INVESTIGATION OF HOW MICROBES INVOLVED IN ANEROBIC DIGESTION OF VINASSE CHANGE AS FUNCTIONS OF TEMPERATURE, VINASSE COMPOSITIONS AND TIME

by

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Abstract

INVESTIGATION OF HOW MICROBES INVOLVED IN ANEROBIC DIGESTION OF VINASSE CHANGE AS FUNCTIONS OF TEMPERATURE, VINASSE COMBINATIONS AND TIME

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Recent fossil fuel supply crunches and price spikes have prompted increased interest in ethanol as an alternative transportation fuel. However, vinasse, the liquid residue left from the distillation of ethanol, poses serious disposal challenges due to its high acidity, chemical oxygen demand, and concentrations of solids, nitrogen, phosphorous, and potassium. On average, 12-14 liters of vinasse are produced per liter of ethanol. Brazilian production of ethanol is estimated as 15.1 billion liters per year, meaning that the vinasse treatment and disposal problem for Brazil is particularly significant.

Degradation via anaerobic microbes has been demonstrated as a viable option for vinasse treatment, which also produces renewable energy in the form of methane as a byproduct. Methane and other gases, produced in the anaerobic digestion of vinasse, are enough to generate 3.6 to 10.60 megawatts of electricity when vinasse BOD ranges from 1.06 to 3.12 lb/cu ft.

The objective of this research was to investigate the microbes involved in the various stages of anaerobic degradation of vinasse, and how the numbers and species of

microbes varied as functions of time, vinasse composition, and reactor temperature. This information will be useful for future seeding of industrial-scale vinasse treatment processes with appropriate microbes.

Vinasse from production of ethanol from corn and milo was obtained from two U.S. companies; additional synthetic vinasse with various concentrations of parameters (COD, N, P, K, S) was prepared in the laboratory. Vinasse was placed in 6.8L glass bioreactors seeded with 10% sewage sludge obtained from an anaerobic process at a wastewater treatment plant. The reactors were operated at three mesophilic temperatures (30, 35 & 40˚C) in batch mode for 15-89 days, depending on methane production. Three molecular techniques, DNA extraction PCR, and Miseq, were used to determine microbial communities in each reactor as a function of time. Post-sequencing microbial community analysis was performed using QIIME software. As temperature increased, the run time generally decreased, as expected. Overall, the synthetic vinasse combinations had higher percentages of Archaea in the runs compared to real vinasse compositions. The synthetic vinasse thus produced higher levels of methane compared to the real vinasse combinations. The synthetic and real vinasse combinations at 30, 35, and 40°C contain higher percentages of Bacteroidetes, Firmicutes, and Proteobacteria and lower percentages of Archaea, Acidobacteria, Tenericutes, Spirochaetes, Synergistetes, Chloroflexi**,** Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, Synergistetes, and Plantomycetes. There were no overall trends of microbial phyla observed with time.

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Chapter 1

Introduction

1.1 Background

According to the National Academy of Engineering, the Grand Challenges of the 21st Century include the search for sustainable energy (NAE, 2008). Recent fossil fuel supply crunches and price spikes have prompted increased interest in ethanol as an alternative transportation fuel. Ethanol derived from renewable sources has brought a host of challenges along with opportunities to the sugar industry. Vinasse, the liquid residue left from the distillation of ethanol, frequently poses serious disposal challenges due to its high chemical oxygen demand (COD) values, up to 100 g/L, and high biochemical oxygen demand (BOD) values, ranging from 17 to 50 g/L. Vinasse, also called stillage, is an acidic liquid (pH 4-5) and also contains high concentrations of solids, nitrogen (N), phosphorous (P), and potassium (K). Vinasse is a mixture of water, organic, and inorganic compounds. Vinasse poses a serious threat to water quality if released untreated. On average, 12-14 liters of vinasse are produced per liter of ethanol. Brazilian production of ethanol is estimated as 15.1 billion liters per year, meaning that the vinasse treatment and disposal problem is significant.

Aerobic and anaerobic microbial processes are options for treating vinasse. When aerobic processes are used for waste treatment, the low energy compounds carbon dioxide and water are formed; much energy is lost to air - about 20 times as much as with an anaerobic process (Deublein and Steinhauser, 2008). Anaerobic processes produce products of high energy like methane. Methane from anaerobic processes is being increasingly utilized as an alternative energy source in developed countries, via large projects that extract methane from landfills or waste water treatment plants.

Methane and other gases, produced in the anaerobic digestion of vinasse, are enough to generate 3.6 to 10.60 megawatts of electricity when vinasse BOD ranges from 1.06 to 3.12 lb/cu ft. (Baez-Smith, 2006).

1.2 Research Objective

The objective of this research is to investigate the microbes involved in the various stages of anaerobic degradation of vinasse. The research will determine how the numbers and species of microbes vary as functions of time, vinasse composition, and reactor temperature. This information will be useful for future seeding of industrial-scale vinasse treatment processes with appropriate microbes. Several previous studies have looked at microbe communities involved in anaerobic degradation of vinasse (Ferreira, 2010; Aguiar et al., 2010; Rodriguez et al., 2011); however, these have not looked at how the communities change as functions of time, temperature and vinasse composition.

1.3 Dissertation Organization

The dissertation is divided into four chapters as summarized below:

Chapter one provides an introduction and presents the problem statement and objectives of the research.

Chapter 2 presents a literature review of the stages of liquid waste (known as Vinasse) and its composition, methods of microbe estimation, factors affecting anaerobic degradation of vinasse, and previous studies of microbial communities in anaerobic bioreactors

Chapter 3 describes the experimental design to study the effect of vinasse composition, temperature, and time for the production of methane gas, setting up laboratory scale reactors, operating and monitoring laboratory scale bioreactors, sample collection and library preparation for identifying microbial communities, and microbial analysis using QIIME software.

Chapter 4 presents the experimental results and discussion of the results.

Chapter 2

Literature Review

2.1 Background on Vinasse

The production of ethanol from biomass, whether from sugar crops (sugar beets, sugar cane, molasses, etc.) dairy products or cellulosic materials, results in the concurrent production of vinasse, or stillage, which exhibits a considerable pollution potential. Vinasse composition depends on upon the raw material used in the fermentation process. Vinasse has a light brown color with solids content from 20,000 to 40,000 mg/l when obtained from straight sugarcane juice and a black-reddish color with total solids ranging from 50,000 to 100,000 mg/l when obtained from sugarcane molasses (Baez-Smith, 2006). Table 2.1 and Table 2.2 below show the different compositions of vinasse produced from different ethanol feedstocks (Wilkie et al., 2000).

Table 2.1 Stillage Characterization for Sugar and Cane Molasses Feedstocks (Wilkie et al., 2000)

Feedstock	**Ethanol production capacity, 10^6 L/yr**	**Ethanol Yield, L/kg feedstock**	**Stillage yield, L/Kg feedstock**	**BOD (COD), g/L**	**COD yield, kg/kg feedstock**	**COD yield, kg/L EtOH**	**References**
Beet fresh and molasses	18.8	0.02	0.22	38(65)	0.014	0.7	Holmes and Sane (1986)
Cane molasses	nd	0.32	3.8	nd	nd	nd	Chamarro (1979)
Corn	7-70.0	0.379	6.29	37	0.349	0.92	Loehr and Sengupta (1985)
Whey	2	0.012	0.02	5.4	nd	nd	Barry (1982)

Where: nd = No Data

Table 2.2 Stillage Characterization for Sugar, Cane Molasses and Starch Feedstocks (Wilkie et al., 2000)

Feedstock	**Stillage yield, L/L EtOH**	**BOD (COD), g/L**	**N (total), mg/L**	**P (total), mg/L**	**K, mg/L**	**Total S as SO_4, mg/L**	**pH**	**References**
Cane juice	20	12 (25)	400	200	800	nd	3.5	Van Haandel and Catunda (1994)
Cane juice	nd	15 (22)	400	58	nd	400	3.5	Driesses et al. (1994)
Apple	nd	22	380	62	nd	nd	3..4	Robertiello (1982)
Barley and sweet potato	nd	nd	nd	9.1	nd	1370	4.2	Shin et al. (1992)
Grapes	nd	nd (26)	nd	nd	800	nd	3-3.2	Henry et al. (1988)
Raisins	nd	30 (57.5)	750	220	nd	480	3.2	Vlissidis and Zouboulis (1985)
Hardwood	nd	nd (22.5)	2800	74	nd	900	nd	Larsson et al. (1997)
Potato	nd	nd (52)	2100	nd	nd	nd	4.2	Temper et al. (1985)
Raspberry	nd	nd (70)	nd	nd	nd	37	2.9-4.8	Stadlbauer et al. (1992)

Where: nd = No Data

Christofoletti et al. (2013) demonstrated the environmental implications of sugarcane vinasse. Although limited application may be beneficial due to provision of nutrients to crops, high application of sugarcane vinasse can create environmental problems in soils and water bodies. Christofoletti et al. (2013) used the vinasse in different forms such as recycling of vinasse in fermentation, fertirrigation, concentration by evaporation, yeast and energy production. Excessive disposal of sugarcane vinasse can change the chemical and physical properties of soils, rivers, and lakes due to its electric conductivity, pH and presence of chemical elements over a longer period of time. Effects of different effluents on plant species are given below in Table 2.3 (Christofoletti et al., 2013).

It is clear from the Table 2.3 that the effects of vinasse on seed germination depend on its concentration and crop. The researchers noticed that germination rate of seeds decreased with increase in the concentration of vinasse. Ramana et al. (2002) observed the germination rates of tomato, onion, bell pepper, pumpkin, and cucumber seeds. They observed that the concentration of vinasse inhibited the germination of five crops above the concentration of 50%. However, ten percent concentration of vinasse helps in the germination of onion seeds. Algur and Kadioglu (1992) explained that growth, biomass, and primary productivity were affected by the toxic effects of the vinasse. Azania et al. (2003) observed that seeds of the Suriname grass and arrowleaf sida were not able to develop and emerge properly due to negative effect of vinasse. However, sugarcane was developed properly perhaps due to sensitivity of seeds to alcohol compounds present in the effluent (Christofoletti et al., 2013).

Srivastava and Jain (2010) compared the effect of raw vinasse, digested vinasse, and diluted vinasse (1.5v/v) on the cytomorphology of 11 genotypes of sugarcane (Saccharum species). They noticed the effect on mitotic index and several genotoxic effects such as adherences and chromosome delays, C-metaphases, multipolarity, bi/multinucleated cells, and

mutagenic effects such as chromosome breaks and micronuclei. They found these changes due to high concentration of K, P, S, Fe, Mn, Zn and Cu and heavy metals such as Cd, Cr, Ni and Pb.

Moraes et al. (2014) explained how vinasse can be used as energy source. Biogas can be produced from anaerobic digestion of vinasse. Energy from biogas could be used for electricity or vehicular fuel replacement. They noticed that the energy produced from a single vinasse biorefinery was comparable to the electricity required by a city (approximately population of 130,000) or the surplus energy from bagasse burning (exported by some sugarcane mills in Brazil). This amount of energy is also comparable to the electricity generated by some hydroelectric plants, reaching 7.5% of the electricity produced by the world's biggest hydroelectric plant on a national level. "Biogas can be used as an alternative fuel because it can replace up to 40% of the annual diesel supply in the agricultural operations of a sugarcane biorefinery and still provide approximately 14 MW h annually from cogeneration" (Moraes et al., 2014).

Santos et al. (2011) did a case study for generating electricity from biogas from stillage in Brazil. They performed this study on a distillery plant which produces 580 m^3/day of ethanol. In Brazil in the harvest 2009/10 would have produced the stillage potential to produce 1.81 billion m^3 of methane and 4.070 GWh of energy. "The case study of sugarcane in the interior of Parana, Cascavel, Brazil produced 5,800 m^3/day stillage, with the potential to generate 2.75 MWh of electricity per month, which results in an income of US$ 218,586.32 a month" (Santos et al., 2011). Hence, vinasse can be used as a large energy source. Table 2.4 (Santos et al., 2011) shows the parameters used in the work to estimate the theoretical potential for electric energy production from vinasse by burning biogas produced by its anaerobic digestion in an upflow anaerobic sludge blanket reactor.

Table 2.3 Effects of Different Effluents in Plant (Christofoletti et al., 2013)

Type of Vinasse and Concentration	Type of Crop	Observed Effects (Beneficial Effect)	Adverse Effects	Authors
Sugar cane vinasse in the concentrations of 1%, 2.5%, 5%, 15%, 30%,50%,75% and 100% (v/v)	Phaseolus radiatus L. (bean)	-Beneficial effects in treatment in the concentration of 5% -Increase in carotenoids in the concentration of 30% in the first and second crops -15 and 5% in the third and fourth crops, respectively	Decrease in % and germination speed with increase in concentration	Sahai et al. (1985)
Sugar cane vinasse in the concentrations of 1%, 2.5%, 5%, 15%, 30%, 50%,75% and 100% (v/v)	Cicer arietinum L. (chickpea)	Positive effect in all growth parameters at the concentration of 5%	-Delay in % and germination speed with increase of concentration -Total inhibition of germination at the concentration of 100%	Srivastava and Sahai (1987)
Beet vinasse in the concentrations of 1%, 2.5%, 5%, 10%, 25%, 50% and 100/% (v/v)	Pisum sativum and Helianthus annuus (pea and sunflower)	-Increase in the length of the stem, leaf area, biomass - net productivity in pea and sunflower at the concentration of 2.5%	-Decrease of these parameters with the increase in concentration	Algur and Kadioglu (1992)
Beet vinasse three mixtures of a concentrated depotassified	Zea mays L., Beta vulgaris L. and Helianthus annuus L. (corn, beet, and sunflower)		No adverse effects were observed on the emergence of plants or phytotoxicity symptoms	Madejon et al. (2001)

Table 2.3—*Continued*

Distillery effluent 5%, 10%,15%, 20%, 25%, 50%, 75% and 100%	Tomato, pepper, pumpkin, cucumber, and onion	-Concentrations of the effluent did not inhibit the germination of pepper, pumpkin, cucumber, and onion seeds - increase in germination rate of onion seeds at the concentration of 10%	-Inhibition of germination in high concentrations (75 and 100%) - Dose-dependent effect on germination speed, rates and peak	Ramana et al. (2002)
Vinasse, flegmass and fusel oil concentrations 12.5%,25%, 50% and 100% (v/v)	Sida rhombifolia, Brachiaria decumbens and sugar cane (variety RB72454) (arrowleaf sida,Suriname grass, and sugar cane)		Negative effect on emergence and development of Suriname grass and arrowleaf sida seeds	Azania et al. (2004)
Distillery effluent 5%, 10%,15%, 20%, 25%, 50%, 75% and 100%	Oryza sativa and Triticum aestivum (rice and wheat)	Increase in germination rate of seeds at lower concentrations (1 and 5%)	Inhibition of seed germination at concentrations of 10 and 25%	Ale et al. (2008)
Distillery effluent 5%, 10%,15%, 20%, 25%, 50%,75% and 100%	Zea mays L. and Oryza sativa L.(corn and rice)		-Inhibition of seed germination and early growth of seedlings of rice and corn due to non-diluted effluent -Significant reduction of the germination %, length of radicle and plumule, and fresh and dry weight -Visible effects of toxicity on seedling leaves	Pandey et al. (2008)
Vinasse, flegmass and fusel oil Concentrations 12.5%, 25%, 50% and 100% (v/v)	Three varieties of sunflower, castor oil, and peanut	Positive effect on castor oil, especially on variables associated to early developmental stages of seedlings	Negative effects on emergence and early development of peanut plants and to a lesser extent sunflower plants, regardless of the variety examined;	Ramos et al. (2008)

Table 2.3—*Continued*

Distillery effluent Concentrations of 1%,5%, 10% and 25% in seeds Crop fertirigation	Vigna angularis, Vigna cylindrical and Sorghum cernum (azuki bean,cowpea, and sorghum)		Decrease in germination rate of the three varieties of seeds tested with the increase of effluent concentration	Doke et al. (2011)

Granato and Silva (2010) performed a case study in a distillery with capacity to produce 600 m^3/day of ethanol, as a result of digestion of stillage generated. They obtained a production of 75,600 Nm^3 biogas per day. “Considering a gas turbine efficiency of 35%, production would be 6,540 kWh of electrical energy alternative” (Granato and Silva, 2010).

Table 2.4 Parameters Used for the Elaboration of the Potential for Energy Production

Parameter	Indicator	Unit	Reference
Vinasse Production	10	M^3/m^3 of Ethanol	UNICA (2010)
Ethanol Production	90	L/t Cane	UNICA (2010)
COD typical of Vinasse	20	Kg/m^3	(Freire & Cortez, 2000)
Concentration of Methane in gas	60	% Methane	Lamonica (2006)
Typical Production of Methane	0.35	Nm^3 /Kg /day COD Removed	(Perez et al, 2006)
Net Calorific Value of Methane	35.558	KJ Nm^3	(N.J.B, 2002)
Overall Efficiency of Motor-Generator	26	% to IBW	(Chevalier & Meunier, 2005)
Energy	9.88	$KWh/m^{3\,M}$ethane	Calculated from PCI Methane
Amount Received from Sale of Energy	79.41	US$ /MWh	EPE, 2010
Dollar Exchange Rate (US $ 1.00)	1.7	R$	Commodities-BMF
Nm^3= normal m^3			

2.2 Anaerobic Process Basics

Anaerobic degradation of organic material (biomass) involves decomposition of bacteria under humid condition in the absence of molecular oxygen. The overall process of anaerobic degradation can be represented as (Deublein and Steinhauser, 2008).

$C_cH_hO_oN_nS_s + y\ H_2O \rightarrow xCH_4 + 9(c-x)\ CO_2 + n\ NH_3 + s\ H_2S$

where $x = 1/8*(4c+h-20-3n-2s)$ and $y = ¼*(4c-h-20+3n+3s)$

The above equation can be used to estimate the theoretical methane (CH_4) yield, if the chemical composition of the substrate is known.

Anaerobic processes are divided into four stages: Hydrolysis, Acidogenesis, Acetogenesis, and Methanogenesis, as shown in Figure 2.1 (Sattler, 2011) shows a schematic of the overall process of anaerobic digestion of organic matter.

Stage 1: Polymer Breakdown (Hydrolysis) – carbohydrates, lipids, and proteins are broken down into soluble monomers by cellulolytic, lipolytic, and proteolytic bacteria.

Stage 2: Acid Production (Acidogenesis) – acid-forming bacteria (acidogens) convert the products of Stage 1, soluble monomers into short chain organic acids volatile fatty acids (lactic, propionic, and butyric acids);

Stage 2.5: Acetic Acid Production (Acetogenesis) - In Stage 2.5, acetogenic microbes convert the volatile fatty acids and ethanol formed in Stage 2 into acetic acid (CH_3COOH)/acetate (CH_3COO), H_2, and CO_2.

Stage 3: Methane Production (Methanogenesis) – Methanogenic bacteria, strictly anaerobic, can use the acetic acid/acetate from Stage 2.5 to form methane, according to:

$CH_3COOH \rightarrow CH_4 + CO_2$

Microbes likely to be found in the various stages of anaerobic digestion of vinasse include (Sattler, 2011):

Hydrolysis- Bacteria of genera *Bacterroides, Lactobacillus, Propioni-bacterium, Sphingomonas, Sporobacterium, Megaspaera, Bifidobacterium* are most common in Hydrolysis, including both facultative and obligatory anaerobes.

Acidogenesis- Facultative and obligate anaerobic fermentative bacteria, including *Clostridium spp., Peptococcus anaerobus, Bifodobacterium spp, lactobacillus, Actinomyces, Staphylococcus, and Escherichia coli*.

Acetogenesis- Mesophilic bacteria Clostridium *aceticum* and *Acetobacterium woodii.*

Methanogenesis- *Methanosarcina* and *Methanosaeta, Methanobacterium, Methanococcus* and *Methanobacillus.*

2.3 Factors Affecting Anaerobic Processes

2.3.1 Temperature

Anaerobic treatment can be done at both mesophilic (30-40°C) and thermophilic temperature (50-60°C). Microbial activity increases at higher temperature and the microbial activity approximately doubles at every 10°C rise in temperature within the optimal range (Khanal, 2008). Thermophilic systems thus produce methane 25-50% faster, depending on the substrate (Henze and Harremoes, 1983). Below 15°C, almost no methane will be generated (FAO, 1984). Obtaining thermophilic anaerobic sludge for use during thermophilic bioreactor start-up may be difficult; thus, Chen (1983) evaluated the metabolic adaptation of mesophilic anaerobic sludge to thermophilic temperatures. Only 9% of the microbes originating in mesophilic sludge were found to be thermophiles, and 1% was obligate thermophiles (Ribas et al., 2009).

The temperature of vinasse as produced is close to 90ºC, which is too high even for thermophilic anaerobic degradation. Therefore, cooling of wastewater is mandatory for biological treatment (Ribas et al., 2009).

2.3.2 pH

Acidogens prefer pH 5.5-6.5; methanogens prefer 7.8-8.2. When both cultures coexist, the optimal pH range is 6.8-7.5 (Khanal, 2008). If the pH drops below 6.6, methanogens are significantly inhibited, and pH below 6.2 is toxic (Metcalf and Eddy, 2004). When acid-forming bacteria of Stage 2 and methanogenic bacteria of Stage 3 have reached equilibrium, the pH will naturally stabilize around 7, since organic acids will be removed as they are produced, unless a problem develops. Normally, alkalinity in anaerobic systems ranges from 1000 to 5000 mg/L, which provides sufficient buffering to avoid large drops in pH (Metcalf and Eddy, 2004). Sodium bicarbonate and calcium carbonate can be used as buffers to maintain the pH (Sattler, 2011).

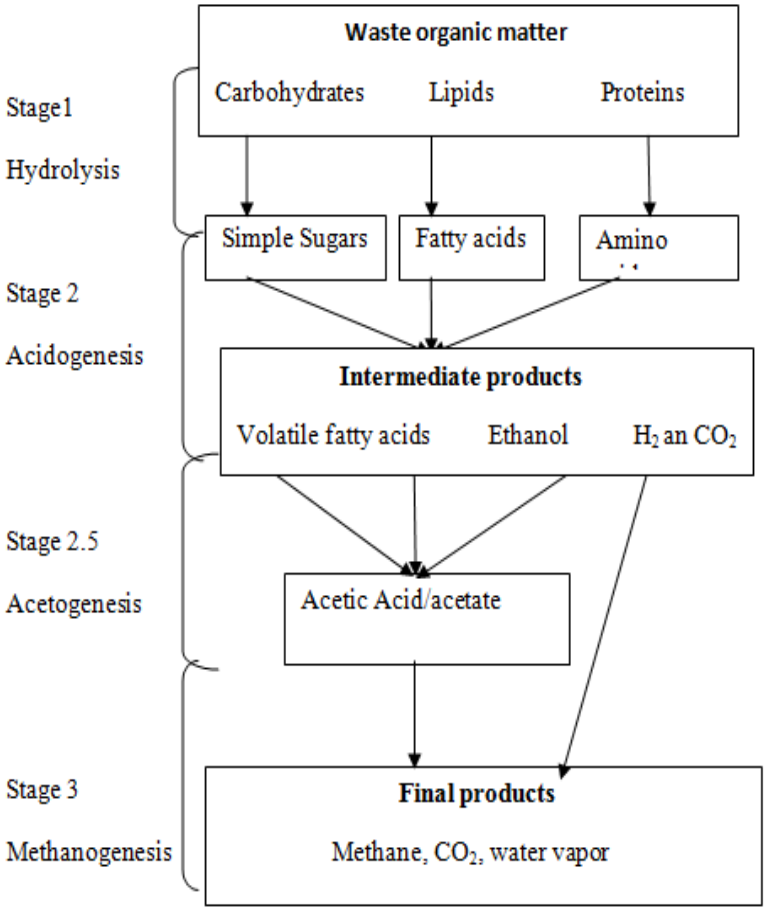


Figure 2.1 Anaerobic Digestion of Waste Organic Matter (Sattler, 2011)

2.3.3 Nutrient Content

Methanogens require macronutrients P and N, as well as micronutrients. The amount of P and N required can be calculated by assuming the empirical formula for a bacterial cell to be $C_5H_7O_2N$ (Speece and McCarty, 1964). P and N requirements can also be estimated using COD/N/P ratios, with a minimum ratio of 350:7:1 COD/N/P needed for highly loaded systems (0.8-1.2 kg COD/(kg VSS*day), and a minimum ratio of 1000:7:1 COD/N/P needed for lightly loaded systems (<0.5 kg COD/(kg VSS*day) (Henze and Harremoes, 1983). Phosphoric acid or phosphate salts are commonly used to supply needed additional phosphorous; urea, aqueous ammonia, or ammonium chloride are used to supply nitrogen (Khanal, 2008). Trace metals that have been found to enhance methane production include iron, cobalt, molybdenum, selenium, calcium, magnesium, sulfide zinc, copper, manganese, tungsten, and boron in the mg/L level and vitamin B12 in μg/L (Speece, 1988). Takashimal et al. (2011) described that trace elements (nickel, iron, cobalt and zinc) are necessary for thermophilic and mesophilic fermentation from glucose.

2.3.4 Toxic Substance Concentrations

High levels of ammonia, soluble sulfides, soluble salts of metals, and alkali and alkaline earth metal salts in solution (e.g. those of sodium, potassium, calcium, or magnesium) can be toxic to methanogens (NAS, 1977). Maximum allowable concentrations of various substances are given in Table 2.5 below. In addition, the methanogens are strict anaerobes; thus, their growth is inhibited by even small amounts of oxygen, or highly oxidized material (like nitrates).

2.3.5 Batch or Continuous Operation

An anaerobic process can be operated in batch or continuous mode. In a batch reactor, a batch or certain volume of waste is introduced and allowed to be degraded completely before any additional waste is introduced. In a continuous reactor, the organic material is added regularly and constantly; the reactor can produce biogas for a long time without the interruption of loading the material and unloading of effluent. A batch bioreactor is easiest and cheapest to build. In a batch bioreactor, the biomass can be mixed properly with good distribution of nutrients

and bacteria; it is thus more robust against inhibitors than a continuous digester, but in a continuous system bacterial flora could acclimated to the inhibitor by slowly increasing the concentration of inhibitor (Carcass Disposal Working Group, 2004). A continuous bioreactor is good for large scale, whereas batch is good for small operations.

Table 2.5 Toxic Substance Concentrations

Cation	**Maximum Recommended Concentration**
Ammonia	1500-3000 mg/L
Calcium	2500-4500 mg/L
Chromium	200 mg/L
Copper	100 mg/L
Cyanide	<25 mg/L
Magnesium	1000-1500 mg/L
Nickel	200-500 mg/L
Potassium	2500-4500 mg/L
Sodium	3500-5500 mg/L
Sodium Chloride	40,000 ppm
Sulfate	5000 ppm

2.3.6 Reactor Mixing

Ghanimeh et al. (2012) examined the effect of mixing and non-mixing strategies on the performance of thermophilic digesters. Both digesters were operated at the same conditions except for mixing. The digester with mixing showed better stability because it minimizes the

production of volatile fatty acids, propionate formation and VFA-to-alkalinity ratio. Mixing provides faster and better start-up of the reactor compared to non-mixing. In addition, mixing provides higher loading capacity.

2.4 Methods for Microbe Determination

Caporaso et al. (2011) demonstrated that 2000 Illumina single-reads are sufficient to recapture the same relationships among samples that they observed with the full dataset. Therefore, thousands of samples can be analyzed together in a single run to predict microbial communities. Caporaso et al. (2011) sequenced diverse array of 25 environmental samples and three known mock communities at a depth averaging 3.1 million reads per sample using Illumina GAIIx platform. A limitation of the Illumina platform is that it can currently only produce relatively short reads (75-100 base pairs, or bp, in a single read); paired ends can produce 150-200 bp from a single molecule. Caporaso et al. (2011) inserted different barcodes and adapters to each sample to differentiate among thousands of samples in a single lane. They used bacteria/Archaea PCR primers (F515/R806) against the V4 region of 16s rRNA, which would yield optimal community clustering with reads of this length.

Illumina has described the next generation tool sequencing for profiling complex microbial communities. The MiSeq system provides the easiest sequencing workflow available. It provides opportunity to researchers to analyze data from sample in only 8 hours. MiSeq delivers more than 1 gigabyte (Gb) of data with quality comparable to that achieved with the HiSeq 2000 platform by using proven sequencing by synthesis (SBS) technology. Illumina used 24 amplicon samples for sequencing in a single MiSeq run using an indexing strategy and overlapping 2× 150 bp reads. Post sequencing microbial community analysis can be performed using the QIIME (Quantitative Insights Into Microbial Ecology) pipeline, an open–source software package which provides several standard community analysis tools. Sequence variation in the 16S ribosomal RNA gene is widely used to find out diffferent taxonomic diversity present in microbial communities. The 16S sequence is composed of nine hypervariable regions. Only one hypervariable region sequence is

sufficient for taxonomic classification, compared to sequencing the entire gene length. Illumina sequenced the V4 region of the 16 S rRNA (http://www.illumina.com/miseq).

Caporaso et al. (2012) performed both HiSeq and MiSeq platforms for ultra-high-throughput microbial community analysis and compared the results with each other. They developed a protocol for community amplicon sequencing on the HiSeq 2000 and MiSeq Illumina platforms. They applied this protocol to sequence 24 microbial communities from host associated and free-living organisms. The samples represented soil and several host-associated environment types: human feces, mouth, skin, and canine feces. Twenty-four samples were sequenced on three paired-end Illumina HiSeq 2000 lanes, and in one paired-end MiSeq run. Three paired-end Illumina HiSeq 2000 lanes and one paired-end MiSeq run provided eight sets of reads, corresponding to 5′ and 3′ reads from each lane. All the sets of reads were studied as independent replicates to determine the reproducibility of the results. The data produced from HiSeq and Miseq was similar but different in scale. Hence, it could be used in different projects. Hiseq can be used in larger projects where cost is more important than completion time of the project. The HiSeq platform can run several lanes at the lowest cost. However, MiSeq is good for smaller projects because it takes less time to process samples and generates comparable data to HiSeq.

Caporaso et al. (2012) previously developed a protocol for high-throughput community sequencing on the Illumina GAIIx, which has been successfully adapted for the HiSeq 2000 and MiSeq platforms, which also reduces the price per sequence of amplicon sequencing to ~15 000 single-end reads per USD$1 on the HiSeq 2000. For example, “based on the lowest high-quality sequence per lane count of 22 928 291 reads (HiSeq 3′ lane 6), if using all 2167 barcodes in each of 15 lanes on the HiSeq2000, leaving one lane for a control, then it is possible to sequence 32 505 samples in a week at a depth of 10 580 sequences per sample, for approximately $22, 000 in sequencing costs” (Caporaso et al., 2012).

Barcode was designed to increase the length of the barcode to run more sequences per sample at a lower depth of sequencing. “On the basis of the lowest high-quality sequence count

on the MiSeq of 1 603 532 reads, MiSeq 3′, if using all 2167 barcodes, it is possible to sequence 2167 samples in a 12 h run at a depth of 740 sequence per sample, for approximately $800 in sequencing costs" (Caporaso et al., 2012).

Lauber et al. (2010) performed microbial community analysis on soil and human-associated samples (human skin and human feces) at different storage conditions for the assessment of DNA-based microbial community analysis. They used barcoded pyrosequencing of bacterial 16S rRNA genes to survey communities. These samples were stored at 20ºC, 4ºC, -20ºC, and -80 ºC for 3 and 14 days before DNA extraction. DNA extraction was performed by MOBIO Power Soil DNA extraction kit. Then, PCR amplification of bacterial 16s RNA was performed at variable regions V1 and V2 using primers. Amplicons produced from PCR reactions were pooled to reduce per-PCR variability and purified with the MOBIO ultra clean PCR clean up kit. The sample was then gel purified and precipitated with ethanol to remove any remaining contaminants. Then, DNA was sequenced using a Roche 454 FLX pyrosequencer. They concluded that bacterial communities composition in the samples were largely unaffected by differences in short-term storage conditions. Pyrosequencing does not identify the reason for changes in bacteria at the species or strain level due to limitations of read length and error rate. Subsample doesn't give same results (same communities), which are stored in different conditions due to changes in inherent difference in DNA extractions and PCR Amplifications methods between sub-samples and insufficient sample homogenization. These sub-samples were stored at room temperature for 14 days to find out the reason of variability. Other reasons for variability are more important than the variability caused by differences in storage temperature and duration between sub-samples even after 14 days. However, the storage conditions can change the relative abundance of specific taxa. They noticed that "the types of samples in this study can be stored and shipped at room temperature without having a significant impact on the assessment of overall community composition or the relative abundances of most major bacterial taxa'' (Lauber et al., 2010).

Rodrigues et al. (2013) followed the different methods for microbial determination in conversion of amazon rainforest to agriculture land. They followed DNA extraction, PCR, and DNA sequencing methods. DNA was extracted in triplicate from each soil sample of Amazon rain forest and agricultural land (pasture) using the Powersoil DNA isolation kit (MOBIO laboratories). They followed the manufacturer's interaction protocol. Extracted DNA from same soil sample was combined to quantify using Nanodrop ND-1000 spectrophotometer. These extracted DNA were stored at -20˚C. The concentration of this DNA from both the treatments had an average of 11.4 ng/µl and 9.8 ng/µl for forest and pasture samples, respectively. These samples were quantified spectrophotometrically with A_{260}/A_{280} ratio averages of 1.78 and 1.84 for forest and pasture soil. "Primers 577F (5′-AYTGGGYDTAAAGNG-3′) and 926R (5′-CCGTCAATTCMTTTRAGT-3′) targeting the region V4 of the 16S rRNA gene were used for PCR, because sequences in that region provide comprehensive coverage and among the highest taxonomical accuracy. Primers were designed with eight-base barcodes and 454 pyrosequencing adapters (Integrated DNA Technology)" (Rodrigues et al., 2013).

DNA was amplified using 50-µL volume containing 1× buffer, 0.2 µM of each primer, 1.8 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 300 ng/µL BSA, 10 ng of template, and 1 µL of the FastStart High Fidelity PCR System enzyme (Roche Applied Sciences). The Polymerage chain Reaction (PCR) used the cycle for DNA amplification with 3 min at 95 °C, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 57 °C for 45 s, extension at 72 °C for 1 min, and final extension for 4 min. All the PCR reactions were performed in triplicate and purified using gel electrophoresis followed by the QIAquick gel extraction kit and the Qiagen PCR purification kit after combining triplicates of each sample together. They performed High-throughput sequencing the 454 GS FLX Sequencer (454 Life Sciences) at the Michigan State University Research Technology Support Facillity and removed reads that contain one or more N's, reads where the lengths lied outside of the main distribution, and those with inexact matches to the primer (Rodrigues et al., 2013).

Mirza et al. (2014) studied the response of free-living nitrogen-fixing Microorganisms due to change in land type in the Amazon forest. They collected the soil samples from three types of land; primary rainforest, a 5-year old pasture originally converted from primary forest, and a secondary forest established after pasture abandonment. They performed DNA extraction PCR amplification, cloning, and DNA sequencing for microbial analysis. Total genomic DNA was extracted from soil using the Power Lyzer PowerSoil DNA isolation kit according to the manufacturer's instructions. “Soil DNA was quantified, diluted to a concentration of 25ng/µl, and used to amplify the *nifH* gene with primers PolF (TGCGAYCCSAARGCBGACTC) and PolR (ATSGCCATCATYTCRCCGGA)” (Mirza et al., 2014). PCR reaction was performed using 0.2 mM each deoxynucleoside triphosphate, 0.4 µM each primer, 1× PCR buffer, 2 mM $MgCl_2$, 50 ng of template DNA, and 2 U of *Taq* DNA polymerase (Gene Script, Piscataway, NJ). “The PCR conditions were 2 min of denaturation, followed by 35 rounds of temperature cycling (95°C for 30 s, 59°C for 30 s, and 72°C for 45 s), and a final extension at 72°C for 7 min’’ (Mirza et al., 2014). After performing PCR, the amplified samples were run on 1% agarose gel to see the amplified product using ethidium bromide. They also tested three other Primer sets—Ueda19F-Ueda470R, PicenoF/R, and Z-primer for *nifH* amplification along with polymerase (Pol) primers. Pol primer set only amplified all 15 samples compared to other primers. Hence, Pol primer was used for further analysis.

Eren et al. (2013) described a method to produce high quality short reads using Illumina Paired-End Technology. “They amplified three different replicates of the V6 region of ribosomal RNAs from *Escherichia coli* (*E. coli*) genomic DNA isolated from pure culture and from 10 metagenomic microbial DNA samples isolated from raw sewage” (Eren et al., 2013). They run 96 samples in one lane after performing PCR using 96 reverse primers and one forward primer. Each primer has different adaptors and barcodes to differentiate among samples. They used total volume of 33 µL per reaction for each library preparation with an amplification cocktail containing 1.0 U Platinum Taq Hi-Fidelity Polymerase, 1X Hi-Fidelity. They performed PCR using DNA concentration of 10–25 ng for each reaction with a no-template control for each primer pair. They

used an initial 94°C, 3 minute denaturation step; 30 cycles of 94°C for 30s, 60°C for 60s, and 72°C for 90s; and a final 10 minute extension at 72°C for all PCR run. Each sample was amplified in triplicate and purified using a Qiaquick PCR 96-well PCR clean up plate (Qiagen, Valencia, CA). 30 µL of Qiagen buffer EB was used for elution of DNA.

Eren et al. (2013) used the picogreen quantitation (Life Technologies, Carlsbad, CA) which provides a basis for pooling equimolar amounts of product. They selected the amplicon products of 200–240 bp on 1% agarose using Pippin Prep (SageScience, Beverly MA), and performed qPCR (Kapa Biosystems, Woburn MA) to measure concentrations of DNA before sequencing on one lane of an Illumina Hiseq 100 cycle paired-end run. 90% of the other lanes was dedicated to PhiX DNA and used to run control. “The combination of CASAVA 1.8.2 to identify reads by index and a custom Python script that resolved barcodes demultiplexed the datasets” (Eren et al., 2013).

Rodriguez et al. (2011) used the method denaturing gradient gel electrophoresis (DGGE) for molecular analysis of the biomass of a fluidized bed reactor treating synthetic vinasse at anaerobic and micro-aerobic conditions. They targeted the V6-V8 regions of the bacterial 16SrRNA genes for amplification by polymerase chain reaction (PCR). They used bacterial primers 968-F-GC and 1401-R (Sigma-Aldrich, St. Louis, MO, USA; Nübel et al., 1996). The V2–V3 regions of the archaeal 16S rRNA genes were amplified for DGGE using the primers A109(T)-F and 515-GC-R (Sigma-Aldrich, St. Louis, MO, USA) (Lane, 1991; Muyzer et al., 1993; Grosskopf et al., 1998).

Rodriguez et al. (2012) prepared the PCR mixture (50 µl) using 1 µl of each primer (10 ng/µl each primer), 10 µl of Taq and GO PCR Mastermix (MP Biomedicals, LLC) containing *Taq* DNA polymerase, PCR reaction buffer and dNTPs, 1 or 2 µl of DNA template, and Milli-Q water. They performed the PCR reaction in iCycler Thermal Cycler with the following thermo-cycling program for bacterial amplification-2 min of pre-denaturation at 95°C, 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and elongation at 72°C for 1 min, with a final 5-min elongation at 72°C. However, they changed the annealing and elongation by 52°C for

40 s and 90 s long, respectively. Therefore, DGGE analysis was performed for microbial (bacterial and archaeal) analysis using urea and foramide.

Eren et al. (2013) described a filtering method to produce high quality short reads using Illumina Paired-End Technology. They also explained about consensus between independent reads which improves the accuracy of genome and transcriptome analysis. “Natural variation of biological significance is showed by lack of consensus between similar sequences in metagenomics studies. They used the overlap analysis, which relies upon consensus between paired-end reads rather than Q-scores, to identify sequences errors in an Illumina HiSeq dataset of V6 amplicon sequences (Eren et al., 2013). This process minimizes the error in shotgun sequencing data. This shotgun sequencing data produced from libraries with small, tightly constrained insert sizes

Zhou et al. (2011) explained the use of overlapping reads to improve amplicon sequence quality and Masella et al (2012) developed a fast aligner (PANDAseq) for overlapping paired ends reds which employs Q-scores to decrease the disagreements between mismatches. It produced more data of high quality reads compared to other methods.

Robertson et al. (2013) used Explicet: graphical user interface software for metadata-driven management, analysis and visualization of microbiome data. Several high performance sequence analysis pipeline such as QIIME, MOTHUR, VAMPS are available for microbiome analysis. The cost associated with DNA sequencing is decreased 10,000-fold from earlier sequencing cost. As a result, the volume of data has increased for analysis. So, they developed software Explicet, “which takes the output data from sequence analysis pipeline and provides metadata-driven management, analysis, and visualization of sequence classification results’’ (Robertson et al., 2013). Explicet reduced the need of personnel with bioinformatics/computational expertise in many laboratories. (Hara et al., 2013; Markle et al., 2013; Robertson et al., 2013).

Werner et al. (2012) compared Illumina paired-end and single-direction sequencing for microbial 16S rRNA gene amplicon surveys. Researchers used mainly 454 pyrosequencing

platform for analyzing microbial community structures in past. However, high-throughput sequencing of 16S rRNA is a new and advanced tool for doing these kinds of analysis among hundreds of samples because Illumina platform can produce large number of reads (1.5 million reads) compared to pyrosequencing platform reads (1 million reads) almost at the same cost. They used the sequences for downstream process which pass the quality threshold in both 5' and 3' direction. However, Illumina produces only 75-150 bp per read compared to 250-400 bp per read on 454 pyrosequencing. They found slightly more alpha diversity in paired end in compare to 5' single direction reads. They also used three beta diversity metrics to find the distance between samples in both paired end data and 5' single direction data: Bray-Curtis (based on abundances of OTUs), unweighted UniFrac (based on phylogenetic structure), and weighted UniFrac (based on phylogenetic structure, weighted by OUT abundances). They calculated these on a scale from 0 to 1. They noticed closely correlated data for Bray-Curtis distance for OTUs picked based on paired end reads and 5' single direction reads and no practical difference in the unweighted UniFrac clustering data (Bartram et al., 2011) obtained overlapping paired end Illumina reads covering the V3 and V6 region of 16S rRNA genes. However, paired ends reads do not overlap with other primer regions (V1-V2 OR V4) which are greater than 200bp in length.

Justin Kuczynski et al. (2011) described the method for analysis of 16S rRNA gene sequences for determining microbial communities using QIIME. They explained how to install QIIME on a computer and use this approach to analyze microbial 16S sequence data from 9 distinct microbial communities. They installed the QIIME virtual box to run the analysis. They described the method how to check the mapping file, assign multiplexed reads to samples based on their nucleotide barcode, quality filtering, picking OTUs, assigning taxonomy, interfering phylogeny, and creating an OTU table.

DeSantis et al. (2006) explained greengenes, a chimera –checked 16S rRNA gene database. Chimeras are DNA sequences of DNA from two or more parents. These are formed during the PCR process. Greengenes has four features a standardized set of descriptive fields, taxonomic assignment, chimera screening, and ARB compatibility which improves the data for

microbial analysis. Greengenes provides a similar multiple-sequence alignment of both archaeal and bacterial 16S small subunit rRNA genes to promote taxonomic placement. “Greengenes offers annotated, chimera-checked, full-length 16S rRNA genes sequences in standard alignment’’ (DeSantis et al., 2006). 16S rDNA amplifications always produce chimeric amplicons as a by-product (Qiu et al., 2001).

Walters et al. (2011) described about PrimeProspector which is an open-source software for de novo design and taxonomic analysis of barcoded polymerase chain reaction. It allows researchers to prepare new primers from the list of available sequences and to estimate existing primers for generating taxonomic data. Hundreds of samples can be distinguished together in a single run using next-generation sequencing methods (Tringe and Hugenholtz, 2008). The primers should amplify at the right position of DNA that is the right length for sequencing and also taxonomically informative (Liu et al., 2008; Wang et. al., 2007). Hence, PrimeProspector is a fast and extensible framework for primer design and analysis.

Cole et al. (2005) explained the ribosomal database project (RDP-II) which provides information about the research community with aligned and annotated rRNA gene sequence as well as analysis services and a phylogenetically consistent taxonomic framework for data. RDP-II data and analysis services can be found at http://rdp.cme.msu.edu/. They revised the RDP analysis services to support the new changes in high-throughput rRNA sequence analysis in microbial ecology and related disciplines. Cole et al. (2009) also described the RDP probe match which analyzed about the phylogenetic distribution of each primer sequence. The aim was to obtain broadly distributed primers that amplify regions of approximately 100-150 bp in length such that the paired end reads from an amplicon would show some overlap.

Cole et al. (2013) released ribosomal database project (RDP) 11.1 in October 2013. “This database contains 2 809 406 aligned and annotated bacterial and archaeal small subunit (SSU) r RNA gene sequences and 62 860 fungal large subunit (LSU) rRNA gene sequences” (Cole et al., 2013). RDP added new methods for browsing and searching the data collections. In additions,

taxonomic classification and nearest neighbor search can also be performed using this database. RDP pipeline is developed for analyzing large volume of amplicon sequence data.

Degnan and Ochman (2012) also explained the Illumina-based analysis of microbial community diversity. Microbes are present in nature with various complexity and diversity. Cultivation-based technology is not enough to give accurate results for microbial diversity. Hence, these problems have been overcome by using a different molecular technique by targeting the universally conserved 16S ribosomal RNA gene. They mentioned about 454 pyrosequencing to sequence thousands of 16S rDNA (pyrotags) sequences together. This technique provided the result of complex microbial communities. Quality trimming of pyrotags is very important for analyzing community composition (Kunin et al., 2010). Hence, Degnan and Ochman filtered the assembled reads based on consensus quality scores estimated by Phrap. 454 pyrotags studies is an advanced method till today. However, Illumina sequencing generated higher number of sequence reads at lower cost. “They used the Illumina sequencing platform to design a method for 16S amplicon analysis (iTags), and studies its generality, practicality, and potential complications” (Degnan and Ochman, 2012). They also explained about sequence paired-end libraries of amplified hyper-variable 16S rDNA fragments. These fragments were produced from different sets of samples which are different in their contents from a single bacterium to highly complex communities. Taxonomic classification could be difficult for short 16S tags and gives limited phylogenetic resolution (Liu et al., 2007). Degnan and Ochman (2012) found that RDP Classifier gives taxonomy classification of all of the 19 expected OTUs. However, it doesn’t provide good success rate for lower taxonomic divisions and gives only 60-90% accuracy. They developed a method to analyze data more efficiently from a large number of sequencing reads. However, Huse et al. (2008) explained about BLAST classification approach which provides more accuracy about full length 16S rRNA sequences. Degnan and Ochman (2012) also evaluated the reason for several potential errors, including sequencing artifacts, amplification biases, non-corresponding paired-end reads and mistakes in taxonomic classification.

2.5 Previous Studies of Microbial Communities in Anaerobic Bioreactors

The microbial communities in anaerobic bioreactors can be identified using molecular probes. The oligonucleotide probes, complementary to conserved tracts of the 16S rRNA of phylogenetically defined groups of methanogens and sulfate-reducing bacteria, can be used to identify microbial community structure (Raskin et al., 1995). Raskin et al. (1995) determined methanogens to be around 8-12% of the total community in healthy mesophilic single phase sludge digesters. *Methanosarcinales* and *Methanomicrobiales* were dominant in the anaerobic sewage digester, whereas only a small number *of Methanococcales* and *Methanobacteriales* were found. Phylogenetic groups of mesophilic, gram negative sulfate-reducing bacteria were consistently present at significant levels (Raskin, 1995). *Desulfovibrio* and *Desulfobulbus spp*. were the dominant sulfate-reducing population, *Desulfobacter* and *Desulfobacterium spp.* were present at lower levels, and *Desulfosarcina, Desulfococcus*, and *Desulfobotulus spp.* were present (Raskin et al., 1995).

Morales el al. (1996) described the methanogenic and acidogenic tests in an anaerobic thermophilic bioreactor. They performed these tests on lab scale wine distillery wastes in the thermophilic range (55ºC). They measured the maximum activity of the different anaerobic microorganism groups in a short span of time: approximately 24 hours in acidogenic assays and 2 days in methanogenic assays. The tests developed can be used to perform standardized inhibition studies and are very important for the operation and control of anaerobic bioreactors.

Bacterial population can also be determined in anaerobic bioreactors using a combination of denaturing gradient gel electrophoresis (DGGE) and real time PCR techniques. Kim et al. (2011) identified bacterial populations in synthetic glucose wastewater, whey permeate, and liquefied sewage sludge for using DGGE and PCR techniques. They found the most common acidogens bacterial populations related to *Aeromonas* spp. and *Clostridium sticklandii* in all reactors. *Aeromonas* populations accounted for up to 86.6–95.3% of total bacterial 16S rRNA genes during start-up, suggesting that, given its capability of utilizing carbohydrate, *Aeromonas* is likely the major acidogen group responsible for the rapid initial fermentation of carbohydrate. *C.*

sticklandii, able to utilize specific amino acids only, occupied up to 8.5–55.2% of total bacterial 16S rRNA genes in the reactors tested. The bacterial 16S rRNA gene fragment was PCR-amplified using the primers BAC338F and BAC805R, targeting the domain bacteria.

Sawayama (2006) measured microbes from an anaerobic bioreactor. They used a 1-L cylindrical glass vessel containing a cylindrical reticular polyurethane foam sheet for immobilization of microbes, thermophilically and anaerobically digested sludge. 16S rRNA gene densities of *Methanosarcina* spp. and *Methanobacterium* spp. immobilized on polyurethane foam were determined as 7.6×10^9 and 2.6×10^8 copies/cm^3, respectively, by the real time PCR analyses, and those freely living in the bioreactor were 7.6×10^7 and 2.6×10^7 copies/cm^3. PCR increases the bacterial rRNA gene copy density 10 times from the original thermophilic sludge to the immobilized microbes, and the methanogenic rRNA gene copy density increased 1000 times. Real-time PCR primer and probe sets used by Sawayama (2006), as well as several other researchers, for measurement of the 16S rRNA gene copy number for some microbes are given in Table 2.6.

Kim et al. (2010) studied the growth condition of bacterial community during anaerobic digestion of food waste- recycling wastewater for maximum hydrolysis of suspended organic materials. They found propionic and butyric acids in the beginning of digestion. They also identified many bacterial communities such as Clostridium thermopalmarium and Clostridium novyi, which are responsible for producing butyric acids and propionic acid. These two hydrogens were present in that reaction. They also found other acidogenic anaerobes such as Aeromonas sharmana, Bacillus coagulans, and Pseudomonas plecoglossicida. Soh et al. (1991) also identified Clostridìum thermopalmarìum sp. as a butyric acid producing bacterium in Palm Wine in Senegal. Cirne et al. (2006) found the microbial communities during anaerobic digestion of energy crops. They performed batch digestions using two substrates, sugar beets and grass/clover. They concluded that solubilization of organic material was faster in the first 10 days with the production of volatile fatty acids (VFAs) and lactate. In both substrates, Archaea started showing up in the hydrolytic phase between 10 to 15 days and then Archaea started decreasing.

They found higher percentage of Firmicutes in grass/clover and Alphaproteobacteria in beets. Limam et al. (2013) found WWE1 in the anaerobic digestion of cellulose at 35°C. The ^{13}C apparent isotopic composition of hybridized WWE1 cells reached the value of about 40% early during the cellulose degradation process in solid municipal waste and mature compost of green waste. This indicates that WWE1 is responsible for hydrolysis of extracellular cellulose and in the absorption of fermentation products.

Ferreira et al. (2010) demonstrated a method for decolorization of vinasse using the *fungus Pleurotus sajor-caju* CCB 020 and degradation of complex vinasse compounds. Melanoidin in vinasse is responsible for dark brown color of vinasse. Melanoidin is produced from Maillard reaction of sugars (carbohydrates) with proteins (amino acids). The reason for discoloration of vinasse is due to higher activities of laccase, manganese-peroxidase and peroxidases. "*P. sajor-caju* demonstrated a rise in biomass production (1.06 g/100 ml), and the enzyme activities such as laccase (varying from 400 to 450 IU/l) reached between the 9th and 10th day of growth and for MnP at the 12th day of cultivation (varying from 60 to 100 IU/l). The basidiomycetes: *P. sajor-caju*, *P. chrysosporium*, *Pleurotus*. sp. 068, *P. shimeji* e *Ganoderma* sp. showed a better potential in the discoloration, followed by the ascomycetes: *A. niger*, *T. reesei* e *M. pusillus*, in the order of 98–48%, when incubated in 100% vinasse for 12 days". (Ferreira et al., 2010).

Table 2.6 Real-Time PCR Primer and Probe Sets Used for Measurement of the 16S rRNA Gene Copy Number (Sawayama, 2006)

Target	Sense primer	Anti-Sense primer	Double dye probe	References
Bacteria	S-D-Bact-0348-S-a-17 (AGGCAGCAGTDRGGAAT)	S-D-Bact-0786-A-a-20(GGACTACYVGGGTATCTAAT)	S-D-Bact 0515-S-a-25(TGCCAGCAGCCGCGGTAATACRDAG)	Takai and Horikoshi (2000)
Methanogenic Archaea	S-P-MArch-0348-S-a-17 (GYGCAGCAGGCGCGAAA)	S-D-Arch-0786-A-a-20(GGACTACVSGGGTATCTAAT)	S-P-MArch-0515-S-a-25(TGCCAGCMGCCGCGGTAAYACCGGC)	Takai and Horikoshi (2000)
Methanobacterium spp.	S-F-Mbac-0398-S-a-20 (CCCAAGTGCCACTCTTAACG)	S-G-Mbac-0578-A-a-22(AGACTTATCAARCCGGCTACGA)	S-G-Mbac-0526-A-a-33(AAYGGCCACCACTTGAGCTGCCGGTGTTACCGC)	Sawayama et al. (2006)
Methanothermobacter spp.	S-F-Mbac-0398-S-a-20(CCCAAGTGCCACTCTTAACG	S-G-Mthb-0589-A-a-25 (GGGATTTCACCAGAGACTTATCAG	Mthb-0549-S-a-32(CGGACGCTTTAGGCCCAATAAAAGCGGCTACC)	Sawayama et al. (2006)
Methanosarcina spp.	S-G-Msar-0450-S-a-19(TAGCAAGGGCCGGGCAAGA	S-G-Msar-0589-S-a-20(ATCCCGGAGGACTGACCAAA)	S-P-Msar-0540-A-a-31(AGACCCAATAATCACGATCACCACTCGGGCC	Sawayama et al. (2006)
Methanosaeta spp.	S-F-Msae-0387-S-a-21(GATAAGGGRAYCTCGAGTGCY	S-F-Msae-0573-A-a-17 (GGCCGRCTACAGACCCT	S-F-Msae-0540-A-a-31(AGACCCAATAAHARCGGTTACCACTCGRGCC	Sawayama et al. (2004)

2.6 Factors Affecting Anaerobic Digestion and Biogas Production

Taherdanak and Zilouei (2014) performed alkaline pretreatment for improving biogas production from wheat plants. Alkaline pretreatment was initiated using 8% (w/v) NaOH solution at five different temperatures (0, 25, 75, and 100°C). The best result was obtained by alkaline pretreatment at 75°C for 60 min.; high amount of glucose was also found at this pretreatment. The cumulative methane yield for wheat plants pretreated at 25, 50 and 75°C was higher than that of the untreated wheat plants by 47.5%, 40.8% and 54.5%, respectively. However, biogas production was not increased effectively at 0 and 100°C pretreatment. Aguiar et al. (2010) also described that the alkaline pre-treatment solubilizes the hemicelluloses and lignin content. The alkali processes tend to promote a high dissolution of the lignin and lower the solubilization/fragmentation of the hemicelluloses (Melo and Azevedo, 1997; Enari, 1983; Esposito and Azevedo, 2004; Kuwahara et al. 1984; Bon et al., 2008). The pre-treatment allows a contact surface and a rupture in the structure of the lignin and cellulose fibers, consequently facilitating the hydrolysis to the simple sugar (Kuhad et al., 1990; Kuwahara *et al.,* 1984; Bon et al., 2008). The pre-treatments can be physical (as defibrillation by pressure and explosion) or chemical (acid and alkaline hydrolysis).

Chandra et al. (2012) demonstrated the experimental methane fermentation study on untreated, NaOH and hydrothermal pretreated substrates of wheat straw at mesophilic temperature (37°C). Substrate concentrations were maintained at 4.45% VS (44.5 g VS/L). The specific methane and biogas production yields of untreated wheat straw substrate were 78.4 L/kg VS_a and 188.4 L/kg VS_a, respectively. However, the specific methane and biogas production yield of NaOH pretreated wheat straw substrate were 165.9 L/kg VS_a and 353.2 L/kg VS_a, respectively. Hydrothermal pretreated wheat straw substrate yielded specific methane and biogas production values of 94.1 L/kg VS_a and 205.7 L/kg VS_a, respectively. NaOH pretreated substrate produced 87.5% higher biogas production and 111.6% higher methane production compared to the untreated wheat straw substrate. Hydrothermal pretreated substrate

resulted in an increase of 9.2% in biogas production and 20.0% in methane production compared to that of untreated wheat straw substrate.

Song et al. (2013) studied the effect of alkaline pretreatment using calcium hydroxide to increase the production of methane from rice straw. Rice straw compounds, such as lignin, cellulose, and hemicelluloses, were significantly degraded with an increase of $Ca(OH)_2$ concentration. They used the different concentrations (4%, 8%, 12%) of $Ca(OH)_2$ to treat rice straw. They added $Ca(OH)_2$ in 500g of rice straw and found the change in the main composition of rice straw after chemical pretreatment. As a result, the concentration of 8% and 12% $Ca(OH)_2$ were more efficient in breaking down the lignocelluloses matrix and supplied organic matter for methanogen. Hence, it increased the methanogenesis process and increased the methane yield, too. Hoon (2004) also demonstrated the effect of alkali pretreatment to increase the biodegradation of complex materials. Kaar and Holzapple (2000) also noticed that slake lime pretreatment enhanced the enzymatic hydrolysis of corn stover by nine times compared to non-treated corn stover.

Thanikal et al. (2007) used polyethylene material (Bioflow 30) as inert media in an anaerobic fixed bed reactor to treat vinasse (wine residue after distillation). They operated this system for 6 months. The specific area and density of the Bioflow 30 were 30 mA^2/mA^3 and 0.93, respectively. They found that the efficiency of the reactor in removal of soluble COD was very good with a maximum organic loading rate of more than 30g of COD/L.D and a COD removal efficiency of more than 80%. They used volume of sludge 10% of the volume of reactor. Polyethylene material has a high capability of biomass retention with 4-6g of dried solids per support. Bioflow 30 is an excellent support and can be used in a high rate anaerobic fixed bed. Hence, the operation of the reactor is possible with a very high loading rate as a result of the increase of the solids in the reactor and maintaining the specific gravity.

Syaichurrozi et al. (2013) described kinetics of biogas production. They operated batch anaerobic digesters at room temperature with retention time of 60 days. They calculated the

biogas fermentation of vinasse within a wide range of COD (chemical Oxygen demand)/N (total nitrogen) ratios. Urea was used as a source of nitrogen to adjust COD/N ratio of 400/7-700/7. They noticed the control variable 400/7, 500/7,600/7,700/7 produced total biogas of 107.45, 123.87, 133.82, 139.17 mL/g COD and had the value of COD removal of 31.274 ± 0.887, 33.483 ± 0.266, 36.573 ± 1.689, 38.088 ± 0.872, 32.714 ± 0.881%, respectively. The found the good result with variable of COD/N ratio of 600/7. On kinetic model of biogas production, COD/N of 600/7 had kinetic constant of *A*, μ and λ of 132.580 ml/g COD, 15.200 mL/g COD/day and 0.213 days, respectively. The kinetic model of biodegradability organic material during production biogas was C (t) = (Co- exp{- exp[μe/A(λ-t) +1]}* Co) + Ce. where, *C*(*t*) is COD at any time (*g*); Co is total COD that can be removed (g) (Co = Ci–Ce, Ci is influent COD (*g*), Ce is effluent COD(g)), A is biogas production potential (mL/g COD), μ is maximum biogas production rate (mL/g COD/day), λ is lag phase period or minimum time to produce biogas (days), t is cumulative time for biogas production (days) and e is mathematical constant. Many authors predicted the kinetic model of biogas production from vinasse using Gompertz equation. Budiyono et al. (2010a) used modified Gompertz equation for the kinetic model of biogas production from cattle manure. Elaiyaraju and Partha (2012) also used modified Gompertz equation to calculate the kinetic model for biogas production potential from orange peel waste and jatropha de-oiled cake. Nopharatana et al. (2007); Zhu et al. (2009); Patil et al. (2012) and Adiga et al. (2012) also modeled the kinetics of biogas production from waste materials using modified Gompertz equation.

Espinoza-Escalantae et al. (2009) studied the effect of three operational parameters - pH, hydraulic retention time (HRT) and growing temperature - on a semi-continuous bioreactor during the anaerobic digestion Tequilla vinasse. These changes were measured by four response variables: total reducing sugars consumption, VFAs, hydrogen and methane production. They also used the mathematical models to explain the effect of the operational parameters based on different variables. They found that hydrogen production is favored at thermophilic temperature

55˚C and mesophilic temperature (35˚C) for methane production, operating the reactor at a slight acidic pH range and at a higher HRT in the boundaries of the experimental design.

Aguiar et al. (2010) cultivated three strains of Pleurotus and Trichoderma reesei in media with pre-treated bagasse and vinasse. They analyzed the cellulolytic and lignolytic activities and biomass production. The *P. sajor-caju* CCB020, *P. ostreatus* and the *T. reesei* showed a higher mycelial biomass when cultivated in the medium containing vinasse, between 30 and 35ºC. Bagasse was treated with 2% H_2O_2 + 1.5% NaOH + autoclave at 121º C for 15 minutes. As a result, the bagasse fibers became more accessible to the culture due to breakage of the fibers and production of exoglucanases and endogluconases. The *T. reesei* had an oxidative activity of manganese-peroxidase when cultivated in medium containing bagasse and vinasse. Hence, the cellulolytic and lignolytic enzyme production can be increased by pre-treatment of the bagasse. "Also, vinasse could be reused as a supplement to the production for these enzymes as well as the production of fungal biomass in combination with bagasse" (Aguilar et al., 2010).

Rodrigues et al. (2013) compared the bacterial communities from pasture soils and forest soils. They studied community diversity and similarity by using both taxonomic and phylogenetic measures. The alpha and beta diversity were analyzed using QIIME software. They noticed the significant difference between bacterial communities from pasture soils and forest soils in both taxonomic and phylogenetic composition, as shown in Figure 2.2. "The largest proportional decrease in response to conversion was by the phylum *Acidobacteria*, which decreased from an average of 21.1% [±0.05; 95% confidence interval (CI)] of the OTUs identified in the forest to 13.4% (±0.08; 95% CI) of those in the pasture, followed by the phyla *Nitrospirae* and *Gemmatimonadetes.* The largest proportional increase in response to conversion was by the phylum *Firmicutes*, which increased from 2.2% (±0.02; 95% CI) in the forest samples to 12.6% (±0.07; 95% CI) in the pasture, followed by slight increases for *Actinobacteria* and *Chloroflexi" (*Rodrigues et al., 2013). They also found bacterial communities are higher in pasture soils than forest soils in alpha diversity.

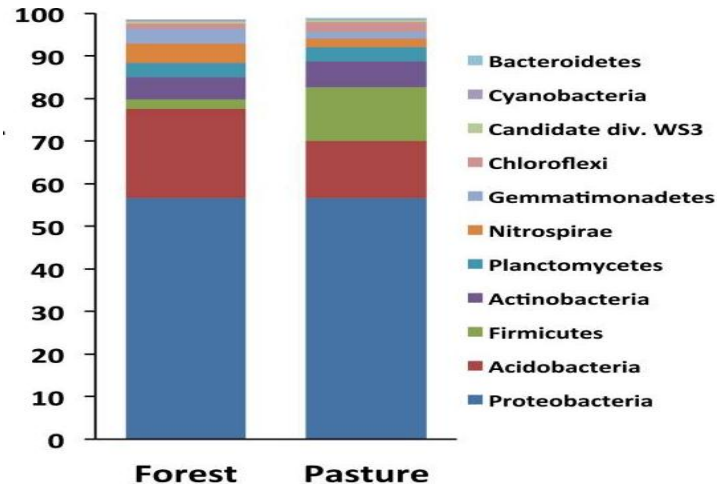


Figure 2.2 Bacterial Community Composition of Pasture and Forest Soil Samples

Rodriguez et al. (2012) performed molecular analysis of the biomass of a fluidized bed reactor treating synthetic vinasse at anaerobic and micro-aerobic conditions. They found the bacteria and archaea in this system by using the method denaturing gradient gel electrophoresis (DGGE). They mainly detected *Methanomethylovorans* and *Methanosaeta* within the Archaea population. Hydrogenotrophic methanogens mainly belonging to the genus *Methanobacterium* were detected at the highest substrate to sulfate ratio but rapidly disappeared by increasing the sulfate concentration.

Several previous studies have looked at microbe communities involved in anaerobic degradation of vinasse as mentioned above. However, these researchers have not looked at how the communities change as functions of time, temperature and vinasse composition. Aguiar et al. (2010) cultivated three strains of Pleurotus and Trichoderma reesei in media with pre-treated bagasse and vinasse. But, they didn't see the change in microbial communities with change in composition. Ferreira (2010) demonstrated a method for decolorization of vinasse using the *fungus Pleurotus sajor-caju* CCB 020 and degradation of complex vinasse compounds. But, they didn't find how these microbes varied with different composition of vinasse with temperature and time. Rodriguez et al. (2012) performed molecular analysis of the biomass of a fluidized bed reactor treating synthetic vinasse at anaerobic and micro-aerobic conditions. They found the

bacteria and Archaea in this system by using the method denaturing gradient gel electrophoresis (DGGE). This method is slow and more time consuming compared to Miseq.

My research focuses on how microbial communities vary with time, temperature, and composition. The microbial communities increased and decreased with time in different composition of vinasse at different temperatures. The percentage of Methanogens can be compared in real and synthetic vinasse. The factors (composition and temperature) which are responsible for producing more biogas are mentioned in Chapter 4. Previous researchers performed alkali treatments and used TMS and NaOH for the better performance of vinasse degraded organisms at single temperature and one composition. However, my research includes every alternate day data to see difference in microbial communities varying with different composition (6 compositions) of vinasse at different temperatures (30, 35, and 40°C). The microbial communities were identified in each step of hydrolysis, acidogenesis, acetogenesis, and methanogenesis phase in my research using Miseq. In addition, the phylogenetic diversity and species richness data are also included with different composition at different temperatures, compared to previous research on vinasse. The microbial data of real and synthetic vinasse are compared in my research using Illumina sequencing technology.

Chapter 3

Materials & Methods

3.1 Introduction

The goal of this research was to investigation of how microbes involved in methane generation during anaerobic degradation of vinasse change as functions of temperature, vinasse composition, and time.

The methodology for this research was divided in 5 tasks.

Task 1: Developing an experimental design for studying the effect of vinasse composition, temperature, and time for the production of methane gas.

