Impact of Simple and Complex Substrates on the Composition and Diversity of Microbial Communities and the End-product Synthesis

by

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Abstract

The effect of simple and complex on the composition and diversity of microbial communities and on end-product (biogas and VFAs) synthesis was investigated using an anaerobic batch respirometer at 37 °C and pH 7.2. These experiments, simple substrates were chemically pure and contain a single carbon source (glucose or α-cellulose), while complex substrates were chemically “impure” substrates containing a mixture of two or three carbon sources (biodiesel-derived glycerol or wheat straw) with a substrate/inoculum ratio 6g chemical oxygen demand (COD)/ g volatile solids (VS) seed and 100g of pre-treated dairy manure digestate (DMD), respectively. Concentrations of hydrogen, carbon dioxide, acetate, butyrate, propionate, and ethanol synthesized by different communities selected by the growth on the different substrates were measured, and confirmed growth of the microbial communities. 16S rDNA illumina sequencing revealed that DMD without substrates was more diverse than the microbiota cultured by fermentation reactions containing D-glucose, glycerol α-cellulose or wheat straw. The data confirmed that substrates play a crucial role in determining the diversity of species in microbial communities. Dominant operational taxonomic units (OTUs) belonging to families Clostridiaceae, Ruminococcaceae, and Enterobacteriaceae, and the genera Clostridium, Ruminococcus, Sporolactobacillus, and Syntrophomonas were potentially responsible for changes in end-product synthesis patterns in communities cultured with simple and complex substrates.
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Dedication

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CHAPTER 1

Introduction

1.1 Anaerobic fermentation

Anaerobic fermentation is a collection of processes in which microorganisms play an important role in breaking down the biodegradable materials in the absence of oxygen. The main advantage of anaerobic fermentation is bioconversion of waste materials into renewable energy. The global economy and our very civilization depend upon low cost energy derived from fossil fuels (petroleum, coal, and natural gas). The combustion of fossil fuels results in the emission of carbon dioxide (CO\textsubscript{2}), a “green house gas” that traps heat energy in the Earth’s atmosphere. Global demand for energy is predicted to increase by 53\% by 2050 (World Energy Council, 2013), driven by the increasing global population, which is predicted to exceed 9 billion people. The increasing population causes increasing demand for energy, which in turn results in increased emission of CO\textsubscript{2} into the atmosphere (Kapdan & Kargi, 2006). The United Nations Intergovernmental Panel on Climate Change (UNIPCC) has predicted that “severe and pervasive impacts of climate change will be felt everywhere” on the planet (UNIPCC, 2014). Mitigation of the climate change requires reduced consumption of fossil fuels. Thus, there is a global effort to develop new sustainable energy sources that can displace the use of fossil fuels (Guo, Trably, Latrielle, Carrère, & Steyer, 2010).

Hydrogen (H\textsubscript{2}) is considered as a viable renewable energy resource that could reduce our dependence on fossil fuels (Valdez-Vazquez & Poggi-Varaldo, 2009). Molecular H\textsubscript{2} has the highest gravimetric energy density of known chemical fuels (143 GJ
tonne\(^{-1}\)). Its gravimetric energy density is 2.7-times greater than petroleum-derived hydrocarbon fuels. Moreover, H\(_2\) is the only fuel that ultimately oxidizes to water (H\(_2\)O) as a combustion product, and thus it can be considered to be a “carbon-free” fuel. In other words, combustion of H\(_2\) does not release CO\(_2\) into the atmosphere (Kotay & Das, 2007).

Currently, hydrogen gas is produced by thermo-chemical processes, such as steam reforming of methane (SRM) or hydrocarbons (SRH) by non-catalytic partial oxidation of fossil fuels (POX). All of these processes require high temperature (850 °C) and are energy consuming (Armor, 1999). Hydrogen can also be produced by electrolysis, which is the cleanest technology, but requires very large amounts of electricity. Biohydrogen is the name given to the production of H\(_2\) gas by microorganisms, such as green algae, bacteria, and archaea. Biological processes, unlike inorganic chemical or electrochemical processes, are catalyzed inside microorganisms at ambient (or near ambient) temperatures and pressures. Biological systems provide a wide-range of approaches to generate H\(_2\), which include: direct biophotolysis; indirect biophotolysis; photo-fermentations, and dark-fermentation. From the above biological processes, researchers prefer dark fermentation reaction because it does not require light source (Fang et al., 2002) and utilize waste materials to produce hydrogen. Furthermore, in dark fermentation the hydrogen production are much higher compared to photo fermentation (Azbar and Levin, 2012).

1.2 Mixed acid fermentation

Mixed acid fermentation is an anaerobic fermentation carried out by microorganisms that convert organic wastes into complex mixture of acids particularly lactate, acetate, succinate and formate as well as ethanol with equal amount of H\(_2\) and CO\(_2\) (Madigan and Martinko, 2006) by series of biochemical reactions: hydrolysis,
acidogenesis, and acetogenesis. Figure 1.1 depicts the flow chart of the three important steps involved in fermentation. A variety of microorganisms plays an important role in fermentation. The most prominent fermentative bacteria are found in the genus *Clostridium*. Spore forming acidogenic bacteria, such as *Clostridium butyricum*, *C. thermolacticum*, *C. pasteurianum*, *C. paraputrificum*, and *C. bifermentans* convert organic materials into volatile fatty acids (VFAs), H₂ and CO₂ under mesophilic conditions (Ntaikou et al., 2010). Hydrogen production by cellulolytic clostridia, such as *C. termitidis* and *C. thermocellum* has also been studied extensively (Levin et al., 2006; Ramachandran et al., 2008; Liu et al., 2008; Islam et al., 2009).

Apart from *Clostridium* species, facultative anaerobic species in the genera *Enterobacter, Bacillus, Thermotogales, Aeromonas, Pseudomonas*, and *Vibrio* have also been reported as mesophilic H₂-producing bacteria (Ntaikou et al., 2010). However, studies of dark fermentation by mixed cultures have attracted research attention because of simple operation, ease of bioprocessing in a non-sterile environment and resulted in the reduction of operational cost (Prakasham et al., 2009). Moreover, mixed cultures containing a variety of different bacterial species have been found to be more efficient in converting the wide-range of molecules found in agri-industrial waste streams to H₂ via dark fermentation (Guo et al., 2010).

Mixed culture biotechnology (MCB) techniques have evolved by the undefined microbial communities derived from wastewater treatment plants, animal waste, and agricultural wastes can result in conversion of complex organic molecules to H₂, and volatile fatty acids which could be used as a source of energy (Wei et al., 2011). Comparisons of biohydrogen by mixed cultures of bacteria have been shown to produce
Figure 1.1 Steps involved in anaerobic fermentation reactions.
higher yields of H$_2$ and other soluble products than pure cultures (Kleerebezem and van Loosdrecht, 2007)

The first stage of dark fermentation, hydrolysis, involves breaking down complex organic substrate into simple monomers. In the second stage, the monomers are further degraded to carbon acids, CO$_2$, and H$_2$ by the acidogenesis process. The final stage of dark fermentation involves further degradation of VFAs or longer chain (C16 and C18) fatty acids into CO$_2$ and H$_2$ through acetogenesis.

### 1.3 Factors that affect anaerobic fermentation

There are a number of factors that influence the biohydrogen production and associated VFAs by dark fermentation, including: inoculum, substrate, temperature, pH, H$_2$ partial pressure, reactor type, and nutrient concentrations (nitrogen, phosphate, metal ions, etc). Among these, pH and temperature are very important parameters to consider. Numerous studies have demonstrated that the pH and temperature of dark fermentation systems must be optimized with respect to the substrate used and the type of bacteria in the mixed culture (Fang and Liu, 2002; Wang and Wan, 2009). Mixed cultures can grow and produce H$_2$ at various temperatures, ranging from 37°C to 60°C (Varrone et al., 2012; Mohammadi and Annuar, 2012; Gupta et al., 2014). pH has a direct impact on both VFA and H$_2$-production in the system. Bacteria favor H$_2$-producing butyrate-acetate pathways at pH 4.5 to 6.0, while at neutral or higher pH conditions, ethanol and propionate tend to be synthesized. In general, pHs ranging from 5.0 to 7.0 are optimal for maximum H$_2$ yield because these pHs favor bacteria with high hydrogenase activity (Wang and Wan, 2009; Kraemer and Bagley, 2007). However, an important point to consider is that when a mixed, undefined microbial community is used for dark fermentation, the temperature, pH, and
substrate type will select specific subsets of bacteria that thrive under these specific conditions, and both the volumetric yield (cumulative production) and molar yield of H₂ will be a function of the microbial community structure selected.

1.3.1 Seed microbiota

Microorganisms capable of producing biohydrogen are generally present in nature. Samples from sewage water treatment plants, soil, anaerobic sludge, and municipal wastes are rich in mixed microbial cultures, which can produce H₂ in higher concentrations than pure culture (Wang and Wan, 2009). Complex organic substrates are degraded effectively by mixed cultures (Angenent and Wrenn, 2008). However, within mixed microbial communities used for fermentative H₂-production, there are also H₂-consuming bacteria, such as methanogens that combine H₂ and acetate or H₂ and CO₂ to synthesize CH₄. Homoacetogenic bacteria utilize the hydrogen to convert CO₂ into acetate (Divergence, 2009; Diekert and Wohlfarth, 1994) and sulfate-reducing bacteria use molecular hydrogen to reduce sulfate (SO₄²⁻) to hydrogen sulfide (H₂S) (Muyzer and Stams, 2008). These methanogenic, homoacetogenic, and sulfate-reducing bacteria must be suppressed in the mixed culture before it is used for fermentative H₂-production.

1.3.2 Pre-treatment of seed micro flora

Several pre-treatment methods to eliminate H₂-consuming bacteria from activated sludge have been described in the literature (Tanaka et al., 1997; Liu et al., 2008; Carrère et al., 2010) Pre-treatment methods include physical treatments, such as heat-shock, aeration, freezing and thawing, and chemical pretreatments, such as acid, base, chloroform, and sodium 2-bromoethanesulfonate (Wang and Wan 2008; Cheong and Hansen, 2006). Various reports have compared different physical and chemical pre-treatment methods for
different forms of sludge, soil, and anaerobic digestates followed by anaerobic fermentation (Kraemer and Bagley, 2007; Rossi et al., 2011).

Heat-shock treatment has been commonly practiced to eliminate the methanogens from mixed cultures, and heat-treatment has been shown to be the most effective method to screen spore forming hydrogen producers from the natural source of inocula (Baghchehsaraee et al., 2008). Pre-treatment of digested sludge by five methods (acid base, heat-shock, aeration, and chloroform) was conducted in batch tests using D-glucose as substrate at 35 °C and pH 7.0 The results showed that dark fermentation carried out with inocula prepared using the heat-shock method resulted in biogas containing 97% H₂ and the cumulative H₂-production was 2.739 g/L, which is 1.36 moles/L (Wang and Wan, 2008). Several researchers have explored preparation of inocula for dark fermentation from different sources, such as cow compost and activated sludge, using a range of temperatures, from 70 °C to 120 °C (Baghchehsaraee et al., 2008 and Zhao et al., 2010).

