC. Due to the higher temperature, the thermophilic condition provides a faster digestion rate and allows for a higher substrate loading rate. High temperature also leads to greater inactivation of pathogens (Koster 1988). Most methanogens function optimally at a pH of 6.7 to 7.5 (Bitton 2005). The acidic environment caused by the organic acidogenic bacteria could lead to failure of methanogensis. Under normal conditions, the pH reduction is buffered by bicarbonate that is added externally or produced by methanogens. However, the buffering capacity can be upset, especially when organic overloading happens, stopping CH_4 production. An increase in volatile organic acid concentration serves as an early indicator of system upset and monitoring

of volatile organic acid level is suggested to ensure system stability. HRT is the average time spent by anaerobic microorganisms in the anaerobic digester. HRT must be long enough to allow metabolism of those microorganisms. The usable substrates for anaerobic microbial communities include carbohydrates, proteins, lipids etc., but a few compounds such as lignin are non-degradable for anaerobic microorganism. Providing nutritionally balanced substrates plays a role in maintaining an adequate anaerobic digestion. The carbon to nitrogen to phosphorus ration for anaerobic bacteria is reported to be 700 to 5 to 1 (Sahm 1984). Sulfur is also required by methanogens, but it is toxic at levels exceeding 150-200 mg/L(Speece 1983). Trace element as iron, nickel and cobalt are also necessary for anaerobic microorganisms to grow. The presence of toxicants is responsible for the occasional failure of anaerobic digestion. Inhibition of methanogenisis is usually accompanied by reduced methane production and accumulation of volatile organic acids. Some of the common inhibitors are oxygen, ammonia and volatile organic acids. Early studies indicate that trace level of oxygen can adversely affect methanogens which are obligate anaerobes (Roberton and Wolfe 1970), but later findings tend to agree that methanogens can tolerate oxygen due to the protection of the sludge aggregates (Kato, Field et al. 1993). Unionized ammonia is toxic to methanogens and the inhibitory level is round 1500 to 3000 mg/L . However, since the formation of unionized ammonia is pH dependent, little toxicity is observed at neutral pH. Volatile organic acids such as acetic acid and butyric acid are also toxic to methanogens and propionic acid display toxicity to both acidogenic bacteria and methanogens. The toxicity of volatile acids is minimal if the pH is maintained near neutrality (Bitton 2005).

Methods for Investigation of Microbial Communities

During the past decade, progress had been made in the development of molecular techniques for determining the numbers and activity of microorganism in anaerobic digestion. These technologies include 16S rRNA gene fingerprinting and sequencing, and fluorescent *in situ* hybridization (Manes, West et al. 2011). In this thesis, 16s rRNA gene sequence analysis was employed to investigate the microbial community structure and diversity. There are 16S rRNA targeted oligonucelotide probes for bacteria or archaea in general as well as for individual species in particular.

Current Technologies for 16S rDNA Sequencing

There are several options of sequencing technology. Sanger sequencing, also known as capillary sequencing, produces long reads (up to 800 bases) which are helpful characterizing gene functions and taxonomic composition of microbial communities (Wommack, Bhavsar et al. 2008). Barcoded pyrosequencing, an innovative sequencing technology initially developed in the 1980s, makes microbial community study orders of magnitude more efficient by being able to generate a much greater number of sequences in a single run (Shoemaker, Lashkari et al. 1996; Hamady, Walker et al. 2008). It also eliminates the laborious step of preparing clone libraries (Ronaghi, Karamohamed et al. 1996). Barcoded pyrosequencing uses molecular barcode techniques to add a unique tag to each primer before PCR amplification (Binladen, Gilbert et al. 2007; Parameswaran, Jalili et al. 2007). Because each sample is tagged with a known primer, an equimolar mixture of samples can be amplified and sequenced and sequences can be assigned to samples based on the unique barcodes. Though shorter sequences are produced in pyrosequencing, the benefit of a large number of short reads outweighs the drawbacks of short read lengths

for many kinds of rRNA-based community analysis: sequence fragments as short as 100 bases, covering only 8% length of full-length 16S rRNA gene, provide results with comparable resolution to 70% as those obtained using full-length sequences (Liu, Lozupone et al. 2007). Given that Sanger sequencing is more expensive than pyrosequencing, requires cloning of DNA fragments into a common host cell (typically *E. coli*), and has remarkably lower output, the latter is a more cost-effective option for investigating the distribution of microbial diversity among samples at this point.

16S rDNA Sequencing Data Analysis

In general, analyses of microbial diversity can be divided in three dimensions (Hamady and Knight 2009). First, an analysis can examine either “alpha diversity” (how many kinds of taxa or lineages are in one sample) or “beta diversity” (how taxa or lineages are shared among samples, e.g., along a gradient). Second, an analysis can be either “qualitative,” examining only presence-absence, or “quantitative,” also taking into account relative abundance (qualitative analyses and quantitative analyses are also called analyses of community membership and community structure, respectively). Third, an analysis can be either “phylogenetic,” building a phylogenetic tree to relate the sequences, or “taxon based,” treating all taxa at a given rank (e.g., orders rank by aboudance). Taxon-based and phylogenetic methods provide different but equally useful insights. In this study, a taxon-based analysis was performed, in which query sequences was matched by similarity to an existing sequence in the 16S rRNA database by BLAST algorithm.

Table 1-1. Comparison of Anaerobic Digesters

Process	Benefit	Drawbacks
CSTR	Suitable for a wide range of wastewaters	Large bioreactor volume required
	Easy to mix	Effluent quality can be poor if large concentration of anaerobic organisms is generated
	Efficiently handles high suspended solid wastewater	Poor performance at short SRT
	Large bioreactor volume to dilute inhibitors	Requires separate mechanical mixing
AC	Performance not dependent on sludge settleability	Biomass settleability critical to digestion performance
	Suitable for concentrated wastewaters	Only suitable for waste with low to moderate levels of suspended solids
	Easy to mix	Mechanically complex system
	Relatively high effluent quality achievable	
	Reduced bioreactor volume compared to CSTR	
UASB	High biomass concentrations and long SRT achievable	Performance dependent on development of dense, settable solids
	Small bioreactor volumes	Little process control possible
	High quality effluent achievable	Low process loading required if wastewater contains suspended solids
	Mechanically simple	
	Compact system	
AF	High biomass concentrations and long SRT achievable	Suspended solids accumulation may negatively impact digestion performance
	Small bioreactor volumes	Not suitable for high suspended solids wastewater
	High quality effluent achievable	Little process control possible
	Mechanically simple	High cost for media and support
	Compact system	
	Performance not dependent on development of dense, settleable solids	

Table 1-1. Continued.

Process	Benefit	Drawbacks
Hybrid UASB/AF	High biomass concentrations and long SRT achievable	Lower process loading required if wastewater contains suspended solids
	Small bioreactor volumes	Little process control possible
	High quality effluent achievable	
	Mechanically simple	
	Compact system	
	Performance partially dependent on development of dense, settleable solids	
	Reduced media cost	
DSFF	High biomass concentrations and long SRT achievable	Biodegradable suspended solids not generally degraded
	Small bioreactor volumes	High cost for media and support
	High quality effluent achievable	Organic removal rate generally lower than other high rate processes
	Mechanically simple	Little process control possible
	Compact system	
	Performance not significantly impacted by wastewaters with suspended solids	
	Performance not dependent on development of dense, settleable solids	
FB	High biomass concentrations and long SRT achievable	Long startup period required
	Small bioreactor volumes	High power requirement for bed fluidization and expansion
	Excellent mass transfer characteristics	Not suitable for high suspended solids wastewater
	High quality effluent achievable	Mechanically complex system
	Most compact of all high rate processes	High cost for carrier media
	Performance not dependent on development of dense, settleable solids	

Adapted from Grady (Grady, Daigger et al. 1999)

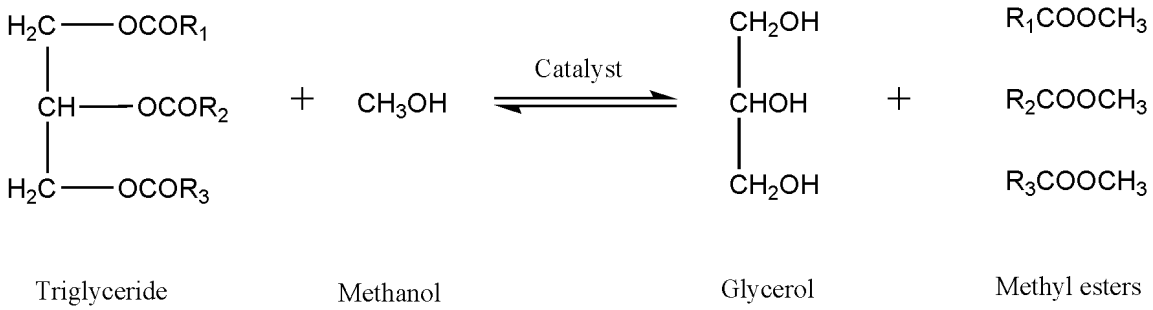


Figure 1-1. Transesterification of triglycerid

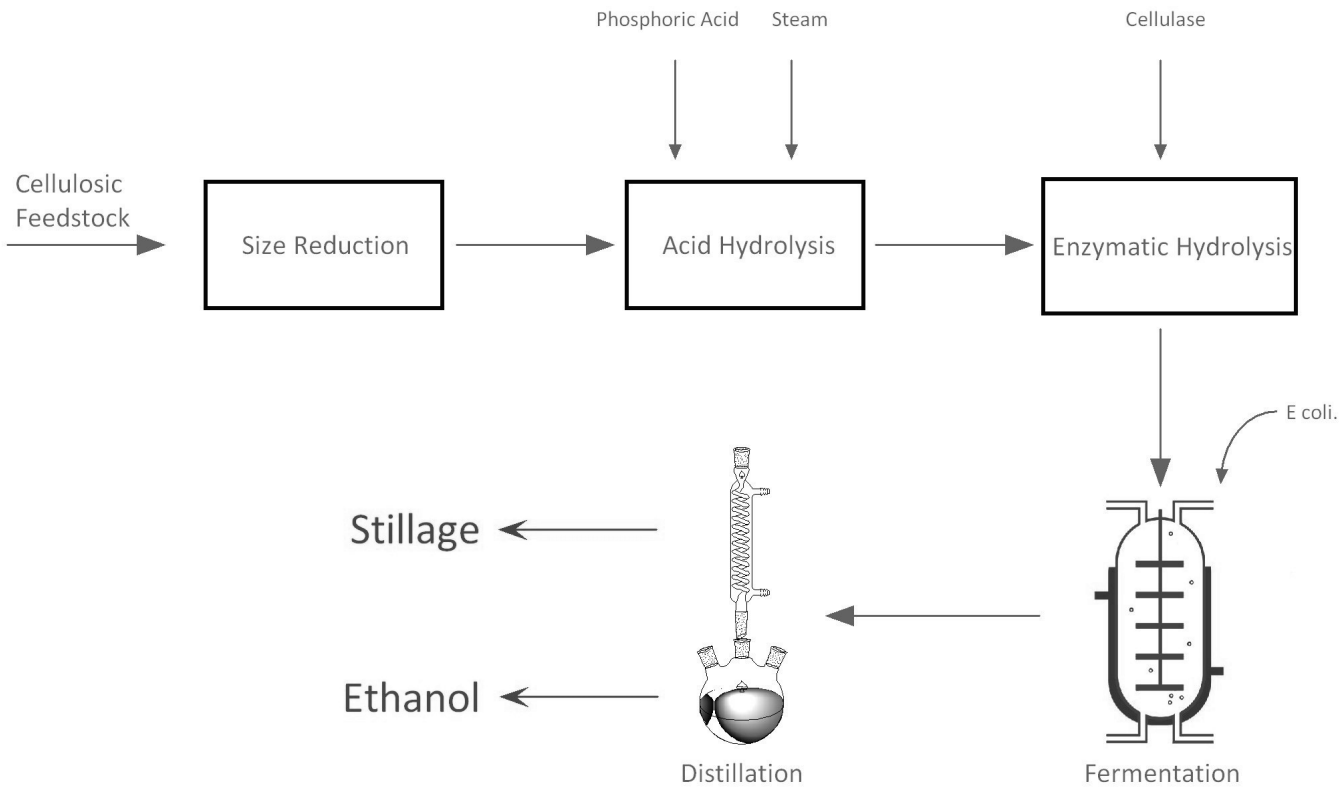


Figure 1-2. Ethanol producton process from sugarcane bagasse

CHAPTER 2
BIOCHEMICAL METHANE POTENTIAL OF GLYCEROL BYPRODUCT FROM BIODIESEL PRODUCTION USING WASTE VEGETABLE OIL

Summary

Biochemical Methane Potential (BMP) assays were performed at mesophilic temperature on the crude glycerol byproduct (GBP) obtained from the settling phase of biodiesel production unit. GBP contained glycerol, methanol, oil residues and some water. The average methane yield of GBP was 455 ml CH4 at STP / ml GBP. This yield was higher than that obtained from pure glycerol, indicating that in addition to glycerol, GBP contained other compounds from which methane was produced. However, the duration of digestion to achieve this yield was long, requiring 100 days of incubation and the amount of glycerol loaded into the assay had an effect on methane production. The average methane yield of GBP at 2 ml loading after the same incubation period was significantly lower. It was determined that the lower production rate at higher loading was not due to the presence of inhibitory compounds in GBP but due to an organic overloading exceeding the glycerol-degradation capacity of the inoculum. Volatile organic acid (VOA) accumulated to much higher concentrations in overloaded assays, with propionic acid being dominant. Presence of a co-substrate with GBP showed improved methane production. Continuous digestion studies were then carried out over 40 days.

Background

Biodiesel refers to monoalkyl transesterfied ester of fatty acid from vegetable oil or animal fat. It is a non-toxic and a renewable diesel fuel which can be used neat or as a blend with petroleum diesel fuel. Compared to fossil fuel, biodiesel holds many advantages like lower carbon monoxide and particulate emissions and higher cetane number (Alptekin and Canakci 2008). Due to concerns of global warming and increases in the price of petroleum in recent years, there is considerable interest in renewable fuels. Biodiesel production is expanding throughout Asia, Europe and America. Several large scale plants, about 120 in European Union and 165 plants in USA, annually produce 14 billion liters of biodiesel combined (Alptekin and Canakci 2008). On the other hand, the relative ease in making biodiesel also encouraged the growth of small scale production facilities, which does not require large investment and complex operation but is able to make sufficient fuel for self-use in households, farms or in communities.

Biodiesel production process involves reacting vegetable oil with methanol in the presence of a catalyst like potassium hydroxide. The process also generates a crude glycerol byproduct (GBP). GBP contains unreacted vegetable oil, methanol, catalyst and other side products like soap and requires purification before it can be marketed. Only large scale plants are able to economically refine the crude glycerol. Alternate options need to be considered for small scale producers.

GBP may be anaerobically digested to biogas consisting of 60-70% methane. Biogas is a good energy source, and can be used in the production process to reduce the fossil energy inputs. Large scale plants can also integrate the anaerobic digestion

process with the biodiesel production facility to improve overall energy efficiency and reduce carbon footprint of the process.

For feeding GBP to anaerobic digesters, it is necessary to evaluate the characteristics of its degradation, namely degradability, methane production potential, degradation rate and inhibitory effect. Anaerobic digestion of glycerol has been reported in the literature. In these studies, either analytical grade pure glycerol (Holm-Nielsen, Lomborg et al. 2008) or refined glycerol from biodiesel production (Chen, Romano et al. 2008) was co-digested with manure. The focus of those studies was to improve volumetric methane productivity of manure digesters by adding glycerol as a supplement to manure digesters. In this study, crude GBP as obtained from the settling tank of a small-scale biodiesel unit at the University of Florida was used as a sole substrate in biochemical methane potential assays. In addition to the methane potential of the substrate, the assay also provided information on the rate of degradation, extent of degradation and the presence of any inhibitory compounds or toxins in the substrate. These batch assays were performed at mesophilic (37 °C) temperature. The feasibility of using a one-stage, continuous digester for anaerobic digestion of GBP was also investigated.

Material and Method

Feedstock

Crude GBP drained from the fatty acid methyl ester settling tank of a small scale biodiesel facility at University of Florida campus was used as feedstock. This facility processes waste cooking oil in a batch reactor using methanol as reactant and KOH as catalyst.

BMP Assay

BMP assays measure the methane potential of feedstocks at optimal conditions for culturing anaerobic digester microbial consortia (Owen, Stuckey et al. 1979; Shelton and Tiedje 1984; Owens and Chynoweth 1993). It was conducted by mixing the substrate with inoculum and nutrients, and incubating the mixture in a sealed bottle at predetermined temperature for a period of time to allow complete degradation of the substrate. The entire set of assays consisted of 18 bottles, and each assay was conducted in 250 ml serum vials at mesophilic temperature of 37^{o}C. Assays contained substrate (GBP or pure glycerol), inoculum and other supportive solutions (micronutrient, macronutrients, co-substrate and pH buffer liquid) with total volume of 154 ml. The inoculum used was collected from a laboratory scale anaerobic digester that had been digesting various biomass feedstock and wastewater at mesophilic temperature for three months. Nutrients stock solution was prepared according to Owens et al. (Owens and Chynoweth 1993). The amount of the substrate added was varied and in some assays a co-substrate was added. The reason being crude GBP may contain unreacted or partially reacted oil and it has been shown that lipids and long chain fatty acids are degraded more efficiently in the presence of co-substrates especially carbohydrates (Kuang, Lepesteur et al. 2002). The co-substrate used in the study was a water extract of sugar beet tailing (tailing water extract). Sugar beet tailing contains a large fraction of readily soluble organic matter and these are extracted easily upon addition of water. The organic components of this extract are primarily carbohydrates and have been shown to degrade readily in an anaerobic digester (Liu, Pullammanappallil et al. 2008). However, the amount of extract added was in small quantities ensuring the extract did not contribute significantly to methane production.

Table 2-1 lists the assays along with its contents and quantities loaded. Assays contained 1 ml or 2ml of GBP. 50 ml of tailings water extract was also added in an assay containing 2 ml GBP. Each assay was duplicated. Three types of control assays each in duplicate, were also analyzed. The first type of control assay contained only inoculum (C-I), the second type contained 1 ml (C-II, 1) or 2 ml (C-II, 2) of analytical grade glycerol (99.5%) with inoculum and the third (C-III) type contained 50 ml of tailings water extract with inoculum. The first type of control assays was set up to provide data on methane production from residual substrates and endogenous metabolism in inoculum. Data using second type of control assay provided information on the nature of degradation of glycerol, and when compared to data from GBP assays was able to provide information on presence of inhibitory compounds in GBP and potential of excess methane production from GBP. The third type of control assay provided information on methane potential of tailings extract.

Semi-continouous Digestion of GBP

The feasibility of anaerobic digestion of GBP was further evaluated in a laboratory scale 5 L semi-continuously fed stirred anaerobic digester. The digester was constructed by modifying a Pyrex glass jar. The height and inner diameter of the digesters were 0.406 m and 0.061 m, respectively. A glass flange fitted with a rubber O-ring was used to seal the top of the digester. The flange was clamped using stainless steel clamps for gas and liquid tightness. The digester was provided with several ports for gas outlet, sample withdrawal and digester liquor circulation. Gas production from the digesters was measured by a positive displacement gas meter which consisted of a clear PVC U- tube filled with anti-freeze solution, a solid state time delay relay (Dayton Off Delay Model 6X153E), a Grainger float switch, a Redington counter and Fabco Air

solenoid valve. More details of the digester and gasmeter can be found in Koppar and Pullammanappallil (Koppar and Pullammanappallil 2008). The gas meter was calibrated in line to determine the volume of biogas per count. An incubator was used to provide 37 °C environment for the digester. The digester was first inoculated with 3 L inoculum and added with 100 grams of sugar beet tailings to activate the inoculum. The digester was allowed to stabilize for 2 days before the feeding was started. GBP was added at 2 ml per day from day 3 to day 18 and increased to 3 ml per day from day 18 to day 40.

Analysis

Methane production, chemical oxygen demand and volatile organic acid concentration were monitored in the BMP assays. Biogas that accumulated in the serum vials was withdrawn periodically using a syringe. Biogas was allowed to fill the syringe until the pressure in vial head space equilibrated with that in the syringe. Methane and carbon dioxide composition was measured using a Fisher Gas Partitioner (model 1200). The gas chromatograph was calibrated with an external standard containing N_2, CH_4, and CO_2 in 25:45:30 volume ratio. Liquid samples were withdrawn from the serum bottles for soluble Chemical Oxygen Demand (SCOD) and Volatile Organic Acid (VOA) measurement. Liquid samples were centrifuged at 8000 RPM for 10 minutes (Fisher Marathon micro H centrifuge), filtered using Millipore filter paper (pore size 1.2 um) for SCOD and VOA analysis. SCOD liquid samples were added in Hach COD vials (range 20-150 ppm) and baked in Hach COD reactor for 2 hours. sCOD was measured using Hach DR/890 Colorimeter. VOA liquid samples were measured using Shimadzu gas chromatograph (GC-9AM equipped with a flame

ionization detector) for acetic, propionic, isobutyric, butyric, isovaleric and valeric acid concentrations.

Daily methane production and pH was measured for the semi-continuous digester. Biogas volume was measured by the displacement gas meter, and methane content of biogas was analyzed using the same gas chromatograph as described above. The pH of digester liquor was measured with an Accumet pH meter.

The performance of the batch biochemical methane potential assays was evaluated by fitting the cumulative methane production data to the modified Gompertz equation (Lay, Li et al. 1998). The modified equation describes cumulative methane production from batch digesters assuming that methane production is a function of bacterial growth and is presented below:

$$M = P \times \exp\left\{-\exp\left[\frac{R_m \times e}{P}(\lambda - t) + 1\right]\right\} \qquad (2.1)$$

Where M is the cumulative methane production, L at any time t, P is the methane yield potential, L, R_m is the specific maximum methane production rate, L d^{-1}, λ is the duration of lag phase, d, and t is the time at which cumulative methane production M is calculated, d. The parameters P, λ and R_m were estimated for each of the 15 data sets by using the 'Solver' feature in MS-Excel. The value of parameters which minimized the sum of the square of errors between fit and experimental data were determined. The model as applied to the anaerobic digestion process assumes that the rate limiting step is methanogenesis and then the parameter R_m is related to the specific growth rate of the methanogens (μ) through the product yield coefficient, Y_{XP} (g biomass /L methane STP),

i.e. $R_m = \mu / Y_{XP}$.

Results

Characterization of the GBP

The density of GBP and pure glycerol (99.5% grade) was determined to be 1.16 $g(ml)^{-1}$ and 1.20 $g(ml)^{-1}$, respectively. GBP was also determined to lose 29.6% weight after it had been dried at 104°C for 24 hours. The lost mass was considered as methanol. In biodiesel production, glycerol phase was reported to contain 62.9% to 76.6% glycerol, based on weight, depending on what feedstock was used. The rest in glycerol phase usually contained methanol, catalyst and oil residues. Specially, for the situation that waste vegetable oil (WVO) was used, methanol content in glycerol phase (GBP) was reported to be 21.6% (Thompson and He 2006). The reported methanol content fell into a close range as the methanol content determined by this study. Using this as a reference, GBP was assumed to contain 29.6% methanol and the rest 70.6% are glycerol.

Methane Potential

The profiles of the average cumulative methane yield of assay A-1 (containing 1 ml GBP), A-3 (containing 2 ml GBP) and C-II, 1 (containing 1 ml pure glycerol) are shown in Figure 2-1. Sample standard deviation is shown as error bars. The methane potential of GBP and pure glycerol over 100 days of incubation was 456 ml CH_4 at STP $(ml\ sample)^{-1}$ and 372 ml CH_4 at STP $(ml\ sample)^{-1}$ from assays A-1 and C-II, 1, respectively. The degradation rate of GBP and glycerol indicated by the slope of curve, initially increased, reached a maximum and then decreased to a minimum as substrate was utilized. The rates of evolution of methane were similar for the first 40 days. Methane production continued in assay A-1 whereas it tailed off in C-II, 1. Eighty five percent of ultimate methane yield of GBP was produced in 60 days. The higher

methane yield from GBP could be because GBP contained a mixture of substrates such as long chain fatty acids, residual oil and methanol that are better energy sources than glycerol itself. Assay A-3 contained 2 ml GBP and the methane potential was determined to be 832 ml CH4 at STP (2 ml sample)$^{-1}$ after 200 days. It took twice as long as assay A-1 did to achieve the ultimate methane yield.

The Gompertz equation was used to fit the cumulative methane production data for assay A-1, C-II, 1 and A-3. Equation parameters, P, λ and R_m were calculated and their values were provided in table 2-2.

The simulation results showed a bi-phase trend (fraction 1 and fraction 2 in Figure 2-1) for assays A-1 and A-3, i.e., the slope initially increased then decreased by day 20 (both assays) but then the methane production began to increase again from day 30 (day 40 for assay A-3) then decrease until methane production completely ceased. Fraction 1 of assays A-1 and A-3 completed methane production in a short period of time, suggesting its easy degradability. Interestingly, both methane yield and methane production rate of fraction 1 doubled in assay A-3 that contained twice as much GBP as in assay A-1. This indicated the prolonged digestion time seen in assay A-3 was not due to the doubled amount of fraction 1. In contrast, fraction 2 of assay A-3 shared similar methane production rate to assay A-1 and took additional 100 days to produce a comparable methane yield. It appeared fraction 2 was the limiting factor in GBP digestion from assay A-3 and caused overloading issue as its quantity increased. The cumulative methane plot from assay C-II, 1 did not show the bi-phasic trend. As digestion of any pure substrate the methane production rate increases at first then

decreases as substrate is consumed until it completely ceases when all substrate is used up.

Fraction 2 in assays A-1 and A-3 had very similar methane production profiles to assay, C-II, 1. Therefore, it was hypothesized that fraction 2 of GBP is glycerol, and fraction 1 is methanol. Table 2-2 indicated 1 ml (1.2 g) pure glycerol capable of producing 394 ml CH4; while glycerol fraction of 1 ml (1.16g) GBP capable of producing 320 ml and 280 ml CH4 from assay A-1 and A-3, respectively. This suggested that glycerol content in GBP averaged 77.1%, based on weight. With the fact that methanol and glycerol fraction made 32% and 68% of total methane production, respectively, methanol content was calculated to be 28.1% based on weight. The two calculated values generally agreed the assumption made about GBP composition.

The methane yield of assay A-2 (containing 2 ml GBP) over 100 days of incubation was considerably less than that of assay A-1. The reason for the low yield was doubted to toxicity of GBP. Since volume of GBP doubled, the amount of toxic compound in assay, if any, could have increased and inhibited methane production. However, methane yield of assay C-II, 2 (containing 2 ml pure glycerol) was also much less than the yield of assay C-II, 1 (containing 1 ml pure glycerol). Therefore, it appeared that the low methanogenic activity in assay A-2 was due to organic overloading instead of the presence of inhibitory compounds. Low methanogenisis caused by organic overload in anaerobic digestion had been reported in some studies (Amon, Amon et al. 2006; Holm-Nielsen, Lomborg et al. 2008; Ruiz, Blazquez et al. 2009). Notably, in Holm-Nielsen et al. (Holm-Nielsen, Lomborg et al. 2008), organic overloading was reported to have occurred in anaerobic digesters when glycerol concentration exceed 5 g L^{-1} – 7 g L^{-1}.

Assay A-2 and C-II, 2 contained 2 ml GBP and 2 ml glycerol respectively dissolved in 150 ml solution. The corresponding concentration was 12 g L^{-1} GBP for assay A-2 and 16.8 g L^{-1} glycerol for assay C-II, 2. These significantly exceed the suggested loading amount resulting in organic overloading.

Assay A-3 (containing 2 ml GBP and co-substrate) exhibited higher methane production and the methane yield over 200 days incubation was 415 ml CH4 at STP (ml sample)$^{-1}$. The methane yield obtained from assay A-1 and A-3 was consistent and verified the methane potential of GBP being around 400 ml CH4 at STP (ml GBP)$^{-1}$. However, assay A-3 took twice as long to reach the yield as assay A-1. This suggested assay A-3 experienced organic overloading as well which possibly caused the lag phase. Assay C-III indicated the methane yield of tailing extract was very small (data now shown). This excluded the possibility that the extra methane production of assay A-3 compared to assay A-2 was from the tailing extract. If longer period of incubation was allowed, assay A-2 and C-II, 2 might have overcome the lag phase and have produced comparable methane yields to those of assay A-1 and A-3. This was not shown in the study presented here, but improved methane production had been shown in assay A-3 with carbohydrate containing co-substrate. Similar results had been reported in anaerobic digestion with co-substrate of oleate in inhibited digesters (Kuang, Lepesteur et al. 2002; Kuang, Pullammanappallil et al. 2006). The anaerobic co-digestion of different organic compounds is a common practice to improve the performance of anaerobic digesters (Bolzonella, Pavan et al. 2006; Siles, Martin et al. 2010). For this reason, GBP was co-digested with tailings extract. The improved methane production from assay A-3 may be that the adding tailings water extract to the sample balanced

nutrients ratio (e.g., carbon to nitrogen ratio) and provided readily degradable organic matters to compensate for low degradability and stimulate activity of microorganisms and degradation of GBP so that the organic overloading problem could be overcome faster (Kuang, Lepesteur et al. 2002; Kim, Han et al. 2003; Krupp, Schubert et al. 2005; Li, Chen et al. 2009).

Soluble Chemical Oxygen Demand Profiles

sCOD was measured periodically in the assays. The sCOD profiles in assay A-1 is shown in Figure 2-1A. Along with measure sCOD values, the expected theoretical COD values is also plotted. The expected values were calculated using stoichiometry that 1 g L^{-1} of COD being capable of producing 0.35 L CH4 at STP. Using the conversion, it is possible to predict the remaining sCOD concentration in the assays by knowing how much methane has been produced. Through comparing the expected sCOD and measured sCOD, consistency of the results can be verified. The initial concentration of sCOD for calculation of expected sCOD was assumed same as the measured COD concentration. As seen in Figure 2-2, the initial sCOD in A-1 was 8,600 mg L^{-1}. This value dropped to 3900 mg L^{-1} on day 45. The expected values showed a faster initial drop compared to the measured and thereafter followed the measured trend closely until day 45. Initially in addition to sCOD, some insoluble matters may have also solubilized and converted to methane, yielding more methane than predicted by dropping measured sCOD. The same method was used to derive the expected sCOD profile for assay A-2. Both expected and measured sCOD profiles showed that sCOD concentration in assay A-2 maintained at the range from 12,000 mg L^{-1} to 14,000 mg L^{-1} for the duration of day 0 to day 100 (Figure 2-2C). Non-degrading sCOD explained the low methane production in assay A-2. Measured sCOD data after day 100 was not

available for assay A-2. Expected and measured sCOD values were compared for assay C-II,1 and C-II,2 as well as shown in Figure 2-2B and 2-2D. sCOD concentration in assay C-II, 1 degraded as methane produced but barely changed in assay C-II, 2 where methane production was low.

Volatile Organic Acid Profiles

Concentration of VOAs, i.e., acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid was measured in assays A-1 and A-2. Propionic acid was found to be the most important contributor to total concentration of VOAs. Acetic acid was the second most important and its concentration was about half of that of propionic acid. Concentration of all other VOAs was negligible. Profiles of propionic acid concentration in assay A-1 and A-2 are shown in Figure 2-3.

Propionic acid concentration in assay A-2 reached 1200 mg L^{-1} and was not degraded up to day 80 whereas its concentration in assay A-1 started to decrease after peaking at 500 mg L^{-1}. Accumulation of higher VOAs concentration in assay A-2 could be responsible for the low methane production. Amon et al. (Amon, Amon et al. 2006) reported that when VOAs exceeded 5000 mg L^{-1}, the anaerobic digestion process was no longer stable and overloading was likely. Propionic acid concentration in assays A-2 was only 1200 mg L^{-1} and less than the reported possible inhibitory threshold. This probably indicated that a small system as used in this study had low tolerance on VOA concentration.

Methane Production from Semi-continuous Digestion of GBP

Semi-continuous digestion was conducted by manually feeding GBP per day to a mesophilic, one-stage lab scale digester. The digestion was started with 100 grams of sugar beet tailings and GBP feeding began at 0.67 ml $(day)^{-1}(L)^{-1}$ from day 3. Due to

that the digester was initially provided with highly degradable substrates, daily methane production increased substantially and started decreasing after 5 days as the substrates were quickly consumed. The daily methane production stabilized at 450 ml CH4 at STP (ml substrate)$^{-1}$ after 11 days. This yield was very similar to the GBP methane yield obtained in BMP assay A-1. GBP loading rate was further increased to 1 0.67 ml (day)$^{-1}$(L) $^{-1}$ from day 18. The daily methane production dropped immediately and stabilized at 0.25 ml CH4 at STP (ml substrate)$^{-1}$ after 7 days. Profiles of daily methane production are shown in Figure 2-4.

It appeared that the digester performance was inhibited when GBP loading rate increased from 0. 67 to 1 ml (day)$^{-1}$(L) $^{-1}$. A study (Chen, Romano et al. 2008) reported a similar result. In their study, a mixture (84% manure and 16% glycerol) was fed to a continuous digester of 18 L. The digestion inhibition was observed when the substrate loading rate increased from 42 g day^{-1} to 82 g day^{-1}. The corresponding glycerol loading rates were calculated to be 0. 29 and 0. 57 ml (day)$^{-1}$(L)$^{-1}$, respectively.

Sugar beet tailings were considered as one of the nitrogen sources because they contained 7.5% crude protein on a Volatile Solid basis (Liu, Pullammanappallil et al. 2008). Nitrogen may also come from digester inoculums that were reported to contain 145 mg L^{-1} total NH4 as N (Koppar and Pullammanappallil 2008). Using the assumptions that GBP contains 70.6% glycerol and 21.6% methanol on a weight basis , the average C/N ratio of semi-continuous GBP digestion over 40 days was calculated to be 58. Since N sources were not provided continuously, real-time C/N ratio was expected to start with a much lower value and slowly increase as N depleted and more C was fed.