Task 2: Setting up laboratory-scale reactors based on experimental design. This task included synthetic vinasse preparation and real vinasse storage and collection from different ethanol production facilities, as well as designing and constructing gas-tight laboratory-scale bioreactors. Synthetic vinasse was prepared in the lab using different chemical reagents which contain nitrogen, phosphorous, potassium, and sulfur nutrients. Trace elements were also added in real and synthetic bioreactors.

Task 3: Operating and monitoring laboratory scale bioreactors. This step involved regular monitoring of pH, temperature and gas fitting connections. In addition, gas volume and gas composition, including as methane (CH_4), carbon dioxide (CO_2), and oxygen (O_2) concentrations, were measured.

Task 4: Sample collection and library preparation for identifying different microbial communities. Vinasse samples were collected every alternate day for six different compositions at three different temperatures. Polymerase chain reaction, DNA extraction, gel extraction and gel purification methods were performed for Illumina Miseq library preparation.

Task 5: Microbial Analysis. QIIME software (Quantitative Insights Into Microbial Ecology) was used for analysis. QIIME is an open source software package which is used for comparing and analysis of diverse microbial communities present in different types of sample. This is mainly

based on high-throughput amplicon sequencing data (such as SSU rRNA) produced on a variety of platforms.

3.2 Task 1 Experimental Design

To study the effects of methane generation, vinasse compositions were varied at three different mesophilic temperatures (30, 35, 40°C). The main constituents of vinasse include organic matter (measured as chemical oxygen demand, or COD), N, P, K, and S. The reactor was fed with vinasse of 6 different compositions, as shown in Table 3.1. The vinasse compositions were made from various combinations of synthetic chemicals and real vinasse. Synthetic vinasse compositions were based on compositions provided in a literature review by Wilkie et al. (2000), which included vinasse from ethanol produced from various feedstocks. Nutrients were added between the higher and lower range of nutrients mentioned in literature review. Initially, 15 different synthetic formulations were tried. The 3 synthetic compositions used in this research were the ones that facilitated microbe growth and gas production.

Synthetic vinasse (compositions 4, 5, and 6) was prepared in the lab using distilled water. Synthetic vinasse compositions 4, 5, and 6 have low, medium, and high COD, respectively. Glucose was used to provide COD, ammonia to provide N, phosphoric acid to provide P, potassium hydroxide to provide K, and sulfuric acid to provide S. The concentrations of N, P, K, and COD varied in different bioreactors, between the higher and lower range of vinasse composition values from literature. A trace mineral solution (TMS) was added to ensure that the microbes had sufficient minor nutrients (Peixoto et al., 2007; Van Lier et al., 1992; Aguiar et al., 2010). 25-30g of buffer (sodium bicarbonate) was added for pH maintenance.

Table 3.1 Experimental Design of Real and Synthetic Vinasse Compositions in Six Bioreactors

			Synthetic and Real Vinasse Composition					
			(g of constituents per L of vinasse)					
		Composition	**COD**	**N**	**P**	**K**	**S**	**Real**
	COD	**Number**	**$C_6H_{12}O_6$**	**NH_3**	**H_3PO_4**	**KOH**	**H_2SO_4**	**Vinasse**
Real Vinasse	Medium	1	27.1	23.7	1.14	0.15	0.62	3.5L
	High	2	32.1	28.6	1.39	0.17	0.62	4L
	Low	3	23.9	40.1	0.67	0.08	0.44	300g
Synthetic Vinasse	Low	4	2.53	0.19	0.16	0.07	0.08	0
	Medium	5	9.83	0.22	0.13	0.02	0.12	0
	High	6	30.5	19.1	1.17	0.01	0.15	0

Real vinasse combinations 1, 2 and 3 were prepared using liquid and solid real vinasse with different amounts of DI water. Real vinasse compositions 1, 2, and 3 was produced from corn and milo.

3.2.1 Temperature

Three different mesophilic temperatures 30ºC (86ºF), 35ºC (95ºF), 40ºC (104ºF) were used for anaerobic degradation of vinasse.

3.3 Task 2 Setting Up Laboratory-Scale Reactors

3.3.1 Vinasse collection

Real vinasse, produced from corn and milo, was shipped from three U.S. companies: White Energy, Plainview, TX; White Energy, Hereford, TX; and MGP Ingredients, KS. Real vinasse compositions 1 and 2 brought from White Energy, Plainview TX and White Energy, Hereford, TX. Composition 1 and 2 real vinasse was produced from corn and milo food grains. Both the facilities of White Energy produced ethanol using corn and milo. Only, the amount of vinasse used in experimental design was 3.5 L and 4 L. Whereas, solid vinasse (composition 3) was brought from MGP ingredients, KS, which were produced from corn only. Alkali treatment was performed before using the liquid form of real vinasse. Alkali treatment is a process in which the pH of vinasse is increased to 12 using sodium hydroxide lentils for 24 hours at room temperature (70ºF). Alkali treatment is helpful in hydrolyzing sugars and other complex organic materials (lipids, proteins) to make them more digestible for microorganisms. After alkali treatment, the pH was decreased to around 7 using hydrochloric acid (Espinoza-Escalante, 2008). Alkali treatment was only performed with real vinasse because it helped in hydrolyzing sugars and other complex organic materials, to make it more digestible for microorganism (Taherdanak and Zilouei, 2014; Aguiar et al., 2010). However, synthetic vinasse does not need alkali treatment because carbon was added synthetically in the form of glucose, which is readily available as a food source for microorganisms. Trace elements were not added in real vinasse because it already contains trace elements; trace elements were added only in synthetic vinasse to provide sufficient nutrients to microorganisms for their proper growth.

3.3.2 Synthetic Vinasse

Synthetic vinasse (compositions 4, 5, and 6) with high, medium, and low COD was prepared in the lab by dissolving different reagents, as mentioned in Table 3.1, using distilled water. Glucose was used to provide COD, ammonia to provide N, phosphoric acid to provide P, potassium hydroxide to provide K, and sulfuric acid to provide S. Real vinasse contains cellulose, hemicelluloses, and lignin which are a food source for microbial activity in anaerobic digestion of vinasse. Glucose was added in synthetic vinasse as a food source. Since glucose is more easily digested than cellulose, hemicellulose, and lignin, it likely was not very representative of actual vinasse. 25g $NaHCO_3$ was added to maintain the pH and 900ml sludge added to synthetic vinasse (McCarty, 1964; Chamy et al., 2007 and Lau & Fang, 1997).

(a) (b)

Figure 3.1 Synthetic Vinasse (a) Vinasse after Adding All the Nutrients without Sludge (b) Vinasse after Adding All the Nutrients with Sludge

The concentrations of vinasse parameters (COD, N, P, K, S) were varied in 3 6.8-L glass bioreactors over ranges corresponding to vinasse produced from ethanol from various feedstocks (sugars and starches). Synthetic vinasse is shown after adding all the nutrients without sludge in

and after adding sludge in Figure 3.1 above. Trace elements were also added in all compositions of synthetic vinasse. Trace elements and quantity (g/L) are listed in Table 3.2**.**

Table 3.2 Trace Nutrients Added to Vinasse

Trace Element	**g/ L (Distilled water)**
Mineral Salts	
Ammonium Chloride (NH_4Cl)	0.53 g
Calcium Chloride ($CaCl_2.2H_2O$)	75 mg
Magnesium Chloride ($MgCl_2.6H_2O$)	100 mg
Ferrous Chloride ($FeCl_2.4\ H_2O$)	20 mg
Trace Metals	
Manganous Chloride ($MnCl_2.\ 4H_2O$)	0.5 mg
Boric Acid (H_3BO_3)	0.05 mg
Zinc Chloride ($ZnCl_2$)	0.05 mg
$CuCl_2$	0.03 mg
$NaMO_4.\ 2H_2O$	0.01 mg
Cobalt Chloride ($CoCl_2.6\ H_2O$)	0.5 mg
Nickel Chloride ($NiCl_2.6\ H_2O$)	0.05 mg
Sodium Selenide Anhydrous (Na_2SeO_3)	0.05 mg

3.3.3 Real Vinasse

Real vinasse composition 3 (produced from corn) was prepared by dissolving 300g semisolid vinasse in distilled water and 30g of glucose and 25g of sodium bicarbonate. The reactor was filled with distilled water till 6.8 L after transferring the semisolid vinasse inside the reactor. Alkali treatment was not performed during this process because 30g of glucose was added as a supplementary food source in this bioreactor. 25-30g sodium bicarbonate ($NaHCO_3$)

was added to buffer the solution against further pH changes after adjusting pH between 6-9 using NaOH (Peixoto et al., 2012; Ryznar-Luty et al., 2008). Alkali treatment was performed in real vinasse compositions 1 and 2 with liquid real vinasse 3.5 L and 4 L. The liquid real vinasse (as shown in Figure 3.2) was transferred to 6.8 L reactor after finishing alkali treatment and DI water was added till 6.8 L. Sodium bicarbonate was not added in the 3.5 and 4.5 L vinasse because it was not helping in maintaining the pH like synthetic vinasse. Since, high amount of sodium is not helpful for microbial growth, it's good to keep it below toxic range. 900 ml sludge was added to both synthetic and real vinasse bioreactors to provide a microbial seed.

Figure 3.2 Real Vinasse after Alkali Treatment without Sludge

3.3.4 Reactors

Three 12 L bioreactors with working volumes of 6.8 L were obtained from VWR International (as shown in Figure 3.3). The flask design gives an optimum inert cell growth environment. A bottom dimple prevents crushing of cells beneath the magnetic impeller and

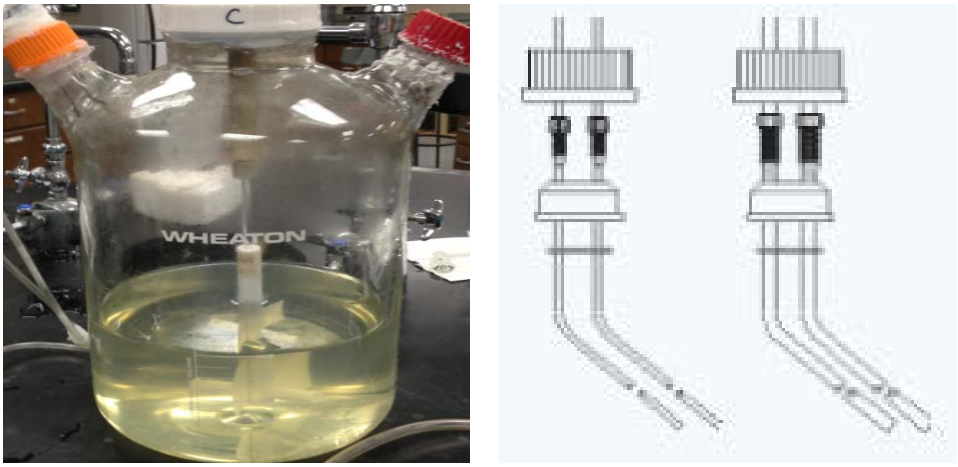
improves cell yield. The reactors are made from borosilicate glass and include a magnetic stirrer to provide homogenous mixing (at a speed of 5-10 rpm) of vinasse (as shown in Figure 3.4). Silicon sealant was used to ensure that the reactors were air-tight. “Gas and media handling fittings as shown in Figure 3.3 below with 3.2 mm (1/8’’) and 6.4 mm (1/4’’) inlets with two long tubes were also ordered from VWR International (as shown in Figure 3.3)”. These fittings were used to connect with gas bags. The reactor with fittings and gas bag is shown below in Figure 3.4. Trace elements, which are important for anaerobic bacteria, were added to the vinasse in the concentrations shown in Table 3.2 above, to ensure that the microbes are not nutrient-deficient. 1L TMS were added in all synthetic bioreactors. 900 ml of anaerobic sludge from the Village Creek Wastewater Treatment Plant, Arlington, TX was added to inoculate each bioreactor with an initial supply of microbes. The total vinasse/sludge volume in each reactor was brought to 6800 ml. A bioreactor of each composition 1-6 was operated at each of three mesophilic temperatures (30, 35, & 40ºC), via placement in constant temperature rooms (as shown in Table 3.4). 5 compositions and 3 temperatures gave a total of 18 reactors. A duplicate set of reactors was also operated for two temperature/composition combinations. The reactors were operated in batch mode for 15-89 days, depending on temperature and composition, until methane generation ceased. During anaerobic treatment, the pH of all reactors (synthetic and real vinasse) was maintained between 6 and 9 by adding sodium hydroxide.

3.3.4.1 Duplicate Reactors and Sample Duplicates

A duplicate set of reactor was also operated at 30ºC and 40ºC with one composition of real and synthetic vinasse. Reactor duplicates of real vinasse (produced from corn and milo) composition 2 (High COD) at 30ºC and synthetic vinasse composition 5 (Medium COD) at 40ºC were operated to see the similarity in microbial composition at same condition.

Sample duplicates of various compositions were also analyzed to compare the microbial diversity at same composition and same temperature. Sample duplicates of real vinasse compositions 1 at 40°C (produced from corn and milo), and 3 (produced from milo) at 30ºC &

40ºC and synthetic vinasse compositions 5 (medium COD) at 40ºC and 6 (High COD) at 30°C were analyzed.



(a) (b)

Figure 3.3 Bioreactor: (a) Bioreactor with Magnetic Stirrer (b) Media Handling Fittings

Figure 3.4 Bioreactor in Operation

3.4 Task 3 Operating and Monitoring Laboratory Scale Bioreactors

3.4.1 Gas Measurement

Gas production was measured by pumping gas out of the collection bag through a standard SKC grab air sampler (SKC Aircheck sampler model 224-44XR) at 1.0 L/min connected

to a calibrator (Bios Defender 510M) to get a minute by minute gas pumping rate. LANDTECH GEM 2000 PLUS (as shown in Figure 3.5) with infrared gas analyzer (±3% accuracy) was used for measuring % Methane (CH_4), % Carbon Dioxide (CO_2), % Oxygen (O_2), and percentage of other gases. In addition, Hydrogen Sulfide (H_2S) and Carbon Monoxide (CO) were recorded. The frequency of gas sampling depended on the amount of gas generated. During the initial stages of degradation, the gas bags were emptied twice a day, to avoid excessive buildup in the gas bags. As degradation progressed, the rate of gas production decreased and the frequency of sampling was reduced accordingly. Gas production rate was reported in STP.

(a) (b)

Figure 3.5 Gas Measurements (a) Composition Using Landtec GEM 2000 (b) Volume Measurement Using SKC Sampler & Calibrator

3.4.2 Monitoring of pH, Temperature and Gas Fitting Connections

pH was measured every day for the first 7- 10 days in the beginning for all synthetic and real vinasse because pH was decreasing during this time due to acidogenesis phase. pH was maintained between 6-9 during anaerobic digestion of real and synthetic vinasse using NaOH to provide optimal condition for the growth of microbes. After that, pH was measured every alternate day using pH meter (as shown in Figure 3.6). pH was dropped below 6 in the first 3-5 days in real vinasse whereas pH dropped below 7 in synthetic vinasse in the first 3-5 days. After that, pH was

stabilized between 6-9. Sometimes, pH decreased below 7 once in a week in real vinasse after first 7 days. In that scenario, little NaOH was added to increase the pH around 7. pH was measured every 4-5 hours for 3-5 days in the beginning of each run. It helped microbes to grow properly at optimum pH.

Temperature was maintained in the hot room using thermostat and also checked manually using hand held thermometer almost every day. Gas fittings were also checked every day to prevent any leak in the system. Hot temperature and improper handling of reactor during the sampling of vinasse may lose the connections. As a result, air can enter inside the reactor and it may delay the anaerobic digestion of vinasse.

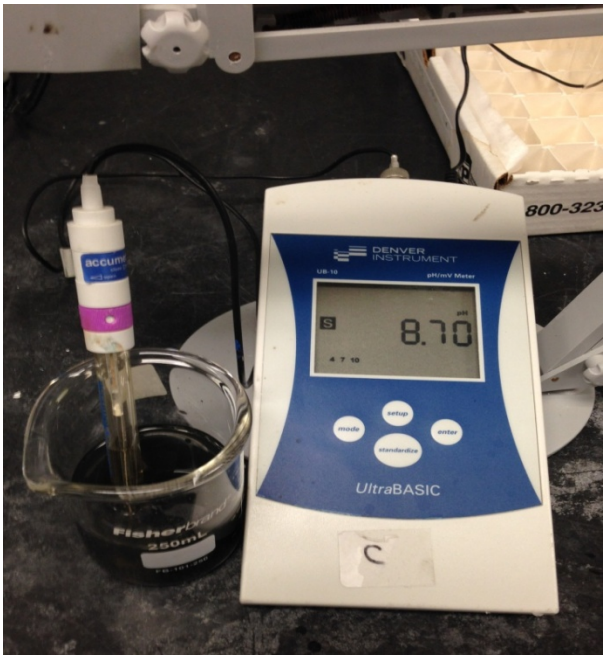


Figure 3.6 pH Meter

3.5 Task 4 Sample Collection

3.5.1 Septa

Liquid samples were collected from the side arm of bioreactor through a butyl rubber septa (shown in Figure 3.7) using a syringe (shown in Figure 3.8) every alternate day. Another student, focusing on physical-chemical characteristics of the vinasse parameters, collected these samples at varying intervals, depending on the parameters. A butyl rubber (0.125 inch of

thickness) sheet was ordered from a local dealer (http://www.rubbersheetroll.com/butyl-rubber.htm) and septa of 8 mm diameter were cut in the UTA workshop. Butyl rubber is a flexible and tolerant material and is used to sustain longevity. Butyl rubber also has great mechanical stability and durability with gases, some solvents and detergent fluids.

3.5.2 Syringes and Needles

Liquid samples of 20-25 ml were collected via syringe every alternate day and refrigerated at -20˚C for DNA extraction. 30 ml luer-Lok PK56 Syringe (Product Number BD 3028321) was ordered from VWR and needles (Product Number 305129) were ordered from BD Medical.

Figure 3.7 Septa

Figure 3.8 Syringe with Needle

3.5.3 Gas Sampling Bag

22 Liter Cali-5-Bond™ 5-Layer gas sampling bags were used for collecting gas from bioreactor (as shown in Figure 3.4). Cali-5-bond™ gas sampling bags are prepared from inert 5.5mil thick material. This thick material is prepared from 5 layers of film. “The layers are comprised of an inner layer of high-density polyethylene, followed by polyamide, an aluminum foil barrier, a polyvinylidene chloride layer and an outer layer of polyester” (Mandel Scientific

Company, Inc). These bags can be used for air sampling such as collection and transportation of gases.

3.5.4 Centrifuge Tubes for Sample Storage

High performance 15 ml centrifuge tubes (as shown in Figure 3.9) with plug caps were ordered from VWR International. These centrifuge tubes (VWR Catalog Number 89039-668) were sterile and made up of polypropylene.

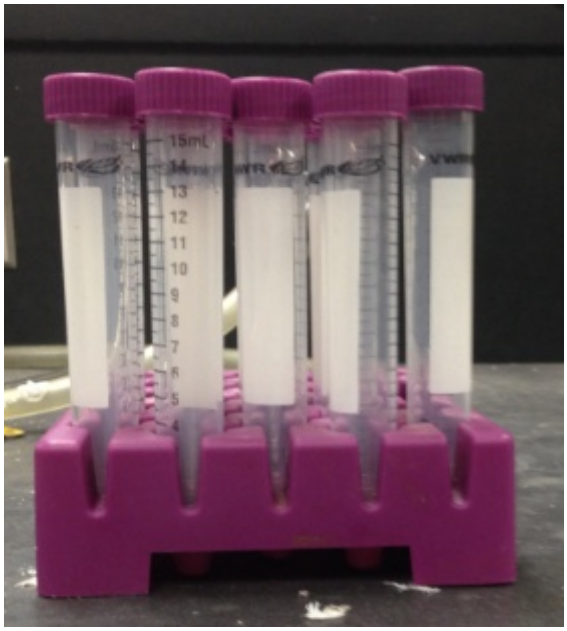


Figure 3.9 Centrifuge Tubes

These tubes have conical based bottom and freestanding tubes. These tubes can be autoclave and freeze. These Conical-Bottom Tubes can be reused. These tubes are RNase-, DNase-, and Endotoxin-Free. Gamma irradiation was used to sterilize these tubes. New tubes were used to collect samples every alternate day. Both synthetic and real vinasse samples were stored in these vials at -20˚C.

3.5.5 Techniques for Microbe Determination

Three molecular techniques, DNA Extraction, PCR, and Gel purification, were used for the determination of microbial communities in different compositions of vinasse at different temperatures.

3.5.5.1 DNA Extraction

DNA extraction is the method to extract deoxyribonucleic acid (DNA) from the cells or viruses in which DNA exists. This method can be used in many diagnostic processes to identify microbes such as bacteria and viruses in the environment. It can also be used in diagnosis of many disease and genetic disorders.

The Powerlyzer Powersoil DNA Isolation kit was used for DNA isolation. MOBIO Experienced user protocol for DNA extraction is given below. This kit can remove PCR inhibitors from different types of environmental wastes. These wastes can be mixed in bead beating tubes and homogenized on the powerlyzer ™ 24 for rapid and thorough homogenization. Cells lysis can be performed by mechanical and chemical interaction. The total DNA was collected in a spin column on a silica membrane. Then, DNA was eluted using solution C6 from the membrane. This DNA can be used PCR analysis and other downstream applications.

3.5.5.2 Experienced User Protocol for DNA Extraction (MOBIO protocol)

The Powerlyzer Powersoil DNA Isolation kit (MO BIO Catalog # 12855) was used for DNA extraction from vinasse samples. This Kit has solution C1, C2, C3, C4, C5, C6, PowerLyzer® Glass Bead Tube, 0.1mm, PowerLyzer™ PowerSoil® Bead Solution, Spin Filters in Tubes, collection tubes. DNA was extracted using Powerlyzer Powersoil DNA Isolation kit. 80 (66 viable) bacterial species and 12 fungal (none viable) were identified using this kit (Stewart et al., 2013).

The protocol for DNA extraction is given in Appendix D. This protocol was adapted from MOBIO experimental protocol (http://www.mobio.com/images/custom/file/protocol/12855.pdf). Sample (vinasse) was centrifuged (as shown in Figure 3.10) 5 times with 1ml vinasse sample at maximum speed of 10,000 x g in glass bead tube to collect the solidified sample between 0.65-0.85 grams. After every run, the supernatant were discarded from glass bead tube. Then, the other steps are followed as given in Appendix D. VWR vortex mixture (as shown in Figure 3.11) was used to gently vortex to mix sample using at a speed of 4-5 RPM during DNA extraction.

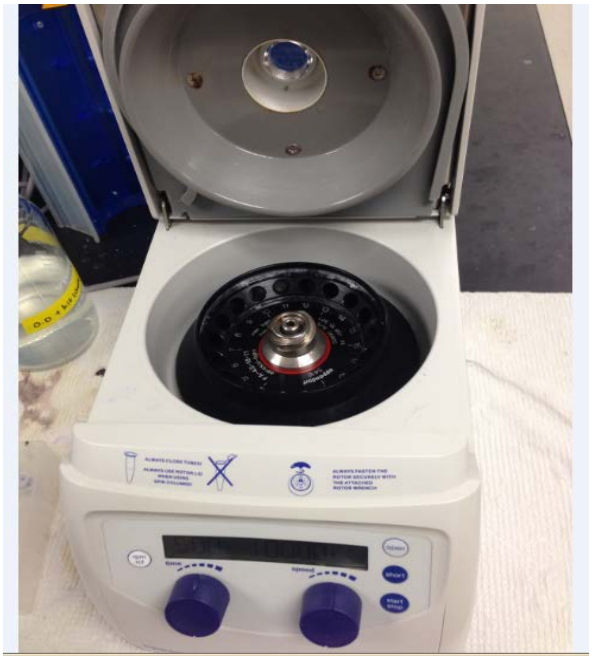


Figure 3.10 Centrifugation Machine

Figure 3.11 Analog Vortex Mixture

After extracting the DNA, the concentration of DNA was measured using Nano Drop spectrophotometer (Figure 3.12). Nano Drop is designed for micro-volume sample size (0.5 µL –

2.0 µL). All samples were spectrophotometrically quantified with A260/A280 ratio of average of 1.78 and 1.87 for all the bioreactors (as shown in Figure 3.13).

Figure 3.12 Nano Drop

Report | Test Type: Nucleic Acid | Date/time: 1/23/2013 6:23 PM | Page #: 1

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
A23	Default	1/23/2013	6:12 PM	78.12	1.562	0.882	1.77	1.66	50.00	230	0.942	0.051
A23D	Default	1/23/2013	6:13 PM	71.23	1.425	0.807	1.77	1.66	50.00	230	0.857	0.050
B23	Default	1/23/2013	6:14 PM	38.70	0.774	0.457	1.69	1.62	50.00	230	0.477	0.042
B23D	Default	1/23/2013	6:14 PM	2.81	0.056	0.055	1.01	0.46	50.00	230	0.122	0.043
C23	Default	1/23/2013	6:15 PM	3.34	0.067	0.065	1.02	0.40	50.00	230	0.167	0.033
C23	Default	1/23/2013	6:16 PM	2.90	0.058	0.037	1.59	0.37	50.00	230	0.155	0.025
C23D	Default	1/23/2013	6:17 PM	74.28	1.486	0.818	1.82	1.77	50.00	230	0.838	0.044
B25	Default	1/23/2013	6:18 PM	88.00	1.760	0.971	1.81	1.98	50.00	230	0.888	0.033
B12/23	Default	1/23/2013	6:19 PM	9.05	0.181	0.143	1.26	0.66	50.00	230	0.275	0.075
B21	Default	1/23/2013	6:19 PM	1.76	0.035	0.035	1.00	0.29	50.00	230	0.120	0.042
B30	Default	1/23/2013	6:20 PM	13.96	0.279	0.195	1.43	0.65	50.00	230	0.430	0.115
B30	Default	1/23/2013	6:22 PM	9.83	0.197	0.130	1.52	0.71	50.00	230	0.276	0.057
C23D	Default	1/23/2013	6:23 PM	75.93	1.519	0.834	1.82	1.81	50.00	230	0.840	0.018

Figure 3.13 DNA Concentration Result From Nano Drop

3.5.5.3 Polymerase Chain Reaction

A few or single copies of a piece of DNA can be amplified to several orders of magnitude and it can produce thousands to millions of copies of a particular DNA sequence by using

Polymerase Chain Reaction (PCR). PCR can be used to identify small amount of sample because PCR amplifies the regions of DNA that it targets. PCR is a promising tool for community analysis in anaerobic processes. The PCR protocols were followed from earth microbiome website for 16S rRNA amplification protocol (Earth Microbiome Website). Primers were used for paired-end 16s community sequencing on the Illumina HiSeq platform using bacterial/archeal primer 515F/806R. Forward and reverse primers are listed in Table 3.3 and Table 3.4. Forward primers have 5´ Illumina adapter, forward primer pad, forward primer linker, forward primer (515f) and reverse primer has reverse complement of 3´ Illumina adapter, golay barcode, reverse primer, reverse primer linker, and reverse primer (806r). All reverse primers have different golay barcodes. Details can be found at Earthmicrobiome website (ftp://ftp.metagenomics.anl.gov/data/misc/EMP/SupplementaryFile1_barcoded_primers_515F_806R.txt).

3.5.5.3.1 Materials and Apparatus Used for Polymerase Chain Reaction

Several materials such as 2 ml tubes, three volume of pipettes (Figure 3.14, 2-20 µl, 20-200 µl, 100-1000 µl), PCR water (MOBIO Labs), Hot Master Mix (MOBIO Labs), Axygen® 0.2 mL thin wall PCR tubes with flat cap were ordered for PCR.

Figure 3.14 Pipettes

3.5.5.3.1.1 Primers Used for PCR

One forward and (515F) and 50 reverse primers (806R) (IDT) were used for PCR. 515F (forward primer) PCR primer sequence contains:

Field number (space-delimited), description:

1. 5' Illumina adapter (5'- AATGATACGGCGACCACCGAGATCTACAC)

2. Forward primer pad (TATGGTAATT)

3. Forward primer linker (GT)

4. Forward primer (GTGCCAGCMGCCGCGGTAA)

Table 3.3 Forward Primer

5'- AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT GT GTGCCAGCMGCCGCGGTAA-3'

806R (reverse primer) PCR primer sequence (each sequence contains different barcode). Each primer is followed by a barcode identifier generated specifically for this set of primers, as shown in Table 3.5.

Reverse primer field number (space-delimited), description:

1. Reverse complement of 3' Illumina adapter

2. Golay barcode

3. Reverse primer pad

4. Reverse primer linker

5. Reverse primer

The primer sequences in this protocol are always listed in the 5’ -> 3’ orientation. This is the orientation that should be used for ordering. One forward primer 515f and 50 806r reverse primers (as shown in Table 3.3 and Table 3.4) with differ barcodes were ordered from IDT. Illumina PCR conditions and complete recipe for 1X PCR reaction are given below in Table 3.5 for 515f-806r region of the 16S rRNA gene. Primer set 515F-806R targeting V4 region of 16S rRNA.

3.5.5.3.2 Preparation of Primers for PCR

All the reverse primers and forward primers were shipped from IDT in the dry form. The molecular weight of each primer was different. Hence, the stock solution (100pmoles/µl) of same concentration of each primer was prepared using Tris EDTA (TE) solution, using TE volumes shown in the appendix. The working concentration (10 pmoles/µl) was prepared from stock solution. The protocol for making stock and working solution of primers is given in the appendix D. Then, all the stock solution and working solution can be stored at -20˚C. The working volume (10 pmoles/µl) of each primer is ready to use for PCR.

Table 3.4 List of Reverse Primers

Reverse primers List	
CAAGCAGAAGACGGCATACGAGAT GCAACACCATCC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc0
CAAGCAGAAGACGGCATACGAGAT GCGATATATCGC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc1
CAAGCAGAAGACGGCATACGAGAT CGAGCAATCCTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc2
CAAGCAGAAGACGGCATACGAGAT AGTCGTGCACAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc3
CAAGCAGAAGACGGCATACGAGAT GTATCTGCGCGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc4
CAAGCAGAAGACGGCATACGAGAT CGAGGGAAAGTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc5
CAAGCAGAAGACGGCATACGAGAT CAAATTCGGGAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc6
CAAGCAGAAGACGGCATACGAGAT AGATTGACCAAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc7
CAAGCAGAAGACGGCATACGAGAT AGTTACGAGCTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc8
CAAGCAGAAGACGGCATACGAGAT GCATATGCACTG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc9
CAAGCAGAAGACGGCATACGAGAT CAACTCCCGTGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc10
CAAGCAGAAGACGGCATACGAGAT TTGCGTTAGCAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc11
CAAGCAGAAGACGGCATACGAGAT TACGAGCCCTAA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc12
CAAGCAGAAGACGGCATACGAGAT CACTACGCTAGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc13
CAAGCAGAAGACGGCATACGAGAT TGCAGTCCTCGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc14
CAAGCAGAAGACGGCATACGAGAT ACCATAGCTCCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc15

Table 3.4—*Continued*

CAAGCAGAAGACGGCATACGAGAT TCGACATCTCTT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc16
CAAGCAGAAGACGGCATACGAGAT GAACACTTTGGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc17
CAAGCAGAAGACGGCATACGAGAT GAGCCATCTGTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc18
CAAGCAGAAGACGGCATACGAGAT TTGGGTACACGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc19
CAAGCAGAAGACGGCATACGAGAT AAGGCGCTCCTT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc20
CAAGCAGAAGACGGCATACGAGAT TAATACGGATCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc21
CAAGCAGAAGACGGCATACGAGAT TCGGAATTAGAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc22
CAAGCAGAAGACGGCATACGAGAT TGTGAATTCGGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc23
CAAGCAGAAGACGGCATACGAGAT CATTCGTGGCGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc24
CAAGCAGAAGACGGCATACGAGAT TACTACGTGGCC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc25
CAAGCAGAAGACGGCATACGAGAT GGCCAGTTCCTA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc26
CAAGCAGAAGACGGCATACGAGAT GATGTTCGCTAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc27
CAAGCAGAAGACGGCATACGAGAT CTATCTCCTGTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc28
CAAGCAGAAGACGGCATACGAGAT ACTCACAGGAAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc29
CAAGCAGAAGACGGCATACGAGAT ATGATGAGCCTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc30
CAAGCAGAAGACGGCATACGAGAT GTCGACAGAGGA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc31
CAAGCAGAAGACGGCATACGAGAT TGTCGCAAATAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc32

Table 3.4—*Continued*

CAAGCAGAAGACGGCATACGAGAT CATCCCTCTACT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc33
CAAGCAGAAGACGGCATACGAGAT TATACCGCTGCG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc34
CAAGCAGAAGACGGCATACGAGAT AGTTGAGGCATT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc35
CAAGCAGAAGACGGCATACGAGAT ACAATAGACACC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc36
CAAGCAGAAGACGGCATACGAGAT CGGTCAATTGAC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc37
CAAGCAGAAGACGGCATACGAGAT GTGGAGTCTCAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc38
CAAGCAGAAGACGGCATACGAGAT GCTCGAAGATTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc39
CAAGCAGAAGACGGCATACGAGAT AGGCTTACGTGT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc40
CAAGCAGAAGACGGCATACGAGAT TCTCTACCACTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc41
CAAGCAGAAGACGGCATACGAGAT ACTTCCAACTTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc42
CAAGCAGAAGACGGCATACGAGAT CTCACCTAGGAA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc43
CAAGCAGAAGACGGCATACGAGAT GTGTTGTCGTGC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc44
CAAGCAGAAGACGGCATACGAGAT CCACAGATCGAT AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc45
CAAGCAGAAGACGGCATACGAGAT TATCGACACAAG AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc46
CAAGCAGAAGACGGCATACGAGAT GATTCCGGCTCA AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc47
CAAGCAGAAGACGGCATACGAGAT CGTAATTGCCGC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc48
CAAGCAGAAGACGGCATACGAGAT GGTGACTAGTTC AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT	806rcbc49

3.5.5.3.3 Reagents Used in PCR

PCR grade water, 5 Primer Hot MM, Forward Primer (10 µM), Reverse Primer (10 µM), and Template DNA were added in a PCR tube. The amount of each reagent is mentioned in Table 3.5. The final primer concentration of master mix was 0.2 µM. Triplicates of each reaction were prepared for PCR. The concentration of DNA was very high (50-100 ng/µl) in synthetic vinasse composition 5 and 6. Hence, the DNA was diluted to half or one third because high concentration of DNA produces more spurious band on gel.

Table 3.5 Complete Reagent Used (Master Mix) for 1x PCR Reaction

PCR Grade H_2O	13µl
5 Primer Hot MM	10µl
Forward Primer (10µM)	0.5µl
Reverse Primer (10µM)	0.5µl
Template DNA	1µl
Total reaction volume	**25µl**

Then, all the samples in triplicate (each 25 µl) were amplified in Thermocycler (as shown in Figure 3.15). During the PCR process, different adapters and barcodes were inserted to differentiate among samples. Thermocycler conditions for 96 well thermocycles are given in Table 3.6.

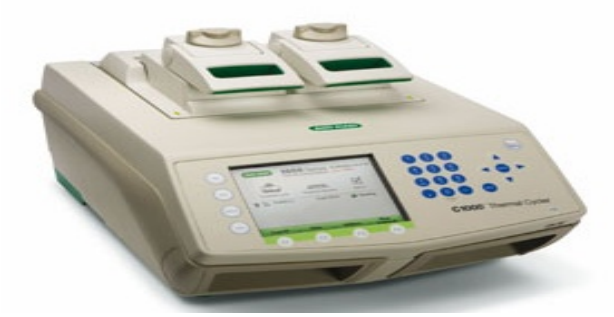


Figure 3.15 C 1000 Thermal Cycler

Table 3.6 Thermocyler Conditions for PCR

94°C 3 minutes
94°C 45 seconds
50°C 60 seconds
72°C 90 seconds
Repeat steps 2-4 35 times
72°C 10 minutes
4°C HOLD

3.5.5.4 Agarose Gel Electrophoresis

Triplicates of each sample were run on Agarose gel after finishing PCR. The gel is prepared by dissolving the agarose powder in a buffer solution TAE. The concentration of gel affects the resolution of DNA separation. For a standard agarose gel electrophoresis, a 0.8% gives good separation or resolution of large 5–10 kb DNA fragments. 1.5% agarose gel was prepared for running all the samples. 1.5 g of agarose gel was added in 1 ml TAE solution. The solution was heated to dissolve agarose in TAE. The melted agarose was allowed to cool sufficiently before pouring the solution into a cast, as the cast may warp or crack if the agarose solution is too hot. Wells were created using a comb for loading sample, and the gel was completely set before use. Once the gel had set, the comb was removed. Then, the DNA samples were loaded to see the DNA band. 1 µL of 6X Loading dye (Figure 3.17) was mixed with the 5 µl DNA sample before the mixture was loaded into the wells. Gene Ruler 1KB Plus DNA ladder (Figure 3.17) was used to compare the size of DNA after gel electrophoresis. Concentration of ladder used for loading into well was 0.1 µg/µl. 4 µl of ladder was mixed with 1 µl of loading dye and then loaded into first well of gel. Then, each sample (mixture of 5 µl DNA sample and 1 µl of loading dye) was loaded into the well. The gel was covered with TAE buffer and samples were run at a voltage of 60 for an hour. DNA is normally visualized by staining with

ethidium bromide (EtBr), which intercalates into the major grooves of the DNA and fluoresces under UV light. Gel was transferred into EtBr solution for 15-20 minutes. When stained with ethidium bromide, the gel was viewed under ultraviolet (UV) using Gel logic 212 PRO (Figure 3.16). The size of DNA in each sample was compared with DNA ladder. Expected band size for 515f/806r was roughly found 375-450 bp.

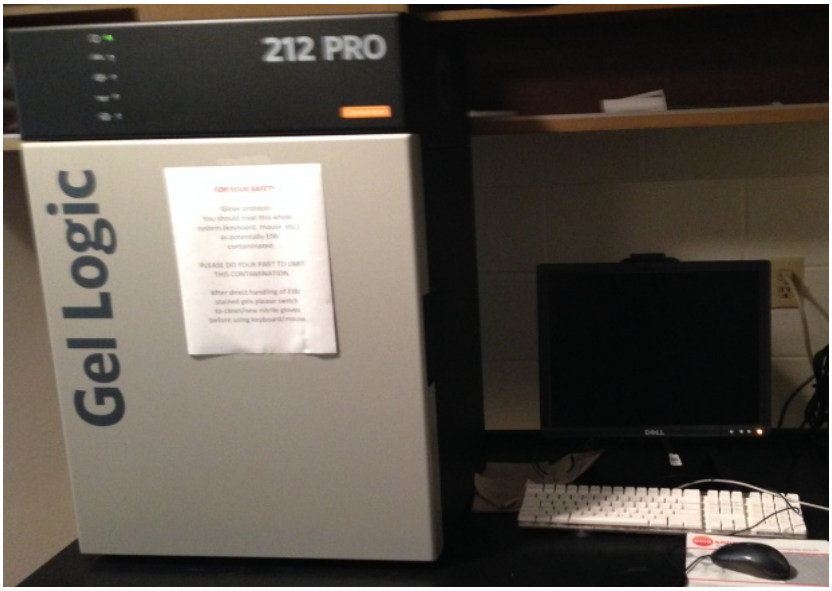
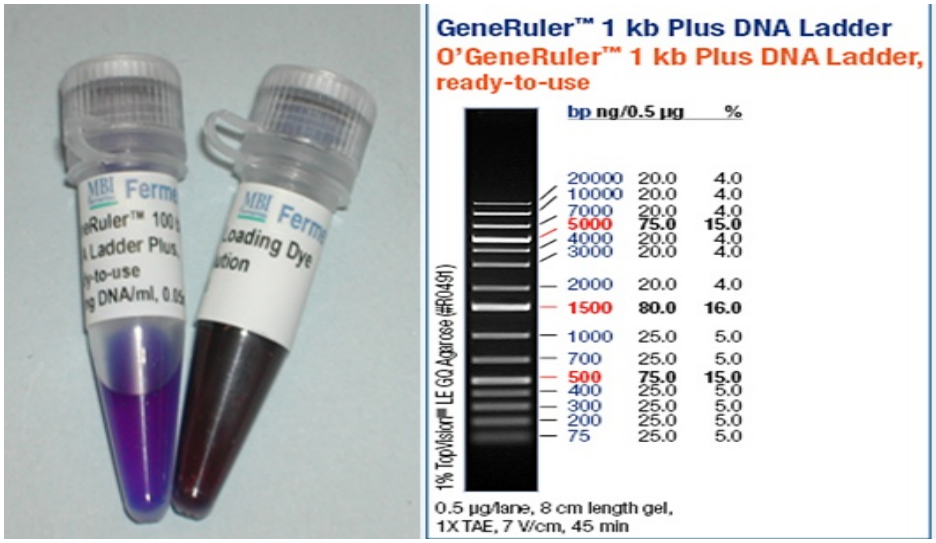


Figure 3.16 Gel Logic 212 PRO



(a) (b)

Figure 3.17 (a) Ladder & Loading Dye (b) Gene Ruler 1Kb Plus DNA Ladder

3.5.5.5 QIAquick Gel Extraction

Triplicates for each sample were combined and run on agarose gel. The total volume of sample was 75 µl after adding triplicate. Hence, 5 µl of loading dye was added with each sample before loading into well. The same amount of ladder (4 µL) and 1 µl of dye was mixed before loading into well. If spurious bands were present in ah sample (as shown in Figure 3.19 & Figure 3.20) other than the target DNA, target band was cut from gel using benchtop UV transilluminator (Figure 3.18). Target band of DNA was cleaned using QIAquick Gel Extraction (Qiagen company website) containing Buffer QG, Buffer PE, and Elution buffer.

Figure 3.18 UV/White Light Transilluminator

3.5.5.5.1 QIAquick Gel Extraction Kit Protocol using a Microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions. For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the new MinElute Reaction Cleanup Kit. The complete steps are mentioned in Appendix D. This protocol was adapted from QIAquick Gel Extraction protocol, available on Qiagen company website (http://sites.bio.indiana.edu/~chenlab/protocol_files/agarose_gel_extraction.pdf).

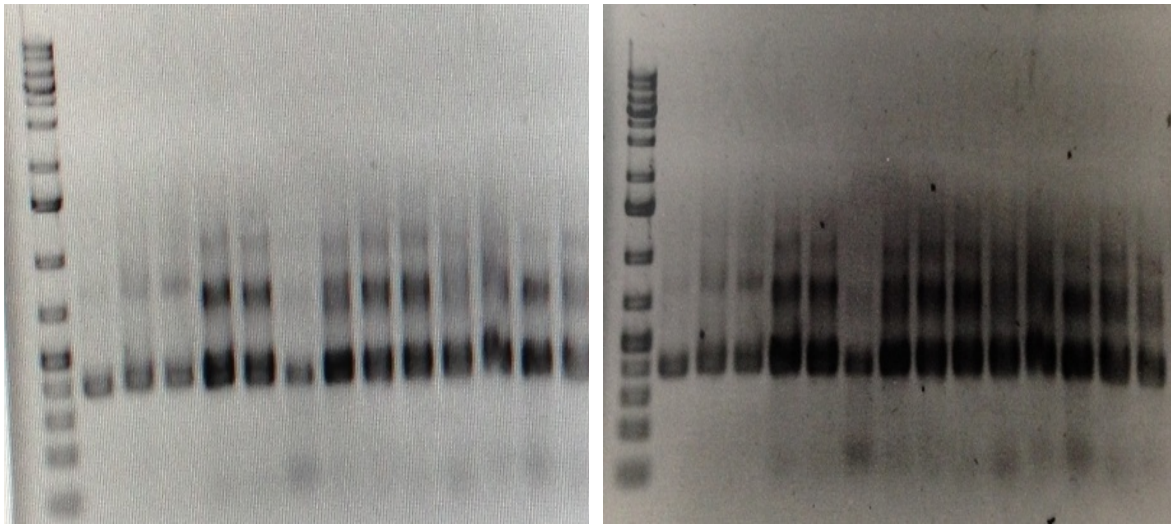


Figure 3.19 DNA Samples after Agarose Gel Electrophoresis

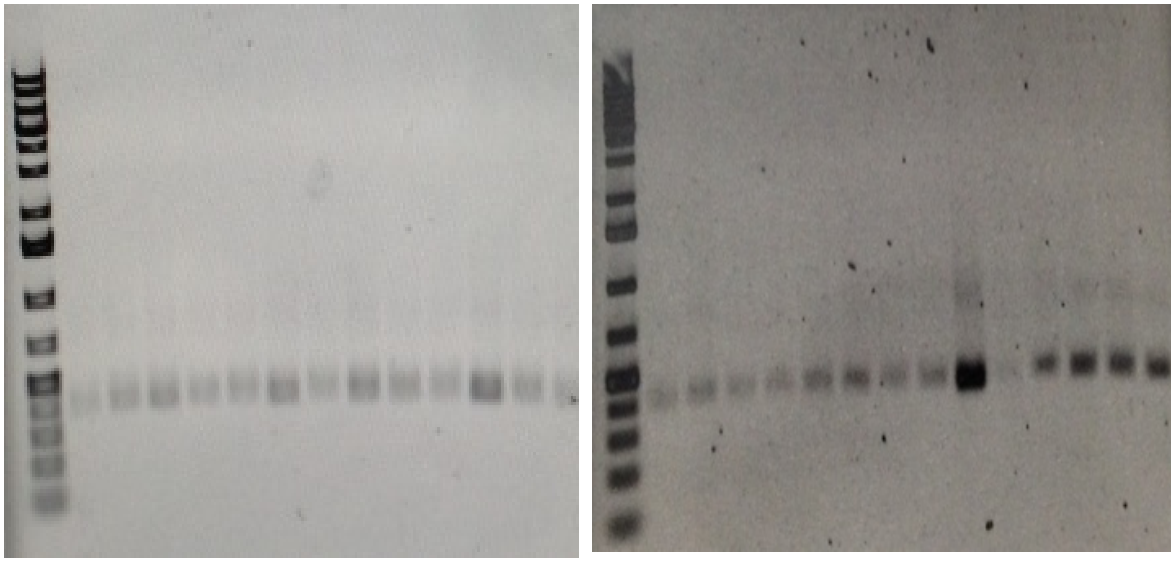


Figure 3.20 DNA Samples after Cleaning from QIAquick Gel Extraction Kit

16S RNA amplification protocol for sequencing is given in Appendix D. All the samples were pooled together based on the Qubit concentration. All the pooling sheets are attached below in Table 3.9, Table 3.10 and Table 3.11**.** Pooled samples (in a single tube) were sent for sequencing along with the sequencing primers (100 µM) as given below in Table 3.7.

Table 3.7 Sequencing Primers

Read 1 sequencing primer	TATGGTAATT GT GTGCCAGCMGCCGCGGTAA
Read 2 sequencing primer	AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT
Index Sequence primer	ATTAGAWACCCBDGTAGTCC GG CTGACTGACT

Three sets of 50 samples each were prepared for MiSeq Illumina sequencing, with a fourth set containing the remaining 5 samples. 12 reactors were analyzed for MiSeq. Details of reactors analyzed for microbial analysis are given in Table 3.8. Duplicates of composition 2 at 30˚C and composition 5 at 40˚C were also analyzed (bolded in Table 3.8).

The samples were pooled and then sent to University of Texas at Arlington Core Facility lab for DNA sequencing to find out different microbial communities at each stage of hydrolysis, acidogenesis, acidogenesis, and methanogenesis during anaerobic treatment of vinasse. All runs were 150 bp Paired end runs, which requires a 300 cycle kit.

Table 3.8 Compositions Used for Miseq Analysis

Temperature (˚C)	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6
30		**yes**	yes			yes
35	yes		yes			
40	yes			yes	**yes**	

The Illumina sequencing Technology MiSeq (as shown in Figure 3.21, Illumina website) was used for analyzing microbes in different stages of anaerobic digestion. The Illumina MiSeq is a Next-Gen DNA sequencer which uses a single –lane flowcell; it can generate 15 million single end or 30 million paired-end reads. Illumina prepared libraries are loaded directly onto the MiSeq, where amplified clusters are generated on the flowcell and sequencing by synthesis occurs. Miseq gives results in hours. The MiSeq system is a small and economical instrument. “MiSeq

uses TruSeq, Illumina's reversible terminator-based sequencing by synthesis chemistry to deliver the fastest time to answer. MiSeq performs the widest breadth of sequencing applications, including highly multiplexed PCR amplicon sequencing, small genome resequencing and de novo sequencing, small RNA sequencing, library quality control, and 16S metagenomics" (http://www.illumina.com/miseq).

Figure 3.21 Illumina MiSeq

Table 3.9 Pooling Sheet for SET-1

Total volume (µl)	Sample ID #	Concentration (ng/µl)	Final amount of DNA (ng/µl)	Group Name	µl per sample	Pool (ng/ul)	Ratio	Target ng	Total µl to Pool 2
30	AF.01	3.32	99.6	Group 1	4.52	2.56	1.25	75	29.3
30	AF.03	3.64	109	at 15 ng/sample	4.12				
30	AF.06	3.82	114		3.93				
30	AF.07	3.24	97.2	10 samples	4.63				
30	AF.08	3.22	96.6		4.66				
30	AF.09	3.41	102		4.40				
30	AF.10	4.01	120		3.75				
30	AF.21	3.69	110		4.07				
30	AF.49	3.51	105		4.27				
30	AF.46	4.01	120		3.74				
30	AF.34	6.92	207	Group 2	2.17	4.24	1	60	14.2
30	AF.35	5.90	177	at 15 ng/sample	2.54				
30	AF.42	6.39	191		2.35				
30	AF.43	5.25	157	8 samples	2.86				
30	AF.12	5.62	168		2.67				

Table 3.9—*Continued*

30	AF.13	6.93	207		2.16				
30	AF.48	5.85	175		2.56				
30	AF.14	7.74	232		2.58				
30	AF.29	4.21	126	Group 3	3.56	3.17	1	60	18.9
30	AF.30	4.92	147	at 15 ng/sample	3.05				
30	AF.18	4.22	126		3.55				
30	AF.27	4.17	125		3.60				
30	AF.20	4.54	136	8 samples	3.30				
30	AF.23	4.27	128		3.51				
30	AF.44	4.38	131		3.42				
30	AF.45	4.64	139		3.23				
30	AF.32	8.17	245	Group 4	2.45	5.99	1.25	75	12.5
30	AF.33	7.54	226	at 20 ng/sample	2.65				
30	AF.36	8.6	258		2.33				
30	AF.37	8.72	261		2.29				
30	AF.39	8.10	243	10 samples	2.47				
30	AF.47	7.54	226		2.65				
30	AF.00	8.95	268		2.23				

Table 3.9—*Continued*

30	AF.02	7.48	224		2.67				
30	AF.11	8.79	263		2.28				
30	AF.22	7.81	234		2.56				
30	AF.04	2.86	85.8	Group 5	5.24	3.06	0.5	30	9.80
30	AF.05	2.59	77.7	at 15ng/sample	5.79				
30	AF.19	2.28	68.4	4 samples	6.58				
30	AF.16	1.65	49.5		9.09				
30	AF.40	11.5	345	Group 6	2.61	8.84	1.25	75	8.48
30	AF.28	12.9	387	at 30 ng/sample	2.33				
30	AF.15	10.7	321		2.80				
30	AF.17	9.72	291		3.09				
30	AF.24	10.1	303	10 samples	2.97				
30	AF.25	12.2	366		2.46				
30	AF.26	14.7	441		2.04				
30	AF.38	9.94	298		3.02				
30	AF.31	17.2	516		1.74				
30	AF.41	14.3	429		2.10				

Table 3.10 Pooling Sheet for SET-2

Total volume(µl)	Sample ID #	Concentration (ng/µl)	Final amount of DNA (ng/µl)	Group Name	µl per sample	Pool (ng/µl)	Ratio	Target ng	Total µl to Pool 2
30	AF.01	3.32	99.6	Group 1	4.52	2.56	1.25	75	29.3
30	AF.03	3.64	109	at 15 ng/sample	4.12				
30	AF.06	3.82	114		3.93				
30	AF.07	3.24	97.2	10 samples	4.63				
30	AF.08	3.22	96.1		4.66				
30	AF.09	3.41	102		4.40				
30	AF.10	4.01	120		3.75				
30	AF.21	3.69	110		4.07				
30	AF.49	3.51	105		4.27				
30	AF.46	4.01	120		3.74				
30	AF.34	6.92	207	Group 2	2.17	4.24	1	60	14.1
30	AF.35	5.9	177	at 15 ng/sample	2.54				
30	AF.42	6.39	191		2.35				
30	AF.43	5.25	157	8 samples	2.86				
30	AF.12	5.62	168		2.67				

Table 3.10—*Continued*

30	AF.13	6.93	207		2.16				
30	AF.48	5.85	175		2.56				
30	AF.14	7.74	232		2.58				
30	AF.29	4.21	126	Group 3	3.56	3.17	1	60	18.9
30	AF.30	4.92	147	at 15 ng/sample	3.05				
30	AF.18	4.22	126		3.55				
30	AF.27	4.17	125		3.60				
30	AF.20	4.54	136	8 samples	3.30				
30	AF.23	4.27	128		3.51				
30	AF.44	4.38	131		3.42				
30	AF.45	4.64	139		3.23				
30	AF.32	8.17	245	Group 4	2.45	5.99	1.25	75	12.5
30	AF.33	7.54	226	at 20ng/sample	2.65				
30	AF.36	8.6	258		2.33				
30	AF.37	8.72	261		2.29				
30	AF.39	8.1	243	10 samples	2.47				
30	AF.47	7.54	226		2.65				
30	AF.00	8.95	268		2.23				

Table 3.10—*Continued*

30	AF.02	7.48	224		2.67				
30	AF.11	8.79	263		2.28				
30	AF.22	7.81	234		2.56				
30	AF.04	2.86	85.8	Group 5	5.24	3.06	0.5	30	9.80
30	AF.05	2.59	77.7	at 15ng/sample	5.79				
30	AF.19	2.28	68.4	4 samples	6.58				
30	AF.16	1.65	49.5		9.09				
30	AF.40	11.5	345	Group 6	2.61	8.84	1.25	75	8.48
30	AF.28	12.9	387	at 30 ng/sample	2.33				
30	AF.15	10.7	321		2.80				
30	AF.17	9.72	291		3.09				
30	AF.24	10.1	303	10 samples	2.97				
30	AF.25	12.2	366		2.46				
30	AF.26	14.7	441		2.04				
30	AF.38	9.94	298		3.02				
30	AF.31	17.2	516		1.74				
30	AF.41	14.3	429		2.10				

Table 3.11 Pooling Sheet for SET-3

Total volume(µl)	Sample ID #	Concentration (ng/µl)	Final amount of DNA(ng/µl)	Group Name	µl per sample	Pool (ng/ul)	Ratio	Target ng	Total µl to Pool 2
35	CF.10	1.03	36.1	Group 1	14.5	2.6	1	85	32.6
35	CF.16	1.27	44.4	at 15 ng/sample	11.8				
35	CF.27	1.49	52.1		10.1				
35	CF.28	0.96	33.6	10 samples	15.6				
35	CF.31	1.69	59.1		8.88				
35	CF.32	1.24	43.4		12.1				
35	CF.46	1.17	40.9		12.8				
35	CF.47	0.74	26.1		20.1				
35	CF.48	1.27	44.4		11.8				
35	CF.49	1.13	39.5		13.2				
35	CF.11	2.79	97.6	Group 2	10.7				
35	CF.15	2.42	84.7	at 30 ng/sample	12.4				
35	CF.00	2.54	88.9	10 samples	11.8				
35	CF.02	2.33	81.5		12.8				
35	CF.04	2.65	92.7		11.3				

Table 3.11—*Continued*

35	CF.22	2.18	76.3		13.76	3.56	1	85	23.8
35	CF.30	2.61	91.3		11.49				
35	CF.33	2.01	70.3		14.93				
35	CF.23	1.81	63.3		16.57				
35	CF.29	1.86	65		16.13				
35	CF.01	3.5	122	Group 3	11.43				
35	CF.03	3.57	124	at 40 ng/sample	11.20	5.28	1	85	16.1
35	CF.05	3.04	106	10 samples	13.16				
35	CF.19	3.45	120		11.59				
35	CF.21	3.5	122		11.43				
35	CF.26	3.9	136		10.26				
35	CF.37	3.55	124		11.27				
35	CF.14	2.84	99.4		14.08				
35	CF.34	2.85	99.7		14.04				
35	CF.25	2.9	101		13.79				
35	CF.06	4.67	163	Group 4	8.57	5.91	1	85	14.3
35	CF.07	5.72	200	at 40 ng/sample	6.99				
35	CF.08	4.02	140		9.95				

Table 3.11—*Continued*

35	CF.13	5.62	196		7.12				
35	CF.17	5.03	176	10 samples	7.95				
35	CF.20	4.67	163		8.57				
35	CF.24	4.62	161		8.66				
35	CF.36	4.61	161		8.68				
35	CF.40	5.27	184		7.59				
35	CF.09	3.76	131		10.64				
35	CF.12	6.01	210	Group 5	9.98	11.5	1	85	7.39
35	CF.18	6.8	238	at 60 ng/sample	8.82				
35	CF.35	6.16	215	10 samples	9.74				
35	CF.38	7.73	270		7.76				
35	CF.39	7.06	247		8.50				
35	CF.41	6.96	243		8.62				
35	CF.42	8.26	289		7.26				
35	CF.43	6.91	241		8.68				
35	CF.44	7.70	269		7.79				
35	CF.45	9.09	318		6.60				

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3.6 Task 5 Microbial Analysis

16S rRNA profiling analysis pipeline (Illumina paired-end) was used for 16S rRNA data analysis. There are several software need to be downloaded before starting the analysis. The name and source of software are mentioned below:

1. QIIME 1.8.0 installed (see:http://qiime.org/index.html)
2. USEARCH 7 (UPARSE) installed (note that only 32 bit version is freely available- http://drive5.com/uparse/)
3. UPARSE Python scripts (see:http://drive5.com/python/)
4. BMP Perl script (https://www.dropbox.com/s/eae7ddnnuy7qaap/bmp-uparse-pipeline.pl)
5. BIOM format scripts (http://biom-format.org/)

Three fastq files forward_reads.fastq (R1.fastq), reverse_reads_fastq (R2.fastq), and barcodes.fastq were generated from Illumina Paired end reads. The protocol was adapted from brmicrobiome.org website (http://www.brmicrobiome.org/#!16s-profiling-pipeline-illumin/c3u5). The detailed protocol for 16S rRNA analysis is given in Appendix D.

It is necessary to prepare a mapping file before starting the analysis. This file contains all of the information about the samples necessary to perform the data analysis. In general, the mapping file should contain the name of each sample, the barcode sequence used for each sample, the linker/primer sequence used to amplify the sample, and a description column. All the instructions and suggestions for creating mapping file are given in qiime website (http://qiime.org/documentation/file_formats.html). This file should be prepared by user. All the mapping files for 3 sets are given in Table 3.12, Table 3.13, and Table 3.14. The core diversity analysis will give average species richness and average phylogenetic diversity (Faith's PD).