In conclusion, heat-shock pre-treatment method is the most cost effective method to eliminate methanogens and at the same time also preserve the H₂-producing bacteria such as Clostridium species in the seed inoculum (Fan 2004; Kawagoshi et al., 2005). Heat-shocked pre-treated seed culture has been widely used in dark fermentation reactions for H₂-production at temperatures from 35 °C to 65 °C and at pH 5.5 to 10 in both batch and continous fermentation reactions ((Ivanova et al., 2009; Hafez et al., 2010; Guo et al., 2010).

1.3.3 Substrates

Dark fermentation is mainly based on the conversion of organic carbon substrates into H₂. Many substrates have been used for dark fermentation processes. These can be
broadly categorized into simple versus complex substrates. Simple substrates include D-glucose, sucrose, and starch, which have been used in dark fermentation reactions with both mixed, undefined cultures and pure defined cultures (Vijayaraghavan et al., 2006). Other studies have focused on utilizing readily available lignocellulosic agricultural wastes, including straw, stover, peelings, cobs, stalks, bagasse, and other materials rich in carbohydrates.

When using complex substrates, it is necessary to breakdown the crystalline nature of the cellulose into soluble, fermentable sugars that can be converted to H₂ and CO₂ (Sims et al., 2010). It has been estimated that 200 billion tons of lignocellulosic biomass may be generated in different parts of the world. This represents an abundant, low cost feedstock for H₂-production (Levin et al., 2004; Levin and Chahine, 2010). Organic waste materials comprised of animal manure, food waste, and industrial wastewaters that are rich in carbohydrates, proteins, and fatty acids, can also be used for dark fermentation with mixed microbial cultures for H₂-production. These wastes are highly biodegradable and contain 75–95% volatile solids, which favors high H₂ yield (Guo et al., 2010). The studies described in this thesis focused on the use of D-glucose and crude-glycerol as models of simple substrates, as well as α-cellulose and wheat straw, as models of complex substrates. The importance of simple and complex substrates is discussed in detail below.

### 1.3.3.1 D-glucose as a substrate for fermentation

D-glucose is widely present in organic wastes, in simple or complex states, and can be converted into useful end-products by microbial fermentation. Fermentation of glucose can yield H₂, CO₂, and organic acids, such as acetic and butyric acid (although under
slightly acidic to neutral pH conditions, these organic acids are in the salt form: acetate or butyrate), by the following equations:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2\text{------------------------ }\text{Equation 1}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \text{------------------------ }\text{Equation 2}
\]

The highest theoretical yields of H\textsubscript{2} are associated with the synthesis of acetate as the fermentation end-product. In practice however, high H\textsubscript{2} yields are associated with a mixture of organic acid fermentation products, which in addition to acetate may include lactate and/or formate, as well as ethanol, which reduce the observed molar yield of H\textsubscript{2} to well below 4 mol H\textsubscript{2}/mol hexose equivalent. Reduced fermentation end-products, like ethanol, butanol, and lactate, contain hydrogen that has not been liberated as the gas. Even lower H\textsubscript{2} yields are associated with butyrate synthesis, which may be accompanied by synthesis of propionate and/or butanol (Levin et al., 2004).

Hydrogen production by anaerobic bacteria is highly dependent on the process conditions such as temperature, pH, hydraulic retention time (HRT), and gas partial pressure, which affect redox balance and thus influence carbon and electron flux through different pathways (Carere et al., 2014 and Rydzak et al., 2014). Thus, fermentation end-products produced by a bacterium depend on the environmental conditions in which it grows. For example, Gupta et al., (2014) carried out batch fermentation experiments to explore the synergetic effect of co-fermentation of D-glucose, sucrose, and cellulose by microbial communities associated with H\textsubscript{2}-production from each substrate, either individually or in combinations. Yields of H\textsubscript{2} were 1.22, 1.00, and 0.15 mol H\textsubscript{2}/mol hexose equivalent added in fermentation reactions containing glucose, starch, and cellulose as mono-substrates. The H\textsubscript{2} yields were greater by an average of 27 ± 4 % than expected in all
the different co-substrate conditions, which affirmed that co-fermentation of different substrates improved the H2 potential. Glucose addition to starch and/or cellulose favored acetate synthesis, while cellulose degradation was associated with the propionate synthesis pathway. These experiments and many others confirm that glucose is a preferred substrate for H2-production.

1.3.3.2 Crude glycerol as a substrate for fermentation

Crude glycerol is a by-product of biodiesel production, and is generated by transesterification of vegetable oils. The ratio of biodiesel: glycerol by-product production is 10:1, and industrial scale production of biodiesel has resulted in the accumulation of massive volumes of waste crude glycerol, which has had a significant negative impact on the economy of biodiesel industries worldwide (Yang et al., 2012). Although pure glycerol has numerous applications in the pharmaceutical and cosmetic industry (as well as many others), crude biodiesel-derived glycerol is very difficult to purify for these applications. Thus, researchers have focused on converting crude biodiesel-derived glycerol into useful products such as 1,3-propanediol, 1,2-propanediol, dihydroxyacetones, hydrogen, polyglycerols, succinic acid, and polyesters using various process. It is interesting to note that due to the high value of renewable biogas and bioethanol, the production of biogas from crude glycerol has been well investigated (Varrone et al., 2012).

Hydrogen can also be produced from glycerol through steam reforming, gasification (partial oxidation), auto-thermal reforming, aqueous-phase reforming (APR), and supercritical water reforming processes (Adhikari et al., 2009; Levin and Chahine, 2010). However, few investigations have concentrated on converting residual glycerol into H2 using microbial consortia. Rossi et al., (2011) evaluated H2-production using waste
crude biodiesel-derived glycerol as the substrate, and observed $5.27 \pm 2.27$ mol H$_2$/g glycerol using heat-shock pre-treated sludge. Varrone et al., (2012) optimized H$_2$ and ethanol production from crude glycerol at 37 °C at an initial pH of 7.9. The yield of H$_2$ and ethanol were 0.96 mol H$_2$/mol glycerol and 7.92 g/L from 15g/L glycerol, respectively.

The above studies have shown that crude glycerol can act as the substrate to produce higher amounts of H$_2$ and other end-products using microbial populations under optimum conditions. In an anaerobic process, bacteria use both oxidation and reduction reactions to metabolize glycerol. The oxidative reaction converts glycerol into pyruvate with intermediate formation of phosphoenolpyruvate.

This pyruvate is further degraded into acetate, lactate, propionate, butyrate, and H$_2$ by the microorganisms, which contain enzymes responsible for consecutive reactions in these pathways. The glycerol degradation pathway is similar to glucose fermentation reaction pathway after the formation of pyruvate in the oxidative reaction (Sarma et al., 2012).

1.3.3.3 Cellulose as a substrate for fermentation

Cellulose is the most abundant biopolymer on Earth. It is an important component of the cell wall of green plants. Cellulose is a linear polysaccharide polymer of D-glucose subunits linked by β-(1,4) glycosidic bonds. The basic subunit of cellulose chains is cellobiose, a disaccharide composed of two-glucose molecules. Although cellulose is more abundant in nature, it always associates with hemicellulose, lignin, and proteins in the matrix of plant cell wall (Leschine, 1995). The great availability of cellulose in the form of agricultural crop residues has gained attention as an abundant feedstock for H$_2$-production via anaerobic fermentation using
mixed microflora (Carere et al., 2008; Levin et al., 2004; Levin, 2010). Mixed microbial populations obtained from activated sludge, dairy compost, and soil contain natural cellulose-degrading microorganisms that can breakdown cellulose chains to oligocellodextrins, cellobiose, and glucose with a concomitant synthesis of H₂, CO₂, and organic acids as show in Figure 1.2. Other saccharolytic microbes in the microbial community can also ferment the cellulose hydrolysis products to H₂, CO₂, and organic acids. In mixed microbial communities that have not been subjected to pre-treatment to eliminate methanogens, H₂ consumers will reduce organic acids into alcohol and CH₄ (Leschine, 1995). Batch fermentation reactions with different concentrations of cellulosic substrates (5–30 g/L), using cow dung compost at 37 °C, and pH 6.8 resulted in volumetric yields that ranged from 828 mL H₂/L culture at 5 g/L cellulose to 1,251 mL H₂/L culture at 10 g/L substrate. However, higher concentrations of substrate did not result in greater H₂-production. Acetate and butyrate were the major aqueous phase fermentation end-products (Ren et al., 2010). Lin and Hung (2008) also evaluated H₂-production from cellulose using enriched cow dung compost at 55 °C, at different pHs (5.5 to 9.0). The data revealed that cow dung sludge is a good seed source of bacteria that can hydrolyze cellulose and generate H₂ and ethanol by fermentation. On the other hand, pure cultures, such as Clostridium thermocellum at thermophilic temperatures, produced 0.62 and 1.6 H₂/mol hexose equivalent from cellulose, respectively (Ramachandran et al., 2008; Levin et al., 2006). Moreover, Liu et al., (2008) investigated H₂-production from cellulose using a co-culture of C. thermocellum
**Figure 1.2** Diagrammatic representation of conversion of cellulose to fermentation end-products by mixed microbial communities.
and Thermoanaerobacterium thermosacchararolyticum. Hydrogen production increased 2-fold, from 0.8 to 1.8 mol H₂/mol glucose in the co-culture compared with each individual mono-culture. The above literatures clearly emphasize that α-cellulose is the suitable complex substrate for biohydrogen production using dark fermentation.

1.3.3.4 Wheat-straw as a substrate for fermentation

Lignocellulosic biomass is rich in hexose (C-6) and pentose (C-5) sugars and can be an attractive source for biofuel production by dark fermentation. Wheat-straw is a lignocellulosic agricultural by-product produced from wheat plants after the grain and chaff have been removed. In recent years, the consumption of wheat by humans has increased drastically which has resulted in the storage of straw (in the form of straw-bales) in huge quantities (Khan and Mubeen, 2012).

Wheat straw can be used for a wide variety of applications, such as feedstock for animals, paper industries, basket making, bedding, craft making, and cosmetics. Still, large amounts of wheat straw are burned in fields by farmers (Wei et al., 2011). In General, wheat straw is composed of cellulose (40–50%), hemicellulose (20–30%), lignin (10-20%) (carbohydrates), and smaller amounts of pectin, protein, minerals, silica, and ash. The chemical composition of wheat straw varies with cultivar and growth conditions in the field (Collins et al., 2014; Antongiovanni and Sargentini, 1991). Hydrogen production from wheat straw could increase the economic value of biomass and reduce the greenhouse emissions globally (Kaparaju et al., 2009).