Discussion

The theoretical methane yield of glycerol at 99.5% grade is 534 ml CH4 at STP (ml glycerol)$^{-1}$. The practical methane yield of the glycerol is 372 ml CH4 at STP (ml glycerol)$^{-1}$ as seen in Figure 2-1 for assay C-II, 1. Low COD and VOA concentration found in assay C-II, 1 at the end of incubation indicated most of the organic contents had been degraded and the difference between the theoretical and practical yield was not due to incomplete substrate degradation. The practical yield of 372 ml recovers 69.6% of the theoretical yield, probably suggesting the confidence of those BMP assays is around 70%.

Thompson and He (Thompson and He 2006) reported that the heat of combustion of the crude glycerol ranged from 18600 kJ /kg to 25200 kJ /kg. It had shown 1 ml GBP was able to yield 450 ml CH4 at STP .Using the heat of combustion of methane (37 kJ L^{-1}), the energy that can be gained from biogasifying GBP to methane was calculated to be 18300 kJ /kg. There was no big difference in directly combusting and anaerobically digesting GBP to obtain energy. However, glycerol combustion will produce highly toxic acrylonitirle if operated inappropriately, and cause server health concern. The advantage of anaerobic digestion is obvious for it is environmental-friendly and conserve most of the energy.

An emergy analysis was conducted for biodiesel production process at small scale (please refer to Appendix A for detailed information). It revealed that 104 KJ electricity and 495 KJ fuel energy (natural gas) is consumed to produce 1 kg biodiesel (Sheehan, Camobreco et al. 1998). Electricity is mainly used in equipment operations and fuel energy is used for heating purpose. Typically 0.1 kg GBP is generated per 1 kg biodiesel produced. According to the BMP assay results, 0.1 kg GBP is able to produce

38.8 L CH4 at STP. Given CH_4 to electricity efficiency of 25%, it would generate 378 kJ electricity (kg biodiesel)$^{-1}$. Electricity consumes 28% of the produced biogas energy, leaving in excess of electricity energy that can be sold to make profits. If biogas were applied in boilers to produce steam, it would produce an energy equivalent of 1058 KJ assuming a combustion efficiency of 70%. This is sufficient to cover fuel energy consumptions as well, suggesting anaerobic digestion has the potential to improve the energy efficiency of biodiesel production. In full scale biodiesel plants, energy requirement is more intensive because byproducts (GBP) purification is usually carried out. In a biodiesel plant with a capacity of 100,000 ton per year, 3064 KJ fuel energy is consumed to produce 1 kg biodiesel (Emiliani and Pistocchi 2006). The produced methane from GBP is able to provide approximately 30% energy consumed. If GBP were digested, the purification would not be needed and the energy consumption would be lower.

Chen et al. (Chen, Romano et al. 2008) reported higher methane production rate of glycerol digestion. Their study obtained methane yield of 390 ml CH4 at STP (ml glycerol)$^{-1}$ using a similar BMP method in 15 days, whereas it took 60 and 90 days for GBP and pure glycerol, respectively, to achieve a comparable methane yield in the experiment conducted in this study. This could be explained as smaller loading quantity leading to faster substrate breakdown rate. In Chen's study, the batch reactors contained 2.8 ml (L) $^{-1}$ glycerol, less than half of the substrate concentration of 6.7 ml GBP (L)$^{-1}$ and 6.7 ml glycerol (L)$^{-1}$in assay A-1 and C-II respectively. Figure 2-1 and 2-2 showed 32% and 40% of the ultimate methane yield were produced in the first 20 days for assay A-1 and C-II, 1. This indicated that assay A-1 and C-II, 1 degraded 2.1 ml (L)$^{-}$

^{1} and 2.68 ml $(L)^{-1}$ glycerol in 20 days. It appeared GBP or glycerol at 2 to 3 ml $(L)^{-1}$ could be degraded as fast as in 15 to 20 days as Chen et al. concluded, but digestion of GBP or glycerol with higher concentration required much longer time. Among those assays, glycerol used in assay A-3 (99.5% grade) was believed to have characteristics closest to the refined glycerol used in Chen's study. Thus, 2.68 ml $(L)^{-1}$ glycerol degraded by assay A-3 within 20 days was closest to glycerol concentration applied in Chen's study. Chen's study also carried out continuous digestion of glycerol along with dairy manures. In trial 1, mixture 1 (containing 16% (%wt) glycerol and 84% manure) was fed and microbial inhabitation was observed when glycerol feeding rate increased from 0.29 ml glycerol $(L)^{-1}$ $(day)^{-1}$ to 0.57 ml glycerol $(L)^{-1}$ $(day)^{-1}$. Further digestion was then carried out using mixture 2 containing less glycerol (9% glycerol and 91% manure) and the methane yield was good for feeding glycerol at 0.43 ml glycerol $(L)^{-1}$ $(day)^{-1}$ and incrementally increasing to 2.57 ml glycerol $(L)^{-1}$ $(day)^{-1}$. The methane yield from digesting mixture 2 was much higher than the combined methane yield of both substrates digested separately in Chen's batch reactors. In order to investigate feasibility of the methane yield, theoretical methane yield of mixture 2 was calculated by proportional combining theoretical methane yield of glycerol and methane yield of manure derived in using the batch reactors. Surprisingly, the practical yield of mixture 2 was so high that it exceeded the calculated theoretical yield by 43% to 72%, depending on mixture 2 loading rate. This may imply that the glycerol used in Chen's study contained other compositions that have higher methane potential than glycerol itself. However, the practical of mixture 1 only accounted for 86% to 105% (depending on mixture 1 loading rate) of the corresponding theoretical methane yield. The low methane

yield from digesting mixture 1 was attributed to high C/N ratio of 20 by the authors. Average C/N ratio was found as high as 58 in the semi-continuous digestion of GBP conducted in this study. Actual C/N ratio at late stage of the digestion (3 ml GBP day^{-1} L^{-1} feeding) was expected even higher, where hampered methane yield was observed. It was hypothesized that low methane yield of semi-continuous digestion of GBP could be due to 1) organic loading problems as found in BMP assays and continuous digestion of mixture 1 conducted in Chen's study; and/or 2) high C/N ratio resulting from N depletion.

Closing Remarks

The BMP assays performed at mesophilic condition showed the methane potential of GBP was 450 ml CH4 at STP $(ml\ GBP)^{-1}$, if organic overloading was avoided. GBP digestion exhibited a bi-phase trend, where digestion of methanol completed fast in phase 1 followed by slow digestion of glycerol in phase 2. Organic overloading problem was seen in assay A-2, A-3 and C-II, 2 as their substrate concentration (GBP or glycerol) considerably exceed the suggested concentration (5 g L^{-1} – 7 g L^{-1}) to use glycerol to produce biogas (Holm-Nielsen, Lomborg et al. 2008). Due to co-digesting with tailing extracts, assay A-3 overcame the lag phase and made a decent amount of methane, whereas assay A-2 and C-II, 2 failed to produce adequate methane at the end of the study. The correlation between the remaining COD concentration and methane production was proved strong as the predicted COD profile was consistent with the measured COD profile. VOA analysis showed degradation of VOAs in the assay A-1 and C-II, 1 and accumulation of VOAs in the assay A-2 and C-II, 2 where organic overloading occurred. Propionic acid had the highest concentration among VOAs monitored, implying the fermentation pathway of GBP could be through

propionic acid, but further studies are needed before drawing any conclusion. Semi-continuous digestion of GBP determined daily methane yield stabilized at 450 ml CH4 at STP (ml substrate)$^{-1}$ at loading rate of 0.67 ml GBP (day)$^{-1}$(L) $^{-1}$. Digestion performance was inhibited as GBP loading rate increased to 1 ml (day)$^{-1}$(L) $^{-1}$.

Table 2-1. Contents and constituents of the assays

Assays	Number of Replicates	GBP	Inoculum	Nutrient Solution	Tailings water extract	DI water
		ml	ml	ml	ml	ml
A-1	2	1	99	4.28	0	50
A-2	2	2	98	4.28	0	50
A-3	2	0	99	4.28	0	51
Control assays	Number of Replicates	Glycerol	Inoculum	Nutrient Solution	Tailings water extract	DI water
		ml	ml	ml	ml	ml
C-I	2	0	99	4.28	0	51
C-II,1	2	1	99	4.28	0	50
C-II,2	2	2	98	4.28	0	50
C-III	2	0	100	4.28	50	0

Table 2-2. Parameters of Gompertz Equation

		P	R_m	λ	Overall methane yield
		ml CH4@STP	ml CH4@STP(days)$^{-1}$	days	ml CH4@STP (ml)$^{-1}$
Assay C-II,1		394.15	6.44	0.52	394.15
Assay A-1	Fraction 1	146.58	17.56	0.00	466.39
	Fraction 2	319.81	8.87	0.64	
Assay A-3	Fraction 1	268.95	30.69	1.61	828.25
	Fraction 2	559.30	10.37	2.14	

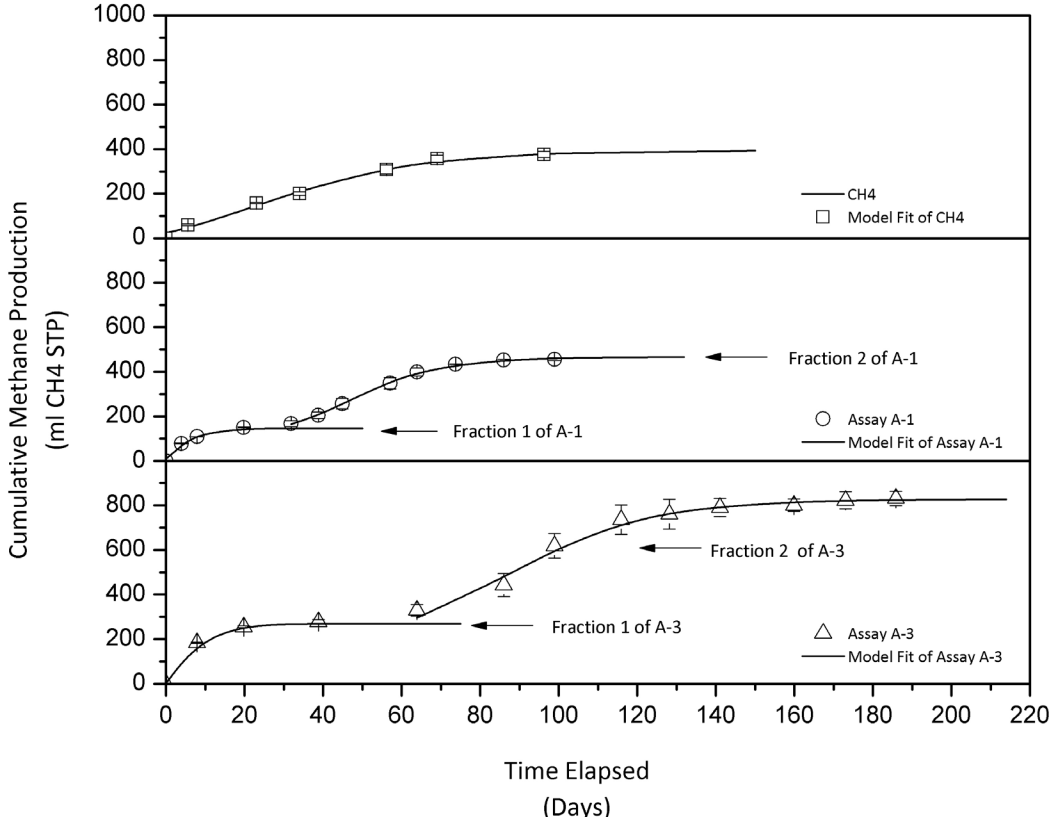


Figure 2-1. Cumulative methane yield from assay A-1, C-II, 1 and A-3

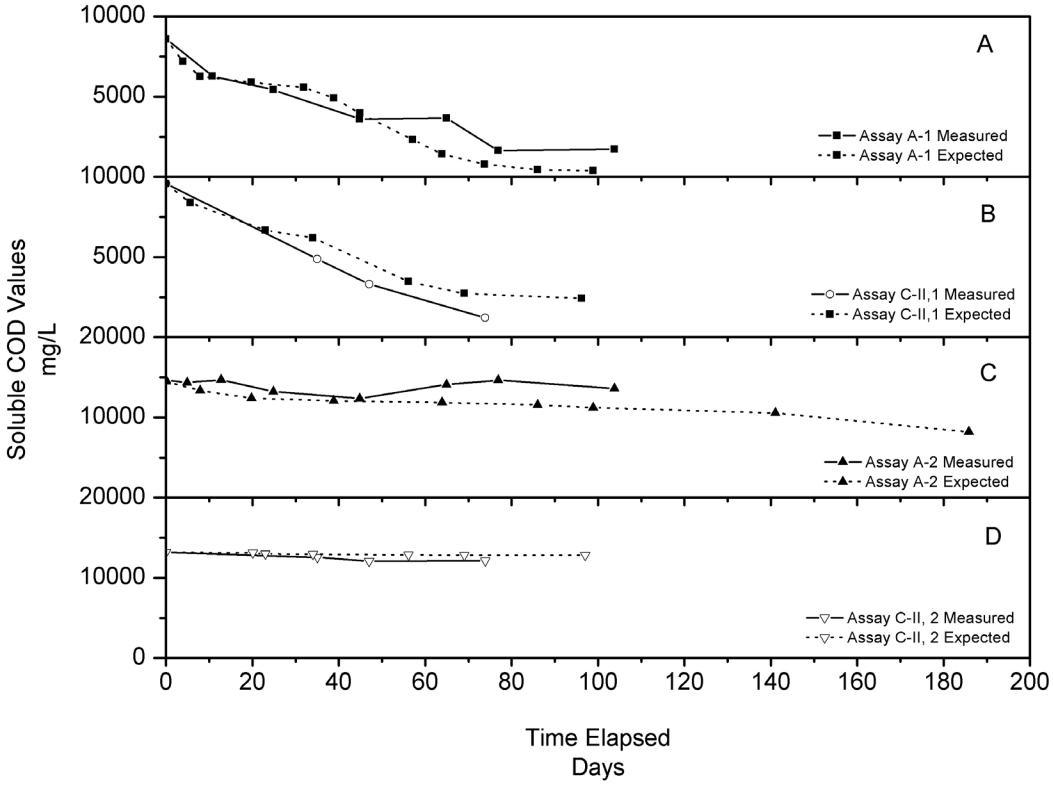


Figure 2-2. sCOD profile in assays A-1, A-2, C-II, 1 and C-II, 2 along with expected sCOD concentrations

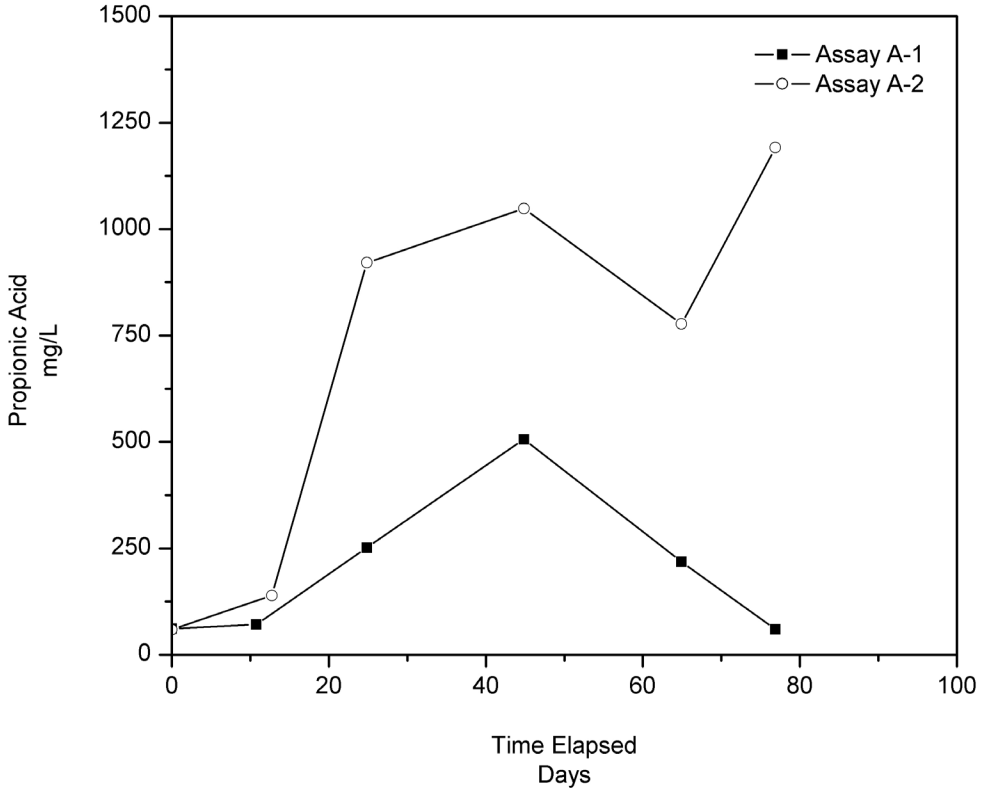


Figure 2-3. Propionic acid profiles from assay A-1 and A-2

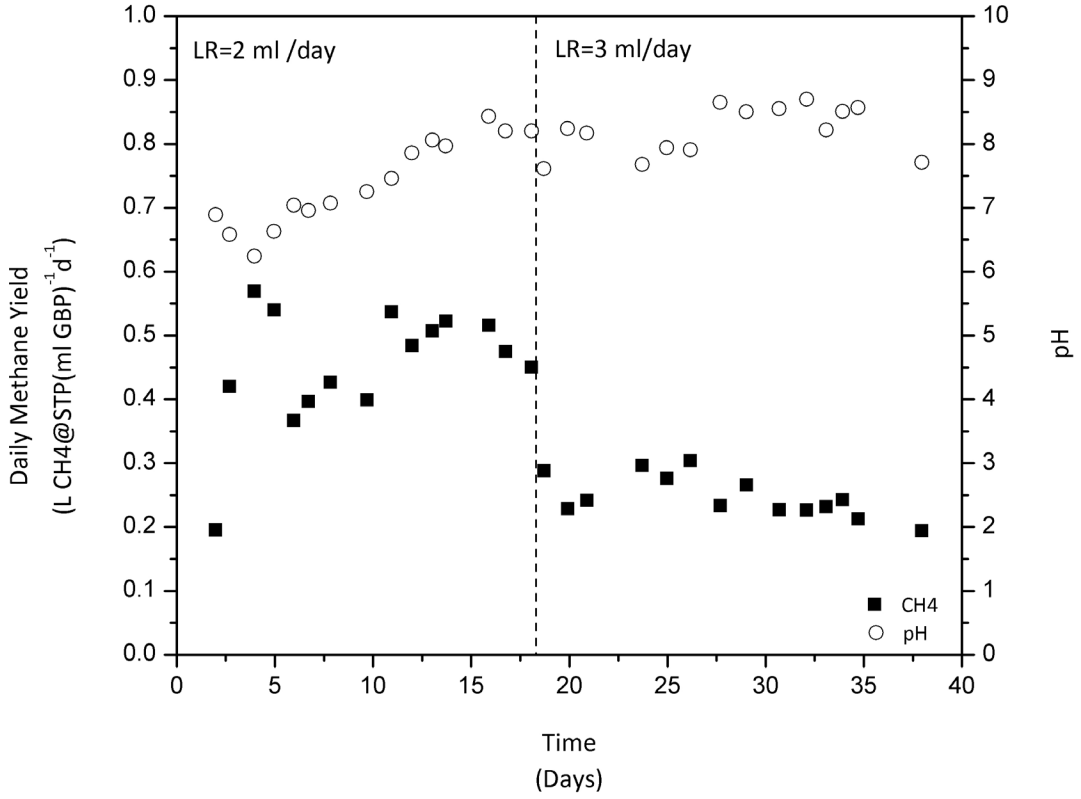


Figure 2-4. Daily methane yields from semi-continuous digestion of GBP

CHAPTER 3
ANAEROBIC DIGESTION FOR TREATMENT OF STILLAGE FROM CELLULOSIC BIOETHANOL PRODUCTION

Summary

Thermophilic anaerobic digestion of stillage from cellulosic ethanol production process was investigated. Methane potential of bagasse and hardwood stillage was determined by conducting Biochemical Methane Potential (BMP) assays. A methane potential of about10 ml CH_4 at STP (ml stillage)$^{-1}$ or 200 ml CH_4 at STP (g VS)$^{-1}$ was obtained for bagasse stillage at concentration of 14.2 and 17.8 g VS (L)$^{-1}$; while 6.5 ml CH_4 at (ml stillage)$^{-1}$ or 100 ml CH_4 (g VS)$^{-1}$ was obtained for hardwood stillage at concentration of 17.2 and 21.5 g VS (L)$^{-1}$. Coarsely separated bagasse stillage (fraction < 0.5 mm size) was successfully anaerobically digested in a laboratory scale (15 L) continuously fed digester which was operated for three months. The digester was operated at HRTs of 21 and 14 days and organic loading rate (OLR) of 1.85 and 2.39 g COD $L^{-1}d^{-1}$. The methane yield from the stillage from the digester was about 90% of the yieldfrom the BMP assays. The influent soluble COD (sCOD) was reduced from between 35.4 – 38.8 gCOD (L^{-1}) to between 7.5 – 8 gCOD (L^{-1}). The soluble chemical oxygen demand (sCOD) removal efficiency was 75- 80% with the sCOD of effluent. The success in anaerobic digestion of bagasse stillage without any dilution was attributed application of long HRTs and low OLRs. A mass and energy balance was developed for an integrated process of cellulosic ethanol production and stilling treatment using anaerobic digestion. It showed the anaerobic digestion being able to produce 70% of the energy required by the ethanol production process.

Background

Currently, commercial bioethanol production processes focus on utilizing classic crops as feedstock like sugar cane and wheat that require high quality agricultural land for growth (Balat 2011). Given that serious problems face the world food supply today, diverting farmland or crops for fuel production is detrimental to the food supply on a global scale (Demirbas 2011). The issue is more pressing in developing countries where food scarcity may happen. An alternate option is to produce ethanol from nonfood biomass resources like forestry and agricultural residues and urban wastes. Ethanol produced from these feedstocks is usually referred as cellulosic ethanol, because in these feedstocks the predominant carbonhydrates converted to ethanol are the polymers cellulose and hemicellulose. Cellulosic materials are first subjected to biological and/or thermochemical pretreatment. Enzymes and/or high temperature along with an acid/base is introduced to hydrolyze cellulose and hemicellulose to sugars. Pretreatment is followed by fermentation of the sugars. For example, at the Biofuels Pilot Plant in University of Florida, a recombinant E. Coli KO11 strain developed in the Microbiology and Cell Science Department is employed to ferment both the hexose and pentose sugars to ethanol (Yomano, York et al. 1998; Yomano, York et al. 2008; Yomano, York et al. 2009).The fermentation is preceded by a dilute acid hydrolysis using phosphoric acid as catalyst and an enzymatic hydrolysis using commercial cellulase enzymes. After fermentation, the broth is distilled to produce ethanol. Distillation generates a by product called stillage, which is generally a high volume, high strength acidic waste that presents significant disposal or treatment problems. Stillage from corn ethanol production is used as animal feed as either distiller's dried grains (DDG) or distiller's dried grains with solubles (DDGS). However, DDGS production

consumes large amount of energy due to the evaporation and drying process (Kaparaju et al., 2010).Stillage produced from nonfood sources as sugarcane bagases usually has poor nutritive quality and is not suitable to use as animal feed.

Utilization of stillage as a fuel or source of biofuel can improve energy efficiency and reduce carbon footprint of ethanol production process. A possible option to add value to the stillage would be to use it as a fuel in bioethanol production process. However, due to its high moisture content, direct combustion would not provide net energy. Anaerobic digestion was found to be a viable and sustainable scheme for simultaneous waste treatment and energy production from stillage (Wilkie, Riedesel et al. 2000). The biogas produced from anaerobic digestion can be used to supplement the energy consumed in the pretreatment and distillation operations of ethanol production process.

Several studies have been carried out on anaerobic digestion of ethanol stillage from conventional sugar or starch based feedstocks such as grain and sugar cane molasses (Sheehan and Greenfield 1980; Shin, Bae et al. 1992; Wilkie, Riedesel et al. 2000; Tang, Fujimura et al. 2007). However, few studies were conducted on digesting ethanol stillage produced from cellulosic materials. Unlike processing of sugar or corn for ethanol, cellulosic materials have to be subjected to harsher processing steps to depolymerize the structural polysaccharides. These processes result in side reaction products that could be potentially inhibitory to microbial growth. So anaerobic digestion of cellulosic ethanol stillage may be fraught with problems. In this study, stillage obtained from the Biofuel Pilot Plant at the University of Florida was used as a sole substrate in biochemical methane potential assays. In addition to the methane potential

of the substrate, the assay also provided information on the rate of degradation, extent of degradation and the presence of any inhibitory compounds or toxins in the substrate. These batch assays were performed at a thermophilic (55 °C) temperature. The feasibility of using a one-stage, continuous digester for anaerobic digestion of stillage was then investigated.

Materials and Methods

Feedstock

Stillage collected from the Biofuel Pilot Plant at University of Florida was used as feedstock for anaerobic digestion. The pilot plant produces ethanol from lignocellulosic biomass, mainly sugarcane bagasse and hardwood chips. The process for ethanol production at the Biofuel Pilot Plant was as follow: The raw material was soaked in 1% phosphoric acid for 4 hours. The excess acid was removed in a screw press. The acid soaked fibers were loaded into a hydrolyser. Dilute-acid steam pretreatment was carried out at 180°C for 10 minutes to solubilize lignocellulosic biomass from its native form, in which it is recalcitrant to cellulase enzyme converting systems, into a form for which enzymatic hydrolysis is effective (Lynd, Weimer et al. 2002; Kumar, Chandra et al. 2010). The pretreated feedstock was hydrolyzed with cellulase. Fermentation of the enzyme-treated slurry was then carried out using a recombinant E.coli strain. The stillage was collected after the distillation process in which ethanol was separated from fermentation broth at 60 °C.

For the experiments reported in this chapter, some of the stillage was also filtered through a sieve with pore size of 0.5 mm for coarse separation of solid and liquid. The unfiltered stillage, filtered stillage, and the fraction retained by the sieve are hereby referred as whole stillage, stillage filtrate and stillage residue, respectively. Two

types of stillages were used in the biochemical methane potential assays – namely stillage produced from sugarcane bagasse as feedstock and stillage produced from hardwood as feedstock. In cellulosic ethanol fermentation process, the lignin in the feedstock passes unconverted through the process and remains in the stillage (Ojeda, Ávila et al. 2011). For value addition to stillage and thereby to improve the economics of the process it has been suggested that the lignin fraction be used as a fuel (Larsen, Petersen et al. 2008) or as a raw material for biocomposites (González, Santos et al. 2011). Lignin is not degraded in an anaerobic digester and moreover microbial hydrolysis of fiber is a slow step. Therefore, by separating the fibers only the quickly degradable portion of the stillage was fermented. If fibers are removed, it is also necessary to quantify the methane potential that would be lost with the fibers by not digesting it.

Biochemical Methane Potential Assay (BMP)

BMP assays measure the methane potential of feedstocks at optimal conditions for culturing anaerobic digestion microbial consortia (Owen, Stuckey et al. 1979; Shelton and Tiedje 1984; Owens and Chynoweth 1993). It was conducted by mixing the substrate (the material for which the methane potential is being determined) with inoculum and nutrients, and incubating the mixture in a sealed bottle at predetermined temperature for a period of time to allow complete degradation of the substrate. Assays contained substrate, inoculum and other supportive solutions (micronutrient, macronutrients, and pH buffer). The inoculum used was collected from a laboratory scale anaerobic digester that had been digesting various biomass feedstock and wastewater at therophilic temperature for over three years. Nutrient stock solution was prepared according to Owens et al. (Owens and Chynoweth 1993). Four types of

assays were carried out, each in duplicate, containing, hardwood whole stillage, bagasse whole stillage, bagasse stillage filtrate or bagasse stillage residue. A control assay containing deionized water was set up to provide data on methane production from residual substrates and endogenous metabolism in inoculum. The Biofuel Pilot Plant used hardwood chips once for a trial operation. As a result, hardwood stillage was available in small quantity that was only sufficient for the BMP assays but not for the lab scale anaerobic digestion, which was later carried out using only the bagasse stillage. Table 3-1 lists the assays along with its contents and quantities loaded. The methane yield was calculated by subtracting the methane produced in the control assays in order to take into account the methane contribution from the inoculum.

Anaerobic digestion process can be carried out at mesophilic (32-38 ^{o}C) or thermophilic (50 – 57 ^{o}C) temperatures. A thermophilic temperature of 55^{o}C was chosen here for the following reason. In industry, distillation is usually conducted at 60 -70 ^{o}C to separate ethanol from the fermentation mixture. Therefore, stillage would be produced at these temperatures. To take advantage of the hot stillage that is available, an onsite anaerobic digester can be operated at a thermophilic temperature. It has been shown that at thermophilic temperatures the rate of degradation is higher and consequently the throughput for a given digester volume can be increased or the volume of digester required for a specified throughput can be decreased (Wang, Ma et al. 2011).

Continuous Digestion of Bagasse Stillage Filtrate

The feasibility of anaerobic digestion of bagasse stillage filtrate was further evaluated in a laboratory scale 15 L semi-continuously fed anaerobic digester. The digester was constructed by modifying a Pyrex glass jar. The height and inner diameter of the digesters were 0.8 m and 0.3 m, respectively. The bottom of the glass bottle was

cut and replaced by a stainless plate with ports. This served as the digester lid and was attached to the bottle with screws and a rubber gasket for air-tight seal. A glass flange fitted with a rubber O-ring was used to seal the top of the bottle. The flange was clamped using stainless steel clamps for gas and liquid tightness. The digester was provided with several ports for gas outlet, sample withdrawal and digester liquor circulation. The bottle was placed upside down on a tripod stand with influent entering through the bottom. Since the digester content was not mechanically mixed, solids settled down and accumulated at the bottom, allowing significant separation of SRT and HRT. This makes the digester resemble a SOLCON reactor to some extent (Srivastava, Biljetina et al. 1989). Figure 3-1 and 3-2 provides schematics of the tripod stand and the continuous digester, respectively. Gas production from the digesters was measured by a positive displacement gas meter which consisted of a clear PVC U- tube filled with anti-freeze solution, a solid state time delay relay (Dayton Off Delay Model 6X153E), a Grainger float switch, a Redington counter and Fabco Air solenoid valve. The gas meter was calibrated in line to determine the volume of biogas per count. The biogas was vented to the building exhaust piping from the exit of the volumetric gas meter.

The digester was operated in a semi-continuous mode with feeding occurring 20 times per day. Feeding was controlled by a two headed MasterFlex pump that was operated by a timer. The timer cycle operated such that each pumping cycle lasted from 5 minutes to 10 minutes to give HTRs of 21 and 14 days. A second timer was used to control the influent substrate mixer so that it would turn on for 10 minutes before influent pumping began and did not turn off until after the pumping cycle was finished. The pump was calibrated to deliver 28 mL stillage per 5 minutes. A second MasterFlex

pump was provided for digester liquor circulation to provide mixing and improve temperature homogeneity and contact of subsrate to microbes. The circulation rate was set at 50 ml per minute. The digester was started up by inoculating with 12 L inoculum and allowed to stabilize for 1 day before the feeding was started. Stillage was filtered through a sieve with 0. 5 mm mesh and the filtrate was fed as the feed. Like the BMP assays, the lab scale semi-continuous digester was maintained at 55 ±2 ^{o}C and the stable temperature was achieved by use of a heating tape controlled by a Campell Scientific datalogger (Model CR10). The temperature was controlled by on-off protocol, i.e., heating tape was turned off when temperature reached 57 ^{o}C and turned on when temperature dropped below 53 ^{o}C. Temperature was monitored using a T-type thermocouple located halfway between center and wall of the digester. The digester was also covered by a layer of insulation material to reduce heat loss.

Monitoring and Analysis

Unfiltered stillage, filtrate and residue samples were analyzed for total solids (TS), volatile solids (VS), soluble COD (sCOD), ammonia-N, phosphate-P, ethanol and forage nutritional compositions.

The BMP assays were monitored daily for biogas production and methane composition of biogas, and periodically for soluble COD and volatile organic acid concentration. The pH of the mixed liquor at the start and end of the assay was also measured. Biogas that accumulated in the serum vials was withdrawn using a syringe. Biogas was allowed to fill the syringe until the pressure in the vial head space equilibrated with that in the syringe. Volume of biogas in the syringe was noted and then used as a sample for compositional analysis.

Biogas production from the continuously fed anaerobic digester was monitored in line using a positive displacement gas meter. The biogas was analyzed for methane content daily. The soluble COD, volatile organic acid, ammonia-N and phosphate-P concentrations of the effluent was analyzed once weekly. A forage nutritional analysis of a composite effluent sample was also carried out.

TS and VS contents were measured using an oven (Fisher Scientific Isotemp model 350G) and a muffle furnace (Fisher Scientific Isotemp), respectively. Samples were centrifuged at 8000 RPM for 10 minutes (Fisher Marathon micro H centrifuge) and filtered using Millipore filter paper (pore size 1.2 μm) for sCOD, ammonia and phosphorus analysis. For sCOD measurement, samples were added in Hach COD vials after appropriate dilution and baked in Hach COD reactor for 2 hours. SCOD was measured using Hach DR/890 Colorimeter. Ammonia and phosphorus concentration was measured using ammonia-selective electrode and ascorbic acid method, respectively (American Public Health, American Water Works et al. 1999) . Ethanol content was measured using an Agilent gas chromatograph (Agilent Technologies, 6890N). Methane and carbon dioxide composition was measured using a Fisher Gas Partitioner (model 1200). The gas chromatograph was calibrated with an external standard containing N_2, CH_4, and CO_2 in 25:45:30 volume ratio. Volatile organic acids were measured using Shimadzu gas chromatograph (GC-9AM equipped with a flame ionization detector). The volatile organic acids detected were acetic, propionic, isobutyric, butyric, isovaleric and valeric acid.