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Table 3.12 Mapping File of SET-1

#SampleID	BarcodeSequence	LinkerPrimerSequence	SampleDescription	Treatment	Description
AF.00	TCCCTTGTCTCC	CCGGACTACHVGGGTWTCTAAT	C(01/21/13)	GL_204_30°C	Composition_6-ReactorC
AF.01	ACGAGACTGATT	CCGGACTACHVGGGTWTCTAAT	C(01/21/13)	GL_204_30°C	Composition_6-ReactorC
AF.02	GCTGTACGGATT	CCGGACTACHVGGGTWTCTAAT	C(01/23/13)	GL_204_30°C	Composition_6-ReactorC
AF.03	ATCACCAGGTGT	CCGGACTACHVGGGTWTCTAAT	C(01/31/13)	GL_204_30°C	Composition_6-ReactorC
AF.04	TGGTCAACGATA	CCGGACTACHVGGGTWTCTAAT	C(2/2/13)	GL_204_30°C	Composition_6-ReactorC
AF.05	ATCGCACAGTAA	CCGGACTACHVGGGTWTCTAAT	C(2/4/13)	GL_204_30°C	Composition_6-ReactorC
AF.06	GTCGTGTAGCCT	CCGGACTACHVGGGTWTCTAAT	C(2/6/13)	GL_204_30°C	Composition_6-ReactorC
AF.07	AGCGGAGGTTAG	CCGGACTACHVGGGTWTCTAAT	C(2/8/13)	GL_204_30°C	Composition_6-ReactorC
AF.08	ATCCTTTGGTTC	CCGGACTACHVGGGTWTCTAAT	C(2/11/13)	GL_204_30°C	Composition_6-ReactorC
AF.09	TACAGCGCATAC	CCGGACTACHVGGGTWTCTAAT	C(2/15/13)	GL_204_30°C	Composition_6-ReactorC
AF.10	ACCGGTATGTAC	CCGGACTACHVGGGTWTCTAAT	C(2/18/13)	GL_204_30°C	Composition_6-ReactorC
AF.11	AATTGTGTCGGA	CCGGACTACHVGGGTWTCTAAT	C(2/20/13)	GL_204_30°C	Composition_6-ReactorC
AF.12	TGCATACACTGG	CCGGACTACHVGGGTWTCTAAT	C(2/22/13)	GL_204_30°C	Composition_6-ReactorC
AF.13	AGTCGAACGAGG	CCGGACTACHVGGGTWTCTAAT	C(2/25/13)	GL_204_30°C	Composition_6-ReactorC
AF.14	ACCAGTGACTCA	CCGGACTACHVGGGTWTCTAAT	C(2/27/13)	GL_204_30°C	Composition_6-ReactorC
AF.15	GAATACCAAGTC	CCGGACTACHVGGGTWTCTAAT	C(3/1/13)	GL_204_30°C	Composition_6-ReactorC

Table 3.12—*Continued*

AF.16	GTAGATCGTGTA	CCGGACTACHVGGGTWTCTAAT	C(3/4/13)	GL_204_30°C	Composition_6-ReactorC
AF.17	TAACGTGTGTGC	CCGGACTACHVGGGTWTCTAAT	C(3/8/13)	GL_204_30°C	Composition_6-ReactorC
AF.18	CATTATGGCGTG	CCGGACTACHVGGGTWTCTAAT	C(3/11/13)	GL_204_30°C	Composition_6-ReactorC
AF.19	CCAATACGCCTG	CCGGACTACHVGGGTWTCTAAT	C(3/15/13)	GL_204_30°C	Composition_6-ReactorC
AF.20	GATCTGCGATCC	CCGGACTACHVGGGTWTCTAAT	C(3/18/13)	GL_204_30°C	Composition_6-ReactorC
AF.21	CAGCTCATCAGC	CCGGACTACHVGGGTWTCTAAT	C(3/25/13)	GL_204_30°C	Composition_6-ReactorC
AF.22	CAAACAACAGCT	CCGGACTACHVGGGTWTCTAAT	C(4/1/13)	GL_204_30°C	Composition_6-ReactorC
AF.23	GCAACACCATCC	CCGGACTACHVGGGTWTCTAAT	C(4/8/13)	GL_204_30°C	Composition_6-ReactorC
AF.24	GCGATATATCGC	CCGGACTACHVGGGTWTCTAAT	C(4/15/13)	GL_204_30°C	Composition_6-ReactorC
AF.25	CGAGCAATCCTA	CCGGACTACHVGGGTWTCTAAT	C(1/25/13)	GL_204_30°C	Composition_6-ReactorC
AF.26	AGTCGTGCACAT	CCGGACTACHVGGGTWTCTAAT	B(12/30/12)	300gRV_40°C	Composition_3-ReactorB
AF.27	GTATCTGCGCGT	CCGGACTACHVGGGTWTCTAAT	B(1/1/13)	300gRV_40°C	Composition_3-ReactorB
AF.28	CGAGGGAAAGTC	CCGGACTACHVGGGTWTCTAAT	B(1/3/13)	300gRV_40°C	Composition_3-ReactorB
AF.29	CAAATTCGGGAT	CCGGACTACHVGGGTWTCTAAT	B(1/3/13)	300gRV_40°C	Composition_3-ReactorB
AF.30	AGATTGACCAAC	CCGGACTACHVGGGTWTCTAAT	B(1/5/13)	300gRV_40°C	Composition_3-ReactorB
AF.31	AGTTACGAGCTA	CCGGACTACHVGGGTWTCTAAT	B(1/5/13)	300gRV_40°C	Composition_3-ReactorB
AF.32	GCATATGCACTG	CCGGACTACHVGGGTWTCTAAT	B(1/9/13)	300gRV_40°C	Composition_3-ReactorB

Table 3.12—*Continued*

AF.33	CAACTCCCGTGA	CCGGACTACHVGGGTWTCTAAT	B(1/11/13)	300gRV_40°C	Composition_3-ReactorB
AF.34	TTGCGTTAGCAG	CCGGACTACHVGGGTWTCTAAT	A(12/27/12)	3.5RV_40°C	Composition_1-ReactorA
AF.35	TACGAGCCCTAA	CCGGACTACHVGGGTWTCTAAT	A(12/30/12)	3.5RV_40°C	Composition_1-ReactorA
AF.36	CACTACGCTAGA	CCGGACTACHVGGGTWTCTAAT	A(1/1/13)	3.5RV_40°C	Composition_1-ReactorA
AF.37	TGCAGTCCTCGA	CCGGACTACHVGGGTWTCTAAT	A(1/3/13)	3.5RV_40°C	Composition_1-ReactorA
AF.38	ACCATAGCTCCG	CCGGACTACHVGGGTWTCTAAT	A(1/3/13)	3.5RV_40°C	Composition_1-ReactorA
AF.39	TCGACATCTCTT	CCGGACTACHVGGGTWTCTAAT	A(1/7/13)	3.5RV_40°C	Composition_1-ReactorA
AF.40	GAACACTTTGGA	CCGGACTACHVGGGTWTCTAAT	A(1/9/13)	3.5RV_40°C	Composition_1-ReactorA
AF.41	GAGCCATCTGTA	CCGGACTACHVGGGTWTCTAAT	A(1/11/13)	3.5RV_40°C	Composition_1-ReactorA
AF.42	TTGGGTACACGT	CCGGACTACHVGGGTWTCTAAT	A(3/22/13)	4L_RV_30°C	Composition_2-ReactorA
AF.43	AAGGCGCTCCTT	CCGGACTACHVGGGTWTCTAAT	A(3/25/13)	4L_RV_30°C	Composition_2-ReactorA
AF.44	TAATACGGATCG	CCGGACTACHVGGGTWTCTAAT	A(3/28/13)	4L_RV_30°C	Composition_2-ReactorA
AF.45	TCGGAATTAGAC	CCGGACTACHVGGGTWTCTAAT	A(3/30/13)	4L_RV_30°C	Composition_2-ReactorA
AF.46	TGTGAATTCGGA	CCGGACTACHVGGGTWTCTAAT	A(4/1/13)	4L_RV_30°C	Composition_2-ReactorA
AF.47	CATTCGTGGCGT	CCGGACTACHVGGGTWTCTAAT	A(4/3/13)	4L_RV_30°C	Composition_2-ReactorA
AF.48	TACTACGTGGCC	CCGGACTACHVGGGTWTCTAAT	A(4/5/13)	4L_RV_30°C	Composition_2-ReactorA

Table 3.13 Mapping File of SET-2

Sample ID	Barcode Sequence	Linker Primer Sequence	Sample Description	Treatment	Description
BF.00	TCCCTTGTCTCC	CCGGACTACHVGGGTWTCTAAT	A_4/8/13	4L_RV_30°C	Composition_2-ReactorA
BF.01	ACGAGACTGATT	CCGGACTACHVGGGTWTCTAAT	A_4/10/13	4L_RV_30°C	Composition_2-ReactorA
BF.02	GCTGTACGGATT	CCGGACTACHVGGGTWTCTAAT	A_4/12/13	4L_RV_30°C	Composition_2-ReactorA
BF.03	ATCACCAGGTGT	CCGGACTACHVGGGTWTCTAAT	A_4/19/13	4L_RV_30°C	Composition_2-ReactorA
BF.04	TGGTCAACGATA	CCGGACTACHVGGGTWTCTAAT	A_4/22/13	4L_RV_30°C	Composition_2-ReactorA
BF.05	ATCGCACAGTAA	CCGGACTACHVGGGTWTCTAAT	A_4/24/13	4L_RV_30°C	Composition_2-ReactorA
BF.06	GTCGTGTAGCCT	CCGGACTACHVGGGTWTCTAAT	A_4/27/13	4L_RV_30°C	Composition_2-ReactorA
BF.07	AGCGGAGGTTAG	CCGGACTACHVGGGTWTCTAAT	B_3/22/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.08	ATCCTTTGGTTC	CCGGACTACHVGGGTWTCTAAT	B_3/25/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.09	TACAGCGCATAC	CCGGACTACHVGGGTWTCTAAT	B_3/28/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.10	ACCGGTATGTAC	CCGGACTACHVGGGTWTCTAAT	B_3/30/13	4L_RV_30°C	Composition_2-ReactorBduplicate

Table 3.13—*Continued*

BF.11	AATTGTGTCGGA	CCGGACTACHVGGGTWTCTAAT	B_4/1/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.12	TGCATACACTGG	CCGGACTACHVGGGTWTCTAAT	B_4/3/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.13	AGTCGAACGAGG	CCGGACTACHVGGGTWTCTAAT	B_4/5/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.14	ACCAGTGACTCA	CCGGACTACHVGGGTWTCTAAT	B_4/8/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.15	GAATACCAAGTC	CCGGACTACHVGGGTWTCTAAT	B_4/10/13	4L_RV_30°C	Composition_2-ReactorBduplicate
BF.16	GTAGATCGTGTA	CCGGACTACHVGGGTWTCTAAT	C_5/11/13	GL_62.78_40°C	Composition_5-ReactorC
BF.17	TAACGTGTGTGC	CCGGACTACHVGGGTWTCTAAT	C_5/13/13	GL_62.78_40°C	Composition_5-ReactorC
BF.18	CATTATGGCGTG	CCGGACTACHVGGGTWTCTAAT	C_5/15/13	GL_62.78_40°C	Composition_5-ReactorC
BF.19	CCAATACGCCTG	CCGGACTACHVGGGTWTCTAAT	C_5/17/13	GL_62.78_40°C	Composition_5-ReactorC
BF.20	GATCTGCGATCC	CCGGACTACHVGGGTWTCTAAT	C_5/19/13	GL_62.78_40°C	Composition_5-ReactorC
BF.21	CAGCTCATCAGC	CCGGACTACHVGGGTWTCTAAT	C_5/21/13	GL_62.78_40°C	Composition_5-ReactorC
BF.22	CAAACAACAGCT	CCGGACTACHVGGGTWTCTAAT	C_5/24/13	GL_62.78_40°C	Composition_5-ReactorC

Table 3.13—*Continued*

BF.23	GCAACACCATCC	CCGGACTACHVGGGTWTCTAAT	C_D_5/24/13	GL_62.78_40°C	Composition_5-ReactorC
BF.24	GCGATATATCGC	CCGGACTACHVGGGTWTCTAAT	C_5/26/13	GL_62.78_40°C	Composition_5-ReactorC
BF.25	CGAGCAATCCTA	CCGGACTACHVGGGTWTCTAAT	C_5/29/13	GL_62.78_40°C	Composition_5-ReactorC
BF.26	AGTCGTGCACAT	CCGGACTACHVGGGTWTCTAAT	C_5/31/13	GL_62.78_40°C	Composition_5-ReactorC
BF.27	GTATCTGCGCGT	CCGGACTACHVGGGTWTCTAAT	C_6/5/13	GL_62.78_40°C	Composition_5-ReactorC
BF.28	CGAGGGAAAGTC	CCGGACTACHVGGGTWTCTAAT	C_6/10/13	GL_62.78_40°C	Composition_5-ReactorC
BF.29	CAAATTCGGGAT	CCGGACTACHVGGGTWTCTAAT	D_5/11/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.30	AGATTGACCAAC	CCGGACTACHVGGGTWTCTAAT	D_5/13/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.31	AGTTACGAGCTA	CCGGACTACHVGGGTWTCTAAT	D_5/15/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.32	GCATATGCACTG	CCGGACTACHVGGGTWTCTAAT	D_5/17/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.33	CAACTCCCGTGA	CCGGACTACHVGGGTWTCTAAT	D_5/19/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate

Table 3.13—*Continued*

BF.34	TTGCGTTAGCAG	CCGGACTACHVGGGTWTCTAAT	D_5/21/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.35	TACGAGCCCTAA	CCGGACTACHVGGGTWTCTAAT	D_5/24/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.36	CACTACGCTAGA	CCGGACTACHVGGGTWTCTAAT	D_5/26/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.37	TGCAGTCCTCGA	CCGGACTACHVGGGTWTCTAAT	D_5/29/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.38	ACCATAGCTCCG	CCGGACTACHVGGGTWTCTAAT	D_5/31/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.39	TCGACATCTCTT	CCGGACTACHVGGGTWTCTAAT	D_6/5/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.40	GAACACTTTGGA	CCGGACTACHVGGGTWTCTAAT	D_6/10/13	GL_62.78_40°C	Composition_5-ReactorD-Duplicate
BF.41	GAGCCATCTGTA	CCGGACTACHVGGGTWTCTAAT	B_11/6/12	3.5RV_35°C	Composition1_-ReactorB
BF.42	TTGGGTACACGT	CCGGACTACHVGGGTWTCTAAT	B_11/8/12	3.5RV_35°C	Composition1_-ReactorB
BF.43	AAGGCGCTCCTT	CCGGACTACHVGGGTWTCTAAT	B_11/12/12	3.5RV_35°C	Composition1_-ReactorB
BF.44	TAATACGGATCG	CCGGACTACHVGGGTWTCTAAT	B_11/16/12	3.5RV_35°C	Composition1_-ReactorB

Table 3.13—*Continued*

BF.45	TCGGAATTAGAC	CCGGACTACHVGGGTWTCTAAT	D_10/23/12	300gRV_35°C	Composition_3-ReactorD
BF.46	TGTGAATTCGGA	CCGGACTACHVGGGTWTCTAAT	D_11/6/12	300gRV_35°C	Composition_3-ReactorD
BF.47	CATTCGTGGCGT	CCGGACTACHVGGGTWTCTAAT	D_11/8/12	300gRV_35°C	Composition_3-ReactorD
BF.48	TACTACGTGGCC	CCGGACTACHVGGGTWTCTAAT	D_11/10/12	300gRV_35°C	Composition_3-ReactorD
BF.49	GGCCAGTTCCTA	CCGGACTACHVGGGTWTCTAAT	D_11/14/12	300gRV_35°C	Composition_3-ReactorD

Table 3.14 Mapping File of SET-3

#SampleID	BarcodeSequence	LinkerPrimerSequence	SampleDescription	Treatment	Description
CF.00	TCCCTTGTCTCC	CCGGACTACHVGGGTWTCTAAT	D_11/16/12	300gRV_35°C	Composition_3-ReactorD
CF.01	ACGAGACTGATT	CCGGACTACHVGGGTWTCTAAT	D_11/18/12	300gRV_35°C	Composition_3-ReactorD
CF.02	GCTGTACGGATT	CCGGACTACHVGGGTWTCTAAT	D_11/20/12	300gRV_35°C	Composition_3-ReactorD
CF.03	ATCACCAGGTGT	CCGGACTACHVGGGTWTCTAAT	B_12/5/12	GL_14.6_40°C	Composition_4-ReactorB
CF.04	TGGTCAACGATA	CCGGACTACHVGGGTWTCTAAT	B_12/8/12	GL_14.6_40°C	Composition_4-ReactorB
CF.05	ATCGCACAGTAA	CCGGACTACHVGGGTWTCTAAT	B_12/10/12	GL_14.6_40°C	Composition_4-ReactorB
CF.06	GTCGTGTAGCCT	CCGGACTACHVGGGTWTCTAAT	B_12/12/12	GL_14.6_40°C	Composition_4-ReactorB
CF.07	AGCGGAGGTTAG	CCGGACTACHVGGGTWTCTAAT	B_12/14/12	GL_14.6_40°C	Composition_4-ReactorB
CF.08	ATCCTTTGGTTC	CCGGACTACHVGGGTWTCTAAT	B_12/18/12	GL_14.6_40°C	Composition_4-ReactorB
CF.09	TACAGCGCATAC	CCGGACTACHVGGGTWTCTAAT	B_12/20/12	GL_14.6_40°C	Composition_4-ReactorB
CF.10	ACCGGTATGTAC	CCGGACTACHVGGGTWTCTAAT	B_12/23/12	GL_14.6_40°C	Composition_4-ReactorB
CF.11	AATTGTGTCGGA	CCGGACTACHVGGGTWTCTAAT	B_12/25/12	GL_14.6_40°C	Composition_4-ReactorB
CF.12	TGCATACACTGG	CCGGACTACHVGGGTWTCTAAT	A_1/19/13	GL_62.78_30°C	Composition_5-ReactorA
CF.13	AGTCGAACGAGG	CCGGACTACHVGGGTWTCTAAT	A_1/12/13	GL_62.78_30°C	Composition_5-ReactorA
CF.14	ACCAGTGACTCA	CCGGACTACHVGGGTWTCTAAT	A_1/23/13	GL_62.78_30°C	Composition_5-ReactorA
CF.15	GAATACCAAGTC	CCGGACTACHVGGGTWTCTAAT	A_1/25/13	GL_62.78_30°C	Composition_5-ReactorA

Table 3.14—*Continued*

CF.16	GTAGATCGTGTA	CCGGACTACHVGGGTWTCTAAT	A_1/28/13	GL_62.78_30°C	Composition_5-ReactorA
CF.17	TAACGTGTGTGC	CCGGACTACHVGGGTWTCTAAT	A_1/31/13	GL_62.78_30°C	Composition_5-ReactorA
CF.18	CATTATGGCGTG	CCGGACTACHVGGGTWTCTAAT	A_2/2/13	GL_62.78_30°C	Composition_5-ReactorA
CF.19	CCAATACGCCTG	CCGGACTACHVGGGTWTCTAAT	A_2/4/13	GL_62.78_30°C	Composition_5-ReactorA
CF.20	GATCTGCGATCC	CCGGACTACHVGGGTWTCTAAT	A_2/6/13	GL_62.78_30°C	Composition_5-ReactorA
CF.21	CAGCTCATCAGC	CCGGACTACHVGGGTWTCTAAT	A_2/8/13	GL_62.78_30°C	Composition_5-ReactorA
CF.22	CAAACAACAGCT	CCGGACTACHVGGGTWTCTAAT	A_2/13/13	GL_62.78_30°C	Composition_5-ReactorA
CF.23	GCAACACCATCC	CCGGACTACHVGGGTWTCTAAT	A_2/15/13	GL_62.78_30°C	Composition_5-ReactorA
CF.24	GCGATATATCGC	CCGGACTACHVGGGTWTCTAAT	A_2/18/13	GL_62.78_30°C	Composition_5-ReactorA
CF.25	CGAGCAATCCTA	CCGGACTACHVGGGTWTCTAAT	A_2/20/13	GL_62.78_30°C	Composition_5-ReactorA
CF.26	AGTCGTGCACAT	CCGGACTACHVGGGTWTCTAAT	A_2/22/13	GL_62.78_30°C	Composition_5-ReactorA
CF.27	GTATCTGCGCGT	CCGGACTACHVGGGTWTCTAAT	A_3/1/13	GL_62.78_30°C	Composition_5-ReactorA
CF.28	CGAGGGAAAGTC	CCGGACTACHVGGGTWTCTAAT	A_3/6/13	GL_62.78_30°C	Composition_5-ReactorA
CF.29	CAAATTCGGGAT	CCGGACTACHVGGGTWTCTAAT	A_3/11/13	GL_62.78_30°C	Composition_5-ReactorA
CF.30	AGATTGACCAAC	CCGGACTACHVGGGTWTCTAAT	A_3/15/13	GL_62.78_30°C	Composition_5-ReactorA
CF.31	AGTTACGAGCTA	CCGGACTACHVGGGTWTCTAAT	B_1/19/13	300gRV_30°C	Composition_3-ReactorB
CF.32	GCATATGCACTG	CCGGACTACHVGGGTWTCTAAT	B_1/22/13	300gRV_30°C	Composition_3-ReactorB

Table 3.14—*Continued*

CF.33	CAACTCCCGTGA	CCGGACTACHVGGGTWTCTAAT	B_1/25/13	300gRV_30°C	Composition_3-ReactorB
CF.34	TTGCGTTAGCAG	CCGGACTACHVGGGTWTCTAAT	B_1/28/13	300gRV_30°C	Composition_3-ReactorB
CF.35	TACGAGCCCTAA	CCGGACTACHVGGGTWTCTAAT	B_1/30/13	300gRV_30°C	Composition_3-ReactorB
CF.36	CACTACGCTAGA	CCGGACTACHVGGGTWTCTAAT	B_2/1/13	300gRV_30°C	Composition_3-ReactorB
CF.37	TGCAGTCCTCGA	CCGGACTACHVGGGTWTCTAAT	B_2/4/13	300gRV_30°C	Composition_3-ReactorB
CF.38	ACCATAGCTCCG	CCGGACTACHVGGGTWTCTAAT	B_2/6/13	300gRV_30°C	Composition_3-ReactorB
CF.39	TCGACATCTCTT	CCGGACTACHVGGGTWTCTAAT	B_2/8/13	300gRV_30°C	Composition_3-ReactorB
CF.40	GAACACTTTGGA	CCGGACTACHVGGGTWTCTAAT	B_2/11/13	300gRV_30°C	Composition_3-ReactorB
CF.41	GAGCCATCTGTA	CCGGACTACHVGGGTWTCTAAT	B_2/13/13	300gRV_30°C	Composition_3-ReactorB
CF.42	TTGGGTACACGT	CCGGACTACHVGGGTWTCTAAT	B_2/15/13	300gRV_30°C	Composition_3-ReactorB
CF.43	AAGGCGCTCCTT	CCGGACTACHVGGGTWTCTAAT	B_2/18/13	300gRV_30°C	Composition_3-ReactorB
CF.44	TAATACGGATCG	CCGGACTACHVGGGTWTCTAAT	B_2/20/13	300gRV_30°C	Composition_3-ReactorB
CF.45	TCGGAATTAGAC	CCGGACTACHVGGGTWTCTAAT	B_2/22/13	300gRV_30°C	Composition_3-ReactorB
CF.46	TGTGAATTCGGA	CCGGACTACHVGGGTWTCTAAT	B_2/22/13_D	300gRV_30°C	Composition_3-ReactorB

Chapter 4

Results & Discussion

4.1 Introduction

The experimental results are presented and discussed in this chapter, which is divided into three sections. The first section includes the amount of DNA extracted from real and synthetic vinasse of 6 compositions. The gas generation data from the laboratory scale bioreactors, along with volume and pH are presented in the second section. The last section of this chapter includes details about microbial data responsible for producing biogas in bioreactors.

4.2 DNA Extraction Result

4.2.1 DNA Extraction Data

DNA extraction was performed on the six different compositions of vinasse. 10 different compositions were analyzed for DNA at three different temperatures (30, 35, and 40°C), plus 2 duplicates, as shown in Table 4.1 (samples with duplicates indicated in bold). DNA extraction results are mentioned below in Table 4.2, Table 4.3, and Table 4.4.

The concentration of DNA was extracted from synthetic vinasse (compositions 4, 5, and 6) was much higher than real vinasse combinations (compositions 1, 2, and 3). Real vinasse DNA concentrations were obtained between 2.68 ng/µl to 75 ng/µl, except some samples which had concentration around 100 ng/µl. However, synthetic vinasse DNA concentration ranged from 16.93 ng/µl to 226.98 ng/µl. Minimum 15 ng/µl DNA concentration is good for PCR. High DNA concentrations were diluted to 15-20 ng/µl for PCR to avoid spurious bands other than target DNA for MiSeq analysis.

DNA extraction of real vinasse compositions 1 and 2 (produced from corn and milo) was more difficult than real vinasse composition 3 (produced from corn). Compositions 1 and 2 produced higher DNA concentration on day 1, but the amount decreased as the reaction progressed further. The microbes may have eaten the easily digestible part of the vinasse on day 1, resulting in high microbial activity and abundant DNA, but leaving less digestible food as the reaction progresses. Alternatively, the kit used for DNA extraction may not have been good for

the type of vinasse produced from food grains milo and corn. Composition 3 had glucose in its composition, which made it better for microbial activity like other synthetic vinasse. Microbes are not able to digest real vinasse due to presence of complex sugars in it.

Table 4.1 Compositions Analyzed for DNA Extraction at Three Temperatures

Temperature (°C)	Composition 1	Composition 2	Composition 3	Composition 4	Composition 5	Composition 6
30		**Analyzed**	Analyzed		Analyzed	Analyzed
35	Analyzed		Analyzed			
40	Analyzed		Analyzed	Analyzed	**Analyzed**	

Table 4.2 DNA Extraction Data for Compositions 1 & 2

COMPOSITION 1 AT 35ºC		**COMPOSITION 1 AT 40ºC**			**COMPOSITION 2 AT 30ºC**		**COMPOSITION 2 AT 40ºC**	
Day	**DNA(ng/µl)**	**Day**	**DNA(ng/µl)**		**Day**	**DNA(ng/µl)**	**Day**	**DNA(ng/µl)**
2	16.2	1	109.43		1	82.03	1	214.76
15	7.18	4	16.69		4	6.97	5	98.84
17	7.45	5	52.52		7	9.31	11	65.54
19	8.16	6	16.17		9	2.68		
21	25.13	8	30.67		11	0.88		
23	16.8	10	17.05		13	9.93		
25	11.38	12	22.13		15	5.59		
		14	45.44		18	4.29		
		16	44.98		20	4.85		
					22	10.5		
					25	6.78		

Table 4.2—*Continued*

					27	8.26		
					29	1.67		
					32	9.74		
					34	16.98		
					37	17.07		

Table 4.3 DNA Extraction Data for Compositions 3 & 4

COMPOSITION 3 AT 30ºC		COMPOSITION 3 AT 35ºC		COMPOSITION 3 AT 40ºC			COMPOSITION 4 AT 30ºC		COMPOSITION 4 AT 40ºC	
Day	**DNA(ng/µl)**	**Day**	**DNA(ng/µl)**	**Day**	**DNA(ng/µl)**		**Day**	**DNA(ng/µl)**	**Day**	**DNA(ng/µl)**
1	21.52	1	64.73	1	70.36		1	226.58	0	130.16
4	44.75	15	33.63	3	35.67		4	188.79	3	53.33
5	38.7	17	45.73	5	42.02		7	144.85	5	97.84
7	18.22	19	53.25	7	20.86		9	127.58	7	84.46
10	61.25	21	51.71	9	32.52		11	141.49	9	25.81
12	18.76	23	61.97	11	30.9		13	120.14	13	32.44
14	19.97	25	57.66	13	19.1		15	103.28	15	23.68
16	37.11	27	34.04				18	96.01	18	21.50
17	30.18	29	44.09				20	64.27	20	88.00
19	31.01						22	62.29	22	24.65
21	40.33						25	35.44		
24	47.62						27	16.93		
26	36.72						29	22.11		
28	44.29						32	50.31		
31	65.84									

Table 4.3—*Continued*

33	29.93									
35	32.46									

Table 4.4 DNA Extraction Data for Compositions 5 & 6

COMPOSITION 5 AT 30ºC		**COMPOSITION 5 AT 40ºC**			**COMPOSITION 6 AT 30ºC**	
Day	**DNA (ng/µl)**	**Day**	**DNA (ng/µl)**		**Day**	**DNA (ng/µl)**
1	275.7	1	110.48		1	23.84
3	86.38	3	218.53		3	74.28
5	78.12	5	74.09		5	47.29
7	88.88	7	68.89		9	26.82
10	53.48	9	65.65		11	77.92
11	51.74	11	67.85		13	49.53
13	52.52	14	84.05		15	40.42
15	55.11	16	46.2		17	29.81
17	59.19	19	56.23		19	42.3
19	63.91	21	59.12		22	38.05
21	55.62				26	36.74
24	68.74				29	52.46
26	71.41				31	27.63
28	55.69				33	40.38
31	57.11				36	53.59
33	36.47				38	32.15
35	36.92				40	33.94
38	73.36				43	39.32
40	42.63				45	41.58
42	44.83				47	37.51
45	45.16				50	55.21
47	52.91				52	89.56

Table 4.4—*Continued*

49	49.96				54	54.02
52	74.77				57	59.25
54	68.5				59	57.49
56	16.33				61	83.07
					64	67.99
					71	37.41
					78	60.94
					85	26.25

4.3 DNA Sequencing Results

As discussed previously, DNA was extracted and PCR performed for Sequencing. Miseq run gave 14 million reads for set-1 and set-3 samples and 11 million reads for set-2, as mentioned in Table 4.5. Fifty samples were run in each set of MiSeq run from 12 different bioreactors of 6 compositions. It includes two duplicates and six sample duplicates. The QIIME software were used for analysing the results (qiime.org).

4.3.1 Quality Filter Results

Quality filter reads after Miseq runs were analysed using QIIME sofware 1.8 version. Each sample has different barcodes so that it can be analyzed and diffrentiated from the others. The quality filter reads for each sample are given below in Table 4.5.

Table 4.5 Quality Filter Reads of Set-1, 2, & 3 Samples

SET-1		SET-2		SET-3	
SAMPLE #	READS	SAMPLE #	READS	SAMPLE #	READS
AF.19	892289	BF.47	616441	CF.33	797097
AF.26	747317	BF.14	458312	CF.09	555662

Table 4.5—*Continued*

AF.37	649535	BF.09	436331	CF.15	525167
AF.38	601182	BF.21	411055	CF.35	482038
AF.46	576592	BF.00	407641	CF.13	471936
AF.48	540283	BF.27	369724	CF.39	470508
AF.28	518466	BF.49	363794	CF.28	437859
AF.14	505828	BF.01	362151	CF.18	424604
AF.47	474128	BF.48	333879	CF.23	413978
AF.36	425035	BF.20	327135	CF.32	410824
AF.49	410846	BF.36	319213	CF.44	403699
AF.45	410617	BF.23	305736	CF.26	391235
AF.24	404988	BF.03	282191	CF.22	383078
AF.25	382607	BF.39	260675	CF.04	375580
AF.39	378503	BF.15	257908	CF.08	362952
AF.29	331446	BF.13	250501	CF.17	358744
AF.30	308335	BF.08	244064	CF.43	349888
AF.44	292262	BF.45	241841	CF.34	332116
AF.13	283243	BF.26	241371	CF.30	326980
AF.20	274856	BF.30	230415	CF.14	323016
AF.35	271239	BF.16	227950	CF.31	318329
AF.27	264064	BF.43	224859	CF.38	305086
AF.23	261920	BF.42	224398	CF.49	298210
AF.43	257557	BF.34	218633	CF.37	297842
AF.03	251873	BF.32	217065	CF.29	253758
AF.31	250228	BF.35	211294	CF.03	249305
AF.40	249906	BF.46	207222	CF.27	240828
AF.15	249805	BF.31	200645	CF.42	236975

Table 4.5—*Continued*

AF.41	204490	BF.33	197967	CF.20	219112
AF.34	196665	BF.17	197516	CF.36	217793
AF.22	194593	BF.37	183856	CF.12	203096
AF.33	186753	BF.07	168677	CF.02	199188
AF.12	184389	BF.44	157252	CF.07	175793
AF.11	183923	BF.10	156148	CF.41	172019
AF.18	183708	BF.25	150722	CF.40	168526
AF.21	176893	BF.41	150699	CF.25	168182
AF.02	166773	BF.02	148460	CF.11	160803
AF.17	165883	BF.40	129294	CF.01	159100
AF.10	148101	BF.18	116577	CF.19	155237
AF.08	134219	BF.12	116567	CF.16	151175
AF.06	120283	BF.22	115330	CF.21	141462
AF.32	109229	BF.29	111520	CF.06	137345
AF.42	97128	BF.28	109533	CF.47	126148
AF.09	88019	BF.38	105516	CF.24	116793
AF.00	86401	BF.06	102703	CF.00	113895
AF.04	79699	BF.05	90355	CF.45	108429
AF.07	79421	BF.19	87897	CF.46	107872
AF.01	41907	BF.04	84978	CF.05	91090
AF.16	28888	BF.24	84145	CF.10	80508
AF.05	27228	BF.11	83190	CF.48	67178
Total number of sequences	14,349,543	Total number of sequences	11,301,346	Total number of sequences	14,038,038

4.3.2 Microbial Results

This section shows the different types of microbes present in real and synthetic vinasse combinations at various stages of anaerobic degradation at mesophilic temperatures 30, 35, and 40°C. Only 10 different compositions out of total 18 combinations were analyzed due to high costs associated with these analyses. Two duplicates and six sample duplicates were also analyzed for microbial analysis. The synthetic-real vinasse combination was selected for analysis based on the high percentage of methane measured and total number of days associated with each reactor.

From the literature, the different phyla which are responsible for performing hydrolysis, acidogenesis, acetogenesis, and methanogenesis are given below in Table 4.6. Bacteroidetes are helpful in hydrolysis (Deublein and Steinhauser, 2010; Ray and Bhunia, 2013). Firmicutes are responsible for all three stages hydrolysis, acidogenesis, and acetogenesis (Drake et al. 2002; Babu et al., 2013; Ray and Bhunia, 2013; Cirne et al., 2006; Sarkar, 2011). Fusobacteria are also responsible for both hydrolysis and acidogenesis (Joubert and Britz, 1987). Spirochaetes are responsible for hydrolysis and acetogenesis (Drake et al., 2002; Deublein and Steinhauser, 2011). Proteobacteria are responsible for both hydrolysis, and acidogenesis (Deublein and Steinhauser, 2011; Cirne et al., 2006; Sarkar, 2011). Some other phyla which help in hydrolysis are Actinobacteria, Chloroflexi, WWE1, and Thermotogae (Deublein and Steinhauser, 2011; Limam et al., 2013; Cirne et al., 2006). Synergistetes and Spirochaetes are responsible for acetogenesis (Drake et al., 2002).

Table 4.6 Phyla of Hydrogens, Acidogens, Acetogens, and Methanogens in 6 Different Compositions of Real and Synthetic Vinasse

Hydrogens	Acidogens	Acetogens	Methanogens
Actinobacteria, Firmicutes, Chloroflexi, Spirochaetes, Thermotogae, Fusobacteria, Proteobacteria, Bacteroidetes, Chloroflexi, WWE1	Firmicutes, Fusobacteria	Firmicutes Synergistetes, Proteobacteria, Spirochaetes	Archaea

Deublein and Steinhauser (2011) found some hydrogens Thermotogae, Bacteriodales, Spirochaetes, Actinobacteria, and Proteobactaeria during the fermentation process in rumen. Limam et

al. (2013) identified WWE1 in the anaerobic digestion of cellulose at 35°C. WWE1 is responsible for hydrolysis of extracellular cellulose and in the absorption of fermentation products. Sarkar (2011) found three acidogenic bacterial isolates viz., Bacillus subtilis, Pseudomonas stutzeri and Lysinibacillus fusiformis related to phyla Firmicutes and Proteobacteria separately. As mentioned later in this section, Bacillus & Lysinibacillus of phylum Firmicutes, Pseudomonas (phylum Proteobacteria) and WWE1 were present in real and synthetic vinasse compositions.

Sawayama (2006) identified Archaea *Methanosarcina* spp. and *Methanobacterium* spp in an anaerobic reactor. España-Gamboa et al. (2012) also detected Methanobacteriales and Methanosarcinales. They found out Archaea are responsible for methanogenesis (Sawayama, 2006; España-Gamboa et al., 2012). *Methanosarcinales*, *Methanomicrobiales,* and *Desulfovibrio* were dominant in the anaerobic sewage digester (Raskin et al., 2005). As metioned later in this section, Methanobacteriales, Methanomicrobiales & Methanosarcinales of phylum Euryarchaeota and Desulfovibrio of phylum Proteobacteria were detected in real and synthetic vinasse compositions.

Kim et al. (2011) identified the most common acidogens bacterial populations related to *Aeromonas* spp. and *Clostridium sticklandii* in all reactors. They also found other acidogenic anaerobes such as Aeromonas sharmana, Bacillus coagulans, and Pseudomonas plecoglossicida. As mentioned later in this section, Aeromonas & Pseudomonas of phylum Proteobacteria and Clostridium & Bacillus of phylum Firmicutes were also present in real and synthetic vinasse compositions.

Sattler (2011) mentioned some bacteria of genera *Bacterroides, Lactobacillus, and Bifidobacterium* to be most common in Hydrolysis. She also mentioned *Clostridium spp., Bifodobacterium spp, Lactobacillus, and Actinomyces* are acidogens and Clostridium *aceticum* and *Acetobacterium woodii* are acetogens. As described later in this section, Bactobacillus & Clostridium of phylum Firmicutes and Bifidobacterium & Actinomyces of phylum Actinobacteria were also identified in real and synthetic vinasse.

Previous research reported that excessive volatile fatty acid accumulation and sudden pH drop occur when waste with high food waste content is started (Shao et al., 2005). Hence, to maintain the pH of the vinasse, buffer ($NaHCO_3$) was dissolved into water and added in the reactor before starting the reactor. It was observed that the real vinasse pH dropped between 5.5- 6.5 within 24 hours after installing

the reactor on day 0. Then, the pH was going down between 6- 7 on day 1. However, the synthetic vinasse pH dropped between 6-7 within 24 hours after installing the reactor on day 0. It was noticed that the rate of pH dropped in real vinasse was greater than synthetic vinasse in the first week of a run. Synthetic vinasse pH stabilized earlier than real vinasse.

In the acidogenic phase, the vinasse pH started dropping, typically below 7.0. The acidogenesis phase typically started between 1 to 7 days in all reactors. If the vinasse pH was found to be below 6, sodium hydroxide (NaOH) was added to the reactors, to avoid excessive acid accumulation. The methane content in gas increased in the third phase, methanogenesis, and stabilized around 25-88% based on the composition of vinasse. During methanogenesis the vinasse pH was found to be between 7.0-8.5. However, even after running the reactor for 16-89 days until methane generation ceased, variation in pH was not observed much after the acidogenesis phase. pH of synthetic compositions was almost same throughout the run, whereas pH of real vinasse dropped little (0.2-0.5) after every 7-10 days. Hence, sodium hydroxide was added to maintain the pH. Once the reactor stopped producing gas, the reactor was assumed to have stopped and was dismantled.

4.3.2.1. Microbial Data of Real Vinasse Composition 1 (Produced from Corn & Milo, White Energy, TX)

Real vinasse composition 1 at 35°C shows high percentages of Bacteroidetes (up to about 31%), Firmicutes (up to about 45%), and Proteobacteria (up to about 40%) at 35°C and intermediate percentage of Fusobacteria (up to about 25%) at 35°C. Presence of Bacteroidetes, Firmicutes, Proteobacteria and Fusobacteria shows hydrolysis, acidogenesis, acetogenesis, and methanogenesis phase in this reactor. Bacteriodetes, responsible for hydrolysis only, decreased in percent between days 15 and 25, as did Fusobacteria. Proteobacteria and Fimicutes increased in percent between days 15 and 25.

It also shows higher percentage of Archaea at the end of run from day 15 to 25. Methanogenesis likely started around day 15 in this composition and continued till the end of run. As shown in Figure 4.2, the methane percent was 1.2% on day 2 and 25% on day 15. Thus, the number of methanogens increases as the percent of methane increases (up to about 2%, as shown in Figure 4.1).

This composition also shows lower percentages of other bacteria such as WWE1, Verrucomicrobia, Tenericutes, Actinobacteria, Spirochaetes, Plantomycytes, Synergistetes Chloroflexi (as shown in Figure 4.1). Spirochaetes, Chloroflexi, and Synergistetes are also responsible for hydrolysis and

acetogenesis. Composition 1 did not produce gas between day 2 to 14. Hence, samples were not stored in the beginning till day 14 of this run.

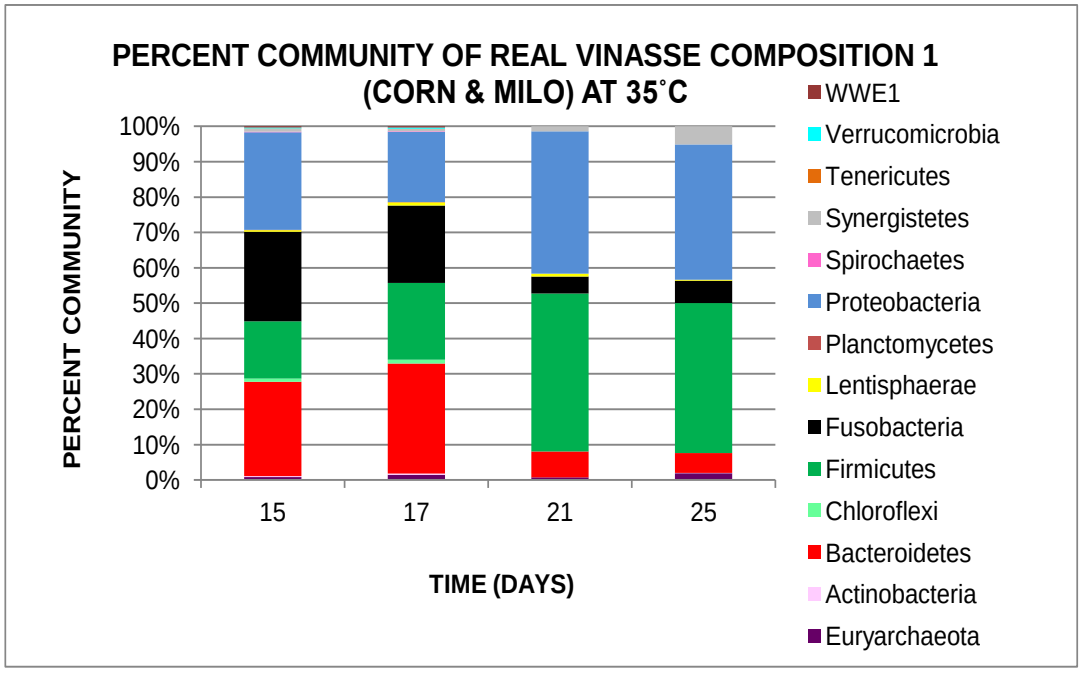


Figure 4.1 Microbial Community Profiling of Real Vinasse Composition 1 (Corn & Milo from White Energy, TX) at 35°C

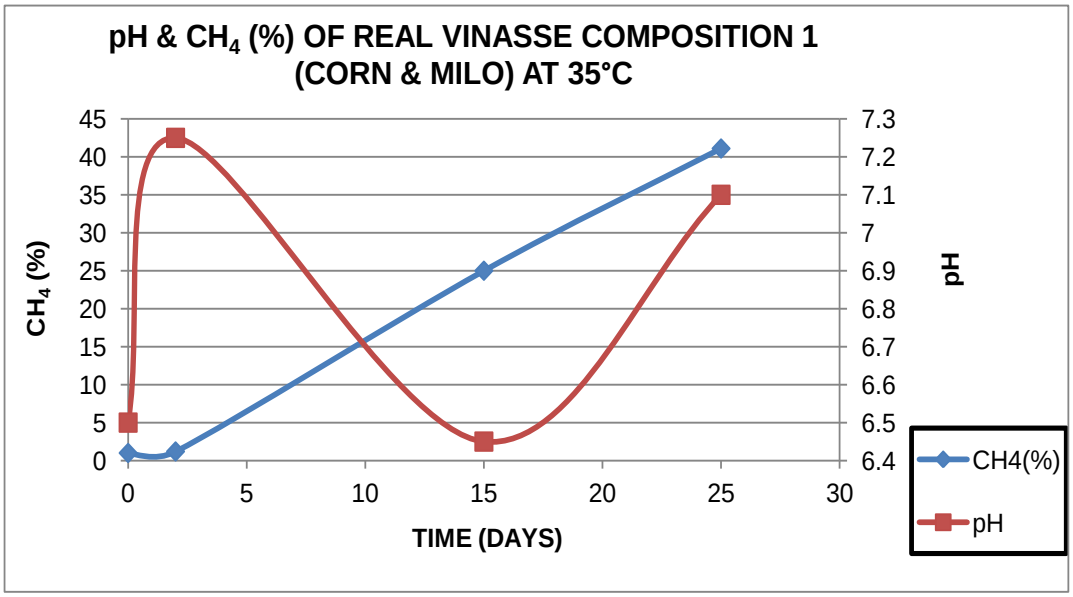


Figure 4.2 pH and CH_4 (%) of Real Vinasse Composition 1 (Corn & Milo from White Energy, TX) at 35°C

4.3.2.1.1 Detailed Listing of Microbe Genus Present

Bacteroidetes, Firmicutes, and Proteobacteria, and Fusobacteria are more abundant than other microbes in Composition 1 at 35°C. These microbes together compose 69% of this composition, as listed below in Table 4.7.

Some other microbes which were present in lower percentage in composition 1 at 35°C are mentioned in Appendix C. These microbes are collectively less than 31% in this composition.

Table 4.7 Microbial Communities Present in Composition1 (Corn & Milo from White Energy, TX) at 35°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

4.3.2.2 Microbial Data of Real Vinasse Composition 1 (Produced from Corn & Milo, White Energy, TX) at 40°C

Real vinasse composition 1 at 40°C shows higher percentage of Fusobacteria (up to 90%) and Bacteroidetes (up to 50%), and lower percents of Firmicutes (up to about 8%) and Proteobacteria (up to about 17%) at 40°C, compared to 35°C. The percentage of Fusobacteria increased enormously at higher temperature compared to 35°C. It might possible that growth of Fusobacteria is more favorable at higher temperature. However, the reason for this change is not clear. Bacteroidetes, responsible for hydrolysis only, peaked early in the run on day 4. Fusobacteria, Fimicutes, and Proteobacteria percents were fairly constant over the duration of the run.

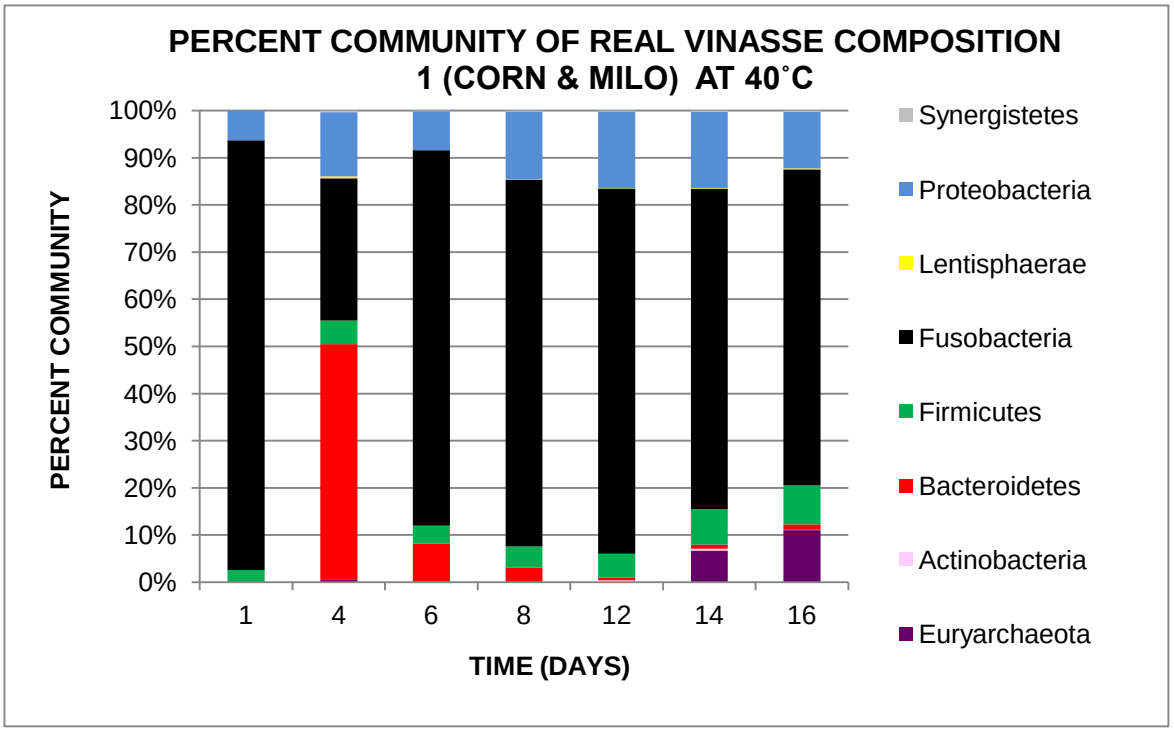


Figure 4.3 Microbial Community Profiling of Real Vinasse Composition 1 (Corn & Milo from White Energy, TX) at 40°C

The percentage of methane started increasing on day 8 to 16 till the reactor stopped producing biogas (as shown in Figure 4.4). Therefore, the Archaea started showing (as shown in Figure 4.3) from day 12 till the end of run. It also shows higher percentage of Archaea on day 16 (over 10%), which is substantially higher than the peak Archaea percent for Composition 1 at 35°C (around 2%). Archaea

started showing earlier at 40°C compared to 35°C for this composition because biological reactions occur faster at higher temperatures, and methanogenesis started earlier.

This composition also shows lower percentage of other bacteria such as Synergistetes, Actinobacteria, Lentisphaerae (as shown in Figure 4.3).

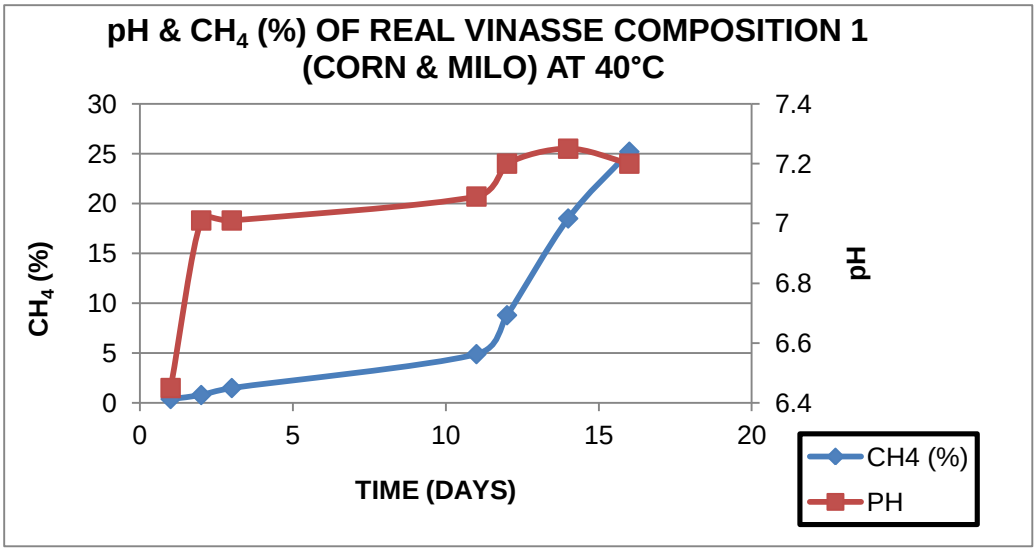


Figure 4.4 pH and CH_4 (%) of Real Vinasse Composition 1 (Corn & Milo from White Energy, TX) at 40°C

4.3.2.2.1 Detailed Listing of Genus Present

Bacteroidetes, Euryarchaeota, Firmicutes, and Proteobacteria, and Fusobacteria are more abundant than other microbes in Composition 1 at 40°C. The microbes listed below are 91% in this composition as mentioned below in Table 4.8.

Some other microbes which are present in lower percentage in composition 1 at 40°C are mentioned in Appendix C. These microbes are 8% in this composition.

Table 4.8 Microbial Communities Present in Composition1 (Corn & Milo from White Energy, TX) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

4.3.2.2.2 Species Richness and Phylogenetic Diversity of Real Vinasse Composition 1 (Produced from Corn & Milo, White Energy, TX) at 35 and 40°C

Real vinasse composition 1 at 35°C (Corn & Milo from White Energy, TX) shows higher species richness (1248) and higher phylogenetic diversity (Faith's PD) 69 on day 17. This indicates high microbial activity in the middle of run, during the methanogenesis phase. However, species richness and Faith's PD values decreased to 425 and 28 at the end of the run (as shown in Figure 4.5).

Real vinasse composition 1 at 40°C (Corn & Milo from White Energy, TX) shows 2 peaks in species richness and PD around days 4, and 12-14, as shown in Figure 4.5 and 4.6. Day 4 was during the hydrolysis phase, based on the high percent of Bacteroidetes. Day 14 was during the methanogenesis phase; methane percent was higher between day 14-16, as shown in Figure 4.4. That means days 9-12 likely were during the acidogenesis/acetogenesis phases. Real vinasse composition 1 at 40°C shows almost half of the peak species richness and higher phylogenetic diversity compared to Composition 1 at 35°C (around 536 for species richness and 31 for PD) , as shown in Figure 4.5 and Figure 4.6.

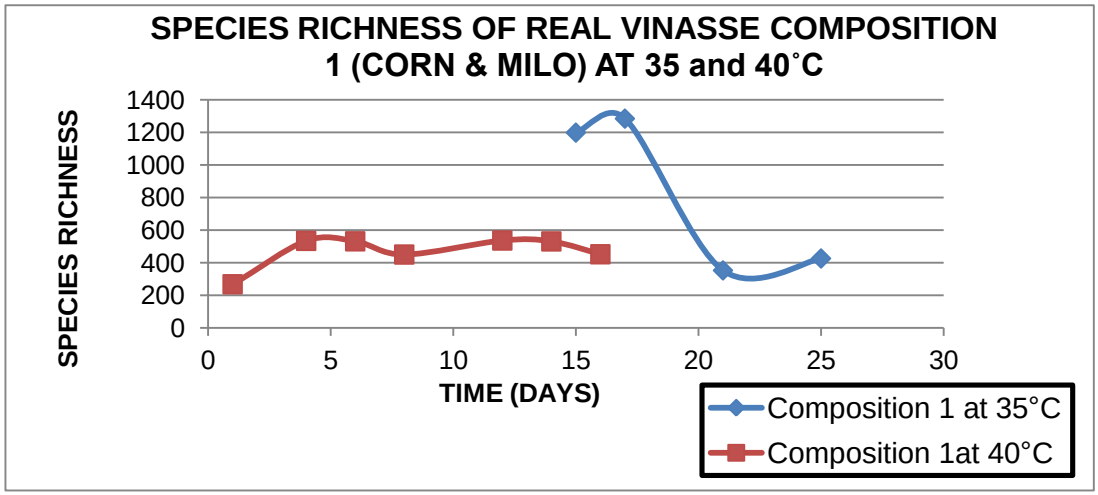


Figure 4.5 Species Richness of Real Vinasse Composition 1 (Corn & Milo, White Energy, TX) at 35 and 40°C

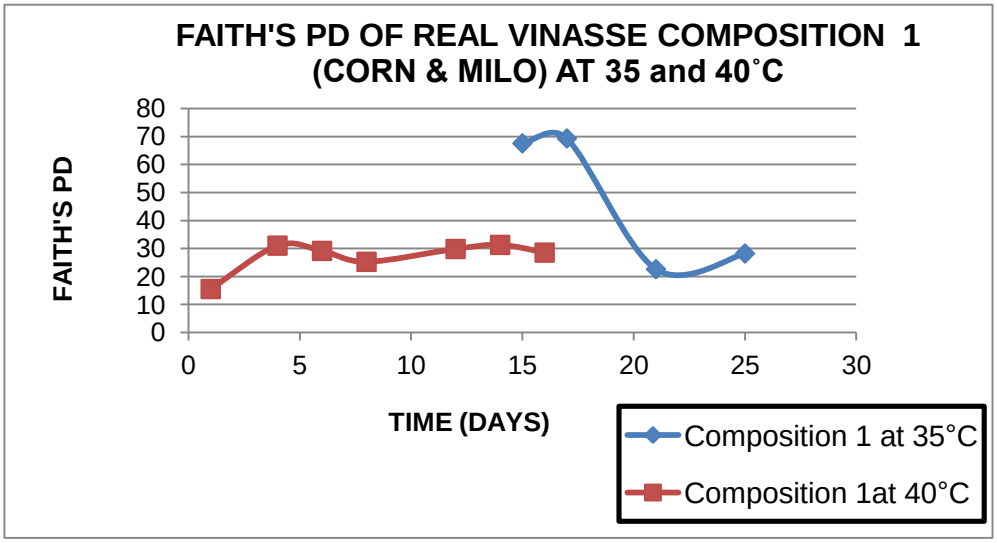


Figure 4.6 Phylogenetic Diversity (Faith's PD) of Real Vinasse Composition 1 (Corn & Milo from White Energy, TX) at 35 and 40°C

4.3.2.2.3 Impact of Temperature for Real Vinasse Composition 1 (Produced from Corn & Milo, White Energy, TX)

Run time was 25 days at 35°C, versus 16 days at 40°C. The shorter run time at higher temperature would be expected, due to faster reactions at higher temperatures.

The percentage of Fusobacteria increased enormously at 40°C compared to 35°C. Methanogens reached over 10% at 40°C, which is substantially higher than the peak Archaea percent for Composition 1 at 35°C (around 2%). Peak species richness and phylogenetic diversity were comparable for the two temperatures.

4.3.2.3 Microbial Data of Real Vinasse Composition 2 (Produced from Corn & Milo White Energy, TX) at 30°C

Real vinasse composition 2 at 30°C shows particularly high percentage of Bacteroidetes (up to 85%) compared to composition 1. Although percents vary by day, Proteobacteria (up to 40%) and Firmicutes (up to 80%) are generally present in moderate percents and Fusobacteria generally in a small percent (up to 60%). Fusobacteria are present in larger percents in the first half of the run compared to the second half; Proteobacteria are present in larger percents in the second half compared to the first half. However, the phyla exhibit no regular trend with time. The maximum percentage of Bacteroidetes and Firmicutes is particularly high in this run, compared to compositions 1 and 3.

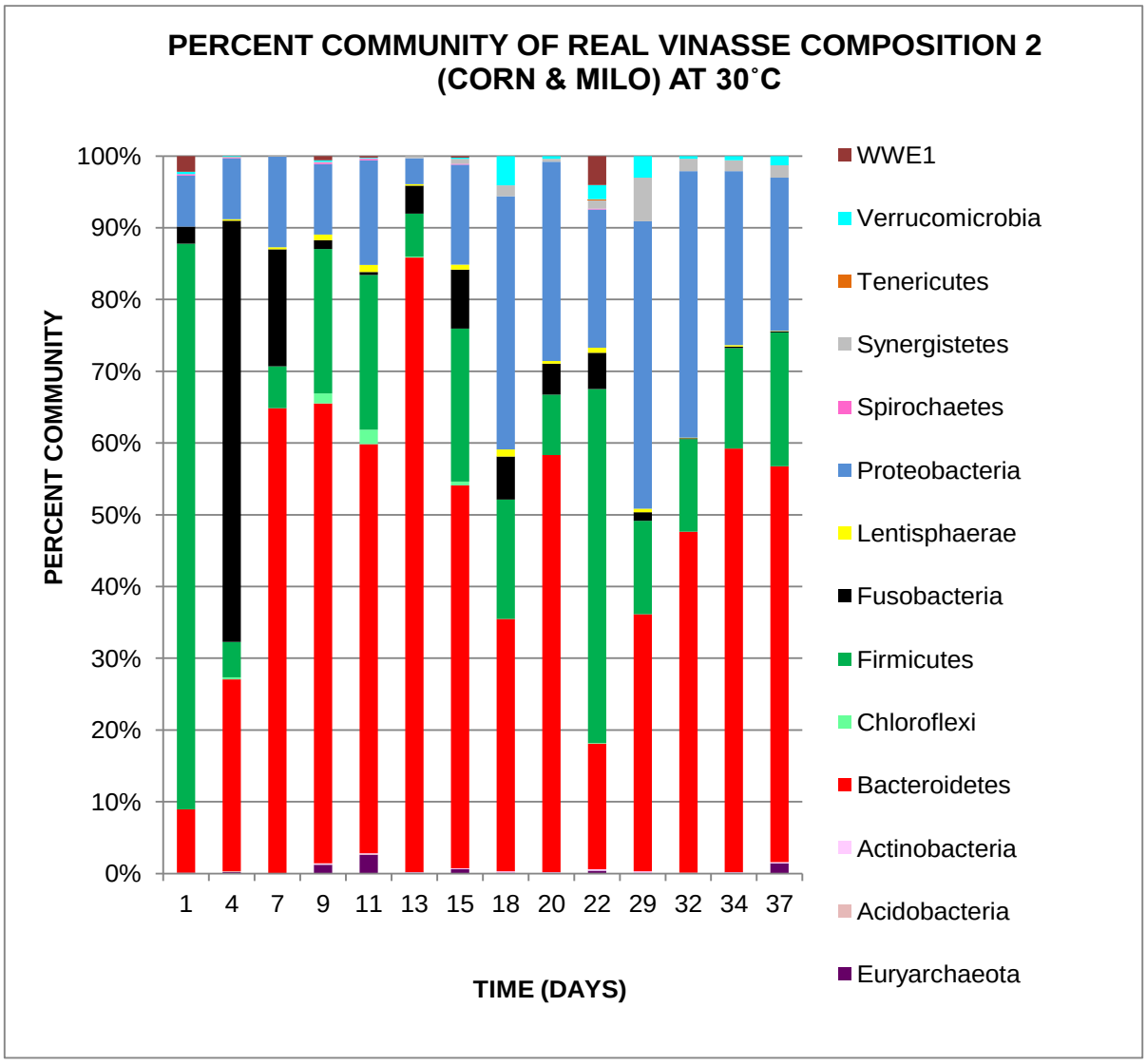


Figure 4.7 Microbial Community Profiling of Real Vinasse Composition 2 (Corn & Milo from White Energy, TX) at 30°C

The percentage of Archaea started increasing on day 9 and was present till the end of run (peak percent about 3% on day 11). Correspondingly, methane percent started increasing on day 11 till the end of reaction (as shown in Figure 4.8).

This composition also shows lower percentage of other bacteria such as Synergistetes, Actinobacteria, Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, Chloroflexi, Actinobacteria (as shown in Figure 4.7). Acidobacteria was found on day 4.

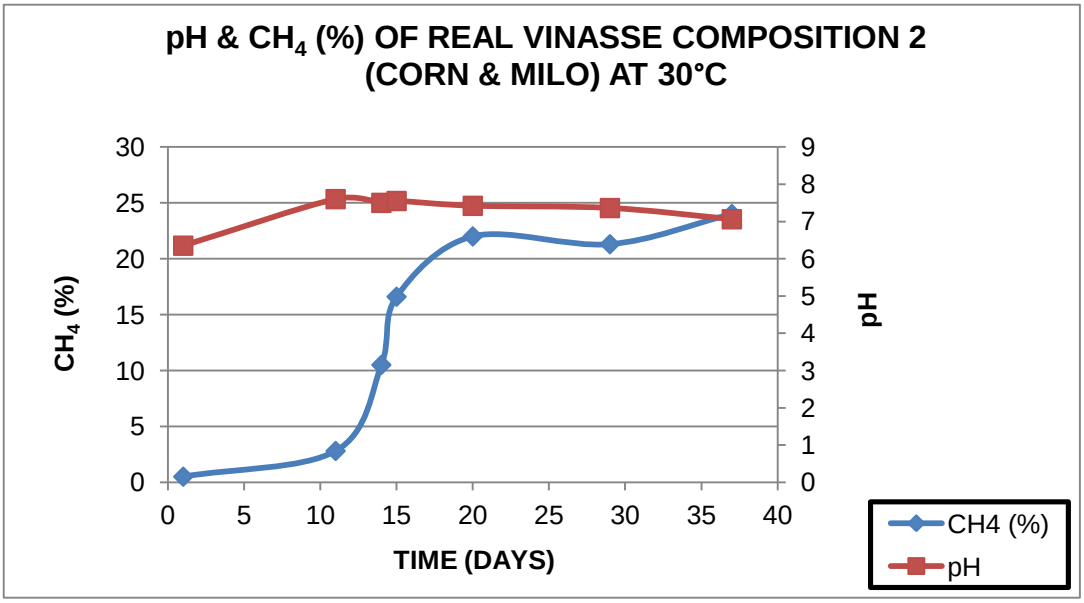


Figure 4.8 pH and CH_4 (%) of Real Vinasse Composition 2 (Corn & Milo from White Energy, TX) at 30°C

4.3.2.3.1 Detailed Listing of Genus Present

Some Bacteroidetes, Firmicutes, and Proteobacteria, Synergistetes, Verrucomicrobia and Fusobacteria are more abundant than other microbes in this composition. These microbes, listed below in Table 4.9, are 77% in this composition.

Some other microbes which are present in lower percentage than above microbes in composition 2 at 30°C are included in Appendix C. These microbes are 22% in this composition.

Table 4.9 Microbial Communities Present in Composition 2 (Corn & Milo from White Energy, TX) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	

4.3.2.3.2 Species Richness and Phylogenetic Diversity of Real Vinasse Composition 2 (Produced from Corn & Milo, White Energy, TX) at 30°C

Real Vinasse Composition 2 at 30°C shows peak (1096) and phylogenetic diversity (Faith's PD) 61 on day 11; these values are comparable to the peak species richness and PD for composition 1 at 35°C (1248 and 69 respectively). This composition shows 4 peaks in species richness and PD throughout the run. The second peak corresponds to an increase in methane percent that occurred around day 11, and likely represents an increase in Archaea. This composition shows a drop in species richness and Faith's PD at the end of run (as shown in Figure 4.9).

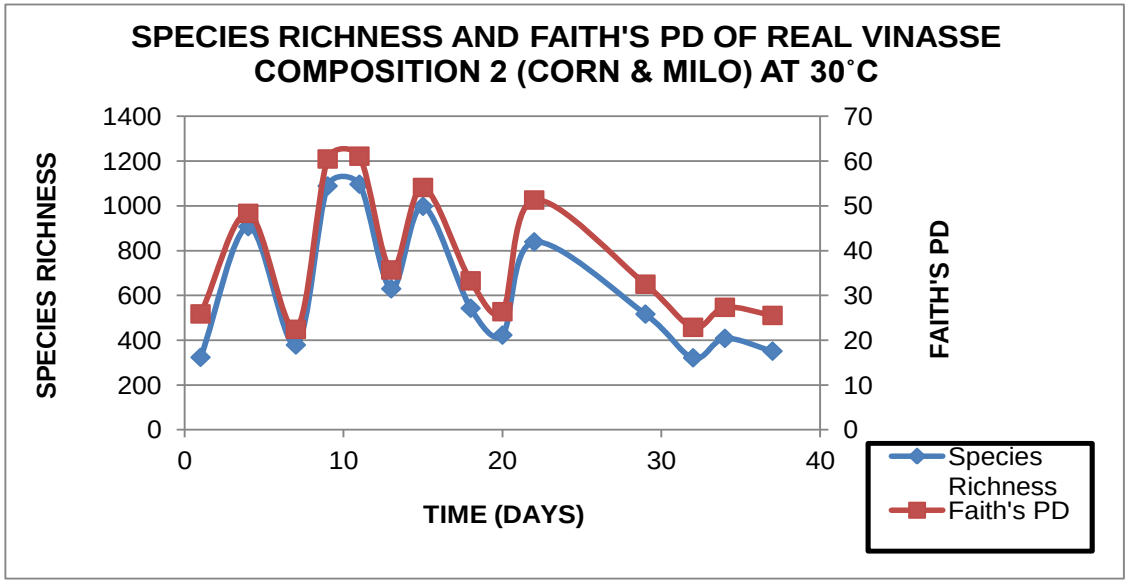


Figure 4.9 Species Richness and Phylogenetic Diversity (Faith's PD) of Real Vinasse Composition 2 (Corn & Milo from White Energy, TX) at 30°C

4.3.2.4 Microbial Data of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 30°C

Real vinasse composition 3 at 30°C shows generally high percentage of Bacteroidetes (maximum over 50%, increasing to days 12-17 and then decreasing), Firmicutes (maximum around 66%, increasing as the run progresses, particularly after day 17), Fusobacteria (maximum over 60%, decreasing as the run progresses) and intermediate percentage of Proteobacteria (66% maximum, also decreasing as the run progresses). The average percent of Firmicutes is particularly high in this run, compared to compositions 1 and 2, as shown in Figure 4.10.

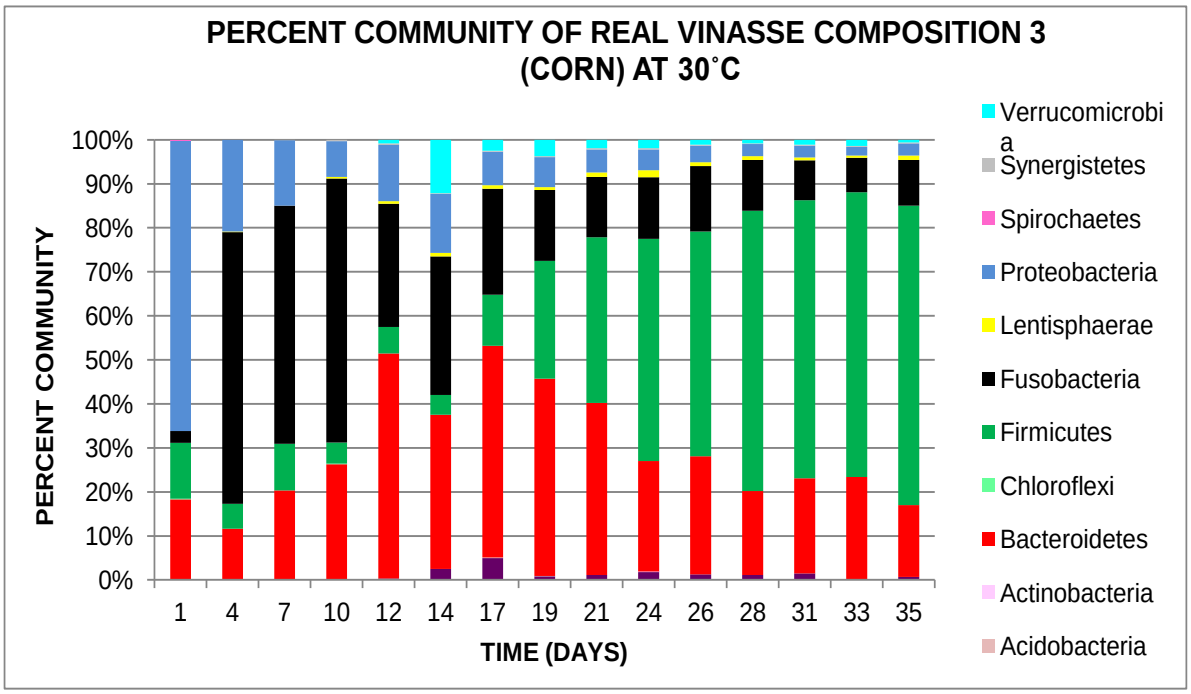


Figure 4.10 Microbial Community Profiling of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 30°C

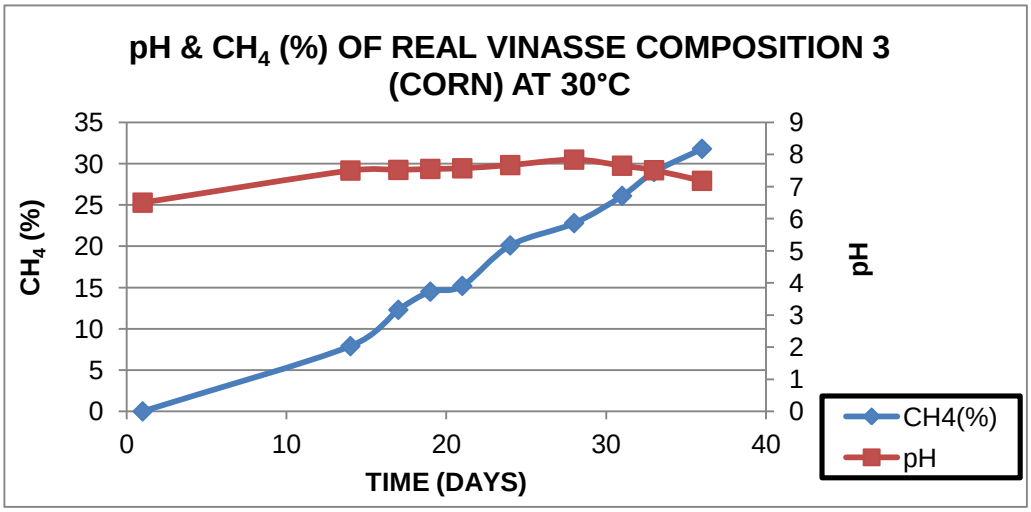


Figure 4.11 pH and CH_4 (%) of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 30°C

Real Vinasse composition 3 at 30°C also contains Acidobacteria and Archaea. Composition 3 shows Archaea on day 12 till the end of run. The percent of Archaea started increasing from day 14, and methane percent also started increasing on this day (as shown in Figure 4.11). The percent of Archaea (Methanogens) peaked at around 5% on day 17.

This composition also contains lower percent of other bacteria such as Verrucomicrobia, Spirochaetes, Chloroflexi, Synergistetes, Lentisphaerae, and Actinobacteria.

4.3.2.4.1 Detailed Listing of Genus Present

Bacteroidetes, Firmicutes, and Fusobacteria are more abundant than other microbes in this composition. These microbes are 86 % in this composition, as given below in Table 4.10.

Some other microbes which are present in lower percentage in composition 3 at 30°C are mentioned in Appendix C. These microbes are 14% in this composition.

Table 4.10 Microbial Communities Present in Composition 3 (Corn from MGP Ingredients, KS) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter

4.3.2.5 Microbial Data of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 35°C

Like real vinasse composition 3 at 30°C, composition 3 at 35°C shows high percentages of Bacteroidetes (maximum over 63%) and Firmicutes (up to 70%) and intermediate percentage of Proteobacteria (up to about 13%). However, the percentage of Fusobacteria is very low (reaching about 4% on day 23 and considerably lower on the other days) compared to the composition 3 at 30°C. These phyla do not, however, show any discernible trends with time. Composition 3 at 35°C also contains Acidobacteria and Archaea. Archaea (Methanogens) are present from day 1 and increased considerably on day 15 (to a peak of about 2.4%), causing a boost in methane production (as shown in Figure 4.13). Composition 3 at 35°C did not produce gas between day 2 to 14. Hence, this composition samples were not stored in the beginning between day 2-14 of this run.