1.4 Microbial community studies and advantages

Most microorganisms live in communities that consist of multiple species. Microbial communities are very complex in their structure and the community members
interact with each other to obtain nutrients and space. The study of microbial communities is important because it can be used to understand the complicated process that take place in natural environments (Handelsman, 2007). Fundamental concepts that have been revealed through characterizing microbial communities in natural ecosystems include: 1) the types of microorganisms present in different environments; 2) discovery of new microorganisms; 3) the role(s) of microorganisms in these communities; and 4) the activities of the microbes related to the ecosystems in which they reside (Rastogi and Sani, 2011).

Traditionally microbes are identified by isolating individual species from the natural environment and study the physiological and morphological characteristic by growing the microbes in a synthetic medium under optimum conditions. However, there are many limitations to culture the microbes. First, lack of knowledge about the requirement of nutrients and substrates to grow in an artificial medium. Second, many microbes grow syntrophically with each other and thus cannot be cultured individually (Pike and Curds, 1971). Lastly, microbes in the communities share similar morphological characteristics and thus cannot be easily differentiated into different taxa. This condition leads to the unreliable identification of microorganisms. To overcome these issues, a number of molecular techniques have been developed for the quantitative and qualitative analysis by extracting the nucleic acids from the microorganisms directly without culturing them in the artificial medium.

1.4.1 Analysis of microbial community studies by molecular techniques

The majority of microorganisms present in ecosystems cannot be cultured with either classical or modern techniques, but nucleic acids and/or proteins of those microorganisms can be isolated and analyzed. Thus, culture-independent molecular
approaches are being used to characterize microbial community structure and/or to identify new microorganisms, which may be present in different environments. Two molecular techniques are commonly employed for identifying microorganisms: i) “Partial community analysis” methods; and ii) “Whole community analysis” methods (Rastogi and Sani, 2011). “Partial community analysis” methods are out-dated and not relevant to this thesis. “Whole community analysis” methods, however, are very relevant to the work described in this thesis, and thus are discussed in detail.

1.4.1.1 Whole community analysis approach

The 16S rRNA genes are the specific targets that are widely used for identifying the composition of microbial communities in ecosystems. Even though 16S rRNA gene sequences are highly conserved, they do not provide sufficient information to differentiate bacteria at species and strain level. Whole genome molecular techniques, however, provide more information about the genetic diversity within microbial communities compared to PCR-based molecular approaches that target specific gene sequences. Whole community analysis can be classified into three different categories based on the molecular targets involved in the study: i) Metagenomics methods utilize genomic DNA sequence data collected from environmental samples, which are analyzed as single large (“meta”) genomes (Handelsman et al., 1998); ii) Metatranscriptomics, or environmental transcriptomics involves the study of global transcription of genes by random sequencing of mRNA transcripts pooled from microbial communities at a particular time and place (Moran, 2010); and iii) Metaproteomics, also known as environmental proteomics, analyzes the total protein molecules isolated from environmental microbial samples. The proteomic analysis of mixed microbial communities is most reliable and provides clear
information of microorganisms in the ecosystem (Wilmes & Bond, 2006). Among the three approaches to whole community analysis, metagenomics study is the easiest and most cost effective method to analyze microbial populations present in open or closed systems. The Metagenomic molecular approach is discussed in detail below.

1.4.1.1.1 *16S rDNA variable region analysis using high-throughput-Illumina sequencing*

The small subunit ribosomal RNA gene (16S rDNA) is widely considered as a standard molecular marker for characterization of bacterial community structure, and has been used to compare microbial communities in a variety of ecological niches (Mizrahi-Man et al., 2013) The 16S rDNA encodes an RNA molecule of 1,542 nucleotides, which contains nine interspersed conserved and variable regions. The variable regions of the 16S rDNA include hyper-variable sequences of 50 to 100 base pairs, which can be used for microbial identification. However, some hyper-variable regions are more effective for differentiating between bacterial species than others. Three different hyper-variable regions of the 16S rDNA (the V2–V3, V4–V5, and V6–V8 regions) have been shown to be effective for differentiating bacterial consortia, while the V2 and V3 regions are most effective for universal identification at the genus level (Petrosino et al., 2009). The main advantages of using 16S rDNA gene sequences for microbial community structure analyses are that these sequences are ubiquitous among prokaryotes, they contain both conserved regions (sequences that accumulate mutations slowly over time), as well as variable and hyper-variable regions, and “universal primers” for amplification of specific target sequences can be used for a wide variety of taxa.
Illumina sequencing is a technique used to determine the single-bases based on reversible dye-terminators that are attached to the DNA stands. This method only uses DNA polymerase as opposed expensive enzymes required by other sequencing techniques like pyro-sequencing. Illumina sequencing offers more advantages than traditional Sanger sequencing. It enables sequencing of multiple strands at a time due to the automated nature in the system. Initially, second-generation sequencing was carried out by large-scale 454-pyrosequencing platforms that amplify longer reads of up to 500 base pairs. Later, Illumina sequencing technology replaced the 454 pyrosequencing with the advantage of analyzing thousands of samples in a single run (although the sequence read length is only 100 to 200 bp). The sequence data generated by Illumina sequencing can be used to construct phylogenetic trees (Caporaso et al., 2010). Figure 1.3 represents the workflow of 16S rDNA amplification and nucleotide sequence analysis via Illumina sequencing technology.

1.4.1.1.2 Metagenomics

Metagenomics is defined as the direct investigation of microbial genomes extracted from the environmental samples without relying on culturing or prior knowledge about the microbiome (Riesenfeld et al., 2004). The broad field may also be referred to as environmental genomics, ecogenomics, or community genomics. Metagenomic investigations have been conducted in the soil, water, and anaerobic digester bioreactors, and have revealed significant phylogenetic diversity. Recovery of DNA (more than 1000 bp) for metagenomic analysis can be carried out by high-throughput sequencing recently. In “High-throughput sequencing”, in which short DNA fragments (~ 400 bp to 700 bp) extracted from the environment samples are sequenced by 454 Pyro sequencing or Illumina Miseq or Hiseq sequencing technologies. The short sequence reads are then analyzed by
Figure 1.3 Workflow of 16S rDNA sequence amplification and nucleotide sequence analysis using illumina-sequencing technology.
bio-informatic tools and compared with Universal DNA sequence databases to identify the microbes present. Xia et al., (2012) investigated the effect of co-substrates and microbial communities enriched by glucose, xylose and starch on thermophilic anaerobic conversion of microcrystalline cellulose using mixed culture enriched from anaerobic digestion sludge (ADS). Illumina high-throughput sequencing of 16S rRNA gene revealed that the thermophilic consortium consisted of species in the genera Clostridium and Thermoanaerobacterium.

1.5 Objectives, hypothesis, and structure of the thesis

The objective of this research was to identify microbial communities in dairy manure digestate (DMD) enriched by simple (D-glucose and crude-glycerol) and complex (α-cellulose and wheat straw) substrates to produce fermentation end-products (H2, CO2, volatile fatty acids, and ethanol) in batch fermentation reactions. The underlying hypothesis of this research was that end-product synthesis patterns are a function of the microbial community structure, which is defined by the carbon source available to the microorganisms. In other words, growth conditions (temperature, pH) and substrate type will select specific subsets of bacteria that thrive under those specific conditions, and the resulting microbial community structure and diversity will determine both the volumetric yield (cumulative production) and/or molar yield of fermentation end-products generated.

This hypothesis was tested in two phases. First, microorganisms were cultivated on D-glucose, crude-glycerol, α-cellulose, and wheat straw in serum bottles at 37 °C and pH 7.2. Gaseous (H2 and CO2) and aqueous phase (volatile fatty acids and ethanol) fermentation end-products were analyzed at the end-point of the fermentation reactions. Second, microbial community structure and diversity in the resulting bacterial populations
were determined by amplification of the 16S rDNA V3 - V4 hyper-variable regions, followed by nucleotide sequence analysis by MiSeq Illumina sequencing technology. The nucleotide sequence data was subjected to bioinformatics to identify the microorganisms, and the data was further subjected to statistical analyses to characterize the microbial diversity. Finally, concentrations of gaseous and aqueous phase fermentation end-products detected in cultures grown with different carbon sources were putatively correlated with the Operational Taxonomic Units (OTUs) identified in the different microbial communities.

This thesis is organized into five chapters. Chapter 1 contains the literature review and a statement of the objectives and a hypothesis of the research. Chapter 2 describes the methods used in the research. Chapter 3 describes the study of microbial populations grown on D-glucose and crude-glycerol to: i) understand the influence of substrates (D-glucose versus crude-glycerol) on microbial community structure and diversity, and correlate the fermentation end-product synthesis patterns with the observed microbial diversity; and ii) compare the microbial diversity enriched by growth on D-glucose versus crude-glycerol at 37°C and pH 7.2.

Chapter 4 describes the influence of α-cellulose on microbial community structure and diversity selected by growth on two different pre-treated dairy manure digestates (DMDs): i) fresh pre-treated DMD; and ii) a mixture of 50% enriched DMD + 50% fresh pretreated DMD (E-DMD). The resulting microbial diversity was then correlated with fermentation end-product synthesis patterns. Chapter 5 describes the influence of raw-wheat straw on microbial community structure and diversity selected by growth of two different pre-treated DMDs: i) fresh pre-treated DMD; and ii) E-DMD, followed by
correlation of the fermentation end-product synthesis patterns with the observed microbial diversity.

Chapter 6 is divided into two parts. The first part describes the comparison of microbial communities selected by growth of DMD on D-glucose, crude-glycerol, α-cellulose, and wheat straw in serum bottles at 37 °C and pH 7.2 using alpha-diversity analysis (rarefaction curves), PERAMANOVA analysis, and diversity indices. The second part presents a bioinformatics analysis of the similarities and differences between microbial communities generated from two different seed inocula- one derived from activated digested sludge (ADS) and the other derived from dairy manure digestate (DMD) - grown on D-glucose and α-cellulose, respectively.
CHAPTER 2

Methodology

2.1. Fermentative micro flora

Dairy manure digestate (DMD) was collected from a continuous stirred tank reactor fed with dairy manure for methane production (Department of Biosystems Engineering in University of Manitoba, Winnipeg, Canada). To eliminate methanogens and select microorganisms that synthesize hydrogen from simple and complex substrates, activated DMD was pre-treated at 70 °C or 100 °C for 30 minutes (min) respectively. The two different temperatures were chosen because of the presence of methanogens at 70 °C pre-treated DMD after 12-15 days of incubation. The pre-treated DMD was then stored in a glass beaker in the refrigerator at 4 °C for future use. The characteristics of pre-treated DMD are listed in Table 2.1.

2.2. Simple and complex substrates

Simple substrates (D-glucose and α-cellulose) and complex substrates (crude glycerol, and raw-wheat straw) were selected as electron donors for hydrogen production in batch experiments. Each substrate was added based on a Substrate:Inoculum ratio of 6 g COD/g VS seed calculated by Equation 1.

\[
\frac{S}{X} \left( \text{g COD/g VS} \right) = \frac{V_f (\text{kg}) \times \text{TCOD (g/kg)}}{V_S (\text{kg}) \times \text{VS seed (g/Kg)}}
\]

(Equation 3)

where: \( V_f \) is the volume of feed; \( V_S \) is the volume of seed; and \( \text{VS} \) is the volatile solid of DMD (Nasr, et al., 2012).
Table 2.1 Characterization of substrates and Dairy Manure Digestate (DMD).