Stillage samples and effluent from digester were also analyzed for forage nutritional compositions, including crude protein, soluble protein, Acid Detergent Fibers

(ADF), Neutral Detergent Fibers (NDF),lignin, Ethanol Soluble Carbohydrates (ESC, including glucose, fructose, sucrose, maltose, and short fructose chains), Digestible Energy (DE), Metabolizable Energy (ME) and Total Digestible Nutrients (TDN). ADF isolates cellulose and lignin and NDF isolates cellulose, hemicellulose and lignin. Knowing the lignin content and by difference, cellulose and hemicellulose contents was determined. These analyses were conducted by a commercial forage testing laboratory (Dairy One, Inc, Ithaca, New York).

Results

Characterization of the Stillage

Selected characteristics of stillage and digested stillage were determined and listed in Table 3-2. This includes TS and VS content, sCOD concentration, cellulose, hemicellulose, lignin, ESC and TDN contents for bagasse whole stillage, bagasse stillage filtrate, bagasse stillage residue and the effluent from the continuous digester. TS and VS content, and sCOD concentration for hardwood whole stillage is also listed. The bagasse and the hardwood stillages had a dry matter content of 6.89 and 8.86 % respectively. The lower solid content is as a result of operating the ethanol fermentor at less than 10% solids content to maintain good mixing. When 100 ml of whole bagasse stillage was filtered in a 0.5 mm sieve, it yielded 19 g residue (retained on the sieve) and 69 ml of filtrate. The soluble COD of bagasse stillage was more than that in the hardwood stillage. The bagasse stillage filtrate had an ethanol content 0f 0.75 g $(L)^{-1}$.DE, ME and TDN values (dry matter basis) of bagasse stillage (bagasse whole stillage, bagasse stillage filtrate and bagasse stillage residue) are in the range of 2500 kcal $(kg)^{-1}$, 2000 kcal $(kg)^{-1}$ and 55%, respectively.

Methane Potential

Profiles of the average methane yield from assay BW-1 (containing40 ml bagasse whole stillage), BW-2 (containing 50 ml bagasse whole stillage), BR (containingbagasse stillage residue), BF (containing stillage filtrate), HW-1 (containing 40 ml hardwood whole stllage) and HW-2 (containing 50 ml hardwood whole stillage) are shown in Figure 3-3. Sample standard deviations are shown as error bars.The methane potential of bagasse whole stillage over 80 days of incubation was 10.25 ml CH_4 at STP (ml stillage)$^{-1}$ and 10.95 ml CH_4 at STP (ml stillage)$^{-1}$ from assays BW-1 and BW-2, respectively. The degradation rate of stillage as indicated by the slope of the curves, initially increased, reached a maximum and then decreased to a minimum as substrate was utilized. The rates of evolution of methane were very similar for the first 20 days. A slightly higher methane production rate was observed after that for assay BW-2, leading to a higher methane yield in the end. In general, the methane yields and evolution rates of assay BW-1 and BW-2agreed with each other quite well. For both assays, 85% of ultimate methane yield was produced in 50 days. The methane yields from assay BW-1 and BW-2 can be also be expressed per gram VS in substrate and this corresponds to 191 ml CH_4 (g VS)$^{-1}$ and 204 ml CH_4 (g VS)$^{-1}$, respectively. The methane potential of hardwood whole stillage was determined to be 6.72 CH_4 at STP (ml stillage)$^{-1}$and 6.18 CH_4 at STP (ml stillage)$^{-1}$ for assay HW-1 and HW-2, respectively.

Assay BR and BF contained bagasse stillage residue and bagasse stillage filtrate, respectively. These assays contained residue and filtrate obtained by coarse filtering 100 ml of whole stillage, i.e., 19 g and 69 ml respectively. The methane yields for assay BR and BF normalized per ml of whole stillage, were calculated as

$$\frac{\text{Individual } CH_4 \text{ Production (residue or filtrate)}}{\text{Whole Stillage Volume (100 ml)}} \quad (3.1)$$

The methane yield from assay BR and BF were 3.05 ml CH4 at STP (ml stillage)$^{-1}$ and 6.91 ml CH4 at STP (ml stillage)$^{-1}$, respectively, after 80 days of incubation. Assay BF and BR had similar methane production profiles for the first 40 days. Methane production continued and increased in assay BF whereas it tailed off in BR.

Soluble COD and Volatile Organic Acids in BMP Assays

Soluble COD was measured periodically in the assays. The soluble COD profiles in assay BW-1, BW-2 and HW-1 and HW-2 are shown in Figure 3-4 and 3-5, respectively. The initial measured SCOD in BW-1 and BW-2 were 11 and 13 gL^{-1} in the assay.These values dropped to around 5 g L^{-1} after 25 days and slowly decreased to 3.6 g L^{-1} in BW-1 and 2 g L^{-1} in BW-2. The maximum SCOD drop rate was seen from day 13 to day 27.This was consistent with the methane yield profiles, which achieved highest methane evolution rate during a similar period (day 17 to day 35). Soluble COD in HW-1 assay dropped from 7 g L^{-1} to 2 g L^{-1} during the assay and in HW-2 it dropped from 10 g L^{-1} to 3.1 g L^{-1}.

Concentration of VOAs, i.e., acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid and valeric acid was also measured in assays BW-1, BW-2, HW-1 and HW-2 and the profiles of acetic acid, butyric acid and propionic acid concentration in assay BW-1, BW-2, HW-1 and HW-2 are shown in Figure 3-4 and 3-5. Acetic acid was found to be the most abundant contributor to total concentration of VOAs. Butyric acid was the second most abundant and its concentration was about half of that of

acetic acid. Concentration of all other VOAs was negligible. The VOA profiles for both assays were similar. For assay BW-1 and BW-2, concentration of acetic acid and butyric acid increased shortly after the assay was initiated. Acetic acid and butyric acid started to degrade after peaking at around 1.3 g/L and 0.7 g/L, respectively at day 10. Accumulation of propionic acid persisted for a longer time and its concentration did not decrease until day 35. The VOA profiles for assay HW-1 and HW-2 were similar, but butyric acid was in much less concentration than in assays BW-1 and BW-2. This could result from different compositions of bagasse and hardwood stillage. In general, degradation of COD and VOAs was evident in assays BW-1, BW-2, HW-1 and HW-2.

Continuous Digestion of Bagasse Stillage Filtrate (BF)

The bagasse stillage filtrate digestion was conducted in 2 stages and in each stage a different HRT was utilized to operate the digester. The digester was operated for 91 days. Table 3-3 summarizes the steady state results. Steady state occurred when the daily biogas production and biogas composition was stable unless the feed (flow rate or composition was changed).Figure 3-6 and 3-7 illustrates profiles of CH_4 production, pH, effluent sCOD, VOA, phosphorus and ammonia concentration with operation time for both stages. During stage 1 (day 1 to day 22), BF was fed at 571 ml/day (hydraulic retention time, HRT =21 days). Daily methane production increased and stabilized at 9.48 L CH_4 (L BF) $^{-1}$ after 12 days. This accounted 95% of CH_4 potential of stillage filtrate as determined by the BMP assay, suggesting conversion of stillage to CH_4 was nearly complete at HRT of 21 days HRT. The sCOD in the digested effluent was 7.8 g L^{-1}. The VOA analysis of digested effluent showed acetic acid and propionic acid were the dominant acids. A sharp increase was seen for acetic acid and propionic acid concentration from day 4 to day 10, and its concentration stabilized at

around 1 gL^{-1} and 0.8 g L^{-1} (acetic acid and propionic acid, receptively) thereafter when the steady state was reached. Phosphate-P concentration in the effluent steadily increased as the digester was operated and by day 21 it had reached 200 mg L^{-1}. Ammonia-N concentration in the effluent fluctuated and was dropping below 200 mg L^{-1} by day 21.

During stage 2 (day 23 to day 90), the feed rate was increased to 857 ml/day (14 days of HRT). The digester acclimated to the change quickly and the CH_4 production reached a new stable state after only one day (day 24). The average CH_4 yield was 10.50 L CH_4 (L stillage) $^{-1}$, slightly increased from the CH_4 yield at stage 1. This indicated that the digester stability was not affected by the increased loading rate. Effluent sCOD and VOA profiles during stage 2 kept low levels, once again indicating a stable digestion performance. Phosphate-P and NH_3–N concentration stabilized around 350 mg L^{-1} and 100 mg L^{-1}, respectively.

Discussion

A forage analysis on bagasse stillage was conducted to determine if it has value as animal feed. Previously, extensive compositional analysis was conducted on distillers dry grains with solubles (DDGS) produced in corn ethanol plants by several researchers. Sophiehs et al. (Spiehs, Whitney et al. 2002) collected 118 DDGS samples from 10 ethanol plants in the Minnesota-South Dakota-region and reported the average DE and ME values (dry matter basis) were 3,990 and 3,749 kcal/kg, respectively. A cellulosic biomass analysis and forage nutritional analysis for DDGS and wet distillers' grains (wet cake produced from centrifuging whole stillage) produced from Big River Resources, LLC, a dry-grind ethanol facility at West Burlington found that DDGS and wet distillers' grain have similar nutritional composition with high crude protein contents

of 30% and TDN of 90% on a dry matter basis (Kim, Mosier et al. 2008). Compared to these values the nutritional value of bagasse stillage was substantially lower. For example DE, ME and TDN were 2,470 kcal/kg, 2060 kcal/kg, and 53% respectively compared to values close to 4000 kcal/kg and 90%. Moreover, crude protein was only 9.9%. So bagasse stillage is nutritionally poor and not suitable for use as animal feed unless supplemented with other components.

When the whole stillage was filtered, some of the fibers also passed through the sieve resulting in 4.8% solids content in the filtrate. The total mass of dry matter retained on the sieve and that which passed through the sieve was 6.8 g ($19 \times 0.1858 + 69 \times 0.048$) in 100 ml which was in close agreement with the solids content of 6.89 % for bagasse whole stillage.

Since the methane yields for assay BR and BF were calculated per whole stillage volume, they were simply added to determine the whole stillage methane potential and then compared to that obtained in assay BW-1 and BW-2. The combined methane production profile of assay BR and BF was shown as dotted line in Figure 3-3. The combined yield was calculated to be 9.97 ml CH_4 at STP (ml stillage)$^{-1}$, which was consistent with the yield obtained from assay BW-1 and BW-2. This confirmed that the methane potential of the stillage was approximately 10 ml CH_4 at STP (ml stillage)$^{-1}$. The stillage filtrate generated approximately 70% CH_4 potential out of the whole stillage. On a dry matter basis the methane yield from BF was 212 ml methane (BF dry matter)$^{-1}$ was much greater than that from BR which was 85ml methane (BR dry matter)$^{-1}$. Since BR dry matter was mainly composed of suspended residual fibers, it can be concluded that very little of the residual fibers in the stillage were digested and that most of the

methane was produced from dissolved organic matter. As discussed later, this was further confirmed from soluble COD measurements in the assay.

It has been reported that the economics of the lignocellulosic ethanol process is highly dependent on the income of co-products (Sassner, Galbe et al. 2008). During the downstream process, stillage stream can be treated with anaerobic digestion and the produced biogas can be incinerated to provide heat and improve overall energy efficiency (Wilkie, Riedesel et al. 2000; Wingren, Galbe et al. 2008). A techno-economic model of anaerobic digestion of stillage for a spruce to ethanol process has been developed (Barta, Reczey et al. 2010) and it concluded that the difference in the production cost of ethanol between using whole stillage and the liquid fraction in anaerobic digestion for producing biogas was negligible (0.4-0.5 euro/liter).

The average methane yield of hardwood stillage was 6.5 ml (ml^{-1}) about 35% less than that of bagasse stillage. This could be attributed to the lower sCOD (measure of dissolved organic matter) of HW stillage. On a VS content basis, BW produced 195 ml CH_4 $(g\ VS)^{-1}$ whereas HW produced 98 ml CH_4 $(g\ VS)^{-1}$. Stillage methane yields from this study was lower than 324 ml $(g\ VS)^{-1}$ obtained from wheat straw stillage (Kaparaju, Serrano et al. 2010). The low CH_4 yield was probably due to that the wheat stillage contained much higher sCOD (61 g/L) than that of the stillage used in this study.

The bagasse stillage still contained about 0.075% ethanol which is less than reported 0.23% ethanol in wheat straw stillage (Kaparaju, Serrano et al. 2010). Ethanol in the stillage will also contribute to the methane potential of stillage. Theoretically, about 0.73 L methane at STP would be produced per g of ethanol, therefore, methane yield from stillage from the ethanol concentration measured above would be 550 ml CH_4

(L stillage)$^{-1}$ or 0.55 ml (ml^{-1}), which is about 5% of the methane yield obtained from bagasse stillage filtrate.

Along with measured sCOD values, the expected theoretical sCOD values were also plotted. The expected values were calculated using stoichiometry that 1 g L^{-1} of sCOD upon mineralization in an anaerobic digester produces 0.35 L CH_4 at STP. Using the conversion, it is possible to predict the remaining sCOD concentration in the assays by knowing how much methane has been produced. The initial values of expected sCOD were assumed the same as the initial measured sCOD in the assay. For all assays, the expected values followed the measured trend closely. Nevertheless, slightly lower values were seen at the end of assay for expected than measured SCOD. If only the soluble COD was mineralized, then the expected value should match the measured value. The lower expected value could mean that some fibers contained in the whole stillage could have been digested to methane as well. Based on the difference between expected and measured it was determined that 2.3 g COD (L^{-1}) in BW-1 and 3.1 g COD (L^{-1}) in BW-2 of fibers was mineralized. Assuming the fibers are cellulosic (therefore 1.0667 gCOD/g) this corresponds to 2.2 g (L^{-1}) and 2.9 g (L^{-1}). In other words, only an additional 3-4% of the dry matter would have been converted to methane. This justified the later use of only bagasse stillage filtrate for continuous digestion. For hardwood, the SCOD profiles (measured and expected) for assay HW-1 and HW-2 showed similar behaviors with sCOD decreasing steadily as methane was produced and difference between measured and expected at the end of the assay was less than that in the BW assays.

The bagasse filtrate stillage was successfully treated in a continuous single stage digester operated for about 70 days at an HRT of 14 days. The bagasse filtrate stillage was fed as received after coarse separation of fibers. The pH in the digester was maintained at around 7.5 without any pH control. The methane yield obtained from the digester was comparable to that obtained from the BMP assays. The phosphate concentration decreased from 530 mg L^{-1} to 350 mgL^{-1} in the effluent and the ammonia from 300 mgL^{-1} to 100 mg L^{-1}. CH_4 yield can be also estimated by knowing the amount of SCOD that has been degraded. At the state where CH_4 yield was constant, sCOD of the digester effluent and stillage filtrate was measured to be 8.0 g/L and 38.8 g/L, respectively. The expected CH_4 yield was 8.96 L CH_4 (L stillage) $^{-1}$, which generally agreed to the actual CH_4 yield.

Anaerobic digestion has been used for treating various types of ethanol stillage (or distillery wastewater) including sugar beet stillage, potato stillage, wheat stillage and shochu stillage, and has resulted in COD removal efficiencies of 75-95% using different reactor configurations (Weiland and Thomsen 1990; Nagano, Arikawa et al. 1992; Wilkie, Riedesel et al. 2000; Hutnan, Hornak et al. 2003; Schaefer and Sung 2008). It should be noted that the stillage was produced from fermentation of sugar or starch based feedstocks and hence contains a higher content of degradable organic carbon. The sCODs of some these stillage was 100 gL^{-1} or higher. In contrast the stillage obtained was from the fermentation of a pretreated cellulosic feedstock with low soluble organic matter. Stillage obtained from such feedstocks is expected to contain lignin and lignin related compounds as phenols (Kaparaju, Serrano et al. 2010), which were reported inhibitory for anaerobic digestion (Sierraalvarez, Field et al. 1994; Torry-Smith,

Sommer et al. 2003). By separating the coarse fibers from the stillage, lignin content of the digester feed in this case was 1.37% (wet weight basis) which was lower than the 7.5% lignin content reported by Kaparaju et al (Kaparaju, Serrano et al. 2010). Successful anaerobic digestion of cellulosic stillage (from wheat straw) at short HRTs (1-2 days) and high OLR (10 – 20 g COD (l d^{-1}) has been reported (Torry-Smith, Sommer et al. 2003; Kaparaju, Serrano et al. 2010). However in these studies, stillage was either diluted by a readily degradable substrate such as animal manure or subject to a series of pretreatment (centrifuge, filtration, autoclaving) before loading to digesters. Digestion of stillage alone as received with very little pretreatment has not been reported. The results of this study demonstrated anaerobic digestion of stillage alone was feasible. The organic loading rate could be increased by lowering HRT if specialized digester designs were used. However, designs like anaerobic filter would be susceptible to clogging due to the fiber content even in the filtered stillage.

The significance of implementing an anaerobic digester for treating cellulosic ethanol stillage was ascertained by determining the potential utilization of biogas as fuel in the ethanol plant. For example, biogas could be used to generate steam for feedstock pretreatment and/or as fuel for distillation. To evaluate energy efficiency of the overall process, a mass balance was developed. Assumption was made that 1 g raw bagasse underwent a series of processes (dilute acid pretreatment, enzymatic hydrolysis, fermentation and distillation) to produce ethanol; the byproduct stillage was anaerobically digested and produced CH_4. The raw bagasse contains 50% moisture and on dry matter basis 48% cellulose, 23% hemicellulose and 27% lignin. A total 4.11 g of 180^{o}C steam, water and cellulase was applied to 1 g raw bagasse and produced

0.11 g of ethanol and 5 g of stillage. The ethanol yield was calculated based on degradation in cellulose and hemicellulose contents between bagasse and stillage. Raw bagasse compositions and quantities of steam, enzyme and water used in the process were provided by the Biofuel Pilot Plant at University of Florida. Analysis of cellulose, hemicellulose and lignin in stillage was conducted by Dairy One. The mass balance illustration is shown in Figure 3-5. In the integrated process, steams were introduced in pretreatment and distillation which were considered as energy intensive steps. For a typical commercialized distillation process, 15 lbs steams are needed per gallon anhydrous ethanol to separate ethanol from 10% ethanol containing mixture (Madson 2003). Using this as a reference, 0.27 g steam was required for 0.11 g ethanol. Assuming the steam was at 250 ^{o}C, it would consume 0.71 KJ energy. In bagasse pretreatment, 1.34 KJ heat was required to generate 0.5 g 180^{o}C steam. As proposed before, energy consumed can be supplemented by the energy generated from anaerobic digestion. The anaerobic digestion process produced 37 ml CH4, equivalent to 1.47 KJ heat given CH4's heat of combustion being 891 KJ/mol. The energy calculation suggested the energy efficiency of cellulosic ethanol production could be improved significantly by integrating anaerobic digestion that produces biogas energy to cover 70% of energy consumed by steams generation. Since only stillage filtrate was digested, a strategy needs to be proposed to manage the leftover stillage residue which has been shown to have relatively low CH4 potential. The stillage residue was produced from fermentation of lignocellulosic biomass and contained relatively high solid contents. The lignin fraction has been subject to pretreatment and fermentation process in the bioethanol production and has been reduced to small particles mechanically and

biologically. This would make lignin a better fuel than traditional biomass and well suited for energy production by combustion (Larsen, Petersen et al. 2008). In fact, lignin is used by some lignite fired power plants as solid biofuels in either dried and wet forms (Kaparaju, Serrano et al. 2009).

Closing Remarks

BMP assayswere conducted on bagasse whole stillage, bagasse stillage filtrate, bagasse stillage residue and hardwood whole stillage. It showed that the CH_4potential of bagasse whole stillage being around 10 ml CH_4 at (ml stillage)$^{-1}$ or 200ml CH_4 (g VS)$^{-1}$, while bagasse stillage filtrate contributed the major CH_4potential of 70%. CH_4 potential of hardwood whole stillage was determined to be around 6.5 ml CH_4 at (ml stillage)$^{-1}$, about 40% less than that of bagasse whole stillage due to less organic matters contained in hardwood whole stillage. Degradation of sCOD and VOA concentration in the assays was evident as CH_4 being produced. The continuous digestion of stillage filtrateoperating at HRTs of 21 and 14 days achieved similar CH_4 yields as obtained in the BMP assays. This suggested the digestion practically obtained complete conversion of stillage to CH_4. sCOD and VOA profiles indicated the digester stability was well maintained at HRTs of 21 and 14 days. During the state where the CH_4 production was stable, the COD removal efficiency was around 80%. The success in the cellulosic stillage digestion can be attributed to the operations of long HRTs and low OLRs. Ammonia and phosphorus analysis, however, determined the digestion effluent contained relatively high concentration of phosphorus. A further process would be necessary to remove the nutrients before discharging the effluent to environment. The mass and energy balance of the integrated process indicated anaerobic digestion

has the potential to conserve most of the energy consumed in steam generation in ethanol production process.

Table 3-1. Contents and constituents of the BMP assays

Assays	Number of Replicates	Bagasse whole stillage	Bagasse stillage filtrate	Bagasse stillage residue	Hardwood whole stillage	Inoculum	Nutrient solution	DI water
		ml	ml	g	ml	ml	ml	ml
BW-1	2	40	0	0	0	100	4.29	10
BW-2	2	50	0	0	0	100	4.29	0
BF	2	0	69	0	0	100	4.83	0
BR	2	0	0	19	0	100	3.4	0
HW-1	2	0	0	0	40	100	4.29	10
HW-2	2	0	0	0	50	100	4.29	0
Control	2	0	0	0	0	100	4.29	50

Table 3-2. Characteristics of bagasse whole stillage, stillage filtrate, stillage residue and hardwood whole stillage.

		Bagasse whole stillage	Bagasse stillage residue	Bagasse stillage filtrate	Effluent	Hardwood whole stillage
TS	% (ww[a])	6.89±0.33	18.58±0.06	4.80±0.17	2.40±0.02	8.86±0.12
VS	% (ww)	5.36±0.31	16.53±0.13	3.11±0.12	ND	6.46±0.15
SCOD	g/L	38.6	ND[b]	38.8		21.8
Ethanol	g/L	ND	ND	0.75	ND	ND
Compositional Forage Analysis (dry matter basis)						
Crude Protein	%	9.90	8.90	10.60	10.50	ND
Soluble Protein	%	4.16	2.23	5.19	6.41	ND
Cellulose	%	23.00	28.90	9.40	2.20	ND
Hemicellulose	%	4.30	8.30	0.40	0.40	ND
Lignin	%	20.00	22.00	17.90	3.90	ND
ESC	%	4.50	4.50%	5.80	1.60	ND
TDN	%	53.00	46.00	65.00	85.00	ND
DE	Mcal/kg	2.47	2.18	2.95	3.73	ND
ME	Mcal/kg	2.06	1.76	2.53	3.32	ND

[a]ww = wet weight based.

[b]ND: Not determined.

Table 3-3. Monitored parameters of anaerobic digestion

HRT, days	21 (day 15-22)	14 (day 22-day 91)
SCOD, g/L		
Influent	38.8	38.4
Effluent	7.5	8
P, g/L		
Influent	0.53	0.53
Effluent	0.19	0.35
NH_3 as N, g/L		
Influent	0.3	0.3
Effluent	0.24	0.1
pH	7.76	7.55
OLR, g COD $(L\ d)^{-1}$	1.85	2.39
VOA (acetic and proponic acid), g COD /L	2.23	1.44
CH4 Yield L $(L\ substrate)^{-1}$	9.48	10.43
CH4 Yield, L $(g\ COD_{removed})^{-1}$	0.30	0.38

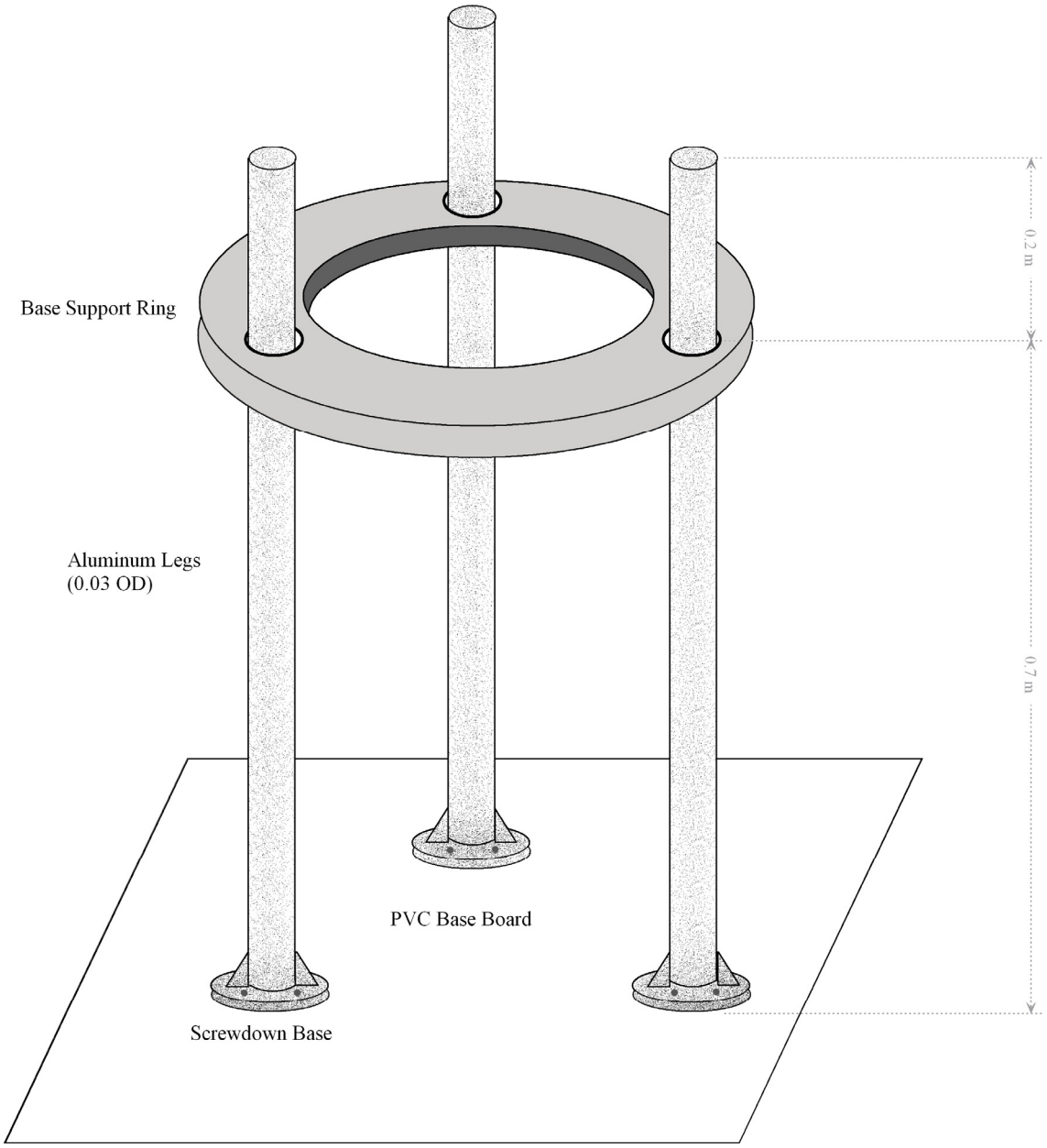


Figure 3-1. Custom-built tripod stand for anaerobic digesters. Adapted from Polematidis (Polematidis, 2007)

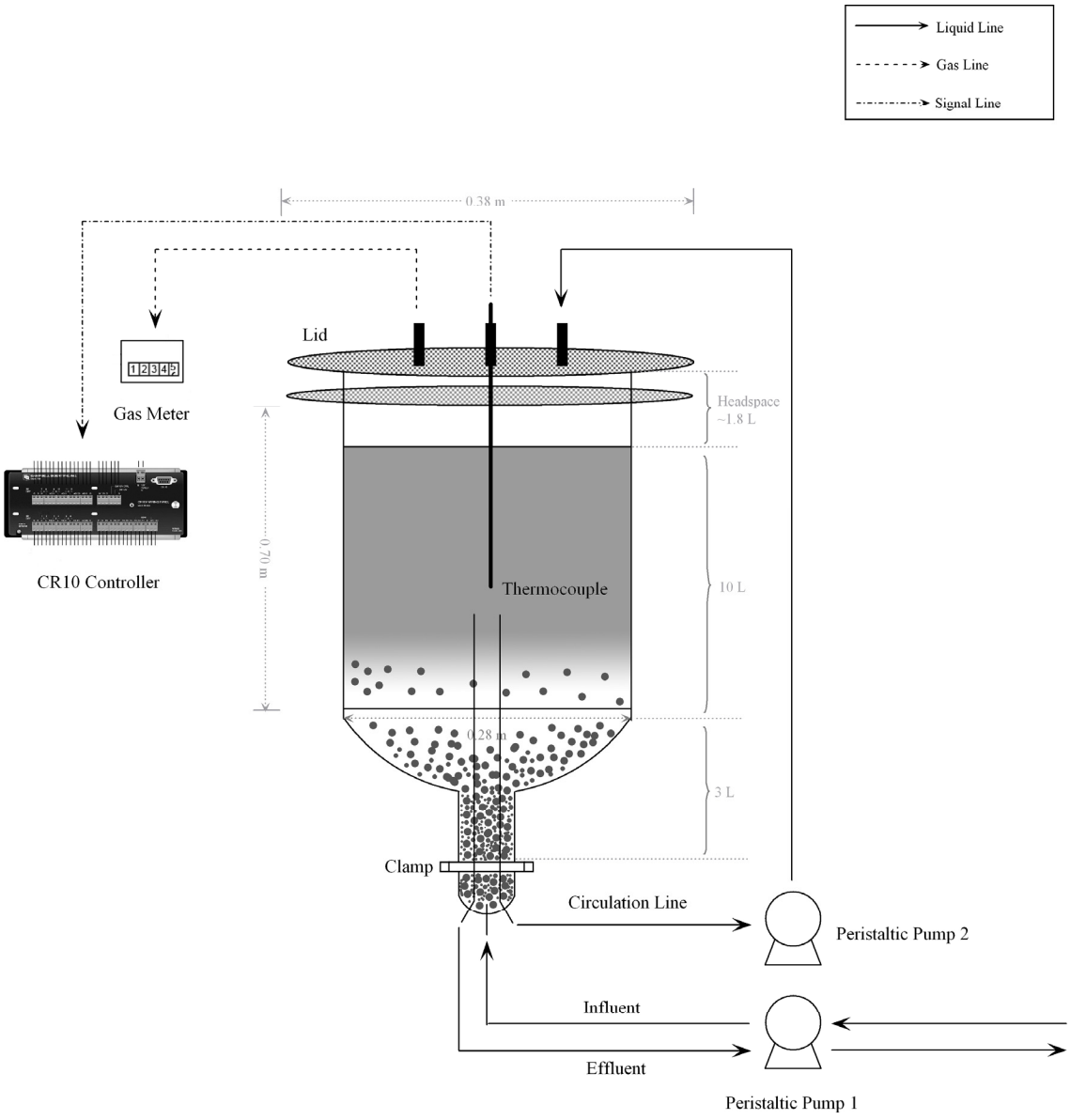


Figure 3-2. Continuous anaerobic reactor schematic. Adapted from Polematidis (Polematidis 2007)

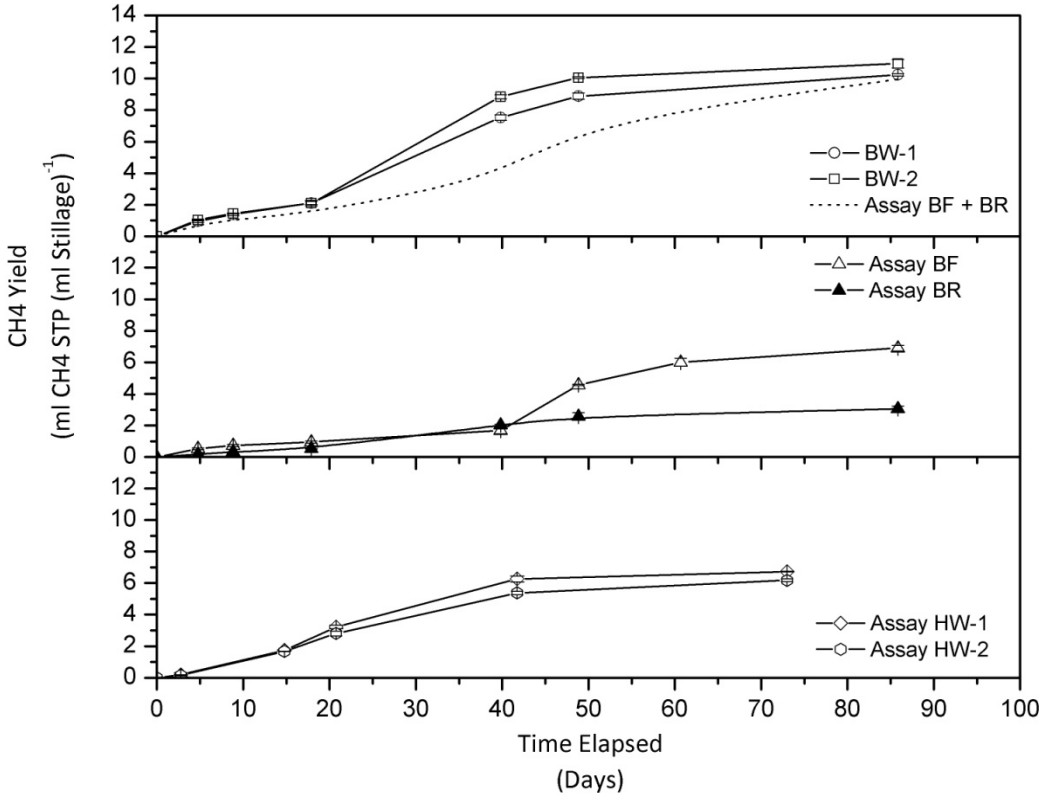


Figure 3-3. Methane yields of Assay BW-1, BW-2, BF, BR, HW-1 and HW-2

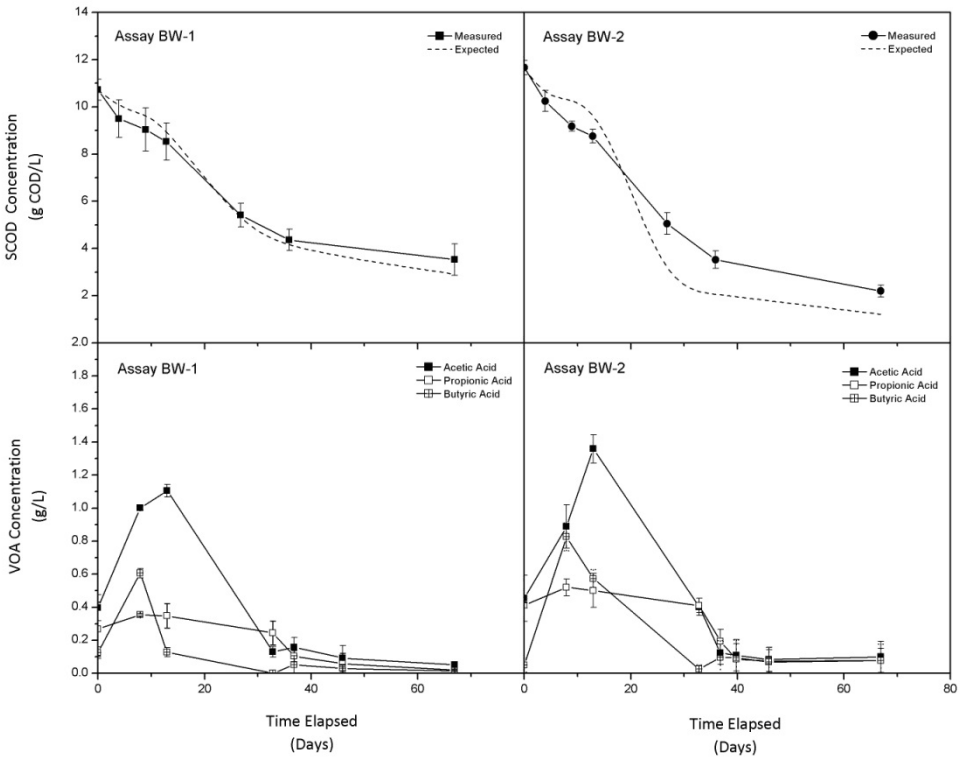


Figure 3-4. COD and VOA profiles of assay BW-1 and BW-2, data represents measured (solid line) and estimated from model (dashed line)