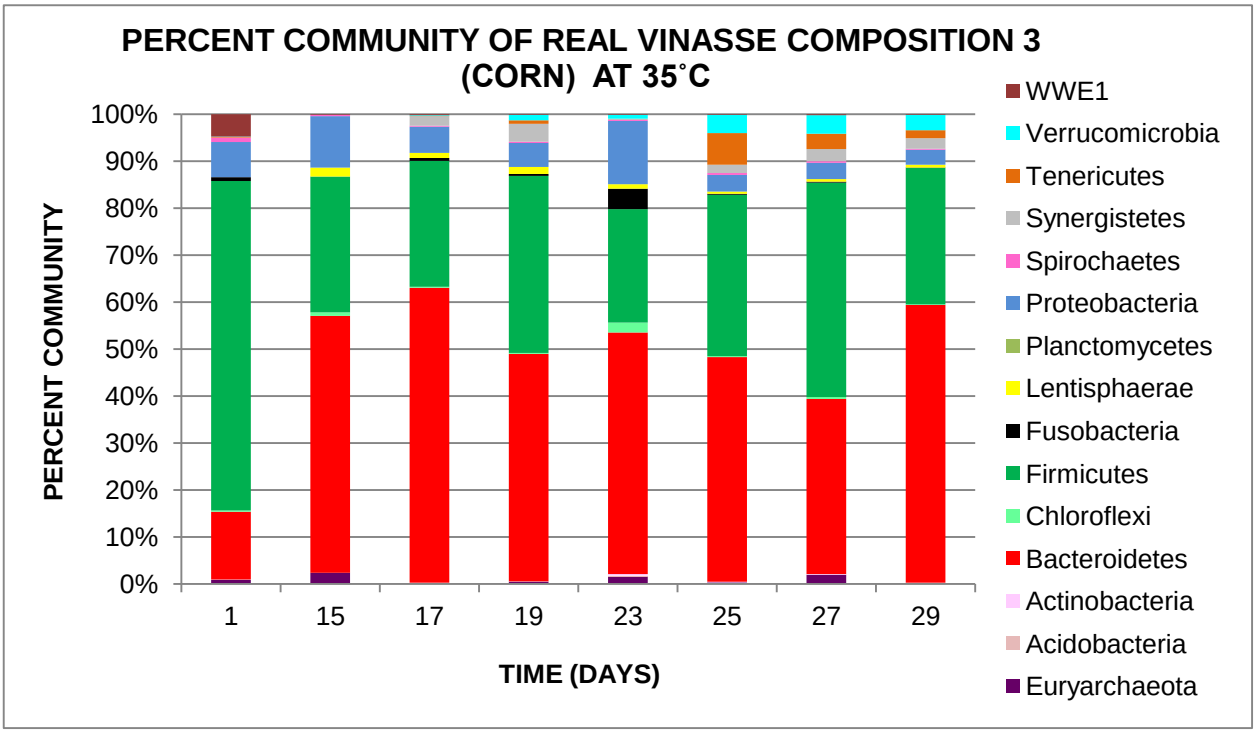


Figure 4.12 Microbial Community Profiling of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 35°C

Real vinasse composition 3 at 35°C also contain lower percent of other bacteria such as Verrucomicrobia, Spirochaetes, Plantomycetes, Chloroflexi, Synergistetes, Lentisphaerae, Actinobacteria,

Tenericutes and WWE1. Tenericutes and WWE1 are present at 35°C which are not present at 30°C (as shown in Figure 4.12). Plantomycetes were present only at temperature 35ºC of real vinasse compositions.

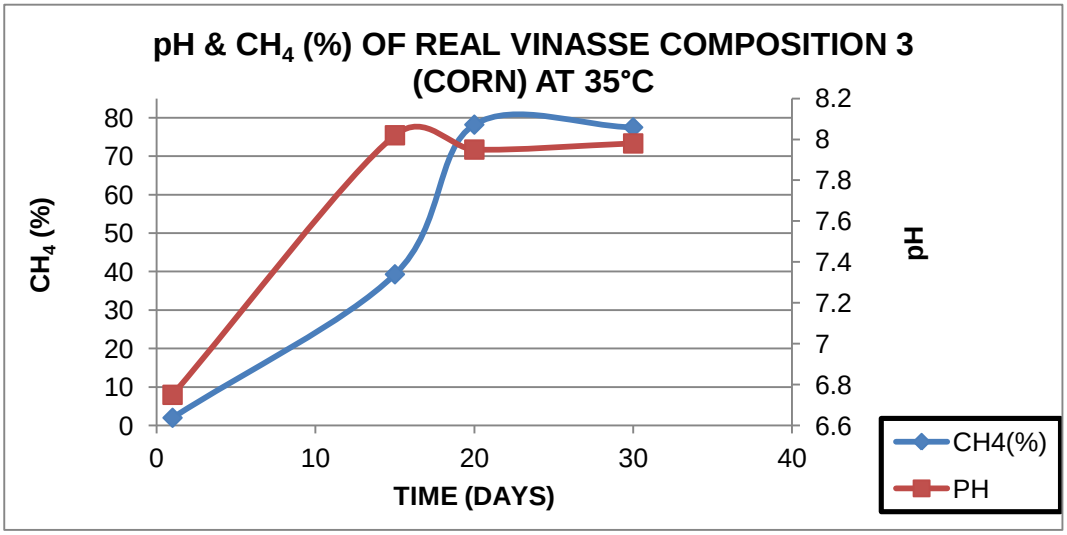


Figure 4.13 pH and CH_4 (%) of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 35°C

4.3.2.5.1 Detailed Listing of Genus Present

Bacteroidetes, and Firmicutes are more abundant than other microbes in this composition (constituting 64%), as given below in Table 4.11.

Some other microbes which are present in lower percentages in composition 3 at 35°C are mentioned in Appendix C. These microbes are 36% in this composition.

Table 4.11 Microbial Communities Present in Composition 3 (Corn from MGP Ingredients, KS) at 35°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira

4.3.2.6 Microbial Data of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 40°C

Like real vinasse composition 1 (Corn and Milo from White Energy, TX) at 40°C, real vinasse composition 3 at 40°C shows a strikingly high percentage of Fusobacteria (up to about 90%), which tends to decrease somewhat during the run with a corresponding increase percentage of Proteobacteria (up to about 37%). The percentages of Bacteroidetes (largest percentage of about 14% the first day, when hydrolytic activity would be expected) and Firmicutes are noticeably lower on this run compared composition 3 at lower temperatures (maximum of about 7%), as shown in Figure 4.14.

This composition does not show Acidobacteria as a separate phylum. It might be possible due to fast reactions at 40°C and the acidogenesis stage thus being shorter at higher temperature. Microbial analysis was done every 2 or 3 days of the run. Hence, Acidobacteria may have been missed during that period.

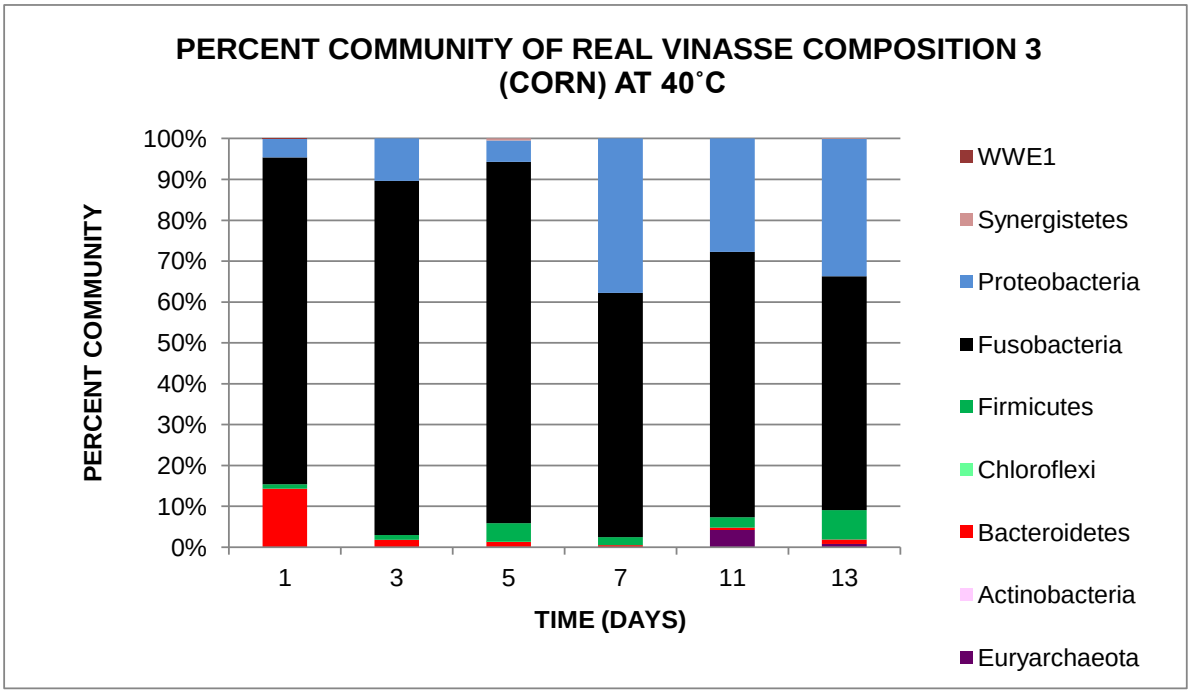


Figure 4.14 Microbial Community Profiling of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 40°C

Real Vinasse composition 3 shows higher percentage of Archaea on day 11 (about 4%). An increase in methane percent had started on day 8 (as shown in Figure 4.15).

Real Vinasse composition 3 at 40°C also contained lower percent of other bacteria such as Chloroflexi, Synergistetes, Actinobacteria, and WWE1.

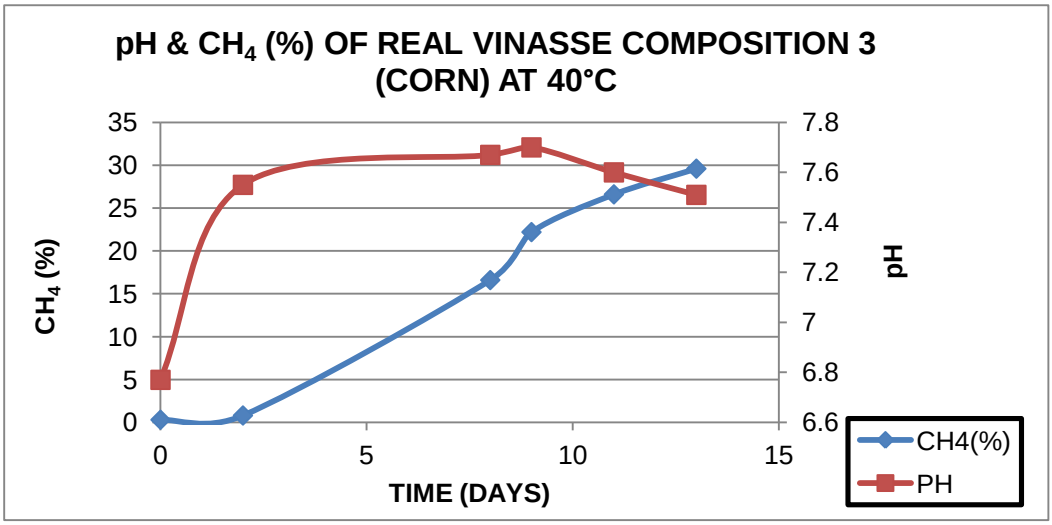


Figure 4.15 pH and CH_4 (%) of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 40°C

4.3.2.6.1 Detailed Listing of Genus Present

Bacteroidetes, Euryarchaeota, Proteobacteria, and Fusobacteria are more abundant than other microbes in this composition. These microbes are 92% in this composition, as given below in Table 4.12.

Some other microbes which are present in lower percentage in composition 3 at 40°C are mentioned in Appendix C. These microbes are 8% in this composition.

Table 4.12 Microbial Communities Present in Composition 3 (Corn from MGP Ingredients, KS) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

4.3.2.6.2 Comparison of Species Richness and Phylogenetic Diversity of Composition 3 (Produced from Corn, MGP Ingredients, KS) at 30, 35, and 40°C

Real Vinasse composition 3 at 30°C shows peak species richness (667) and higher phylogenetic diversity (Faith's PD) 38 on day 10; smaller peaks occur on day 1 and day 24 (as shown in Figure 4.16). The peak species richness and PD for this composition are considerably lower than for compositions 1 at 35°C and 2 at 30°C (668 vs. 1100-1250, and 39 vs. 61-70). All 3 contain real vinasse, but composition 3 contained solid vinasse with glucose added. Composition 3 at 30°C maximum species richness and phylogenetic diversity is comparable to composition 1 at 40°C.

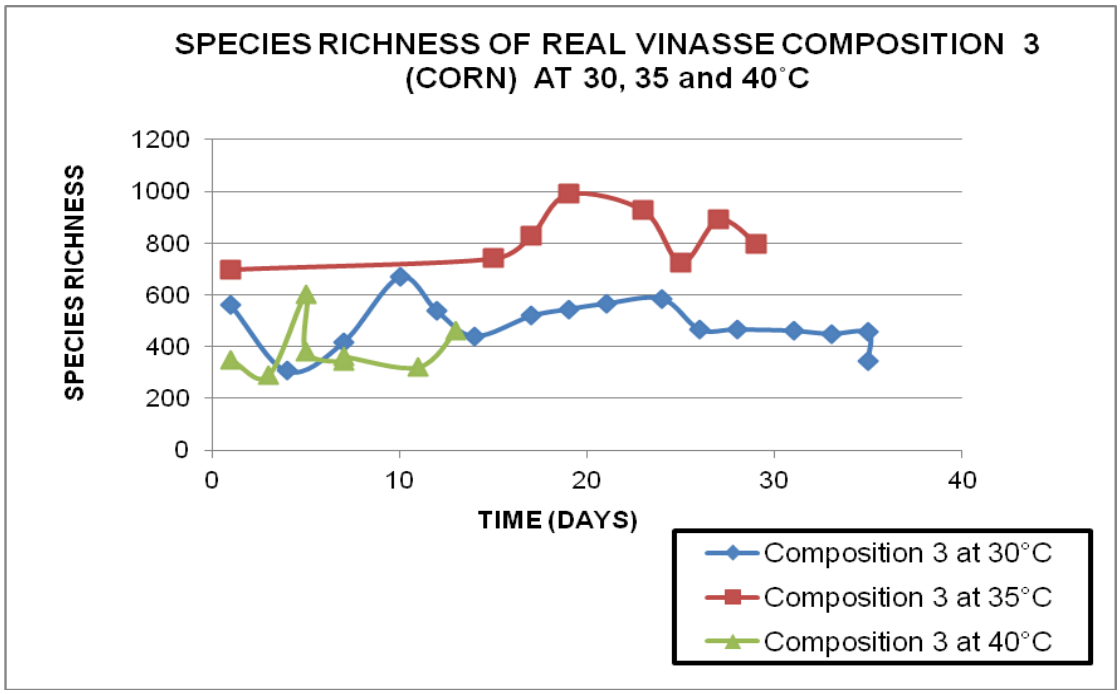


Figure 4.16 Species Richness of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 30, 35 and 40°C

Real Vinasse composition 3 at 35°C shows higher species richness (989) on day 19 and higher phylogenetic diversity (Faith's PD) 59 on day 23, with secondary peaks around day 27, as shown in Figure 4.17). Higher phylogenetic diversity on day 23 shows different types of microbes Bacteroidetes, Firmicutes, Archaea, Proteobacteria, Fusobacteria, Lentisphaerae, Chloroflexi, Verrucomicrobia are present in high percentage in compare to other days. The primary and secondary species richness and PD peaks occurred after the onset of methanogenesis and substantial increase in methane percent.

Composition 3 at 35°C shows higher species richness and phylogenetic diversity compared to Composition 3 at 30°C; the values are comparable to compositions 1 at 35°C and 2 at 30°C.

Real Vinasse composition 3 at 40°C shows higher species richness (602) and higher phylogenetic diversity (Faith's PD) 32 on day 5 (prior to the methanogenesis phase), with secondary peaks of 463 and 28 at the end of the run (day 13). This indicates little drop in species richness and Faith's PD. Composition 3 at 35°C shows higher species richness and higher phylogenetic diversity compared to 35 and 40°C, as shown in Figure 4.16 and Figure 4.17.

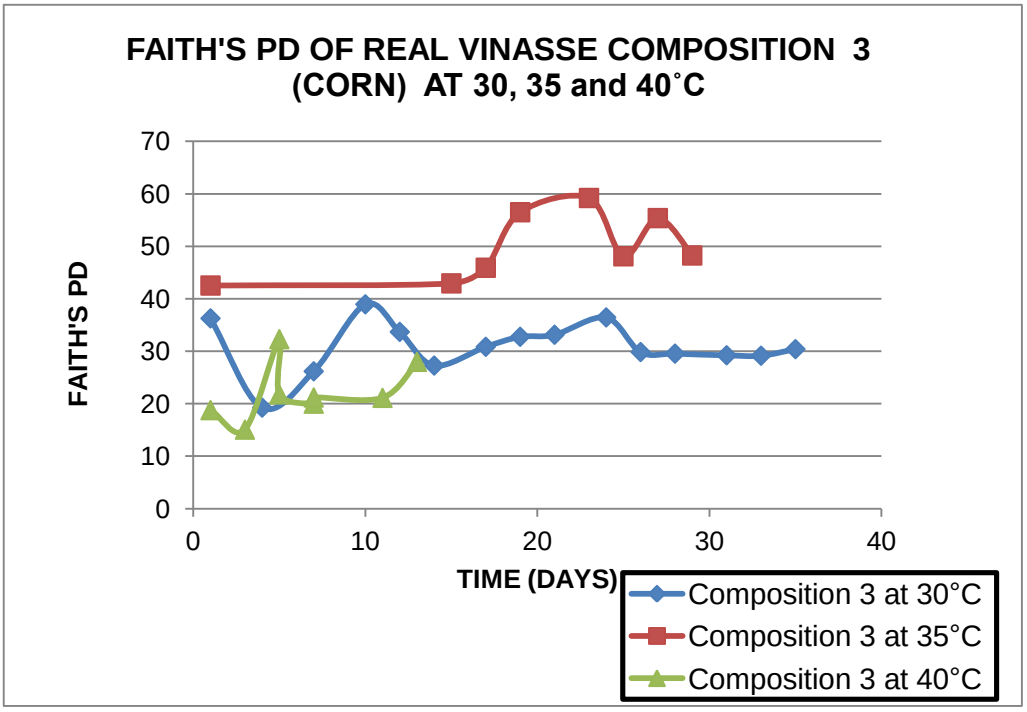


Figure 4.17 Phylogenetic Diversity (Faith's PD) of Real Vinasse Composition 3 (Corn from MGP Ingredients, KS) at 30, 35 and 40°C

4.3.2.6.3 Impact of Temperature for Real Vinasse Composition 3 (Corn from MGP Ingredients, KS)

The runs lasted 35, 29, and 13 days at 30, 35, and 40°C, respectively. Methanogenesis started earlier at 40°C than lower temperatures of this composition.

The real vinasse contains higher percent of Bacteroidetes, Firmicutes, and Proteobacteria at 30 and 35ºC and lower percent at 40ºC, whereas the percent of Fusobacteria was much higher at 40ºC compared to lower temperatures. It might be possible that higher temperature is more favorable for

Fusobacteria than lower temperature. It may also be that excessive addition of Na is helpful for growing Fusobacteria. However, the reason is unclear.

Real vinasse composition 3 at 35°C produced higher methane percentage compared to composition 3 at 30 and 40°C. This indicates 35°C is the most favorable environment for microbial growth for this vinasse composition. The low percent for 40°C may have been due to excessive addition of Na to maintain pH drop and alkali treatment stopped microbes to reach till methanogenesis phase at higher temperature.

Real Vinasse composition 3 at 35°C also had higher species richness and phylogenetic diversity compared to composition 3 at 30 and 40°C.

4.3.2.7 Microbial Data of Synthetic Vinasse Composition 4 (Low COD) at 40°C

Synthetic vinasse Composition 4 at 40°C generally shows high percentages of Bacteroidetes (up to about 60%), Proteobacteria (up to about 67%), moderate percentages of Chloroflexi (22%), WWE1 (31%)**,** and low percents of Firmicutes (6%), Verrucomicrobia, Spirochaetes, Archaea and Thermotogae. However, the species exhibit no clear trends with time. Chloroflexi and WWE1 are also hydrogens. WWE1 was found in previous research to be effective in degrading lignin; the synthetic vinasse, however, contains only glucose and no lignin. It also shows low percentages of Acidobacteria. Composition 4 at 40°C also shows lower percent of other bacteria such as Chloroflexi, Synergistetes, Actinobacteria, WS3, Caldiserica, Lentisphaerae, Plantomycetes, NKB19, OP11, OP3, OP8, OP9, Cyanobacteria (as shown in Figure 4.18). Hydrolytic, acidogenic, and acetogenic bacteria are present in significant percents throughout reactor operation. Fusobacteria are present in negligible amounts in synthetic vinasse combinations compared to real vinasse combinations at 40°C.

Methanogenic bacteria percent increases on day 4, corresponding to the increase in methane percent from day 4-14 and then methane percent decreased little from day 16-22 due to leak in the reactor (as shown in Figure 4.19). The percent of Archaea (Methanogens) peaks at around 4% on days 6 and 21.

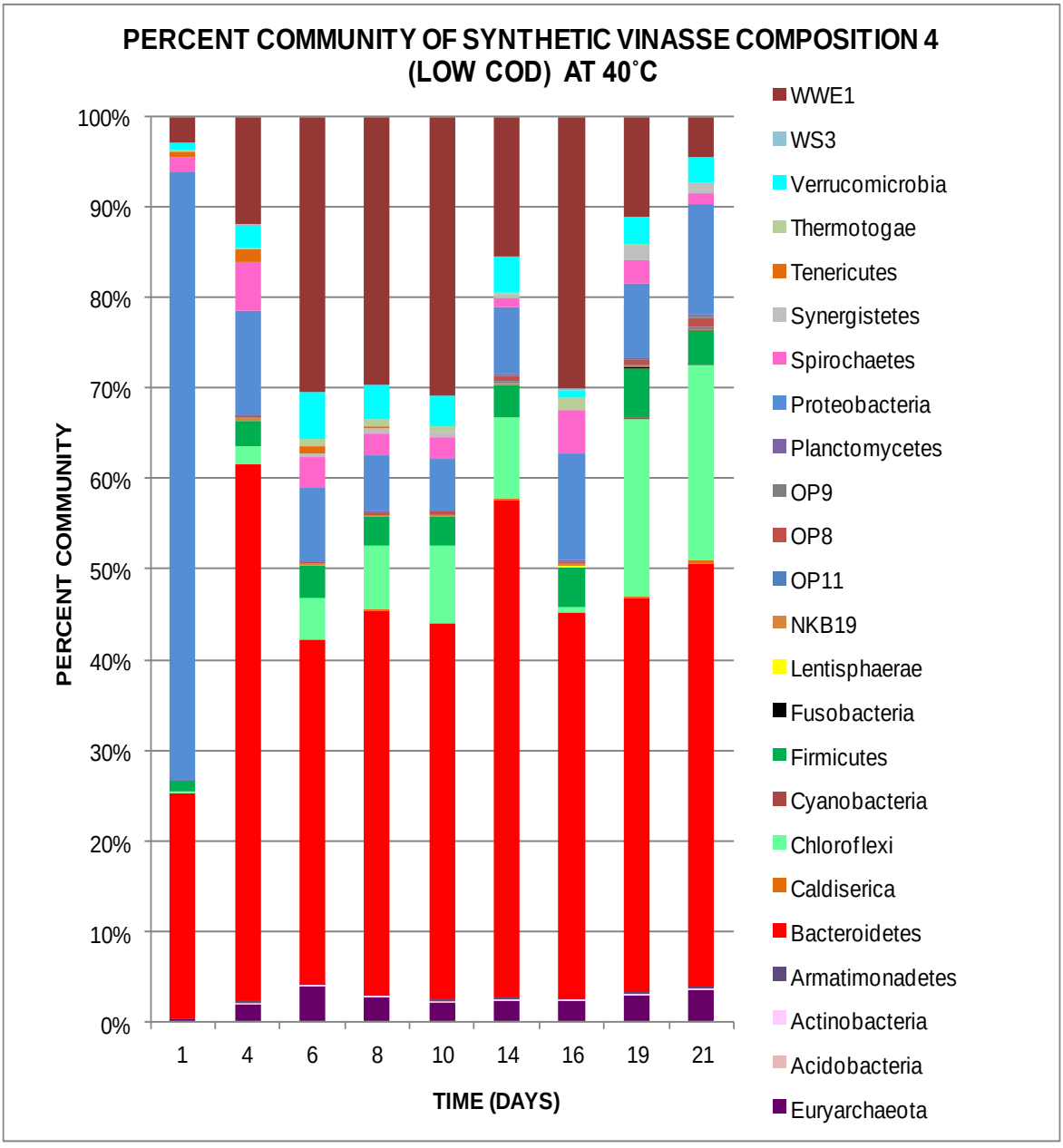


Figure 4.18 Microbial Community Profiling of Synthetic Composition 4 (Low COD) at 40°C

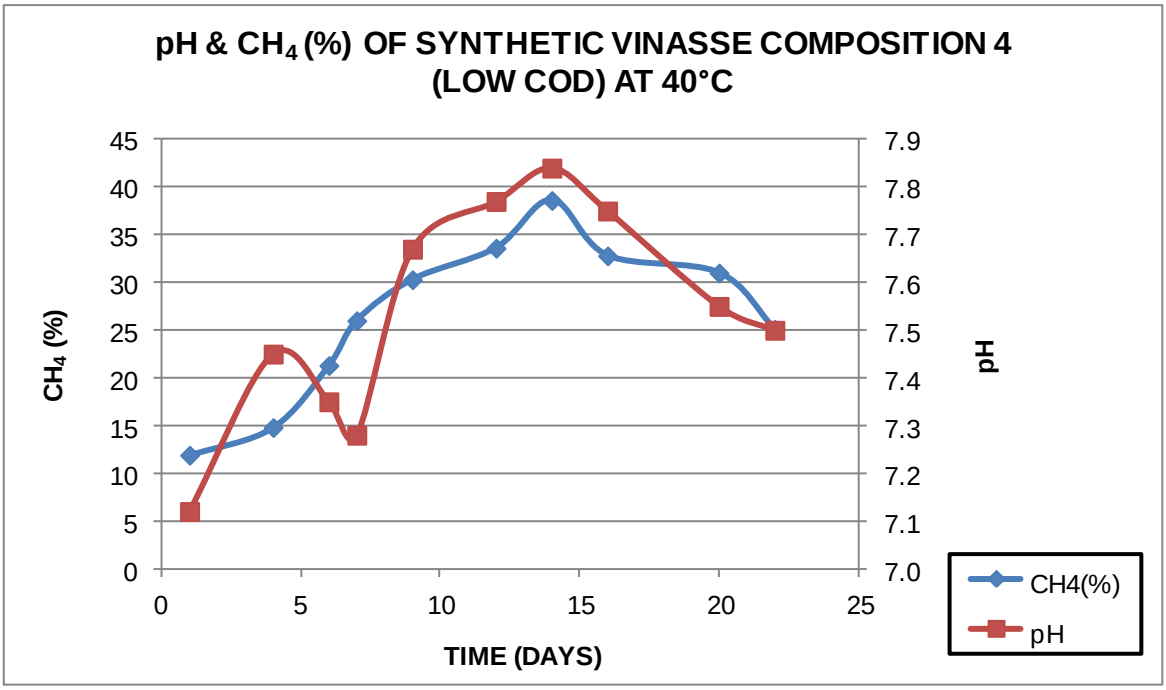


Figure 4.19 pH and CH_4 (%) of Synthetic Vinasse Composition 4 (Low COD) at 40°C

4.3.2.7.1 Detailed Listing of Genus Present

Bacteroidetes, Euryarchaeota, Chloroflexi, Verrucomicrobia, WWE1, and Firmicutes are more abundant than other microbes in this composition. These microbes are 82% in this composition, as listed below in Table 4.13.

Some other microbes which are present in lower percentages than above microbes in composition 4 at 40°C are mentioned in Appendix C. These microbes are 18% in this composition.

Table 4.13 Microbial Communities Present in Composition 4 (Low COD) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

4.3.2.7.2 Species Richness and Phylogenetic Diversity (Faith's PD) of Synthetic Vinasse Composition 4 (Low COD) at 40°C

Synthetic vinasse composition 4 at 40°C shows peak (1255) and higher phylogenetic diversity (Faith's PD) 71 on day 14, with comparable peaks on day 4 (when methanogenesis began) and day 22 (as shown in Figure 4.20). Both values are almost same throughout the run after day 14. This shows high microbial activity in this reactor.

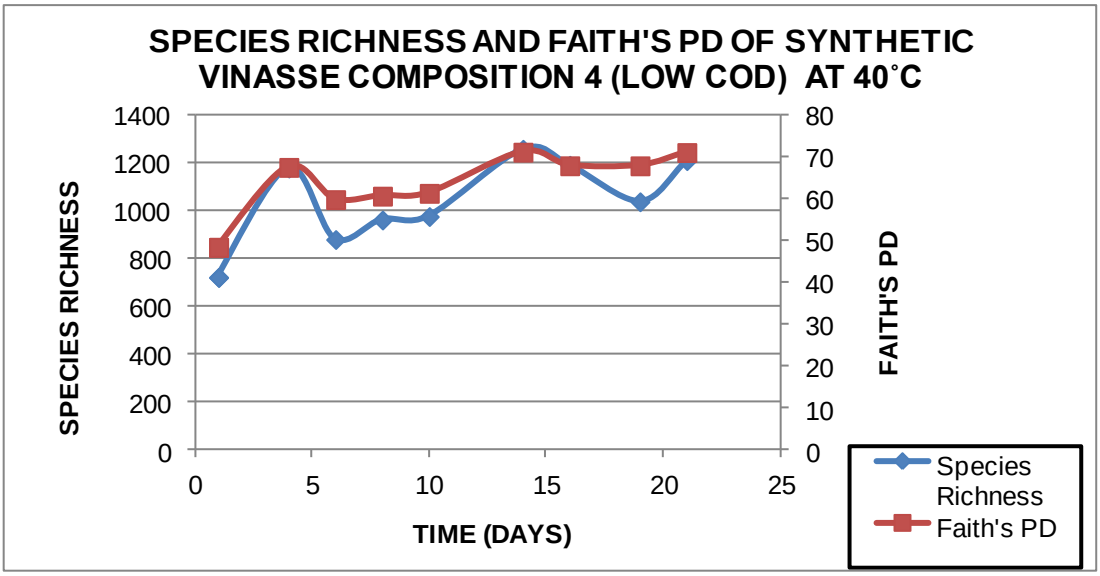


Figure 4.20 Species Richness and Phylogenetic Diversity (Faith's PD) of Synthetic Vinasse Composition 4 (Low COD) at 40°C

4.3.2.8 Microbial Data of Synthetic Vinasse Composition 5 (Medium COD) at 30°C

Similar to composition real vinasse composition 2 at 30°C, synthetic vinasse Composition 5 at 30°C shows a strikingly high percentage of Bacteroidetes (>90% on day 26) and generally moderate percentages of Firmicutes (up to about 56%) and Proteobacteria (up to about 75%) and Chloroflexi (39%). The phyla show no discernable trends with time. It also shows lower percentages of Acidobacteria, Lentisphaerae, and Archaea. Higher percentage of Acidobacteria is present on day 10 and decreases later. It shows microbes are going from acidogenesis phase to next phases of cycle (as shown in Figure 4.21). Synthetic vinasse composition 5 at 30°C also contains very small percentages of other bacteria such as WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, and Synergistetes.

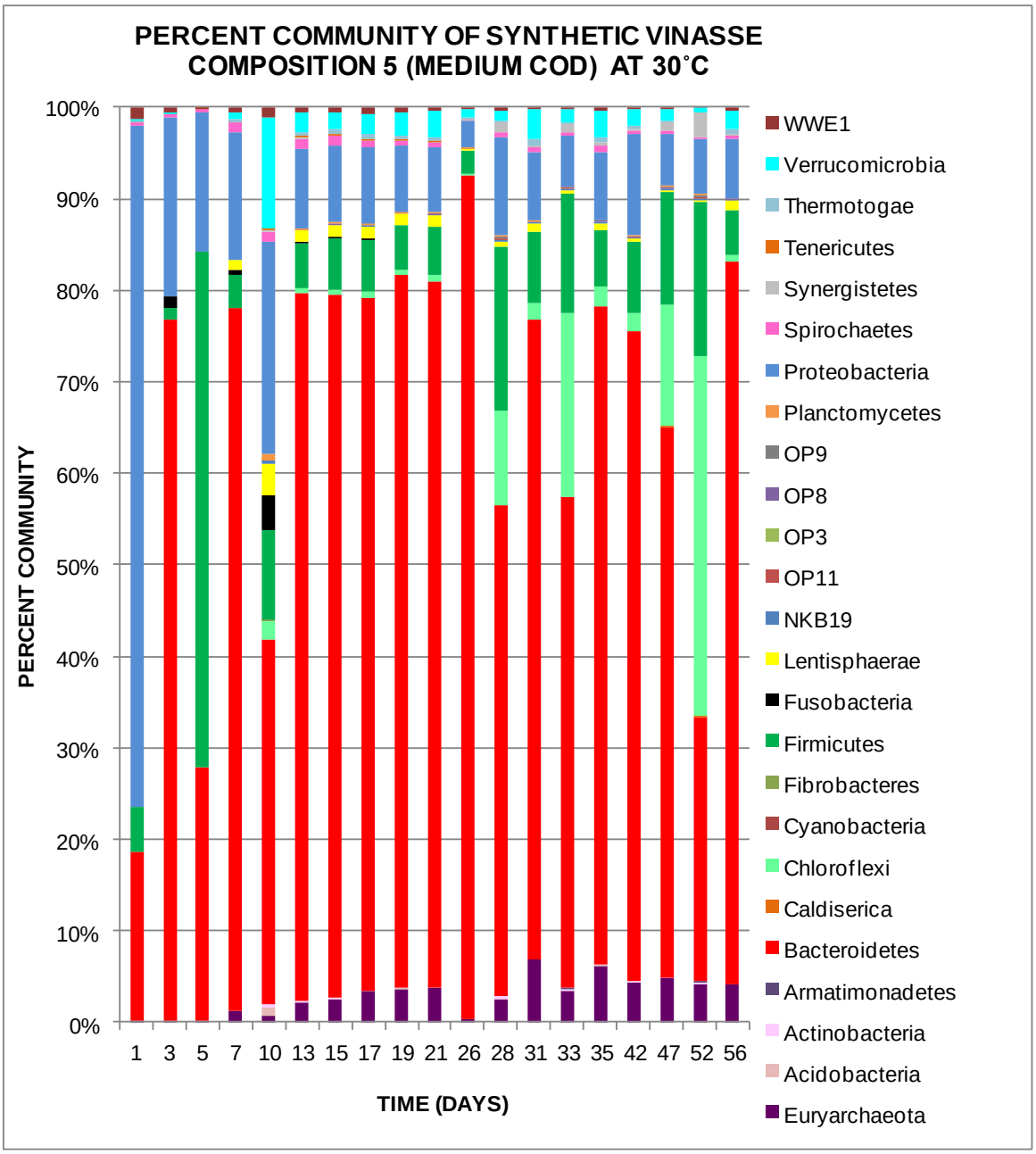


Figure 4.21 Microbial Community Profiling of Real Vinasse Composition 5 (Medium COD) at 30°C

Archaea started increasing around day 7 as the methane generation increases till the end of cycle and higher percentage of Archaea (up to about 7%) is present throughout the run after day 7. Methane percent started increasing on day 13, corresponding to an increase in the percent of Archaea (Methanogens), as shown in Figure 4.22.

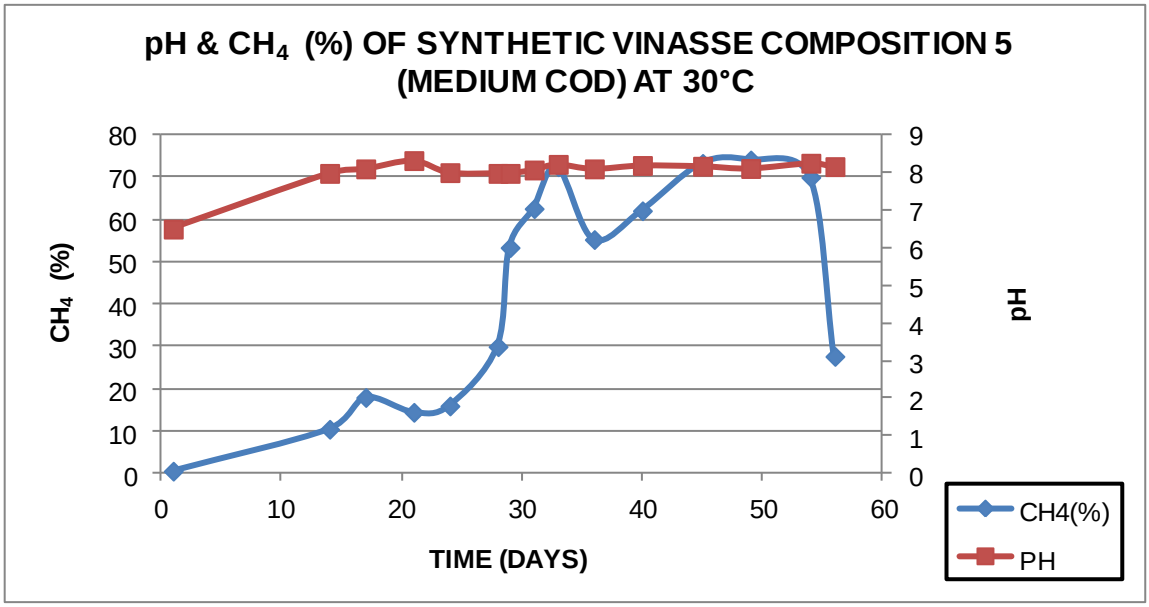


Figure 4.22 pH and CH_4 (%) of Synthetic Vinasse Composition 5 (Medium COD) at 30°C

4.3.2.8.1 Detailed Listing of Genus Present

Bacteroidetes, Euryarchaeota, Proteobacteria, Chloroflexi, Verrucomicrobia, and Firmicutes are more abundant than other microbes in this composition, as listed below in Table 4.14. These microbes are 85% in this composition.

Some other microbes which are present in lower percentages than other microbes in composition 5 at 30°C are mentioned in Appendix C. These microbes are 15% in this composition.

Table 4.14 Microbial Communities Present in Composition 5 (Medium COD) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

Table 4.14—*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	

4.3.2.9 Microbial Data of Synthetic Vinasse Composition 5 (Medium COD) at 40°C

Synthetic vinasse Composition 5 at 40°C shows generally high percent of Proteobacteria (up to 83%), moderate percentage of Bacteroidetes (up to 54%) and Firmicutes (up to about 52%) and Chloroflexi (29%), and low percentage of Fusobacteria (3%). The percent of Proteobacteria in particular is higher than other runs. It also shows lower percentage of Lentisphaerae, Verrucomicrobia and Archaea, Spirochaetes, Synergistetes, Lentisphaerae, WWE1, Tenericutes, and Plantomycetes (as shown in Figure 4.23).

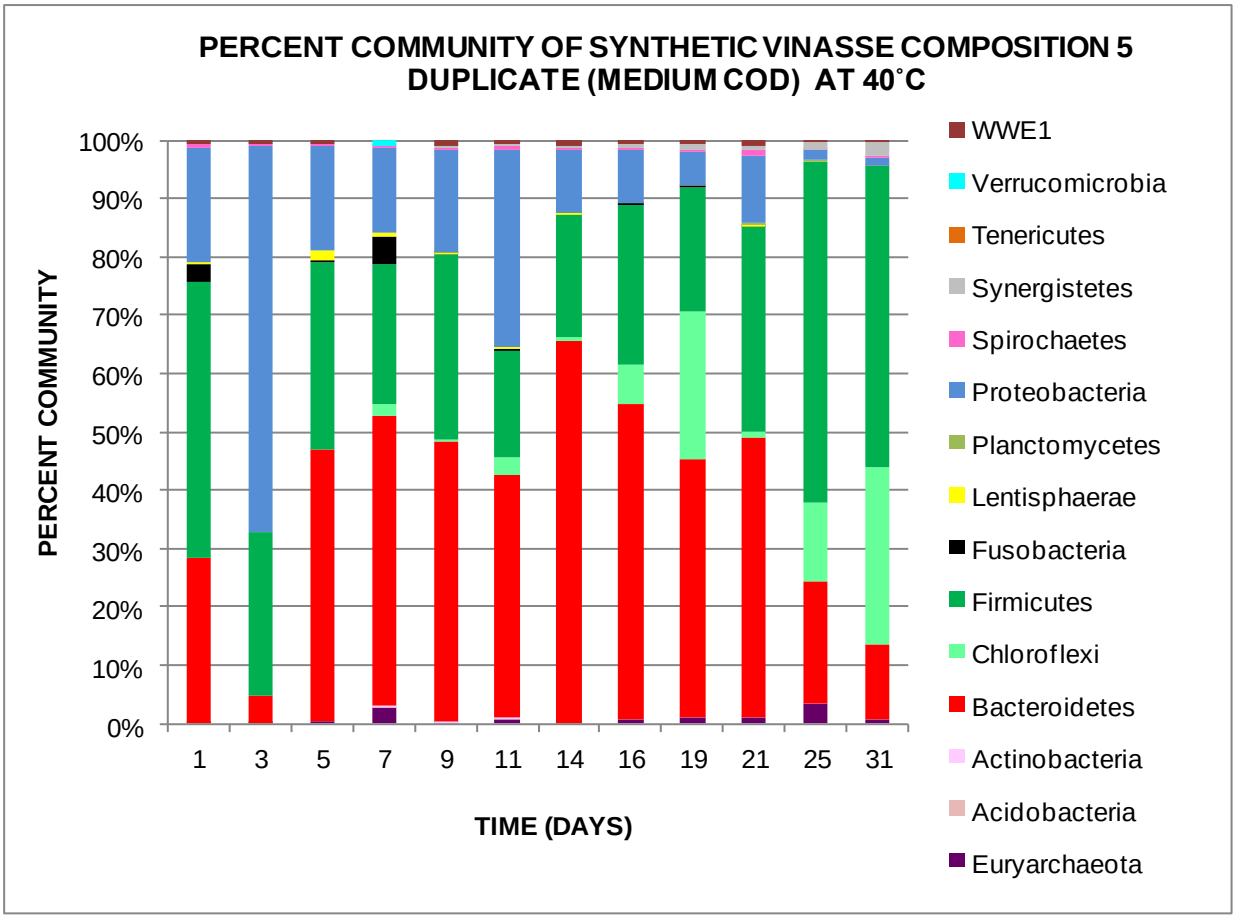


Figure 4.23 Microbial Community Profiling of Synthetic Composition 5 (Medium COD) at 40°C

This composition does not show Acidobacteria as a separate phylum. It might be possible due to fastest reaction at 40°C and acidogenesis stage will be shorter at higher temperature. Microbial analysis was done every 2 or 3 day of the run. Hence, Acidobacteria may have been missed during that period. However, other acidogens were present in this composition.

Methanogens are present from day 9 till the end of reaction, but at low percents compared to other synthetic compositions, with the exception of the 7% peak on day 25. This composition produced high methane throughout the run, despite the fact that the methanogens were not high in percent (as shown in Figure 4.23 and Figure 4.24). The reason is not clear. If the bacteria were high in numbers for this run, the methanogens may have been present in high numbers despite their low percent.

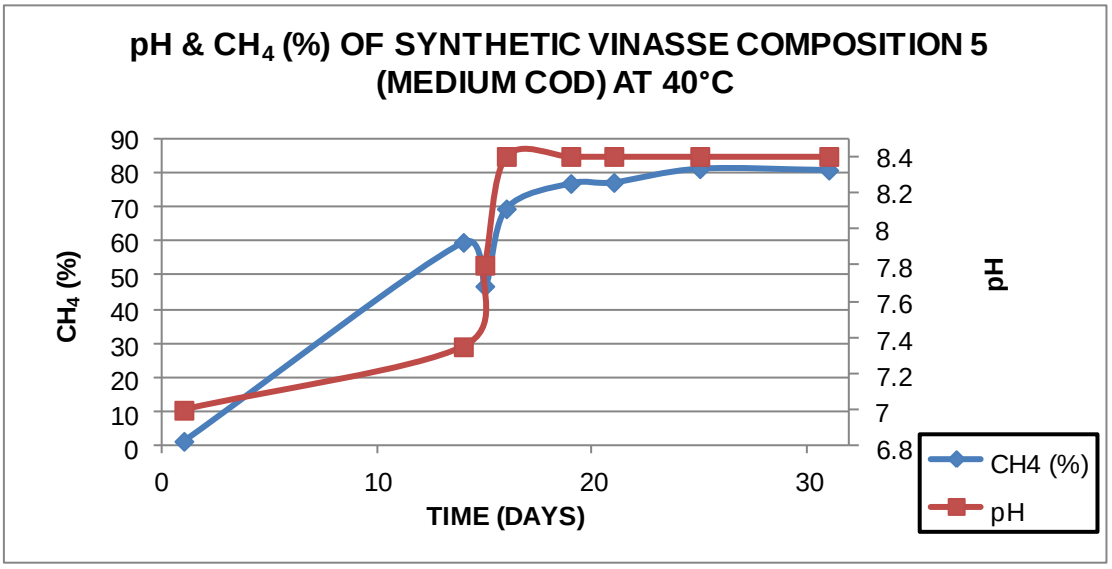


Figure 4.24 pH and CH_4 (%) of Synthetic Vinasse Composition 5 (Medium COD) at 40°C

4.3.2.9.1 Detailed Listing of Genus Present

Bacteroidetes, Chloroflexi, Euryarchaeota, Proteobacteria and Firmicutes are more abundant than other species in this composition, as listed below in Table 4.15. These microbes are 89% in this composition.

Some other microbes which are present in lower percentages than above microbes in composition 5 at 40°C are mentioned in Appendix C. These microbes are 11% in this composition.

Table 4.15 Microbial Communities Present in Composition 5 (Medium COD) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

4.3.2.9.2 Species Richness and Phylogenetic Diversity of Composition 5 (Medium COD) at 30 and 40°C

Composition 5 at 30°C shows a peak in species richness (1768) and phylogenetic diversity (Faith's PD) 107 on day 10 after methanogenesis starts. (as shown in Figure 4.25 and Figure 4.26). Composition 5 at 30°C shows highest species richness and higher phylogenetic diversity among all the six compositions. The reason is unclear. .

Composition 5 at 40°C shows peak species richness (1042) and higher phylogenetic diversity (Faith's PD) 54 on day 11, with several secondary peaks throughout the run compared to Composition 5 at 30°C (as shown in Figure 4.25 and Figure 4.26). These values are comparable to the other compositions. There was little drop in species richness and Faith's PD at the end of run, perhaps because a high amount of food was available for microbes for a longer period of the run

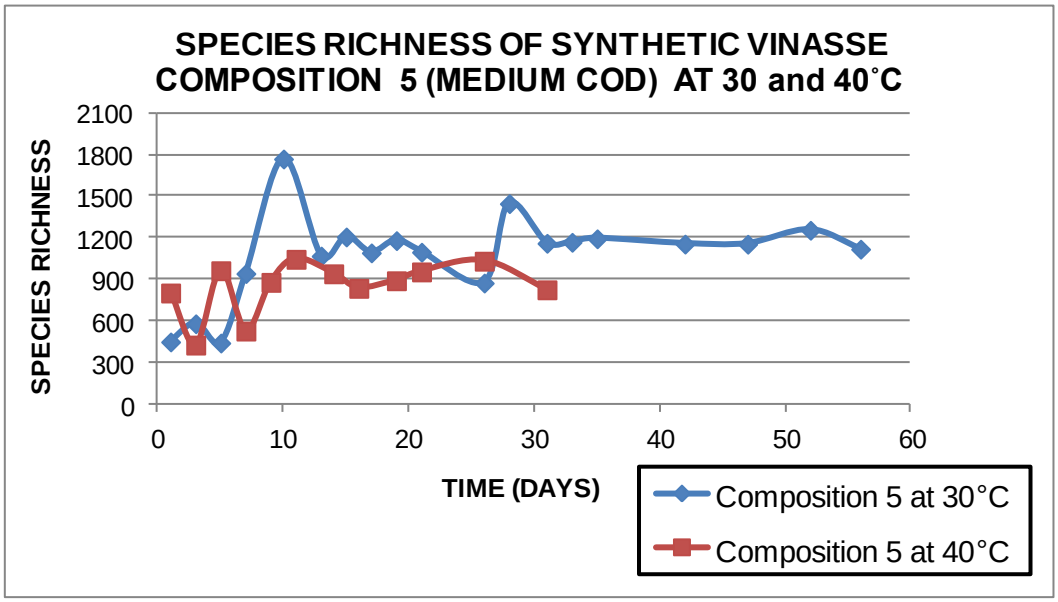


Figure 4.25Species Richness of Synthetic Vinasse Composition 5 (Medium COD) at 30 and 40°C

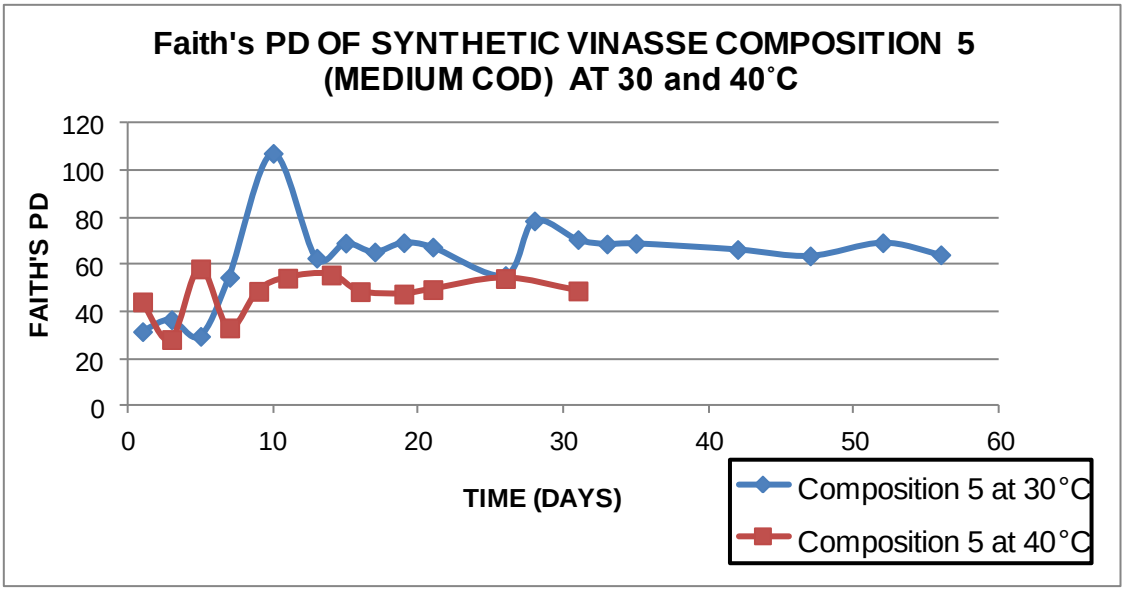


Figure 4.26 Phylogenetic Diversity (Faith's PD) of Synthetic Vinasse Composition 5 (Medium COD) at 30 and 40°C

4.3.2.10 Microbial Data of Synthetic Vinasse Composition 6 (High COD) at 30°C

Synthetic vinasse Composition 6 at 30°C shows generally high percentage of Bacteroidetes (over 75% on day 3), moderate percents of Firmicutes (up to 37%) and Proteobacteria (up to 66%), and low percentages of Lentisphaerae,Verrucomicrobia and Archaea. The phyla show no discernible trends with time. It also shows lower percentages of Acidobacteria, Tenericutes, Spirochaetes, Synergistetes, Chloroflexi**,** Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes and Plantomycetes. Substantial percentage of Acidobacteria is present on day 26. Hydrolytic, acidogenic, and acetogenic bacteria were present in substantial percents throughout reactor operation (as shown in Figure 4.27).

Archaea started increasing on day 13 of the cycle, peaking on day 40 at about 7% (a percent similar to the peak for synthetic vinasse composition 5). Methane percent was high between day 29 to 87. (as shown in Figure 4.28).

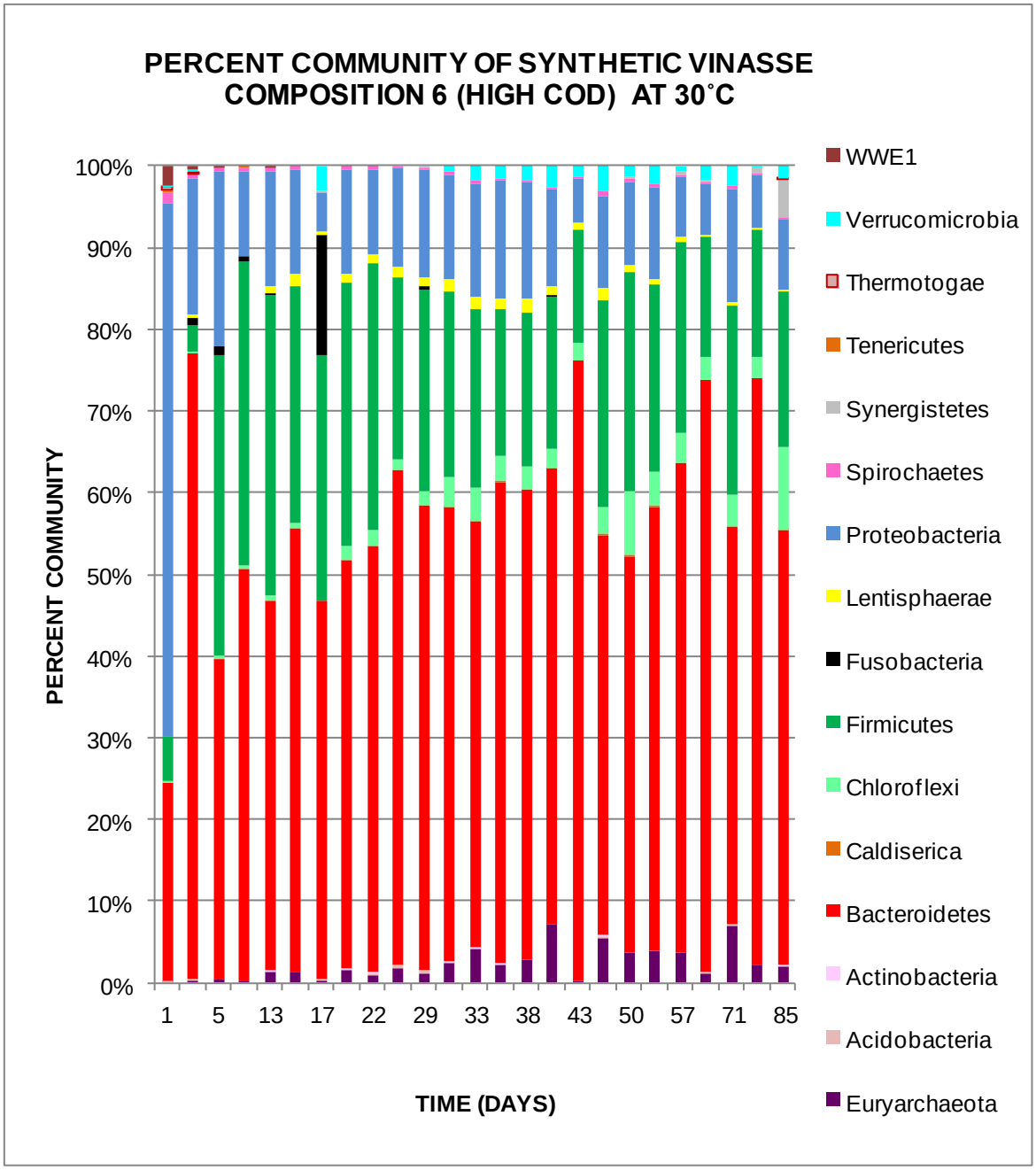


Figure 4.27 Microbial Community Profiling of Synthetic Composition 6 (High COD) at 30°C

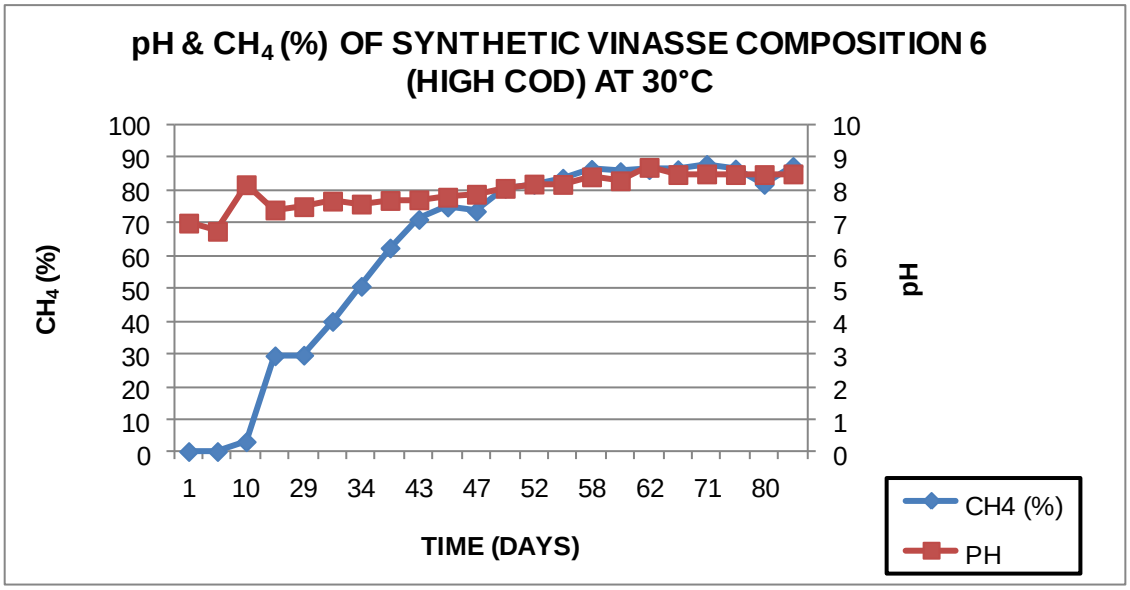


Figure 4.28 pH and CH_4 (%) of Synthetic Vinasse Composition 6 (High COD) at 30°C

4.3.2.10.1 Detailed Listing of Genus Present

Some species of Bacteroidetes, Chloroflexi and Firmicutes are more abundant than other species in this composition. These microbes are 94% in this composition. A list of these microbes is given below in Table 4.16.

Some other microbes which are present in lower percentage than above microbes in composition 6 at 30°C are given in Appendix C. These microbes are 6% in this composition.

Table 4.16 Microbial Communities Present in Composition 6 (High COD) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium

Table 4.16—*Continued*

Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		

4.3.2.10.2 Species Richness and Phylogenetic Diversity of Synthetic Vinasse Composition 6 (High COD) at 30°C

Synthetic vinasse composition 6 at 30°C shows peak species richness (1092) and higher phylogenetic diversity (Faith's PD) 59 on day 50, which is comparable to the other compositions (as shown in Figure 4.29). This run showed generally high species richness and Faith's PD throughout the run, perhaps due to higher COD of this composition.

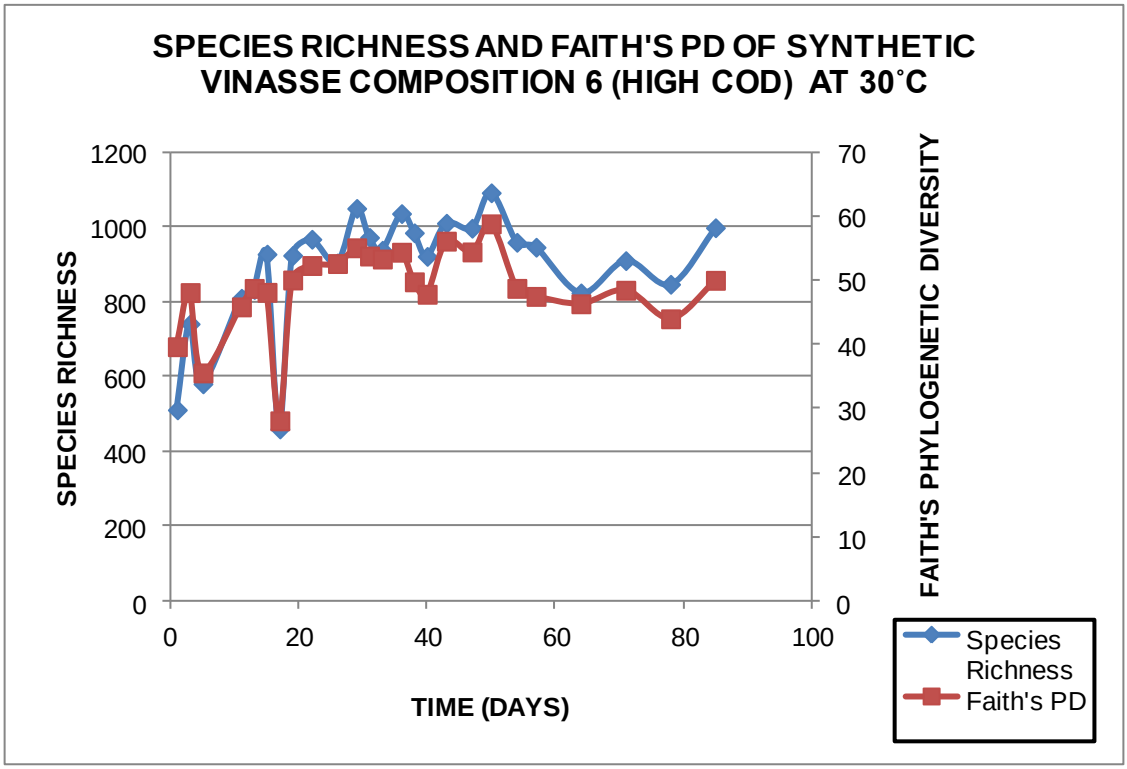


Figure 4.29 Species Richness and Phylogenetic Diversity of Real Vinasse Composition 6 (High COD) at 30°C

4.3.2.10.3 Comparison of Species Richness and Phylogenetic Diversity of Real and Synthetic vinasse at 30, 35, and 40°C

Since the COD of real vinasse is higher than synthetic vinasse Species Richness and Phylogenetic Diversity should be higher than synthetic. However, Species Richness and Phylogenetic Diversity for synthetic vinasse 6 at 30°C is much higher than other real and synthetic vinasse compositions (as shown in Figure 4.30 and Figure 4.32). It might possible that microbes are not able to digest real vinasse compositions. Real vinasse composition 1 at 35°C (corn & milo) shows higher Species

Richness and Phylogenetic Diversity compared to 3 at 35°C (as shown in Figure 4.32 and Figure 4.33). However, synthetic vinasse composition 5 (Medium COD) shows higher Species Richness and Phylogenetic Diversity at 40°C compared to real vinasse compositions 1 and 3 (corn & milo) and synthetic vinasse (low COD compared to real vinasse) compositions 4 and 5 (as shown in Figure 4.34 and Figure 4.35).