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Total Solids (TS) %</th>
<th>Volatile Solids (VS) %</th>
<th>Chemical Oxygen Demand (COD) (mg/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-glucose</td>
<td>97.59 ± 2</td>
<td>97.70 ± 2.1</td>
<td>10670</td>
</tr>
<tr>
<td>Crude Glycerol</td>
<td>80.05 ± 0.5</td>
<td>76.13 ± 0.5</td>
<td>12200</td>
</tr>
<tr>
<td>Cellulose</td>
<td>97.59 ± 2</td>
<td>72.99 ± 0.2</td>
<td>11800</td>
</tr>
<tr>
<td>Wheat straw</td>
<td>97.70 ± 0.1</td>
<td>90.91 ± 0.1</td>
<td>28100</td>
</tr>
<tr>
<td>DMD</td>
<td>4.6 ± 0.1</td>
<td>3.3 ± 0.57</td>
<td>43000</td>
</tr>
</tbody>
</table>
The Total Chemical Oxygen Demand (TCOD) of the substrate to be added was calculated to be 18.5 g COD per 100 g of seed DMD added to each bottle. The Total Solids (TS), Volatile Solids (VS), and Chemical Oxygen Demand (COD) of the simple and complex substrates are displayed in Table 2.1.

2.3. System set up and operation conditions

Biological hydrogen production experiments were conducted in batch tests using a respirometer system, which enables continuous monitoring of biogas accumulation in the headspace of the reaction vessels. The system contains eight 500 mL serum bottles closed with rubber stoppers. Each bottle was connected to a bubble counter and gas production was monitored by on-line software system that measured the volume of gas produced during each time interval.

A volume containing 100 g of seed was added to each bottle and made up to a 400 mL working volume using nutrient media with stock solution 1 and stock solution 2 as described in (Moody et al., 2011). The initial pH was adjusted to 7.2 by adding sodium bicarbonate to nutrient medium. The headspace was flushed with N₂ gas for a period of 8 min and capped tightly with a rubber stopper. Six independently replicated (biological replicates) were conducted simultaneously in six separate bottles, along with a control bottle, which contained 100 g of DMD seed, but no carbon source substrate. The bottles were mixed at a constant speed of 150 rpm using magnetic stir bars within the bottles and magnetic stir plates built into the water bath of the respirometer system. The system was operated anaerobically at 37 °C and atmospheric pressure for 10–25 days, based on substrate type.
2.4. Analytical methods

Biogas production was measured using suitable sized glass syringes in the range of 0.1–1.0 mL. Concentrations of H₂, CO₂, CH₄, and N₂ in the biogas were determined by Gas Chromatography (SRI Instruments, Model 8610C or Agilent 7890A GC System). The cumulative H₂ and CO₂ gas concentrations were calculated from the bottle headspace measurements and the total volume of biogas synthesized in each interval using Equation 2.

\[
V_{H,i} = V_{H,i-1} + C_{H,i} (V_{G,i} - V_{G,i-1}) + V_H (C_{H,i-1} - C_{H,i-1})
\]

Equation 4

where: \( V_{H,i} \) and \( V_{H,i-1} \) are cumulative hydrogen gas volume at the current (i) and previous (i-1) time intervals; \( V_{G,i} \) and \( V_{G,i-1} \) are the total gas volumes in the current and previous time intervals; \( C_{H,i} \) and \( C_{H,i-1} \) are the fraction of hydrogen gas in the headspace of the bottle measured using gas chromatography in the current and previous intervals; and \( V_H \) is the total volume of headspace in the bottle (Logan et al., 2002). The total cumulative H₂ and CO₂ gas production were calculated by subtracting the value of Treatment 1 (DMD without substrate) from the net gas production by Treatments containing different substrates inoculated with pre-treated DMD.

Volatile fatty acids (VFAs) were analyzed using a High Performance Liquid Chromatography (Waters Breeze™ 2 HPLC system) with the mobile phase consisting of 5mM H₂SO₄. Chemical oxygen demand (COD) was measured using HACH methods and test kits (HACH Odyssey DR/2500 spectrophotometer manual). Total solids (TS) and Volatile solids (VS) were analyzed using standard methods (Eaton et al., 1995).
2.5. Microbial community analysis

2.5.1. Sampling and DNA Extraction

DMD was collected from each of the six bottles for each experiment that contained different substrates: D-glucose, crude-glycerol, raw-wheat straw, α-cellulose. Each replicated experiment also contained two blank bottles (that contained DMD but no substrate), which were collected from the respirometer with the 6 test bottles at the end of each experiment. DNA was extracted from each DMD sample, and each sample was subjected to Illumina sequencing. DNA was extracted from approximately 1.0 g of sludge sample using E.Z.N.A. Soil DNA kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer’s instructions and laboratory manuals (Dineen, Aranda, Anders, & Robertson, 2010). The quantity of DNA was determined using a Nano Drop ND1000 UV-Vis spectrometer at 260 nm. The DNA purity was estimated spectrophotometrically by calculating the A260/A280 and A260/A230 ratios.

2.5.2. Library construction and Illumina sequencing

The isolated DMD DNA was used as a template for polymerase chain reaction (PCR) amplification of V3-V4 hyper-variable regions of 16S rRNA genes using a modified F338 primer (5’AATGATACGGCGACCACCAGATCTACACTATGGTAAATTGTACTCCTACTCGGAGGAGCAG-3’) as the forward primer and modified barcoded R806 primers as described by (Caporaso et al., 2012). In brief, PCR reactions for each sample were performed in duplicate and contained 1.0 µL of pre-normalized DNA, 1.0 µL of each forward and reverse primer (10 µM), 12 µL HPLC grade water (Fisher Scientific, ON, Canada) and 10 µL 5-Prime Hot MasterMix® (5-Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial denaturing step at 94 °C for 3 min followed by 35
amplification cycles at 94 °C for 45 seconds (sec), 50 °C for 60 sec, and 72 °C for 90 sec; finalized by an extension step at 72 °C for 10 min in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs, and reaction components. PCR reaction conditions were optimized as following: an initial denaturing step at 94 °C for 3 min followed by 30 amplification cycles at 94 °C for 45 sec, 62°C for 60 sec, and 72 °C for 90 sec, followed by an extension step at 72 °C for 10 min.

The V3-V4 libraries were then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, NY, USA) and diluted to a final concentration of 5 pm, as measured by a Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). In order to improve the unbalanced and biased base composition of the generated 16S rRNA libraries, 15% of PhiX control library was spiked into each amplicon pool. Customized sequencing primers (Table 2.2) were designed and added to the MiSeq Reagent Kit V3 (600-cycle) (Illumina, CA, USA). All the primers used in this study were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA). The 150 and 300 paired-end sequencing reactions were performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

2.5.3. Bioinformatics and statistical analysis

Initially, the MiSeq Illumina sequencer generated a total of 1,669,266 sequences from the 42 biological replicate samples obtained from the anaerobic batch experiments. The PANDAseq assembler (Masella et al., 2012) was used to merge overlapping PE Illumina
fastq files and all the sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output FASTq files were then analyzed using the open source software package QIIME (Caporaso et al., 2010). Assembled reads were de-multiplexed according to the barcode sequences and exposed to additional quality-filters so that reads with more than 3 consecutive bases with quality scores below 1e-5 were truncated. Chimeric reads were filtered using UCHIME (Edgar et al., 2011) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME implementation of UCLUST (Edgar, 2010) at 97% pair-wise identity threshold. Taxonomies were assigned to the representative sequence of each OTU using RDP classifier (Wang et al., 2007) and aligned with the Greengenes Core reference database (DeSantis et al., 2006) using PyNAST algorithms (Caporaso et al., 2010).

2.5.3.1. Alpha- and Beta-diversity

Alpha-diversity was calculated using QIIME scripts within each community. Alpha rarefaction curves were generated using Chao 1 estimator of species richness (Chao, 1984) with ten sampling repetitions at each sampling depth. An even depth of approximately 5000 sequences per sample was used for calculation of richness and diversity indices. Beta-diversity between microbial communities was compared using permutational
Table 2.2 Customized sequencing primers added to Miseq V2 and V3 reagent kits to support paired-end sequencing reactions.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
<th>Cartridge’s well #</th>
<th>Volume (µl)</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read 1 (V4)</td>
<td>TATGGTAATTGTGTGCCAGCMGCCGCGGTAA</td>
<td>12</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Read 1 (V3-V4)</td>
<td>TATGGTAATTGTACTCCTACGGGAGGCAG</td>
<td>12</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Read 2</td>
<td>AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT</td>
<td>14</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Index</td>
<td>ATTAGAWACCCBDGTAGTCCGGCTGACTGACT</td>
<td>13</td>
<td>4</td>
<td>100</td>
</tr>
</tbody>
</table>
multivariate analysis of variance (PERMANOVA; Anderson, 2005) was used to calculate P-values and test for differences between microbial communities.

2.5.3.2. **Statistical analyses**

Statistical analyses were performed as described by (Caporaso et al., 2010). In brief, Partial Least Square-Discriminant Analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on Genus data to identify the microbial population enriched by the substrates such as D-glucose, crude-glycerol, α-cellulose, and raw-wheat straw.

The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the categories of a categorical variable on X. In this case, the X variables were bacterial taxa, and the Y variables were observations of different Treatments: i.e. the microbial populations that were identified when DMD and E-DMD were grown on different substrates versus the microbial populations that were identified when DMD was grown without substrates in the batch experiments. Unit Variance in SIMCA P+ was used for the purpose of scaling the data. The number of significant PLS components (PC1 and PC2) were determined by Cross-Validation (Li et al., 2012).

The PLS regression co-efficient of taxa were used to identify the significance towards the treatments and positively correlated taxa clustered where the treatments show a high degree of association (indicated by a triangle displayed in the loading plot). To avoid over parameterization of the model, Variable Influence on Projection value (VIP) were estimated for each taxon, and taxa with VIP < 0.1 were removed from the final model. The R2 estimate was then used to evaluate the goodness of fit, and the Q2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to
identify taxa that were most characteristic for each of the different substrates used, and the results were visualized by PLS-DA loading plots.