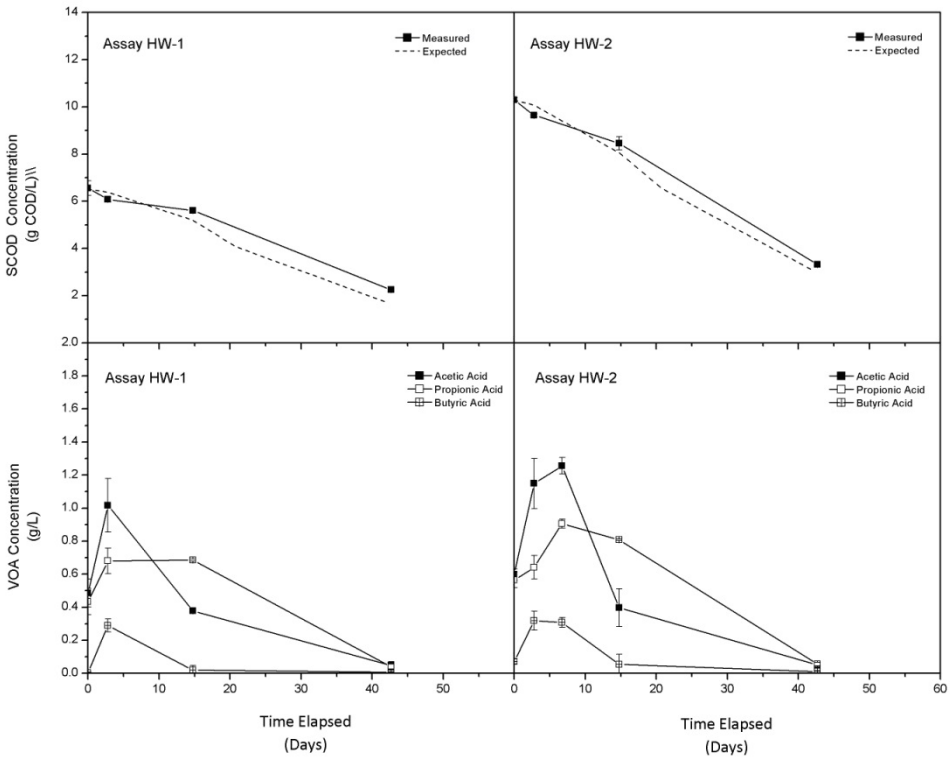


Figure 3-5. COD and VOA profiles of assay HW-1 and HW-2, data represents measured (solid line) and estimated from model (dashed line)

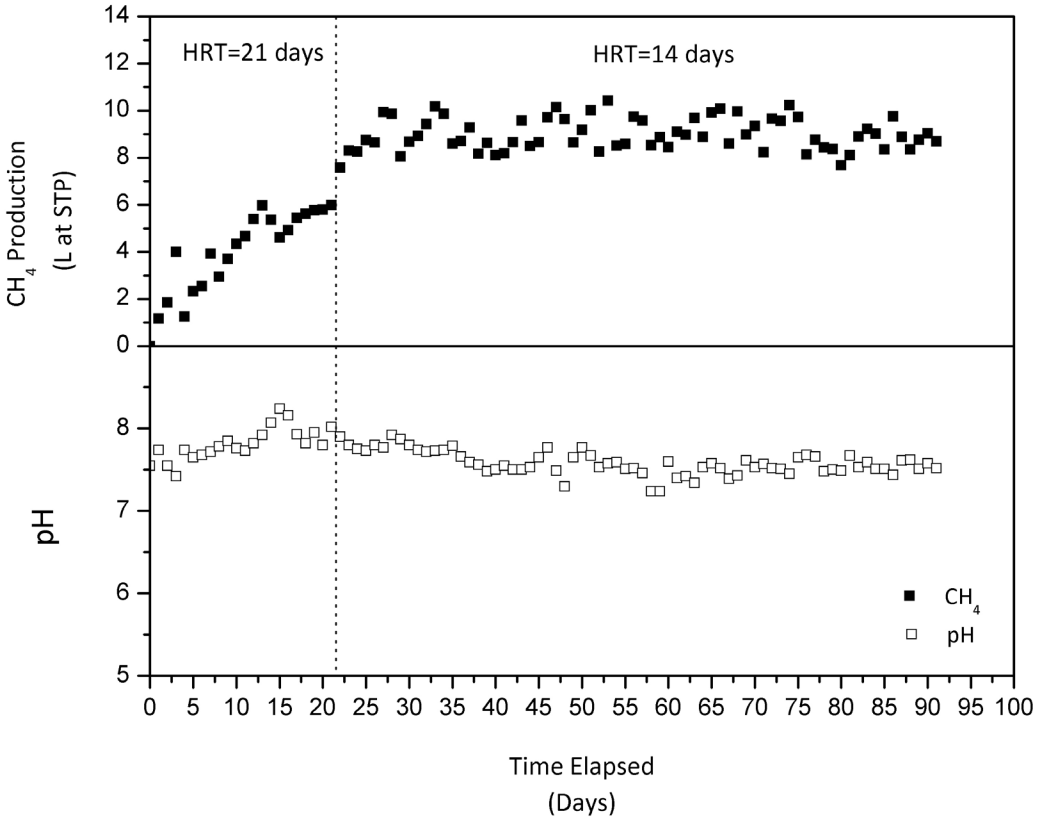


Figure 3-6. Daily methane yield from continuous digestion of stillage

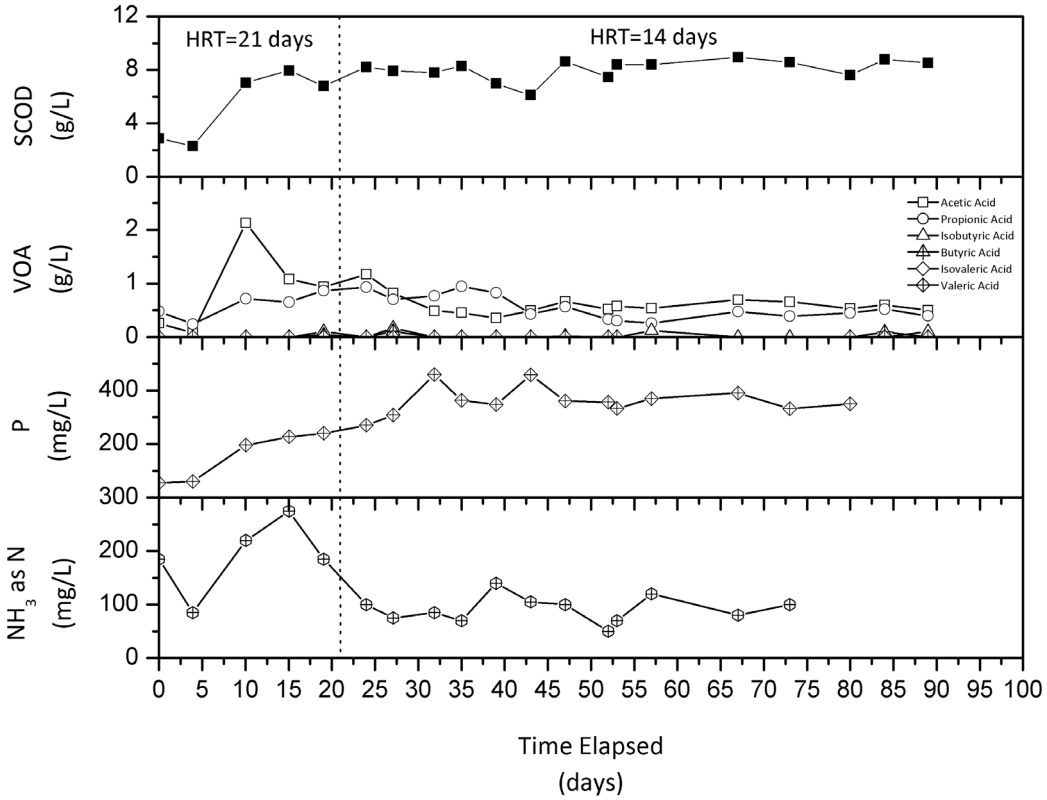


Figure 3-7. Profiles of SCOD, VOA, phosphorus and ammonia as N of the semi-continuous anaerobic digester

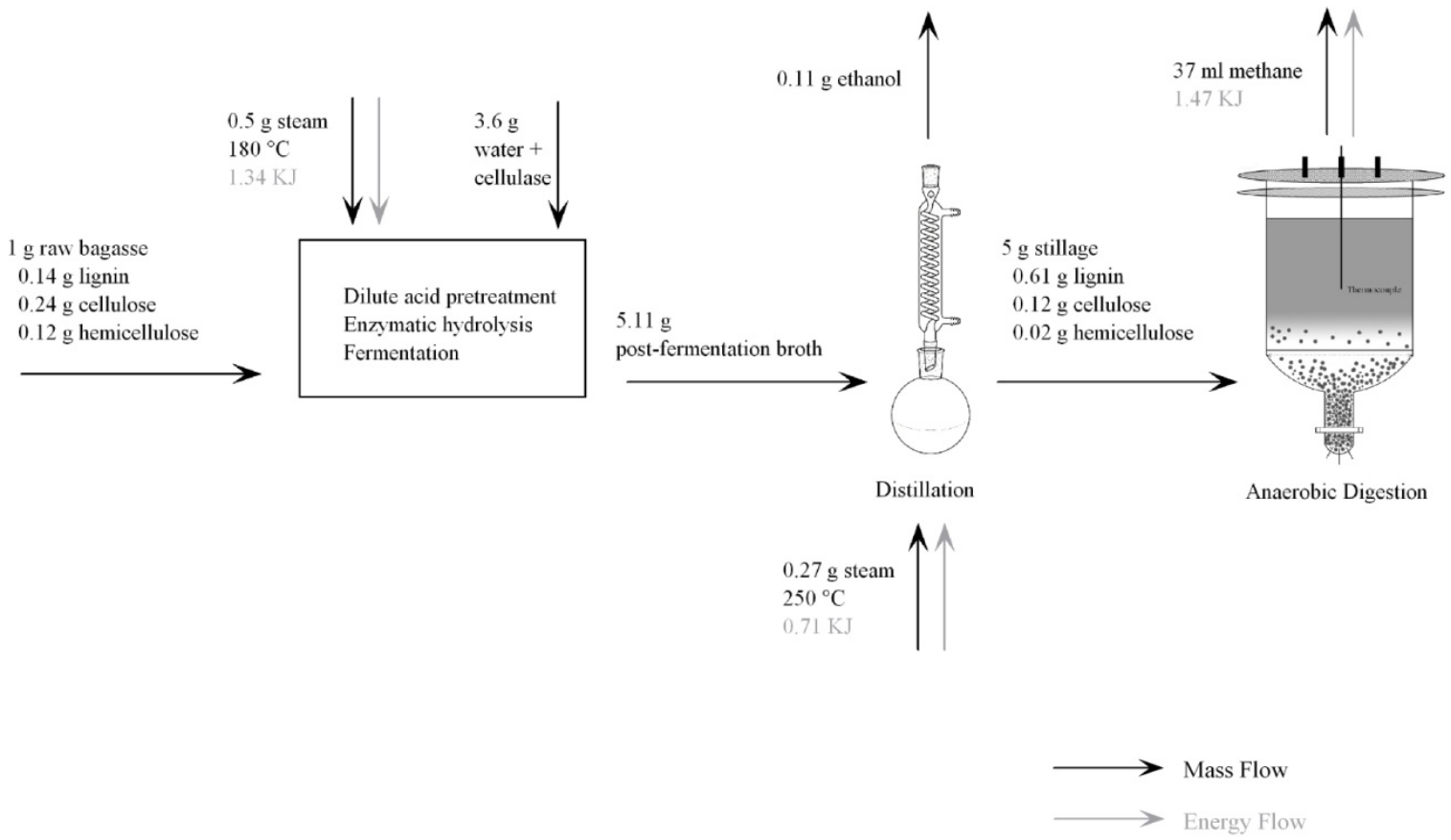


Figure 3-8. Mass balance of the integrated process of integrating anaerobic digestion with ethanol production.

CHAPTER 4
COMPARISON OF BATCH ANAEROBIC DIGESTION OF SUGARBEET TAILINGS AT MIXED AND NON-MIXED CONDITIONS

Summary

Thermophilic anaerobic digestion of sugar beet tailings at non-mixed and mixed conditions was conducted. Six experiments each were carried out in two identical digesters with a working volume of 3 L. The tailings in a non-mixed digester was bulked with lava rocks while the mixed digester contents were continuously mixed at 100 RPM. The mixing intensity of 180 RPM was normalized to a unit power input of 6.42 W/m^3. Though it was not regarded as a high intensity reported to interrupt syntrophic interactions, the mixed digester showed lower CH4 yield and slower CH4 production rate. The average methane yields from the non-mixed and the mixed digester were 0.34 L CH4 at STP $(g\ VS)^{-1}$ and 0.25 L CH4 at STP $(g\ VS)^{-1}$, respectively. Higher sCOD and VOA accumulation was detected in the mixed digester which confirmed its depressed digestion performance. The 16s rRNA clone library analysis revealed a diverse microbial community for the non-mixed digester with phylotypes *Methanoculleus* and *Methanosarcina* being dominant methanogens. However, *Methanosaeta* was identified as the only methanogens at a very low abundance (2%) and a hydrogen-producing bacterial phylotype *Petrotoga* was detected with high abundance (70%) in the mixed digester. Dominance of *Petrotoga* was speculated to limit the substrate supply for aceticlastic *Methanosaeta* resulting in inadequate growth. The mixed digester showed higher methane production rate when inoculated with the non-mixed digester mixed liquor though the overall methane yield was not improved. A COD balance calculation suggested that a fraction of substrate may have been mineralized to hydrogen along with methane.

Background

Among various technologies that are available for anaerobic digestion, continuously stirred tank reactors (CSTR) are typically used to process high solid wastewater. Thorough mixing of digester contents helps particle size reduction and evolution of biogas, distributes microorganism uniformly and improves mass and heat transfer, and therefore is regarded as essential in efficient anaerobic digestion (Sawyer and Grumbling 1960; Meynell 1978). Mixing is usually accomplished by mechanical mixers, slurry recirculation or biogas recirculation (Karim, Hoffmann et al. 2005). The significance of mixing in anaerobic digestion has been reported in many studies (Smith, Elliot et al. 1996; Kim, Kim et al. 2000; McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001; Vavilin and Angelidaki 2005; Hoffmann, Garcia et al. 2008; Suryawanshi, Chaudhari et al. 2010). Factors affecting digesters mixing include mixing strategy (continuous or intermittent), duration and intensity, among which, the effect of mixing intensity on anaerobic digestion are found contradictory. While some studies showed mixing improved biogas production for anaerobic digestion (Karim, Hoffmann et al. 2005; Vavilin and Angelidaki 2005; Kaparaju, Buendia et al. 2008), opposite results were reported by others (Ghaly and Benhassan 1989; Chen, Chynoweth et al. 1990; Vedrenne, Beline et al. 2008). Mixing at high intensity was shown to result in delayed methane (Vavilin and Angelidaki 2005) production whereas no significant difference was found in methane production for digesters with a broad range of mixing intensity (Hoffmann, Garcia et al. 2008). In spite of these disagreements, most studies tended to agree that anaerobic digestion could be interfered by excessive mixing while it may benefit from moderate mixing. The negative effect caused by intense mixing was interpreted as high shear forces disrupting microbial flock structures and disturbing syntrophic

relationship between bacteria and methanogenes (McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001; Vavilin and Angelidaki 2005; Kaparaju, Buendia et al. 2008). Anaerobic digestion and mixing studies was mostly conducted on animal manure (in which most of the degradable portion is present as soluble organic matter), no studies were found investigating effect of mixing on anaerobic digestion of solid feedstocks.

On a per hectare basis, sugar beet is one of the most efficient sources of ethanol and is widely used in Europe for ethanol fermentation (Kaffka 2009; Vries, Ven et al. 2010). However, no firm in United States has begun prodcuing ethanol from sugar beet (Outlaw, Ribera et al. 2007). Effective anaerobic digestion of sugar beet tailings using non-mixed system has been reported (Liu, Pullammanappallil et al. 2008). The result showed a net saving of treating sugar beet tailings with anaerobic digestion was around 4000 USD per day for the East Grand Forks plant, American Crystal Sugar Company which spends 1,000,000 USD annually to dispose the tailings (Teixeira, Chynoweth et al. 2005). Non-mixed anaerobic digestion conserves energy and does not require fine shredding of the substrates. It can be conducted at ambient pressure and at both mesophilic and thermophilic temperatures. (Pullammanappallil, Clarke et al. 2005). However, when carrying out anaerobic digestion of sugar beet tailings, Polematidis (Polematidis 2007) observed substrate compaction and/or flotation at non-mixed conditions and this negatively impacted digestion performance. A solution was found by providing bulking materials along with tailings. This approach eliminated substrate compaction and improved methane production rate substantially (Polematidis 2007). Substrate compaction and flotation could also have been eliminated by providing gentle agitation of digester contents. The objective of this study was to evaluate and compare anaerobic

digestion of sugar beet tailings at non-mixed and mixed conditions. The experiments were carried out in two single-stage batch systems operated at thermophilic temperature (55°C). The non-mixed digester was added with bulking agents whereas the mixed digester was provided with low-speed continuous mixing. Digestion performance at both conditions, including CH_4 yield, CH_4 production rate and sCOD concentration were measured and compared. To further explore the digestion at mixed and non-mixed conditions, the microbial community structure was studied. Comparative analysis of bacterial and archael 16s rRNA was carried out for the two digesters and the major microbial phylotypes were identified.

Material and Method

Feedstock

The sugar beet tailings was provided by America Crystal Sugar Company, Minnesota and stored at 4 °C before the experiment. The tailings were washed two times with tap water to soluble organic matter mainly sugars. Wash water was discarded.

Anaerobic Digesters

Two identical anaerobic digesters (digester 1 and 2) were constructed by modifying 5 L Pyrex glass bottles. The digester was sealed on top with a glass lid fitted with a rubber ring. The lid was clamped using a stainless steel clamp for gas tightness. The height and inner diameter of the digester were 0.406 m and 0.061 m, respectively. The digester was provided with several ports for gas and liquid sample withdrawal and biogas exhaust. Biogas production was measured by a positive displacement gas meter, which consists of a clear PVC U-shaped tube filled with anti-freeze solution, a solid state time delay relay (Dayton Off Delay Model 6X153E),

a Grainger float switch, a Redington counter and a Fabco Air solenoid valve. The gas meter was calibrated in line to determine the volume of biogas per count.

Experiment Description

The study was carried out in two sets, consisting of six experimental runs which were conducted in succession with digester liquors from the previous run being used to initiate the next run, as shown in Figure 4-3. Experiment set 1 involved mixing digester liquors and exchanging between digester 1 and 2 while set 2 kept the digester liquor unchanged for the individual digester. In each run, digester 1 and 2 were operated at non-mixed and mixed condition, respectively. At beginning of a run, digester 1 was added 0.3 kg washed sugar beet tailings on a wet weight basis along with 200 g bulking materials (lava rocks from a landscaping supplier, 0.025 m in diameter averagely) to prevent substrate compaction and/or floatation. The tailings and bulking materials were added in the following alternative manner with 3 layers: tailing-rock-tailing-rock-tailing rock. The same amount of washed sugar beet tailings (0.3 kg on a wet weight basis) was added in digester 2. A 50.8 mm x 9.5 mm PTFE coated polygon bar was placed in the digester and the mixing of digester content was achieve by using a large volume magnetic stirrer (Siceneware Cool Stirrer). The mixing intensity was set as 100 revolutions per minutes (RPM). No bulking materials were used for digester 2. Both digesters were kept in a 55 °C incubator throughout the experiments.

Three experimental runs were conducted in set 1. In run 1, each digester was inoculated with 3 L inoculumn taken from an anaerobic digester that has been fed with desugarized molasses at thermophilic temperature for 2 years. Run 1 was ended when the gas production was low and the digesters were emptied and washed thoroughly. Residual sugar beet tailings were discarded, and digester liquor

was saved for run 2. Digester 1 and 2 liquors from run 1 were mixed and divided into equal amount and used as the inoculum for run 2. As for run 1, run 2 was initialed by adding 0.3 kg fresh washed tailings and inoculated with 3 L inoculumn. When the biogas production stopped run 2 was ended and the digesters were again unloaded and washed. Residue tailings were discarded while digester liquors were kept for the next run. In run 3, new fresh substrates were added and digester liquors from run 2 were exchanged and used as the inoculum, that is, digester 1 was inoculated with digester 2 liquor and digester 2 was inoculated from digester 1 liquor from the last run. Run 3 was considered complete when the biogas production was low. Additionally, three experimental runs were conducted in set 2. Digester 1 liquors was recovered from run 3 and diluted by an equal volume of deionized water. The diluted digester 1 liquor was then divided equally and used to inoculate digester 1 and 2, respectively. In run 5 and 6, digester 1 and digester 2 were added with 0.3 kg fresh washed tailings and inoculated with 3 L inoculum as before. Digester 1 liquors from run 4 and 5 were used to inoculate digester 1 to start run 5 and 6, respectively. Digester 2 was inoculated in the same manner. Each experimental run was operated for 15 to 20 days.

Temperature Monitor

A large-volume magnetic stirrer was used to provide mixing for digester 2. According to the manufacturer, the stirrer motor is insulated to prevent the heating from being transmitted to thermolabile solution. To demonstrate that digester 2 liquor was not overheated by the use of the stirrer, a simple temperature monitoring experiment was carried out. Three mercury thermometers were used to measure the temperature in the incubator, digester 1 and digester 2. Temperatures were read manually. Observations were made periodically, ranging from minutes to hours.

Chemical Analysis

Total Solids (TS) and Volatile Solids (VS) contents were determined for sugar beet tailing. Tailing samples were dried at 105°C for 24 hours using a Fisher Scientific Isotemp Oven (Model 350G) and combusted at 550 °C for 3 hours using a muffle furnace (Fisher Scientific Isotemp) for TS and VS measurement, respectively.

Daily biogas production, biogas composition, pH and soluble chemical oxygen demand (sCOD) concentration were monitored for digester 1 and 2. Biogas volume was measured by the displacement gas meter and biogas composition was analyzed using Fisher Gas Partitioner (Model 1200). The gas chromatograph was calibrated with an external standard containing N_2, CH_4, and CO_2 in 25:45:30 volume ratio. Digester 1 and 2 liquor samples were collected periodically for pH, sCOD. pH was measured using an Accument pH meter. For sCOD analysis, liquid samples were centrifuged at 8000 RPM for 10 minutes (Fisher Marathon micro H centrifuge) and filtered using Millipore filter paper (pore size 1.2 um). Prepared samples were added in Hach COD vials (range 20-150 ppm), baked in Hach COD reactor for 2 hours and measured using Hach DR/890 Colorimeter for sCOD concentration. Volatile organic acid (VOA) concentration was monitored from run 1 through run 3. The analysis was conducted using Shimadzu gas chromatograph (GC-9AM equipped with a flame ionization detector) for acetic, propionic, isobutyric, butyric, isovaleric and valeric acid concentrations.

Microbial Community Analysis

Microbial communities were investigated for the original incolumn (run 1) and digester 1 and digester 2 liquors in run 6. Digester liquors were sampled for both digesters at day 3 when the methane production was active. DNA was extracted and purified by using FastDNA Kit and PowerClean DNA kit, respectively. The quality of

DNA was verified by agar gel electrophoresis running at 100V for approximately 50 minutes. Results were visualized in UV light. Extracted DNA was stored at -20 C before using as a template for Polymerase Chain Reaction (PCR) amplification.PCR reaction was triplicated for each sample and was done in a mixture of 28 ul of RNase free water (provided by QIAGEN), 0.5 ul of forward primer, 0.5 ul of reverse primer , 20 ul of HotMaster solution (provided by 5 Prime) and 0.5 to 2 ul of template depending on its concentration. The total amount of template added was below 20 ng. Primers Archaea A1 (5'-GCCTTGCCAGCCCGGCTCAGAAGCCGTTTCATTAGATACCCA-3') and Archaea Rev (5'-GCCTCCCTCGCGCGATCAGTCTTMGGGGCATTCNKACCT-3') were used for archaeal 16S rDNA amplification. Primers 454B 27F (GCCTTGCCAGCCCGCTCAGTCAGAGTTTGATCCTGGCTCAG-3') and 454A 338R (5'-GCCTCCCTCGCGCCATCAGCATGCTGCCTCCCGTAGGAGT-3') were used for bacterial 16S rDNA amplification. All primers were provided by Invitrogen. The PCR reaction was conducted using Eppendorf Mastercycler at the following program: an initial denaturation at 94 C for 2 mininutes; 30 cycles of denaturing at 94 C for 20 seconds, annealing at 53 C for 20 seconds, extension at 65 C for 1 min; end of extension at 65 C for 6 min. Amplified products were purified using QIAquick PCR purification kit and the quality was verified by agar gel electrophoresis. The purified fresh PCR products were ligated into pCR4-TOPO vectors and then transformed into chemically competent E.coli DH5α-T1 cells by using Invitrogen TOPO TA Cloning Kit. E.coli colonies were screened on Luria Broth agar plates with 50 ug/ml kanamycin. 96 colonies for each sample were randomly selected into a 96 welled plate preloaded with 200 ul LB medium (50 ug/ml kanamycin). Archaea population was expected to have less diversity and only half as many as clones as for bacteria

were developed for archaea. Samples were sent to Interdisciplinary Center for Biotechnology Research (ICBR) at University of Florida for 16s rRNA sequencing. Sequence similarity searches were performed using BLAST in the RDP (Ribosomal Database Project) database. Phylotype was defined as a group of cloned sequences with 90% identity (taxonomic order level). The percentage of coverage was calculated using the equation [1-(n/N)], where n is the number of phylotypes represented by a single clone and N is the total number of clones obtained (Good 1953).

Results

Characterization of Substrates

TS and VS contents of sugar beet tailings were determined for 6 runs and shown in table 4-1. The volatile matter content of tailings used in run 1, run 2, run 3, run 4, run 5 and run 6 were 10.68%, 9.23% , 8.19%, 10.53%, 10.17% and 10.26% in run 1, run 2, run 3, run 4, run 5 and run 6 respectively. Substrate loading quantities and digester packing density on wet weight and dry matter basis were calculated for each run and also listed in Table 4-1.

Temperature Profiles

The temperature profiles of incubator, digester 1 and digester 2 are illustrated in Figure 4-3 There was no noticeable difference between digester 1 and digester 2 temperature. Temperatures of incubator, digester 1 and digester 2 dropped when measurements were taken within a short time interval of 10 to 20 minutes. This was because incubator had to be opened frequently to make measurements which cooled the chamber, thus lowering the temperature.. Both digester 1 and digester 2 showed slightly lower temperatures than that of incubator possible due to the slow

evaporation of digesters liquors. Generally, temperatures in digester 1 and 2 were maintained well within thmermophilic range.

CH_4 Production

The profiles of cumulative methane yield for digester 1 and 2 in six runs are shown in Figure 4-4. In run 1, digester 1 achieved CH_4 yield of 0.36 CH_4 at STP kg VS^{-1} and digester 2 achieved 0.23 m^3 CH_4 at STP kg VS^{-1}. The maximum CH_4 production rate of digester 1 and 2 was 1.3 m^3 m^{-3} d^{-1} and 0.61 m^3 m^{-3} d^{-1}, respectively. The maximum rate was achieved sooner in digester 1 (at day 3) than in digester 2 (at day 4). Run 1 was ended when daily CH_4 production rate was low (0.035 m^3 m^{-3} d^{-1} for digester 1 and 0.030 m^3 m^{-3} d^{-1} for digester 2). Run 2 was started by flooding digester 1 and 2 with mixed digester 1 and 2 liquors from run 1. The CH_4 yields obtained were similar to those obtained in run 1. In this run digester 1 and 2 achieved CH_4 yields of 0.35 m^3 CH_4 at STP kg VS^{-1} and 0.27 m^3 CH4 at STP kg VS^{-1}, respectively. Daily methane production rate peaked at 1.24 m^3 m^{-3} d^{-1} on day 3 for digester 1 and 0.48 m^3 m^{-3} d^{-1} on day 5 for digester 2. In run 3, the CH_4 yields of digester 1 and 2 were 0.34 m^3 and 0.25 m^3 CH_4 at STP kg VS^{-1}, respectively. Daily CH_4 production rate reached maximum on day 3 for digester 1 (0.90 m^3 m^{-3} d^{-1}) and day 4 for digester 2 (0.47 m^3 m^{-3} d^{-1}). For run 4, 5 and 6, digester obtained CH_4 yields round 0.36 STP kg VS^{-1} and maintained the maximum CH_4 production rate at the level from 0.8 to 1.1 m^3 m^{-3} d^{-1}. On the contrary, digester 2 showed lower CH_4 production rates than in first 3 runs, though the cumulative yields CH_4 were similar. Its maximum production rate at run 4, 5 and 6 were calculated around 0.25 to 0.32 m^3 m^{-3} d^{-1}. In all six runs digester 1 exhibited noticeably higher CH_4 yield and CH_4 production rate than digester 2.

Profiles of sCOD Degradation Comparison

The profiles of sCOD for digester 1 and 2 are shown in Figure 4-5. sCOD comparisons were made between digester 1 and 2 within a certain run and also for a digester in different runs. In general, sCOD concentration of both digesters initially increased, reached a maximum and decreased to a minimum. The initial increase of sCOD was due to the solubilization of sugar beet tailings. Digester 1 exhibited similar sCOD profiles in all 6 runs whereas in digester 2 much higher sCOD accumulations was seen in run 4, 5 and 6 than in run 1, 2 and 3. In the first 3 runs, sCOD concentration in digester 2 increased to the range of 5 to 6 g L^{-1}. However, for run 4, 5 and 6, the sCOD concentration in digester 2 reached a maximum of 6 to 10 g L^{-1} and seemed to persist for longer time before noticeable degradation. Within a run, digester 2 had accumulation of higher sCOD concentration and slower sCOD degradation than digester 1. sCOD accumulation of digester 1 was around 4 g L^{-1}, and degradation became evident after 2 to 3 days.

Profiles of Volatile Organic Acid

The profiles of VOA for digester 1 and 2 in run 1, 2 and 3 are shown in Figure 4-6 Acetic acid, propionic acid and butyric acid were dominant among the organic acids detected. The concentration of isobutyric acid, isovaleric acid and valeric acid were negligible and are not shown in Figure 4-5. Acetic acid had the highest concentration among VOAs in digester 1. The concentration reached around 1 g L^{-1} and then quickly decreased as CH_4 was produced. High concentration of acetic acid was also observed in digester 2 and the degradation appeared slower than in digester 1. Profiles of propionic acid were distinct between digester 1 and digester 2. Much higher propionic acid accumulation (maximum around 0.8) was seen in digester 2 than in digester 1. The persistence of propionic acid probably

indicated the hindered digestion. Concentration of propionic acid of digester 1 in run was initially high, because the digester was inoculated with digester 2 liquor recovered from run 2. The propionic acid started degrading shortly after run 3 began.