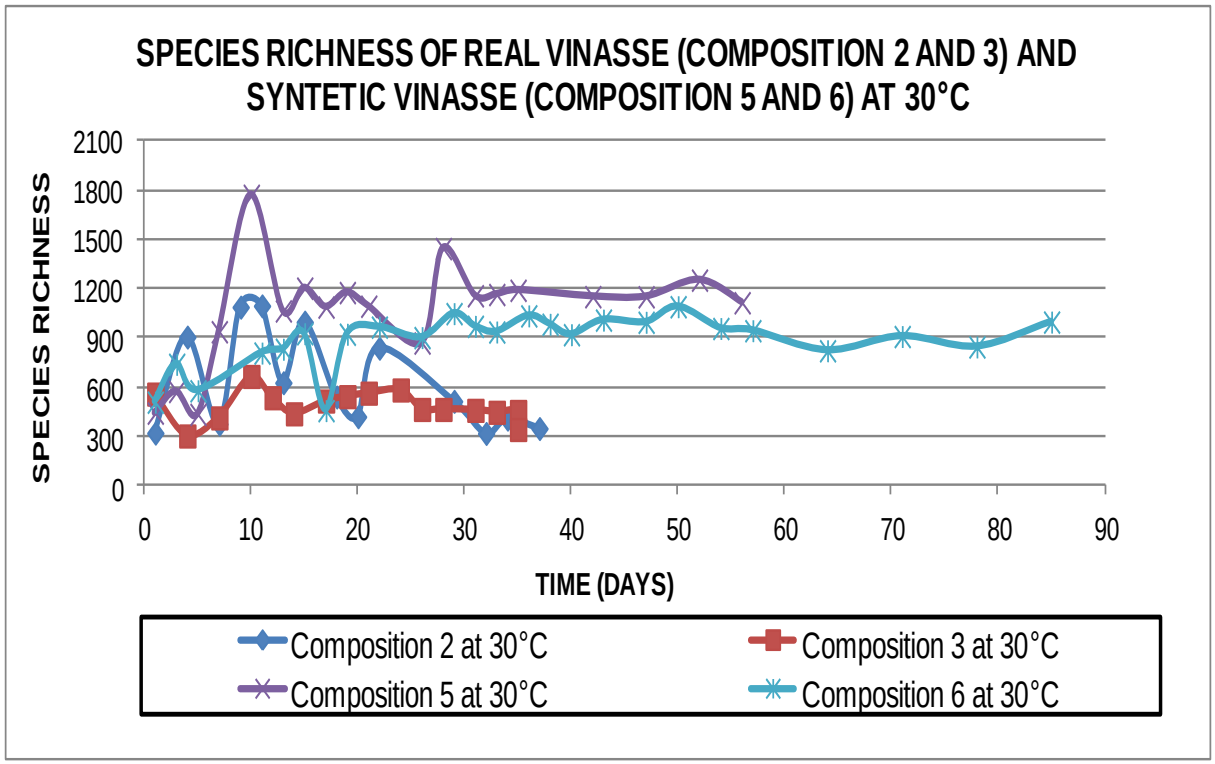


Figure 4.30 Species Richness of Real (Composition 2 & 3) and Synthetic Vinasse (Composition 5 & 6) at 30°C

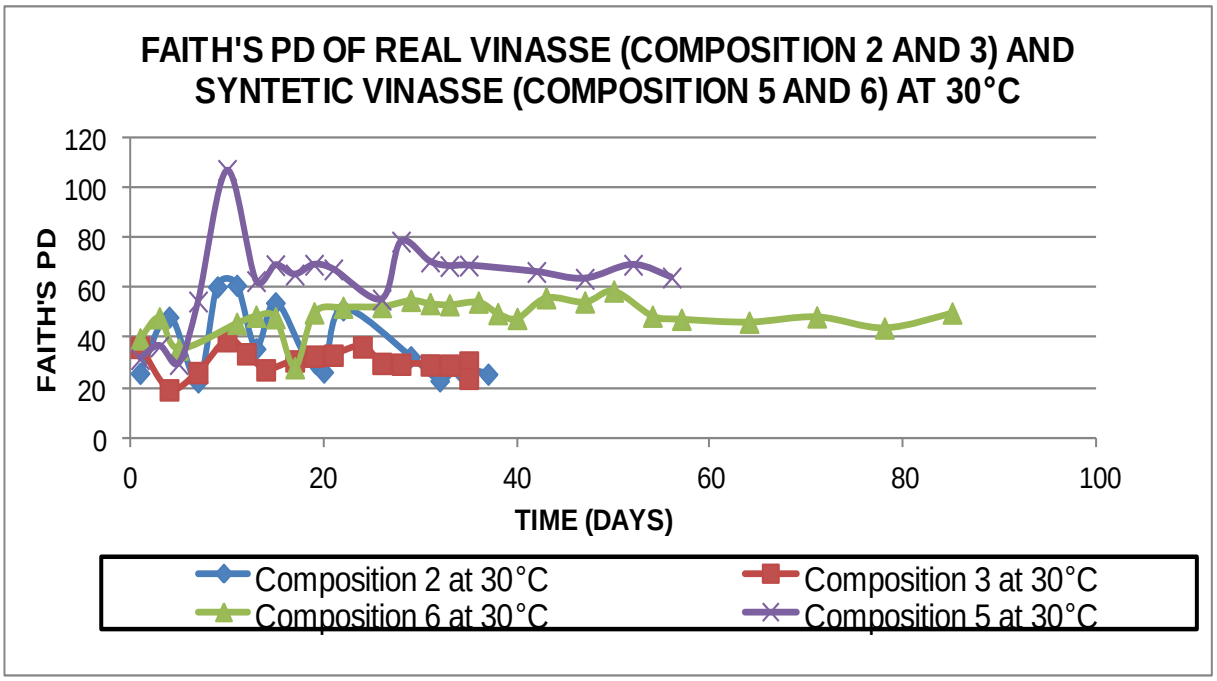


Figure 4.31 Phylogenetic Diversity (Faith's PD) of Real (Composition 2 & 3) and Synthetic Vinasse (Composition 5 & 6) at 30°C

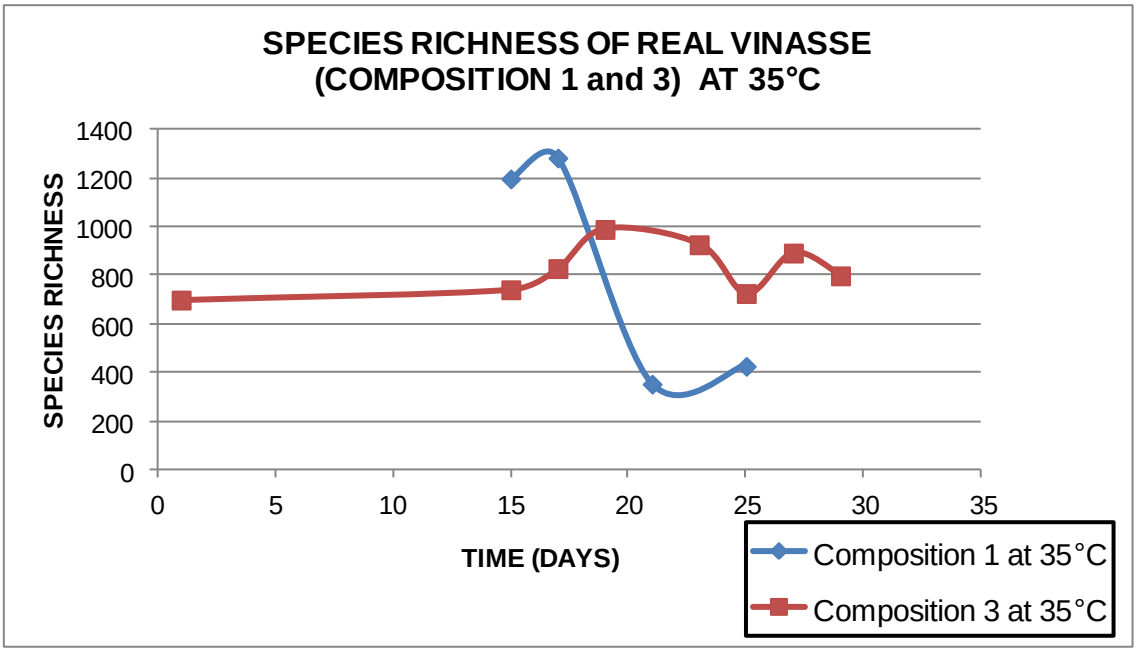


Figure 4.32 Species Richness of Real Vinasse Compositions 1 and 3 at 35°C

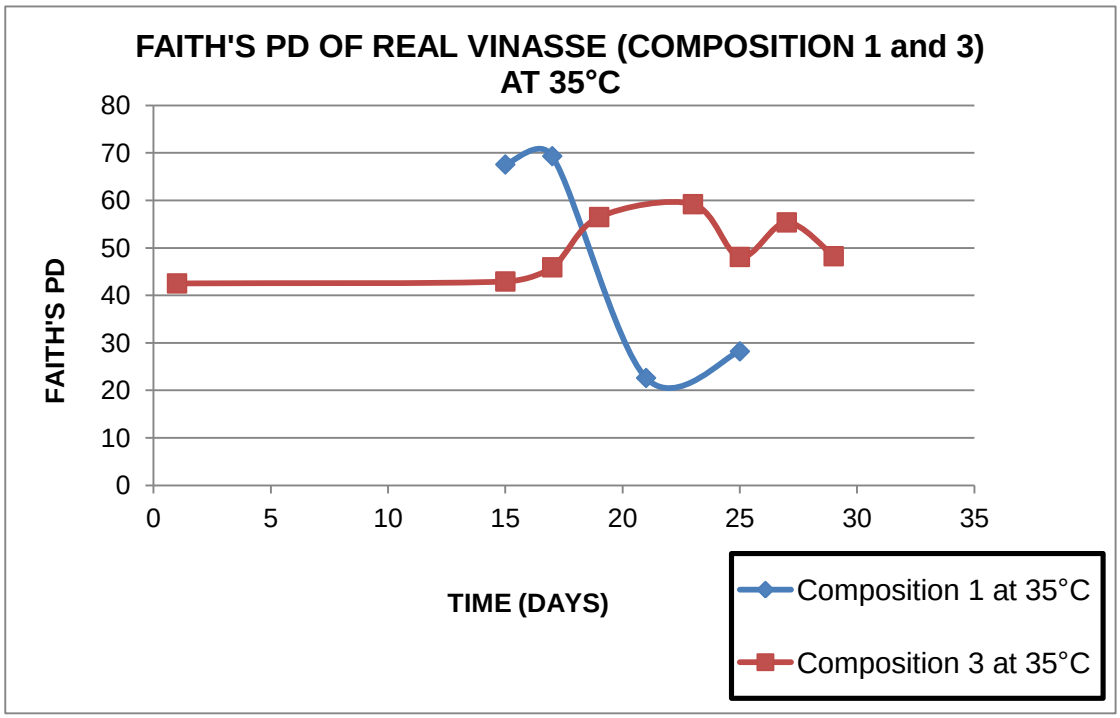


Figure 4.33 Phylogenetic Diversity (Faith's PD) of Real Vinasse Compositions 1 and 3 at 35°C

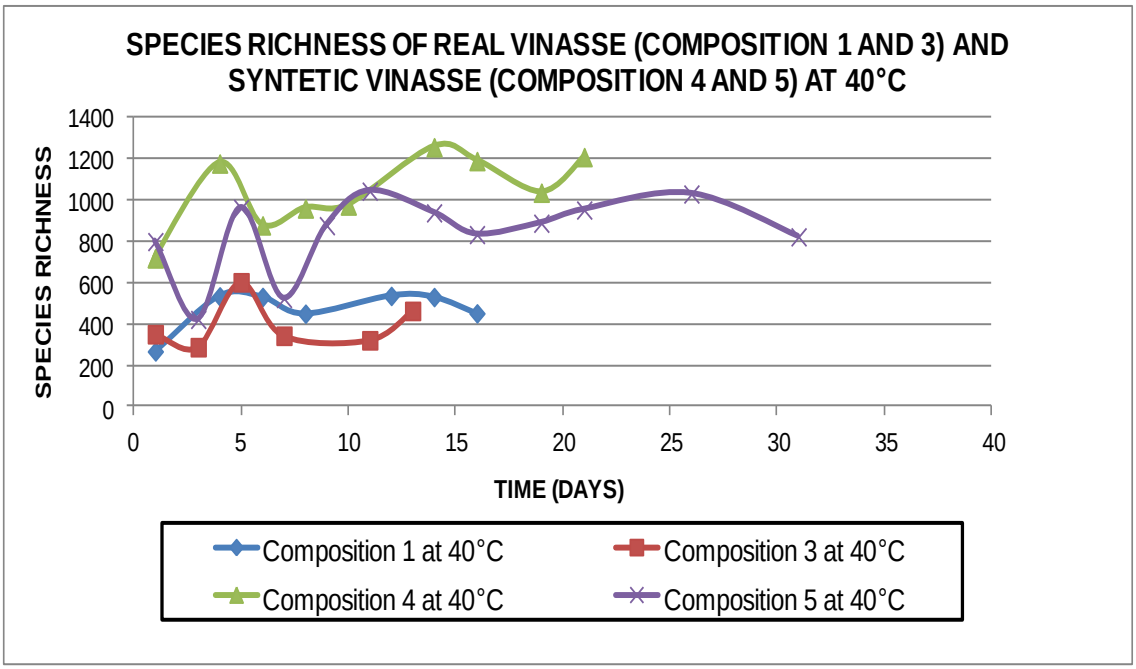


Figure 4.34 Species Richness of Real Vinasse (Composition 1 and 3) and Synthetic Vinasse (Composition 4 and 5) at 40°C

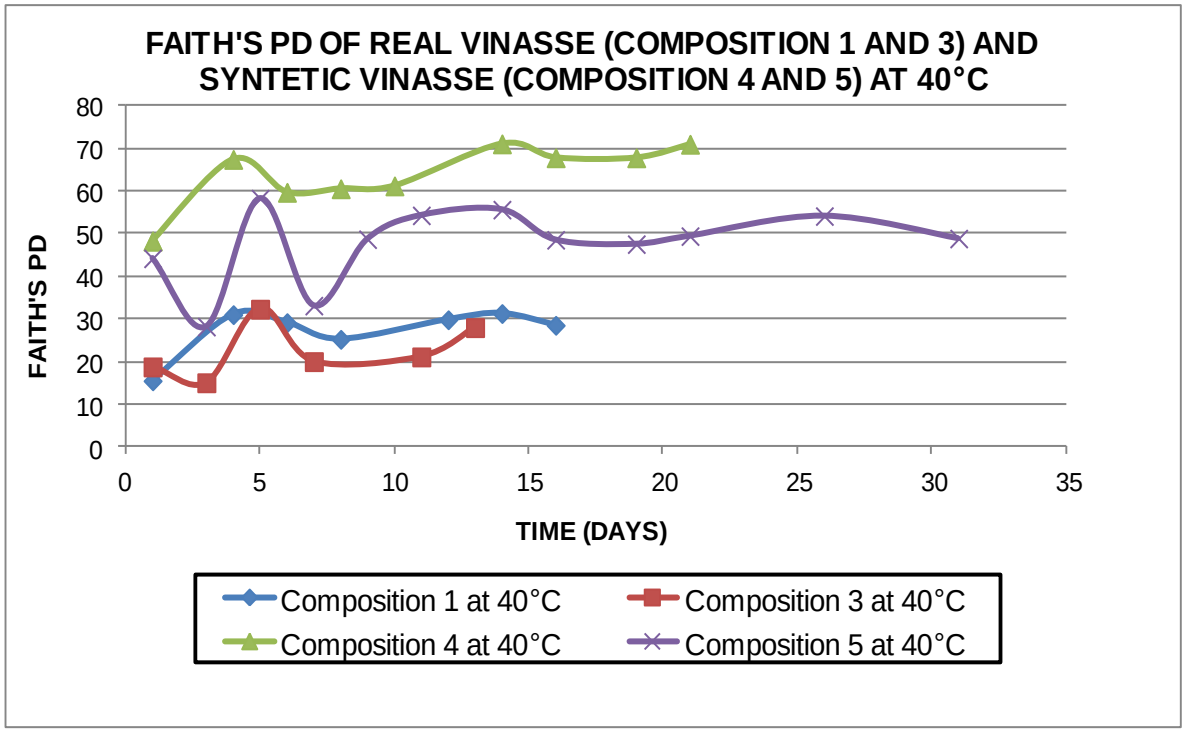


Figure 4.35 Phylogenetic Diversity of Real Vinasse (Composition 1 and 3) and Synthetic Vinasse (Composition 4 and 5) at 40°C

4.3.2.11 Microbial Data for Real Vinasse Composition 2 (Produced from Corn and Milo, White Energy, TX) at 30°C and its Duplicate

Real vinasse composition 2 at 30°C and its duplicate (as shown in Figure 4.31 and Figure 4.32) show high percentages of Bacteroidetes (peaking at over 80% for both), and moderate percentages of Firmicutes, Proteobacteria, and Fusobacteria, and lower percentages of Chloroflexi, Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, Synergistetes, Archaea, Acidobacteria, and Actinobacteria at 30°C. However, the percent of microbial community differs each day. This is because the total run time was different for both duplicates. Composition 2 at 30°C had lesser percentage of Bacteroidetes and higher percentage of Firmicutes and Fusobacteria in the beginning, but Bacteroidetes started increasing till day 13 and then decreasing till day 22 and started rising again till the end of reaction. Composition 2 at 30°C (duplicate) had higher percentage of Bacteroidetes and less percentage of Firmicutes and Fusobacteria in the beginning, as shown in Figure 4.36 and Figure 4.37. Bacteroidetes started increasing till day 9 and then decreased till the end of the run. Composition 2 at 30°C (duplicate)

had higher percentage of Fusobacteria and Archaea in comparison to Composition 2 at 30°C. This was likely because the real vinasse composition and sludge were not homogenous and microbial growth depends on these factors. Archaea started increasing on day 9 in Composition 2 at 30°C and day 11 in Composition 2 at 30°C (duplicate) with corresponding increase methane percentage after day 10, as shown in Figure 4.38 and Figure 4.39. Archaea peaked at 20% for the duplicate, compared with around 3% for the original. Composition 2 at 30°C (Duplicate) produced higher percent methane (as shown in Figure 4.39) in less time, perhaps due to high species richness and higher phylogenetic diversity.

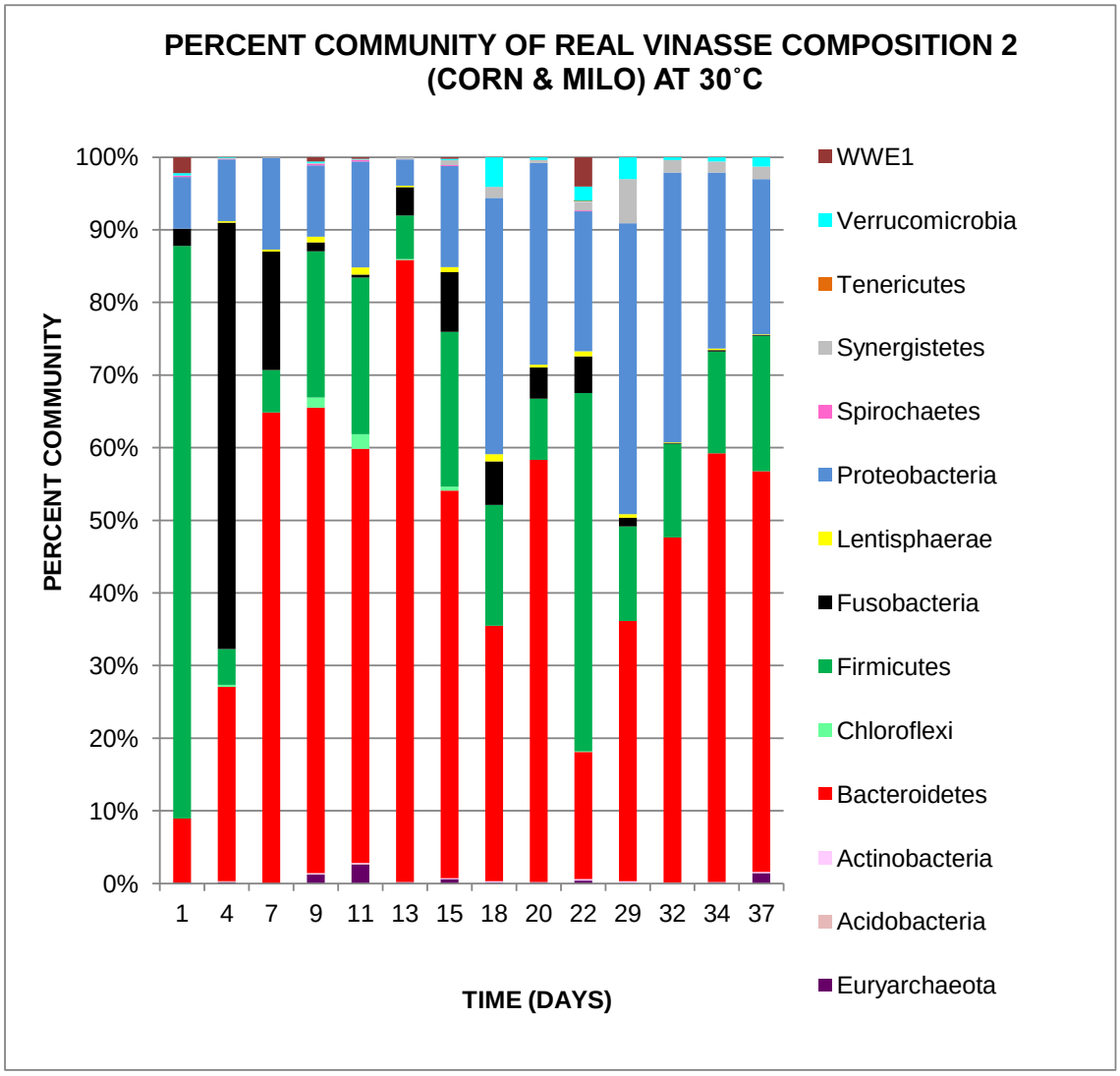


Figure 4.36 Microbial Community Profiling of Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) at 30°C

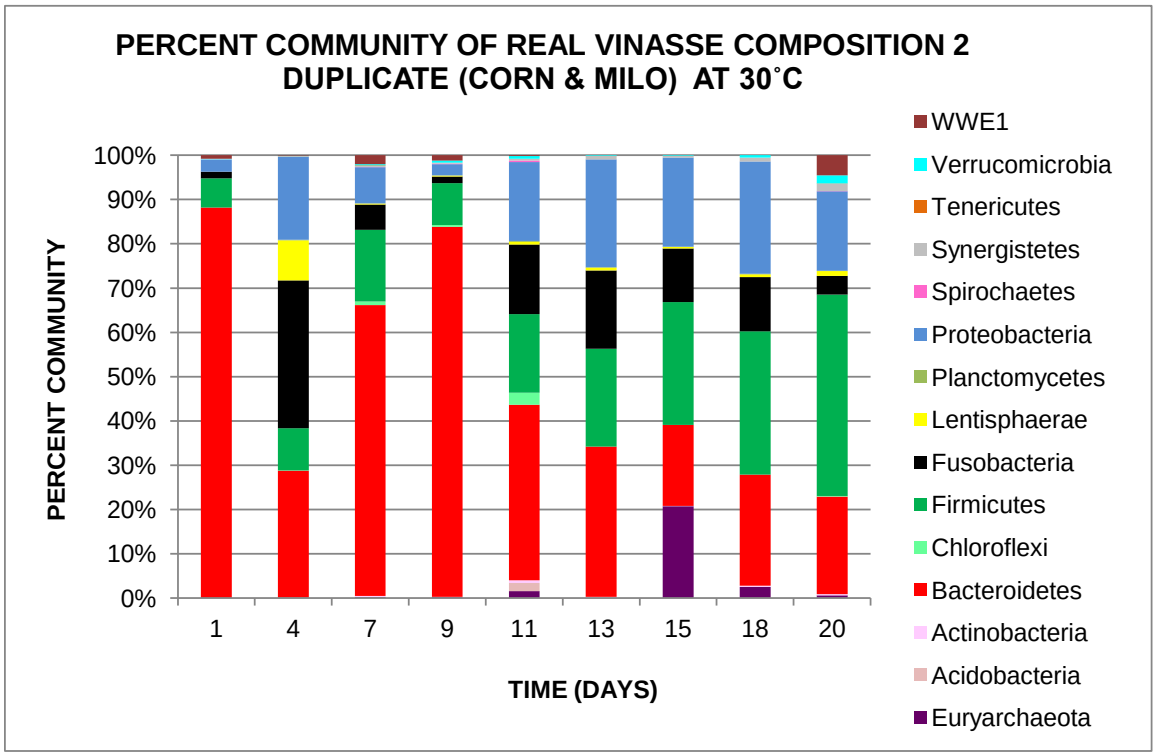


Figure 4.37 Microbial Community Profiling of Real Vinasse Composition 2 Duplicate (Corn and Milo, White Energy, TX) at 30°C

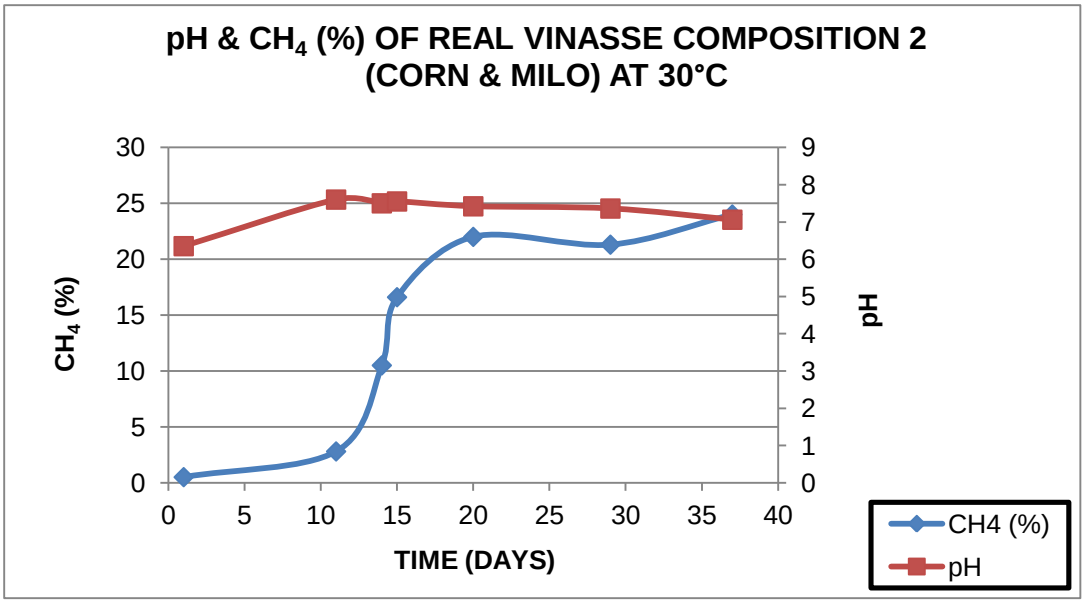


Figure 4.38 pH and CH_4 (%) of Real vinasse Composition 2 (Corn and Milo, White Energy, TX) at 30°C

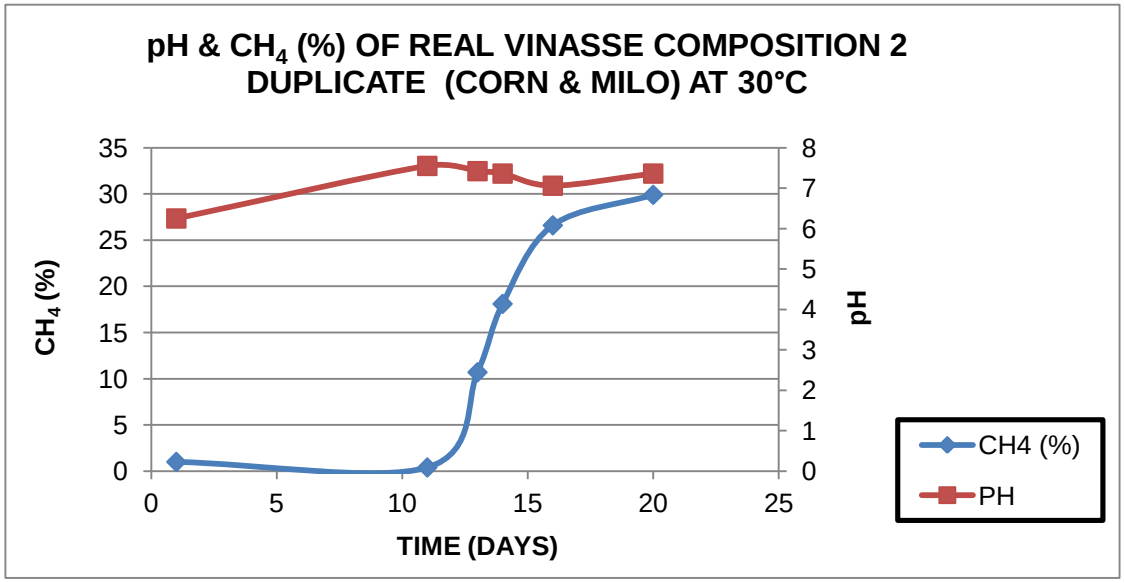


Figure 4.39 pH and CH_4 (%) of Real vinasse Composition 2 Duplicate (Corn and Milo, White Energy, TX) at 30°C

4.3.2.11.1 Species Richness and Phylogenetic Diversity Real Vinasse of Composition 2 (Produced from Corn and Milo, White Energy, TX) and its Duplicate

Real vinasse composition 2 at 30°C shows highest species richness (1097) and phylogenetic diversity (Faith's PD) 61 on day 11, with several secondary peaks. Composition 2 at 30°C (duplicate) shows the highest species richness (1252) and phylogenetic diversity (Faith's PD) 80 on day 11, with 2 secondary peaks. For both composition 2 and its duplicate, species richness oscillates between around 400 and 1200 (as shown in Figure 4.40 and Figure 4.41). The percent differences between highest species richness and Faith's PD of Composition 2 at 30°C and Composition 2 at 30°C (Duplicate) are 13% and 26%, respectively.

The total run times for real vinasse composition 2 at 30°C and Composition 2 at 30°C (duplicate) were 37 and 20 days, respectively. Both composition 2 at 30°C and its duplicate contain some Archaea and bacteria in higher percentages than other microbes, as given below in Table 4.17. These microbes are 79% in this composition.

Some other microbes which are present in lower percentage than other microbes in composition 2 at 30°C (Duplicate) are given in Appendix C. These microbes are 21% in this composition.

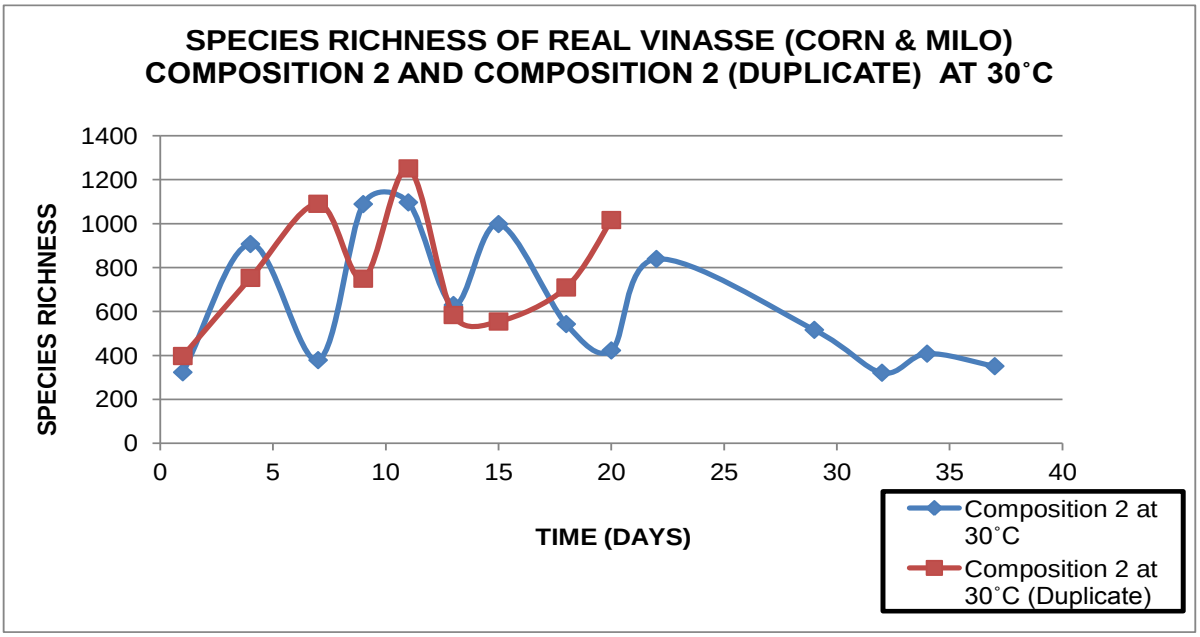


Figure 4.40 Species Richness of Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) and its Duplicate at 30°C

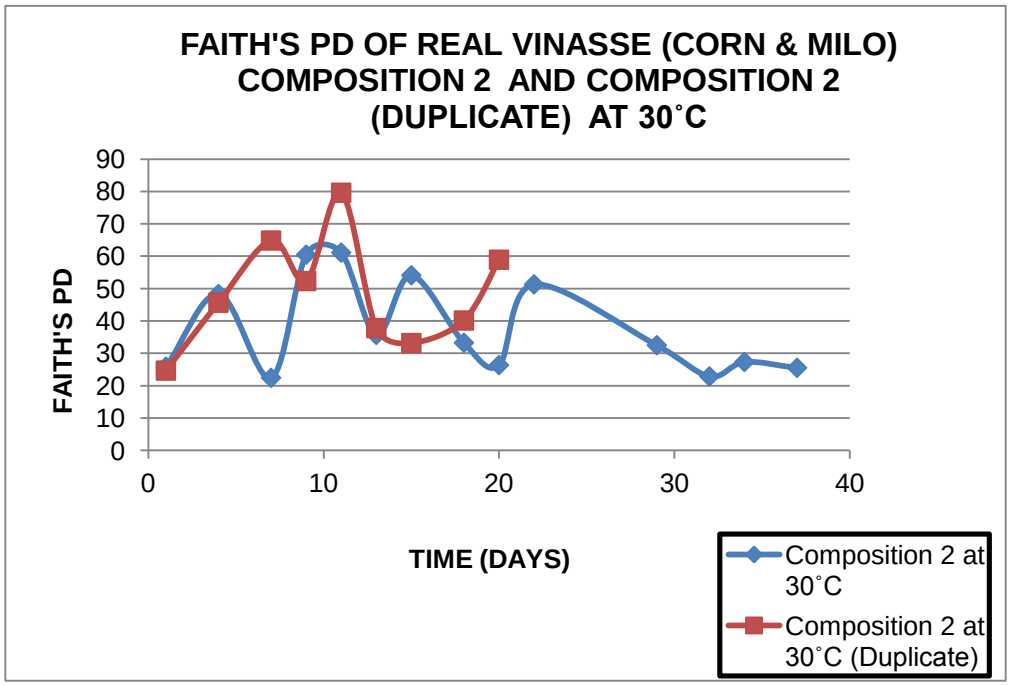


Figure 4.41 Phylogenic Diversity (Faith's PD) of Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) and its Duplicate at 30°C

Table 4.17 Microbial Communities Present in Composition 2 (Corn and Milo, White Energy, TX) and it's Duplicate at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio

4.3.2.12 Microbial Data of Synthetic Vinasse Composition 5 at 40°C (Medium COD) and its Duplicate

Composition 5 had a slightly higher percentage of Bacteroidetes and Firmicutes than it's duplicate, whereas Composition 5 at 40°C (duplicate) has little higher percent of Proteobacteria. Acidobacteria was present only in composition 5 at 40°C (Duplicate). Both show Bacteroidetes generally increasing to the mid-point of the run, and then decreasing, Proteobacteria decreasing toward the end of the run, and Firmicutes increasing. Both show a low percentage of Chloroflexi. The microbial communities were comparable because the same nutrients were added synthetically in these compositions (as shown in Figure 4.42 and Figure 4.43).

Composition 5 at 40°C does not show Acidobacteria. It might be possible due the fast reaction at 40°C and acidogenesis stage being shorter at higher temperature. Microbial analysis was done every 2 or 3 days of the run. Hence, Acidobacteria may have been missed during that period. However, it was present at composition 5 at 40°C (duplicate).

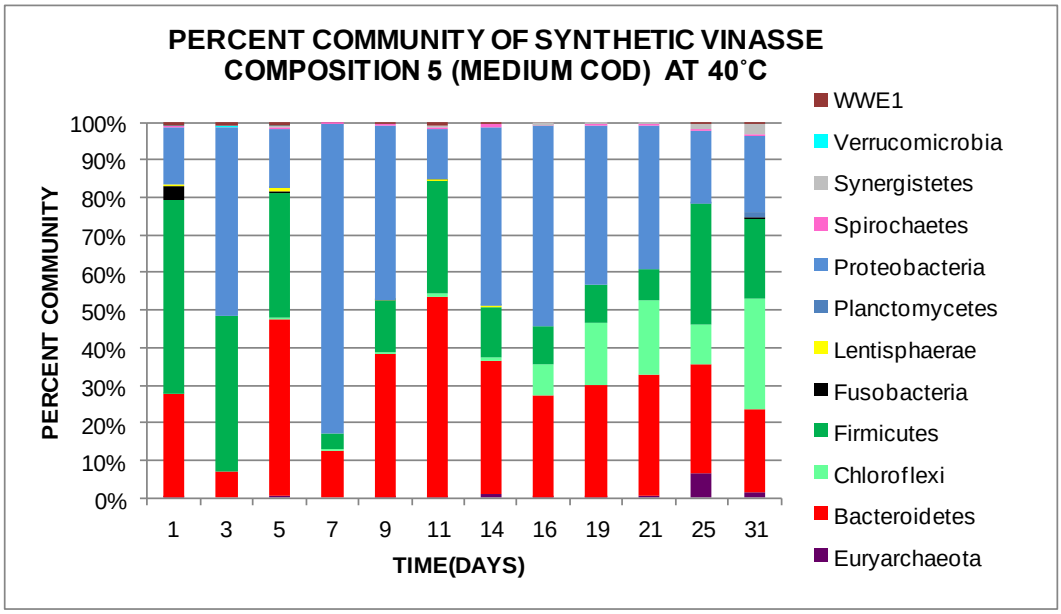


Figure 4.42 Microbial Community Profiling of Synthetic Composition 5 (Medium COD) at 40°C

Both compositions produced maximum percentage of Archaea on day 25, although the percent was about 6% for the original and 3% for the duplicate. Archaea appeared on day 5 in Composition 5 at 40°C and day 7 in composition 5 at 40°C (duplicate), with an increase in methane generation. Methane

percent started increasing on day 14 till the end of reaction (as shown in Figure 4.44 and Figure 4.45). Both reactors produced the same percent methane. The total run time was the same for both reactors.

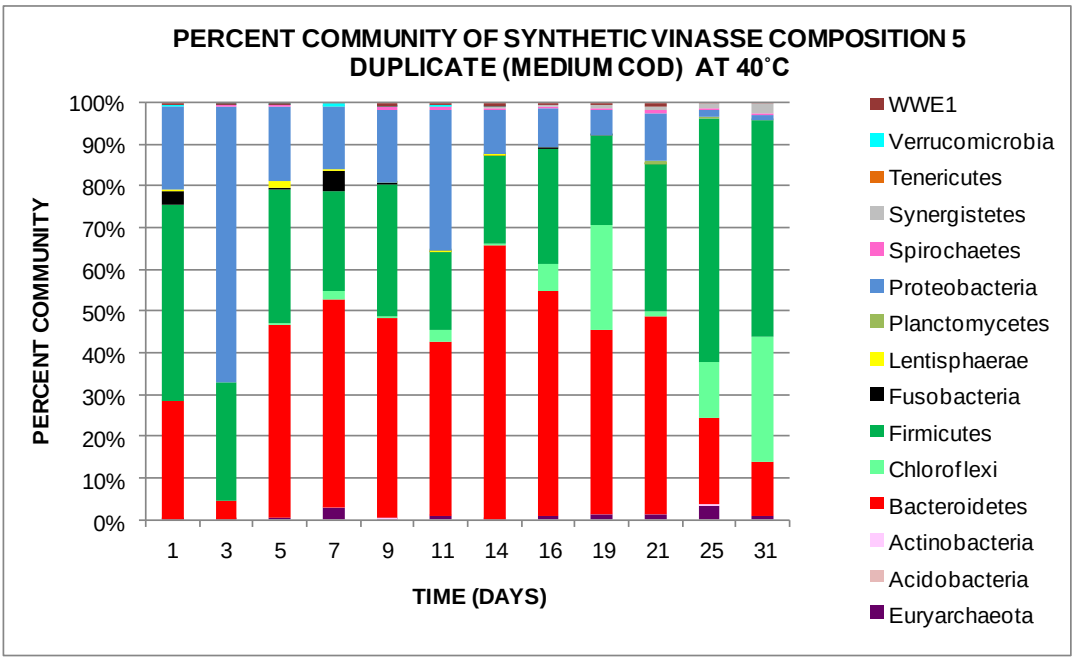


Figure 4.43 Microbial Community Profiling of Synthetic Composition 5 (Medium COD) Duplicate at 40°C

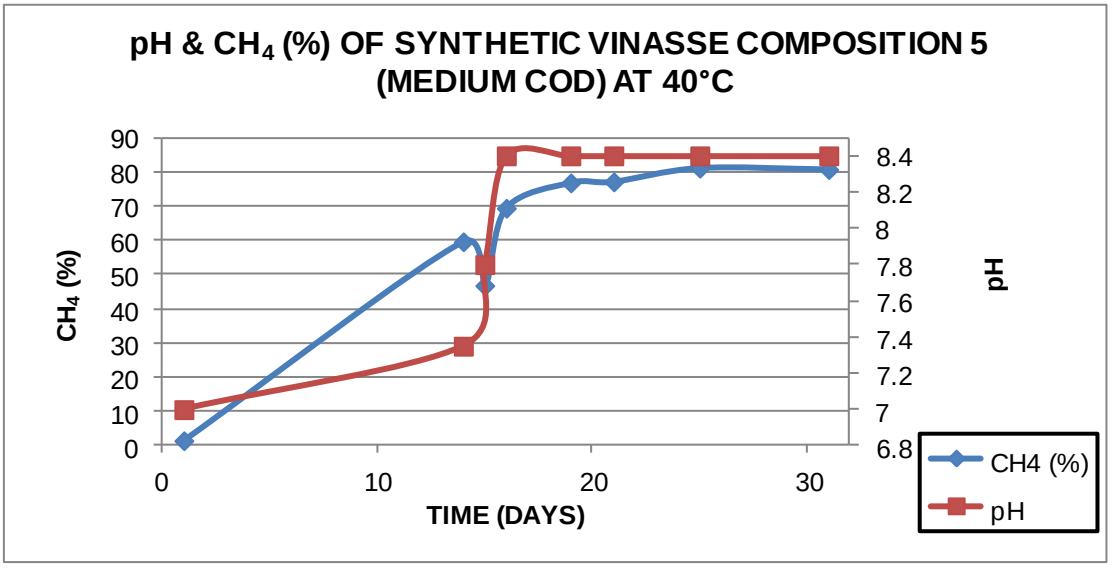


Figure 4.44 pH and CH_4 (%) of Synthetic Vinasse Composition 5 (Medium COD) at 40°C

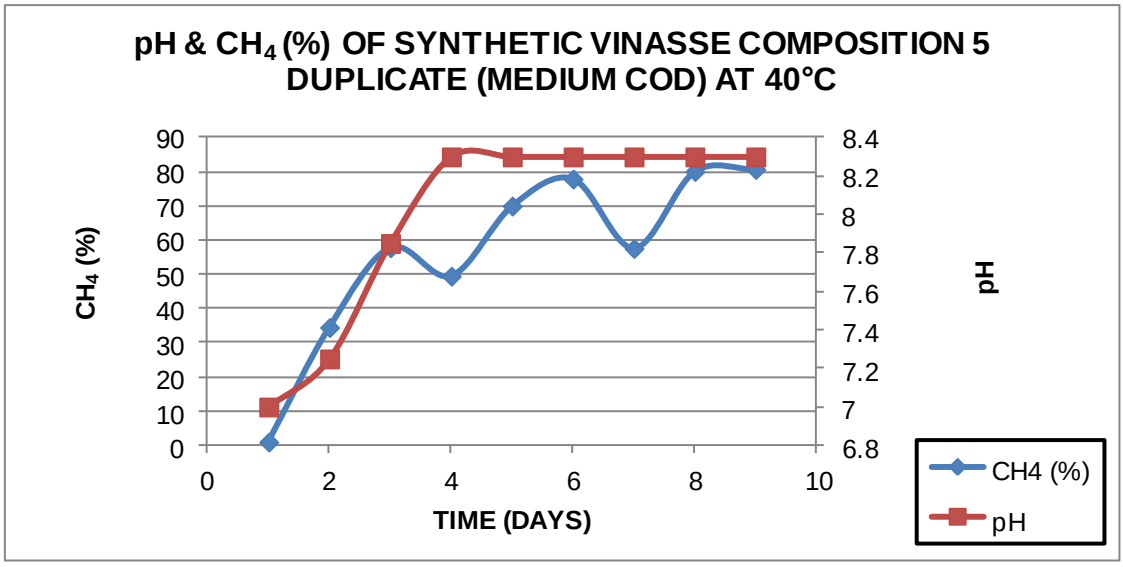


Figure 4.45 pH and CH_4 (%) of Synthetic Vinasse Composition 5 Duplicate (Medium COD) at 40°C

pH and CH_4 (%) of rest of the real and synthetic vinasse compositions, which were not analyzed for microbial analysis, are given in Appendix A.

4.3.2.12.1 Species Richness and Phylogenetic Diversity of Synthetic Vinasse Composition 5 at 40°C (Medium COD) and its Duplicate

Composition 5 at 40°C shows higher species richness (1043) and higher phylogenetic diversity (Faith's PD) 54 on day 11 (as shown in Figure 4.46). Composition 5 at 40°C (Duplicate) shows higher species richness (1121) and higher phylogenetic diversity (Faith's PD) 59 on day 16 (as shown in Figure Figure 4.46 and Figure 4.47). The percent differences between highest species richness and Faith's PD of Composition 2 at 30°C and Composition 2 at 30°C (Duplicate) are 12 and 16%, respectively.

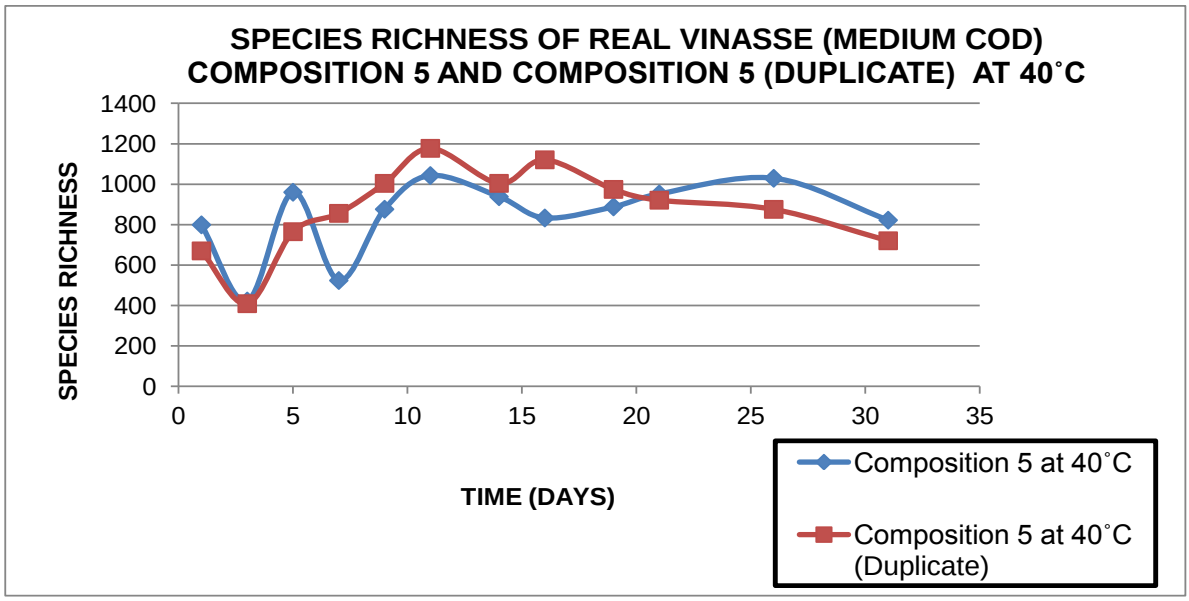


Figure 4.46 Species Richness of Synthetic Vinasse Composition 5 (Medium COD) and its Duplicate at 40°C

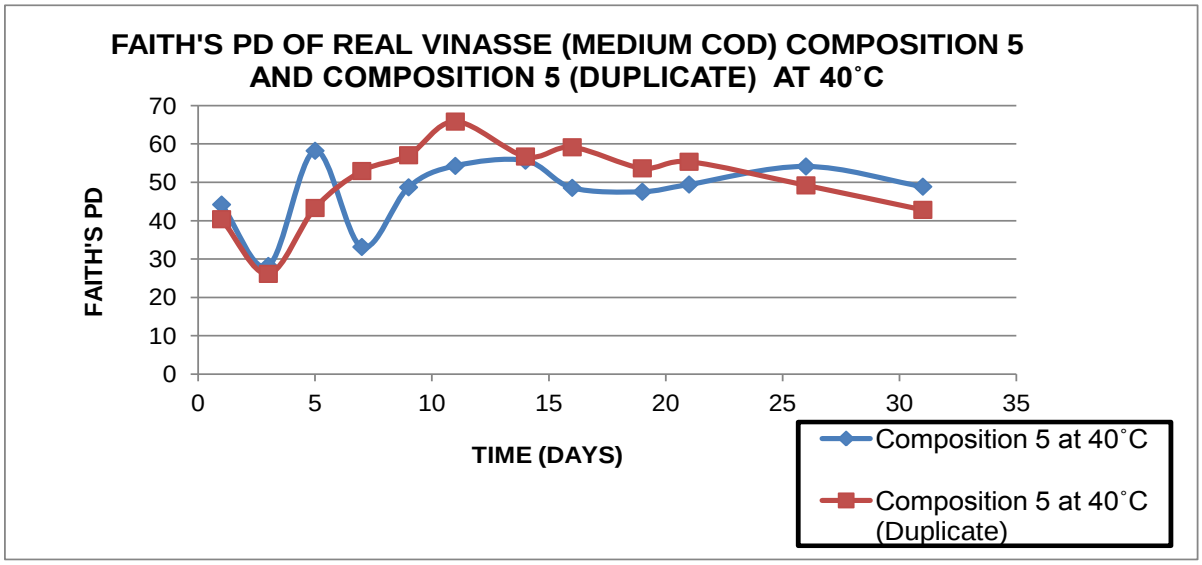


Figure 4.47 Phylogenetic Diversity (Faith's PD) of Synthetic Vinasse Composition 5 (Medium COD) and its Duplicate at 40°C

Both the composition 5 at 40°C and its duplicates contain microbes in higher percentages as given below in Table 4.18. These microbes are 79% in this composition. Some other microbes which are present in lower percentages than other microbes in composition 5 at 40°C (Duplicate) are mentioned in Appendix C. These microbes are 21% in this composition.

Table 4.18 Microbial Communities Present in Composition 5 (Medium COD) and its Duplicate at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

4.3.2.13 Sample duplicates of Real Vinasse composition 1 (Produced from Corn and Milo, White Energy, TX) at 40°C on Day 8

Sample duplicates of real vinasse composition 1 at 40°C (8) and its duplicate (8_D) were analyzed on day 8. Both reactors show the same types of microbes and almost the same percentage of microbes in their composition. Both the reactors show highest percentage of Fusobacteria, moderate percentages of Firmicutes and Proteobacteria, and lower percentage of Bacteroidetes. They also show very small percentage of Lentisphaerae, Synergistetes, and Actinobacteria (as shown in Figure 4.47).

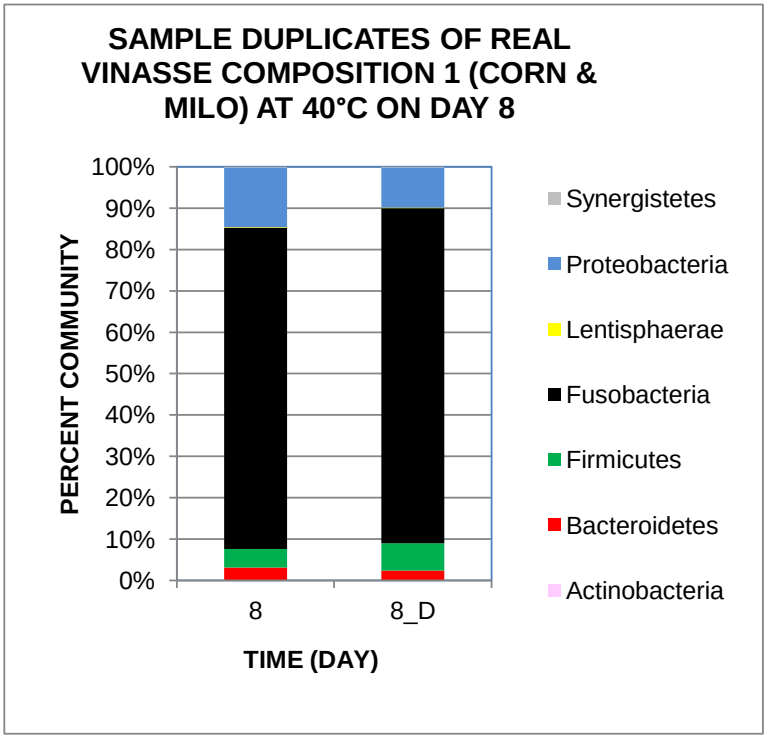


Figure 4.48 Microbial Community Profiling of Real Vinasse Sample Duplicate of Composition 1 (Corn and Milo, White Energy, TX) at 40°C on Day 8

Fusobacteria are more abundant than other microbes in sample duplicates of composition 1 at 40°C. These microbes are 80 and 85% in 8 and 8_D, respectively. These microbes are 80- 85% in both the compositions. A list of these microbes is given below in Table 4.19.

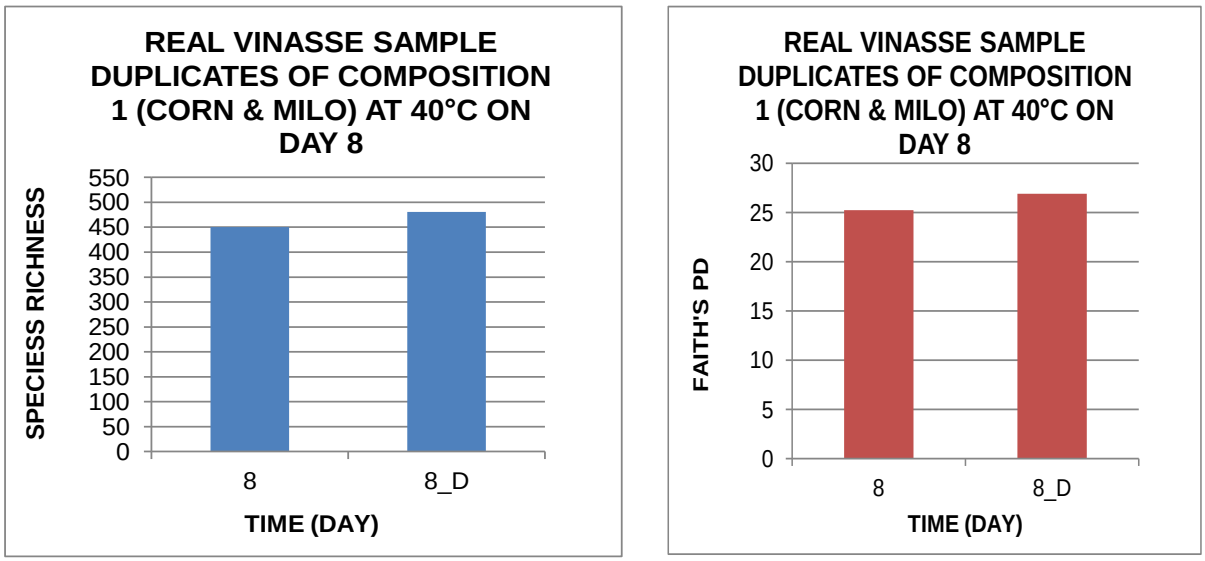
Some other microbes which are present in both reactors of composition 1 at 40°C and its Duplicate are mentioned in Appendix C. These microbes are 15 and 20 % in 8 and 8_D, respectively.

Table 4.19 Microbial Communities Present in of Real Vinasse Sample Duplicate of Composition 1 (Corn and Milo, White Energy, TX) at 40°C on Day 8

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium

4.3.2.13.1 Species Richness and Phylogenetic Diversity of Real Vinasse composition 1 (Produced from Corn and Milo, White Energy, TX) at 40°C on Day 8

Composition 1 at 40°C (8) and its duplicates (8_D) show species richness 450 & 481 and phylogenetic diversity (Faith's PD) 25 & 27 on day 8, respectively. The percent difference in species richness and Faith's PD between Composition 1 at 40°C (8) and its duplicates (8_D) are 6.5 and 6.3%. This indicates little difference in species richness and Faith's PD (as shown in Figure 4.49).



(a) (b)

Figure 4.49 (a) & (b) Species Richness and Phylogenetic Diversity (Faith's PD) of Real Vinasse Sample Duplicates Composition 1 (Corn and Milo, White Energy, TX) at 40°C on Day 8

4.3.2.14 Sample duplicates of Real Vinasse composition 3 (Produced from Corn, MGP Ingredients, KS) at 30°C on Day 35

Sample duplicates of Real Vinasse composition 3 at 30°C (35) and its duplicate (35_D) were analyzed on day 35. Both the reactors show the same types of microbes and almost the same percentage of microbes in their composition. Both the reactors show higher percentage of Firmicutes, moderate percentages of Fusobacteria and Bacteroidetes, and lower percentages of Proteobacteria and Lentisphaerae. It also shows very small percentage of Synergistetes, Verrucomicrobia, Chloroflexi and Archaea (as shown in Figure 4.49).

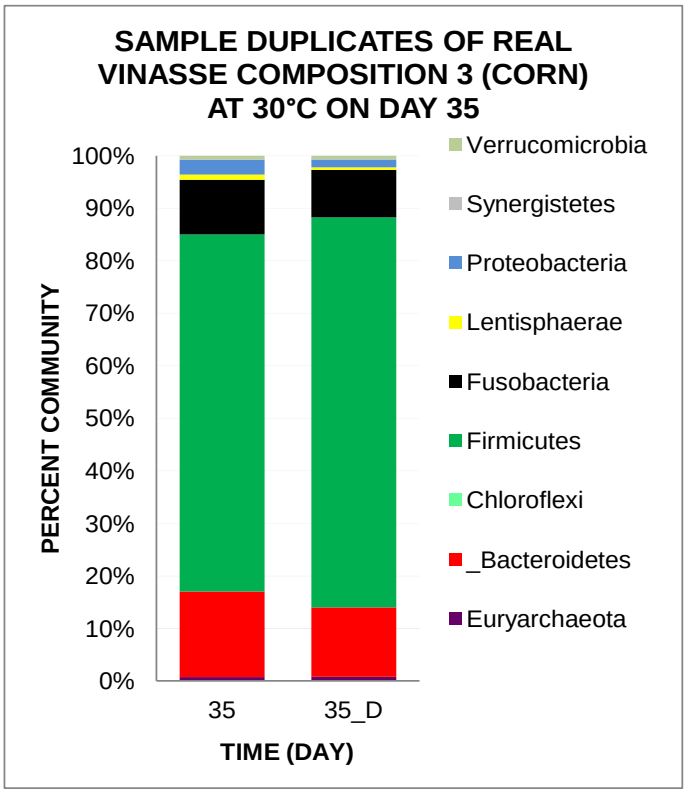


Figure 4.50 Microbial Community Profiling of Real Vinasse Composition 3 Sample Duplicate (Corn, MGP Ingredients, KS) at 30°C on Day 35

Fusobacteria, Bacteroidetes, and Firmicutes are more abundant than other microbes in sample duplicates of composition 3 at 30°C. These microbes are 78 and 87% in 35 and 35_D, respectively. A list of these microbes is given below in Table 4.20.

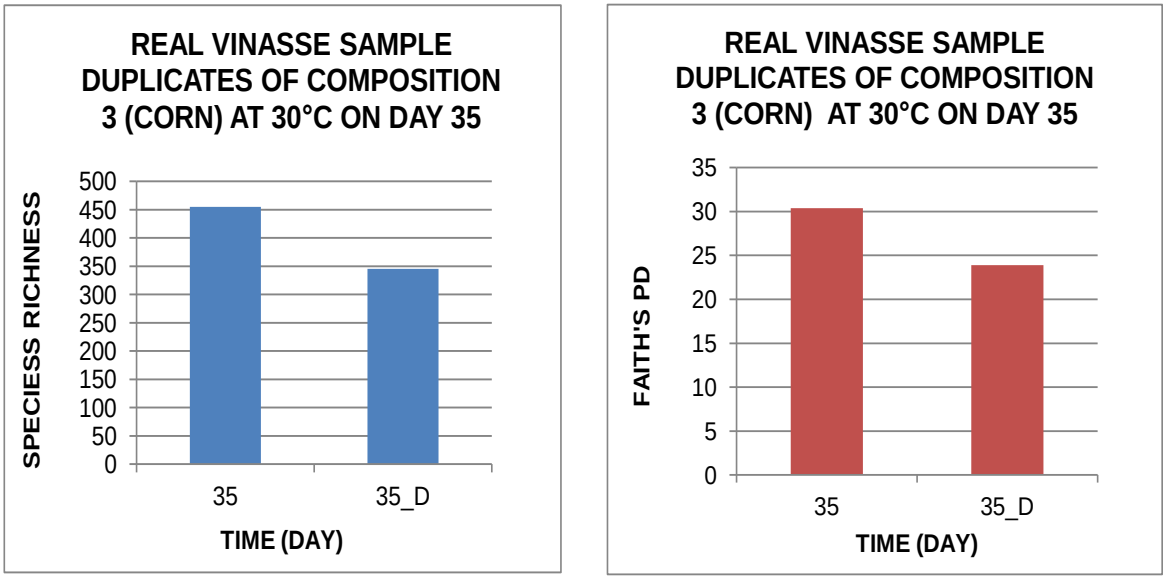
Some other microbes which are present in both the reactors of composition 3 at 30°C and its Duplicate are mentioned in Appendix C. These microbes are 22 and 13% in 35 and 35_D, respectively.

Table 4.20 Microbial Communities Present in Real Vinasse Composition 3 Sample Duplicate (Corn, MGP Ingredients, KS) at 30°C on Day 35

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira

4.3.2.14.1 Species Richness and Phylogenetic Diversity of Real Vinasse composition 3 (Produced from Corn, MGP Ingredients, KS) at 30°C

Composition 3 at 30°C (35) and its duplicates (35_D) show species richness 455 & 345 and phylogenetic diversity (Faith's PD) 30 & 24 on day 35, respectively. The percent difference in species richness and Faith's PD between Composition 3 at 30°C (35) and its duplicates (35_D) are 27 and 24%. This indicates higher species richness and Faith's PD in Composition 3 at 30°C than its duplicate (as shown in Figure 4.51). This may be due to the non-homogeneous nature of the real vinasse, although continuous mixing was employed.



(a) (b)

Figure 4.51 (a) & (b) Species Richness and Phylogenetic Diversity (Faith's PD) of Real Vinasse Sample Duplicates Composition 3 (Corn, MGP Ingredients, KS) at 30°C on Day 35

4.3.2.15 Sample Duplicates of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 40°C on Day 5

Sample duplicates of Real Vinasse composition 3 at 40°C (5) and its duplicate (5_D) were analyzed on day 5. Both the reactors show higher percentage of Fusobacteria, moderate percentage of Proteobacteria and lower percentages of Firmicutes and Bacteroidetes. However, the first sample contains almost 90% Fusobacteria, compared with 50-60% for the duplicate, and the first sample contains around 5% Proteobacteria, compared with almost 40% for the duplicate. This could be due to the

heterogeneity of the real vinasse, although continuous mixing was employed. Both show very small percentage of Synergistetes, Chloroflexi and Actinobacteria (as shown in Figure 4.52).

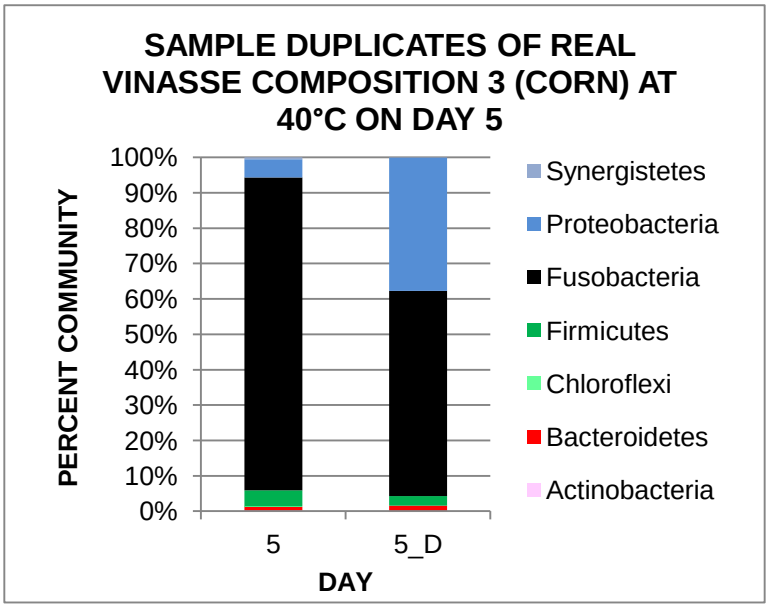


Figure 4.52 Microbial Community Profiling of Real Vinasse Sample Duplicates of Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 5

Fusobacteria are more abundant than other species in both the reactors of composition 3 at 40°C. These microbes are 90 and 58 % in 5 and 5_D, respectively. A list of these microbes is given below in Table 4.21.

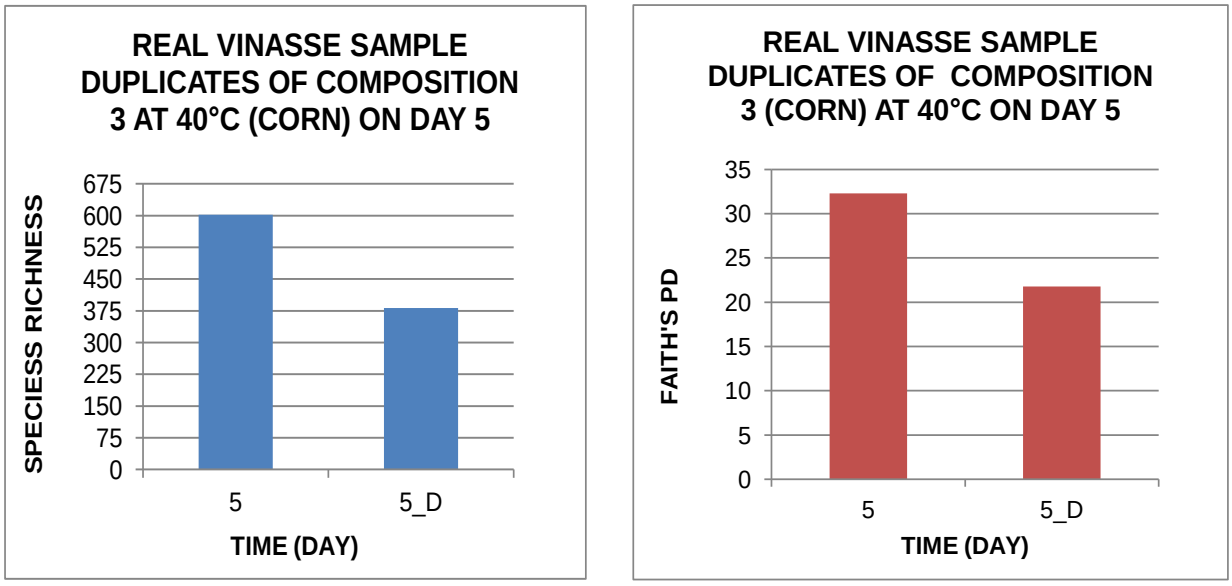
Table 4.21 Microbial Communities Present in Real Vinasse Sample Duplicates of Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 5

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium

Some other microbes which are present in both the reactors of composition 3 at 40°C and its Duplicate are given mentioned in Appendix C. These microbes are 10 and 42% in 5 and 5_D, respectively

4.3.2.15.1 Species Richness and Phylogenetic Diversity of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 40°C

Composition 3 at 40°C (5) and its Composition 3 at 40°C (5_D) show species richness 602 & 382 and phylogenetic diversity (Faith's PD) 32 & 22 on day 5, respectively. The percent differences in species richness and Faith's PD between Composition 3 at 40°C (5) and its Composition 3 at 40°C (5_D) are 44 and 38%. This indicates higher species richness and Faith's PD in Composition 3 at 30°C compared to its duplicate (as shown in Figure 4.53), perhaps because the real vinasse mixture was not homogenous.



(a) (b)

Figure 4.53 (a) & (b) Species Richness and Phylogenetic Diversity (Faith's PD) of Real Vinasse Sample Duplicates Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 5

4.3.2.16 Sample Duplicates of Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 40°C on Day 7

Sample duplicates of Real Vinasse composition 3 at 40°C (7) and its duplicate (7_D) were analyzed on day 5. Both the reactors show the same types of microbes in their composition. Both the reactors show higher percentage of Fusobacteria, moderate percentage of Proteobacteria and lower

percentages of Firmicutes and Bacteroidetes (as shown in Figure 4.54). However, the first reactor shows almost 40% Proteobacteria, compared with 20% for the duplicate; the first reactor shows about 60% Fusobacteria, compared with about 80% for its duplicate.

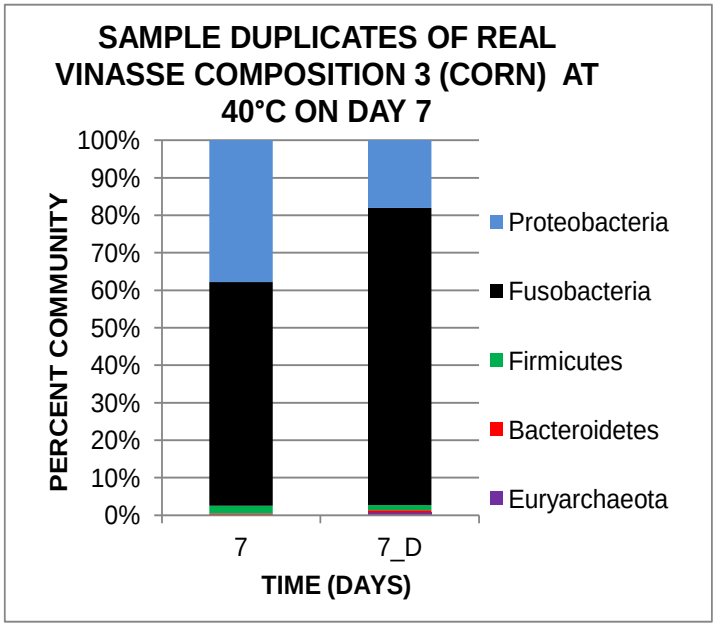


Figure 4.54 Microbial Community Profiling of Real Vinasse Sample Duplicates of Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 7

Bacteria (Fusobacteria and Proteobacteria) are more abundant (>93%) than other species in both the reactors of composition 3 at 40°C on day 7. A list of these microbes is given below in Table 4.22.