The UNIVARIATE procedure of SAS (SAS 9.3, 2012) was used to test the normality of residuals for Alpha biodiversity data. Data that were not normally distributed were log transformed and then used to assess the effect of co-substrates using MIXED procedure of SAS. Operational Taxonomic Unit (OTU) percentage data were also used to evaluate statistical differences among the microbial communities that were generated from different substrates. The MIXED procedure of SAS was utilized, as described above, to test for significant changes in the proportions of different Phyla among the groups of substrates. The differences between groups were considered significant at P < 0.05, while trends were observed at P < 0.01.
Chapter 3

Influence of substrate type on microbial community structure and fermentation end-product synthesis patterns I:

D-glucose and crude-glycerol

3.1. Microbial diversity and community structure selected by growth on D-glucose and crude-glycerol

D-glucose and crude-glycerol which are simple organic substrates were widely used as a source to produce bio hydrogen and VFAs using microorganisms through fermentation reaction. Fang and Liu (2002) studied the effect of pH (from 4.0 to 7.0) on the diversity of microbial populations and on the conversion of glucose to H$_2$, by a mixed culture at 36 °C in a batch reactor. The result of 16S rDNA analysis by DGGE revealed that microbial diversity increased with pH. Hung et al., (2007) and Hafez et al., (2010) investigated H$_2$-production by microbial communities in agitated granular sludge bed bioreactors (AGSB) and a continuous stirred tank reactor (CSTR) equipped with a clarifier system (Biohydrogenator) using glucose as substrate. DGGE analysis of PCR amplified 16S rDNA indicated that Clostridium sp., Clostridium pasteurianum, Clostridium acetobutyricum, Klebsiella oxytoca, and Streptococcus sp., were dominant in the both reactor systems.

Recently, Gupta et al., (2014) carried out a batch experiment to explore the synergetic effects of co-fermentation of glucose, sucrose, and cellulose, and characterized the associated microbial communities involved in H$_2$-production from each substrate, individually or in combination, using pre-treated wastewater treatment sludge. Glucose
alone enriched the growth of operational taxonomic units (OTUs) in the order Clostridiales, the families Clostridiaceae, and Ruminococcaceae, and in the genera *Clostridium*, *Ethanologenens*, all of which are known H₂ producers. The microbial community generated by growth on glucose produced greater amounts of H₂ than all other substrates. Rossi et al., (2011) and Varrone et al., (2013) studied H₂-production by microbial populations grown on crude glycerol. The experiments revealed that OTUs in the genera *Klebsiella*, *Pantoea*, *Escherichia*, *Shigella*, and *Cupriavidus* were the dominant species.

Microbial community analysis by 16S rDNA sequence analysis provides much greater detail about mixed microbial communities that are enriched by growth on glucose and crude-glycerol and may identify new microorganisms for future studies of biohydrogen production which are currently not explored in the literature. The objective of Chapter 3 is to determine the microbial diversity selected by growth on glucose versus glycerol, and the resulting impact on biohydrogen production and associated VFAs. Anaerobic batch respirometer experiments were performed with dairy manure digestate (DMD) pre-treated by incubation at 70 °C for 30 minutes, as source of inoculum and D-glucose and crude-glycerol (REG-80) as substrates.

3.2. Experimental design

The differences in the microbial community structure and diversity, and differences in gas production (H₂ and CO₂) by each microbial community resulting from growth on D-glucose versus glycerol were examined in batch experiments conducted using a respirometer system. To eliminate methanogens, the DMD was pre-treated at 70 °C for 30 minutes, as described in Chapter 2, Section 2.1. Anaerobic batch experiments were
conducted as described in Chapter 2, Sections 2.2 and 2.3. Based on the substrate/inoculum ratio 6 g COD/g VS seed was calculated for 100 g of seed, and a substrate:inoculum ratio of 6 g COD/g VS was maintained in each bottle. The TCOD of D-glucose and crude glycerol were calculated to be 18 g COD for 400 mL working volume. The bottles were purged with nitrogen gas for 8 minutes to create an anaerobic condition. The bottles were incubated in respirometer water bath at 37 °C for 15–20 days. Gas production in each bottle was monitored each day and the final VFAs were analyzed at the end of experiments. The composition of biogas generated from each substrate was evaluated for every 24 hours. Concentrations of H₂ and CO₂ and of VFAs were determined as described in Chapter 2, Section 2.4. The experiments were conducted with six biological replicates, and the data were plotted by taking an average of three best replicate bottles in the batch experiments. The microbial community structure and diversity resulting from growth on D-glucose or glycerol were determined as described in Chapter 2, Section 2.5.

3.3. Results and Discussion

3.3.1. Metagenomic analysis of bacteria grown on D-glucose

3.3.1.1. Partial least square analysis

The operational taxonomic units (OTUs) present in the DMD seed (without substrate) were compared with the DMD OTUs selected by growth on D-glucose using a multivariate, Partial Least Squares-Discriminant Analysis (Figure 3.1). The loading plot reveals the partition between Treatment 1 (DMD microbes grown without D-glucose) and Treatment 2 (DMD microbes selected by growth on D-glucose) with two components; $R^2_Y = 0.99$ and $Q^2_X = 0.99$. A total of 63 OTUs with VIP > 0.01 were selected. Those OTUs were distributed among phyla Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, OP9,
**Figure 3.1** Partial Least Square–Discriminate Analysis of microbial communities in the pretreated DMD grown without substrate (Treatment 1) or grown with D-glucose (Treatment 2). Bacterial taxa (OTUs) were plotted according to their correlations to Treatment 1 and Treatment 2. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation with Treatment 2. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
Proteobacteria, Spirochaetes, Synergistetes, TM7, Tenericutes, and WS-6. From the above, phyla Chloroflexi, OP9, Spirochaetes, TM7, and WS-6 contain only 1 family and 1 order, respectively. The phyla, Bacteroidetes, Proteobacteria and Synergistetes contributed each 5 OTUs (at the level of families and genus) and phylum Actinobacteria contributed 2 OTUs (at the level of 1 family and 1 genus) from total population. However, the phyla Firmicutes contributed 5 orders; 10 families and 24 genera from the total OTUs represented in the microbial community.

The OTUs of microbes grown without substrates (Treatment 1) were clustered on the left hand side of the Figure 3.1. The dominant microbes selected by Treatment 1 were bacteria in the phylum Bacteroides, and were represented by OTUs in the families Porphyromonadaceae (5.6%) and Bacteroidaceae (2.8%), and the order Bacteroidales (3.6%). Bacteria in the phylum Firmicutes were also dominant, and were represented by OTUs in the families Planococcaceae (4.2%) and Ruminococcaceae (2.57%), the orders BSA2B08 (5.48%) and MBA08 (2.1%), and the genera Coprococcus (2.3%) and Butyrivibririo (1.5%) respectively. OTUs in other phyla contributed less than 2% of total population microbial population grown without any substrates (Treatment 1).

D-glucose (Treatment 2-DMD grown on D-glucose) selected very few species from the total population of pre-treated-DMD at 37 °C. The dominant microbes selected by Treatment 2 were bacteria in the Firmicutes phylum represented by (% of total OTUs detected in the population) families Clostridiaceae (20.42%), Peptostreptococcaceae (2.16%), and the genera Clostridium (12.21%), Ruminococcus (0.9%), and Sporolactobacillus (10.1%). OTUs in the family Enterobacteriaceae (5.3%) within the
phylum Proteobacteria, and the genus Acholeplasma (0.1%) within the phylum Tenericutes were also enriched by Treatment 2.

3.3.1.2. Statistical analysis

Least Square means of OTUs were used to determine the significance of microbes which are actively involved in mixed fermentation using the MIXED procedure in SAS program. The fold-enrichment of OTUs was calculated from the least square mean values of each taxon of two Treatments 1 and 2 (Table 3.1). The genus Sporolactobacillus in the phylum Firmicutes and the family Enterobacteriaceae in the phylum Proteobacteria were enriched 159-fold and 125-fold, respectively, when D-glucose was used as the carbon source. Additionally, the second most dominant OTUs in the family Clostridiaceae were enriched 9-fold, while OTUs in the genera Clostridium, and Ruminococcus increased 4-fold.

3.3.2 Microbial diversity and end-product distribution patterns as a consequence of growth on D-glucose

3.3.2.1. Hydrogen and carbon dioxide synthesis by the selected microbial communities

The cumulative H₂ and CO₂ production by DMD seed incubated with D-glucose were 2,905 mL and 1,725 mL, respectively, at 216 hours post-inoculation (h pi; Figure 3.2). The total biogas production (H₂ + CO₂) was 6,095 mL. Thus, the microbial communities generated from pre-treated DMD cultured with glucose as the carbon source generated approximately double the amount of H₂ than CO₂ compared with DMD seed incubated without a carbon source (Treatment 1). Logan et al., (2002) conducted a similar anaerobic batch experiment using pre-treated tomato soil as source of inoculum and D-glucose, sucrose,
Table 3.1 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pre-treated DMD seed with D-glucose. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 2.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 1 (Blank)</th>
<th>Treatment 2 (D-glucose)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporolactobacillus (G)</td>
<td>0.11</td>
<td>17.51</td>
<td>159</td>
</tr>
<tr>
<td>Enterobacteriaceae (F)</td>
<td>0.08</td>
<td>10.57</td>
<td>124</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>4.02</td>
<td>35.36</td>
<td>9</td>
</tr>
<tr>
<td>Ruminococcus (G)</td>
<td>0.39</td>
<td>1.75</td>
<td>4</td>
</tr>
<tr>
<td>Clostridium (G)</td>
<td>4.70</td>
<td>17.85</td>
<td>4</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
Figure 3.2 Cumulative $\text{H}_2$ and $\text{CO}_2$ production by DMD grown on D-glucose (Treatment 2).
lactose and potato starch as the substrate, at 26 °C at pH 6.0. A higher concentration of H₂ was produced when the pre-treated soil sample was incubated with D-glucose compared with sucrose, lactose, and potato starch that were used in the experiment.

The fermentation reaction performed with DMD seed and D-glucose as substrate was conducted for 9 days. The DMD yielded 1.11 mol H₂/mol D-glucose at 37 °C and pH 7.2. This result was consistent with Davila-Vazquez et al., (2008), who observed 1.46 mol H₂/mol substrate (5 g glucose/L), using anaerobic granular sludge at 37 °C at pH 7.5 in batch experiments. Furthermore, Salerno et al., (2006) and Lin and Chang (1999) also observed a yield of 1.7 mol H₂/mol glucose, at substrate concentrations of 18 g/L and pH 5.7, as well as at a glucose concentration of 3.75 g/L and pH 6.2.