Microbial Communities Structure

Bacterial and archaeal 16S rRNA gene clone libraries were constructed for the original incolum and digester 1 and 2 liquors at run 6 and presented in Table 4-3 and 4-4. A total of 215 bacterial phylotypes were obtained from 271 16S rRNA cloned sequence and 125 archaeal phylotypes were obtained from 128 clones with the criterion greater than 90% identity. The comparative 16S rRNA gene analysis revealed that microbiological communities were distinct among the inoculum, digester 1 liquors and digester 2 liquors. Bacteria phyla *Actinobacteria*, *Firmcutes*, *Synergistales* and *Thermotogae* were abundant in the inoculum with *Thermotogae* being dominant (52%), which has been identified in thermophilic anaerobic digesters (Riviere, Desvignes et al. 2009; Weiss, Jerome et al. 2009). The phylum *Thermotogae* was also dominant in digester 2 (70%) and abundant in digester 1 (22%). Its closest relative was identified as *Petrotoga olearia* with a low similarity of 90%. In digester 1, bacterial phylum *Firmcutes* was found to be dominant and genera *Caloramator*, *Clostridium* and *Sporobacterium* were identified within the phylum. *Firmcutes* have been commonly observed in anaerobic processes treating organic wastes (Hatamoto, Imachi et al. 2007; Narihiro, Terada et al. 2009; Sasaki, Hori et al. 2011). Some phylotypes detected in digester 1 were either not found (classified in the phylum *Proteobacteria*) or detected with low abundance (classified in the phylum *Synergistets*) in digester 2. As expected, the identified archaeal phylotypes were less diverse than bacteria, and all the archaea clones are member in phyla *Crenarchaeota* and *Euryarchaeota*. The dominant archaeal phylotypes for

inoculum, digester 1 and digeser 2 was surprisingly found to be *Crenarchaeota*, to which methanogens does not belong. Phylotypes.within *Euryarchaeota* were *Pyrobaculum*-like phylotypes, which were reported to be facultative anaerobes and grow at thermophilic temperature and neutral pH. However, they have not been reported found in anaerobic reactors. In spite of *Crenarchaeota* being dominant, methanogenic phylotypes were still found abundant in the inoculumn (37%) and digester 1 (46%). Methanogenic phylotypes in digester 2 were identified with high identity (95%-99%) and classified in order *Methanobacteriales*, *Methanomicrobiales* and *Methanosarchinales* while phylotypes in the inoculum were all classified in the order *Methanomicrobiales*. In contrast, methanogenic phylotypes in digester 2 were detected at much less abundance of 2%. They were assigned to the order *Methanobacteriales*. Overall, digester 1 showed more diverse microbial communities than digester 2. This may explain the low methanogenic activity observed in digester 2. The rank abundances of bacterial and archaeal phylotypes are shown in Figure 4-7.

Discussion

Determination of Mixing Intensity

Utilization of mixing at high intensity has been shown to be detrimental to anaerobic digestion (McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001; Vavilin and Angelidaki 2005; Kaparaju, Buendia et al. 2008). The mixing rate used in digester 2 was carefully chosen based on recommendations in literature. Hoffman (Hoffmann, Garcia et al. 2008) investigated the effect of mixing at different intensities of 50, 250, 500 and 1500 RPM on anaerobic digesters (4.5 L working volume) fed with 5% VS based cow manure slurry. Differences in the CH_4 yields at four mixing intensities were statistically insignificant. Computer automated radioactive particle

tracking (CARPT) in conjunction with computational fluid dynamics (CFD) was utilized to map shear distribution throughout the digesters and estimate local velocities. As expected, the digester mixed at 250 RPM showed lowest vertical, horizontal and azimuthal velocities (CARPT was not successful for the 50 RPM digester due to the low mixing intensity). Shear stresses were also lowest and uniformly distributed in the 250 RPM digester. High local shear stresses were present near the mixer region for all analyzed digesters. Using these results as references, the mixing rate employed in this study was chosen to be 100 RPM. The rate only developed a small vortex and dispensed the tailings well (no floatation or settlement) in digester 2. It was expected to aid in particle size reduction and mass transfer but avoid excessive mixing. However, digester 2 showed lower CH_4 yield and CH_4 production rate than the non-mixed parallel (digester 1), though the 100 RPM was close to the low mixing intensity used in Hoffman's study. Since digester 2 has a working volume smaller than the digester used by Hoffman, it is possible that 100 RPM could still introduce intense mixing to the smaller system used here. To compare the mixing intensities used in different studies, it is necessary to develop a method for normalization because mixing in anaerobic digestion at different scales has been achieved by various mechanical ways such as impeller mixing and shaking (using a shaking table). The comparison was made between this study and other four studies (Karim, Hoffmann et al. 2005; Vavilin and Angelidaki 2005; Hoffmann, Garcia et al. 2008; Kaparaju, Buendia et al. 2008). The mixing intensities was normalized to power supply per unit volume, W/m^3 according to equation 4.1 and 4.2, which are used for impeller mixing and shaking, respectively.

$$P = N_P^{'} \rho N_i^3 D_i^5 \qquad (4.1)$$

where $N_P^{'}$ = Power number, dimensionless

ρ = density of digester liquor, kg/m^3

N_i =Rotational speed, s^{-1}

D_i =Impeller diameter, m

$$P = \int_0^s mads \tag{4.2}$$

where m = mass, kg

a = acceleration, m/s^2

s = displacement, m

$$E = \frac{1}{2}mv^2 \tag{4.3}$$

where E=kinetic energy

m= mass

v = velocity

The power numbers were assumed to be 0.35 (paddle impeller) for the impeller used in Hoffman and Karim's studies (Karim, Hoffmann et al. 2005; Hoffmann, Garcia et al. 2008) and also for the mixer (a polygon bar) used in this study. This assumption was made as the power number is independent of Reynolds number and only depends on impeller geometry when turbulence was achieved. Reynolds numbers were calculated and showed turbulence was achieved, except for the 50 RPM-mixed digester used by Hoffman. Although the 50 RPM digester was determined being at transition state, Reynolds number of 0.35 was still assigned. All digester liquors were assumed to have the same density and viscosity as water for Reynolds number calculation. Equation 4.2 was used to normalize mixing intensity reported in Kaparaju's study (Kaparaju, Buendia et al. 2008). Kaparaju carried out experiments in 1 L serum bottles (450 ml working volume) and the bottle contents

were mixed by using a shaker table. Mixing was provided in three intensities, vigorous (100 times per minute with a 3.5 cm stroke), gently (35 times per minute with a 1.2 cm stroke) and minimum (manually shaking the bottles every time biogas sample were withdrawn). Normalization was calculated for vigorous and gentle mixing only. Shaking bottles were assumed to have a symmetric parabola shaped velocity profile against time, where velocity is zero at beginning and end and reaches maximum at midpoint of a vibrating cycle. Using this assumption, acceleration of serum bottles can be calculated. The experiment (digestion of diluted municipal household solid wastes) carried out in Vavilin's study (Vavilin and Angelidaki 2005) was very similar to Kaparaju's method. Shaker tables were used to provide vigorous (105 times per min with a 5-cm length) and gentle mixing (58 round per minutes with a 17 cm radius) while minimal mixing was conducted by hand shaking for 1 minute every 1 to 2 days. The only difference was that bottles at gentle mixing were shaking in a pattern of approximate circular motion instead of linear vibration. Equation 4.2 and the same assumption were first used to calculate power input per unit volume for bottles at both gentle and vigorous mixing in Vavilin's study. The results showed gentle mixing provided orders of magnitude more power than vigorous mixing. It clearly overestimated the power supply of gentle mixing. It is true that bottles at gentle mixing moved much longer distance (a circle with 0.17 cm radius) that the bottles at vigorous mixing (0.05 m) in a shaking cycle, but velocity of circular motion was expected to be relatively constant which would consume much less energy as required by linear vibration to change the velocity frequently. Therefore, a different assumption was made that the bottles at gentle mixing in Vavilin's study underwent uniform circular motion, and the kinetic energy can be simply calculated using Equation 4.3. To calculate the corresponding power input, the kinetic energy was

assumed to be consumed in 3 shaking cycles (3 rounds or 2.9 seconds). The normalization results are shown in Table 4-4. In Karim's study (Karim, Hoffmann et al. 2005), the power supply per unit volume has been provided for mixed digesters. The equation $P=T\times A$ (where T is torque and A is angular speed) was used to calculate the power input per unit volume (8 W/m^3) for the digester that was provided with impeller mixing. Though a different equation was used in this study to calculate the unit power input (9.12 W/m^3) for the same digester, the result was in accordance with Karim's calculation. The power input per unit volume of digester 2 was calculated to be 1.10 W/m^3, which was not regarded as a high value compared to other results. In Karim's study, the impeller mixed digester produced 22% more CH_4 than the unmixed one when 10% (TS based) manure slurry was fed. This suggested that the low CH_4 production seen in digester 2 was not due to the excessive mixing as reported in some studies (McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001; Vavilin and Angelidaki 2005; Kaparaju, Buendia et al. 2008). The unit power input of 1.10 W/m^3 was also close to the unit power input at gentle mixing in Vavilin's study. However, in Kaparaju's studies, the gentle mixing showed a very low unit power input of 0.06 W/m^3. In comparison to that, 100 RPM used in digester 2 exceeded degree of gentle mixing though it was regarded as low-intense mixing according to Karim, Hoffman and Vavilin. Contradiction was found in comparing the mixing intensities between shaking and impeller mixing. Kaparaju reported a digester mixed at 12.67 W/m^3 showed low and delayed CH_4 production compared to digestion with less intense mixing (0.06 W/m^3 or no mixing); while Karim reported improved CH_4 production in digestion mixed at 9.12 W/m^3 compared to the non-mixed counterpart. It appeared that shaking and impeller-like mixing could have opposite effects on anaerobic digestion even though the unit power inputs were

comparable. A pilot scale anaerobic digestion of cow manure was further carried out using an impeller equipped reactor in Kaparaju's study. The mixing was provided at unit power input of 312.5 w/m^3, significantly exceeding the unit power input for the vigorous mixing (12.67 and 28.69 w/m^3), which was suggested to be avoided in anaerobic digestion by Kaparaju and Vavilin. Though there was a minor discrepancy in determining the mixing used in digester 2 whether being high-intense or low-intense, many studies agreed that mixing around 100-200 RPM was not viewed as excessive mixing and was typically applied to facilitate anaerobic digestion (Karim, Hoffmann et al. 2005; Hoffmann, Garcia et al. 2008; Alkaya and Demirer 2011; Alkaya and Demirer 2011; Penteado, Santana et al. 2011; Yu, Ma et al. 2011). In fact mixing power provided by the magnetic mixer for digester 2 (1.10 w/m^3) is well within the range of typical volumetric power input (1 to 10 w/m^3) recommended for anaerobic digesters (Grady, Daigger et al. 1999; Stroot, McMahon et al. 2001). The mixing intensity applied in digester 2 was therefore expected not to cause the disruption of spatial associations between microorganisms and ineffective transfer of hydrogen and acetate from syntrophic acetogens to neighboring methanogens as reported in vigorously mixed systems (Conrad, Phelps et al. 1985; Whitmore, Lloyd et al. 1987; Stroot, McMahon et al. 2001; Hoffmann, Garcia et al. 2008; Kaparaju, Buendia et al. 2008).

Another explanation for the hindered performance at the mixed condition could relate to the difference in substrate distribution. Mixing may promote rapid hydrolysis and fermentation, while methanogens and syntrophis may not have been able to turn over fermentation products at the rate of formation due to the inhibitory effect of some VOAs, resulting in poorer digestion performance (McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001). Digester 2 was observed to accumulate

propionic acid at concentrations which has been reported to inhibit methanogenesis (Barredo and Evison 1991).

In addition to mixing intensity, mixing frequency was also expected to affect anaerobic digestion. Continuous mixing (mixing using a shaker table) has been shown to be detrimental for anaerobic reactors digesting municipal waste (Grady, Daigger et al. 1999; McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001). Similar to digester 2, continuously mixed digesters in these studies exhibited persistent accumulation of propionic acid and less abundant of methanogens compared to the minimally mixed counterparts (manual shaking for 2 min everyday). McMahon and Stroot concluded continuous mixing appeared to disrupt spatial juxtaposition of syntrophic bacteria and their aceticlasic and hydrogenotrophic partners and resulted in unstable digestion performance. In comparison with this study, persistence of propionic acid was much more severe in McMahon and Stroot's study. This can be attributed to that they used a significantly higher mixing power of 1500 W/m^3. However, the effect of mixing intensity was not discussed in their studies. While several studies has showed anaerobic digestion at non-mixed or less mixed conditions (i.e., intermittent mixing instead of continuous mixing) exhibited higher CH4 yield than the continuously mixed digestion others has found no significant difference in performance between completely and minimally mixed digestion (Dague, McKinney et al. 1970; Chen, Chynoweth et al. 1990). Furthermore, mixing has showed to hinder industrial scale anaerobic digestion as well (Pfeffer 1987). These findings seemed to support the results obtained in this study, suggesting practice of continuous mixing for high solid digesters may not be always feasible since the possible inhibitory effect and operational problems.

Digestion Performance Comparison

In all six runs, digester 1 showed consistent CH_4 yield, CH_4 production rate and sCOD profiles. Digester 2 produced lower CH_4 yield and CH_4 production rate compared to that of digester 1. In run 4, 5 and 6, CH_4 production rate of digester 2 was further decreased though the final yield CH_4 was kept similar. It seemed that digester 2 performance deteriorated in the last 3 runs where the digester liquor was reused as the inoculum for the following run. This was confirmed by the high sCOD accumulation and long persistence period before degradation (6-8 days) observed for digester 2 in run 4, 5 and 6. Digester 2 appeared in capable of degrading sCOD to the extent achieved in digester 1.

During anaerobic digestion, solid substrate (sugar beet tailings) is first hydrolyzed to soluble forms by extracellular enzyme before cellular uptake. Fermentation of soluble organic matter produces a mixture of volatile organic acids. sCOD measurements include both the hydrolyzed organic matter as well as organic acids. Digester 2 was only able to convert a fraction of sCOD (large difference seen between initial and final sCOD) and the rest remained in the digester liquor. Because in run 4, 5 and 6, the digester liquor was used for inoculation for the next run, the unconverted sCOD from the previous run was detected as initial sCOD in the following run. Approximately 2 g L^{-1} of sCOD was left non-degraded in each run and accounted for the initial sCOD, resulting in higher and higher sCOD accumulation from run 4 through run 6. This phenomena was not seen for digester 2 in run 1, 2 and 3, because digester 1 and 2 liquors were either mixed or exchanged before inoculation. Providing digester 2 with digester 1 liquor (partial or all) also seemed to improve the rate of sCOD degradation, which started after about 5 days in run 1, 2 and 3. This agreed with generally with higher CH_4 production rate in run 1, 2 and 3

than in run 4, 5 and 6 for digester 2. In run 3, despite the fact that digester 1 was inoculated with digester 2 liquor, it neither showed delayed CH_4 production rate nor poor sCOD degradation. Speculation was made that microorganisms involved in sCOD degradation and methanogensis could quickly adapt to a stagnant environment and help to recover CH_4 production quickly in spite of having been exposed to the mixing conditions. A study has showed anaerobic digestion can be improved by providing a quiescent environment for bacteria (Stroot, McMahon et al. 2001). Addition of the bulking agents in digester 1 was also expected to facilitate the development of microbes by preventing compaction and providing surface for concentrated growth.

Microbial Community

In the development of 16s rRNA library, the number of singletons (phylotypes represented by a single clone) were found few, leading to high coverage values (Good 1953). Bacteria phyla *Proteobacteria* and *Synergistets* were identified in digester 1. Species in these phyla are known as syntrophic bacteria that play critical roles in methanogensis. For instance, *Syntrophobacter* classified in *Proteobacteria* was identified as a key player in syntrophic propionate degradation (Botsch and Conrad 2011). While the function of *Synergistets* in anaerobic ecosystem remains largely unknown, they are suspected to be involved in amino acid degradation and function as a biological electron acceptor in syntrophy with hydrogenotrophic methanogens (Godon, Moriniere et al. 2005). More than 50% bacterial phylotypes in digester 1 were members of phylum *Firmcute*, in which, phytotypes *Caloramator*, *Clostridium* and *Sporobacterium* were found with high abundance. Studies have reported that some *Clostridium* species were capable of syntrophic long chain fatty acid degradation (Hatamoto, Imachi et al. 2007) while *Sporobacterium olearium* (the

close representative of phylotype *Sporobacterium*) utilized aromatic compounds to produce acetate and butyrate (Mechichi, Labat et al. 1999). This may indicated those microbes played an intermediate role in anaerobic digestion, providing substrates for small-molecule degraders. *Caloramator fervidus* (the close representative of phylotype *Caloramator*) was found as one of the dominant hydrogen-producing bacteria in anaerobic digesters (Kongjan, O-Thong et al. 2011). It is likely to provide hydrogen to hydrogetrophic methanogens as genera *Methanoculleus* and *Methanosarcina* (both aceticlastic and hydrogenrophic) found in digester 1. Some *Clostridium* species were also reported to utilize acetate in syntrophic association with hydrogenotrophic methanogens (Schnurer, Schink et al. 1996; Hattori, Kamagata et al. 2000). Phylotype *Thermotogales* were found dominant (70%) in digester 2 with its closest representative being *Petrotoga*, which was also found high abundance(53%) in a lab-scale thermophilic continuous-flow stirred-tank (CFTR) reactor fed with a commercial dog food (Sasaki, Hori et al. 2011). Phylotype *Petrotoga*, as well as another *Thermotogales* member *Thermotoga* have been known as fermentative and sulfur reducing bacteria. Some *Petrotoga* and *Thermotoga* species were reported to produce H_2 through sugar fermentation (Lien, Madsen et al. 1998; Balk, Weijma et al. 2002). Inhibition of H_2 at high partial pressure has been showed inhibitory on growth of a *Petrotoga* species. The inhibition was alleviated by adding element sulfur or thiosulfate because the bacterium was able to use them as electron acceptors to produce hydrogen sulfide (H_2S) and reduce H_2 concentration. The presence of abundant *Petrotoga*-like bacteria in both digester 1 and 2 could be attributed to the inoculum, in which the bacteria were already found dominant. The incolumn was taken from an anaerobic reactor digesting desugarized molasses that has been reported to contain relatively

high sulfate concentration (Fang, Boe et al. 2011). This was speculated to favor the bacteria growth in the inoculumn. Interestingly, a *Petrotoga* relative, *Thermotoga* lettinga was reported to grow through H_2 fermentation in the absence of electron acceptors (as sulfur) by taking advantage of the syntrophic relation with a hydrogenotrophic methanogen *Methanothermobacter* (Balk, Weijma et al. 2002). Though *Petrotoga* was remotely related to (belong to the same order) *Thermotoga*, it could share a similar syntrophic relation with methanogens as well. However, hydrogenotrophic methanogens were not identified in digester 2. The only methanogenic phylotype was *Methanosaeta*, which has been determined obligately aceticlastic (Patel and Sprott 1990). This indicated in digeser 2 *Petrotoga* was unable to gain metabolic advantages in the community because *Methanosaeta* cannot maintain a low H_2 partial pressure. Therefore, syntrophic relation between *Petrotoga* and *Methanosaeta* was not expected for digester 2. As a result, *Petrotoga* had to grow through H_2 fermentaion pathway. The high H_2 generation ability of *Petrotoga* has been reported in several studies (Lien, Madsen et al. 1998; L'Haridon, Miroshnichenko et al. 2002; Miranda-Tello, Fardeai et al. 2007; Kano, Mukaidani et al. 2009). This could affect VOA degradation which requires very low H_2 concentration (Bitton 2005). It was possible that acetate formation was reduced in digester, leading to insufficient growth (3% relative abundance to all archaea) of *Methanosaeta* and low CH_4 yield and production rate. Some *Petrotoga* related bacteria (*Thermotoga neapolitana*) exhibited stimulated growth and greatly improved hydrogen production in a agitated environment (75 rpm, 50 ml medium) (Van Ooteghem, Jones et al. 2004), which could exacerbate the acetate scarcity in digester 2. In contrast, digester 1 can benefit from *Petrotoga* since hydrogentrophic methanogens use H_2 as the substrate. As discussed above, sulfur reducing bacteria

are also autotrophic and they could compete with methanogens for H_2 utilization. Dar et al reported methanogens was outcompeted by sulfur reducing bacteria in a co-culture with sulfate concentratioin at 1.5 g/L. The competitive yet syntrophic relation between sulfur reducing bacteria and methanogens makes their interaction quite complex and not well understood (Dar, Kleerebezem et al. 2008). Discovery of sulfur reducing bacteria in anaerobic digestion was confirmed in a similar study, in which sugar beet pulp was utilized as feedstock (Labat and Garcia 1986). Archaeal phylotype *Pyrobaculum* was found dominant in digester 2 with a relative abundance of 97%. Amo et al. isolated a *Pyrobaculum* speices (*Pyrobaculum calidifontis*) from a hot spring and demonstrated significantly stimulated growth and in a 200 ml medium with 120 rpm shaking (Amo, Paje et al. 2002). This probably suggested the high abundance of *Pyrobaculum* was due to the mixing provided by digester 2. *Pyrobaculum* species were reported to grow heterotrophically via sulfur/sulfate oxidation (Amo, Paje et al. 2002). This could explain the high abundance of *Pyrobaculum* in the inoculum and digester 1 as well, since the incoculum was from a digester fed substrate containing high sulfate concentration

COD Balance

During anaerobic digestion, the degradable components of tailings were solubilized (hydrolyzed) and then converted to CH_4. sCOD was measured in the experiments. CH_4-COD was calculated using the conversion factor that 1 L CH_4 at STP has a COD equivalent of 2.86 g. A previous study determined the degradable COD of 0.3 kg sugar beet tailings (average 10% VS content) to be around 31.67 g using BMP assays (Polematidis 2007). This value was used as the initial degradable COD in the solid substrates for all 6 runs. The remaining degradable COD in the solid at a specified time was the difference between initial degradable COD and sum

of measured CH_4-COD and sCOD in the same period. By plotting, solid degradable COD, sCOD and CH_4-COD against time, the COD balance profiles of digester 1 and 2 were developed for experiment set 1 and 2, respectively (Figure 4-8). The tailings used in run 3 were determined to have relatively low VS contents and produced an approximately 20% less CH_4 (yield times VS) than in run 1 and 2. COD balance for experiment set 1 was calculated using the data from run 1 and 2 only to avoid large errors. Digesters from run 1 were inoculated with the liquor taken from a digester fed with different substrates (desugarized molasses). When run 1 liquors were used as inoculum for run 2, they had been adapted to degrade the tailings. Therefore, higher initial CH_4 production rates (day 2 to day 4) were seen for digesters in run 2 than in run 1, especially for digester 1. The variation in initial CH_4 productions mainly resulted in errors seen at day 2 and day 4 for experiment set 1. In both experiment sets (run 1, 2 and run 4, 5, 6), hydrolysis seemed to proceed well for both digester 1 and 2, since most of the degradable COD was converted (either as sCOD or CH_4-COD) at the end. While degradable COD left in digester 1 was negligible, 2-3 g of degradable COD stayed in digester 2. The comparative 16s rRNA library analysis has showed presence of a hydrogen-producing bacterial phylotype *Petrotoga* with high abundance (73%) in digester 2. The production of hydrogen could account for the discrepancy since hydrogen content of biogas was not measured. Hydrogen oxidization stoichiometry shows 1 L hydrogen consumes 0.72 g oxygen. To compensate for the missing COD of 2-3 g, digester 2 would produce 3-4 L hydrogen gas, or around 30% of biogas would be hydrogen (volume/volume, CO_2 excluded). *Petrotoga* was reported to have a doubling time of 12 hours (Lien, Madsen et al. 1998) and had the potential to outpace the growth of *Methanosaet*a (the only methanogen in digester 2) with doubling time being 3 to 5 days (Janssen 2003;

Yoochatchaval, Ohashi et al. 2008). Therefore, hydrogen production would be faster when usable substrates as glucose became available to *Petrotoga*. This agreed with the observation of a rapid H_2 production during an anaerobic digestion of waste lactose (Banks, Zotova et al. 2010). As seen in the sCOD profiles, COD balance also showed high sCOD accumulation in digester 2. VOA made up approximately 2.5 g COD equivalent at the end of the experiment. The rest of sCOD was expected to be other fermentation intermediates such sugars. It appeared the presence of H_2 not only affected VOA degradation but also sugar fermentation.

Closing Remarks

Anaerobic digestions of sugar beet tailings were performed at non-mixed and mixed conditions for 6 runs using two batch anaerobic reactors. The mixed condition caused delayed CH_4 production rate and overall low CH_4 yield in mixed digester (digester 2). The comparative 16s rDNA library analysis revealed *Petrotoga* and *Pyrobaculum* dominated in bacteria and archaea community, respectively, in digester 2. The abundance of methanogens (*Methanosaeta* as the only identified phylotype) was very low. In contrast, digester 1 was detected with a diverse group of methanogens including hydrogenotrophic *Methanoculleus* and acetocalstic *Methanosarcina* (also hydrogenotrophic). The mixing intensities in digester 2 were not considered as high intensity. The hindered digestion performance was attributed to the inhibition by H_2. Digester 1 and 2 were added the same quantities of substrate at the beginning of each run, but the COD balance calculation showed 2 - 3 g substrate COD missing in digester 2 (neither converted to sCOD nor CH_4). It was hypothesized that digester 2 produced hydrogen which was not measured in this study, resulting in the discrepancy.

Table 4-1. Substrate characteristics and loading amounts for digester 1 and 2

Sugar beet tailings	Unit	Run 1	Run 2	Run 3
TS	% (wt/wt)	11.50%±0.39%	10.22%±0.23%	8.97%±0.43%
VS	% (wt/wt)	10.68%±0.56%	9.23%±0.19%	8.19%±0.46%
Digestion loading	Unit			
Wet weight	kg	0.3	0.3	0.3
Dry weight	kg	0.034	0.031	0.027
Volatile matter	kg	0.032	0.029	0.025
Inoculumn added	L	3	3	3
Packing density	kg/m^3, wet weight basis	100	100	100
Packing density	kg/m^3, dry weight basis	11.42	10.42	8.97
Sugar beet tailings	Unit	Run 4	Run 5	Run 6
TS	% (wt/wt)	11.22%±0.49%	10.88%±0.72%	11.00%±0.17%
VS	% (wt/wt)	10.53%±0.45%	10.17%±0.66%	10.26%±0.14%
Digestion loading	Unit			
Wet weight	kg	0.3	0.3	0.3
Dry weight	kg	0.034	0.033	0.033
Volatile matter	kg	0.032	0.031	0.031
Inoculumn added	L	3	3	3
Packing density	kg/m^3, wet weight basis	100	100	100
Packing density	kg/m^3, dry weight basis	11.22	10.88	11

Table 4-2. Power input per unit volume

	Digester	Working volume	Temperature	Mixing method	Rotational speed	Normalized intensity
		L	ºC		RPM	W/m^3
[a]Kaparaju et al.	Batch	0.4	55	Shaking	100	12.76
					35	0.06
[b]Vavilin et al.	Batch	0.5	37	Shaking	105	28.69
					58	1.28
[c]Hoffman et al.	Continuous	4.5	35	Impeller mixing	50	0.041
					250	11.13
					500	91.74
					1500	1109
[d]Karima et al.	Continuous	3.37	35	Impeller mixing	275	9.12
[e]This study	Batch	3	55	Magnetic mixing	100	1.10

[a] Substrate, fresh cow manure; substrate to inoculum ratio, 0.11
[b] Substrate, municipal household solid wate; substrate to inoculum ratio, 0.125
[c] Feed, cow manure slurry 5.8% TS based;
[d] Feed, cow manure slurry 10% TS based;
[e] Substrate, sugar beet tailings; substrate to inoculum ratio, 0.10

Table 4-3. Bacterial 16S rRNA gene library of the inoculum, digester 1 and digester 2 liquors.

	Incoulumn	Digester 1	Digester 2
Clone Analyzed	94	90	87
Phylotypes	90	58	76
Coverage	90.42%	93.33%	95.40%
Bacteria			
[a]*Actinobacteria*	[b]1.11	1.72	-
[c]*Rubrobacterales*	[d]1.11	-	-
Coriobacteriales	-	1.72	-
Bacteroidetes	1.11	-	1.32
Bacteroidales	1.11	-	1.32
Firmcutes	32.22	51.72	23.68
Bacillales	-	6.90	-
Clostridiales	23.33	44.83	18.42
Thermoanaerobacteriales	8.89	-	5.26
Proteobacteria	-	3.45	-
Betaproteobacteria	-	1.72	-
Hydrogenophilales	-	1.72	-
Synergistetes	13.33	20.69	3.95
Synergistales	13.33	20.69	3.95
Tenericutes	-	-	1.32
Acholeplasmatales	-	-	1.32
Thermotogae	52.22	22.41	69.74
Thermotogales	52.22	22.41	69.74

[a] Bacterial phyla are named according to National Center for Biotechnology Information (NCBI) taxonomy database
[b] Phylum frequencies are calculated as a phylogenenic group in percentage of the total sequences analyzed
[c] Bacterial orders are named according to National Center for Biotechnology Information (NCBI) taxonomy database
[d] Order frequencies are calculated as a phylogenenic group in percentage of the total sequences analyzed

Table 4-4. Archaeal 16S rRNA gene library of the inoculum, digester 1 and digester 2 liquors

	Incoulumn	Digester 1	Digester 2
Clone Analyzed	44	41	43
Phylotypes	42	41	42
Coverage	100.00%	97.73%	97.67%
Archaea			
[a]*Crenarchaeota*	[b]62.8	53.66	97.62
[c]*Thermoproteales*	[d]62.8	53.66	97.62
Euryarchaeota	38.1	46.36	2.38
Methanobacteriales	-	7.32	2.38
Methanomicrobiales	37.2	24.39	-
Methanosarchinales	-	14.63	-

[a] Archaeal phyla are named according to National Center for Biotechnology Information (NCBI)t axonomy database

[b] Phylum frequencies are calculated as a phylogenenic group in percentage of the total sequences analyzed

[c] Archaeal orders are named according to National Center for Biotechnology Information (NCBI) taxonomy database

[d] Order frequencies are calculated as a phylogenenic group in percentage of the total sequences analyzed