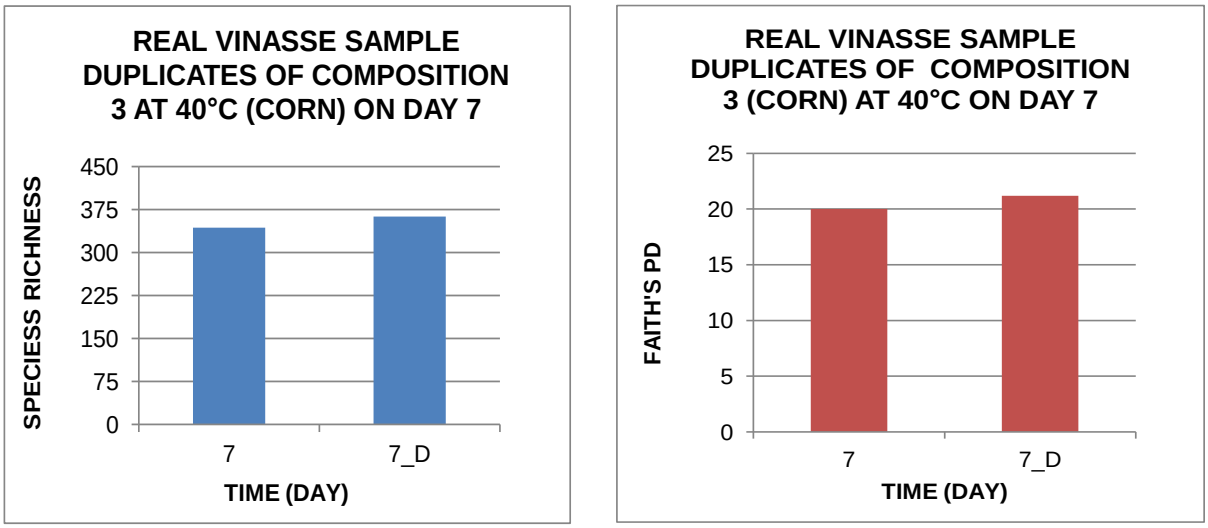
Some other microbes which are present in both the reactors of composition 3 at 40°C and its Duplicate are mentioned in Appendix C. These microbes are less than 7% in this composition.

Table 4.22 Microbial Communities Present in Real Vinasse Sample Duplicates of Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 7

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio

4.3.2.16.1 Species Richness and Phylogenetic Diversity Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 40°C

Composition 3 at 40°C (7) and its Composition 3 at 40°C (Duplicate) 7_D show species richness 343 & 363 and phylogenetic diversity (Faith's PD) 20 & 21 on day 7, respectively. The percent difference in species richness and Faith's PD composition 3 at 40°C (7) and its duplicate (7_D) are 5.60 and 5.68%.This indicates little difference in species richness and Faith's PD in Composition 3 at 40°C compared to its duplicate (as shown in Figure 4.55). Archaea was only present in very small percentage in composition 3 at 40°C.



(a) (b)

Figure 4.55 (a) & (b) Species Richness and Phylogenetic Diversity (Faith's PD) of Real Vinasse Sample Duplicates Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 7

4.3.2.17 Sample Duplicates of Real Vinasse Composition 5 (Medium COD) at 40°C on Day 14

Sample duplicates of Real Vinasse composition 5 at 40°C (14) and its duplicate (14_D) were analyzed on day 14. Both the reactors show the same types of microbes in their composition. Both the reactors show higher percentages of Proteobacteria and Bacteroidetes, moderate percentage of Firmicutes and lower percentages of Chloroflexi, WWE1**,** Spirochaetes, Archaea Synergistetes, Lentisphaerae, and Plantomycetes (as shown in Figure 4.56).

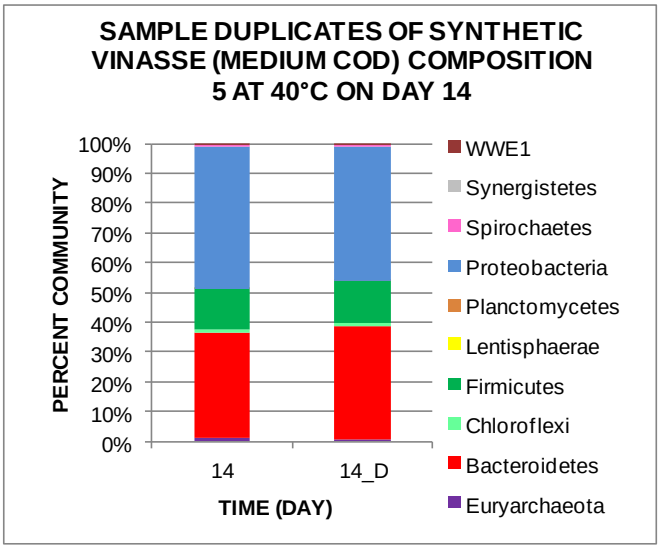


Figure 4.56 Microbial Community Profiling of Synthetic Vinasse Sample Duplicates of Composition 5 (Medium COD) at 40°C on Day 14

Firmicutes, Bacteroidetes, Fusobacteria and Proteobacteria are more abundant (>90%) than other species in both the reactors of composition 5 at 40°C on day 14. A list of these microbes is given below in Table 4.23.

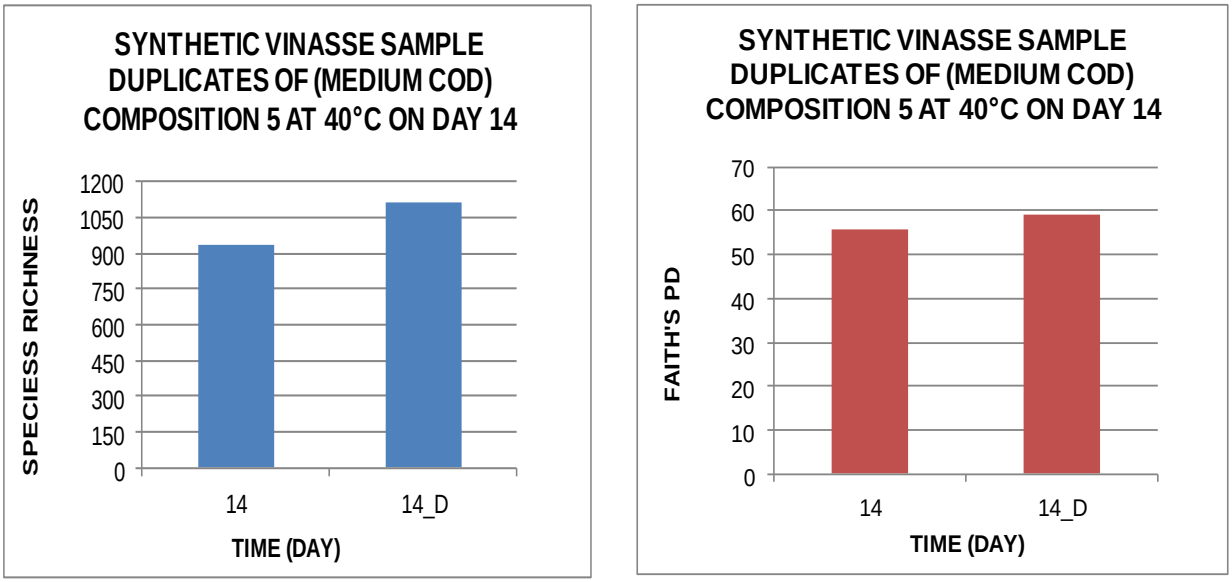
Some other microbes which are present in both the reactors of composition 5 at 40°C and its Duplicate are mentioned in Appendix C. These microbes are less than10% in this composition.

Table 4.23 Microbial Communities Present in Synthetic Vinasse Sample Duplicates of Composition 5 (Medium COD) at 40°C on Day 14

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus

4.3.2.17.1 Species Richness and Phylogenetic Diversity of Synthetic Vinasse composition 5 (Medium COD) at 40°C

Composition 5 at 40°C (14) and its Composition 5 at 40°C (14_D) show species richness 938.02 & 1110.25 and phylogenetic diversity (Faith's PD) 56 & 59 on day 14, respectively. This indicates little difference in species richness and Faith's PD in Composition 5 at 40°C compared to its duplicate (as shown in Figure 4.57. The percent difference in species richness and Faith's PD in Composition 5 at 40°C (14) and its Composition 5 at 40°C (14_D) are 16 and 5.8%.



(a) (b)

Figure 4.57 (a) & (b) Species Richness and Phylogenetic Diversity (Faith's PD) of Synthetic Vinasse Sample Duplicates Composition 5 (Medium COD) at 40°C on Day 14

4.3.2.18 Sample Duplicates of Synthetic Vinasse composition 6 (High COD) at 30°C on Day 1

Sample duplicates of Real Vinasse composition 6 at 30°C (1) and its duplicate (1_D) were analyzed on day 1. Both the reactors show the same types of microbes in their composition. Both the reactors show higher percentages of Proteobacteria and Bacteroidetes, moderate percentage of Firmicutes and lower percentages of Chloroflexi, WWE1**,** Spirochaetes, Verrucomicrobia, Thermotogae, Archaea Synergistetes, Lentisphaerae, and Plantomycetes (as shown in Figure 4.58). Acidobacteria was only present in a very small percentage in composition 6 at 30°C.

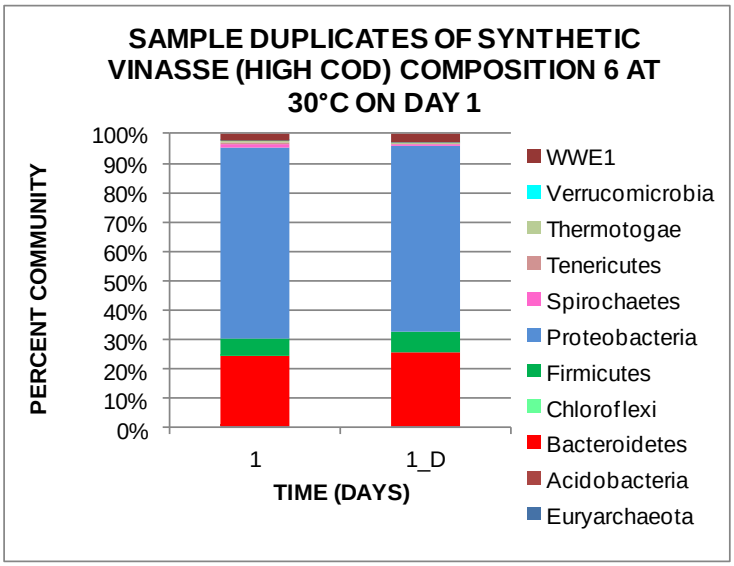
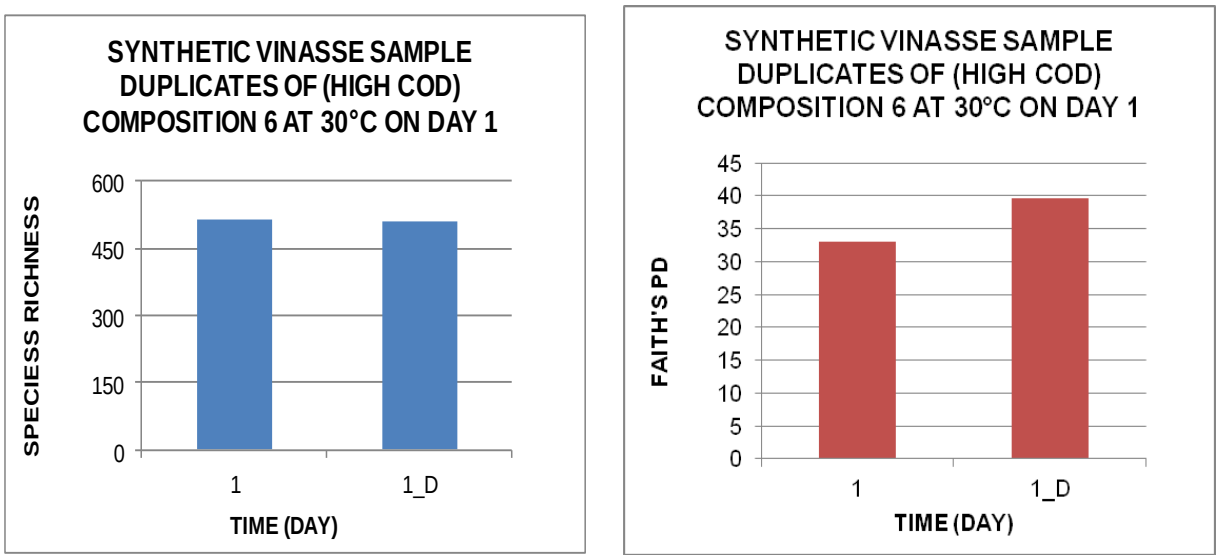


Figure 4.58 Microbial Community Profiling of Synthetic Vinasse Sample Duplicates of Composition 6 (High COD) at 30°C on Day 1

4.3.2.18.1 Species Richness and Phylogenetic diversity of Synthetic Vinasse composition 6 (High COD) at 30°C on Day 1

Composition 6 at 30°C (1) and its Composition 6 at 30°C (Duplicate) 1_D show species richness 515 & 511 and phylogenetic diversity (Faith's PD) 33 & 39 on day 1, respectively. This indicates little difference in species richness and Faith's PD in Composition 6 at 30°C compared to its duplicate (as shown in Figure 4.59). The percent difference in species richness and Faith's PD in Composition 6 at 30°C (1) and its Composition 6 at 30°C (Duplicate) 1_D are 0.78 and 18%.



(a) (b)

Figure 4.59 (a) & (b) Species Richness of Synthetic Vinasse Sample Duplicates Composition 6 (High COD) at 30°C on Day 1

Bacteroidetes and Proteobacteria are more abundant (>75%) than other phylum in both the reactors of composition 6 at 30°C on day 1. A list of these microbes is given below.

Some other microbes which are present in both the reactors of composition 5 at 40°C and its Duplicate mentioned in Appendix C. These microbes are 25% in this composition.

Table 4.24 Microbial Communities Present in Synthetic Vinasse Sample Duplicates of Composition 6 (High COD) at 30°C on Day 1

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	

4.4 Cumulative Methane Generation and Methane Generation Rate

The methane generation rate is expressed in terms of ml of methane at STP/L of vinasse/day. The cumulative methane generation is computed by adding the daily methane generation rate and is expressed in terms of ml of methane at STP/L of vinasse. Comparisons of methane generation rates and cumulative methane generation from each composition at three different temperatures are presented in this section.

4.4.1 Comparison of Methane Generation Rates and Cumulative Methane Generation of real vinasse composition 1 (Produced from Corn and Milo, White Energy, TX) at 30 and 40°C.

Figure 4.60 and Figure 4.61 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from composition 1 at 30 and 40°C, respectively. The volume of methane was not measured for composition 1 at 35°C because the calibrator was sent for repair.

Real vinasse composition 1 at 30°C methane generation rate on day 2 was 1.14 and increased to 1.36 ml/L/day on day 27. The total run time was 35 days for this composition. The volume of methane was not measured on day 35 because there was a very small amount of volume left after measuring the concentration of gas. This reactor shows leak in the system because it produced only 2.3% maximum methane and 8% of oxygen percentage at 30°C on the last day at 30°C, unlike same composition at 35°C and 40°C, which produced higher percentage of methane. Hence, it produced lesser methane and cumulative methane in compare to composition 1 at 35°C and 40°C. The cumulative methane generated from composition 1 at 30°C increased from 7.78 to 36 ml/L at the end. This value is low, also due to the leak.

For real vinasse composition 1 at 40°C, the volume of methane was not measured till day 10 because the calibrator was sent for repair. Composition 1 at 40°C methane generation rate on day 11 was 3.27 ml/L/day and increased to 20 ml/L/day on day 14. Then, it decreased to 13 ml/L/day on day 16. The percentage of methane started increasing on day 11 till day 16. This indicates methanogenesis phase started on day 11. Methanogenesis phase started at low rate on day 11 and continued higher methane generation rate till day 14 and then started decreasing due to decrease in COD in the vinasse.

The methanogens did not have enough food to generate more methane after day 16 and the batch stopped generating biogas. The cumulative methane generated from composition 1 at 40°C increased from 22 to 280 ml/L. The cumulative methane for this reactor cannot be compared to the reactor at 30°C because all days of data were not available.

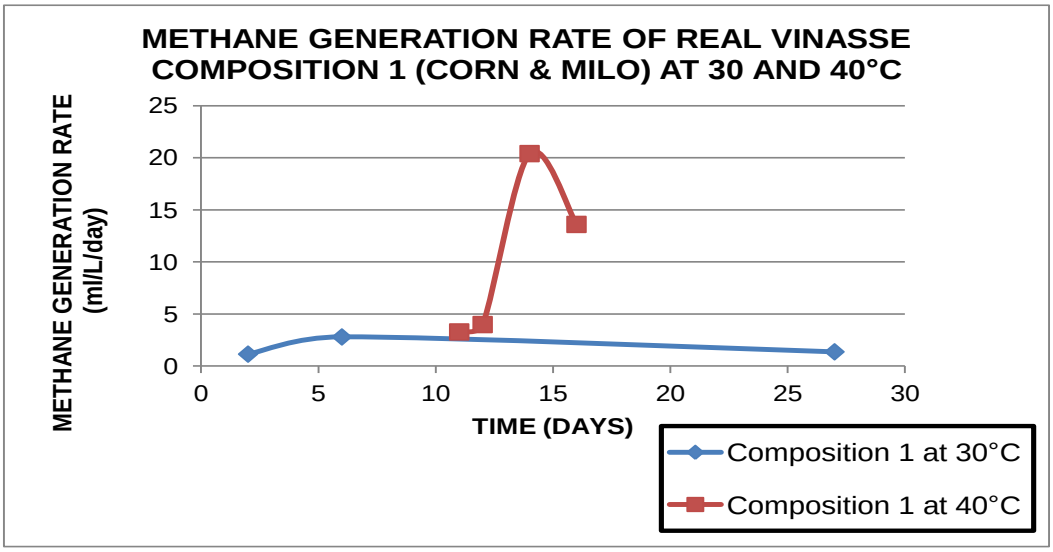


Figure 4.60 Methane Generation Rate of Real Vinasse Composition 1 (Corn and Milo, White Energy, TX) at 30 and 40°C

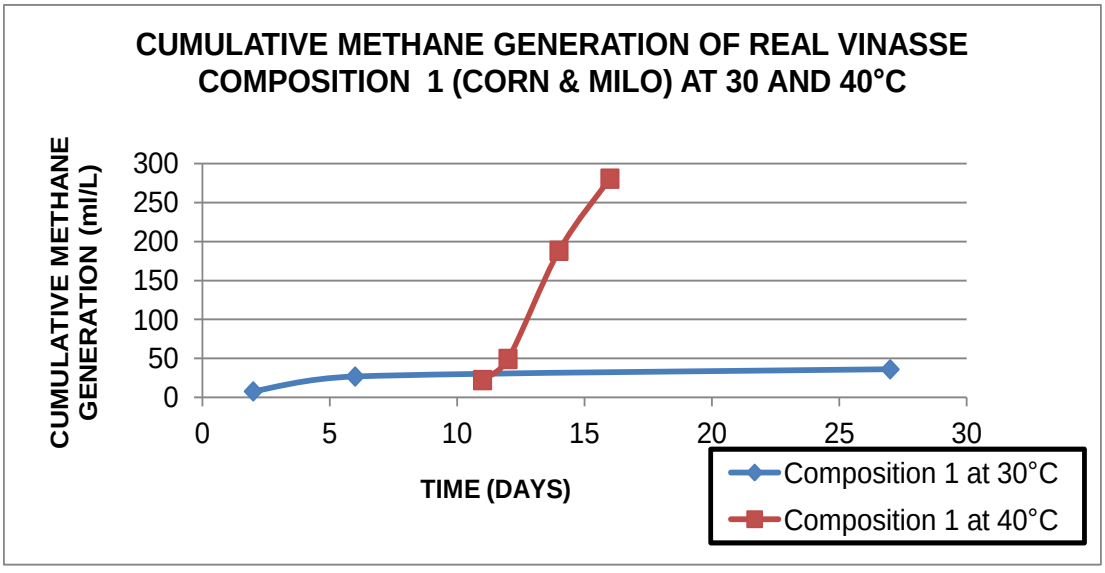


Figure 4.61 Cumulative Methane Generation of Real Vinasse Composition 1 (Corn and Milo, White Energy, TX) at 30 and 40°C

4.4.2 Comparison of Methane Generation Rates and Cumulative Methane Generation of real vinasse composition 2 (Produced from Corn and Milo, White Energy, TX) at 30, 35 and 40°C

Figure 4.62 and Figure 4.63 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from composition 2 at 30, 35 and 40°C, respectively.

Real vinasse composition 2 at 30°C methane generation rate increased substantially on day 15 till day 20 and then decreased on day 29 and started increasing till day 37. The methanogens did not have enough food to generate more methane after day 37 and the batch stopped generating more biogas. The cumulative methane generated from composition 2 at 30°C increased from 35 to 565 ml/L at the end of run.

Real vinasse composition 2 at 35°C methane generation rate on day 1 was 9 and increased to 47 ml/L/day on day 22. The methanogens did not have enough food to generate more methane after day 22 and the batch stopped generating more biogas. The cumulative methane generated from composition 2 at 35°C increased from 68 to 477 ml/L at the end of run.

Real vinasse composition 2 at 40°C methane generation rate on day 1 was 14 ml/L/day and increased to 57 ml/L/day on day 11. The cumulative methane generated from composition 2 at 40°C increased from 100 on day 1 to 598 ml/L on day 11.

The microbial activity should have increased with temperature, according to theory. Real vinasse composition 2 at 40°C did show the highest generation rate (57 ml/L/day) of the 3 reactors. Also, its run time was the shortest (11 days compared with 22 days for the 35°C reactor and 37 days for the 30°C reactor), indicating that the microbes used up all the food sooner at 40°C than lower temperatures.

Since the same amount of food (glucose) was added to all 3 reactors, the cumulative methane generation should have been the same. The cumulative methane generated was comparable for the three reactors (565, 477, and 598 ml/L), as would be expected since each contained equal amounts of COD.

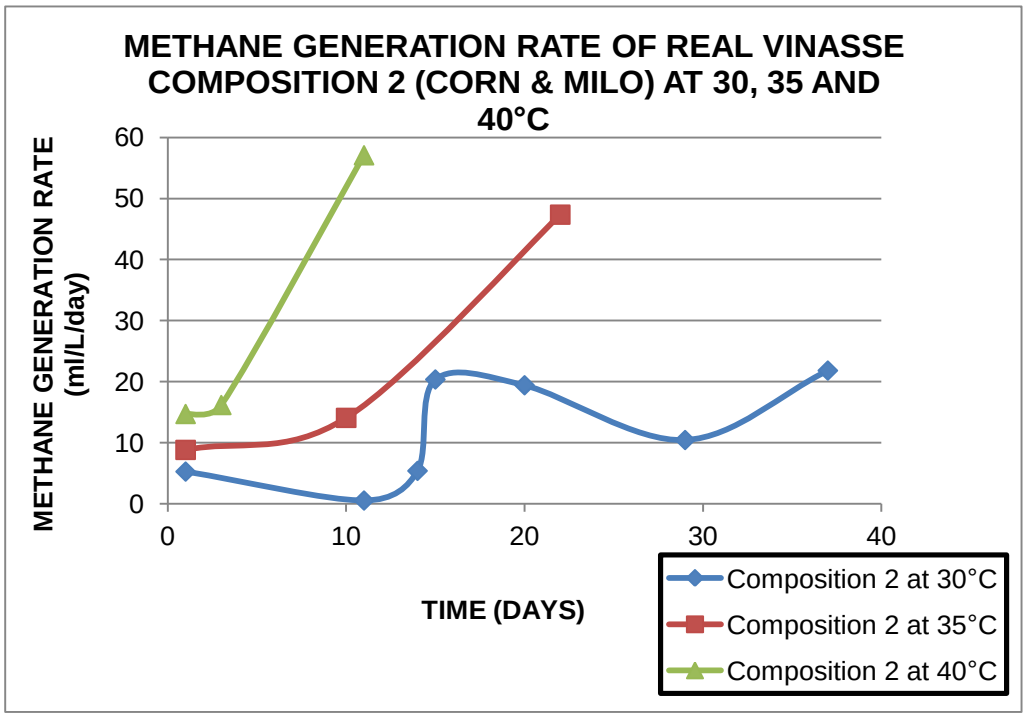


Figure 4.62 Methane Generation Rate of Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) at 30, 35 and 40°C

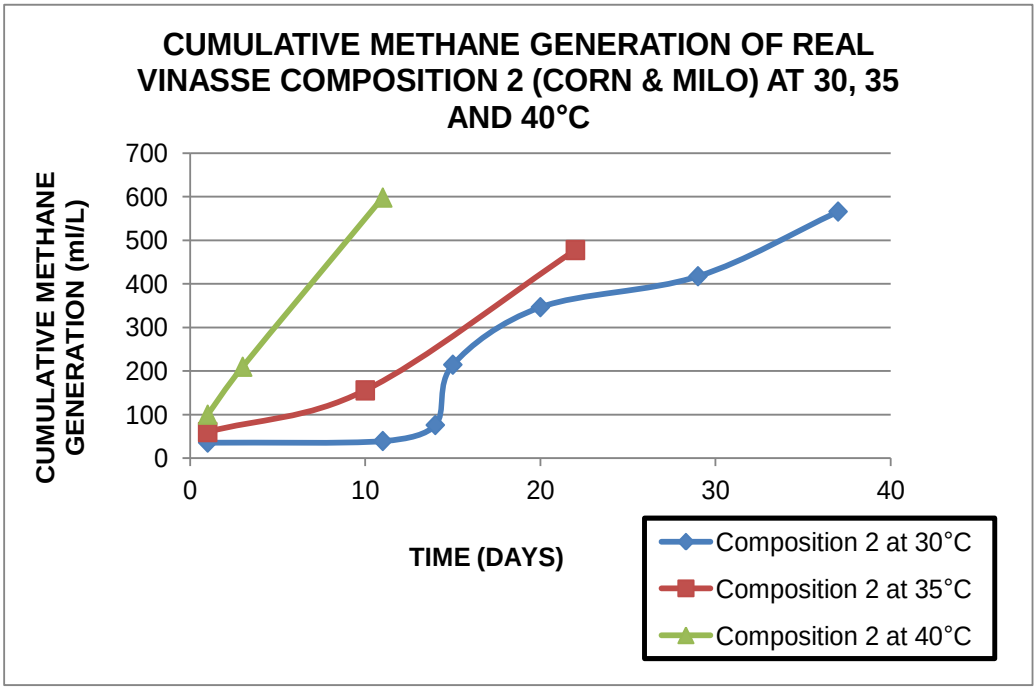


Figure 4.63 Cumulative: Methane Generation of Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) at 30, 35 and 40°C

4.4.3 Comparison of Methane Generation Rates and Cumulative Methane Generation of real vinasse composition 3 (Produced from Corn, MGP Ingredients, KS) at 30 and 40°C.

Figure 4.64 and Figure 4.65 show methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from real vinasse composition 3 at 30°C. The cumulative methane generation and methane generation rate generated from real vinasse composition 3 at 35°C was not measured because the calibrator was sent for repair.

Real vinasse composition 3 at 30°C shows a substantial increase in methane generation on day 31, with generation peaking between days 31-36. The cumulative methane generated from composition 3 at 30°C increased from 0.47 to 771 ml/L at the end of run.

Real vinasse composition 3 at 40°C shows a substantial increase in methane generation on day 9, with generation peaking on day 11. The total run time was 13 days which was much faster than 35 days of composition 3 at 30°C. The cumulative methane generated from composition 3 at 40°C was increased from 12 to 821 ml/L at the end of run.

Real vinasse composition 3 at 40°C took only 13 days to complete the run, compared to 36 days of Composition 3 at 30°C, due to increased microbial activity at the higher temperature. Also, the peak methane generation rate was higher at 40°C (48 ml/L/day) compared to 30°C (around 25 ml/L/day). The cumulative methane generated was comparable for the two reactors (771 and 821 ml/L), as would be expected since each contained equal amounts of COD.

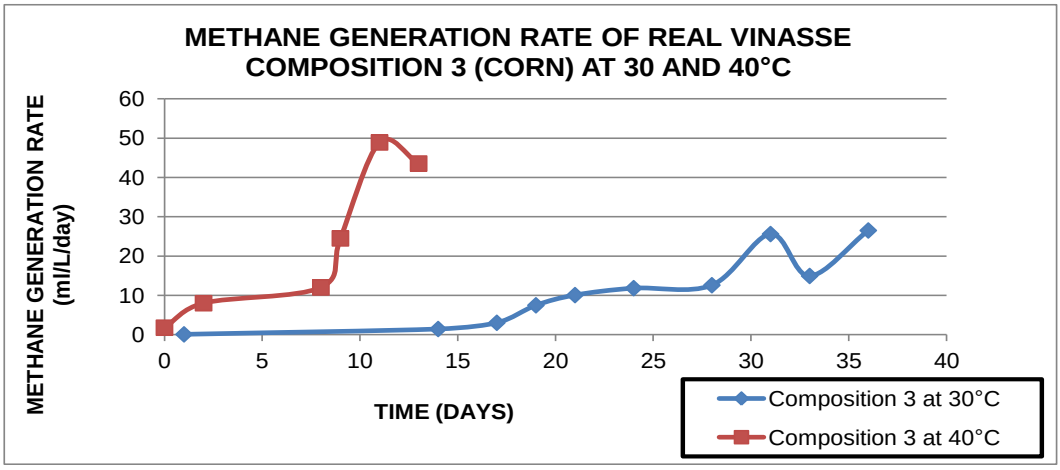


Figure 4.64 Methane Generation Rate of Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 30 and 40°C

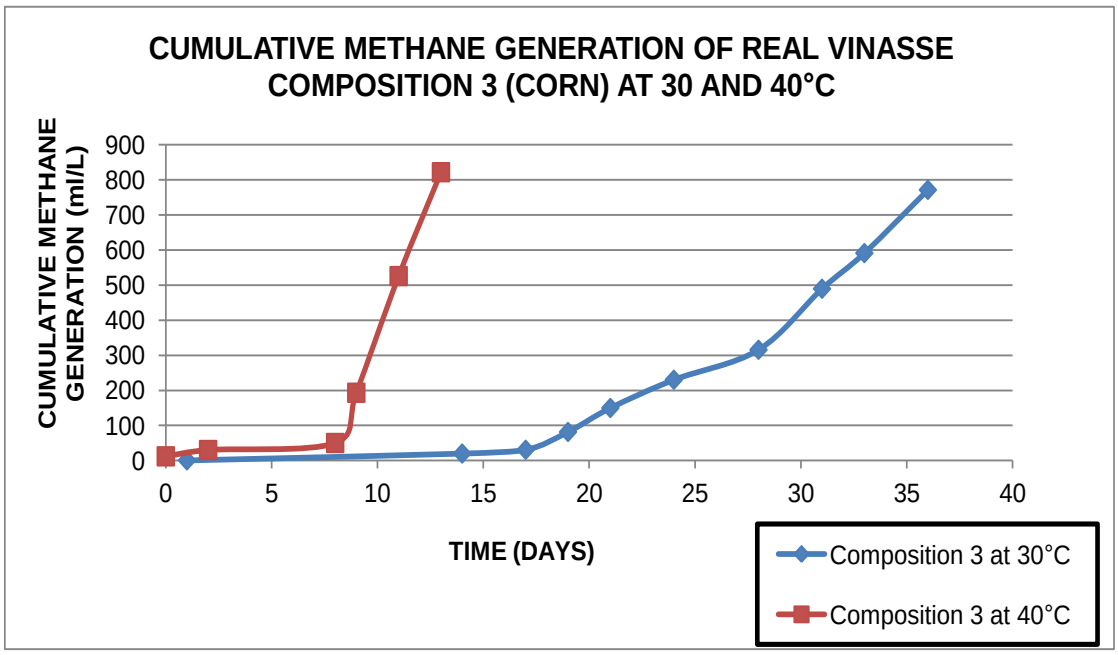


Figure 4.65 Cumulative Methane Generation of Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 30 and 40°C

4.4.4 Comparison of Methane Generation Rates and Cumulative Methane Generation of Synthetic Vinasse Composition 4 (Low COD) at 30 and 35°C.

Figure 4.66 and Figure 4.67 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from synthetic vinasse composition 4 at 30 and 35°C. The cumulative methane generation, and methane generation generated from composition 4 at 40°C was not measured because calibrator was sent for repair. Composition 4 at 30°C methane generation rate on day 2 was 0.7 ml/L/day and peaked on day 20 at 18 ml/L/day. Finally, methane generation rate decreased to 16 ml/L/day at the end on day 32. Then, it stopped producing more gas because all food was used up on day 32. The cumulative methane generated from composition 4 at 30°C increased from 5 to 334 ml/L.

Synthetic vinasse composition 4 at 35°C methane generation rate on day 1 was 2.42 ml/L/day and increased to 20 ml/L/day on day 12 and decreased to 7.3 ml/L/day on day 16. The cumulative methane generated from composition 4 at 35°C increased from 16.5 to 268 ml/L.

Synthetic vinasse composition 4 at 35°C took only 16 days to complete the run, compared to 32 days of composition 4 at 30°C, due to increased microbial activity at the higher temperature. Also, the peak methane generation rate was higher at 35°C (20 ml/L/day) compared to 30°C (around 16 ml/L/day). The cumulative methane generated was comparable for the two reactors (271 and 296 ml/L), as would be expected since each contained equal amounts of COD. The methane generation rate decreased from 20 to 7 ml/L/day at the end because the sludge used for this composition at 35°C was too thin. This composition produced lowest cumulative methane generation because the COD was too low in compare to other compositions.

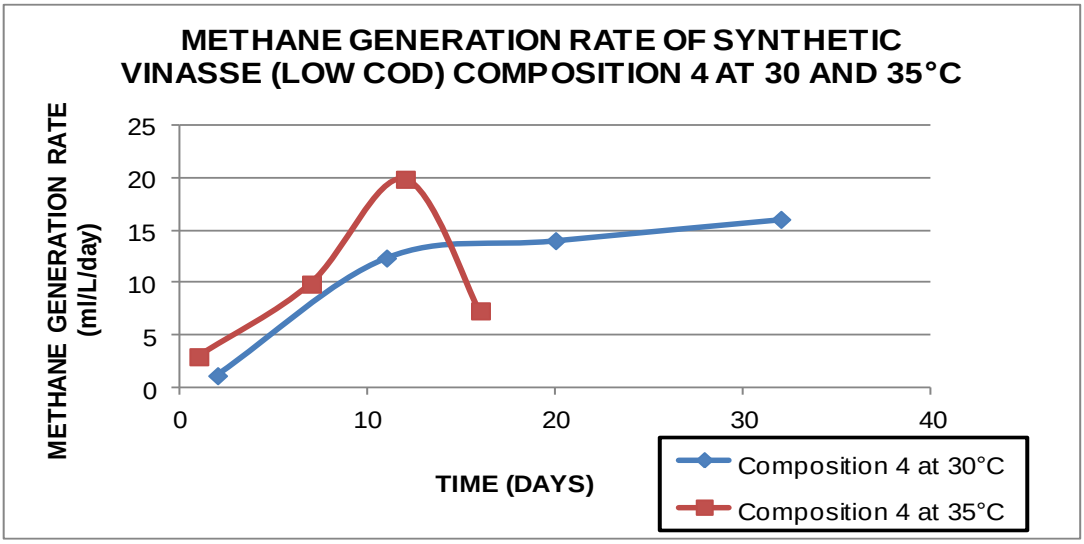


Figure 4.66 Methane Generation Rate of Synthetic Vinasse Composition 4 (Low COD) at 30 and 35°C

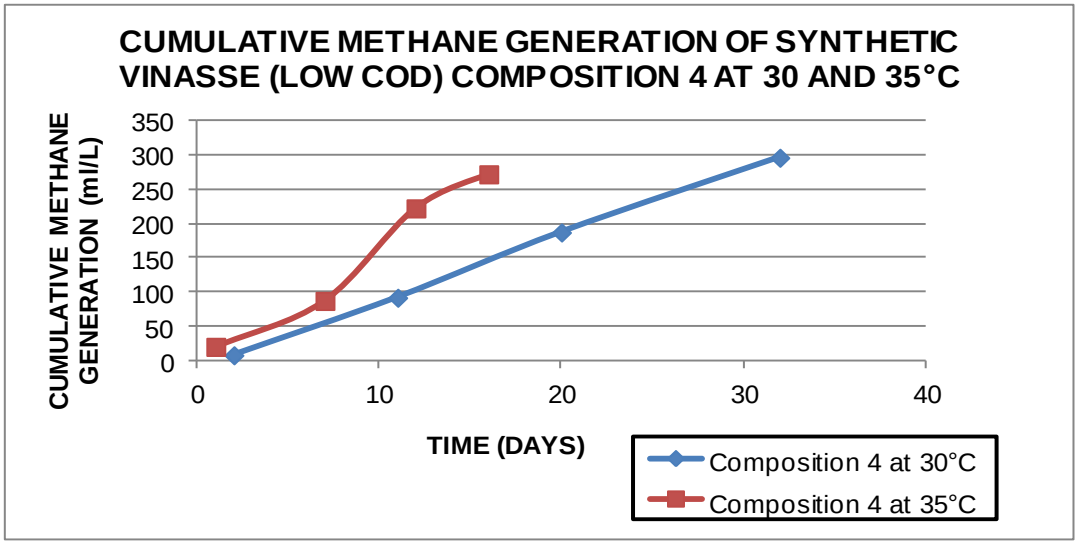


Figure 4.67 Cumulative Methane Generation of Synthetic Vinasse Composition 4 (Low COD) at 30 and 35°C

4.4.5 Comparison of Methane Generation Rates and Cumulative Methane Generation of Synthetic Vinasse Composition 5 (Medium COD) at 30, 35 and 40°C.

Figure 4.68 and Figure 4.69 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from composition 5 at 30, 35, and 40°C. Composition 5 at 30°C methane generation rate on day 1 was 3.2 ml/L/day and peaked at 45 on day 49. The cumulative methane generated from composition 5 at 30°C increased from 21 to 1655 ml/L.

Synthetic vinasse composition 5 at 35°C methane generation rate on day 1 was 8.23 ml/L/day and increased to 112 ml/L/day on day 39. The cumulative methane generated from composition 5 at 35°C increased from 56 to 1305 ml/L. This composition shows higher methane percentage throughout the run in compare to Composition 5 at 35°C.

Synthetic vinasse composition 5 at 40°C methane generation rate on day 1 was 13 ml/L/day and peaked on day 25. The cumulative methane generated from composition 5 at 40°C increased from 90 to 4846 ml/L. This composition also shows higher methane percentage throughout the run in compared to Composition 5 at 35°C.

Synthetic vinasse composition 5 at 40°C took only 31 days to complete the cycle in comparison to 56 days cycle of composition 5 at 30°C and 39 days cycle of composition 5 at 35°C. Increased microbial activity is expected at higher temperatures. The peak methane generation rate was highest at

40°C (149 ml/L/day), intermediate at 35°C (112 mL/L/day), and lowest at 30°C (45 ml/L/day), as would be expected.

The cumulative methane generation is higher for synthetic vinasse composition 5 compared to synthetic vinasse composition 4 because a higher amount of glucose was added. However, the cumulative methane generation should have been the same for all 3 reactors with composition 5; however, the reactor at 40°C generated very high volume of methane in compare to reactors at 30°C and 35°C. This may have been due to leaks in the reactor at 30 and 35°C. Composition 5 at 30°C shows 16.5% methane on last day, which indicates leaks in this reactor.

Since the same amount of food (glucose) was added to all 3 reactors, the cumulative methane generation should have been the same. However, the total methane generated for 40°C is much higher than 30, 35°C. The microbes may have functioned better and been able to degrade a larger fraction of the COD at 40°C. In addition, there were operational difficulties in maintaining the pH in the beginning of the real vinasse combinations. As a result, microbes likely died in the beginning, which could have reduced reactor performance. Also, the amount of microbes present in the sludge may have varied among runs because sludge was collected from the wastewater treatment plant for different runs on different days. Moreover, the real vinasse was changing its color and odor while stored at cold room temperature; it may have changed between the beginning of one run and the next. Furthermore, the real vinasse compositions were not homogenous and the amount of nutrient varied in each run. Finally, sometimes the connections came off from composition 2 due to high temperature in hot room and moving of the reactor at the time of sampling.

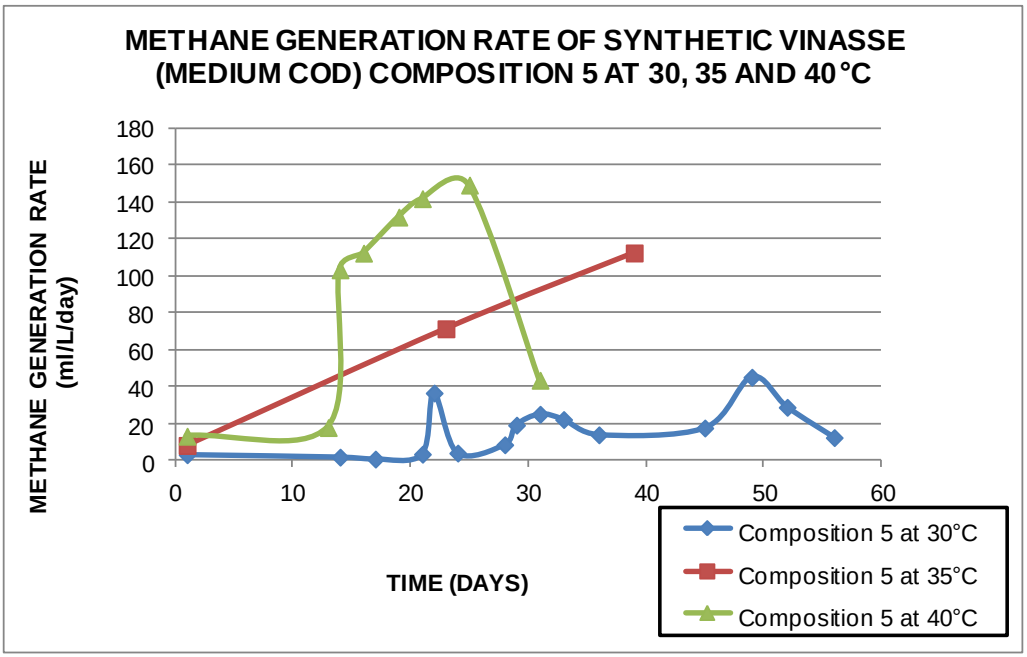


Figure 4.68 Methane Generation Rate of Synthetic Vinasse Composition 5 (Medium COD) at 30, 35 and 40°C

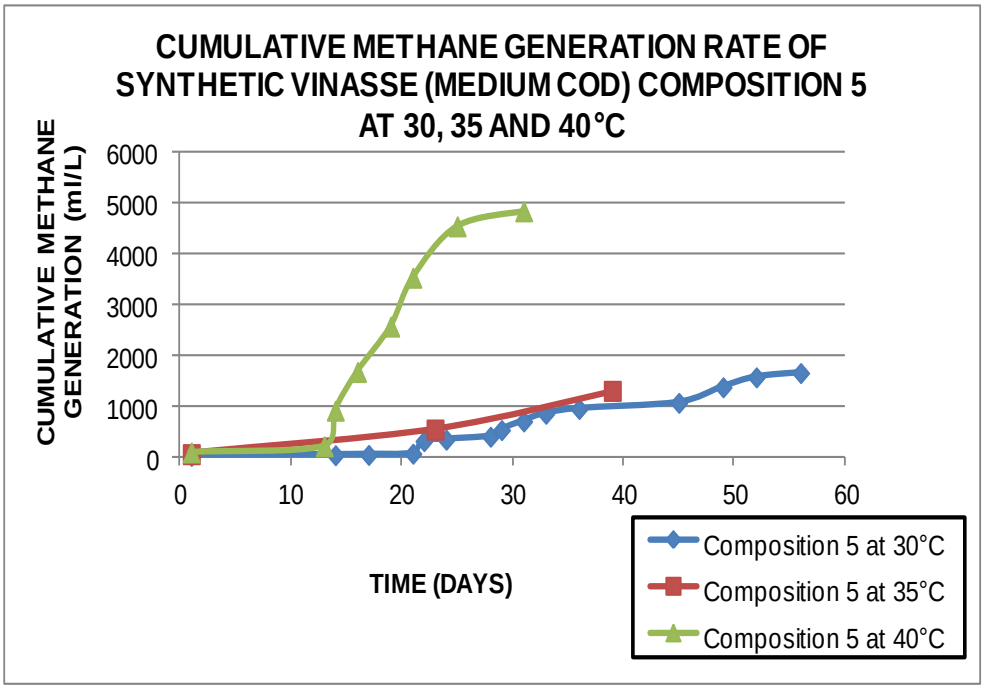


Figure 4.69 Cumulative Methane Generation of Synthetic Vinasse Composition 5 (Medium COD) at 30, 35 and 40°C

4.4.6: Comparison of Methane Generation Rates and Cumulative Methane Generation of synthetic vinasse composition 6 (High COD) at 30 and 35 and 40°C.

Figure 4.70 and Figure 4.71 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from composition 6 at 30, 35, and 40°C.

Synthetic vinasse composition 6 at 30°C methane generation rate on day 2 was 6.8 ml/L/day, peaked on day 50 at 568 ml/L/day, and then decreased to 108 on day 87. The cumulative methane generated from composition 6 at 30°C increased from 46 to 14706 ml/L. Archaea was present in the overall reaction with high methane generation rate.

Synthetic vinasse composition 6 at 35°C methane generation rate on day 1 was 12.35 ml/L/day, peaked on day 48 at 309 ml/L/day, and decreased to 238 ml/L/day on day 89. The cumulative methane generated from composition 6 at 35°C increased from 84 to 7935 ml/L.

Synthetic vinasse composition 6 at 40°C methane generation rate on day 1 was 14 ml/L/day and increased to 57 on day 11. The cumulative methane generated from composition 6 at 40°C increased from 96 to 1042 ml/L.

The methane generation rate and cumulative methane generation were highest for this composition (for 30°C and 35°C) compared to other composition because highest amount of glucose was added. Also, the total run time was longer at 30°C and 35°C compositions.

The methane generation rate and cumulative methane generation were unusually low for the 40°C reactor, compared to the 30 and 35°C reactors for this composition. Also, this composition produced twice the cumulative methane at 30°C in compare to 35°C.This indicates that the reactor at 35 and 40°C may have had some leaks. Synthetic vinasse composition 6 at 35 and 40°C produced 6.3 and 4.5 oxygen on last day, which increased from 1.5% oxygen from previous day. This indicates leak in those reactors. Alternately, the microbes may have functioned better and been able to degrade a larger fraction of the COD at 40°C. Also, the amount of microbes present in the sludge may have varied among runs because sludge was collected from the wastewater treatment plant for different runs on different days. Finally, sometimes the connections came off from composition 6 due to high temperature in hot room and moving of the reactor at the time of sampling.

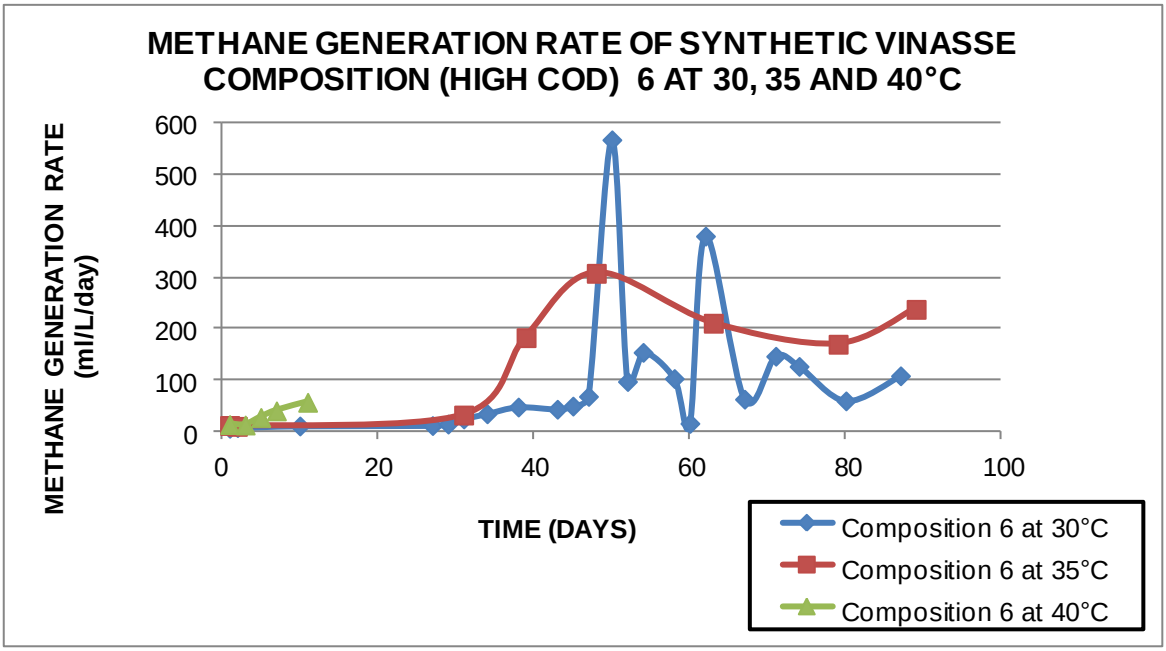


Figure 4.70 Methane Generation Rate of Synthetic Vinasse Composition 6 (High COD) at 30, 35 and 40°C

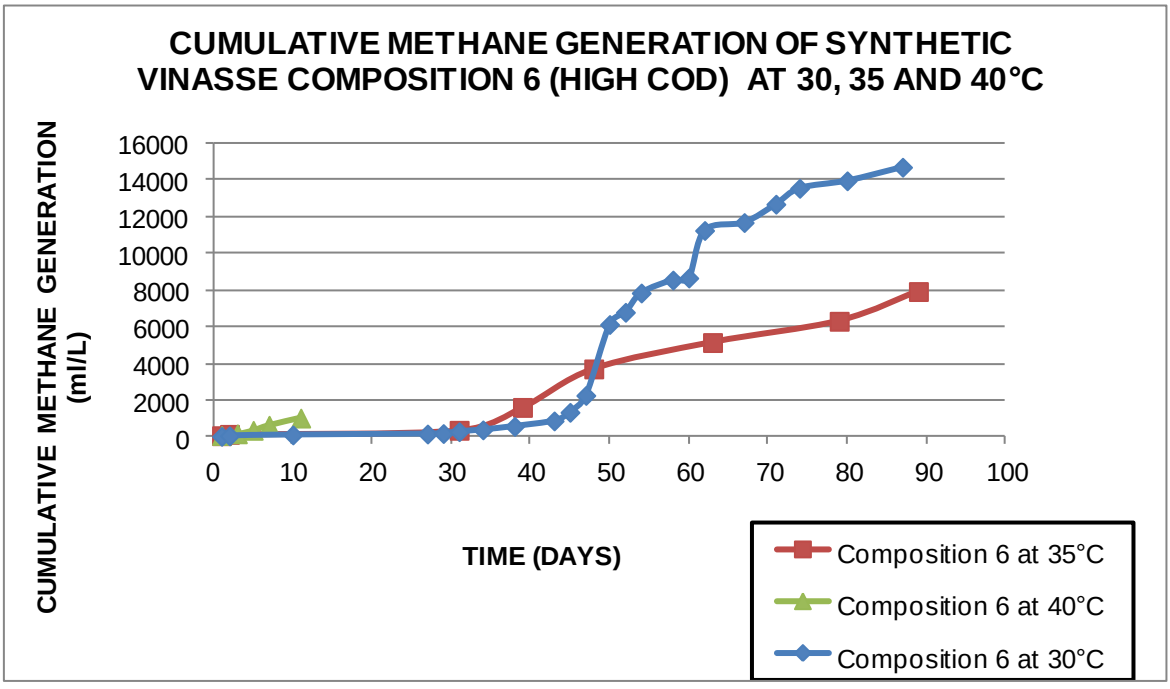


Figure 4.71 Cumulative Methane Generation of Synthetic Vinasse Composition 6 (High COD) at 30, 35 and 40°C

4.4.7 Impacts of Vinasse Compositions on Methane Generation Rate and Cumulative Methane Generation at 30, 35, and 40°C

Since the COD of real vinasse is higher than synthetic vinasse, the cumulative methane generation and methane generation rate should be higher than synthetic. However, the total methane generated and cumulative methane produced for 30°C is much higher in synthetic vinasse. It might possible that microbes are not able to digest real vinasse. Synthetic vinasse composition 6 (high COD) produced maximum methane generation and cumulative methane generation at 30 and 35°C (as shown in Figure 4.72 to Figure 4.75). However, synthetic vinasse composition 5 (Medium COD) produced maximum methane generation and cumulative methane generation at 40°C (as shown in Figure 4.76 and Figure 4.77).

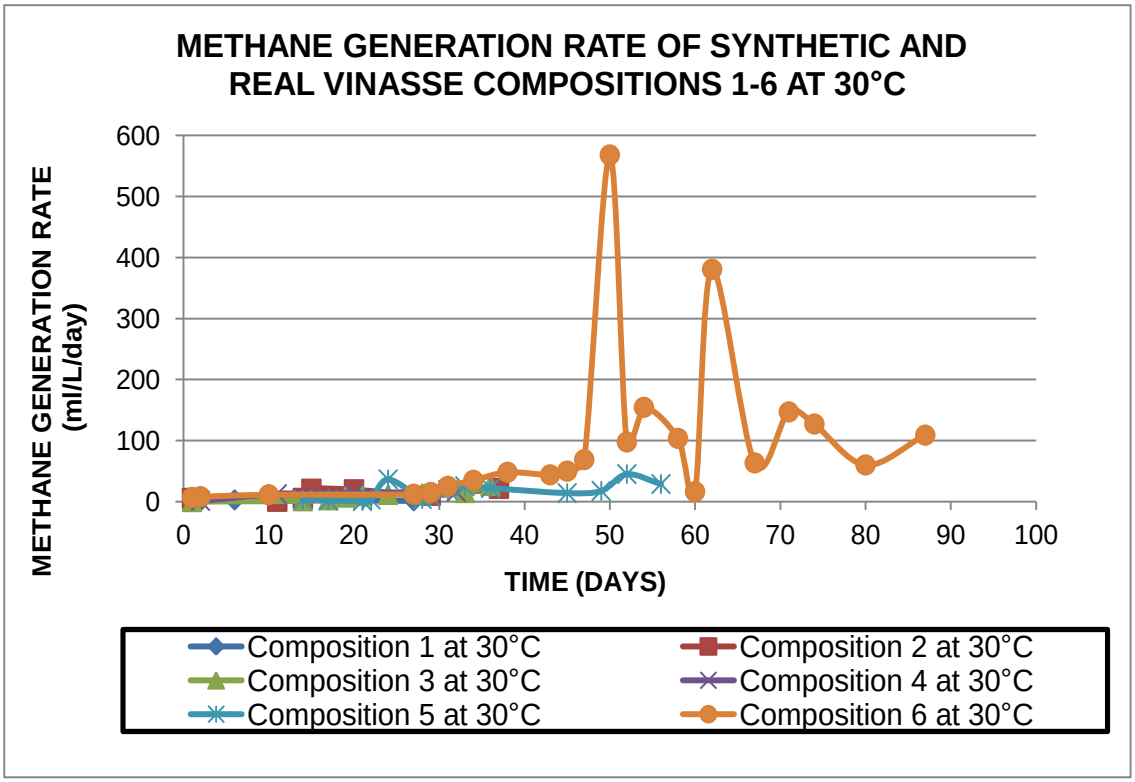


Figure 4.72 Methane Generation Rate of Synthetic and Real Vinasse Composition 1- 6 at 30°C

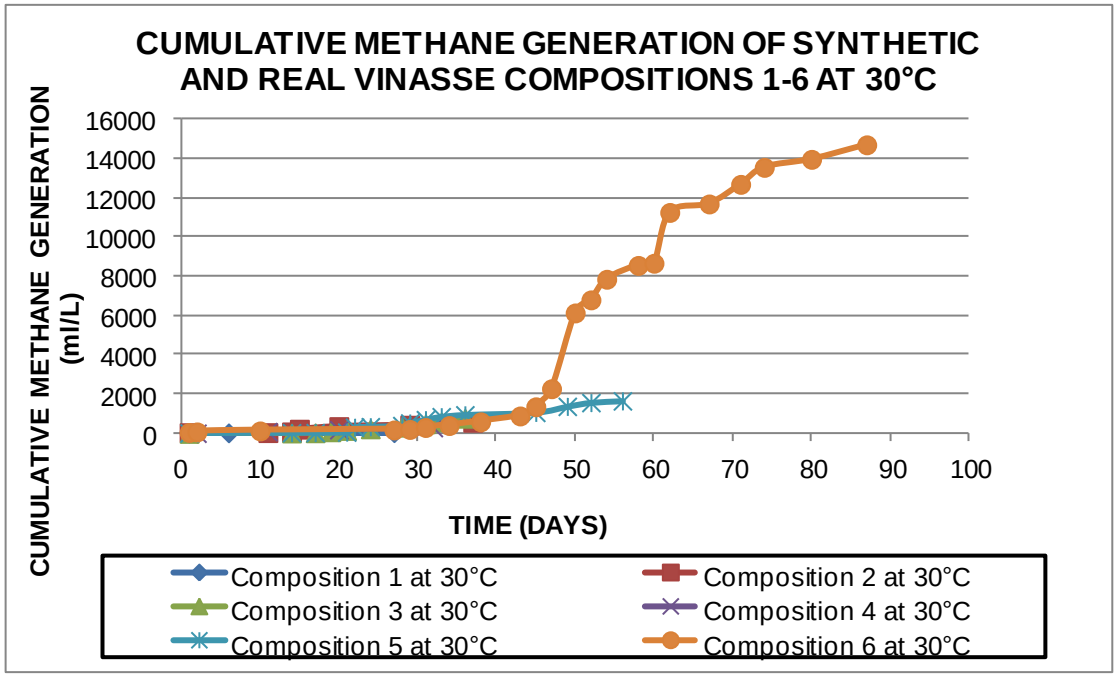


Figure 4.73 Cumulative Methane Generation of Synthetic and Real Vinasse Composition 1- 6 at 30°C

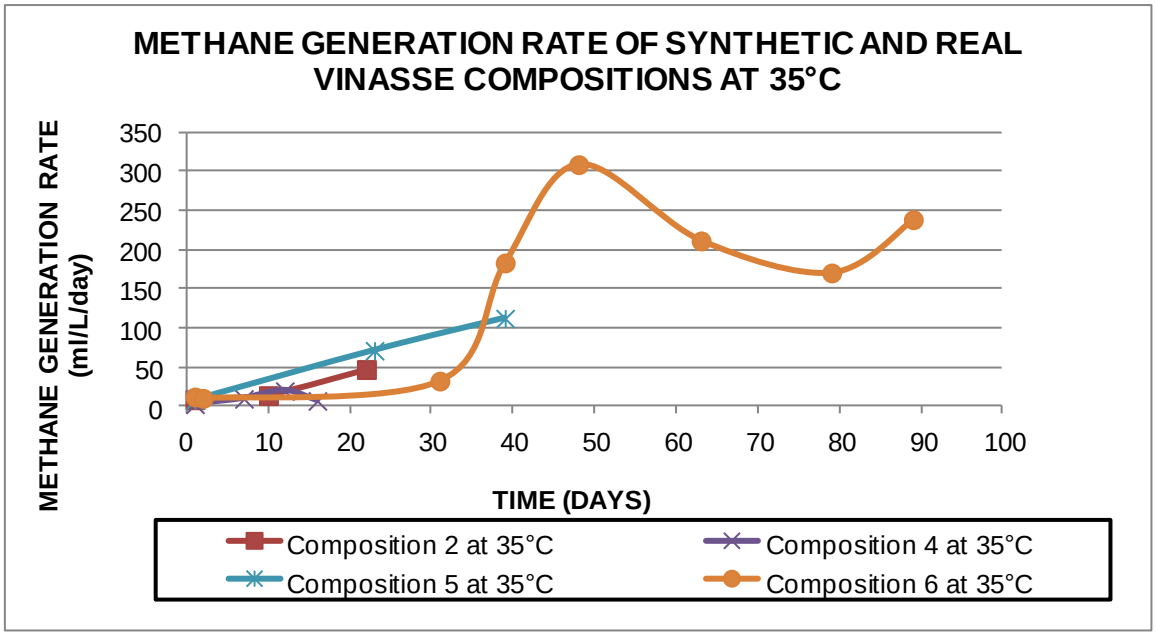


Figure 4.74 Methane Generation Rate of Real (Composition 2) and Synthetic Vinasse (Composition 4, 5 & 6) Compositions at 35°C

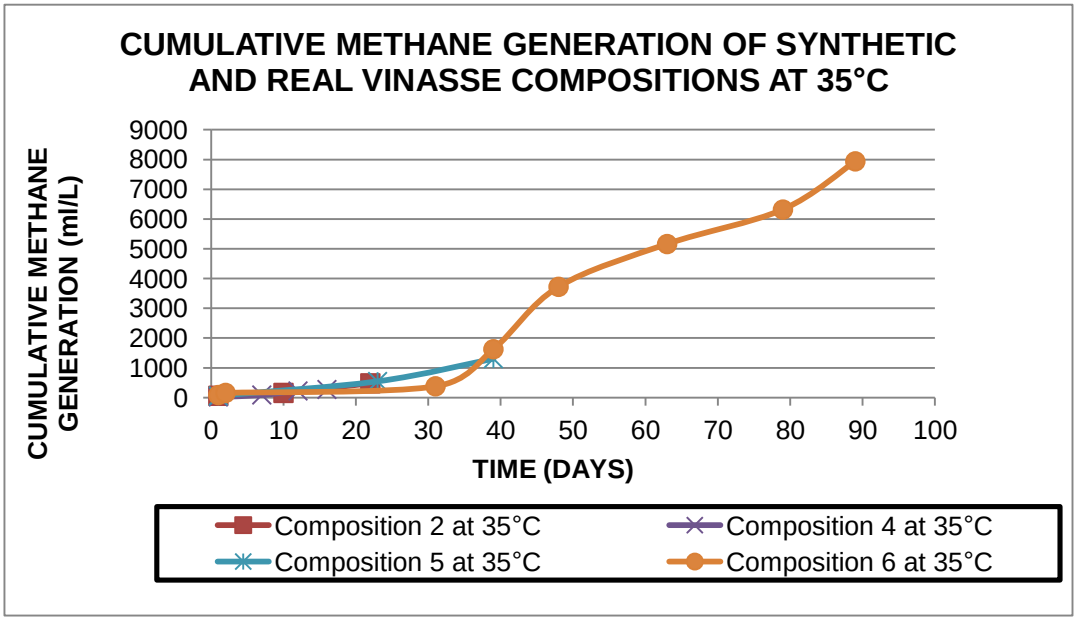


Figure 4.75 Cumulative Methane Generation of Real (Composition 2) and Synthetic Vinasse (Composition 4, 5 & 6) Compositions at 35°C

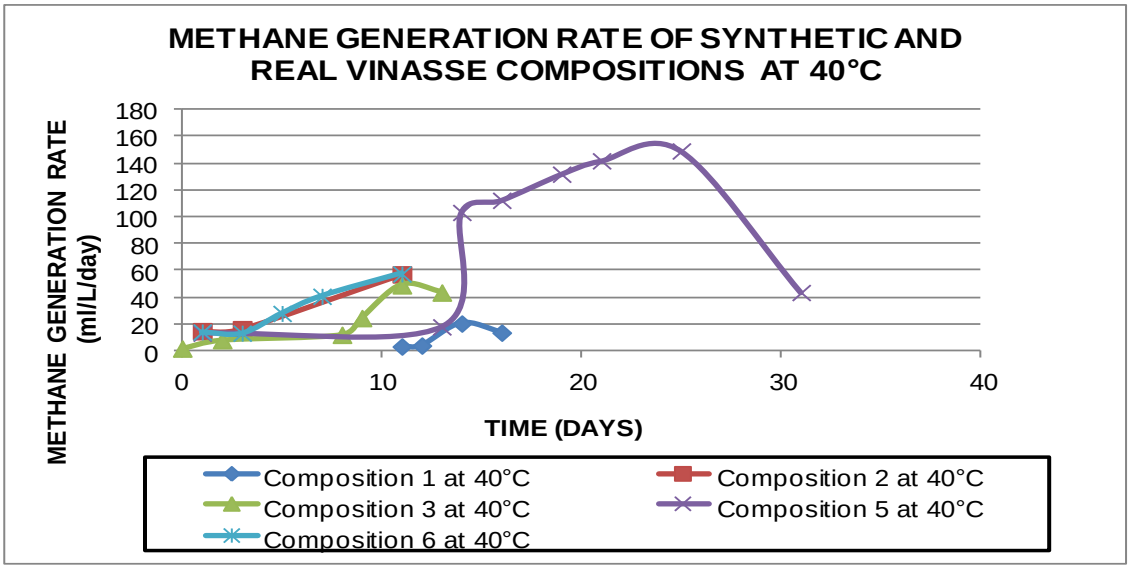


Figure 4.76 Methane Generation Rate of Real (Composition 1, 2 & 3) and Synthetic Vinasse (Composition 5 & 6) Compositions at 40°C

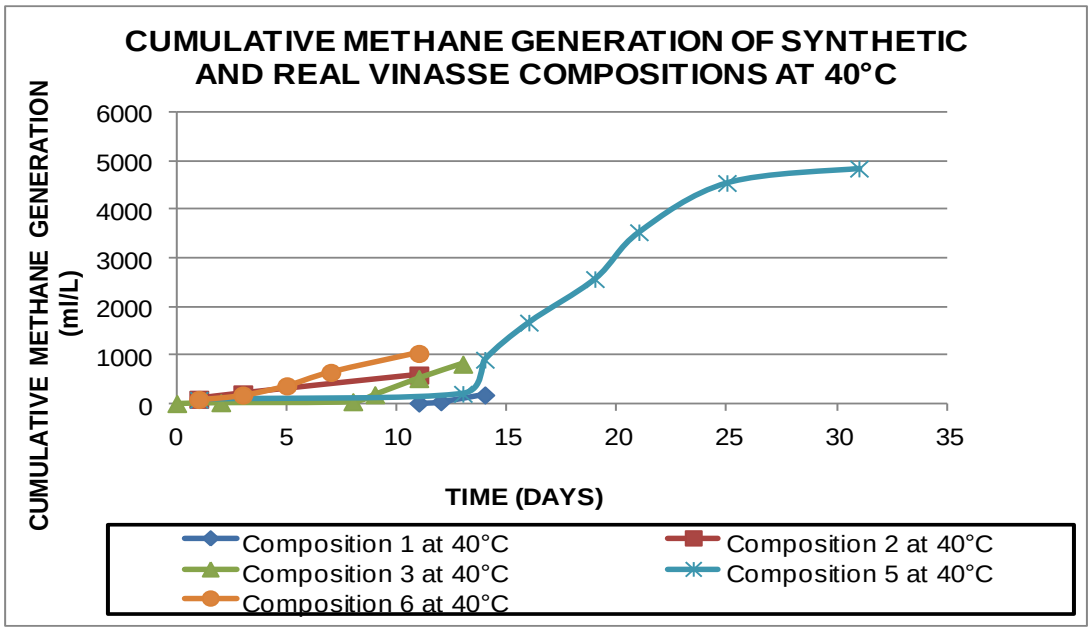


Figure 4.77 Cumulative Methane Generation of Real (Composition 1, 2 & 3) and Synthetic Vinasse (Composition 5 & 6) Compositions at 40°C

4.4.8 Comparison of Methane Generation Rates and Cumulative Methane Generation of Composition 2 (Produced from Corn and Milo, White Energy, TX) at 30°C and its Duplicate.