In the microbial community selected by growth of pre-treated DMD on D-glucose (Treatment 2), OTUs in the genus *Sporolactobacillus* and in the family Enterobacteriaceae were found in higher numbers compared to other microorganisms. These bacteria are known as H₂-producers. Fang et al., (2002) identified *Sporolactobacillus racemicus* in sludge granules grown on sucrose, and Kumar and Das (2001) identified *Enterobacter cloacae* (in the family Enterobacteriaceae) in sludge grown on D-glucose as H₂-producing bacteria under mesophilic conditions. Kim et al., (2006) reported that *Clostridium spp.* were the dominant microorganisms grown on D-glucose, as determined by Denaturing Gradient Gel Electrophoresis (DGGE) analysis. Furthermore, the majority of the species identified in mesophilic anaerobic sludge contain *Clostridium spp* and these bacteria have been studied extensively with different substrates and under different operational conditions (Fang and Liu, 2002). Bacteria in the genus *Ruminococcus*, present in the rumen of cattle, can ferment sugars and synthesize H₂ and CO₂ (Ntaikou et al., 2008).
3.3.2.2. Volatile Fatty acids and alcohols

VFAs and alcohols are aqueous phase end-products synthesized during the breakdown of D-glucose into simpler molecules by mixed culture. Acetate, butyrate, and propionate are usually the dominant VFAs products observed in mixed culture anaerobic fermentation reactions reported in the literature. In the batch experiments described in this thesis, lactate and ethanol were dominant at concentrations of 14.28 ± 4.4 g/L and 28.0 ± 1.9 g/L, respectively, while acetate and butyrate were the end-products with the next highest concentrations (Figure 3.3). In contrast formate, propionate, and iso-butyrate were produced in very low quantities. Zhao et al., (2010) reported lactate production by Sporolactobacillus sp. in batch fermentation cultures grown on D-glucose. The high numbers of Sporolactobacillus sp. in the microbial population selected by Treatment 2 may explain the large amounts of lactic acid produced from D-glucose. Bond and Russell (1998) reported that the gram-positive bacterium, Streptococcus bovis, utilized the lactate synthesis pathway to increase ATP synthesis and cell growth under culture conditions with low pH and high glucose concentrations. Similarly, in our batch experiments, when DMD was grown on D-glucose, the decrease in the pH may have induced the growth of a gram-positive, lactate synthesizing species of Sporolactobacillus.

Fan (2004) also observed high ethanol production using anaerobes present in cow dung compost, and reported that Clostridium sp. produced VFAs and ethanol during the exponential growth phase if a positive H₂ partial pressure was maintained in the reaction bottles. Our results are also consistent with microbial populations that contained Clostridium species and other OTUs in the family Clostridiaceae, which were shown to produce acetate and butyrate (Cummins and Johnson, 1971; Mizuno et al., 2000).
Low concentrations of formate (0.81 ± 0.01g/L) and propionate (0.23 ± 0.3 g/L) were observed in our batch experiments, suggesting that some bacteria in the microbial community may have utilized the propionate, resulting in reduced concentrations of propionic acid. Horiuchi et al., (2002) also observed a similar organic acid production pattern (high acetic and butyric acid production, with low concentrations of propionic acid) at pH 7.0. Thus, detection of H2, CO2, lactate, acetate, butyrate, and ethanol in the fermentation reactions containing pre-treated DMD grown on D-glucose correlated well with the presence of OTUs in the genera *Clostridium*, *Ruminococcus*, and *Sporolactobacillus*, and in the families Clostridiaceae and Enterobacteriaceae.
Figure 3.3 Volatile fatty acids (VFAs) produced by DMD grown on D-glucose (Treatment 2).
3.3.3 Metagenomic analysis of bacteria grown on crude-glycerol

3.3.3.1. Partial least square analysis

The OTUs present in the DMD seed (without substrate; Treatment 1) were compared with the DMD OTUs selected by growth on crude-glycerol (Treatment 3) using PLS-DA, and a loading plot was used to characterize and quantify the statistical significance of OTUs in the microbial communities selected by these Treatments (Figure 3.4). The diversity of taxa that were supported by crude glycerol from DMD was separated by two components and with $R^2_Y = 0.99$ and $Q^2_X = 0.99$.

A total of 63 OTUs with VIP > 0.01 were selected to analyze the phylotypes present in DMD which were distributed among 11 phyla: Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, OP9, Proteobacteria, Spirochaetes, Synergistetes, TM7, Tenericutes, and WS-6. From the above phyla, Chloroflexi, OP9, Spirochaetes, TM7, and WS-6 shared 1 family and 1 order, respectively. The phyla, Bacteroidetes, Proteobacteria and Synergistetes contributed each 5 OTUs along with the phylum Actinobacteria, which consist of least number 2 OTUs (at the level of 1 family and 1 genus) from total population. Out of the 63 total OTUs represented in the microbial population, the phylum Firmicutes contributed 5 orders, 10 families, and 24 genera.
**Figure 3.4** Partial Least Square Discriminate Analysis of microbial communities in the pre-treated DMD seed without substrate (Treatment 1) versus the pre-treated DMD seed cultured with crude glycerol (Treatment 3). Bacterial taxa were plotted according to their correlations to Treatment 1 and Treatment 3. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 3. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
Microbes that were significantly correlated to Treatment 1 (DMD microbes grown without substrate) clustered on left side of Figure 3.4, whereas OTUs that were significantly correlated to Treatment 3 (DMD microbes grown on crude glycerol) clustered on right side of the figure. The dominant OTUs present in DMD grown without substrates (Treatment 1) were described in detail in Section 3.3.1.1 above.

DMD microbes selected by growth on crude-glycerol (Treatment 3) were significantly dominated by OTUs of phylum Firmicutes, and were represented by OTUs in the Family Clostridiaceae (10.58%), and the genera Clostridium (7.17), Ruminococcus (4.56%), and Syntrophomonas (2.76%) of the total population. The second most abundant phyla Bacteroides shared the OTUs between order Bacteroidales (7.1% of the total population) and the family Bacteroidaceae (5.8% of the total population), respectively. Family of Caulobacteraceae (3.6%) in the Proteobacteria phylum was the only OTU enriched in the microbial community along with Firmicutes and Bacteroides phyla growth on crude-glycerol.

3.3.3.2. Statistical analysis

Least Square means of OTUs were used to determine the significance of microbes which are actively involved in mixed fermentation using the MIXED procedure in SAS program. Families of Caulobacteraceae; Clostridiaceae and genera of Clostridium; Syntrophomonas and Ruminococcus in the Firmicutes phylum were observed to be dominant in Treatment 3 (DMD grown on crude-glycerol). The other OTUs of order Bacteroidales and family Bacteroidaceae are also statistically significant members of the microbial community selected by growth on crude-glycerol. OTUs in the family Caulobacteraceae and the genus Syntrophomonas and Ruminococcus increased 62-fold, 9-
fold, and 15-fold, respectively, in Treatment 3 compared to Treatment 1, and OTUs in the genera *Clostridium* and family Clostridiaceae were increased 2-fold and 4-fold in the presence of crude-glycerol (Treatment 3) compared with the pre-treated DMD seed (without substrate). OTUs in the phylum Bacteroides were also enriched when the pre-treated DMD seed was grown with crude-glycerol (Table 3.2).

### 3.3.4 Microbial diversity and end-product distribution patterns as a consequence of growth on crude-glycerol

#### 3.3.4.1. Hydrogen and carbon dioxide synthesis by the selected microbial communities

Culture of pre-treated DMD seed with crude-glycerol as the substrate resulted in a high volume (1,162 ± 149 mL) of biogas (Figure 3.5). At 24 h pi, the cultures produced very little H₂ (< 2 mL) and CO₂ (79 mL). However, by 48 h pi, gas production increased significantly and the cumulative H₂ and CO₂ concentrations at the end of the experiment (312 h pi) were 142 mL and 702 mL, respectively (Figure 3.5). Methane gas was not observed throughout experiment, indicating that all the methanogens were eliminated by the heat pre-Treatment of the DMD sludge.

Fountoulakis and Manios (2009) examined the effect of crude glycerol on the performance of single-stage anaerobic reactor treating different types of organic waste, and found that the mean daily H₂-production increased from 113 mL/d to 263 mL/d after the addition of crude glycerol into the reactor. Fountoulakis and Manios (2009) reported a yield 70 mmol H₂/mol of crude glycerol substrate, but this was lower than H₂ produced from ultra-pure glycerol (3.9 mol H₂/mol) and B-glycerol (1.8 mol H₂/mol), produced from transesterification of soybean oil, as reported by Selembo et al., (2009).
OTUs in the families Clostridiaceae, Caulobacteraceae, Bacteroidaceae, and in the order Bacteroidales were enriched in crude-glycerol fermentation at 37 °C. Wongtanet et al., (2007) also observed microorganisms in these families in a CSTR reactor that produced H₂ from D-glucose using an anaerobic mixed culture at 35 °C and pH 5.5 using RFLP analysis followed by 16S rDNA sequencing. Additionally, OTUs in the genera Clostridium, Syntrophomonas, and Ruminococcus were enriched and converted glycerol into H₂ and CO₂ (Fang and Liu 2002; Ntaikou et al., 2008 and Sieber et al., 2010), although there is no clear evidence from the literature that these bacteria grow on crude-glycerol.

3.4.4.2. Volatile Fatty acids

The aqueous phase fermentation end-products synthesized by the microbial community selected by growth of the pre-treated DMD seed on crude glycerol included acetate, propionate and butyrate (Figure 3.6). The most abundant VFA synthesized by the microbial community selected by growth on crude glycerol was propionate (15.53 g/L), followed by acetate (4.8 g/L) and butyrate (0.46 g/L). Rossi et al., (2011) studied the effect of different pre-Treatment of environmental microbial consortia for H₂-production from crude-glycerol. They found that acetate (1.86 ± 0.03 g/L), butyrate (2.53 ± 0.21 g/L), and 1,3-propanediol (4.55 ± 0.12 g/L) were the dominant aqueous fermentation end-products.
Figure 3.5 Cumulative H$_2$ and CO$_2$ production by DMD seed grown crude glycerol (Treatment 3).
Table 3.2 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pre-treated DMD seed with crude-glycerol. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 3.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 1 (Blank)</th>
<th>Treatment 3 (Crude-Glycerol)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caulobacteraceae (F)</td>
<td>0.11</td>
<td>7.09</td>
<td>62</td>
</tr>
<tr>
<td>Ruminococcus (G)</td>
<td>0.39</td>
<td>5.83</td>
<td>15</td>
</tr>
<tr>
<td>Syntrophomonas (G)</td>
<td>0.76</td>
<td>4.46</td>
<td>9</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>4.02</td>
<td>14.35</td>
<td>4</td>
</tr>
<tr>
<td>Clostridium (G)</td>
<td>4.70</td>
<td>7.40</td>
<td>2</td>
</tr>
<tr>
<td>Bacteroidaceae (F)</td>
<td>4.16</td>
<td>6.08</td>
<td>1</td>
</tr>
<tr>
<td>Bacteroidales (O)</td>
<td>5.84</td>
<td>7.81</td>
<td>1</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
Jitrwung and Yargeau (2011) also reported the presence of acetate, 1,3-propandiol and high concentrations of ethanol while optimizing the media composition for the production of H₂ from waste glycerol using bacteria in the family Caulobacteraceae (Phylum Proteobacteria), and noted that a significant presence of these bacteria (62-fold enrichment) in DMD could have been responsible for the high propionic acid concentrations from crude-glycerol. Zhang and Yang (2009) successfully produced 0.55 g propionate / g crude glycerol with *Propionibacterium acidipropionici*, and found that glycerol, as a more reduced substrate, shifted the metabolic pathways of *P. acidipropionici* towards propionate synthesis to maintain the redox balance. In this pathway, 1 mole of glycerol is converted into 1 mole of pyruvate plus 2 moles of NADH, which maintains reducing power in the cell. Donkin (2008) and Young Lee et al., (2011) also observed production of higher concentrations of propionate than acetate, butyrate, and valerate by ruminant bacteria using crude glycerol as a carbon source.