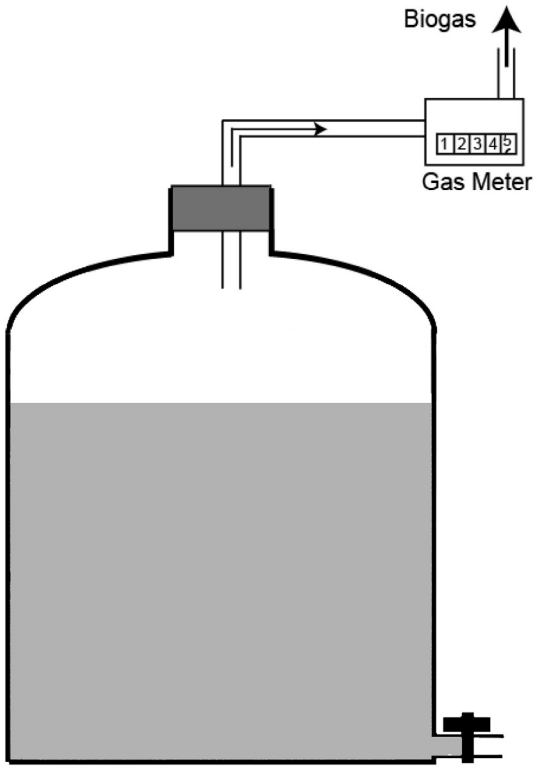


Figure 4-1. Digester and gas meter configuration

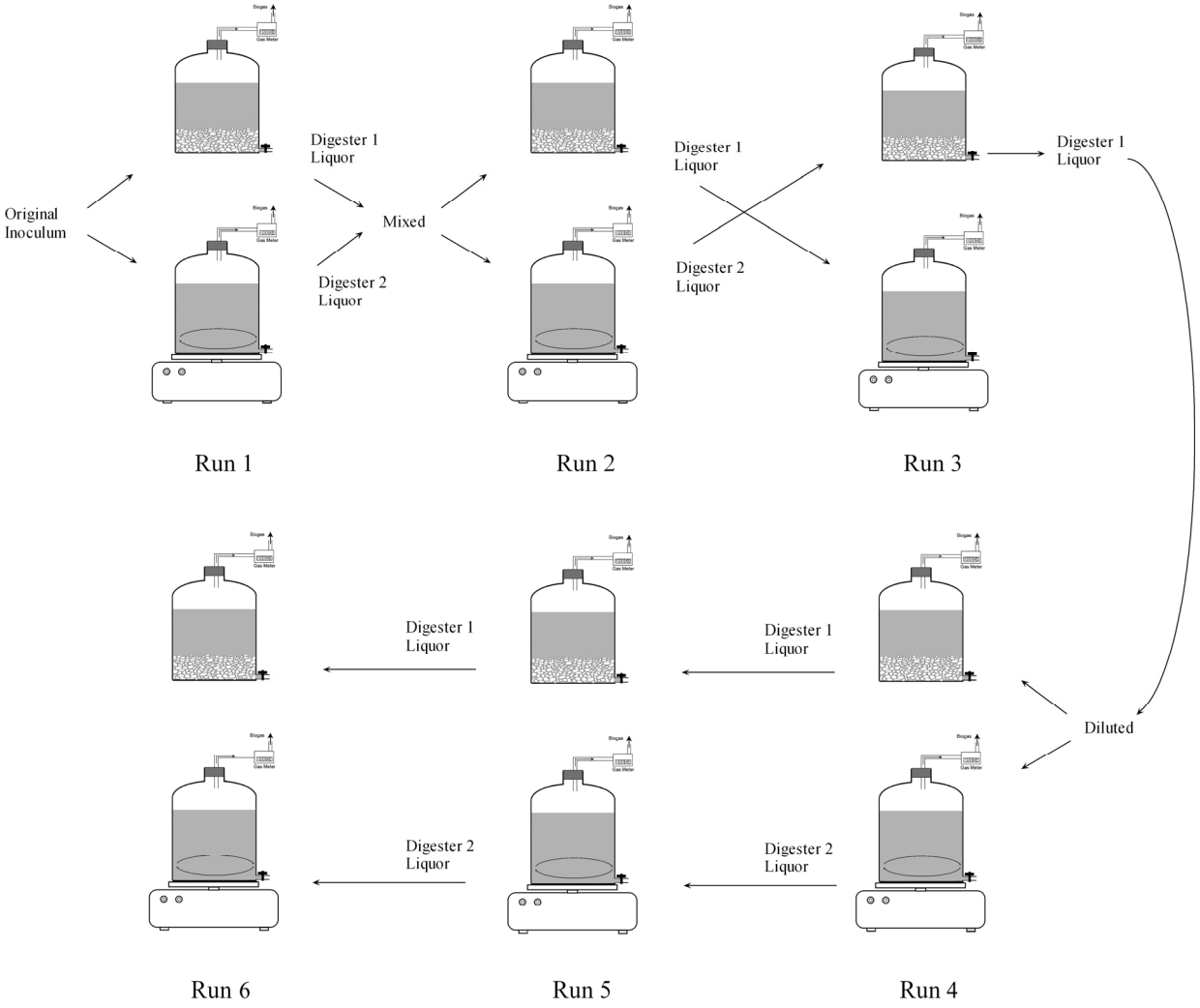


Figure 4-2. Inoculum used for experimental set 1 and 2

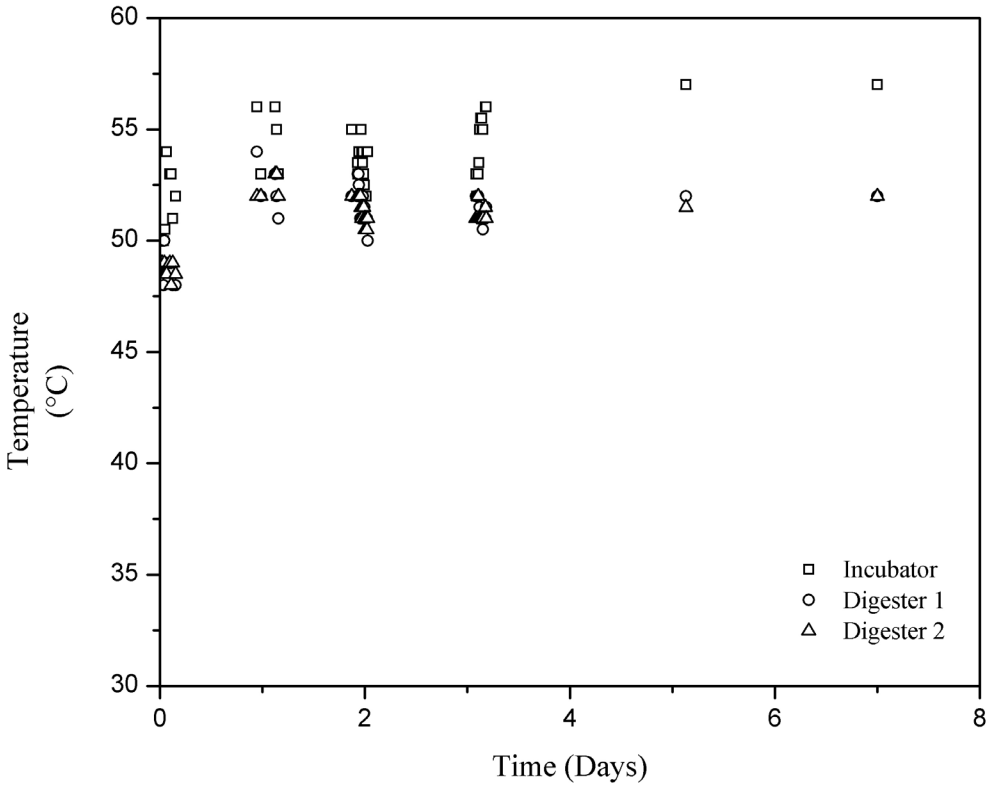


Figure 4-3. Temperature profiles of incubator, digester 1 and digester 2

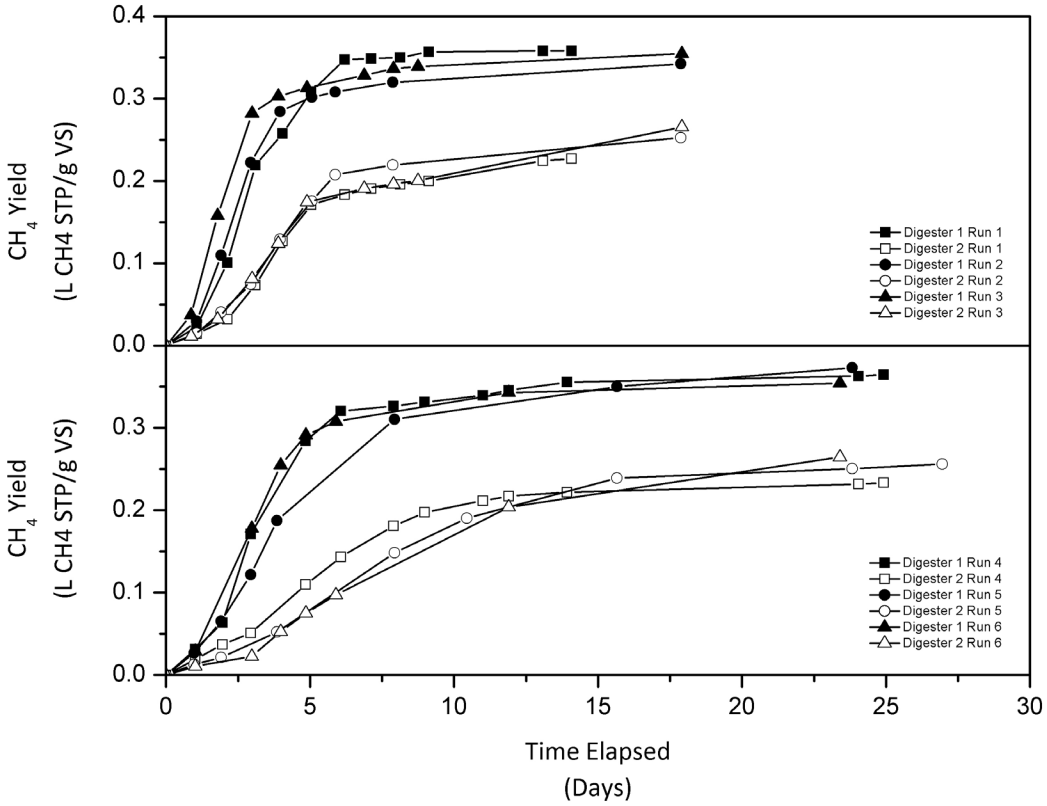


Figure 4-4. Methane yield in digester 1 and 2 from all 6 runs

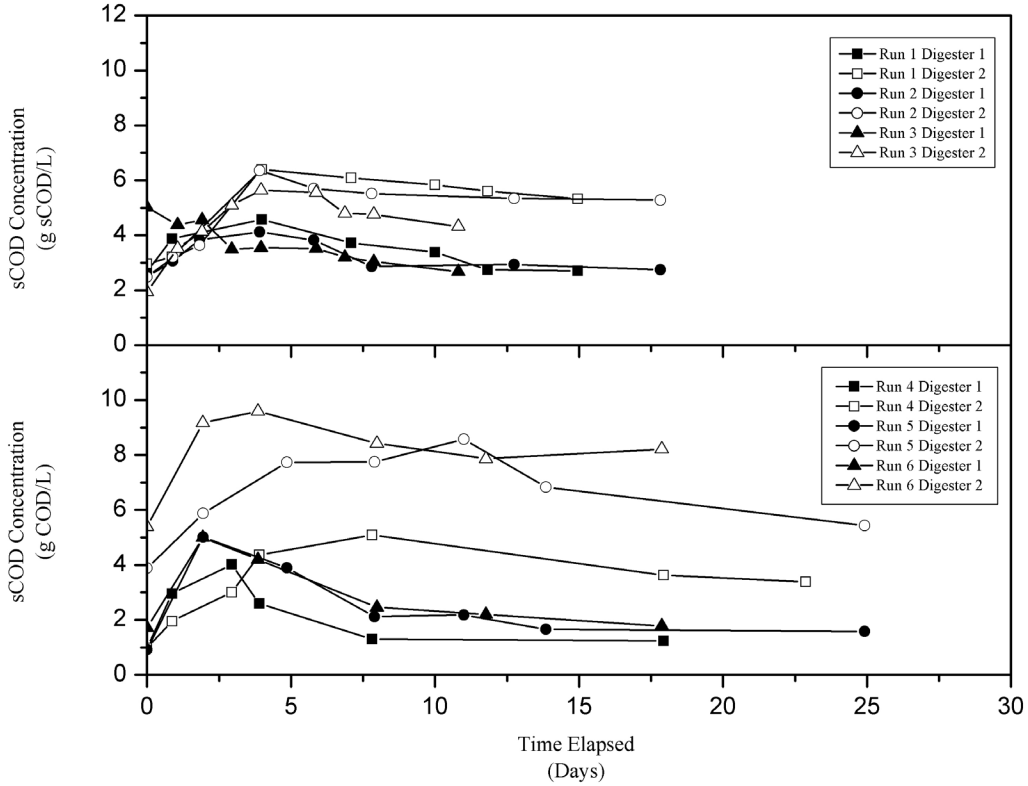


Figure 4-5. SCOD concentration in digester 1 and 2 from 6 runs

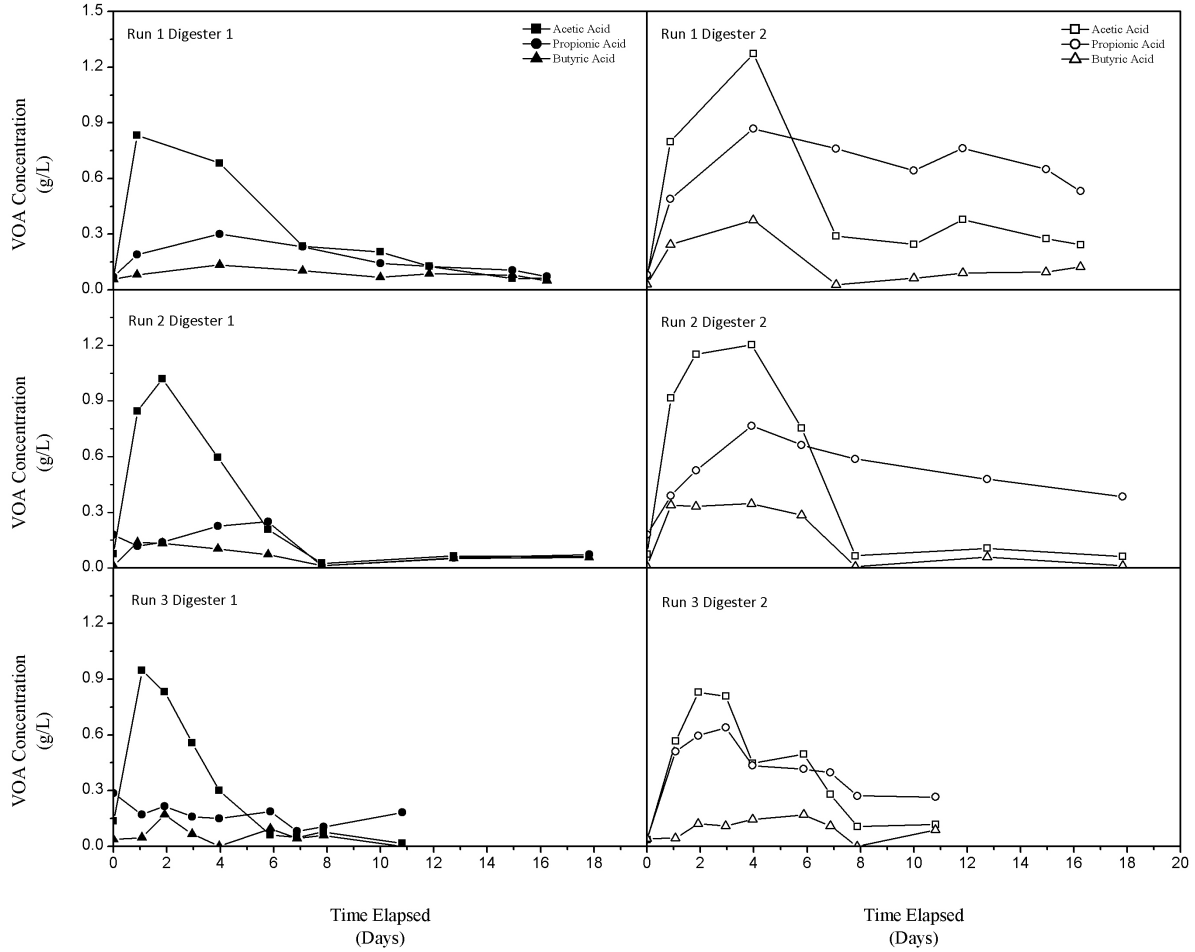


Figure 4-6. VOA concentration in digester 1 and 2 from three runs

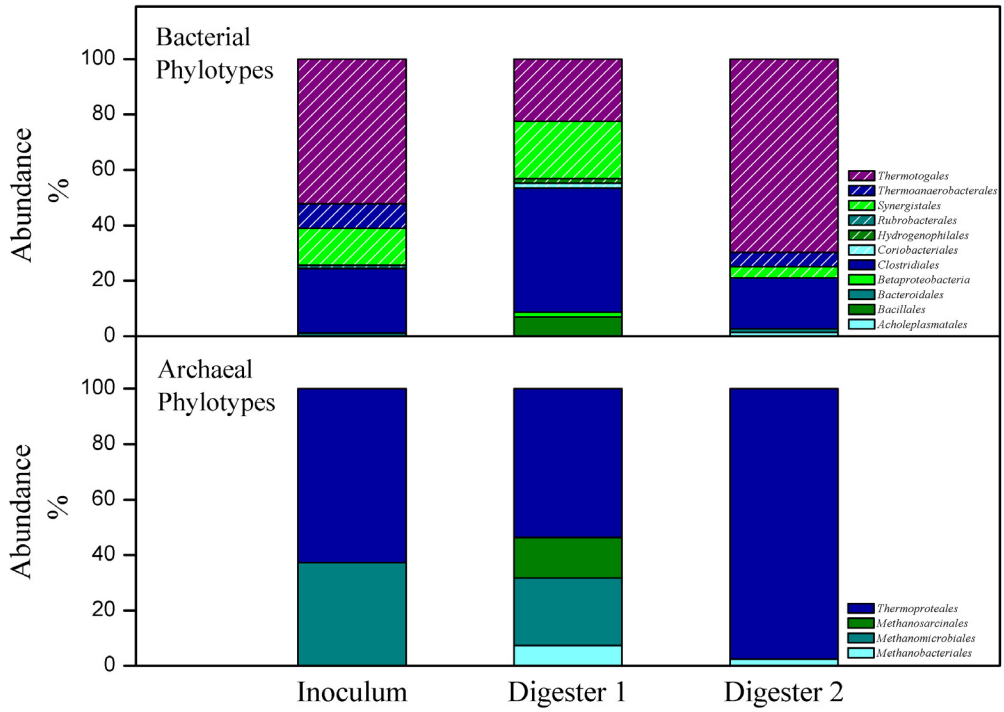


Figure 4-7. The rank abundances of bacterial and archaeal phylotypes in the original inoculum, and digester 1 and digester 2 liquors

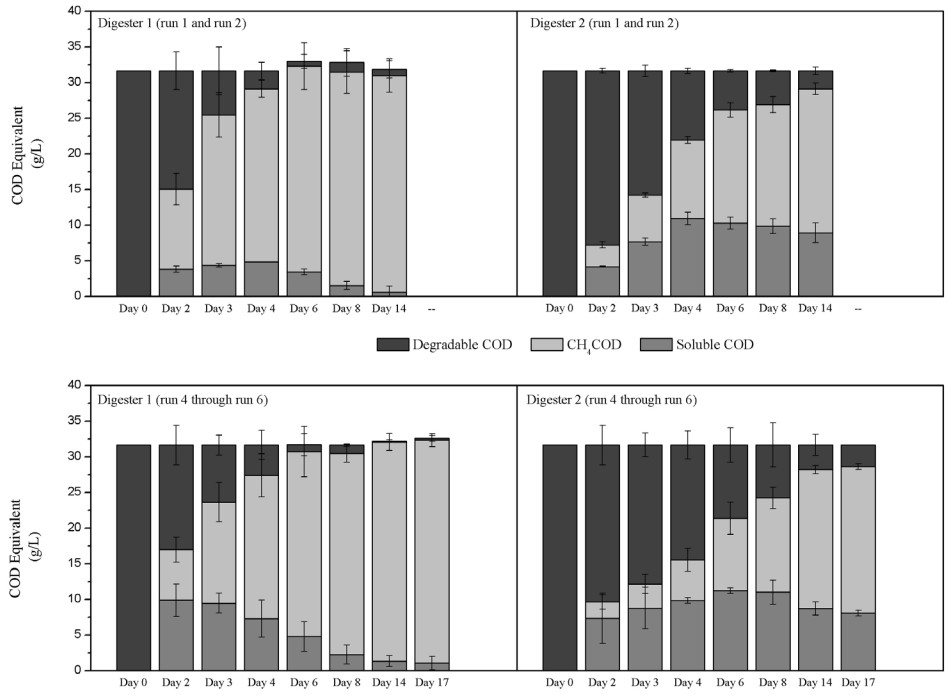


Figure 4-8. Average COD balance of digesters 1 and 2

CHAPTER 5
MICROBIAL POPULATION IDENTIFICATION AND DYNAMICS DURING BATCH THERMOPHILIC ANAEROBIC DIGESTION AT MIXED AND NON-MIXED CONDITIONS

Summary

Two trials of anaerobic digestion of sugar beet tailings at mixed and non-mixed condition were carried out in addition to those reported in Chapter 4. Digestion conditions were similar to that conducted in Chapter 4. The results confirmed digestion performance was affected by mixing, where lower methane yield and delayed methane production rate were observed. This was further verified by the accumulation of high sCOD and persistence of VOAs (particularly propionic acid) in the mixed digester. To elucidate the different digestion performance, microbial community analysis was conducted using 454 pyrosequencing. The microbial community of the mixed and non-mixed digester shifted significantly from that of the inoculum and adapted to a stable structure swiftly. Methanogens were more abundant and diverse in the non-mixed digester, including *Methanobacterium*, *Methanoculleus*, *Methanosarcina* and *Methanothermobacter*. Syntrophic bacteria such as *Desulfotomaculum*, *Pelotomaculum* and *Syntrophomonas* were also identified. They were known to play a crucial role in propionate and butyrate degradation. In comparison, relative abundance of syntrophic bacteria and methanogens, particularly hydrogenotrophic methanogens, were noticeably lower in the mixed digester. This agreed with the observation of low methane yield and accumulation of propionic acid. Hydrogen producing bacteria were identified in relatively abundant numbers in the mixed digester. Among them, *Ruminococcus* species were of special interest, which were reported to produce hydrogen in the presence of hydrogen scavengers. It was hypothesized that presence of hydrogen inhibited VOA degradation and resulted in lower methane yield in the mixed digester.

Background

Classically, environmental microbial communities are analyzed by construction of 16S rRNA clone libraries and subsequent sequencing of individual clones. The approach, termed Sanger sequencing, has been applied for many biogas-producing microbial communities (Huang, Zhou et al. 2002; Huang, Zhu et al. 2005; Klocke, Mahnert et al. 2007; Klocke, Nettmann et al. 2008) and was used in Chapter 4. Traditional Sanger sequencing technology has its limitations in revealing the whole complexity of microbial community because a relatively small amount of clones are sequenced. New development of high-throughput sequencing technologies such as 454 barcoded pyrosequencing not only eliminates the laborious step of preparing clone libraries, but also makes large scale environmental sequencing cost effective and keeps the bias small (Ronaghi, Karamohamed et al. 1996). However, phylogenetic assignments based on barcoded pyrosequencing could be less precise due to the short read lengths compared to relatively larger sequence lengths resulting from classical 16S rRNA sequencing. Barcoded pyrosequecing has been reported to be applied in microbial population identification in anaerobic digestion and one of those studies revealed that *Clostridia* is the most prevalent taxonomic class, and *Methanomicrobiales* is dominant among methanogenic *Archaea (*Krause, Diaz et al. 2008). 16s rDNA library analysis has revealed distinct microbial community structures between mixed and non-mixed anaerobic digesters digesting sugar beet tailings. However, due to the limitation of generating small number of sequencing results, the analysis was carried out once for each digester and did not provide the information of the changing of microbial communities with time. Thus, 454 pyrosequencing was utilized as the approach for further exploration of the microbial community structure and its dynamics. Similar to

Chapter 4, two runs of anaerobic digestion of sugar beet tailings was carried out at mixed and non-mixed condition. Digestion performances such as CH_4 production, SCOD and VOA concentrations were monitored and compared. The microbial communities were identified and characterized by analyzing sequence data obtained from using 454 barcoded pyrosequencing.

Method and Materials

Feedstock

The sugar beet tailings was provided by America Crystal Sugar Company, Minnesota and stored at 4 C before using. The tailings were washed using tap water before loading to the digesters. Wash water was discarded.

Anaerobic Reactors and Operation

Two digesters (digester 1 and 2) of 5 L were constructed by modifying Pyrex glass jars. Gas production from the digesters was measured by a positive displacement gas meter. Refer to Chapter 4 for detailed information about the anaerobic digesters and gas meters. Digester 1 was operated at non-mixed condition and added with a bulking agent. Digester 2 content was continuously mixed. A 50.8 mm x 9.5 mm PTFE coated polygon bar was placed in the digester and the mixing of digester content was achieve by using a large volume magnetic stirrer (Siceneware Cool Stirrer). Digester 1 and 2 were placed in a 55°C incubator throughout the experiments. Two experimental trials were carried out. In trial 1, digester 1 and 2 were loaded with 0.3 kg (wet weight) of washed tailings and maintained at 55 °C. 2 kg of bulking materials (lava rocks from landscaping supplier, 0.025 m in average size) were added into digester 1 along with substrates to prevent substrate compaction and floatation. Digester 2 was not added bulking materials and the digester content was continuously mixing at 100 RPM. Each digester was

inoculated with 3 L inoculumn taken from an anaerobic digester that has been digesting with sugar beet tailings for months. Trial 1 was ended when the gas production from both digesters was low. Digester 1 and 2 were then emptied and washed thoroughly. Residual substrates were discarded while the digester liquor from trial 1 were saved and used as inoculums to initiate trial 2. About 3 L digester 1 and 2 liquors were recovered and used to inoculate digester 1 and 2 in trial 2, respectively.

Additionally 3 runs were carried out in the mixed digester (digester 2). In the 3 runs, digester 2 was fed with only propionic acid (analytical grade 99.5%). This was to demonstrate whether propionic acid degradation was affected by the mixing intensity used digester 2. Refer to Appendix B for detail.

Chemical Analysis

Total Solids (TS) and Volatile Solids (VS) contents were determined for the feedstock sugar beet tailings. Gas composition (CH4 and CO2) was analyzed using Fisher Gas Partitioner (Model 1200). sCOD was determined using Hach COD vials and measured using Hach DR/890 Colorimeter. VOA analysis was conducted using Shimadzu gas chromatograph (GC-9AM equipped with a flame ionization detector) for acetic, propionic, isobutyric, butyric, isovaleric and valeric acid concentrations. For analyses details, refer to analysis section in Chapter 4.

Molecular Biological Analysis

Microbial community analysis was conducted for the original inoculums and digester 1 and 2 liquors. Digester liquors were sampled at day 3, 15 and 18 for trial 1 and day 0, 4, 8, 11, 12 and 14 for trial 2. A total 17 samples were analyzed.

DNA Extraction and Purification

Total DNA was extracted and purified by using FastDNA Kit and PowerClean DNA kit, respectively, according to the manufactures's instruction . The quality of DNA was verified by agar gel electrophoresis running at 100V for approximately 50 minutes. Results were visualized in UV light. Extracted DNA was stored at -20 °C before using as a template for Polymerase Chain Reaction (PCR) amplification.

Polymerase Chain Reaction Amplification

For each sample, 16S rRNA gene was amplified using a composite forward primer and a reverse primer containing a unique 8-base barcode used to tag each PCR product (Hamady, Walker et al. 2008). Three independent PCR reactions were carried out for each of 17 samples. The reactions were performed in a 50µl volume, containing 20 to 30 ng of DNA template and 20µL of HotMasterMix™ [0.5U Taq DNA Polymerase, 45 mM KCl, 2.5 mM Mg^{2+}, and 200 µM of dNTP (5Prime GmbH)] and 1µL of barcoded primer (100 pmoles). The amplification protocol was as follows: one initial denaturation cycle at 94^{o}C for 3 minutes, 30 denaturation cycles at 94^{o}C for 45 seconds, annealing at 50^{o}C for 30 seconds, and extension at 65^{o}C for 90 seconds, and one final extension cycle at 65^{o}C for 10 minutes. The Prokaryote primer information is provided as follows.

Original primers (yellow tubes)

515 GTGTGCCAGCMGCCGCGGTAA

806R GGGGACTACVSGGGTATCTAAT

454 Primers (blue tubes)

F515 GCCTTGCCAGCCCGCTCAGGTGTGCCAGCMGCCGCGGTAA

A1-806R GCCTCCCTCGCGCCATCAG**AACGCACGCTAG**GGGGACTACVSGGGTATCTAAT

A2-806R GCCTCCCTCGCGCCATCAG**AACTCGTCGATG**GGGGACTACVSGGGTATCTAAT

A3-806R GCCTCCCTCGCGCCATCAG**AACTGTGCGTAC**GGGGACTACVSGGGTATCTAAT

A4-806R GCCTCCCTCGCGCCATCAG**AAGAGATGTCGA**GGGGACTACVSGGGTATCTAAT

A5-806R GCCTCCCTCGCGCCATCAG**AAGCTGCAGTCG**GGGGACTACVSGGGTATCTAAT

A6-806R GCCTCCCTCGCGCCATCAG**AATCAGTCTCGT**GGGGACTACVSGGGTATCTAAT

A7-806R GCCTCCCTCGCGCCATCAG**AATCGTGACTCG**GGGGACTACVSGGGTATCTAAT

A8-806R GCCTCCCTCGCGCCATCAG**ACACACTATGGC**GGGGACTACVSGGGTATCTAAT

A9-806R GCCTCCCTCGCGCCATCAG**ACACATGTCTAC**GGGGACTACVSGGGTATCTAAT

A10-806R GCCTCCCTCGCGCCATCAG**ACACGAGCCACA**GGGGACTACVSGGGTATCTAAT

A11-806R GCCTCCCTCGCGCCATCAG**ACACGGTGTCTA**GGGGACTACVSGGGTATCTAAT

A12-806R GCCTCCCTCGCGCCATCAG**ACACTAGATCCG**GGGGACTACVSGGGTATCTAAT

underline = 454 primers

in red = barcodes

Because each sample was amplified with a known tagged primer, an equimolar mixture of the PCR-amplified DNA from each sample can be sequenced and sequence can be assigned to samples based on the unique barcodes. Since there were 12 pairs of primers available, 17 samples were amplified in two batches (set 1 and 2) as specified in Table 5-1. Samples (1 inoculum sample, 6 trial 1 samples and 2 trial 2 samples) in Set 1 were tagged and amplified with primers A1 through A9. Samples (8 trial 2 samples) in Set 2 were tagged and amplified with primers A1 though A5 and primes A10, A11 and A12 . Three replicated PCR products were combined for each sample and purified using the QIAquick PCR Purification Kit (Qiagen, CA) and quantified using on-chip gel electrophoresis with Agilent 2100 Bioanalyzer and DNA Lab Chip® Kit 7500. The quantity of each PCR product was made equal within a set. Set 1 contained 3888 ng DNA in total with

each PCR product being 432 ng. Set 2 contained 3840 ng DNA with each PCR product being 480 ng. Set 1 and set 2 were then sent to Interdisciplinary Center for Biotechnology Research (ICBR) at University of Florida for pyrosequencing. Total 11536 sequence reads with average alignment length of 239 bp and 3237 sequence reads with average alignment length of 238 bp were produced for set 1 and set 2, respectively. To identify 16 rDNA reads information, a homology search to the RDP (Ribosomal Database Project) database was conducted by means of BLAST. Search results were grouped into Operational Taxonomic Units (OTUs) at different levels (Phylum, Class, Order, Family and Genus) according to the nearest neighbor (best matching BLAST-hit) in the RDP database. Phylotype was defined as a group of 16s rDNA sequences with 95% identity (taxonomic genuslevel). A non-parametric estimation of maximum species richness was derived using Chao1 estimate (Schloss, Westcott et al. 2009). The sampling coverage was then defined as number of OTUs by Chao1 estimated richness. The following dissimilarity cut-offs were used to represent OTUs at different taxonomic ranks, 5%, genus; 10%, family, order and classes, and 20% , phylum (Hong, Bunge et al. 2006).

Results

Characteristics of Feed Substrates

TS and VS contents of sugar beet tailings, loadedquantities and packing density were determined for 2 trials and presented in Table 5-1. The average TS and VS contents of sugar beet tailings were 10.90% (wt/wt) and 9.68% (wt/wt), respectively.

Methane Production

Cumulative CH_4 yield for digester 1(non-mixed) and 2 (mixed) in two trials are shown in Figure 5-1. CH_4 production rate for digester 1 peaked at 0.70 m^3 d^{-1} (kg

VS)$^{-1}$ on day 5, and 0.34 m^3 d^{-1} (kg VS)$^{-1}$ on day 11 for digester 2. Digester 1 achieved CH_4 yield of 0.37 m^3 CH_4 at STP (kg VS)$^{-1}$ and digester 2 achieved CH_4 yield of 0.24 m^3 CH_4 at STP (kg VS)$^{-1}$ at the end of trial 1. Trial 2 was started by flooding digester 1 and 2 with digester liquor left in trial 1. Digester 1 showed higher CH_4 production rate than in run 1 whereas digester 2 showed similar production rate. Maximal CH_4 production rate for digester 1 and 2 were 0.94 m^3d^{-1}(kg VS)$^{-1}$ on day 4 and 0.35 m^3d^{-1}(kg VS)$^{-1}$ on day 7, respectively. The CH_4 yield was 0.35 m^3 CH_4 at STP kg VS^{-1} for digester 1 and 0.26 m^3 CH_4 at STP kg VS^{-1} for digester 2 and the end of run 2.

Degradation of Organic Matters

Profiles of soluble COD (sCOD) and VOA concentration of digester 1 and 2 were shown in Figure 5-2. sCOD concentration of both digesters initially accumulated, reached a maximum and decreased to a minimum. Digester 1 showed less sCOD accumulation and faster degradation rate than digester 2 in both trials. This was consistent with the higher CH_4 production rate of digester 1 seen in Figure 5-1. Acetic acid was detected with the highest concentrations among VOAs in digester 1. The concentration decreased rapidly to a negligible amount as methane was produced. Butyric and propionic acid were detected with the second and third highest concentrations, respectively. As for acetic acid, degradation of butyric acid was rapid and started from day 3. Though accumulation of propionic acid was low in trial 1, the degradation of was delayed and did not begin until near the end of the trail 1. In trial 2, propionic acid degraded along with acetic and butyric acid which occurred shortly after day 4. In digester 2, acetic acid, propionic acid and butyric acid have dominant concentrations over other VOAs. The initial increase and degradation of the three acids were noticeably slower in digester 2 than in digester 1.

Concentration of acetic acid and butyric acid peaked between day 8 to day 11 and it dropped thereafter. This time frame coincided with the occurrence of maximal CH_4 production rate in digester 2. Distinct from other VOAs, there was no evidence of degradation of propionic acid throughout trail 1 and 2 for digester 2. Accumulation of propionic acid was around 400 mg/L in trial 1 and was further increased to above 1000 mg/L in trial 2

Microbial Community Analysis

All 16 rDNA reads were taxonomically classified by means of BLAST search to RDP reference database. Sampling coverage at dissimilarity cut-off of 5%, 10% and 20% (corresponding to genus, family/order/class and phylum, respectively) was compared and showed in Figure 5-3. At higher taxonomic ranks (phylum, class and order), a defined OTU tended to contains a cluster of 16s rDNA reads whereas number of singleton OTUs increased significantly at the low rank (genus), resulting in a low Chao1 richness. Therefore, the microbial community was analyzed at order rank (sequence identity of 90% or more) the relative abundance (appearance frequency) of detected OTUs were showed in Table 5-3. Chao1 estimation at order rank gave coverage of approximately 70% or more for most samples. The structure of microbial community in digester 1 and digester 2 shifted significantly from the inoculum community. Bacterial phyla *Proteobacteria* and *Firmcutes* were found dominant in inoculum microbial community with relative abundance being 41% and 33%, respectively. Numbers of OTUs at 10% cut-off belonged to *Proteobacteria* were much more than that of *Firmcutes*, making *Proteobacteria* the most diverse phylum. Phylotypes *Hydrogenophaga*, *Arcobacter* and *Pseudomonas* were found abundant within phyla *Proteobacteria* while phylotypes *Bacillus*, *Clostridium*, *Coprothermobacter*, *Cryptanaerobacter* and *Proteiniborus* were abundant within

phylum *Firmcutes*. As the digestion began, the relative abundance of *Proteobacteria* quickly diminished in both digesters. In contrast, the abundance of *Firmcutes* was maintained and was identified with similar phylotypes as in the inoculum. Bacteria phyla *Bacteroidetes* and *Thermotogae* were seen increasing abundance in both digesters as the digestion was progressing. Typical phylotypes identified in phylum *Bacteroidetes* included *Bacteroides* and *Petrimonas* (for both digesters). No defined phylotypes (95% identity, genus level) were not detected in phylum *Thermotogae*. The closest relative was identified as *Thermotogales* (order level). Although the microbial community of digester 1 and 2 shared aforementioned similarities at phylum rank (e.g., both abundant Firmcutes), they were distinct at genus rank (defined phlotype).Phylotypes *Acetanaerobacterium* and *Ruminococcus* in phylum and phylotype *Ruminofilibacter* in phylum *Bacteroidetes* were detected in digester 2 and exhibited increasing abundance with the development of the digestion. However, these phylotypes were either not detected or detected at a very low abundance in digester 1. Phylotype *Bacteroides* was detected in both digesters, but the abundance was significantly greater in digester 2 (40% versus 10%). Likewise, its relative abundance low at beginning (inoculum) showed an increase. Furthermore, digester 1 and digester 2 showed different distribution of phylum *Synergistales*. While *Synergistales* gradually developed and reached the relatively high abundance of 15% in digester 1 (after 18 days), it was detected with constantly low abundance in digester 2. *Anaerobaculum* was identified as the dominant phylotype in *Synergistales*. Compared to bacteria, archaea was identified at low abundance for inoculum digester 1 and digester 2 samples. As expected, methanogens were found abundant within phylum *Euryarchaeota* and the main phylotypes included *Methanobacterium*, *Methanoculleus*, *Methanosarcina* and *Methanothermobacter*.