Figure 4.78 and Figure 4.79 show the methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from composition 2 at 30°C and it's duplicate, respectively. The peak methane generation rates were 22 and 33.22 ml/L/day; cumulative methane generation was 565 and 586 ml/L; and run times were 37 and 20 days for Composition 2 at 30°C and its duplicate, respectively. All values should have been the same, but were around 2 times higher for run time in real vinasse composition 2 compared to its duplicate. This discrepancy may have been due to leak in the duplicate reactor: because methane percentage of methane was almost same till day 14. The oxygen percentage was increased from 1 to 4 % in duplicate (20 days) reactor. In addition, the discrepancy may have been due to the fact that the real vinasse mixture was not homogenous and might contain different nutrients in both reactors.

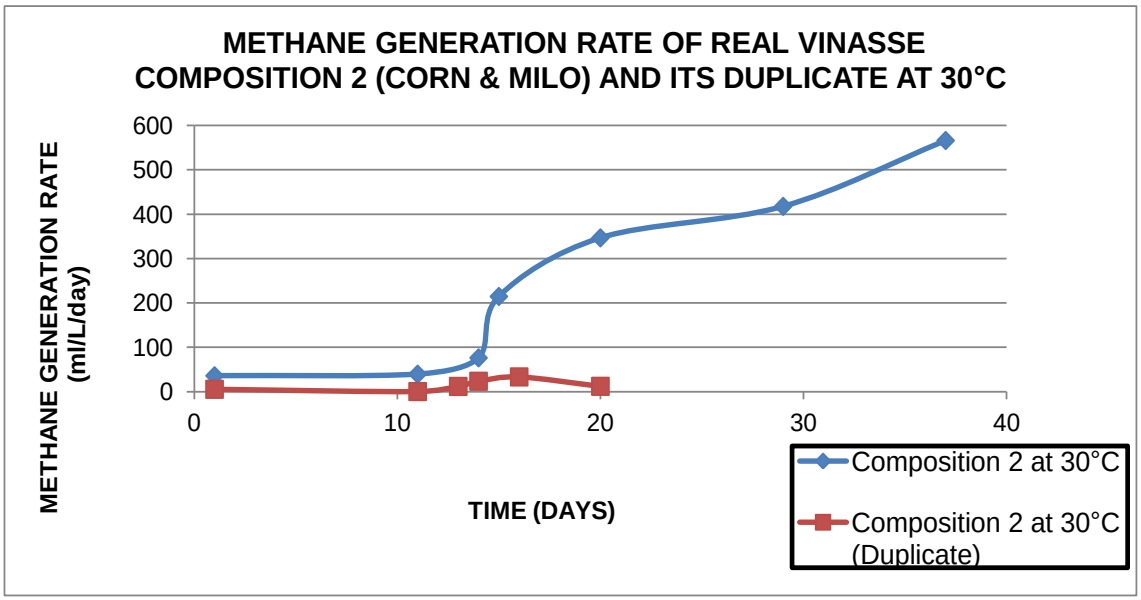


Figure 4.78 Methane Generation Rate of Composition 2 and its Duplicate (Corn and Milo, White Energy, TX) at 30°C

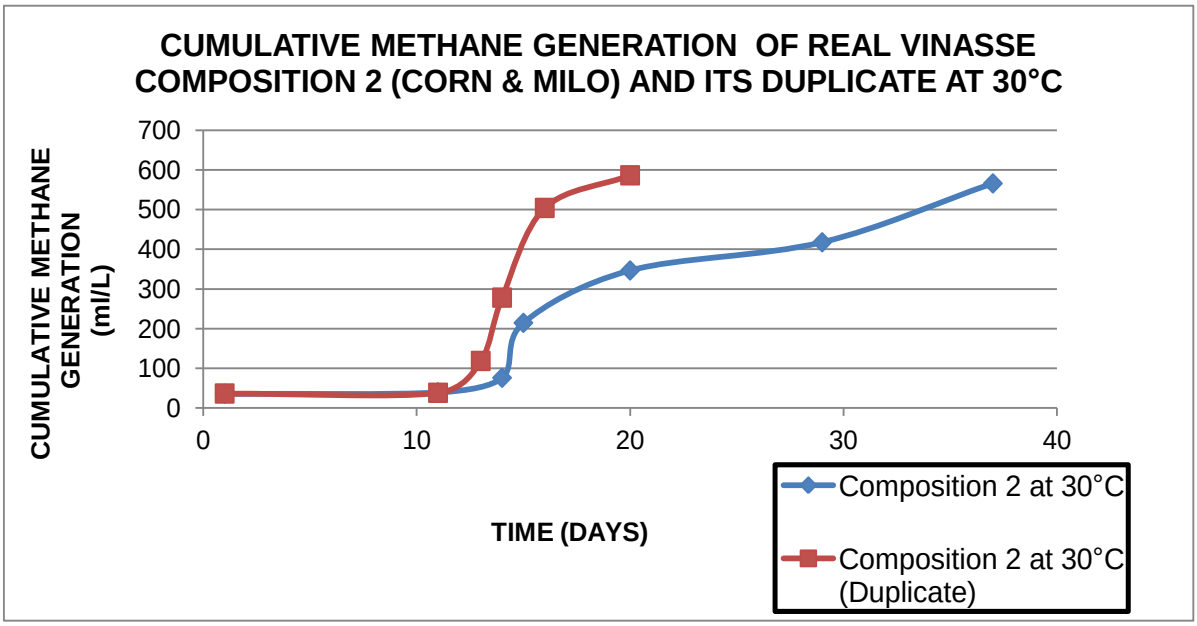


Figure 4.79 Cumulative Methane Generation of Composition 2 and its Duplicate (Corn and Milo, White Energy, TX) at 30°C

4.4.9 Comparison of Methane Generation Rates and Cumulative Methane Generation of Synthetic Vinasse Composition 5 (Medium COD) at 40°C and its Duplicate

Figure 4.80 and Figure 4.81 show methane generation rate (ml/L/day) and cumulative methane generation, at STP, per Liter of vinasse (ml/L), generated from synthetic vinasse composition 5 at 40°C and its duplicate, respectively. The maximum methane generation rates were 149 and 132 ml/L/day on day 25; the cumulative methane generated was 4846 and 4937 ml/L; and the run times were 31 days and 31 days for synthetic vinasse composition 5 at 40°C and its duplicate, respectively. These are very similar. The reason for this similarity is obvious that same amount of nutrients were added synthetically. So, the vinasse was homogenous and it gave the same environment for growing microbes inside the reactor.

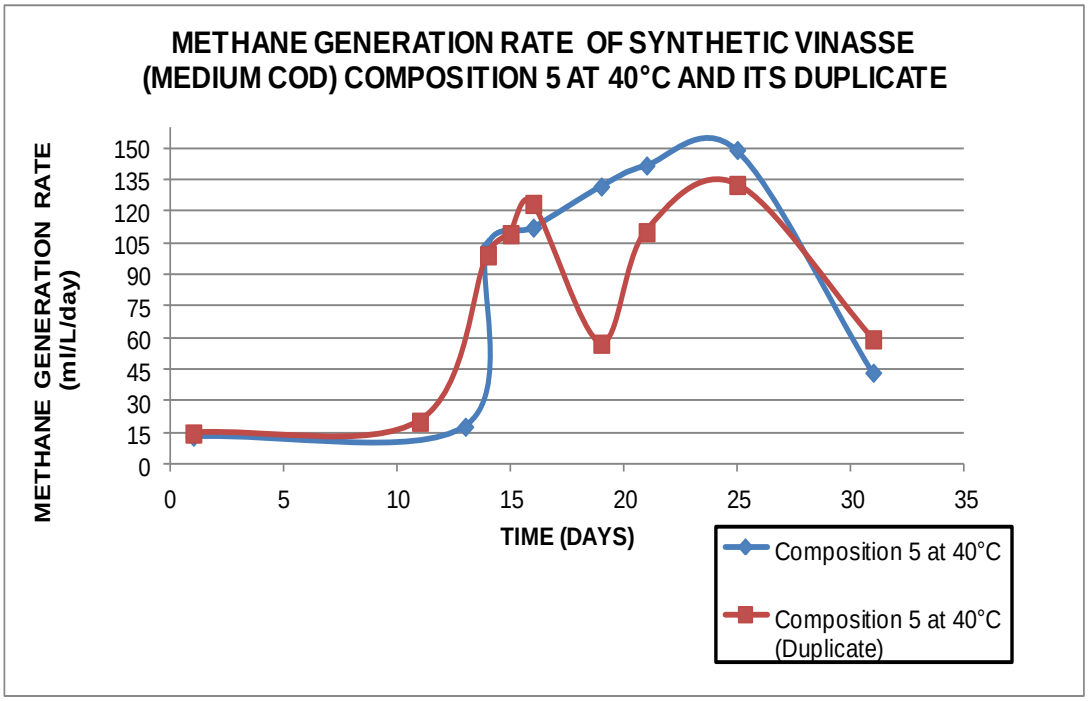


Figure 4.80 Methane Generation Rate of Composition 5 and its Duplicate (Medium COD) at 40°C

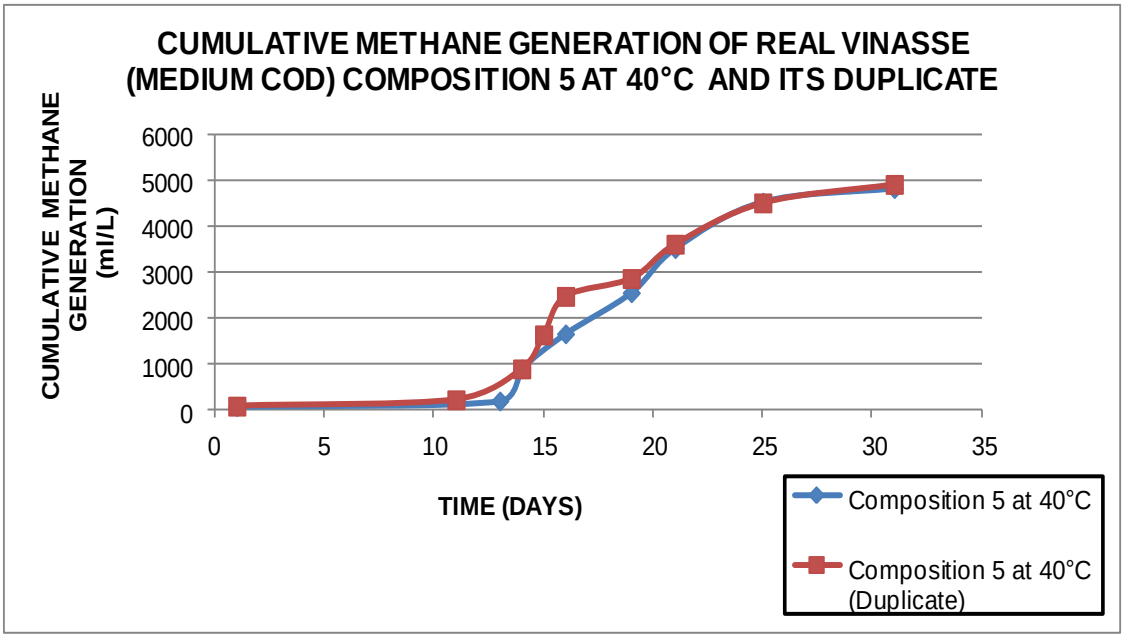


Figure 4.81 Cumulative Methane Generation of Composition 5 and its Duplicate (Medium COD) at 40°C

4.5 Data Synthesis

4.5.1 Impacts of Time on Microbial Communities

As shown in Table 4.25, there were no overall trends of microbial phyla with time. For certain runs, phyla may have increased or decreased, but the majority of the runs showed no clear trends. Even for a specific composition, there were no consistent trends with time across runs conducted at different temperatures.

One observation regarding time was that Methanogens generally survived for a longer time in synthetic vinasse composition, likely due to higher COD and the fact that the microbes were able to digest synthetic vinasse easier than real vinasse.

Table 4.25 Phyla Trends with Time for the Experimental Runs

Composition #	Temp. (°C)	Phyla Trends with Time				
		Euryarchaeota	Bacteroidetes	Firmicutes	Proteobacteria	Fusobacteria
1	35	Peaked days 17 and 25	Decreased	Increased	Increased	Decreased
	40	Peaked day 16	Peaked day 4	Fairly constant	Fairly constant	Fairly constant
2	30	Peaked on day 11	No clear trend	No clear trend	No clear trend	No clear trend
3	30	Peaked on day 17	Increased to days 12-17 and then decreased	Increased	Decreased	Decreased
	35	Peaked on day 15	No clear trend	No clear trend	No clear trend	No clear trend
	40	Peaked on day 11	Largest percent on first day	No clear trend	Increased	Decreased
4	40	Peaked on days 6 and 21	No clear trend	No clear trend	No clear trend	No clear trend
5	30	Peaked on day 31	No clear trend	No clear trend	No clear trend	No clear trend
	40	Peaked on day 25	No clear trend	No clear trend	No clear trend	No clear trend
6	30	Peaked on day 40	No clear trend	No clear trend	No clear trend	No clear trend

4.5.2 Impacts of Temperature on Run Duration and Microbial Communities

Table 4.26 and Figure 4.82 show total run time for each of the composition/temperature combinations. As temperature increases, the run time generally decreases. It was very clear that all compositions had their shortest duration at 40°C except synthetic vinasse composition 4 (as shown in Figure 4.82). As expected, the higher temperature promoted rapid microbiological activities as well as rapid nutrient consumption. The rate of reaction increases with increase in temperature. Reactors operated at higher temperatures converted COD to methane at a faster rate and ceased operation sooner.

Synthetic vinasse composition 4 at 35°C had sludge which was very thin compared to other reactors. As a result, microbes died earlier than expected. Synthetic vinasse composition 6 at 40°C shows fewer days compared to 30 and 35°C because there was a leak in the system. The percentage of oxygen increased from 1.8% (day 7) to 4.5% (day 11) in synthetic vinasse composition 6 at 40°C.

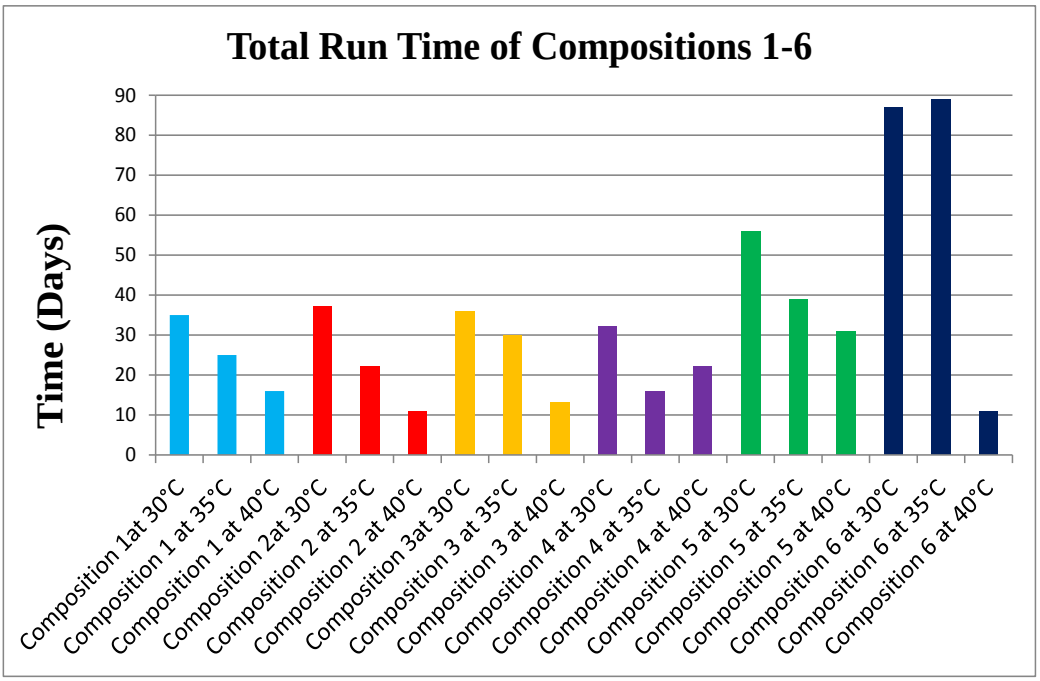


Figure 4.82 Total Run Time of Real and Synthetic Vinasse Compositions 1-6 at 30, 35, and 40°C

Table 4.26 Total Run Time of All Compositions at 30, 35, and 40°C

Composition	Temperature (°C)	Total Run Time (Days)
1 Real	30	35
	35	25
	40	16
2 Real	30	37
	35	22
	40	11
3 Real	30	36
	35	30
	40	13
Real Average		**25.6**
4 Synthetic	30	32
	35	16
	40	22
5 Synthetic	30	56
	35	39
	40	31
6 Synthetic	30	87
	35	89
	40	11
Synthetic Average		**42.6**

The total run times were generally shorter for the real vinasse combinations (average 25.6 days) than synthetic vinasse combinations (average 42.6) because real vinasse reactors microbes were not able to decompose food easily even at higher initial COD. This may have been due to the high amount of Na was added to the real vinasse compositions to maintain the pH in and during alkali treatment; it may

have caused the microbes to die because high Na which is toxic to microbes. Alternatively, the alkali treatment of real vinasse may not have been enough to breakdown complex sugars in the real vinasse into simple sugars. As a result, food may not have been readily available for microbes despite the high COD value.

As shown in Table 4.29 below, the maximum species richness and PD for compositions 1 and 3 is greater at 35°C than at 30°C or 40°C. For composition 5, the maximum species richness and PD is greater for 30°C compared to 40°C. 35°C, the midpoint of the mesophilic range, might be favorable to more microbes.

From Table 4.27, there do not appear to be any trends of maximum phyla percent with temperature. Synthetic vinasse combinations 5 and 6 produced much higher percentage of Archaea for a longer time at 30°C than higher temperature 40°C. This may have been because the rate of reaction is faster at higher temperature and methanogenesis phase was shorter and microbes used up all food faster at higher temperature. So, methanogens may have survived for a longer time at 30°C due to availability of food.

4.5.3 Impacts of Vinasse Composition on Microbial Communities

Table 4.27 and Table 4.28 show maximum and average percent of bacterial phyla for the experimental runs of different composition at three different temperatures. Table 4.29 shows the species richness and phylogenetic diversity for the experimental runs of different composition.

Table 4.27 Maximum Percent of Phyla for the Experimental Runs

Composition #	**Temp. (°C)**	**Maximum Percent of Phyla**				
		Euryarchaeota (%)	Bacteroidetes (%)	Firmicutes (%)	Proteobacteria (%)	Fusobacteria (%)
1 Real	35	1.9	31	45	40	25
1 Real	40	11	50	8	17	90
2 Real	30	2.6	85	80	40	60
3 Real	30	5.0	51	66	66	61

Table 4.27—*Continued*

3 Real	35	2.4	63	70	13	4
3 Real	40	4.2	14	7	37	90
4 Syn.	40	3.8	60	6	67	0
5 Syn.	30	6.8	90	56	75	4
5 Syn	40	6.5	54	52	83	3
6 Syn.	30	7.2	77	37	66	15

Table 4.28 Average Percent of Phyla for the Experimental Runs

Composition #	**Temp. (°C)**	**Average Percent of Phyla**				
		Euryarchaeota (%)	Bacteroidetes (%)	Firmicutes (%)	Proteobacteria (%)	Fusobacteria (%)
1 Real	35	1.23	18	31	32	15
	40	2.63	9	5	12	70
2 Real	30	0.49	48	21	20	8
3 Real	30	1.23	28	35	11	23
	35	1.03	47	38	7	1
	40	.0.71	3	3	22	72
4 Syn.	40	2.37	44	4	15	1
5 Syn.	30	2.87	63	10	13	1
	40	0.91	30	22	37	1
6 Syn.	30	2.30	55	23	14	1

Table 4.29 Species Richness and Phylogenetic Diversity Present in 6 Compositions

Composition	Temp. (°C)	MAXIMUM SPECIES RICHNESS	DAY	MAXIMUM FAITH'PD	DAY
1 Real	35	1248	17	69	17
	40	536	12	31	12
2 Real	30	1096	11	61	11
3 Real	30	668	10	39	10
	35	989	19	59	23
	40	602	5	32	5
4 Syn.	40	1255	14	71	14
5 Syn.	30	1768	10	107	10
	40	1042	11	54	11
6 Syn.	30	1092	50	59	50

The maximum species richness for compositions 3 (real vinasse) and 5 (synthetic vinasse) at 30°C is higher than 40°C and real vinasse composition 1 at 35°C is also higher in compare to composition 1 at 40°C. Hence, the maximum species richness for each composition is greater at lower temperature than higher temperature. It is possible that microbes survived for a longer time due to slow rate of reaction and higher COD at lower temperature as compare to faster reaction at higher temperature.

All synthetic vinasse compositions at 30, 35, and 40°C contain higher percentages of Bacteroidetes, Firmicutes, and Proteobacteria and lower percentages of Acidobacteria, Tenericutes, Spirochaetes, Synergistetes, Chloroflexi**,** Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, Synergistetes, and Plantomycetes. Synthetic vinasse contains NKB19, OP11, OP3, OP8, OP9 in lower percents. However, these microbes were not present in real vinasse. Real vinasse contains high percentages of Fusobacteria but these microbes were present in very small amount in synthetic vinasse.

The synthetic vinasse (compositions 4, 5, and 6) had higher percentages of Archaea in the overall runs compared to real vinasse compositions. The synthetic vinasse thus produced higher levels of methane compared to the real vinasse combinations. This may have been due to the high amount of Na was added to the real vinasse compositions to maintain the pH in and during alkali treatment; it may have caused the microbes to die because high Na which is toxic to microbes. Alternatively, the alkali treatment of real vinasse may not have been enough to breakdown complex sugars in the real vinasse into simple sugars. As a result, food may not have been readily available for microbes despite the high COD value. Small percents of the phyla Acidogens were present in compositions 4 and 6 of synthetic vinasse. Acidobacteria were not present at 40ºC of real vinasse combinations and 40ºC of composition 5.

As shown in Table 4.29, Composition 5 has lower COD but it shows highest species richness and Faith's PD. The reason is not clear. Real vinasse compositions shows lower species richness and Faith's PD at 40°C compared to synthetic vinasse compositions at 40°C. It might be possible that large number of microbes were not able to reach (or survive) into next phase due to addition of high amount of NaOH during alkali treatment and maintaining the pH and died earlier at higher temperature. It is possible that alkali treatment was not enough to breakdown complex sugars into simple sugars. As a result, food was not readily available as a food for microbes even after high COD value. However, alkali treatment was not performed with synthetic vinasse and pH drop was less in synthetic vinasse combinations. Glucose was easily digestible for microbes. Hence, microbial activity was observed for a longer period of time in synthetic vinasse. As a result, high species richness and phylogenetic diversity was observed in synthetic vinasse combinations at higher temperature.

4.5.4 Impacts of Vinasse Composition on Microbial Methane Generation

Anaerobic decomposition of liquid waste occurs in four stages: (i) aerobic phase, (ii) acidogenesis (acid formation), (ii) methanogenesis (methane formation), (iv) decelerating methane phase. From Appendix A, it can be observed that initially the percent oxygen in the reactors was high (>10%), and reduced rapidly. This being the aerobic phase, gas mainly consisted of carbon dioxide and other gases (H_2S, and nitrogen compounds). In this phase, methane content in all the reactors was less than 2%.The maximum methane percent, cumulative methane generation, and maximum methane generation rate of all six compositions at three different temperatures are given in Table 4.30. The red color in Table 4.30

indicates the maximum methane percent of the same composition among 3 different temperatures. The gas compositions (CO_2, O_2, CH_4 and others) of all real and synthetic vinasse compositions are given in Appendix B.

Compositions 3, 5 and 6 produced maximum percent of methane compared to compositions 1, 2, and 4 and Methanogens survived for a longer period of time in compositions 3, 5 and 6. The reason is not clear. The methane percents for composition 5 were fairly consistent for all 3 temperatures, ranging from 74.1% to 81.5%. However, for the other compositions, methane percentages varied by a factor of 2 or more among temperatures. The reason for this is not known. It can be seen from the methane generation Table 4.30 that the percentage of methane generated from composition 6 at 30 and 35°C was higher than that from other compositions. This could be due to higher amount of glucose present in synthetic vinasse composition 6. Microbes had more food than other compositions. However, synthetic vinasse combination 5 also produced high percentage of methane which has less COD (1/3rd) compared to composition 6. This indicates that right amount of glucose (9.5-31 g/L) may play an important role in anaerobic digestion of vinasse which helps in methanogenesis phase.

The linear regression line was also plotted between percentages of Archaea (Methanogens) versus % of methane of composition 6 at 30°C (as shown in Figure 4.83). It shows the correlation between them. The linear regression line was also plotted between average percentages of Euryarchaeota (Methanogens) versus maximum % of methane, Maximum methane generation rate, and Cumulative methane generation. However, it does not show any correlation between them. It is likely that the absolute number of methanogens, which was not measured in this study, was more critical in determining the methane percent, maximum generation rate, and cumulative methane generation than the percent of methanogens.

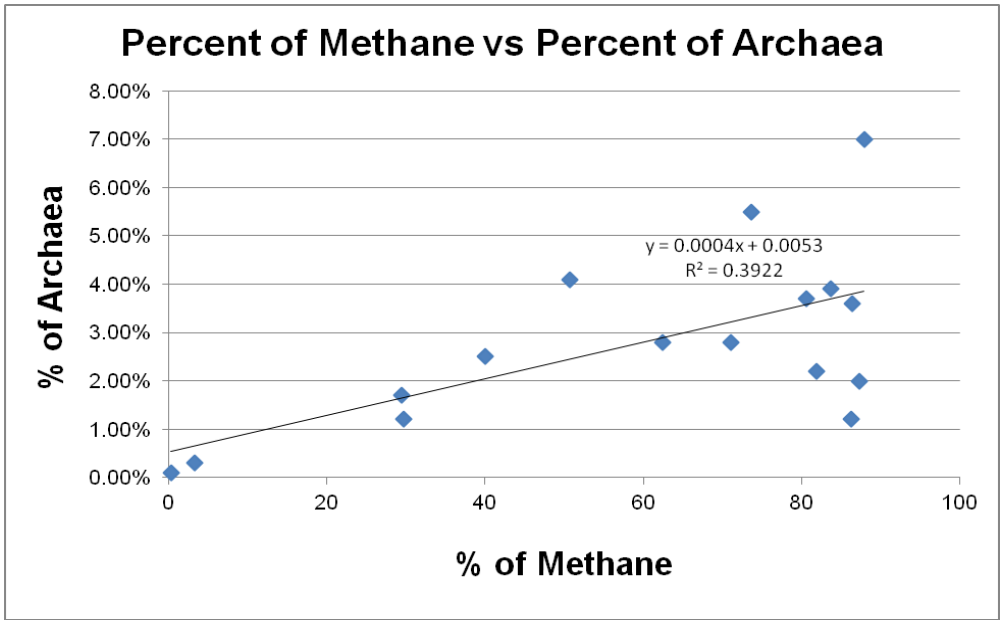


Figure 4.83 Percent of Methane vs Percent of Archaea of Composition 6 (High COD) at 30°C

Table 4.30 Maximum % Methane, Cumulative Methane Generation, and Maximum Methane Generation Rate for Real and Synthetic Vinasse Compositions 1-6 at Different Temperatures

Composition #	Temp. (°C)	Max. Percent Euryarchaeota	Methane Maximum				Cumulative Methane Generation (ml/L)	Maximum Methane Generation Rate (ml/L/day)
			Day	%	pH			
1 Real	30	N/A	35	2.3	7.2		36	2.79
	35	2%	25	41.1	7.1		N/A	N/A
	40	>10%	16	25.2	7.2		280	20
2 Real	30	3%	37	24	7.06		565	22
	35	N/A	22	48.3	7.55		477	47
	40	N/A	11	34.5	7.98		598	57
3 Real	30	5%	36	31.8	7.18		771	25
	35	2%	20	78.2	7.95		N/A	N/A
	40	4%	13	29.6	7.51		821	48
4 Synthetic	30	N/A	32	37.4	8.24		296	16.04
	35	N/A	16	19.9	7.75		271	20
	40	4%	14	38.6	7.84		N/A	N/A

Table 4.30—*Continued*

5. Synthetic	30	7%	49	74.1	8.11		1655	45
	35	N/A	39	81.5	8.24		1305	112
	40	7%	31	80.7	8.4		4846	149
6 Synthetic	30	7%	71	87.9	8.5		14706	568
	35	N/A	63	87.7	8.3		7935	309
	40	N/A	11	44.6	7.87		1042	57

4.5.5 Reactor Duplicates

As discussed earlier, reactor duplicates were run for real vinasse composition 2 (Corn and Milo, White Energy, TX) at 30°C and synthetic vinasse composition 5 (Medium COD) at 40°C. Table 4.31 gives the difference between maximum species richness and maximum Faith's PD composition 2 and 5 with their duplicates. Composition 2 shows higher percent differences in maximum species richness (13%) and maximum Faith's PD (26%) compared to composition 5 maximum species richness (12%) and maximum Faith's PD (16%), as shown in Table 4.31.

As shown in Table 4.32, Composition 2 produced higher percentage of methane and maximum methane generation (22 and 33 ml/L/day) rate than its duplicate. However, the cumulative methane generation was almost same (565 and 586 ml/L), as expected due to equal COD. Duplicate reactors of composition 2 produced 24% methane and 30% methane and duplicate reactors of composition 5 produced same percent of methane 80.7%. Duplicate results for composition 2 (real vinasse) produced the maximum percentage of methane on 37^{th} and 20^{th} day because the color and odor of vinasse changed with time in cold storage room (4°C).

The differences in species richness, PD, methane percent, and methane generation rate for composition 2 and its duplicate might be due to the amount of microbes present in the sludge varying among runs because sludge was collected from the wastewater treatment plant for different runs on different days. In addition, the vinasse mixture for composition 2 (real vinasse) was likely not homogenous. As a result, the amount of nutrients was likely not similar in these reactors. This variation in composition of vinasse may cause these changes.

However, duplicate results for composition 5 (synthetic vinasse) were similar. Duplicates reactors of synthetic vinasse (composition 5) produced the same percentage of methane on the same day (day 31). The maximum methane generation (149 and 132 ml/L/day) rate and cumulative methane generation (4846 and 4937 ml/L) was almost same, as expected due to equal amount of COD. Synthetic vinasse contained the same amount of nutrients which were added synthetically in composition 5. Hence, the microbes had the same amount of food and nutrients to grow in the bioreactor.

Table 4.31 Percent Different in Maximum Species Richness and Maximum Faith's PD of Composition 5 and 2 with its Duplicates

Composition #	**Temperature (°C)**	**Maximum Species Richness (% difference)**	**Maximum Faith PD (% difference)**
Composition 2 and Composition 2 (Duplicate)	30	13%	26
Composition 5 and Composition 5 (Duplicate)	40	12%	16%

Table 4.32 Cumulative Methane Generation, Maximum Methane Generation Rate, and Total Run Time for Duplicate Compositions at Different Temperature and pH

Composition #	**Temperature (°C)**	**Day**	**Maximum Methane (%)**	**pH**	**Total Run Time (days)**	**Cumulative Methane Generation (ml/L)**	**Maximum Methane Generation Rate (ml/L/day)**
Composition 2	30	37	24	7.06	37	565	22
Composition 2 (Duplicate)	30	20	29.9	7.36	20	586	33
Composition 5	40	31	80.7	8.4	31	4846	149
Composition 5 (Duplicate)	40	31	80.7	8.3	31	4937	132

4.5.6 Sample Duplicates

The sample duplicates of real and synthetic vinasse compositions 1, 3, 5 and 6 at different temperatures show the same types of microbes in its composition. The samples duplicates of synthetic vinasse shows almost same percentage of microbes in it composition, whereas sample duplicates of real vinasse composition shows same types of microbes with little variation in percentage of phyla. It might be possible due to real vinasse mixture was not homogeneous during different run. The percent difference between maximum species richness and maximum Faith's PD of each sample duplicates are given below in Table 4.33. This table shows large percent difference in species richness and Faith's PD of composition 3 (35) and Composition 3 (35_D) and Composition 3 (5) and Composition 3 (5_D) compared to others. This might be possible because real vinasse mixture was not homogenous in each run.

Table 4.33 Percent Difference of Sample Duplicates Between Species Richness and Faith's PD

Sample Duplicates	Temperature (°C)	Day	SpeciesRichness (% Difference)	Faith's PD (% Difference)
Composition 1 (8) and Composition 1(8_D)	40	8	6.5	6.3
Composition 3 (35) and Composition 3 (35_D)	30	35	27	24
Composition 3 (5) and Composition 3 (5_D)	40	5	44	38
Composition 3 (7) and Composition 3 (7_D)	40	7	5.6	5.68
Composition 5 (14) and Composition 5 (14_D)	40	14	16	5.8
Composition 6 (1) and Composition 6 (1_D)	30	1	0.78	18

Chapter 5

Conclusions & Future Recommendations

5.1 Conclusions

This research shows the different types of microbes present in real and synthetic vinasse at various stages of anaerobic degradation at mesophilic temperatures 30, 35, and 40°C.

Impacts of Time on Microbial Communities

- There were no overall trends of microbial phyla with time.

Impacts of Temperature on Run Duration and Microbial Communities

- As temperature increased, the run time generally decreased, as expected.
- The total run times were generally shorter for the real vinasse combinations (average 25.6 days) than synthetic vinasse combinations (average 42.6) because microbes were not able to decompose real vinasse easily.
- The maximum species richness and PD for compositions 1 and 3 is greater at 35°C than at 30°C or 40°C. For composition 5, the maximum species richness and PD is greater for 30°C compared to 40°C. 35°C, the midpoint of the mesophilic range, might be favorable to more species.
- There were not any trends of maximum phyla percent with temperature.

Impacts of Vinasse Composition on Microbial Communities

- Composition 5 synthetic vinasse, medium COD, had the highest species richness and Faith's phylogenetic diversity.
- All synthetic and real vinasse combinations at 30, 35, and 40°C contain higher percentages of Bacteroidetes (except 3 at 40°C), Firmicutes (except 3 and 4 at 40°C), and Proteobacteria (except 3 at 35°C) and lower percentages of Acidobacteria, Tenericutes, Spirochaetes, Synergistetes. Chloroflexi,

Lentisphaerae, WWE1, Verrucomicrobia, Tenericutes, Spirochaetes, Synergistetes, and Plantomycetes.

- The synthetic vinasse (combinations 4, 5, and 6) had higher percentages of Archaea in the overall runs compared to real vinasse compositions. The synthetic vinasse thus produced higher levels of methane compared to the real vinasse combinations.

Impacts of Vinasse Composition on Microbial Methane Generation

- The percentage of methane generated from composition 6 at 30 and 35°C was higher than that from other compositions. This could be due to higher amount of glucose present in synthetic vinasse composition 6.
- There were no correlations between microbial phyla percents and maximum methane percent, maximum methane generation rate, or cumulative methane generation except percentage of Archaea versus percentage of methane.

5.2 Recommendations for Future Research

- Identify microbes present in sludge before seeding into reactor.
- Use automatic pH controller to avoid leaks in the system.
- Develop a method to maintain the pH without adding high amount of sodium hydroxide which cannot be toxic to microbes. Also, it would be expensive to add NaOH in large scale bioreactor.
- Identify methods to use the liquid waste resulting after anaerobic digestion of vinasse.
- Use sludge from different treatment plants to see the effect on methane generation.
- Use of Real Vinasse from different food grains (other than milo and corn) to see the methane generation rate.

Appendix A

pH & CH_4 (%) of Real and Synthetic Vinasse

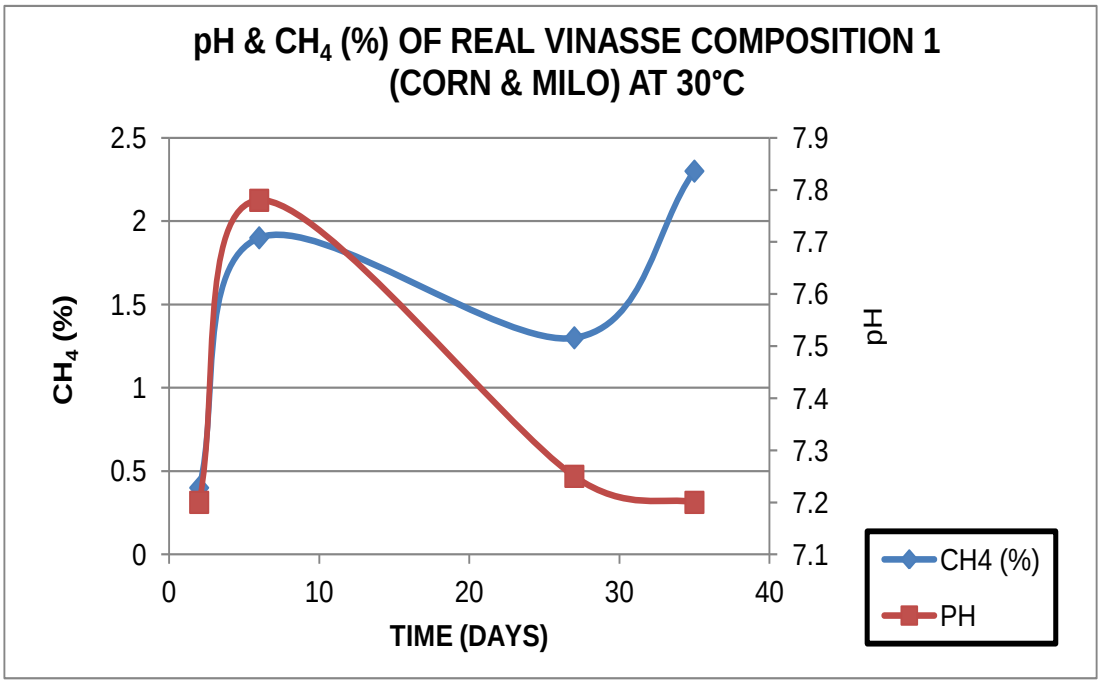


Figure A.1: pH and CH_4 (%) of Real Vinasse composition 1 (Corn & Milo from White Energy, TX) at 30°C

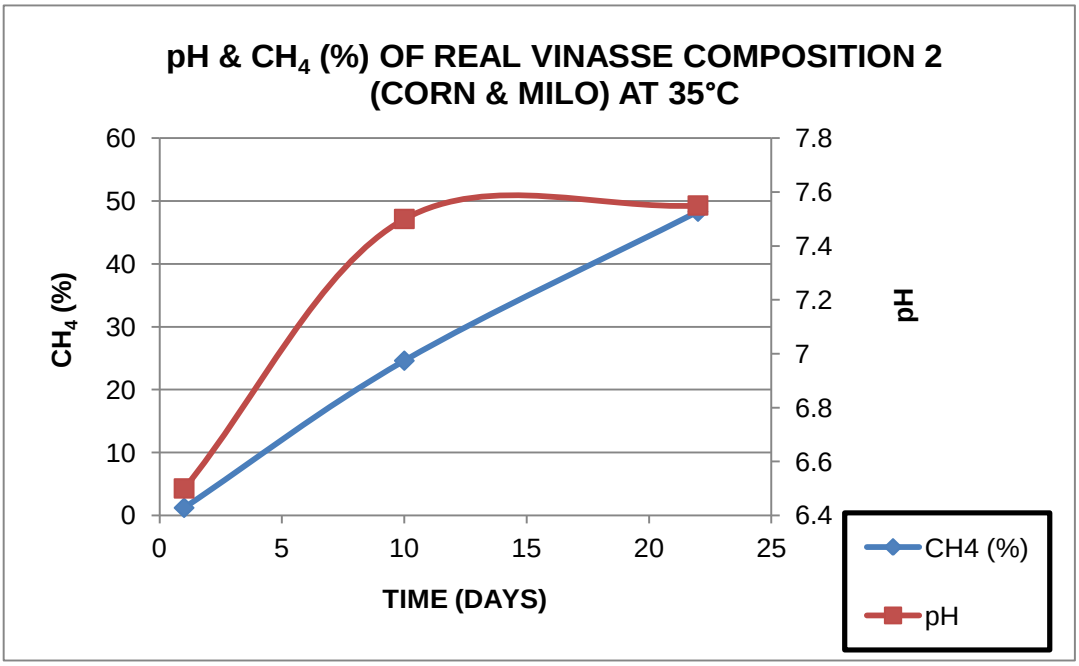


Figure A.2: pH and CH_4 (%) of Real Vinasse composition 2 (Corn & Milo from White Energy, TX) at 35°C

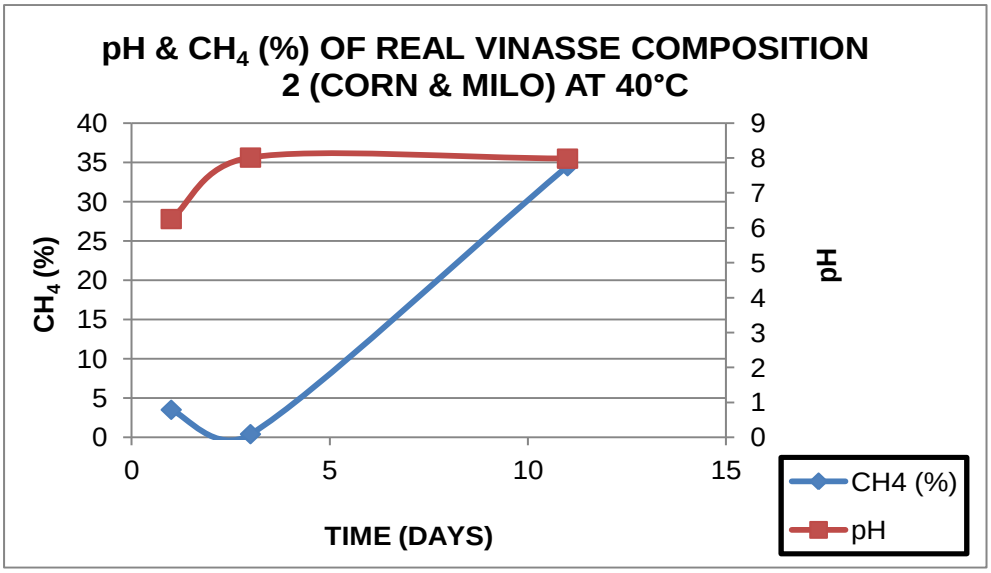


Figure A.3: pH and CH_4 (%) of Real Vinasse Composition 2 (Corn & Milo from White Energy, TX) at 40°C

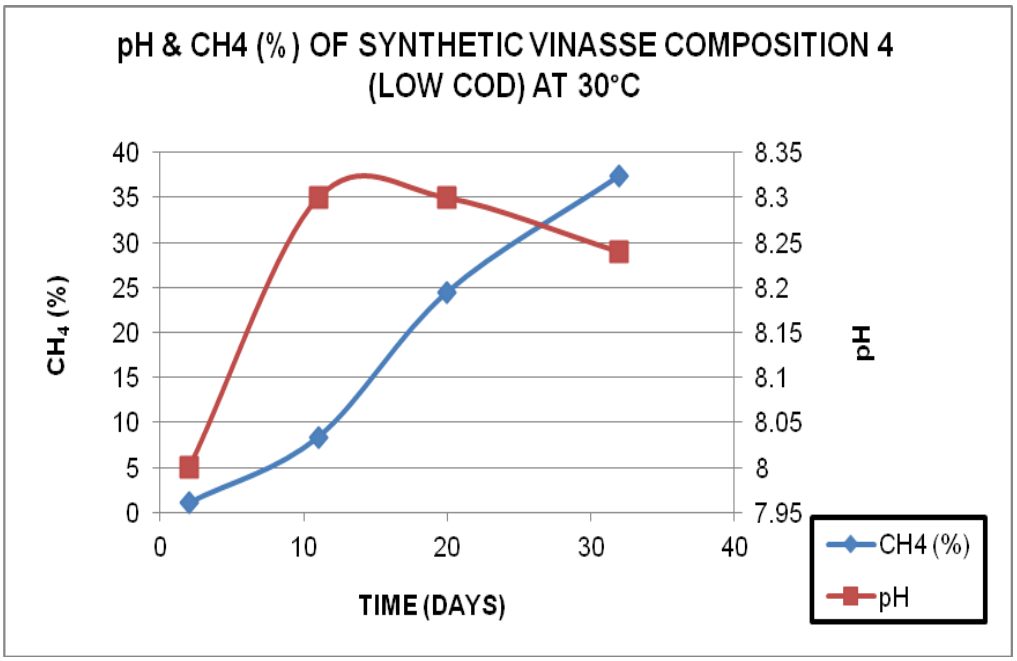


Figure A.4: pH and CH_4 (%) of Synthetic Vinasse Composition 4 (Low COD) at 30°C

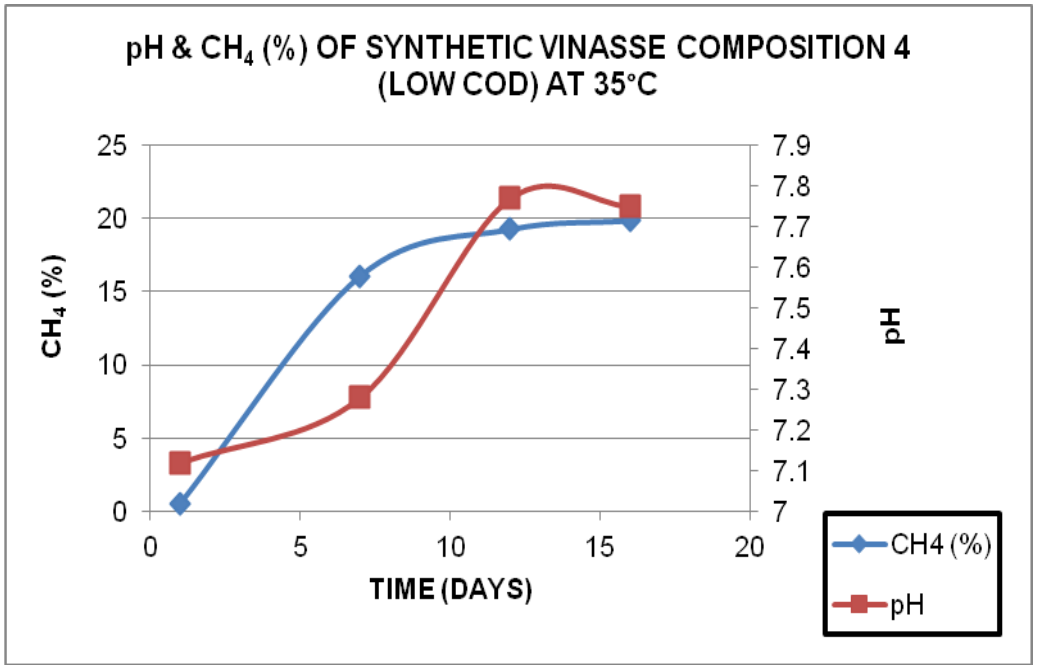


Figure A.5: pH and CH_4 (%) of Real Vinasse Composition 4 (Low COD) at 35°C

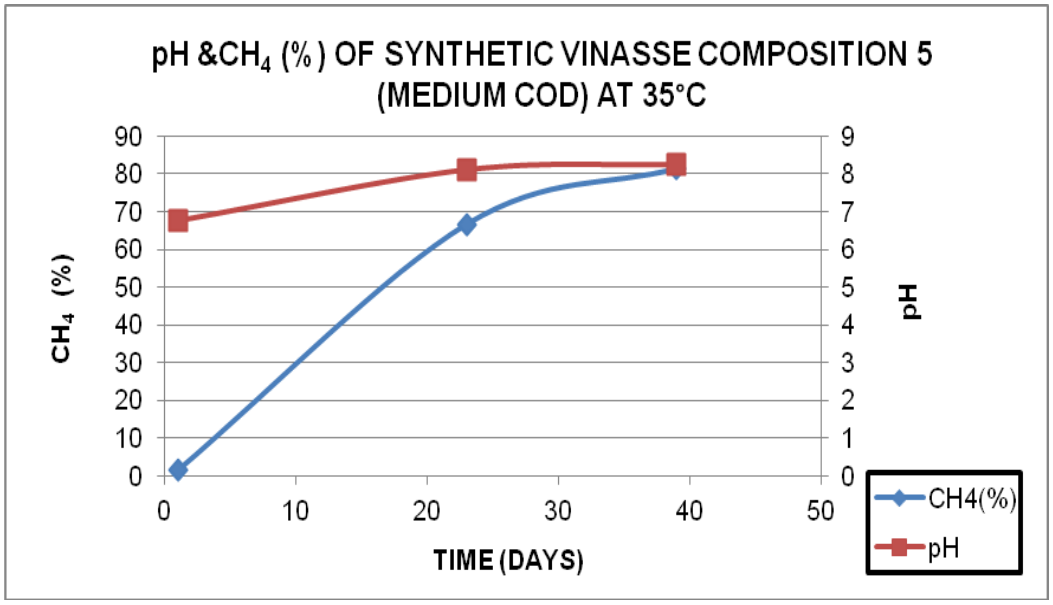


Figure A.6: pH and CH_4 (%) of Real Vinasse Composition 5 (Medium COD) at 35°C

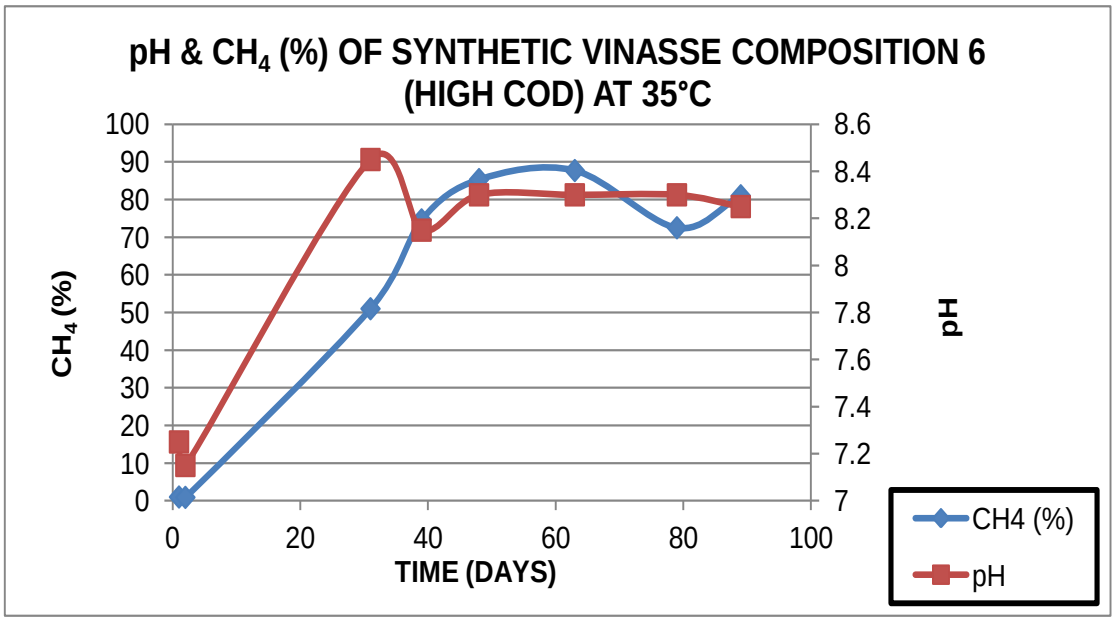


Figure A.7: pH and CH_4 (%) of Synthetic Vinasse Composition 6 (High COD) at 35°C

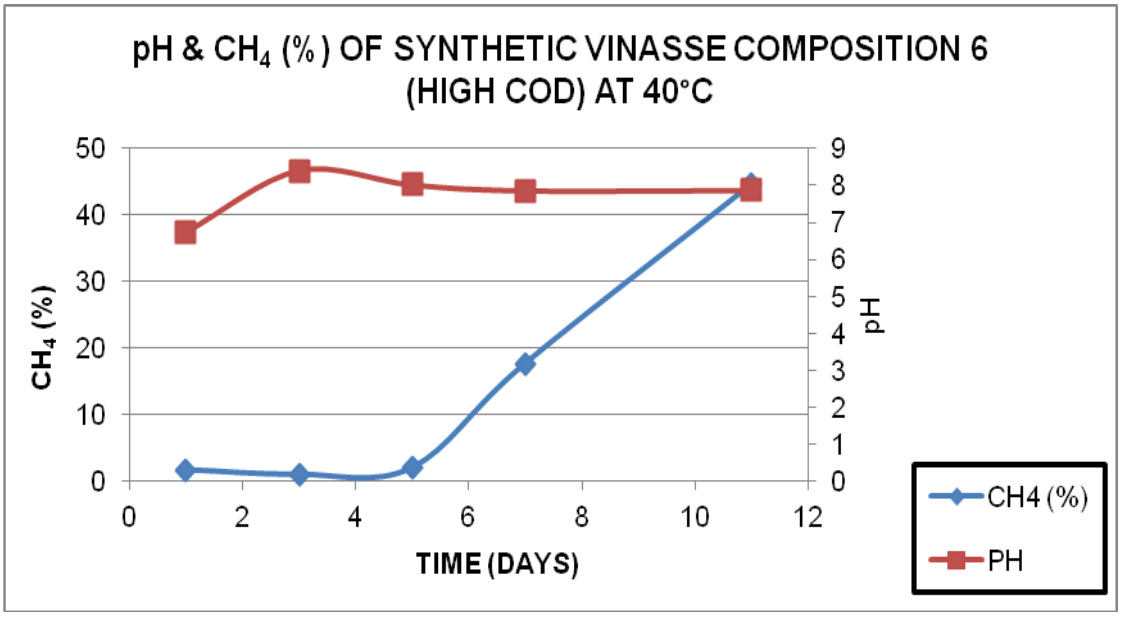


Figure A.8: pH and CH_4 (%) of Synthetic Vinasse Composition 6 (High COD) at 40°C

Appendix B

Gas Compositions of Real And Synthetic Vinasse

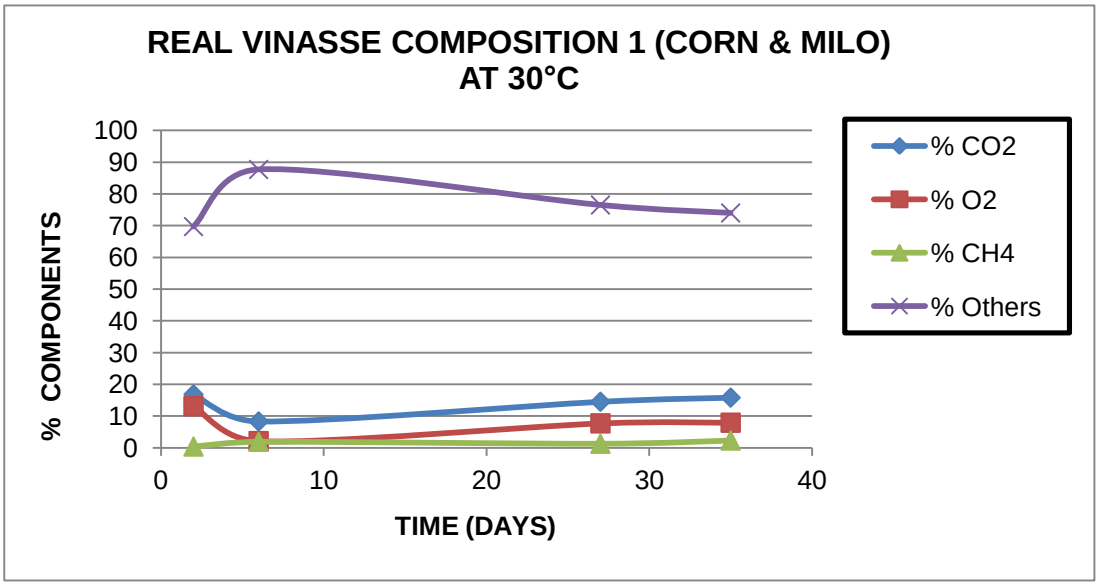


Figure B.1: Gas Compositions in Real Vinasse Composition 1 (Produced from Corn and Milo, White Energy, TX) at 30°C

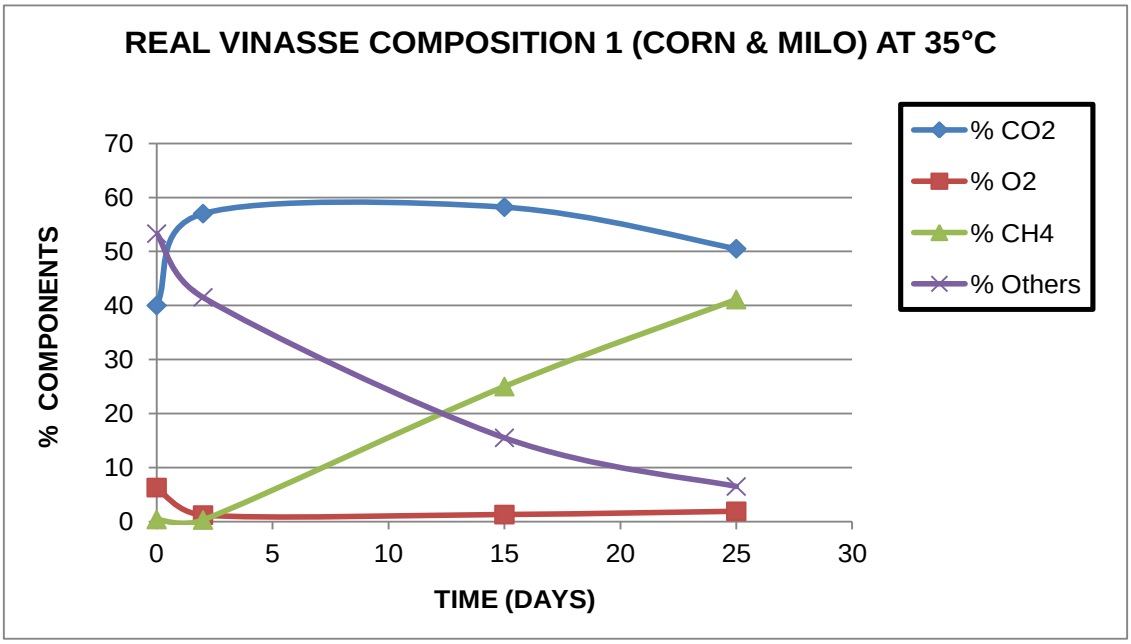


Figure B.2: Gas compositions in Real Vinasse Composition 1 (Produced from Corn and Milo, White Energy, TX) at 35°C

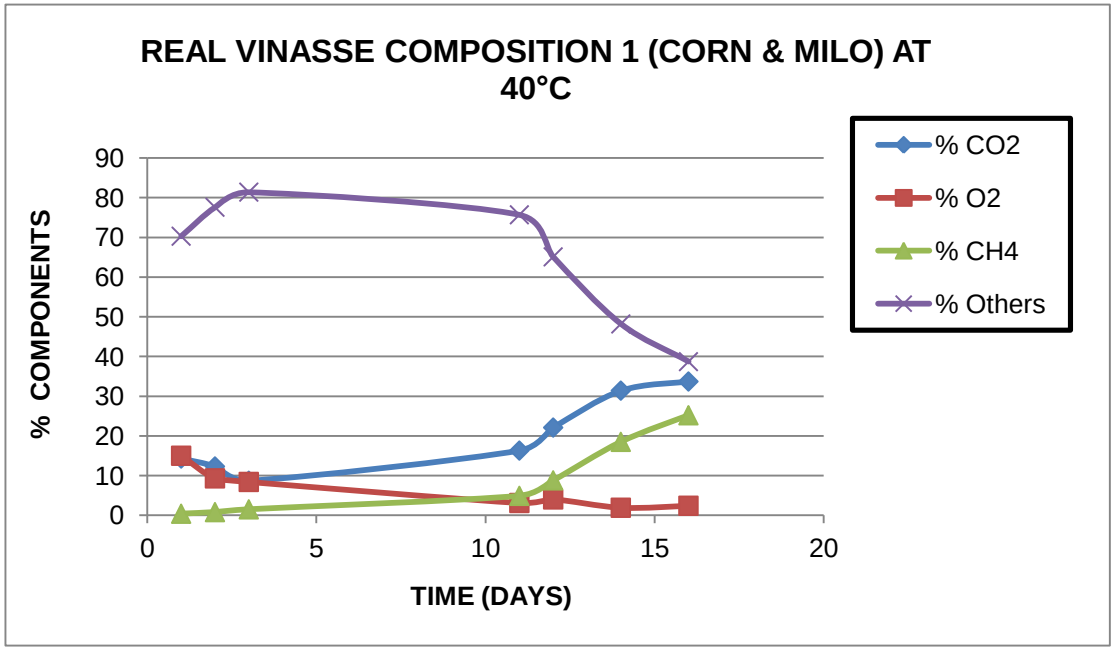


Figure B.3: Gas compositions in Real Vinasse Composition 1 (Produced from Corn and Milo, White Energy, TX) at 40°C

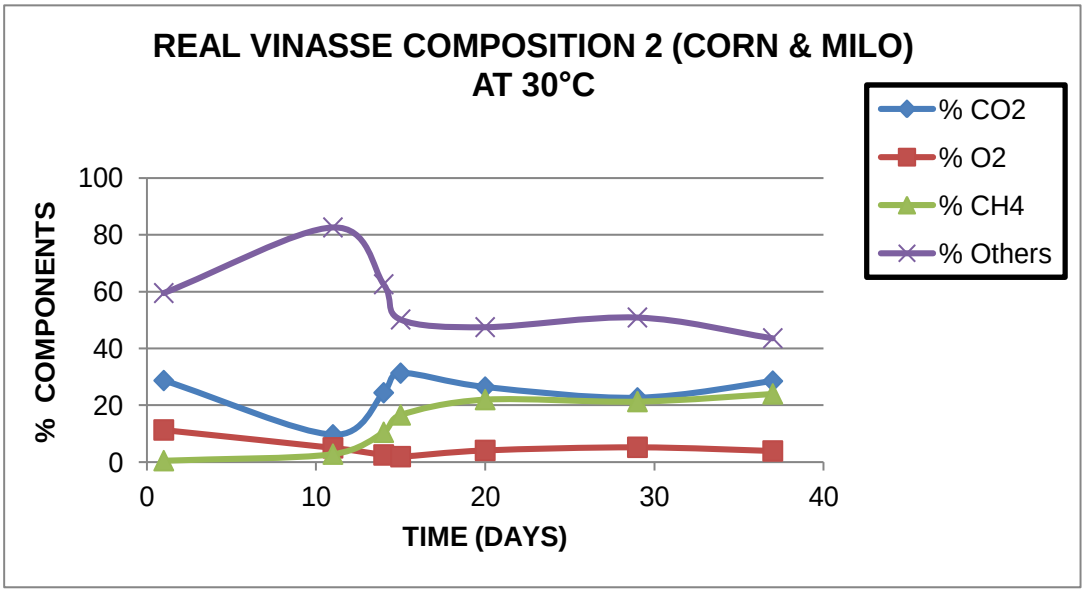


Figure B.4: Gas compositions in Real Vinasse Composition 2 (Produced from Corn and Milo, White Energy, TX) at 30°C

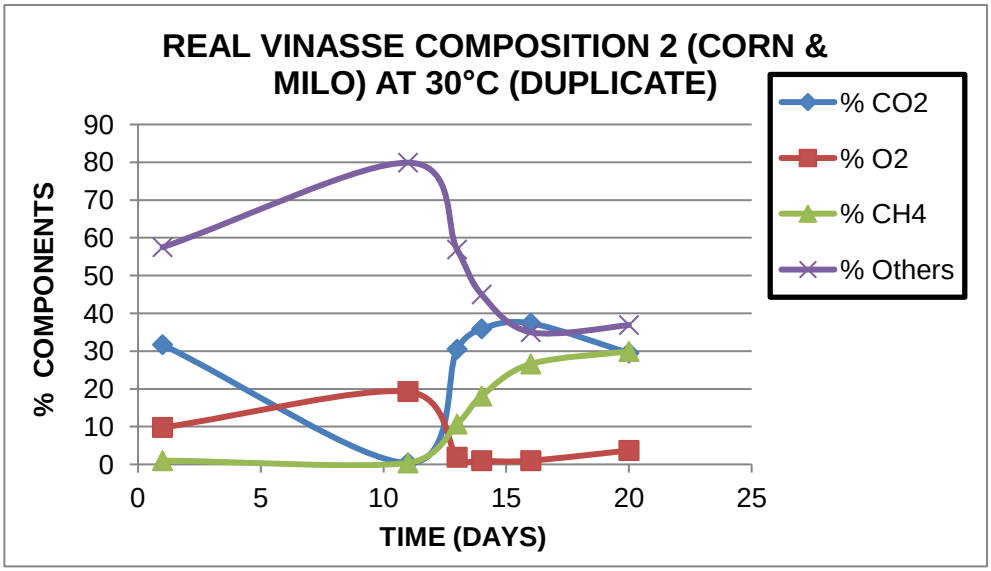


Figure B.5: Gas compositions in Real Vinasse Composition 2 Duplicate (Produced from Corn and Milo, White Energy, TX) at 30°C