Mcinerney et al., (1981) and Sieber et al., (2010) discovered that *Syntrophomonas wolfei*, a new syntrophic bacterium, has the ability to convert butyrate into H₂, CO₂, and CH₄. Therefore, the presence of bacteria in the genus *Syntrophomonas* in the microbial community selected by growth on crude-glycerol may have utilized the butyrate produced by other microorganism, resulting in lower detected concentrations. Our results of pre-treated DMD seed cultured with crude glycerol are consistent with these previous observations, and suggest that growth of the pre-treated DMD with crude glycerol resulted in selection of propionate synthesizing bacteria, which would also explain the low level of H₂-production.
Figure 3.6 Volatile fatty acids produced by DMD seed grown crude glycerol (Treatment 3).
3.3.5 Comparison of microbial communities selected by growth of pre-treated DMD seed with D-glucose and crude-glycerol

D-glucose and glycerol are metabolized in a similar manner during anaerobic fermentation. They are both metabolized via glycolysis and share a similar stoichiometry. However, the distribution of fermentation end-products synthesized from glucose and glycerol are different. Fermentation of glucose typically results in synthesis of H₂, acetate or butyrate, and lactate in higher concentrations than fermentation of glycerol. In contrast, fermentation of glycerol can result in synthesis of 1,3-propanediol as major end-product, with synthesis of lower concentrations of acetate and lactate, and a CO₂:H₂ molar ratio less than 1. Fermentation of pre-treated DMD seed with D-glucose resulted in synthesis of lactate, ethanol, acetate, and butyrate, and greater amounts of H₂ than CO₂, whereas fermentation reactions with crude-glycerol resulted in synthesis of very high concentrations of propionate and greater amounts of CO₂ than H₂.

In this study, we assessed and compared the composition of bacterial communities selected by growth of the pre-treated DMD seed on D-glucose (Treatment 2) versus crude-glycerol (Treatment 3). A comparison of microbial populations resulting from Treatment 2 and Treatment 3 is shown in Figure 3.7. The PLS-DA scatter plot displays significant correlations of OTUs with each particular Treatment with two components, R²Y = 0.98 and Q²X = 0.91. The microbial community selected by growth of pre-treated DMD seed with D-glucose (Treatment 2) was enriched by the OTUs of family Enterococcaceae (phylum Proteobacteria) and the genera *Paenibacillus*, *Sporolactobacillus*, and *Clostridium*. The microbial community selected by growth of pre-treated DMD seed with crude-glycerol (Treatment 3), however, displayed significantly greater diversity, and
contained OTUs in the phyla Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes, Synergistetes, and Tenericutes.

OTUs from the phylum Bacteroides were significantly correlated with the pre-treated DMD seed cultured with glycerol as carbon source (Treatment 3) compared with the DMD seed cultured with D-glucose (Treatment 2). OTUs in the phylum Bacteroidetes identified in the microbial community resulting from Treatment 3 represented approx. 8.3% of the total population, with OTUs in order Bacteroidales representing 4.4%, OTUs in the family Bacteroidaceae representing 3.9%. In addition to Bacteroides, phylum Firmicutes also enriched with OTUs of families Ruminococcaceae (1.3%); Porphyromonadaceae (1.29%); Order BSA2B08 (2.46%) and genus Syntrophomonas represented 2.49% of total population.

Well known H₂ synthesizing bacteria in the family Clostridiaceae, and the genera Clostridium and Ruminococcus were present in the microbial communities selected by both Treatment 2 and Treatment 3, but were greatly enriched in the community selected by Treatment 2, with Clostridial OTUs making up 46.33% of the total microbial population (0.14% identified as order Clostridiales, 26.72% identified as family Clostridiaceae, 5.07% identified as the genus Ruminococcus, and 14.4% identified as the genus Clostridium).
Figure 3.7 comparative analyses of microbial communities selected by growth of pre-treated DMD seed on D-glucose (Treatment 1) versus crude-glycerol (Treatment 3). Bacterial taxa were plotted according to their correlations to Treatment 2 and Treatment 3. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 2 and Treatment 3. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
The “Mixed Procedure” of statistical analysis was used to determine the significance of the OTUs enriched by growth of pre-treated DMD seed in D-glucose versus crude-glycerol (Table 3.3). OTUs identified in the genus *Sporolactobacillus* were enriched 17,672-fold in D-glucose grown DMD cultures (Treatment 2) compared to DMD grown in crude-glycerol (Treatment 3). Enrichment of OTUs in the genus *Sporolactobacillus* in D-glucose grown DMD may have been responsible for the higher concentrations of lactate produced, as there no lactate was detected in the crude-glycerol grown DMD culture. OTUs of families Clostridiaceae, Enterobacteriaceae, and Peptostreptococcaceae, and in the genus *Clostridium* were enriched 2- to 7-fold in DMD D-glucose cultures. The molar yield of H$_2$ (1.11 mol H$_2$/mol substrate) in DMD D-glucose cultures was 15-fold greater than the H$_2$ yield (0.07 mol H$_2$/mol substrate) in DMD crude-glycerol cultures.
Table 3.3 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pre-treated DMD seed with crude-glycerol versus D-glucose. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 3 versus Treatment 2.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 2 (D-glucose)</th>
<th>Treatment 3 (Crude-Glycerol)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporolactobacillus (G)</td>
<td>0.001</td>
<td>17.51</td>
<td>17,672</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>4.56</td>
<td>35.36</td>
<td>7</td>
</tr>
<tr>
<td>Clostridium (G)</td>
<td>2.48</td>
<td>17.85</td>
<td>7</td>
</tr>
<tr>
<td>Peptostreptococcaceae (F)</td>
<td>0.94</td>
<td>2.39</td>
<td>3</td>
</tr>
<tr>
<td>Enterobacteriaceae (F)</td>
<td>4.44</td>
<td>10.57</td>
<td>2</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
3.4. Conclusions

Several conclusions can be drawn from this work: 1) Dairy manure digestate (DMD) contained a wide diversity of bacteria; 2) fermentation reactions in the presence of D-glucose and crude-glycerol selected for subsets of the microbial community within the DMD seed; 3) very different sets of microorganisms were enriched in the DMD seed cultured with D-glucose versus glycerol; 4) a greater diversity of bacteria was selected when the DMD seed was cultured with a complex substrate (crude-glycerol) compared with a chemically pure, simple substrate (D-glucose) because most of the D-glucose was utilized by OTUs in the genus *Sporolactobacillus* and family Enterobacteriaceae; and 5) the fermentation end-products (H₂, CO₂, organic acids, and alcohols) synthesized, and their level of synthesis were determined by the carbon source used to select the microbial community structure.
Chapter 4

Influence of substrate type on microbial community structure and fermentation end-product synthesis patterns II:

\( \alpha \)-Cellulose

4.1. Microbial diversity and community structure selected by growth on \( \alpha \)-cellulose

\( \alpha \)-Cellulose, a polymer composed of D-glucose subunits, is one of the most naturally abundant polymers on the planet. Hydrolysis of cellulose into simple 6 carbon sugars is the first step in anaerobic fermentation to produce \( \text{H}_2 \), \( \text{CO}_2 \), and volatile fatty acids (Wang et al., 2011; see Chapter 1, Figure 1.5). In recent studies, the use of naturally available cellulolytic degrading microorganisms in soil, cow dung compost, or anaerobic digestate have been used as seed source for fermentation reactions to convert cellulose into biohydrogen and soluble products.

Ueno et al., (2001) studied the anaerobic, thermophilic, \( \text{H}_2 \)-producing microflora enriched from sludge compost grown on cellulose with artificial medium using the \( V_3 \) variable region of 16S rDNA using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis. PCR-DGGE analysis revealed that dominant microorganisms to be *Thermoanaerobacterium thermosaccharolyticum*, *Clostridium thermocellum*, and *Clostridium cellulosi*, and together they produced 1.0 mol \( \text{H}_2 \) / mol hexose equivalent, with acetate and butyrate as by-products. Similarity, Gadow et al., (2013) characterized hyper-thermophilic cellulolytic degrading microorganisms in an
continuous anaerobic sewage sludge digester using 16S rRNA gene cloning analysis, and found that the cellulolytic H$_2$-producing bacteria were closely associated with the genus *Thermoanaerobacterium* and yielded $7.07 \pm 0.23$ mmol H$_2$ / g cellulose. Gupta et al., (2014) studied the synergistic effects of co-fermentation of glucose, starch, and cellulose using anaerobic digester sludge on H$_2$-producing and associated microbial communities at 37 °C and pH 6.5, using 16S rDNA Illumina sequencing, and showed that the dominate species belonged to the families Clostridiaceae and Ruminococcaceae. These studies revealed mixed microbial cultures derived from natural source contain a number of species that can convert cellulose via anaerobic digestion to H$_2$, CO$_2$, and organic acids. The amount and molar yield of H$_2$ produced, and the ratio of various other end products synthesized are determined by the microbial community structure and diversity that are selected by the growth conditions (temperature, pH, etc) and the nature of the substrate used.

The objective of Chapter 4 is to study the structure and diversity of microbial communities enriched in fermentation reactions containing a complex substrate (cellulose) at mesophilic temperatures, and determines the relationship between microbial community structure and fermentation end-product synthesis patterns.

### 4.2. Experimental design

Anaerobic batch respirometer experiments were performed with dairy manure digestate (DMD) pre-treated by incubation at 100 °C for 30 minutes, as source of inoculum and $\alpha$-cellulose was chosen as the model complex substrate, as described in Chapter 2, Section 2.1. Anaerobic batch experiments were conducted as described in Chapter 2, Sections 2.2 and 2.3. A substrate/inoculum ratio 6 g COD/g VS$_{seed}$ was used for 100 g of
seed, and this substrate:inoculum ratio was maintained in each bottle. The TCOD of \( \alpha \)-cellulose was calculated as 16 g COD for the 400 mL working volume of each fermentation reaction. The bottles were purged with nitrogen gas for 8 minutes to create anaerobic condition. The bottles were incubated in respirometer water bath at 37 °C for 21 days.

Gas production in each bottle was monitored each day and the final VFAs were analyzed at the end of experiments. The composition of biogas generated from each substrate was evaluated for every 24 hours. Concentrations of \( \mathrm{H}_2 \) and \( \mathrm{CO}_2 \), and of volatile fatty acids (VFAs) were determined as described in Chapter 2, Section 2.4. The experiments were conducted with six biological replicates, and the data were plotted by taking average of three best replicate bottles in the batch experiments.

Samples for DNA extraction were collected from the six replicate serum bottles at 96 hours of incubation and also at the end of the experiment separately, based on the mimic experiment result, which showed a biphasic growth pattern for hydrogen production. DNA was extracted individually from each sample and 16S rDNA sequencing was carried out as described in Chapter 2, Section 2.5.