Abundance of methanogens increased with the digestion proceeding. The abundance was found highest at day 5 and day 4 (cumulative time day 32) for digester 1 in trial 1 (abundance 6%) and trial 2 (11%), respectively. Development of methanogens seemed delayed in digester 2 with the highest abundance (4%) seen at day 18 in trial 1. In trial 2, the abundance of methanogens continuously reduced and no significant detections was obtained near the trial end.

Discussion

Digestion Performance Comparison

As concluded in Chapter 4, digester 1 (nom-mixed) achieved higher CH_4 yield and CH_4 production rate than digester 2 (mixed). Digester 1 in trial 2 was inoculated with digester 1 liquor recovered from trial 1, which had been adapted to tailings decomposition. Therefore, digester 1 showed higher CH_4 production rate in trial 2 than in trial 1. This was consistent with the higher sCOD degradation rate of digester 1 in trial 2 though the initial increase of sCOD in both trials was both around 8000 mg/L. The adapted digester 1 has a maximum daily CH_4 production rate of 0.94 $m^3d^{-1}(kg\ VS)^{-1}$, which agreed well with the maximum daily CH_4 production rate of the nonmixed digester in Chapter 4. The initial sCOD accumulation of digester 2 was around 10000 to 12000 mg/L, and a lagged period of 8 to 10 days was observed before degradation. As for digester 1, digester 2 inoculum was adapted in trial 2, but neither CH_4 production nor substrate decomposition showed accelerated rate. This suggested the mixing affected digester 2 performance adversely.

The VOA profiles revealed that the initial accumulation of acetic acid of both digesters (trial 1 and trial 2) was in a close range of 1.3 g/L. However, acetic acid in digester 2 persisted for 10 to 12 days before degradation whereas acetic acid in digester 1 degraded substantially. Conversion of propionic acid appeared

problematic in digester 2 as high accumulation was observed. In anaerobic digestion, acetogenic bacteria ferment propionate to acetate which was then utilized by methanogens to CH_4. The pathway plays an important role in methanogenesis (Fukuzaki, Nishio et al. 1990). The high accumulation of propionic acid in digester 2 probably suggested propionate degradation was inhibited. The accumulation was further increased in trial 2, indicating digestion inhibition could be exacerbated if the digester liquor was exposed to mixing and used for inoculation repeatedly. It was well accepted that accumulation of propionic acid indicated an anaerobic process instability (Hill, Cobb et al. 1987; Fox, Suidan et al. 1988; Nielsen, Uellendahl et al. 2007) , but it can also be considered as the cause of the process failure as its accumulation had been reported inhibitory for methanogens (Barredo and Evison 1991).

Microbial Community

16s rDNA sequences reads were compared to the entries of RDP database, and grouped to OTUs at 5 taxonomic ranks according to their nearest neighbors (Best BLAST hits). Most reads could only be assigned to higher taxonomic ranks. Number of BLAST hits obtained at genus rank was significantly reduced, suggesting most members of microbial community in anaerobic digestion are unexplored.

Microbial community shift from the inoculum (day 0) was observed for digester 1 and 2. The shifting occurred swiftly. At day 3 the community structure was significantly different from the original while the changes were small thereafter for digester 1 and 2, respectively. The swift change was expected as the result of quick adaption to the new environment of the microbial consortium in the inoulum. The newly adapted consortium managed to maintain their community structure throughout the experiment period.

Though the microbial community between digester 1 and 2 were mostly distinct, they shared some similarity. *Proteobacteria* phylotypes *Hydrogenophaga* and *Pseudomonas* were identified in the inoculum but reduced to low abundance in both digesters shortly after digestion began. *Hydrogenophaga* and *Pseudomonas* are recognized for their degradative abilities in the presence of oxygen (Willems, Busse et al. 1989; Juwarkar, Singh et al. 2010). In trial 1, the digesters were inoculated with stored incolum that may have been exposed to aerobic environment and thus favored the growth of some aerobes. *Hydrogenophaga* and *Pseudomonas* were taken over quickly by anaerobes as anaerobic digestion became active. Relative abundance of some facultative bacteria as *Acrobacter* (Vandamme, Falsen et al. 1991) also decreased significantly in digester 1 and 2. *Firmcutes* Phylotypes *Bacillus*, *Clostridium*, *Coprothermobacter* and *Proteiniborus* were also abundant in the original inoculum. Their relative abundance maintained throughout the digestion for digester 1 and 2. Abundance of *Clostridium* was increased at early stage of the digestion (day 3 to day 5) and was the dominant species in the period. These bacteria are commonly identified in anaerobic digesters and involved in degradations of a wide range of carbohydrates and proteins and producing acetic acid, hydrogen and carbon dioxide as typical fermentation products (Etchebehere, Pavan et al. 1998; Niu, Song et al. 2008; Cardinali-Rezende, Moraes et al. 2011; Kim, Shin et al. 2011). Hydrolysis is usually considered as the first step in anaerobic digestion and this could explain the increase of hydrolytic bacteria like *Clostridium* at beginning of the experiment. Phylotypes *Bacteroides* and *Petrimonas* within phylum *Bacteroidetes* were seen in greatly increased abundance after digestion started. These bacteria were reported to play important roles in hydrolysis and acidogenesis as well , among which, *Bacteroides* are known to be cellulolytic and were expected

to help into degrading cellulose which was detected as one of the main components in sugarbeet tailings (Grabowski, Tindall et al. 2005; Liu, Pullammanappallil et al. 2008; Russell, Muck et al. 2009; Ziganshin, Schmidt et al. 2011).

Bacteria order *Synergistetes* showed differences in dynamics between digester 1 from digester 2. Only one bacteria phylotype *Anaerobaculum* was identified within the order. Its relative abundance gradually increased, reached and maintained at 15% in digester 1. Digester 2 had a constantly lower abundance of *Anaerobaculum* compared to that of digester 1 at the same period. The abundance exhibited a decreasing pattern in digester 2 from trial 1 to trial 2, and in trial 2 it was kept overall low. Identification of *Anaerobaculum* in anaerobic digestion were reported and they were known to degrade peptide and a limited number of carbohydrates (Rees, Patel et al. 1997; Baena, Fardeau et al. 1998; Manes, Fernandez et al. 2001; Menes and Muxi 2002; Sousa, Smidt et al. 2007; Weiss, Jerome et al. 2008). Sugihara et al studied the propionate degrading ability of a microbial consortium by subjecting the enriched culture to sequencing fed batch cultivation with a periodical impulse of propionate (Sugihara, Shiratori et al. 2007). The consortium population analysis revealed an *Anaerobaculum* related species being the major bacterial constituent. Though *Anaerobaculum* was not recognized as a syntrophic propionate utilizing bacterium, Sugihara's study seemed to imply they play a role in propionate degradation. Therefore, it was postulated that the fewer growth of *Anaerobaculum* probably resulted in accumulation of propionic acid in digester 2. *Anaerobaculum sp.* related species were also reported to persist in a LCFA (long chain fatty acid) enriched cultures, suggesting a possible LCFA degrading ability (Manes, Fernandez et al. 2001; Hatamoto, Imachi et al. 2007; Palatsi, Viñas et al. 2011). Furthermore, hydrogen (H_2) inhibition was reported on

glucose utilization by *Anaerobaculum mobile* (Menes and Muxi 2002). This could explain the low growth of in digester 2 where free H_2 were speculated producing (discussed later).

Phylotype *Ruminofilibacter* was identified in digester 2. The relative abundance increased greatly after the digestion began (for both trials) and then depressed slowly after reaching a maximum. The phylotype was observed with constantly low abundance in digester 1. *Ruminofilibacter* was detected in anaerobic digesters and shown to have pronounced xylanolytic activity (Kröber, Bekel et al. 2009; Weiß, Zankel et al. 2011). It might suggest that xylan being released from tailings in digester 2 as a result of hydrolysis.

Bacterial phylotypes *Desulfotomaculum*, *Pelotomaculum* and *Syntrophomonas* were detected in both digester 1 and 2 at low abundance (below 2%). Species in these genera are well known as syntrophic bacteria that play crucial role degradation of short chain fatty acid such as propionate and butyrate (Plugge, Balk et al. 2002; de Bok, Harmsen et al. 2005; Müller, Worm et al. 2010). The presence of syntrophic bacteria at low levels agreed with the study of McMahon et al. (McMahon, Zheng et al. 2004). This might indicate effective propionate or butyrate degradation does not require high abundance of syntrophic bacteria. Similar to syntrophic bacteria, methanogens appeared at low abundance throughout the digestion (compared to the total bacteria). This generally agreed with the reported methanogen abundance ranging from 0.1% to 15% (relative abundance of the total microbial population) (Solera, Romero et al. 2001; Huang, Chen et al. 2003; Dar, Kleerebezem et al. 2008; Shin, Lee et al. 2010). Identified methanogens were closely related to *Methanobacterium*, *Methanoculleus*, *Methanothermobacter*, *Methanosarcina* and an uncharacterized methanogenic archaeon CH1270. Among

them, members in *Methanobacterium*, *Methanoculleus*, *Methanothermobacter* are hydrogentrophic (Xun, Boone et al. 1989; Maestrojuan, Boone et al. 1990; Elberson and Sowers 1997; Valentine, Blanton et al. 2000; Shcherbakova, Rivkina et al. 2011). *Methanosarcina* species are mostly aceticlasic but are also able to use H_2 (Kotsyurbenko, Chin et al. 2004). Metabolic pathway of the uncharacterized methanogen is unknown, which was detected in adult chicken ceca (Saengkerdsub, Anderson et al. 2007). Methanogens population dynamics exhibited changing abundance over time. Increased abundance was usually associated with acetic acid degradation. For instance, a marked increase in methanogen abundance was seen from day 3 to day 5 (trial 1) for digester 1 and during the same period (day 4) acetic acid was decreased sharply. This indicated CH_4 was mainly produced through aceticlastic way. In average, the abundance of methanogens and syntrophic bacteria in digester 2 was lower than in digester 1, specially hydrogenotrophic methanogens. The phenomenon could be the consequence of disrupted spatial juxtapositions of syntrophic bacteria and methanogens resulting from continuous mixing (Conrad, Phelps et al. 1985; Dolfing 1992; McMahon, Stroot et al. 2001). However, the mixing utilized for digester 2 was not regarded intense as discussed in Chapter 4. The disruption was expected to be limited. Therefore, an alternative hypothesis of H_2 inhibition was made and discussed as follows.

Bacteria phylotypes *Acetanaerobacterium* and *Ruminococcus* were found to be relatively abundant in digester 2. They are recognized to degrade cellulose and produce hydrogen as one of the fermentation products (Ren, Xing et al. 2007; Jindou, Brulc et al. 2008; Saraphirom and Reungsang 2010; Mosoni, Martin et al. 2011). Application of species in *Ruminococcus* has been widely used in hydrogen production on a variety of feedstock (Antonopoulou, Ntaikou et al. 2007; Ntaikou,

Gavala et al. 2008; Ivanova, Rákhely et al. 2009; Ntaikou, Koutros et al. 2009; Zhang, Banaszak et al. 2009; Ntaikou, Gavala et al. 2010). Though *Ruminococcus* species were reported to be mesophilic, their appearance has been confirmed in theromphilic anaerobic digesters as well (Yang, Tsukahara et al. 2008). This supports the detection of *Ruminococcus* in thermophilic digesters in this study. In anaerobic digestion, hydrogen could be generated through fermentation of intermediate products as sugars or VOAs (Lettinga, Field et al. 1997; Grady, Daigger et al. 1999; Angenent, Karim et al. 2004). Unfortunately, hydrogen (H_2) production from organic substrate is energetically unfavorable due to proton being a poor electron acceptor. The low midpoint redox potential of redox couple H^+/H_2 of -414 mV suggests the energetic difficulty in reducing H^+. Therefore, production of H_2 requires a strong reducing agent (Angenent, Karim et al. 2004). Common redox mediators involved in fermentation include NADH and $FADH_2$, and the redox potential of redox couples NAD^+/NADH and $FADH/FADH_2$ is -320 and -220 mV, respectively. The high redox potentials re expected to cause an energetic problem in reducing H^+ to H_2 (Stams and Plugge 2009). To make NADH oxidation and $FADH_2$ oxidation coupled to H^+ reduction energetically feasible, the partial pressure of H_2 has to be very low. Assuming the intracellular concentrations of the oxidized and reduced forms of NADH (or other electron carriers) are about equal, the maximum H_2 partial pressure that allow the process of hydrogen producing is determined by:

$$P_{H_2 \max} \leq \exp\left(\frac{2F(E^0_{H_2} - E^0_x)}{RT}\right) \quad \text{(Angenent, Karim et al. 2004)} \qquad (5.1)$$

where $P_{H_2 \max}$ is the allowed maximum H_2 partial pressure, E_x^0 is the redox potential of the electron carrier, $E_{H_2}^0$ is the redox potential of redox couple H^+/H_2,F is Faraday's constant, R is the ideal gas constant and T is the Kelvin temperature. The low H_2 partial pressure can be achieved in the presence of H_2 scavengers as homoacetogens and hydrogenotrophic methanogens. This is a syntrophic relation in which bacteria produce H_2 that is consumed as the substrate by methanogens, which in turn keep low H_2 partial pressure for the bacteria to grow through H_2 producing pathway. This process is referred to interspecies hydrogen transfer (Thauer, Jungermann et al. 1977). The development of syntrophic communities allows H_2 production to become energetically favorable and sustain degradation of organic compounds and production of CH_4. Bacteria that are only able to oxidize NADH coupled to hydrogen formation are considered obligate syntrophs. Production H_2 through NADH oxidation plays a crucial role in their energy metabolism as this is the only manner in which they can dispose the electron derived from substrate oxidation (Reddy, Wolin et al. 1972). Some well known obligate syntrophs include species belonging to genera *Syntrophomonas* (butyrate degrading), *Syntrophus* (benzoate degrading), *Syntrophobacter* (propionate degrading) and *Pelotomaculum* (propionate degrading) (Harmsen, Van Kuijk et al. 1998; Jackson, Bhupathiraju et al. 1999; Imachi, Sekiguchi et al. 2002; McInerney, Rohlin et al. 2007). Presence of H_2 scavengers as hydrogenotrophic methanogens typically existing in anaerobic digestion is essential for their survival. However, many fermenting bacteria can also oxidize NADH by reducing intracellular metabolites and their syntrophic relation with H_2 scavengers is facultative. A classic example is the study of fermentation product in a rumen bacterium pure culture *Ruminococcus albus*, which is known to have two alternative pathways for NADH oxidization: either by reducing acetyl-coenzyme A to

ethanol or by reducing H^+(Iannotti, Kafkewit.D et al. 1973). Since NADH oxidization through proton reduction is energetically difficult, energy metabolism of the bacterium mainly proceeds through ethanol fermentation pathway in the absence of H_2 scavengers. H_2 is still produced through the decarboxylation of the fermentation intermediate pyruvate. Ferrodoxin (Fd) in the key redox mediator in the conversion. The redox potential of the redox couple Fd (ox)/Fd (red) is -400 mV or lower depending on the source (Stams and Plugge 2009). The relative low potential suggests H_2 production through oxidation of reduced from of ferrodoxin is energetically easier. For this reason, anaerobic processes were developed for H_2 production using fermenting bacteria with similar metabolic pathways. In fact, most H_2 production observed in biological system can be attributed to decarboxylation of pyruvate (Angenent, Karim et al. 2004). Mixed cultures are usually preferred over pure cultures for H_2 production because process with mixed culture are simpler to operate and control, and may have a boarder choice of feedstocks (Valdez-Vazquez, Ríos-Leal et al. 2005). Yet in a mixed culture H_2 produced from fermentative bacteria could be readily converted CH_4 to due to the energetically favorable syntrophic pathway. A pretreatment process is needed to suppress the activity of methanogens, including heating, acidic treatment, adding inhibiting chemicals and so forth (Danko, Pinheiro et al. 2008). pH control is crucial and readily achievable for H_2 production because methanogenic activity drops sharply in an acidic environment (Chen, Lin et al. 2002). Though H_2 production is mostly carried out at pH 4 to 6 for H_2 production, H_2 was also detected in anaerobic processes operated at neural or near neutral pH (Fang and Liu 2002; Valdez-Vazquez, Ríos-Leal et al. 2005; Zhu, Parker et al. 2009). In these studies, a mixed culture was used to produce H_2 at either thermophilic or mesophilic temperature without applying a pretreatment to inhibit H_2

consuming microorganism. H_2 production was recorded at pH 7 (or slightly higher), though this pH was not determined optimal. Fang and Zhu reported a lower H_2 production (30% H_2 of biogas) and yield of 30 ml (g VS) $^{-1}$, respectively; whereas Valdez-Vazquez derived a much higher H_2 yield of 100 and 300 ml (g VS) $^{-1}$ from an anaerobic digester operated at mesophilic and thermophilic temperature, respectively. This contradicts with the syntrophic metabolism of H_2 producing bacteria. At neutral pH, methanogenic activity was expected (since methanogens was not suppressed) and the produced H_2 would be a substrate for CH_4 production. However, a recent study seemed to support the observation of free H_2 production in the presence of methanogens. Rychlik and May studied the effect of *Methanobrevibacter smithii* on growth rate, organic acid production and specific ATP activity of *Ruminococcus albus* in a co-ulture (Rychlik and May 2000). The result indicated no increase in the growth rate, acetate or ATP production, suggesting *Ruminococcus albus* did not receive energetic advantage from co-culturing with the methanogen. This disagreed with the experiment of Iannotti et al, in which *Ruminococcus albus* was shown to gain more ATP in the presence of a different H_2 consuming species *Vibrio succinogenes*. The discrepancy probably suggests the syntrophic interaction between H_2 producers and scavengers could be species dependent. Another indirect evidence came from Zhou et al, who investigated effect of methanogenic inhibitors on methanogens and three rumen bacteria *Fibrobacter succinogenes, Ruminococcus albus* and *Ruminococcus flavefaciens* (Zhou, Meng et al. 2011). While the anti-methanogen compounds effectively reduced the population of methanogens, the inhibiting effect was insignificant on none of the bacterial population, suggesting the syntrophic relation was weak or did not exist. Bacteria phylotype *Ruminococcus* along with *Acetanaerobacterium* were detected only in

digester 2 with high abundance. They were speculated to produce H_2 even at neutral pH (average pH 7.22 for digester 2, pH profile not shown), particularly for *Ruminococcus*, because it had been specifically proven to lack energetic advantage growing with methanogens. These bacteria could then choose ethanol fermentation for energy metabolism and produce H_2 through ferredoxin oxidation. Presence of free H_2 was expected to have adverse effect on anaerobic digestion. Obligate syntrophic bacteria are extremely sensitive to H_2. Even at low partial pressure, H_2 can inhibit syntrophic metabolism and thereby limit the substrate supply to methanogens. This could explain the low abundant methanogens detected in digester 2. This inhibition seemed to be cumulative as digester 2 community dynamics showed decreasing methanogen abundance with time. Inhibition of syntrophic interactions could also result in accumulation of VOAs because many syntrophs are known to degrade VOAs. This was verified by the high accumulation of propionic acid observed for digester 2, particularly in trial 2. Interestingly, some species of *Ruminococcus* were reported to produce propionate other than ethanol as fermentation products, which could also lead to propionic acid accumulation (Ren, Xing et al. 2005). The discussions above appeared to justify the possible H_2 production from digester 2. A fraction of substrate COD was converted to H_2 and led to a lower CH_4 yield. Presence of free H_2 inhibited growth of syntrophic bacteria, limiting acetate production and methanogensis. The lowered CH_4 yield could be attributed to both sCOD loss (H_2 production was not monitored in the study) and the inhibiting effect of H_2. Further investigations are required to verify these hypotheses. Support for the hypotheses were found in a similar case, where simultaneous H_2 and CH_4 production was observed in a single-stage anaerobic digestion though H_2 was produced under slightly acidic pH (6.3-6.6) (Banks, Zotova et al. 2010). The

produced gas mixture (CO_2 excluded) consisted of 20% of H_2 and 80% of CH_4. The estimate of H_2 content of biogas produced in the mixed digestion conducted in Chapter 4 is close to Banks' results.

The microbial community analysis conducted in Chapter 4 using Sanger sequencing showed less diversity. Some bacterial phylotypes at low abundance were detected by 454 pyrosequencing but were not seen in the classic identification method. This indicated 454 sequencing provided a better coverage of the microbial community (Huang, Chen et al. 2003). For instance, low abundant syntrophic bacteria (*Desulfotomaculum*, *Pelotomaculum* and *Syntrophomonas*) were not identified by the 16s rDNA clone library analysis. Nevertheless, detection of bacteria with high abundance was generally consistent (For the same period, i.e., day 3 after digestion began). Bacteria order *Clostridiales*, *Bacillales* and *Thermotogales* were detected abundant by both methods. Species in *Clostridiales* and *Bacillales* are mostly known to be involved in carbohydrates and protein degradation (Kim, Song et al. 2010). Both methods revealed low abundance of bacterial order *Synergistales* in digester 2 while marked higher abundance in digester 1. The Identified phylotypes of *Synergistales* were reported in association with syntrophic propionate-degrading community (Sugihara, Shiratori et al. 2007).

Closing Remarks

This study was conducted to further investigate the effect of mixing on anaerobic digestion, focusing on the microbial community dynamics. Two anaerobic digestion trials were carried out at mixed and non-mixed condition. The mixed digester 2 exhibited lower CH_4 yield, delayed CH_4 production and persistence of propionic acid. This confirmed the results obtained in Chapter 4. The microbial community analysis revealed the abundance of methanogens, syntrophic bacteria

and an *Anaerobaculum* species in the non-mixed digester but were markedly lower in the mixed digester. Their abundance showed a declining pattern over time, suggesting the mixed digestion could deteriorate if the digester liquor were reused for inoculation. Some bacteria known to produce H_2 through sugar fermentation were detected at in digester 2, among which, phylotype *Ruminococcus* were abundant. *Ruminococcus* species were found in many anaerobic processes for H_2 production and reported to not change fermentation pathways when co-cultured with methanogens. Therefore, it is possible that that methanogensis was affected in the mixed digester due to the presence of minor amount of H_2 which could have inhibited syntrophic interactions and VOA degradation.

Table 5-1. PCR amplifications of 16s rRNA genes using 12 barcoded primers (an uniform forward primer F515 and 12 different reverse primers A1 through A12)

Cumulative time		Day 0	Day 3	Day 15	Day 18		
Trial 1		Day 0 (inoculum)	Day 3	Day 15	Day 18		
	Digester 1	Primer A1	Primer A2	Primer A4	Primer A6		
	Digester 2	N/A	Primer A3	Primer A5	Primer A7		
Cumulative time		Day 28	Day 32	Day 36	Day 39	Day 40	Day 42
Trial 2		Day 0	Day 4	Day 8	Day 11	Day 12	Day 14
	Digester 1	Primer A8	Primer A10	Primer A12	N/A	Primer A3	N/A
	Digester 2	Primer A9	Prime A11	Primer A1	Primer A2	Primer A4	Primer A5
Set 1							
Set 2							

Table 5-2. Substrate characteristics and loaded quantities for digesters 1 and 2

Sugar beet tailings	Unit	Run 1	Run 2
TS	% (wt/wt)	10.91%±0.20%	10.90%±0.35%
VS	% (wt/wt)	9.39%±0.75%	9.94%±0.31%
Digestion loading	Unit		
Wet weight	kg	0.3	0.3
Dry weight	kg	0.033	0.033
Volatile matter	kg	0.028	0.030
Inoculumn added	L	3	3
Packing density	kg/m^3, wet weight basis	100	100
Packing density	kg/m^3, dry weight basis	10.91	10.9

Table 5-3. Analysis of 16S-rDNA sequence at taxon rank of order.

Time	Day 0	Day 3		Day 5		Day 18		Day 28		Day 32		Day 36		Day 39	Day 40		Day 42
	[a]I	[b]D1	[c]D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D2	D1	D2	D2
[d]*Bacteria*	[g]99.66	99.58	100.00	95.43	99.46	98.54	98.30	98.89	99.01	92.45	99.39	96.48	100.00	100.00	98.40	100.00	100.00
[e]*Acidobacteria*	0.27	0.00	0.00	0.00	0.00	0.15	0.00	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
[f]*Acidobacteriales*	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinomycetales	0.20	0.00	0.00	0.00	0.00	0.15	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bifidobacteriales	0.00	0.00	0.00	1.83	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chloroflexi	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anaerolinaeles	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	4.53	2.12	33.64	7.47	46.48	17.72	52.30	28.39	53.96	23.27	48.78	24.62	40.74	41.37	34.80	45.23	42.20
Bacteroidales	3.92	2.12	33.64	7.32	46.48	17.13	51.62	27.64	53.63	23.27	48.78	23.62	40.74	41.37	34.80	45.23	42.20
Flavobacteriales	0.14	0.00	0.00	0.15	0.00	0.59	0.00	0.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingobacteriales	0.47	0.00	0.00	0.00	0.00	0.00	0.68	0.00	0.33	0.00	0.00	1.01	0.00	0.00	0.00	0.00	0.00
Firmcutes	32.81	80.04	59.29	58.38	42.14	42.90	24.19	29.31	17.82	31.76	20.12	37.19	37.04	26.10	20.00	22.11	21.97
Bacillales	6.22	17.62	25.23	17.99	6.23	8.05	8.18	2.60	0.83	5.97	2.44	8.04	0.00	1.20	2.00	0.50	0.58
Clostridiales	20.09	52.87	33.33	30.49	35.37	27.82	14.82	21.71	15.51	24.21	17.68	26.13	36.11	24.10	17.60	20.10	19.65
Erysipelotrichales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Halanaerobiales	0.54	0.85	0.10	1.37	0.00	0.44	0.00	0.93	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactobacillales	0.00	0.42	0.31	0.15	0.00	0.15	0.00	0.00	0.00	0.31	0.00	0.50	0.00	0.00	0.00	0.50	0.00
Natranaerobiales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thermoanaerobacterales	5.82	8.28	0.31	8.38	0.54	6.44	1.19	3.90	1.49	0.94	0.00	2.51	0.93	0.80	0.40	1.01	1.73
Thermolithobacterales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Planctomycetes	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.00	0.00
Planctomycetales	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00	0.00	0.00	0.00	0.00	0.00	0.80	0.00	0.00
Proteobacteria	40.80	11.46	5.92	9.15	2.85	11.42	6.98	18.00	7.92	5.03	0.61	6.53	0.93	2.81	6.80	2.01	1.73
Alteromonadales	0.07	0.00	0.00	0.15	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Burkholderiales	32.81	8.92	4.88	6.40	1.76	8.78	3.75	8.72	5.45	2.52	0.61	4.02	0.93	2.41	2.80	2.01	1.73
Campylobacterales	1.56	1.27	0.00	0.30	0.00	0.15	0.00	0.56	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00
Caulobacterales	0.00	0.00	0.00	0.15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chromatiales	0.41	0.00	0.00	0.00	0.14	0.15	0.00	0.74	0.17	0.31	0.00	0.50	0.00	0.00	0.40	0.00	0.00
Desulfovibrionales	0.41	0.21	0.00	0.00	0.00	0.00	0.00	0.19	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Desulfuromonadales	0.20	0.00	0.00	0.00	0.00	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 5-3. Continued.

Time	Day 0	Day 3		Day 5		Day 18		Day 28		Day 32		Day 36		Day 39	Day 40		Day 42
	[a]I	[b]D1	[c]D2	D1	D2	D1	D2	D1	D2	D1	D2	D1	D2	D2	D1	D2	D2
Enterobacteriales	0.07	0.00	0.00	0.15	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.40	0.40	0.00	0.00
Hydrogenophilales	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Methylococcales	0.07	0.00	0.00	0.00	0.14	0.15	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Myxococcales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.40	0.00	0.00
Pseudomonadales	1.76	0.21	0.73	0.00	0.41	0.15	0.00	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhizobiales	0.07	0.00	0.00	0.46	0.00	0.15	0.17	0.37	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodobacterales	1.76	0.00	0.10	0.15	0.00	0.44	0.17	2.41	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Rhodocyclales	0.27	0.00	0.21	1.22	0.14	0.73	1.87	0.37	1.16	1.26	0.00	0.50	0.00	0.00	0.00	0.00	0.00
Rhodospirillales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sneathiellales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingomonadales	0.07	0.21	0.00	0.00	0.00	0.00	0.00	2.60	0.00	0.94	0.00	1.51	0.00	0.00	2.40	0.00	0.00
Syntrophobacterales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thiotrichales	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Xanthomonadales	0.81	0.64	0.00	0.15	0.27	0.73	0.00	1.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spirochaetes	3.25	1.49	0.10	5.64	0.00	4.69	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spirochaetales	3.25	1.49	0.10	5.64	0.00	4.69	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Synergistetes	13.19	1.70	0.31	2.90	1.49	12.45	8.69	7.98	6.27	12.89	1.22	12.56	0.93	3.21	18.80	3.02	0.58
Synergistales	13.19	1.70	0.31	2.90	1.49	12.45	8.69	7.98	6.27	12.89	1.22	12.56	0.93	3.21	18.80	3.02	0.58
Tenericutes	0.07	0.64	0.10	0.76	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Acholeplasmatales	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Haloplasmatales	0.00	0.64	0.10	0.76	0.00	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thermotogae	4.53	2.12	0.62	11.13	6.50	8.78	5.96	14.29	13.04	19.18	28.66	15.58	20.37	26.51	17.20	27.64	33.53
Thermotogales	4.53	2.12	0.62	11.13	6.50	8.78	5.96	14.29	13.04	19.18	28.66	15.58	20.37	26.51	17.20	27.64	33.53
4Archeae	0.34	0.42	0.00	2.74	0.54	1.46	1.70	1.11	0.99	7.55	0.61	3.52	0.00	0.00	1.60	0.00	0.00
5Euryarchaeota	0.34	0.42	0.00	2.74	0.54	1.46	1.70	1.11	0.99	7.55	0.61	3.52	0.00	0.00	1.60	0.00	0.00
6Methanobacteriales	0.27	0.21	0.00	0.00	0.54	0.15	0.00	0.56	0.00	0.63	0.61	0.00	0.00	0.00	0.00	0.00	0.00
Methanomicrobiales	0.07	0.21	0.00	0.46	0.00	0.88	0.00	0.56	0.17	2.83	0.00	3.02	0.00	0.00	1.60	0.00	0.00
Methanosarcinales	0.00	0.00	0.00	2.29	0.00	0.44	1.70	0.00	0.83	4.09	0.00	0.50	0.00	0.00	0.00	0.00	0.00

[a] Inoculum . [b] Digester 1, [c] Digester 2, [d] Taxonomic domain

[e] Bacterial and archaeal phyla are assigned according to National Center for Biotechnology Informatin (NCBI) Taxonomy database

[f] Bacterial and archaeal orders are assigned according to National Center for Biotechnology Informatin (NCBI) Taxonomy database

[g] Relative abundance are calculated as a phylogenenic group in percentage of the 16s rDNA reads analyzed