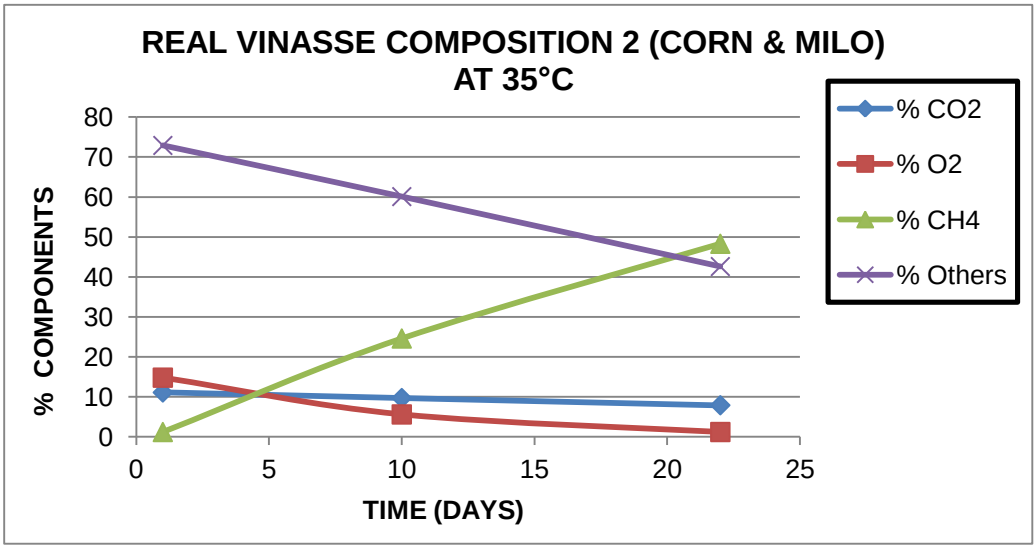


Figure B.6: Gas compositions in Real Vinasse Composition 2 (Produced from Corn and Milo, White Energy, TX) at 35°C

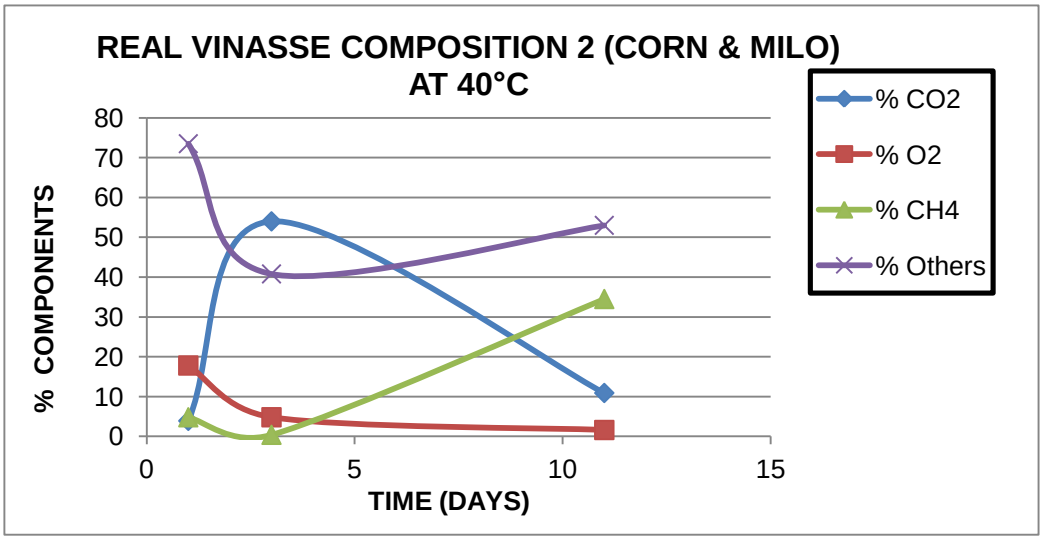


Figure B.7: Gas compositions in Real Vinasse Composition 2 (Produced from Corn and Milo, White Energy, TX) at 40°C

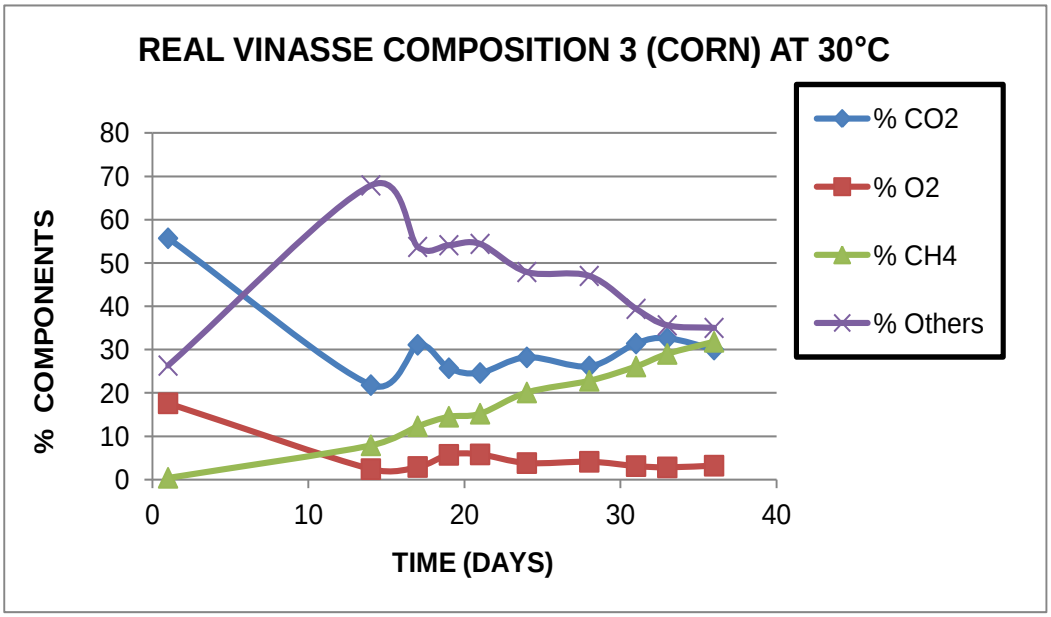


Figure B.8: Gas compositions in Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 30°C

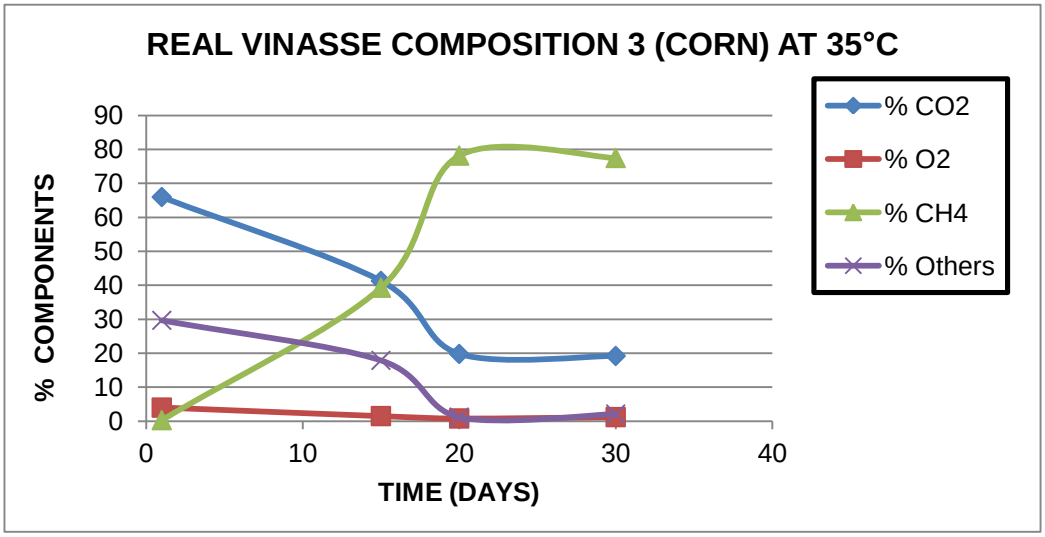


Figure B.9: Gas compositions in Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 35°C

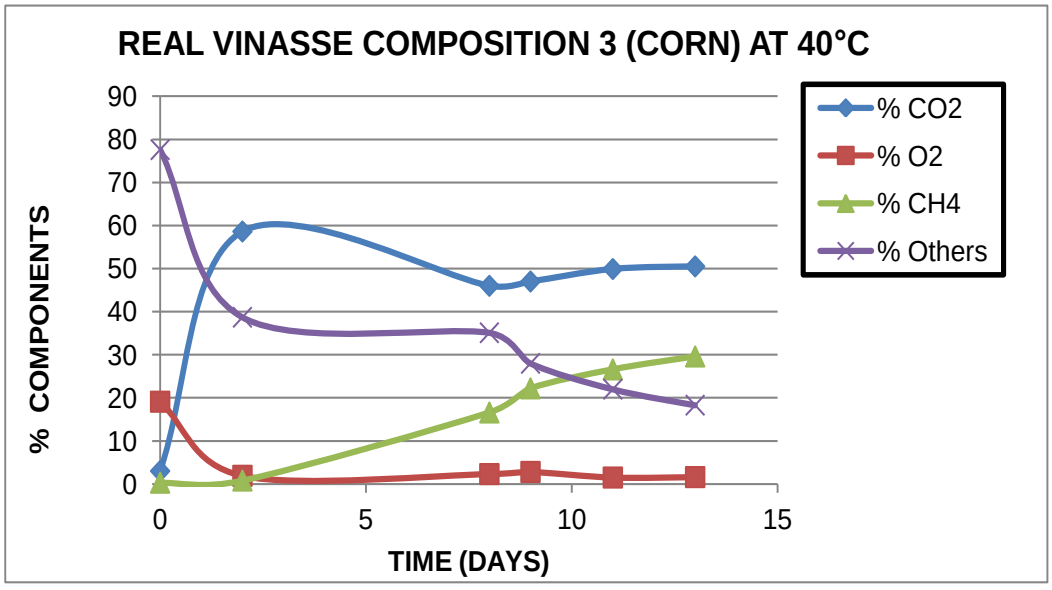


Figure B.10: Gas compositions in Real Vinasse Composition 3 (Produced from Corn, MGP Ingredients, KS) at 40°C

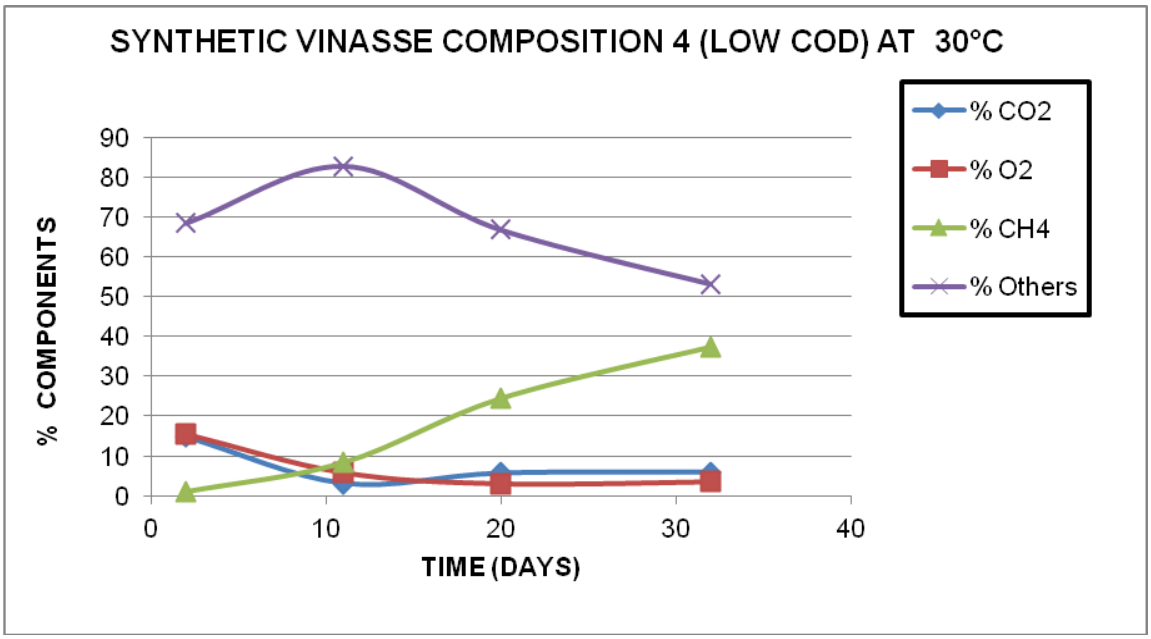


Figure B.11: Gas compositions in Synthetic Vinasse Composition 4 (Low COD) at 30°C

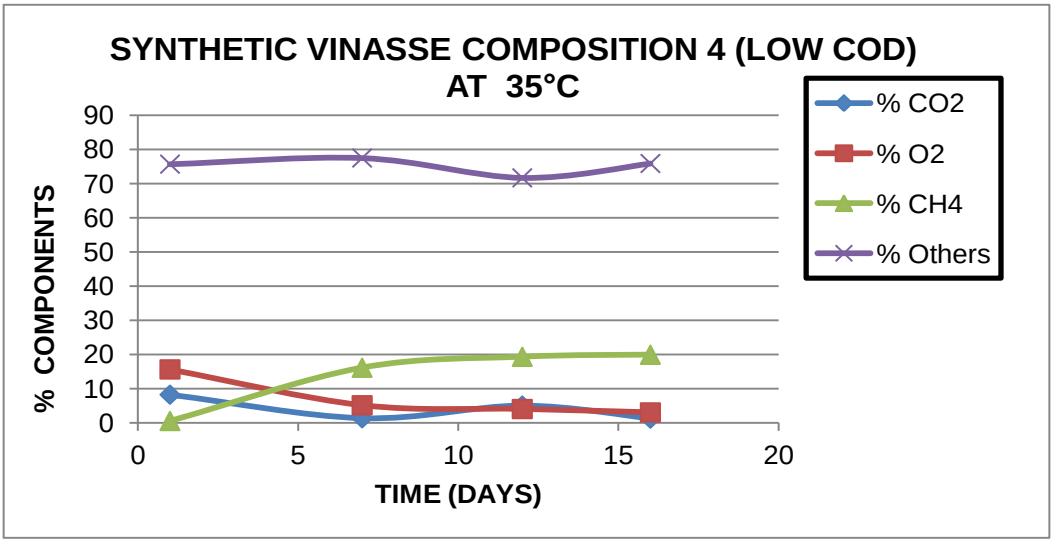


Figure B.12: Gas compositions in Synthetic Vinasse Composition 4 (Low COD) at 35°C

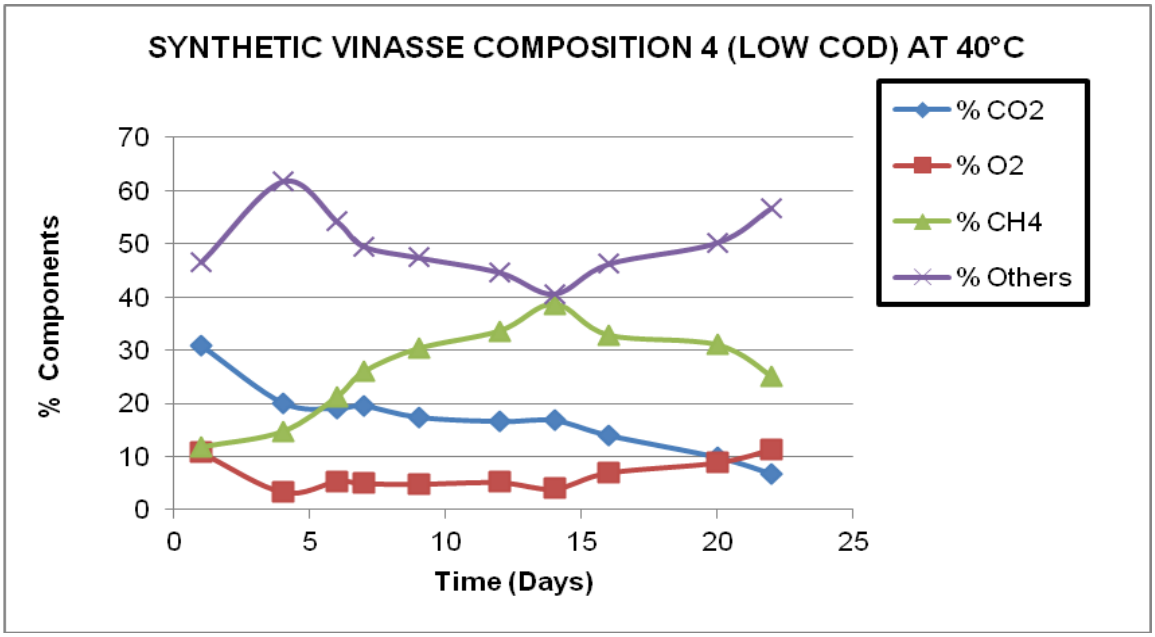


Figure B.13: Gas compositions in Synthetic Vinasse Composition 4 (Low COD) at 40°C

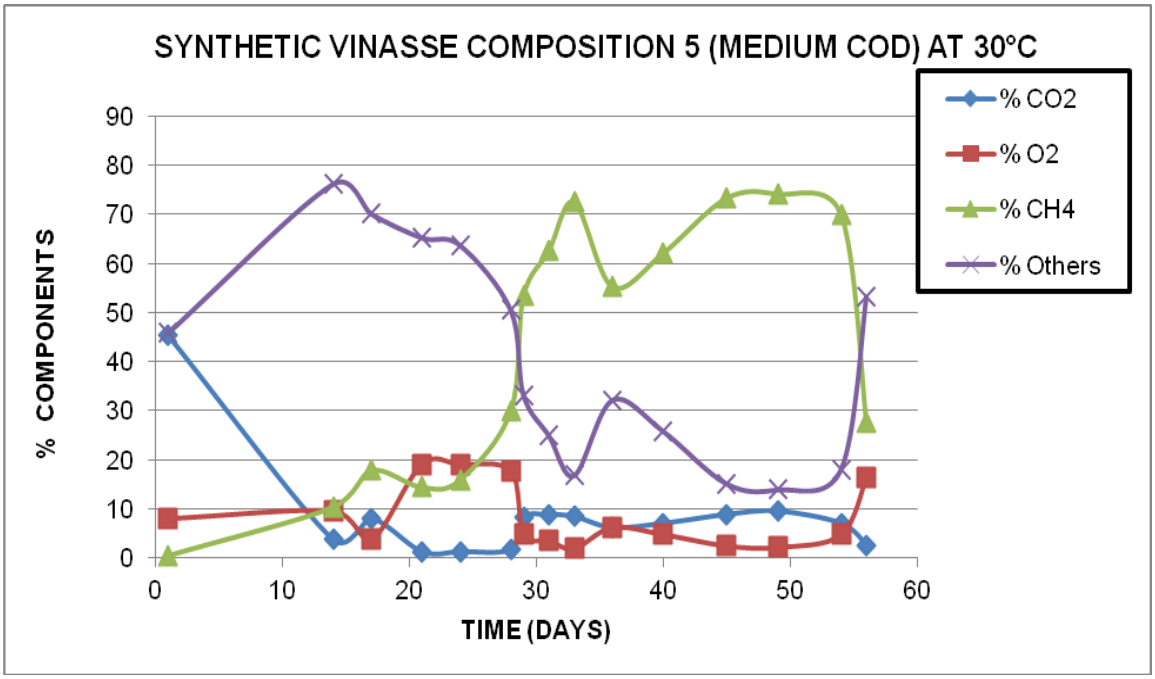


Figure B.14: Gas compositions in Synthetic Vinasse Composition 5 (Medium COD) at 30°C

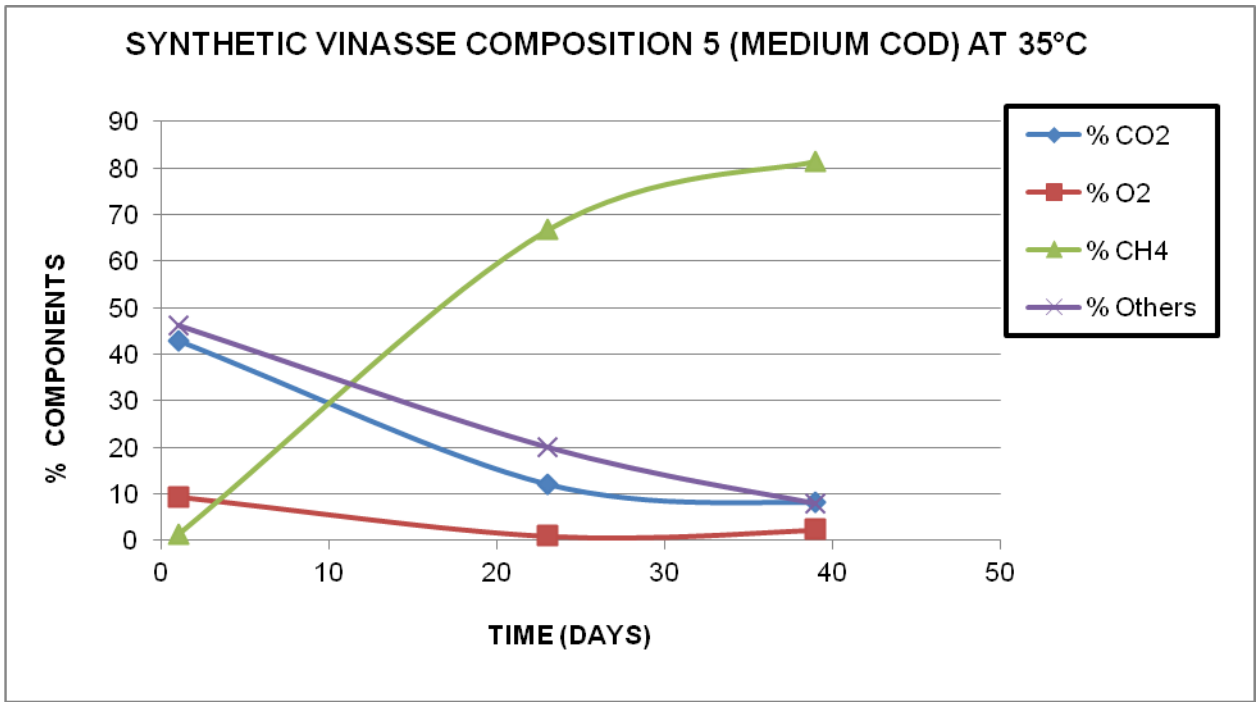


Figure B.15: Gas compositions in Synthetic Vinasse Composition 5 (Medium COD) at 35°C

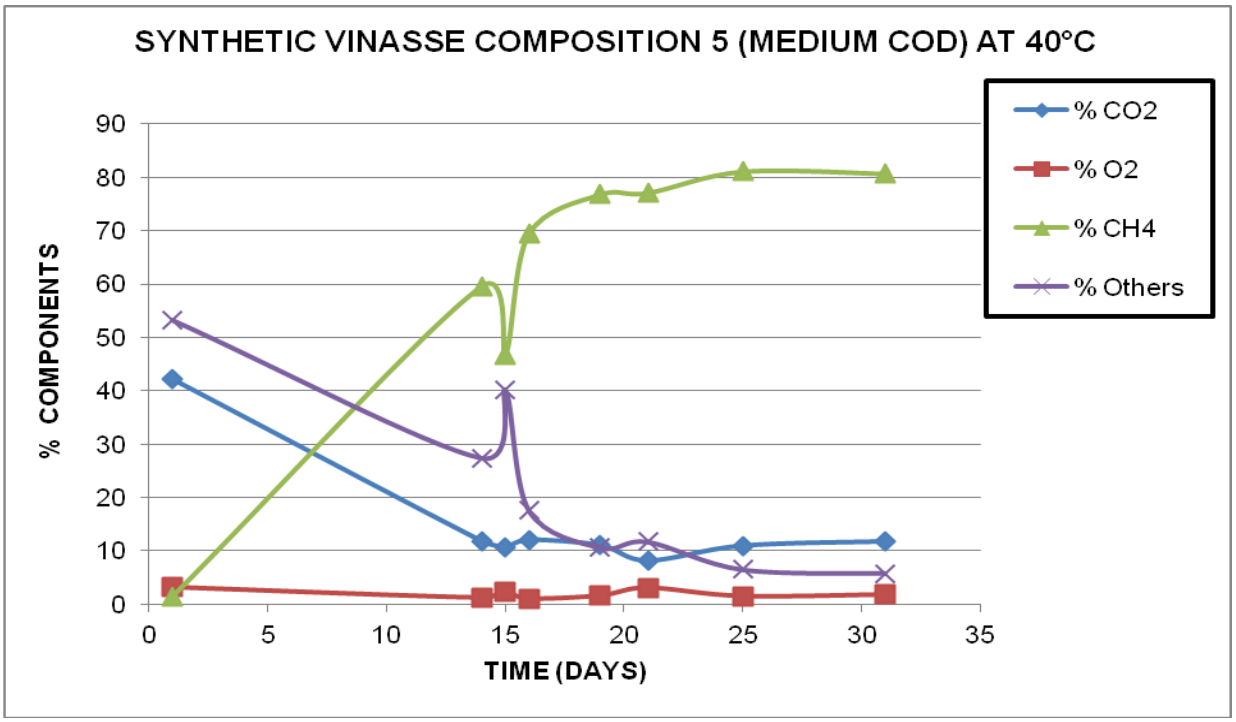


Figure B.16: Gas compositions in Synthetic Vinasse Composition 5 (Medium COD) at 40°C

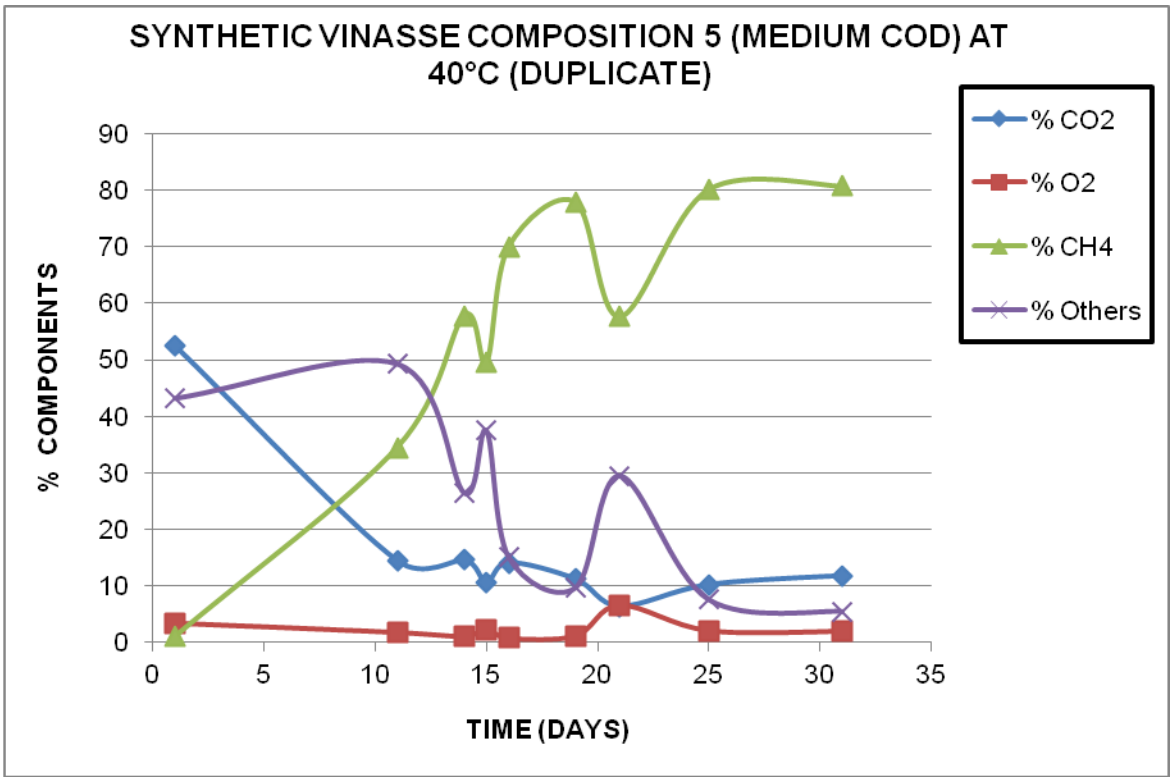


Figure B.17: Gas compositions in Synthetic Vinasse Composition 5 (Medium COD) at 40°C (Duplicate)

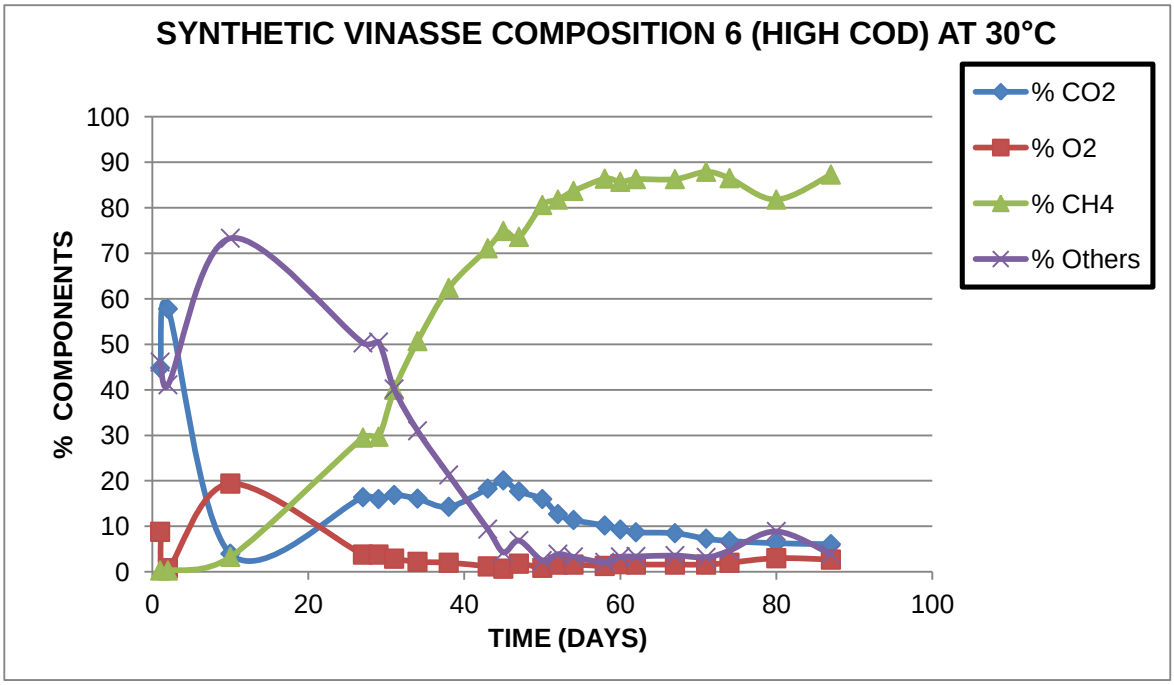


Figure B.18: Gas compositions in Synthetic Vinasse Composition 6 (High COD) at 30°C

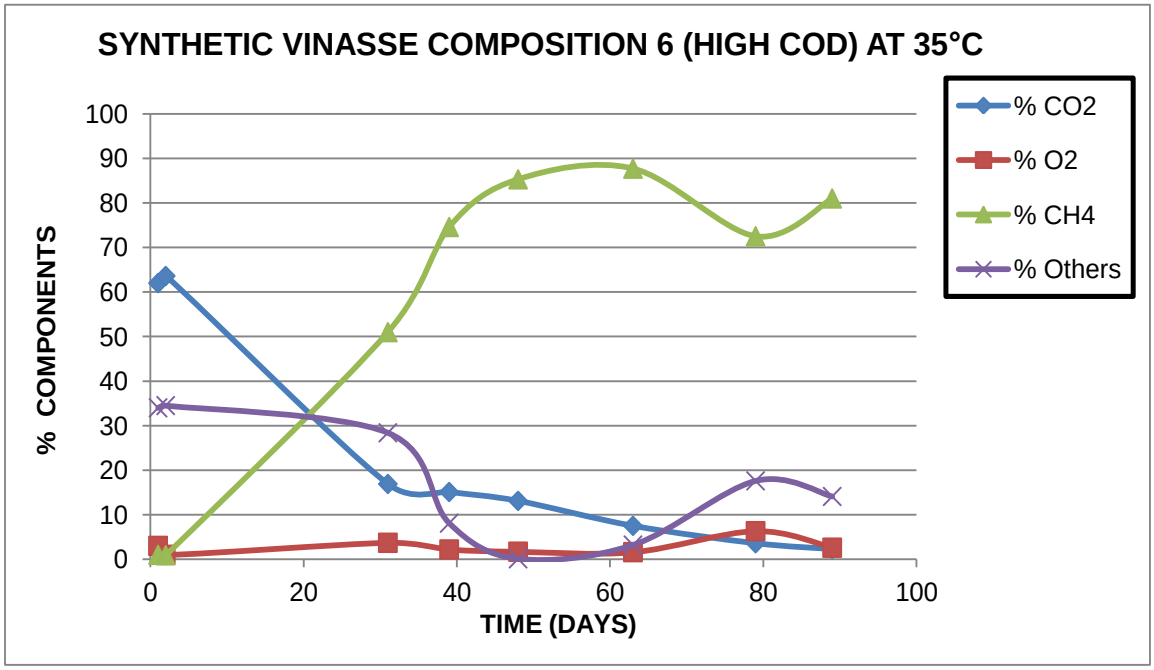


Figure B.19: Gas compositions in Synthetic Vinasse Composition 6 (High COD) at 35°C

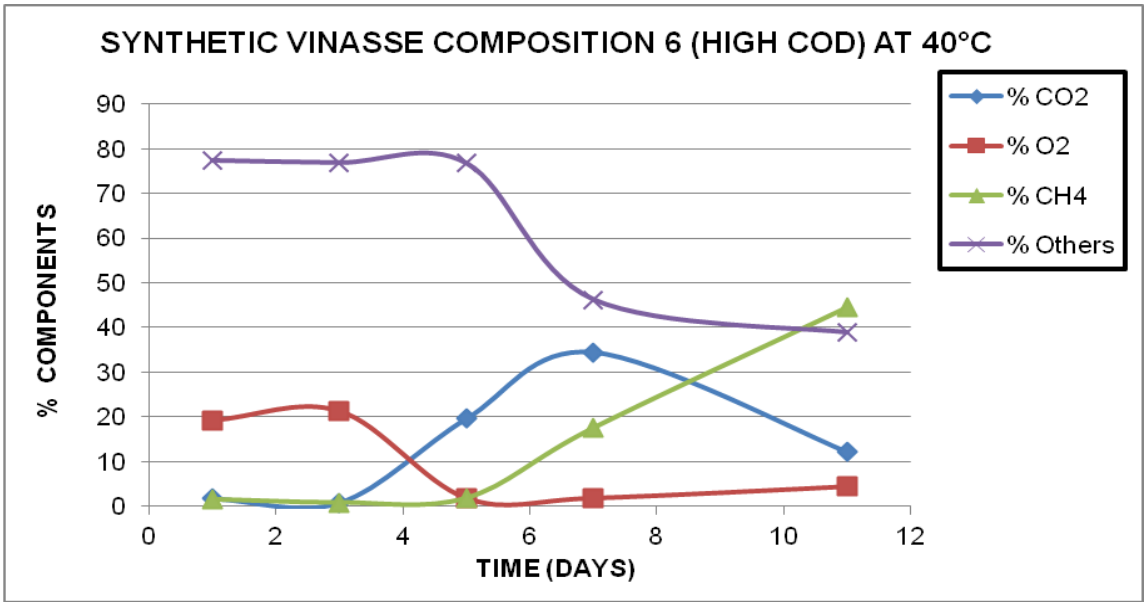


Figure B.20: Gas compositions in Synthetic Vinasse Composition 6 (High COD) at 40°C

Appendix C

Microbial Communities of Real and Synthetic Vinasse Compositions

Table C.1 Microbial Communities Present in Real Vinasse Composition 1 (Corn & Milo, White Energy, TX) at 35°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Candidatus Methanoregula
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	vadinCA11
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales		
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28

Table C.1 —*Continued*

Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	Trichococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter _Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus

Table C.1 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Acidaminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megasphaera
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Mitsuokella
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Succiniclasticum
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium

Table C.1 —*Continued*

Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenes
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema

Table C.1 —*Continued*

Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Tenericutes	RF3	ML615J-28		
Bacteria	Verrucomicrobia	Verruco-5	LD1-PB3		
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.2 Microbial Communities Present in Real Vinasse Composition 1 (Corn & Milo, White Energy, TX) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Bacteria	Actinobacteria	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Barnesiellaceae]	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	YRC22
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium

Table C.2 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megamonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	

Table C.2 —*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73

Table C.3 Microbial Communities Present in Real Vinasse Composition 2 (Corn & Milo, White Energy, TX) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Carnobacteriaceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Clostridia	Other		
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	

Table C.3 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Natronincola_Anaerovirgula
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Peptoniphilus

Table C.3 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Planctomycetes	Phycisphaerae	MSBL9		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacter
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rubellimicrobium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Telmatospirillum
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	

Table C.3 —*Continued*

Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Tenericutes	RF3	ML615J-28		
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.4 Microbial Communities Present in Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	Methanomassiliicoccus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	YRC22
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter

Table C.4 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	vadinHB04
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax

Table C.4 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Peptoniphilus
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
Bacteria	Firmicutes	Clostridia	MBA08		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio

Table C.4 —*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.5 Microbial Communities Present in Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 35°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	Methanomassiliicoccus
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	vadinCA11
Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
Bacteria	Acidobacteria	DA052	Ellin6513		
Bacteria	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Butyricimonas

Table C.5 —*Continued*

Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	

Table C.5 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megamonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Mitsuokella
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Selenomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	vadinHB04
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Mogibacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium

Table C.5 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella _Soehngenia
Bacteria	Firmicutes	Clostridia	MBA08		
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenes
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	

Table C.5 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales		
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	SC-I-84		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Providencia

Table C.5 —*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Bacteria	Spirochaetes	MVP-15	PL-11B10		
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Pyramidobacter
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.6 Microbial Communities Present in Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Anaerovibrio
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megamonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella_Soehngenia
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	

Table C.6 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium

Table C.7 Microbial Communities Present in Synthetic Vinasse Composition 4 (Low COD) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
Bacteria	AD3	JG37-AG-4			
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales		
Bacteria	Armatimonadetes	SJA-176	RB046		
Bacteria	Armatimonadetes	SJA-176	TP122		
Bacteria	BRC1	NPL-UPA2			
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Candidatus Azobacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	[Weeksellaceae]	Cloacibacterium

Table C.7 —*Continued*

Bacteria	Caldiserica	OP5	WCHB1-02	SHBZ1169	
Bacteria	Caldiserica	TTA-B1			
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1_B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Cyanobacteria	4C0d-2	MLE1-12		
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Gracilibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas

Table C.7 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales		
Bacteria	Lentisphaerae	[Lentisphaeria]	Z20	R4-45B	
Bacteria	NKB19				
Bacteria	NKB19	TSBW08			
Bacteria	NKB19	noFP			
Bacteria	OP11	WCHB1-64	d153		
Bacteria	OP8	OP8	OPB95		
Bacteria	OP9	OPB46	SHA-1		
Bacteria	Planctomycetes	Phycisphaerae	AKAU3564		
Bacteria	Planctomycetes	Phycisphaerae	MSBL9		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans

Table C.7 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Thiomonas
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azovibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
Bacteria	Proteobacteria	Betaproteobacteria	SC-I-84		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfomicrobiaceae	Desulfomicrobium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	

Table C.7 —*Continued*

Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	125ds10	
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Spirochaetes	MVP-15	PL-11B10		
Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Spirochaetes	[Brevinematae]			
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Synergistetes	Synergistia	Synergistales	TTA	E6
Bacteria	Tenericutes	RF3	ML615J-28		
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	AUTHM297

Table C.7 —*Continued*

Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Fervidobacterium
Bacteria	Verrucomicrobia	Verruco-5	LD1-PB3		
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	
Bacteria	WS1				
Bacteria	WS3	PRR-12	Sediment-1		

Table C.8 Microbial Communities Present in Synthetic Vinasse Composition 5 (Medium COD) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Crenarchaeota	MBGA	NRP-J		
Archaea	Crenarchaeota	Thaumarchaeota	Cenarchaeales	SAGMA-X	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanofollis
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanospirillaceae	Methanospirillum
Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosaetaceae	Methanosaeta
Archaea	Euryarchaeota	Methanomicrobia	Methanosarcinales	Methanosarcinaceae	Methanosarcina
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	Methanomassiliicoccus
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	vadinCA11
Archaea	[Parvarchaeota]	[Parvarchaea]	WCHD3-30		
Bacteria					
Bacteria	Acidobacteria	Acidobacteriia	Acidobacteriales	Koribacteraceae	
Bacteria	Acidobacteria	DA052	Ellin6513		
Bacteria	Acidobacteria	Solibacteres	Solibacterales		
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales		

Table C.8 —*Continued*

Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	p-2534-18B5	
Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Saprospiraceae	
Bacteria	Caldiserica	TTA-B1			
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Chloroflexi	Anaerolineae	GCA004		
Bacteria	Chloroflexi	Anaerolineae	SBR1031		

Table C.8 —*Continued*

Bacteria	Chloroflexi	Ktedonobacteria	Thermogemmatisporales	Thermogemmatisporaceae	
Bacteria	Fibrobacteres	Fibrobacteria	Fibrobacterales	Fibrobacteraceae	Fibrobacter
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Acetobacterium_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira

Table C.8 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Acidaminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Anaeromusa
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	GAL15				
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Lentisphaerae	[Lentisphaeria]	Z20		
Bacteria	Lentisphaerae	[Lentisphaeria]	Z20	R4-45B	
Bacteria	NKB19				
Bacteria	NKB19	TSBW08			

Table C.8 —*Continued*

Bacteria	NKB19	noFP			
Bacteria	Nitrospirae	Nitrospira	Nitrospirales		
Bacteria	OP3	koll11	GIF10		
Bacteria	OP8	OP8	OPB95		
Bacteria	OP9	OPB46	SHA-1		
Bacteria	Planctomycetes	Phycisphaerae	AKAU3564		
Bacteria	Planctomycetes	Phycisphaerae	MSBL9		
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales		
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenes
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax

Table C.8 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales		
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azovibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
Bacteria	Proteobacteria	Betaproteobacteria	SC-I-84		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfomicrobiaceae	Desulfomicrobium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfuromonadales	Geobacteraceae	Geobacter
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	

Table C.8 —*Continued*

Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Tolumonas
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Kushneria
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
Bacteria	Spirochaetes	MVP-15	PL-11B10		
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema

Table C.8 —*Continued*

Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	Cloacibacillus
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Tenericutes	RF3	ML615J-28		
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	AUTHM297
Bacteria	Verrucomicrobia	Opitutae	[Cerasicoccales]	[Cerasicoccaceae]	
Bacteria	Verrucomicrobia	Verruco-5	WCHB1-41	RFP12	
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	
Bacteria	Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	DA101
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.9 Microbial Communities Present in Synthetic Vinasse Composition 5 (Medium COD) at 40°C

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	Methanomassiliicoccus
Bacteria					
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Caldiserica	TTA-B1			
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05

Table C.9 —*Continued*

Bacteria	Chloroflexi	Anaerolineae	SBR1031		
Bacteria	Chloroflexi	Anaerolineae	SBR1031	SHA-31	
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Anaerovirgula
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Natronincola
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Proteiniclasticum_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	

Table C.9 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Finegoldia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	Natranaerobiales		
Bacteria	Firmicutes	Clostridia	Natranaerobiales	Anaerobrancaceae	
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	

Table C.9 —*Continued*

Bacteria	OP8	OP8_1	OPB95		
Bacteria	OP9	OPB46	SHA-1		
Bacteria	Planctomycetes	Phycisphaerae	Pla1		
Bacteria	Planctomycetes	Phycisphaerae	WD2101		
Bacteria	Proteobacteria	Other			
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azovibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera

Table C.9 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Spirochaetes	[Brevinematae]			

Table C.9 —*Continued*

Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	PD-UASB-13
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Kosmotoga
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.10 Microbial Communities Present in Synthetic Vinasse Composition 6 (High COD) at 30°C

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Butyricimonas
Bacteria	Caldiserica	TTA-B1			
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Proteiniclasticum
Bacteria	Firmicutes	Clostridia	Clostridiales	EtOH8	
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Garciella
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	

Table C.10 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	Pelotomaculum
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella_B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus

Table C.11 Microbial Communities Present in Real Vinasse Composition 2 (Corn and Milo, White Energy, TX) at 30°C (Duplicate)

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Crenarchaeota	MBGA	NRP-J		
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	vadinCA11
Bacteria	Acidobacteria	Acidobacteriia	Acidobacteriales	Koribacteraceae	
Bacteria	Acidobacteria	Acidobacteriia	Acidobacteriales	Koribacteraceae	Candidatus Koribacter
Bacteria	Acidobacteria	DA052	Ellin6513		
Bacteria	Acidobacteria	Solibacteres	Solibacterales	Solibacteraceae	
Bacteria	Acidobacteria	TM1			
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales		
Bacteria	Actinobacteria	Acidimicrobiia	Acidimicrobiales	Microthrixaceae	
Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales	Gaiellaceae	
Bacteria	Actinobacteria	Thermoleophilia	Solirubrobacterales	Conexibacteraceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	

Table C.11 —*Continued*

Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Candidatus Azobacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	SB-1	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Butyricimonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	YRC22
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Chloroflexi	Ktedonobacteria	Thermogemmatisporales	Thermogemmatisporaceae	
Bacteria	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Mucispirillum

Table C.11 —*Continued*

Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium_Anaerovirgula
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Natronincola
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Dorea
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	

Table C.11 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	vadinHB04
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	Anaerovorax
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Peptoniphilus
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	

Table C.11 —*Continued*

Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	
Bacteria	GAL15				
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Lentisphaerae	[Lentisphaeria]	Z20	R4-45B	
Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Gemmataceae	
Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Planctomyces
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacterium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Telmatospirillum
Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales		
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	

Table C.11 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenes
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Tepidimonas
Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Nitrosomonadales		
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Tenericutes	RF3	ML615J-28		
Bacteria	Verrucomicrobia	Verruco-5	WCHB1-41	WCHB1-25	
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	Ellin515	

Table C.11 —*Continued*

Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	
Bacteria	WPS-2				
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.12 Microbial Communities Present in Synthetic Vinasse Composition 5 (Medium COD) at 40°C (Duplicate)

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	Methanomassiliicoccus
Archaea	Euryarchaeota	Thermoplasmata	E2	[Methanomassiliicoccaceae]	vadinCA11
Bacteria					
Bacteria	Actinobacteria	Thermoleophilia	Gaiellales		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	[Odoribacteraceae]	Butyricimonas
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	B004

Table C.12 —*Continued*

Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	C1
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanomicrobiaceae	Methanoculleus
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Chloroflexi	Anaerolineae	SBR1031		
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
Bacteria	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	
Bacteria	Firmicutes	Bacilli	Bacillales	[Thermicanaceae]	Bacillus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Caldicoprobacteraceae	Caldicoprobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenella
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium_Thermotalea
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Geosporobacter_Anaerovirgula

Table C.12 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Natronincola
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Proteiniclasticum_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium_Soehngenia

Table C.12 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	Natranaerobiales		
Bacteria	Firmicutes	Clostridia	Natranaerobiales	Anaerobrancaceae	
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Alcaligenes
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	

Table C.12 —*Continued*

Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azospira
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azovibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Dechloromonas
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Syntrophobacter
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter

Table C.12 —*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Proteus
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Providencia
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	vadinCA02
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Kosmotoga
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	Candidatus Cloacamonas
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W22
Bacteria	WWE1	[Cloacamonae]	[Cloacamonales]	[Cloacamonaceae]	W5

Table C.13 Microbial Communities Present in Sample Duplicates of Real Vinasse Composition 1 (Corn & Milo, White Energy, TX) at 40°C on Day 8

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium

Table C.14 Microbial Communities Present in Sample Duplicates of Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 30°C on Day 35

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	MBA08		
Bacteria	Firmicutes	OPB54			
Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Cetobacterium
Bacteria	Lentisphaerae	[Lentisphaeria]	Victivallales	Victivallaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	

Table C.14 —*Continued*

Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila

Table C.15 Microbial Communities Present in Sample Duplicates of Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 5

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Megamonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tepidimicrobium_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	Thermoanaerobacterales		
Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	

Table C.15 —*Continued*

Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter

Table C.16 Microbial Communities Present in Sample Duplicates of Real Vinasse Composition 3 (Corn, MGP Ingredients, KS) at 40°C on Day 7

Kingdom	Phylum	Class	Order	Family	Genus
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sporanaerobacter
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter

Table C.17 Microbial Communities Present in Sample Duplicates of Synthetic Vinasse Composition 5 (Medium COD) at 40°C on Day 14

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobacterium
Archaea	Euryarchaeota	Methanomicrobia	Methanomicrobiales	Methanoregulaceae	Methanolinea
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Candidatus Azobacteroides
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Dysgonomonas
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Blvii28
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	SHD-231
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	T78
Bacteria	Chloroflexi	Anaerolineae	Anaerolineales	Anaerolinaceae	WCHB1-05
Bacteria	Firmicutes	Bacilli	Bacillales		
Bacteria	Firmicutes	Clostridia	Clostridiales		
Bacteria	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	

Table C.17 —*Continued*

Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium
Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Proteiniclasticum_Eubacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Pseudoramibacter
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Gracilibacteraceae	Lutispora
Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Sedimentibacter_Soehngenia
Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Tissierella
Bacteria	Firmicutes	Clostridia	Natranaerobiales		

Table C.17 —*Continued*

Bacteria	Firmicutes	Clostridia	Natranaerobiales	Anaerobrancaceae	
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Firmicutes	Clostridia	SHA-98	D2	
Bacteria	Firmicutes	OPB54			
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Limnohabitans
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Methylibium
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Azovibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibrio
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Thauera
Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Zoogloea
Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	GW-28		
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	
Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophaceae	Syntrophus

Table C.17 —*Continued*

Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophorhabdaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Spirochaetes	Spirochaetes	Spirochaetales	Spirochaetaceae	Treponema
Bacteria	Spirochaetes	[Brachyspirae]	[Brachyspirales]	Brachyspiraceae	
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	Aminobacterium
Bacteria	Synergistetes	Synergistia	Synergistales	Dethiosulfovibrionaceae	HA73
Bacteria	Thermotogae	Thermotogae	Thermotogales	Thermotogaceae	Kosmotoga

Table C.18 Microbial Communities Present in Sample Duplicates of Real Vinasse Composition 6 (High COD) at 30°C on Day 1

Kingdom	Phylum	Class	Order	Family	Genus
Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	WSA2	
Bacteria					
Bacteria	Acidobacteria	DA052	Ellin6513		
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	
Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiaceae	
Bacteria	Firmicutes	Clostridia	SHA-98		
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Arcobacter
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Sulfurospirillum
Bacteria	Proteobacteria	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Aeromonas
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Tolumonas
Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio
Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	125ds10	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobacter
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Edwardsiella
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia

Table C.18 —*Continued*

Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyella
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	Cloacibacillus
Bacteria	Synergistetes	Synergistia	Synergistales	Synergistaceae	Synergistes
Bacteria	Verrucomicrobia	Opitutae	Puniceicoccales	Puniceicoccaceae	
Bacteria	Verrucomicrobia	Opitutae	Puniceicoccales	Puniceicoccaceae	Coraliomargarita
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	R4-41B	
Bacteria	Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	auto67_4W	
Bacteria	Verrucomicrobia	[Spartobacteria]	[Chthoniobacterales]	[Chthoniobacteraceae]	

Appendix D

DNA Extraction Protocol, Gel Extraction Protocol, Microbial Analysis Protocol, Calculation and Protocol for Preparing Primer Stock and Working Solution

D.1 DNA Extraction Protocol

This protocol was adapted from MOBIO experimental protocol (http://www.mobio.com/images/custom/file/protocol/12855.pdf).

1. Properly identify each Glass Bead Tube on both the cap and on the side.

2. To the PowerLyzer® Glass Bead Tube, 0.1 mm provided, add 0.65-0.85 grams of sample.

Sample (vinasse) was centrifuged times with 1ml vinasse sample at maximum speed of 10,000 x g in glass bead tube to collect the solidified sample between 0.65-0.85 grams. After every run, the supernatant were discarded from glass bead tube.

3. Add 750 ml of Bead Solution to the Glass Bead Tube. Gently vortex to mix sample using VWR vortex mixture at a speed of 4-5 RPM.

4. Check Solution C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use.

5. Add 60 ml of Solution C1 and invert several times or vortex briefly.

6. Bead Beating Options:

Vortex: Secure the PowerLyzer® Glass Bead Tubes horizontally using the VWR Vortex

Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with

tape. Vortex at maximum speed for 10 minutes.

7. Make sure the Glass Bead Tubes rotate freely in your centrifuge without rubbing. Centrifuge Bead Tubes at 10,000 x g for 30 seconds at room temperature.

CAUTION: Be sure not to exceed 10,000 x g or tubes may break.

8. Transfer the supernatant to a clean 2 ml Collection Tube.

Note: Expect between 400 to 500 ml of supernatant. Supernatant may still contain some soil particles.

9. Add 250 ml of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.

10. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

11. Avoiding the pellet, transfer up to, but no more than, 600 ml of supernatant to a clean 2 ml Collection Tube.

12. Add 200 ml of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.

13. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.

14. Avoiding the pellet, transfer up to, but no more than, 750 ml of supernatant into a clean 2 ml Collection Tube.

15. Shake to mix Solution C4 before use. Add 1200 ml of Solution C4 to the supernatant and vortex for 5 seconds.

16. Load approximately 675 ml onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 ml of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. A total of three loads for each sample processed are required.

DNA is selectively bound to the silica membrane in the Spin Filter device in the high salt solution. Contaminants pass through the filter membrane, leaving only DNA bound to the membrane.

17. Add 500 ml of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g.

Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the Spin Filter. This wash solution removes residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane.

18. Discard the flow through from the 2 ml Collection Tube.

This flow through fraction is just non-DNA organic and inorganic waste removed from the silica Spin Filter membrane by the ethanol wash solution.

19. Centrifuge at room temperature for 1 minute at 10,000 x g.

This second spin removes residual Solution C5 (ethanol wash solution). It is critical to remove all traces of wash solution because the ethanol in Solution C5 can interfere with many downstream DNA applications such as PCR, restriction digests, and gel electrophoresis.

20. Carefully place Spin Filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

Note: It is important to avoid any traces of the ethanol based wash solution.

21. Add 100 ml of Solution C6 to the center of the white filter membrane.

Note: Placing the Solution C6 (sterile elution buffer) in the center of the small white membrane will make sure the entire membrane is wetted. This will result in a more efficient and complete release of the DNA from the silica Spin Filter membrane. As Solution C6 (elution buffer) passes through the silica membrane, DNA that was bound in the presence of high salt is selectively released by Solution C6 (10 mM Tris) which lacks salt.

Alternatively, sterile DNA-Free PCR Grade Water may be used for DNA elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10). Solution C6 contains no EDTA. If DNA degradation is a concern, Sterile TE may also be used instead of Solution C6 for elution of DNA from the Spin Filter.

22. Centrifuge at room temperature for 30 seconds at 10,000 x g.

23. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

DNA should be stored frozen (-20 °C to -80 °C). Solution C6 does not contain any EDTA. Concentration of DNA was measure using Nano Drop spectrophotometer

D.2 QIAquick Gel Extraction Kit Protocol using a microcentrifuge

This protocol was quoted from QIAquick Gel Extraction protocol, available on Qiagen company website (http://sites.bio.indiana.edu/~chenlab/protocol_files/agarose_gel_extraction.pdf).

All centrifugation steps are carried out at ≥10,000 x g (~13,000 rpm) in a conventional table-top microcentrifuge. 3 M sodium acetate, pH 5.0, may be necessary.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose. The DNA fragment was cut using

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl). For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix.

For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, keep the solution in the refrigerator for 10 minutes, and centrifuge for 1 min. If more solution is present in the tube, load the remaining solution onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.

8. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube. Collection tubes are re-used to reduce plastic waste

9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x g (~13,000 rpm).

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the natively, for increased DNA concentration, add 30 µl elution buffer to the center of the alquick membrane and centrifuge the column for 1 min at maximum speed. QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average elute volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

D.3 Microbial Analysis Protocol

This protocol was adapted from brmicrobiome.org website (http://www.brmicrobiome.org/#!16s-profiling-pipeline-illumin/c3u5).

1. Take forward and reverse Illumina reads (R1.fastq and R2.fastq files) and joins them using the method fastq-join, together with updating the barcode file **on** QIIME 1.8.0.). File locations are represented here as $PWD/.

   Join_paired_ends.py –f $PWD/forward_reads.fastq –r $PWD/reverse_reads.fastq –b $PWD/barcodes.fastq –o $PWD/joined.fastq

2. Demultiplex fastq sequence data, when barcodes and sequences are contained in two separate .fastq files. Here, we are turning off filter parameters, and storing the demultiplexed *fastq* file (quality filtering will be done in the next step).

   split_libraries_fastq.py – i $PWD/fastqjoin.join.fastq –b $PWD/fastqjoin.join_barcodes.fastq –o $PWD/slout -m $PWD/map.txt --rev_comp_mapping_barcodes --store_demultiplexed_fastq -r 0 -q 0 -n 100

where map.txt is the mappling file. This file contains all of the information about the samples necessary to perform the data analysis. In general, the mapping file should contain the name of each sample, the barcode sequence used for each sample, the linker/primer sequence used to amplify the sample, and a description column. One should also include in the mapping file any metadata that relates to the samples (for instance, health status or sampling site) and any additional information relating to specific samples that may be useful to have at hand when considering outliers (for example, what medications a patient was taking at time of sampling).(qiime.org). All the instructions and suggestions for creating mapping file are given in qiime website (http://qiime.org/documentation/file_formats.html). This file is created by user. All the mapping files for 3 sets are given in Table 3.11, 3.12, 3.13.

Download usearch 7 and add usearch to Qiime path using the command below (a-e).

a. mv usearch7.0-5.1090_i86osx32 usearch7
b. chmod +x usearch7

c. $PATH

d. echo "export PATH=`pwd`:$PATH">>~/.bashrc source ~/.bashrc

e. usearch --version

If usearch is installed properly, it will give the version name (usearch v7.0.1090_i86osx32) of usearch 7 after following step a-e.

1. Quality filtering, length truncate, and convert to FASTA <<<USING USEARCH 7>>>

   usearch –fastq filter $PWD/seqs.fastq -fastq_maxee 0.5 -fastq_trunclen 240 –fastaout reads.fa.

4. Change sequence header to make file compatible with further UPARSE steps <<<USING BMP PERL SCRIPT>>>. This script will generate your converted FASTA file.

   perl bmp-uparse-pipeline.pl –i $PWD/reads.fa -o $PWD /reads_uparse.fa

5. Dereplication <<<USING USEARCH 7>>>

   usearch -derep_fulllength $PWD/reads_uparse.fa -output derep.fa –sizeout

6. Abundance sort and discard singletons <<<USING USEARCH 7>>>

   usearch –sortbysize $PWD/derep.fa -output sorted.fa -minsize 2

7. OTU clustering using UPARSE method <<<USING USEARCH 7>>>

   Usearch -cluster_otus $PWD/sorted.fa -otus $PWD/otus1.fa

8. Chimera filtering using reference database <<<USING USEARCH 7>>> (Download Gold.fa dataset from http://drive5.com/uchime/gold.fa)

   usearch -uchime_ref $PWD/otus1.fa -db $PWD/gold.fa -strand plus -nonchimeras otus2.fa

9. If the sizes of reads are bigger in one set, it would not pass through usearch 32 bit in step 3. Hence, reads of one set was divided into two separate sets by dividing mapping file in two different files. Divided sets were run from step 3 to step 9. Two different sets were joined in one set using cat command in step 11.

10. Add reads_uparse.fa and otus2.fa of two different sets using cat command.

    cat reads.uparse.fa1 reads_uparse.fa2 > mergeuparse.fa

    cat otus.fa1 otus2.fa2 > mergeotus2.fa

11. Label OTU sequences OTU_1, OTU_2... <<<UPARSE Python script>>>

    python $PWD python_scripts/fasta_number.py $PWD/mergeotu2.fa OTU_ > otus.fa

12. Map reads (including singletons) back to OTUs <<<USING USEARCH 7>>>

    usearch -usearch_global $PWD/mergeuparse.fa -db $PWD/otus.fa -strand plus -id 0.97 -uc map.uc

13. Create OTU table <<<UPARSE Python script>>>

    python /$PWD/python_scripts/uc2otutab.py $PWD/map.uc > otu_table.txt

14. Convert OTU table in the .txt format to BIOM format (used by QIIME) <<<USING BIOM SCRIPT>>>

    biom convert -i /Users/madhurani/Desktop/software/set3/combined_sets/otu_table.txt –o $PWD/table.from_txt.biom --table-type="otu table"

15. Assign taxonomy to OTUS using uclust method on QIIME (use the file "otus.fa" from UPARSE as input file). Greengenes (gg_13_8_otus/rep_set/99_otus.fasta and gg_13_8_otus/taxonomy/99_otu_taxonomy.txt) can be downloaded from http://qiime.org/home_static/dataFiles.html. 13_8 (most recent) was used for analysis.

    assign_taxonomy.py –i $PWD/otus.fa -o $PWD/output –r $PWD/gg_13_8_otus/rep_set/99_otus.fasta –t $PWD/gg_13_8_otus/taxonomy/99_otu_taxonomy.txt

17. Add metadata (taxonomy) to OTU table <<<USING BIOM SCRIPT>>>

    biom add-metadata –I $PWD/table.from_txt.biom -o $PWD/table_tax.biom --observation-metadata-fp $PWD/otus_tax_assignments.txt --observation-header OTUID,taxonomy,confidence --sc-separated taxonomy --float-fields confidence

18. Align sequences on QIIME, using greengenes reference sequences (use the file "otus.fa" from UPARSE as input file)

    align_seqs.py -i $PWD/otus.fa –o rep_set_align –t $PWD/gg_13_8_otus/rep_set_aligned/99_otus.fasta

19. Filter alignments on QIIME

    filter_alignment.py -i $PWD/otus_aligned.fasta -o $PWD/filtered_alignment

20. Make the reference tree on QIIME

    make_phylogeny.py –i $PWD/otus_aligned_pfiltered.fasta -o $PWD/rep_set.tre

21. Check OTU Table on QIIME

    biom summarize-table -i $PWD/table_tax.biom –o $PWD/results_biom_table

22. Run diversity analyses on QIIME (or any other analysis of your choice). The parameter "-e" is the sequencing depth to use for even sub-sampling and maximum rarefaction depth. You should review the output of the 'biom summarize-table' (step 21) command to decide on this value (xxxx).

    core_diversity_analyses.py -i $PWD/table_tax.biom -m $PWD/mapping_file.txt -t $PWD/rep_set.tre -e xxxx -o $PWD/core_output

Core diversity analysis gave the data of alpha and beta diversity. This script calculates alpha diversity, or within-sample diversity, using an otu table. The QIIME pipeline allows users to conveniently calculate more than two dozen different diversity metrics. This script gives the detailed information about species richness (chao1), phylogenetic diversity (PD_whole tree), and Observed species. e is the minimum number of reads obtained from results biom table after running step 21. If e is greater than the minimum number of reads, it will not show the result of sample which has values lower than minimum number. Hence, it is advisable to take those values for getting results of all samples included in mapping file (as shown in Table 3.13, 3.14, and 3 15.

D.4 Calculation for preparing primer stock solution

Forward/Reverse primers	Amount of primer (pmoles/µl)	Amount of TE added to make a Stock Solution (100pmoles/µl)
515F	22.3	223
806rcbc0	80.1	801
806rcbc1	91.8	918
806rcbc2	72	720
806rcbc3	73.7	737
806rcbc4	80.9	809
806rcbc5	82.3	823
806rcbc6	81.5	815
806rcbc7	79.8	798
806rcbc8	79.2	792
806rcbc9	88.9	889
806rcbc10	68.4	684
806rcbc11	75.8	758
806rcbc12	78.8	788
806rcbc13	84.9	849
806rcbc14	89.8	898
806rcbc15	75.9	759
806rcbc16	73.9	739
806rcbc17	62.6	626
806rcbc18	74.4	744
806rcbc19	85	850
806rcbc20	75.1	751

D.4 —*Continued*

806rcbc21	87.3	873
806rcbc22	76.6	766
806rcbc23	82	820
806rcbc24	75.7	757
806rcbc25	81.8	818
806rcbc26	67.3	673
806rcbc27	68.9	689
806rcbc28	75.5	755
806rcbc29	70.5	705
806rcbc30	69.9	699
806rcbc31	98.2	982
806rcbc32	74.4	744
806rcbc33	90.2	902
806rcbc34	70.2	702
806rcbc35	73.9	739
806rcbc36	64.7	647
806rcbc37	103.3	1033
806rcbc38	75.9	759
806rcbc39	82.1	821
806rcbc40	83.8	838
806rcbc41	67.6	676
806rcbc42	77.4	774
806rcbc43	86.1	861
806rcbc44	75.3	753
806rcbc45	73.3	733

D.4—*Continued*

806rcbc46	66.5	665
806rcbc47	81.7	817
806rcbc48	70.2	702
806rcbc49	62.7	627

D.5 Stock Solution Preparation Protocol

1) Centrifuge primer tubes for a minute at a speed of 14000RPM.

2) Carefully transfer all the tubes on ice.

3) Add Tris EDTA (TE) buffer solution (pH 8.0) to each tube based on the volume calculated below in Table 3.3.

4) Vortex all tubes briefly (15-20 seconds).

5) Put all the primers back on ice.

D.6 Working Volume Preparation Protocol

1) Label all the tubes (primer name and date).

2) Add 180µl of PCR grade water to each tube.

3) Add 20µl of stock solution to each tube.

4) Vortex all tubes briefly after adding water and stock solution.

5) Put all the tubes back on ice.

D.7 16S RNA amplification protocol

1. Samples were amplified in triplicate (each 25 µl PCR reactions).
2. Run amplicons for each sample on an agarose gel. Expected band size for 515f/806r is roughly 375-450bp.
3. Quantify amplicons with EtBr. 1Kb Plus DNA ladder and 6X DNA loading dye was used for running on agarose gel.
4. Combine all the triplicates (each 25µl PCR reactions) into a single tube and perform the gel extraction method if the spurious bands are present. Cut the target band from the gel using benchtop UV transilluminator.
5. Clean the amplicon using QlAquick Gel Extraction Kit (Catalog # 28704) according to manufacturer's protocol.
6. Run the amplicon for each sample to check the clean band on agarose gel and measure the concentration of each sample separately by Qubit quantitation.
7. Pooled all the samples together based on the Qubit concentration.
8. Use this pooled sampled (all the samples in single volume/tube) for sequencing along with the sequencing primers (100 µM).

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Madhu Sabnis received her B.Sc (Bachelor of Science) in Botany, Zoology and Chemistry, in May 2002 from Banaras Hindu University, Varanasi, India. Then, she obtained her Master's in biotechnology from Jiwaji University, Gwalior, India. She also worked as a Project Assistant at National Institute of Oceanography, Mumbai, India for 2 years. Madhu Sabnis finished her M.S. (Master of Science) program in Environmental and Earth Sciences from University of Texas at Arlington, Arlington, Texas, in August 2009. Her master's thesis was on removal of butylene and propylene gas using biofiltration. After completing her master's degree, Madhu worked as a research assistant for a year in the University of Texas at Arlington.

In 2011, she joined The University of Texas at Arlington (UTA) as a doctoral student in Earth and Environmental Sciences and earned doctoral degree in Environmental and Earth Sciences in August 2014. Her doctoral dissertation was on investigation of how microbes involved in anaerobic digestion of vinasse change as functions of temperature, vinasse composition, and time. Her research interests are in the areas of renewable energy, climate change, and oil and gas projects.