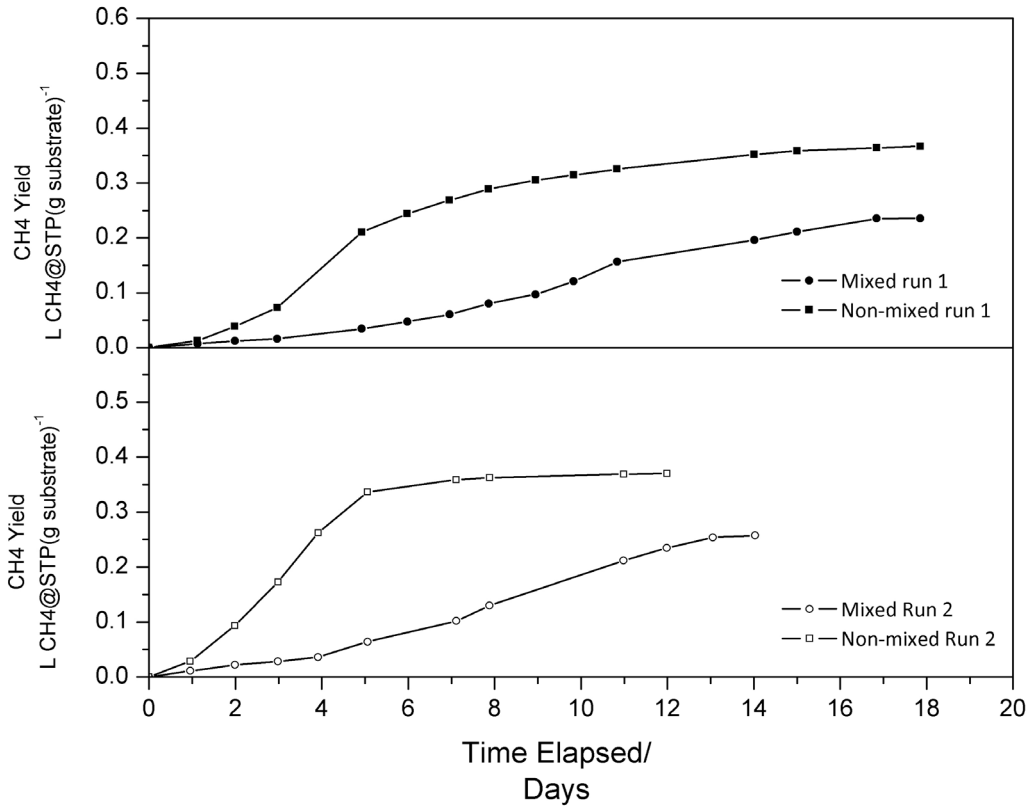


Figure 5-1. Methane yield from digesters 1 and 2

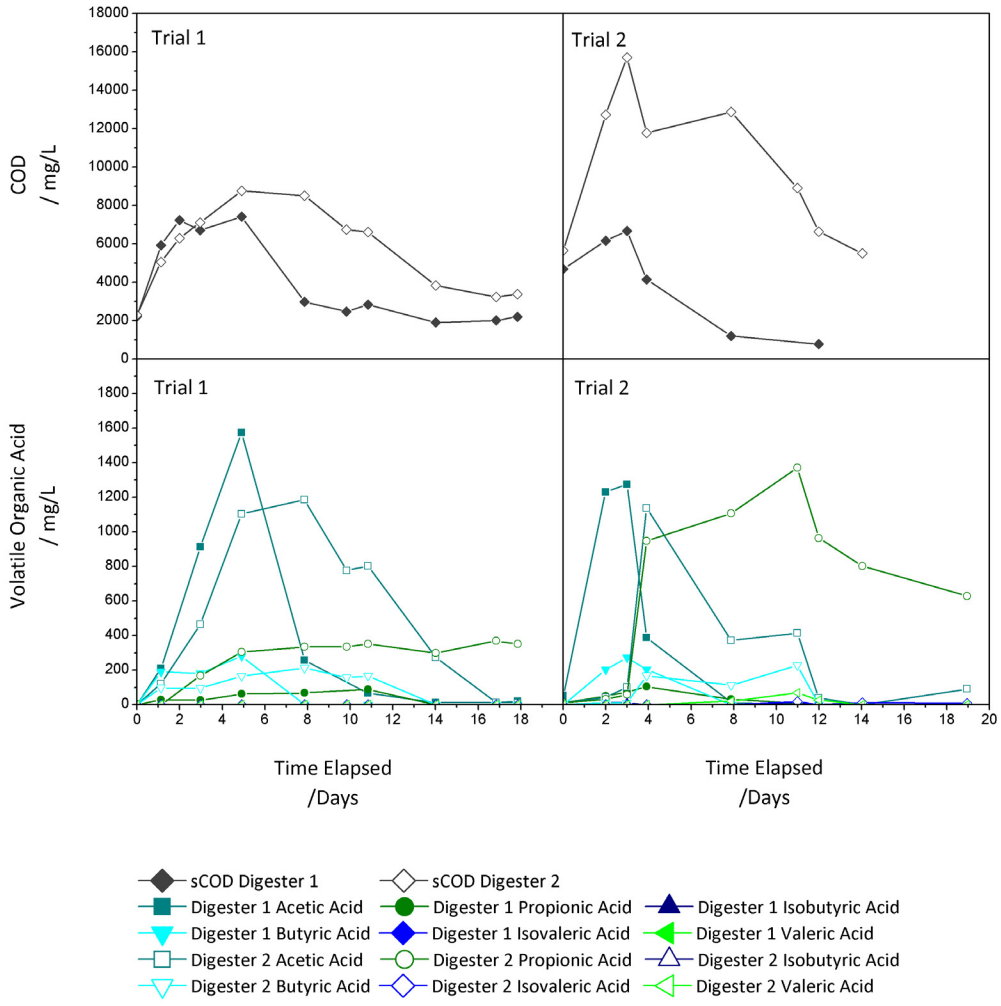


Figure 5-2. SOCD and VOA profiles of digester 1 and digester 2

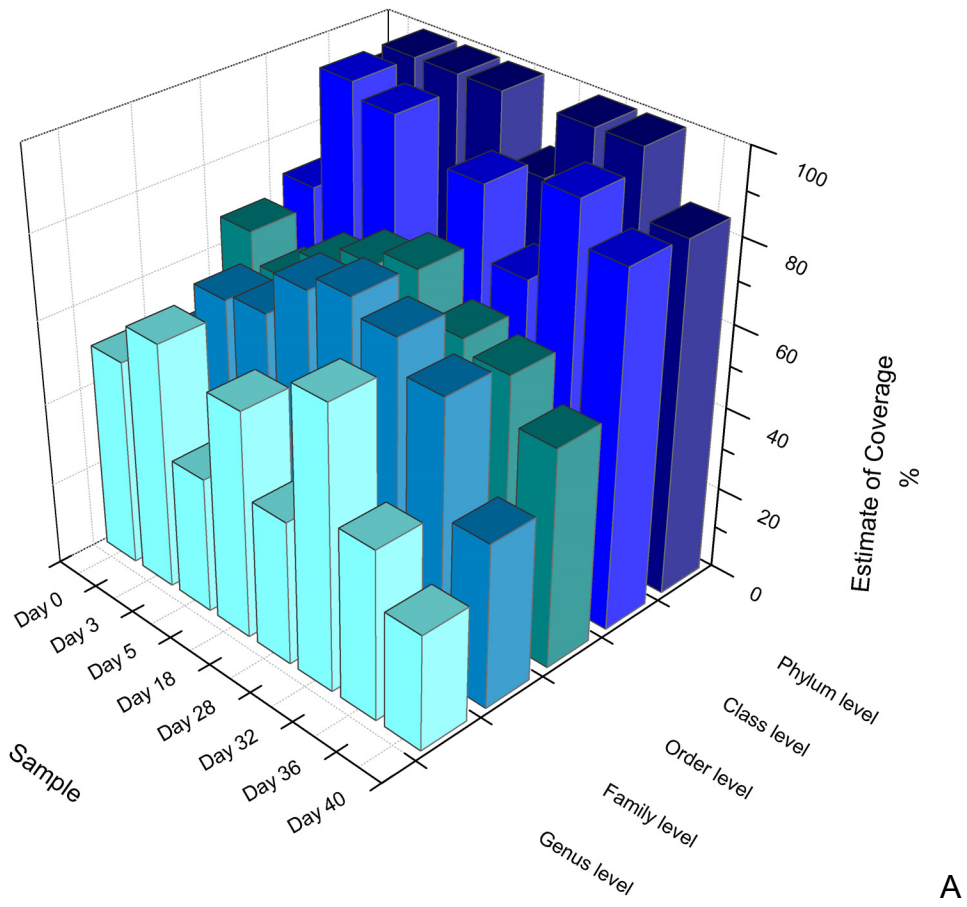
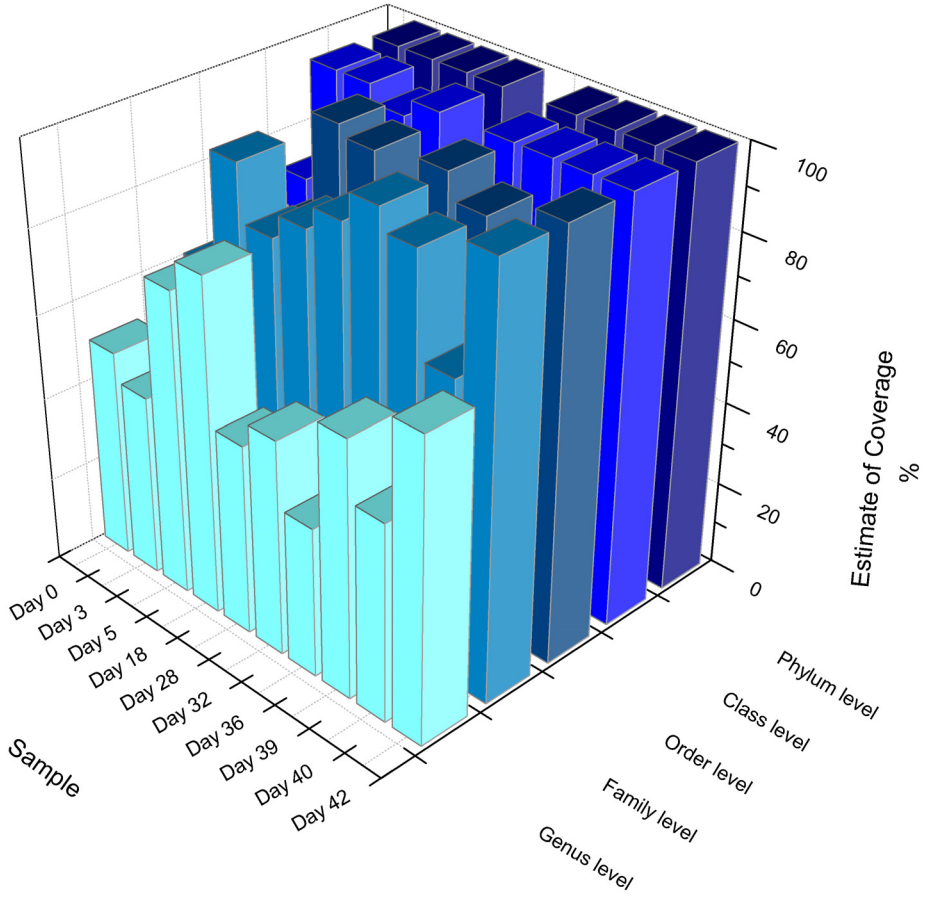


Figure 5-3. Estimate of Chao1 coverage at taxonomic ranks of phylum, class, order, family and genus: A) digester 1 B) digester 2



B

Figure 5-3. Continued

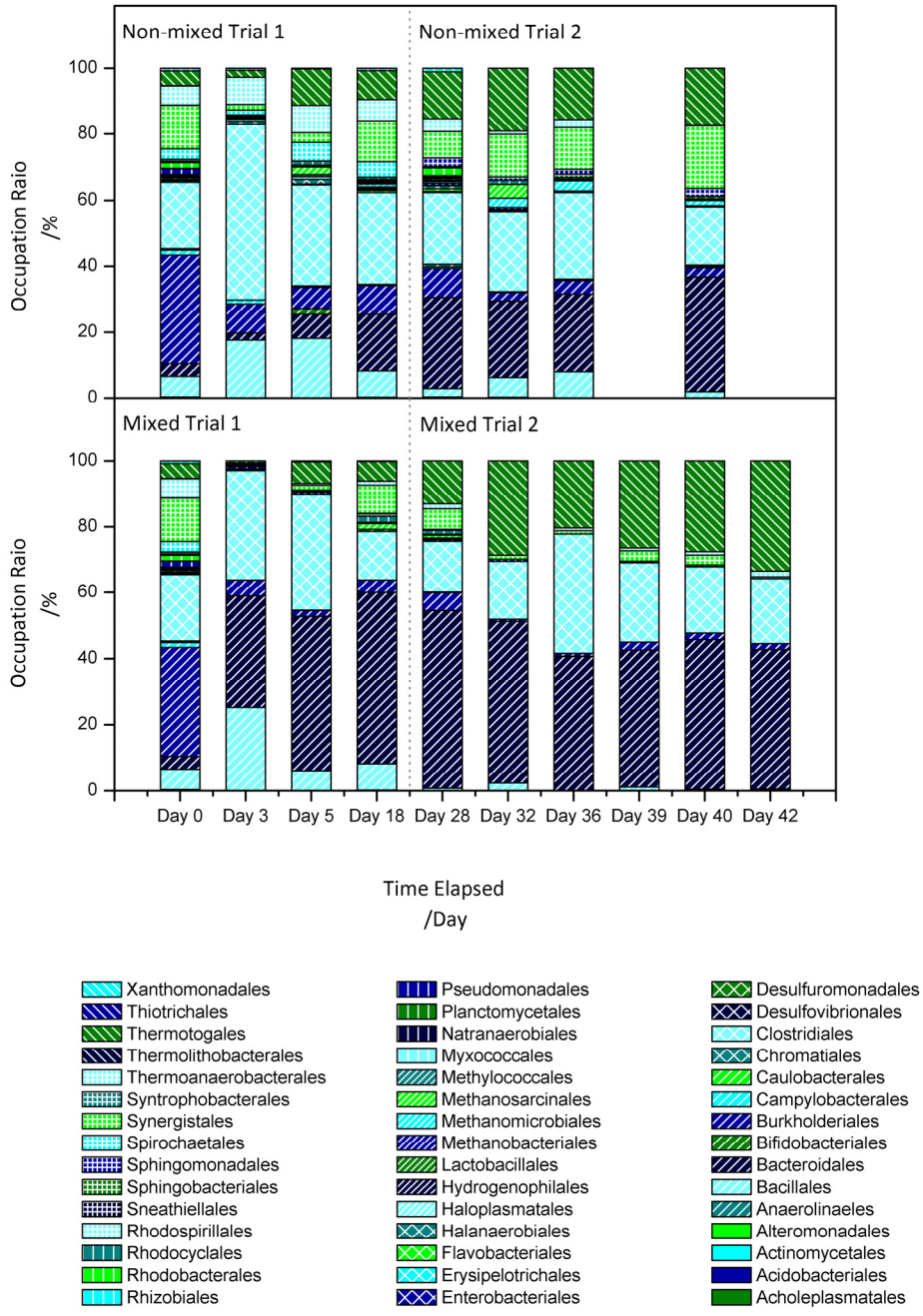


Figure 5-4. Microbial community dynamics of digester 1 and 2

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

Conclusions

The conclusions from this research work are presented in two parts. Part one focuses on the value addition through anaerobic digestion of byproducts from common liquid biofuel production process. The objective to develop a process for simultaneous waste treatment and energy production was fulfilled. The BMP assays determined high methane producing potential of the byproducts and the following long term laboratory scale anaerobic digestion studies provided the feasibility and economics to apply a full scale process to biodiesel and cellulosic ethanol industry.

The glycerol byproduct was collected from a pilot biodiesel plant at University of Florida. Mesophilic BMP assays were conducted on glycerol byproduct and compared with BMP of 99.5% grade glycerol. GBP methane potential was 456 ml CH4 at STP (ml sample)$^{-1}$ compared to 372 ml CH4 at STP (ml sample)$^{-1}$ for pure glycerol. The higher methane yield from glycerol product was attributed to the mixture containing long chain fatty acids, residual oil and methanol that are better energy sources than glycerol itself. The energy that could be recovered from biogasifying glycerol byproduct was calculated to be 18300 kJ /kg using the heat of combustion of methane of 37 kJ L^{-1}. This is comparable to the energy that can be obtained from directly combusting glycerol. The Gompertz equation was used to fit the cumulative methane production data for the glycerol byproduct. The simulation results exhibited a biphasic trend (fraction 1 and fraction 2 in Figure 2-1). Fraction 1 completed methane production in a short period of time, suggesting its ease of degradability; while fraction 2 has a similar methane production profile and methane yield to that of 99.5% grade glycerol. Fraction 2 is

glycerol and fraction 1 is methanol. Overloading issues were found in some BMP assays containing 2 ml glycerol byproduct as seen from low methane production and accumulation of volatile organic acids. Co-digestion of 2 ml of glycerol byproduct with a readily degradable substrate appeared to alleviate the overloading issue, but it still took twice as long to reach a similar yield achieved in assays loaded with 1 ml of glycerol byproduct. Continuous digestion of glycerol byproduct was further evaluated in a laboratory scale 5 L anaerobic digester using GBP as the sole substrate. Methane yield at a feed rate of 0.67 ml $(L)^{-1}$ $(day)^{-1}$ was around 450 ml CH_4 at STP $(ml\ substrate)^{-1}$, which was comparable to the yield obtained from the BMP assays. However, when feeding rate was then increased to 1 ml $(L)^{-1}(day)^{-1}$ the yield decreased to 200 ml CH_4 at STP $(ml\ substrate)^{-1}$. However, feeding glycerol byproduct as sole substrate to anaerobic digesters may easily cause an organic overloading problem. Therefore, dilution or co-digestion with another substrate is recommended.

A similar approach was used to investigate the anaerobic digestion of stillage produced from cellulosic ethanol production. BMP assays were first carried out to determine the methane potential and later stillage was fed to a laboratory scale (15 L) continuous anaerobic digester for over two months to observe long term digestibility of stillage. In industry, distillation is usually conducted at 60-70^{o}C to separate ethanol from the fermentation mixture. To take advantage of the high temperature, an onsite anaerobic digester can be operated at thermophilic temperature for high substrate degradation rates. Thus, the BMP assays and anaerobic digestion of stillage were conducted at 55^{o}C to simulate this situation. Since fibers contained in the stillage were expected to be difficult to degrade and would cause a clogging problem for anaerobic

digestion. The stillage was filtered through a sieve with 0.5 mm mesh. The filtered stillage and the fraction retained by sieve are referred as stillage filtrate and stillage residue. The BMP assays showed stillage filtrate and residue produced 70% and 30% of the methane potential of the whole stillage, respectively. The stillage residue contains only 18% solids, and calculation showed only 50% of methane was produced from the solid fraction. In combination, only 15% of total methane was produced from the solid fraction. In a continuous digester the methane yield of stillage filtrate was between 9 to 10 L CH_4 (L substrate) $^{-1}$(day)$^{-1}$. The sCOD removal efficiency was around 80%. Nutrient analysis showed ammonia and phosphate was not limiting in the digester. A mass and energy balance was developed for integrating anaerobic digestion to the cellulosic ethanol production process in the Biofuel Pilot Plant at University of Florida. It showed that the biogas can cover 70% of the energy consumed for process heat and steam.

The second part of this research work studied the effect of mixing on anaerobic digestion under thermophilic conditions. Digestion performance at mixed and non-mixed conditions was compared, including methane yield, methane production rate, sCOD and VOA degradation. To elucidate the differences, the microbial communities were investigated using 16S rDNA analysis. A total of eight experimental runs were carried out at laboratory scale for mixed and non-mixed digestion, respectively. Sugar beet tailings were used as the feedstock. The non-mixed digester was added with a bulking agent to prevent substrate floatation and compaction. In all eight runs, the mixed digester consistently showed lower methane yield, delayed methane production and higher accumulation of sCOD and VOA than the non-mixed counterpart. More sCOD

and VOA accumulated in the mixed digester as the digester liquor recovered from the last run was used for inoculation in the next run. This suggested constant exposure to mixing might result in loss of activity of anaerobic bacteria. The microbial community structure was then investigated using classical 16s rDNA clone analysis and 454 pyrosequencing. Both techniques showed that methanogens are in lower relative abundance in the mixed digester, explaining the low methane production. The relative abundance of bacteria *Anaerobaculum* was also found to be lower in the mixed digester but high in the non-mixed digester. The bacterium was reportedly associated with a propionate degrading microbial consortium. Lack of *Anaerobaculum* could be one of the reasons for proipionic acid accumulation in the mixed digester. The microbial communities analyzed using 454 pyrosequencing showed higher diversity. Some bacteria that were not detected with 16s rDNA clone analysis were detected by 454 pyrosequencing, such as *Pelotomaculum*. The bacterium is known to syntrophically grow with methanogens and play an important role in propionate degradation. The relative abundance of syntrophic bacteria and methanogens decreased with time in the mixed digester and was overall lower than in the non-mixed digester. It was generally agreed that mixing may inhibit methane production by disrupting the syntrophic relation even at low intensities of 1.1 W/m^3.Therefore, an alternative hypothesis was made that hydrogen was producing during the mixed digestion and hindered the methane production. This was verified by the identification of high abundant hydrogen producing bacteria as *Ruminococcus* and *Acetanaerobacterium* in the mixed digester. To further demonstrate the syntrophic propionate degradation was not affected under the mixing condition used in the study, propionic acid was provided as the only substrate. The VOA

analysis showed steady propionic acid degradation within 15 days for consecutive 3 runs. The study of effect of mixing suggested practice of continuous mixing for high solid digesters may not be always feasible. This is supported by some studies that reported high methane yield at non-mixed or less mixed condition than in continuously mixed digesters.

Future Work

This research work attempted to investigate the microbiological nature of anaerobic digestion and included the application of the process in treating wastes from production of common biofuels. The study presented here opens some areas for further research. The following topics of interest can be addressed:

- Understanding the role of co-substrate in promoting GBP degradation
- Exploring an effective strategy to manage stillage residue containing high content of fibers and lignin that are difficult to degrade in anaerobic digestion. Possible options for example are using the fiber residues (mainly lignin) as fuel or as feedstock for bioproducts.
- Develop a secondary process to treat the effluent after the anaerobic digestion of stillage to further reduce the sCOD concentration and remove nutrients (primary phosphate) to be able to recycle the water within the biorefinery.
- Investigating the effect of mixing mode in addition to mixing intensity on anaerobic digestion, i.e., comparing digestion performance at continuous mixing, intermittent mixing and no-mixing condition.
- Investigating the metabolism of hydrogen producing bacteria (e.g., *Ruminococcus*) in co-culturing with methanogens and syntrophic bacteria to provide a better understanding of hydrogen inhibition on methanogensis.

APPENDIX A
EMERGY ANALYSIS OF BIODIESEL PRODCTION FROM WASTE VEGETABLE OIL

To evaluate the energy economics of a biodiesel production process in a pilot plant at University of Florida, emergy analysis was conducted. Waste vegetable oil was used as the feedstock to produce biodiesel. The results are showed in Table A-1.

Table A-1. Emergy evaluation of biodiesel production

Item	Quantity	Unit /batch	Unit Emergy Values (sej/unit)	Emergy (E10 sej/batch)
Renewable inputs				
None				
Non-renewable storage				
None				
Sum of free inputs				0
Purchased inputs				
Infrastructure				
Storage tank	1		1.11E+15	212.32
Steel tank	3		2.40E+14	138.18
Steel drum	5		1.21E+14	115.83
Tote tank	1		4.42E+14	84.76
Pump	3		3.15E+14	181.52
PVC pipe	15		2.84E+13	81.77
Heating tape	11		6.48E+13	136.91
Pickup truck	1		2.27E+16	4360.84
Drum insulator	1		1.85E+14	35.43
Sum of infrastructure input				5347.58
Operational inputs				
Waste vegetable oil	284	L	1.01E+11	2854.66
Methanol	62.5	L	5.63E+11	3517.17
KOH	1590	g	5.68E+10	9031.20
Electricity	33458	KJ	2.00E+08	669.16
Natural gas	159237	KJ	2.86E+07	454.96
Water	151.4	L	3.39E+08	5.13
Transport	2.58	tkm	1.10E+12	283.80
Labor	4	hour	1.46E+10	5.84
Sum of operational cost				15822.41
Total emergy				21169.99
Output	284	L	7.45E+11	21169.99
Biodiesel				
UEV w/labor	284	L	7.45E+11	
UEV w/o labor	284	L	7.45E+11	

APPENDIX B
DEGRADATION OF PROPIONIC ACID IN MIXED ANAEROBIC DIGESTION

Chapter 4 and Chapter 5 presented results from several (eight) trials of sugarbeet tailings digestion. The results consistently showed lower methane yield and slower methane production rate from the mixed digester, compared to the non-mixed digester. The mixing speed of 100 RPM provided a volumetric power input at 1.1 W/m^3, which was within the typical range of mixing intensity used in anaerobic digestion (Grady, Daigger et al. 1999). Different hypotheses have been proposed to interpret the adverse effect of mixing. Most studies tend to agree mixing force may interfere with the syntrophic associations between bacteria and methanogens by disrupting their spatial juxtaposition (Conrad, Phelps et al. 1985; Whitmore, Lloyd et al. 1987; Dolfing 1992; McMahon, Stroot et al. 2001; Stroot, McMahon et al. 2001; Vavilin and Angelidaki 2005; Hoffmann, Garcia et al. 2008; Kaparaju, Buendia et al. 2008). The microbial community analysis identified abundant hydrogen producing bacteria in the mixed digester. Based on this result, an alternative hypothesis was postulated that hydrogen was produced in the mixed digester and inhibited the intermediate fermentation, particularly volatile organic acid (VOA) degradation. Acetic acid and propionic acid had pronounced concentration among VOAs monitored. While most of accumulated acetic acid eventually degraded propionic acid still persisted after 20 days of digestion. To investigate whether the propionic acid degradation was hindered by the mixing, further experiments were conducted by providing propionic acid as the sole substrate. Since the main substrate (sugar) for hydrogen producing bacteria was not available, the inhibiting effect from hydrogen was limited. Therefore, if the propionic acid still degraded, it could be attributed to the syntrophic associations that survived under the mixing condition.

The sugar beet tailings was provided by America Crystal Sugar Company, Minnesota and stored at 4 C before using. The tailings were washed using tap water before loading to the digesters. Wash water was discarded. Analytical grade (99.5% pure) propionic acid was purchased from Fisher Scientific.

The digester is the same as that used in Chapters 4 and 5. It was constructed by modifying a 3 L Pyrex glass jar. Gas production from the digesters was measured by a positive displacement gas meter. Refer to Chapter 4 for detailed information about the anaerobic digesters and gas meters. The digester contents werecontinuously mixed at 80 RPM. A 50.8 mm x 9.5 mm PTFE coated polygon stirrer bar was placed in the digester and the digester was mixed by a large volume magnetic stirrer (Scienceware Cool Stirrer). The digester was placed in a 55°C incubator throughout the experiments. The digester was initially loaded with 0.3 kg sugar beet tailings and inoculated with 3 L leachate taken from a pilot scale anaerobic digester digesting food wastes. When the digestion of sugarbeet tailings was completed, 1.3 L of digester liquor was recovered and used as inoculum in the following experiments. Three experimental trials were carried out. A solution was prepared by mixing propionic acid in 100 ml deionized water and dosed to the digester. The solution pH was adjusted between 5 and 6 by adding potassium hydroxide. In trial 1, 1.3 g propionic acid was dosed at the beginning. The trial was ended when propionic acid was degraded to low concentration. The digester liquor was kept and 1.4 g propionic acid was dosed again to start trial 2. Likewise, when propionic acid degraded, trial 2 was considered complete and the digester was again dosed with 2 g propionic acid to initiate trial 3. Each trial was operated for 12 to 20 days.

The digester liquor was sampled periodically for pH, volatile organic acid (VOA) analysis. pH was measured using an Accument pH meter. Samples were centrifuged at 8000 RPM for 10 minutes (Fisher Marathon micro H centrifuge) and filtered using Millipore filter paper (pore size 1.2 um). VOA analysis was conducted using Shimadzu gas chromatograph (GC-9AM equipped with a flame ionization detector) for acetic, propionic, isobutyric, butyric, isovaleric and valeric acid concentrations.

The digester content was mixed at 80 RPM. The speed was reduced (compared to 100 RPM used in Chapter 4 and 5) due to the decreased volume. Eighty RPM gave the volumetric power input of 1.3 w/m^3 in 1.3 L digester liquor. This is comparable to the volumetric power input (1.1 w/m^3) used in Chapter 4 and 5. For calculation of volumetric power input of the digester, please refer to Chapter 4.

The VOA and pH profiles of the digester are shown in Figures 6-1 and 6-2. Three experimental trials were carried out over 53 days. pH of all trials was kept above 7.5. The dosing quantities were determined to provide a close concentration as observed in the mixed digester in Chapter 4 and 5. VOA analysis revealed that initial concentration of propionic acid in trial 1, 2 and 3 was 1 g/L, 1 g/L and 1.6 g/L, respectively consistent with the amount dosed. In trial 3, the initial concentration of propionic acid was higher than in trial 1 and 2 because more propionic acid was dosed. In all trials, propionic acid exhibited a steady decrease, indicating the syntrophic community could survive at the mixing intensity of 1.3 w/m^3. In trial 1, propionic acid spiked at 1 g/L and decreased to 0.4 g/L after 4 days. Degradation of propionic acid was delayed in trials 2 and 3. Degradation was not noticeable until 8 days. Acetic acid was produced from the degradation of propionic acid. Though the digester in trial 3 was pulsed a higher dose, most propionic acid degraded in 14 days, suggesting methanogensis was not inhibited at propionic acid concentration of

1.6 g/L. In Chapter 5, the mixed digester accumulated higher concentration of propionic acid (1.4 g/L) than the non-mixed digester. This was speculated to result in low methane production in the mixed digester because the propionic acid accumulation being reported inhibitory to methanogens. However, the observed propionic acid degradation in trial 3 may exclude the speculation

In summary, the three experimental trials showed consistent propionic acid degradation and provided the evidence that syntrophic relation between methanogens and propionate utilizing bacteria was not disrupted by the mixing used in Chapter 4 and 5.

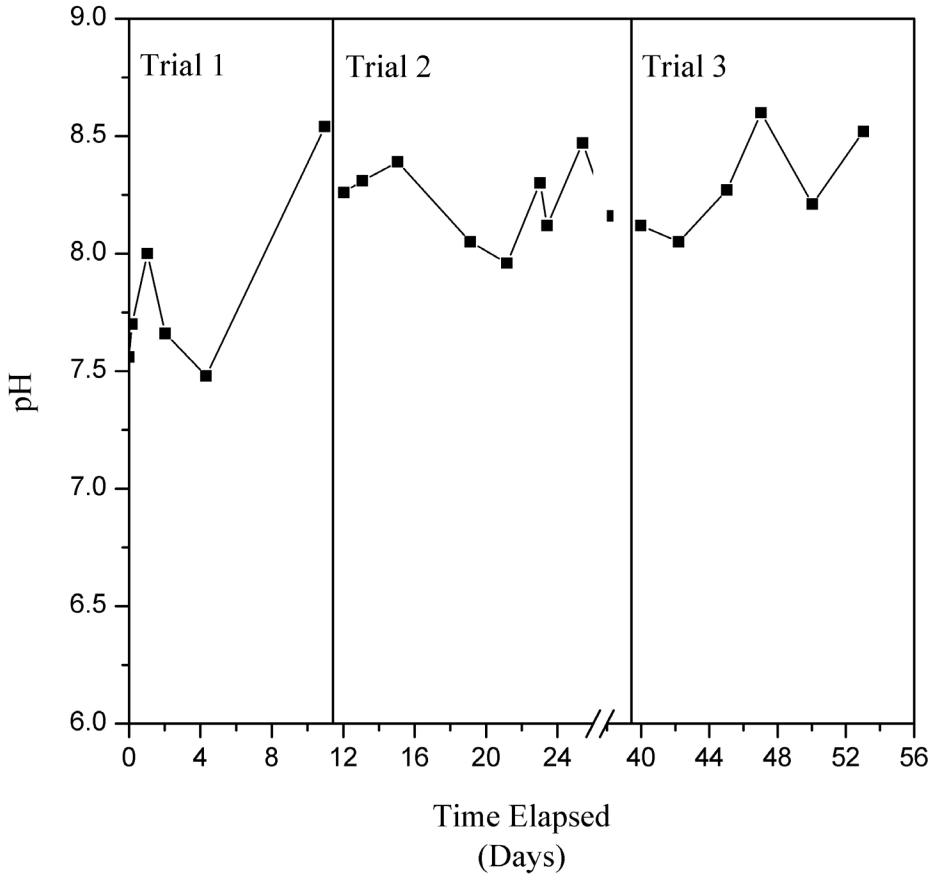


Figure B-2. pH profile

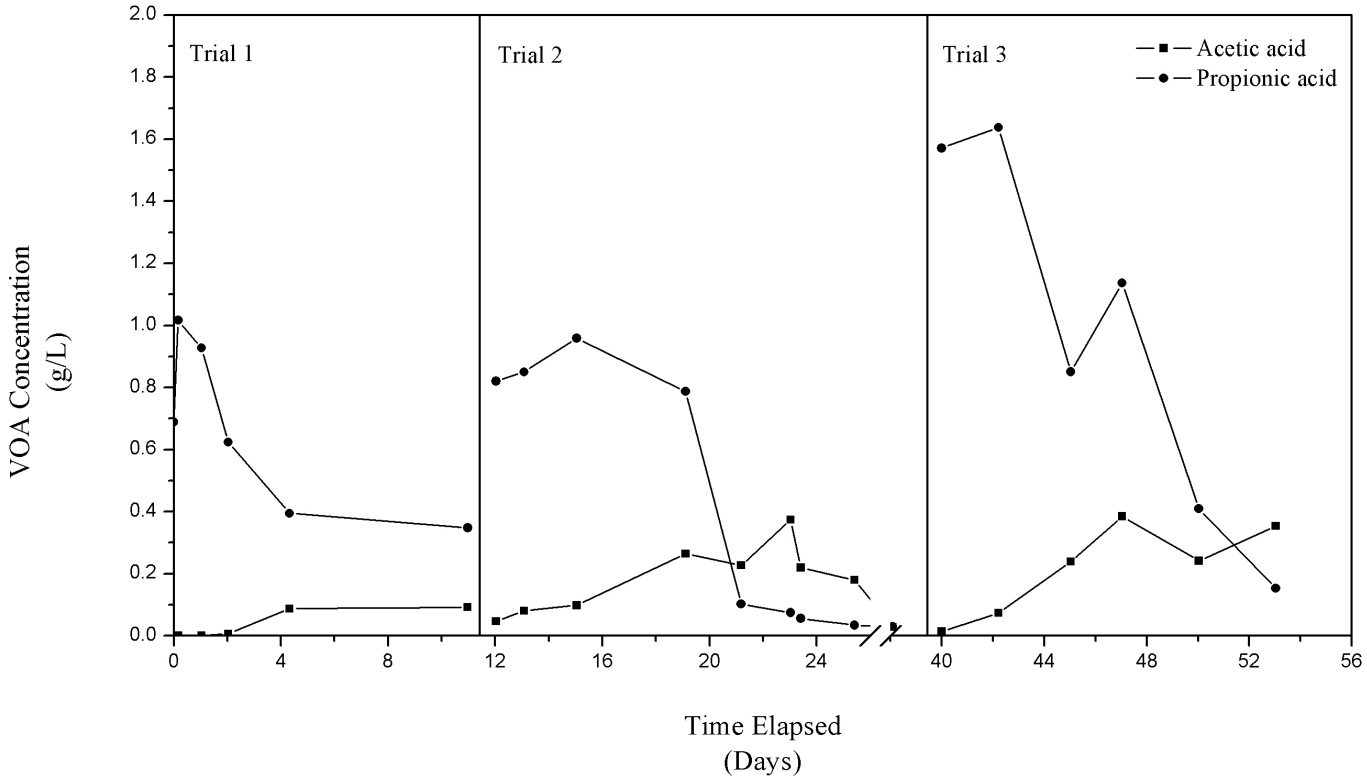


Figure B-3. VOA profile

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BIOGRAPHICAL SKETCH

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