To evaluate the effect of a “pre-selected” microbial community, a second batch experiment was conducted by mixing DMD cultured on \( \alpha \)-cellulose for 22 days at 37 °C with fresh pre-treated DMD at a ratio of 1:1 v/v. was used as source of inoculum (E-DMD; Treatment 5). The extended DMD (E-DMD) experiment was carried out for 22 days at 37°C and at pH 6.9, instead of 7.2, due to the addition of 50 g of the previously fermented DMD (which had a pH 6.4)
4.3. Results and Discussion

4.3.1. Metagenomic analysis of bacteria grown on α-cellulose

4.3.1.1. Partial least square analysis

The OTUs in the microbial population of DMD grown without a carbon source (Treatment 1) were compared with the microorganisms enriched by incubation of the DMD with α-cellulose (Treatment 4). Partial Least Square-Discriminant Analysis (PLS-DA) revealed the associations of these microorganisms (Figure 4.1) into two groups (components), with $R^2_Y = 0.99$ and $Q^2_X = 0.91$, respectively. The Y-variable represents the OTUs, while the X-variable represents the two Treatments (DMD grown without a carbon source versus DMD grown with α-cellulose).

A total of 41 OTUs were distributed among phyla Bacteroidetes, Firmicutes, Proteobacteria, Synergistetes, and WS-6. Of these, phyla Synergistetes and WS-6 contain only 1 genus and 1 order, respectively. The second highest number of shared OTUs were identified in the phyla Bacteroidetes and Proteobacteria, which were represented by 3 OTUs (3 at the level of genus) and 5 OTUs (4 at the level of genus and 4 at the level of family), respectively, whereas the phylum Firmicutes contributed OTUs representing 5 orders, 4 families, and 21 genera from the total OTUs presented in the microbial community. The OTUs of microbes grown without substrates (Treatment 1) and OTUs correlated to Treatment 4 (DMD grown on α-cellulose) were clustered on left and right side of Figure 4.1, respectively.

OTUs in the family of Ruminococcaceae (21.27%) and the genus Ruminococcus (18.01%) were the dominant OTUs and significantly correlated to growth on α-cellulose. On the other hand, OTUs in the genera Clostridium and Coprococcus, the family
Figure 4.1 Partial Least Square-Discriminate Analysis of microbial communities in the pretreated DMD seed without substrate (Treatment 1) versus grown on α-cellulose (Treatment 4). Bacterial taxa were plotted according to their correlations to Treatment 1 and Treatment 4. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 4. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
Clostridiaceae, and the order Natranaerobiales contributed 6.10%, 6.54%, 3.56%, and 2.36% of the total population of microorganisms grown on α-cellulose, respectively. Bacteria in the family Ruminococcaceae (4.87%), the genera Ruminococcus and Dorea (8.89% and 1.3%, respectively), and the order Natranaerobiales (2.67%), in the phylum Firmicutes, the genus Actinobacillus (1.29%) in the family Enterobacteriaceae (phylum Proteobacteria), and the genus Prevotella in the phylum Bacteroidetes were the dominant OTUs observed in the first phase (0 to 96 h pi) of H2-production.

4.3.1.2. Statistical analysis

A list of the OTUs enriched in DMD grown on α-cellulose (Treatment 4) compared to DMD grown without any substrate (Treatment 1) is shown in Table 4.1. OTUs in the order Natranaerobiales, and the genera Ruminococcus and Sporanaerobacter were enriched 481-, 35-, and 26-times, respectively, when DMD was grown with α-cellulose. OTUs in the genus Clostridium, the family Clostridiaceae, the family Ruminococcaceae, and the order Thermoanaerobacterales were also enriched in DMD grown on α-cellulose. OTUs in the genera Actinobacillus, Dorea, and Prevotella increased their numbers 1,506-, 542-, and 715-times by 96 h pi of the pretreated DMD into fermentation reactions containing α-cellulose (Table 4.2).
Table 4.1 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pretreated DMD seed with α-cellulose. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 4.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 1 (Blank)</th>
<th>Treatment 4 (α-Cellulose)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natranaerobiales (O)</td>
<td>0.0098</td>
<td>4.73</td>
<td>481</td>
</tr>
<tr>
<td><em>Ruminococcus</em> (G)</td>
<td>0.98</td>
<td>35.05</td>
<td>35</td>
</tr>
<tr>
<td><em>Sporanaerobacter</em> (G)</td>
<td>0.064</td>
<td>1.66</td>
<td>26</td>
</tr>
<tr>
<td><em>Clostridium</em> (G)</td>
<td>1.35</td>
<td>10.94</td>
<td>8</td>
</tr>
<tr>
<td>Ruminococcaceae (F)</td>
<td>9.93</td>
<td>28.61</td>
<td>3</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>2.27</td>
<td>4.87</td>
<td>2</td>
</tr>
<tr>
<td><em>Coprococcus</em> (G)</td>
<td>5.45</td>
<td>7.64</td>
<td>1</td>
</tr>
<tr>
<td>Thermoanaerobacterales (O)</td>
<td>2.18</td>
<td>2.71</td>
<td>1</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
Table 4.2 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pretreated DMD seed with α-cellulose at 96 hours post-inoculation. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 4.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 1 (Blank)</th>
<th>Treatment 4 (α-Cellulose)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinobacillus (G)</td>
<td>0.0016</td>
<td>2.38</td>
<td>1506</td>
</tr>
<tr>
<td>Dorea (G)</td>
<td>0.0044</td>
<td>2.38</td>
<td>542</td>
</tr>
<tr>
<td>Prevotella (G)</td>
<td>0.0065</td>
<td>4.62</td>
<td>715</td>
</tr>
<tr>
<td>Enterobacteriaceae (F)</td>
<td>0.14</td>
<td>7.17</td>
<td>50</td>
</tr>
<tr>
<td>Natranerobiales (O)</td>
<td>0.23</td>
<td>4.70</td>
<td>20</td>
</tr>
<tr>
<td>Ruminococcus (G)</td>
<td>3.14</td>
<td>13.70</td>
<td>4</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
4.3.2 Microbial diversity and end-product synthesis patterns as a consequence of growth on α-cellulose

4.3.2.1. Hydrogen and carbon dioxide synthesis by the selected microbial communities

Figure 4.2 show the cumulative H\(_2\) and CO\(_2\) production from cellulose using pretreated DMD at 37 °C and pH 7.2. The doubling-time of different microbes in the community in DMD resulted in diphasic growth pattern on the cellulose substrate. At 72 h pi, there was a slight increase in the volume of H\(_2\) produced (5.2 mL) followed by a gradual decrease in the volume of H\(_2\) (3 mL) up to 96 h pi. Between 192 and 216 h pi, the volume of H\(_2\) increased rapidly to 16mL and gradually increased thereafter. In contrast, the volume of CO\(_2\) produced increased moderately in the fermentation reaction in a more or less steady rate. The total biogas production (H\(_2\) + CO\(_2\)) from cellulose was 376 mL, which contained a maximum of 77 mL H\(_2\) and 124 mL CO\(_2\).

Lin and Hung (2008) conducted batch tests to enhance the H\(_2\)/EtOH productivity using two pre-treated cow dung microflora (heat pre-treated and enriched mixed culture in nutrient medium) to ferment cellulose. They observed greater cumulative H\(_2\)-production (42 ± 16 mL) in the enriched microflora reactions compared to the heat-treated cow dung compost reactions (4.5 ± 1.7 mL). Our batch experiment conducted with pretreated DMD and α-cellulose generated 1.7-times more H\(_2\) than reported by Lin and Hung (2008). In another set of experiments, Wang et al., (2011) investigated H\(_2\)-production using a mixed culture developed from rotten wood crumbs collected from a forest in an integrated hydrogen production system consisting of a fermentation reactor and three microbial fuel cells. The molar yield of H\(_2\) from this system was reported as 10 mmol H\(_2\)/mol cellulose.
Figure 4.2 Cumulative $H_2$ and $CO_2$ production by DMD grown on $\alpha$-cellulose (Treatment 4).
The molar yield of H$_2$ in our batch experiment with pretreated DMD and α-cellulose was 4-time higher (40 mmol H$_2$/mol cellulose) that that reported by Wang et al., (2011).

During first phase (0-96 h pi) of microbial community growth, OTUs in the genera Actinobacillus, Dorea, and Prevotella, and OTUs in the family Enterobacteriaceae multiplied and converted α-cellulose into H$_2$, CO$_2$, and organic acids. Mariakakis et al., (2011) analyzed the microbial communities in sewage treatment plant sludge grown with sucrose in a continuous reactor system by 16S rDNA PCR-DGGE analysis, and found the dominant bacteria to be in the genera Prevotella and Clostridium. OTUs in the genus Clostridium and Enterobacter play a major role in H$_2$-production from complex substrates such as cellulose and starch (Liu et al., 2008; Palazzi et al., 2000). OTUs in the genera Actinobacillus and Dorea are facultative anaerobes, and were also observed to increase in the microbial population selected by growth of DMD with α-cellulose, but neither of these bacteria is reported in the literature reported as H$_2$-producing microbes.

OTUs in the order Natranaerobiales, the families Ruminococcaceae and Clostridiaceae, and the genera Ruminococcus and Clostridium increased significantly during the second phase of the Treatment 4 experiment (pretreated DMD grown with α-cellulose). This observation is consistent with other reports in the literature. (Li et al., 2014) compared the microbial communities of a solid-state anaerobic digester operated under mesophilic and thermophilic temperatures for H$_2$-production using 16S rRNA gene sequencing. They observed that, there was a two-fold greater increase in the population of OTUs in the order Natranaerobiales in the mesophilic digesters compared to the thermophilic digesters. Ueno et al., (2001) studied H$_2$-production in relation to the microbial community of thermophilic anaerobic microflora enriched from sludge compost...
grown on cellulose powder. 16S rDNA PCR-DGGE analysis revealed that the one of the dominant OTUs was *Ruminococcus albus* (family Ruminococcaceae; genus *Ruminococcus*). These results support the presence of a large number of OTUs related to these species in our anaerobic respirometer experiment.

### 4.3.2.2 Volatile Fatty acids

Along with H₂-production, volatile fatty acids (VFAs) were also observed in the batch fermentations at the end of the experiment (22 days post-inoculation). The liquid from the bottles was centrifuged at 4,000 × g for 15 minutes and supernatant was analyzed for acetate, butyrate, and valerate. VFA analysis revealed that after 22 days of incubation with α-cellulose, the microbial communities selected produced 6.81 ± 0.20 g/L of acetate, 2.3 ± 0.5 g/L of butyrate, and low concentrations (0.12 g/L) of valerate (Figure 4.3). These data are also consistent with reports in the literature.

Ren et al., (2010) detected a similar pattern of greater amounts of acetic acid (1,280 mg/L) than butyric acid (1,010 mg/L) produced by a cow dung compost enriched culture grown on cellulose. The higher production of acetate and butyrate suggest selection of OTUs in the families Clostridiaceae and Rumiococcaceae, the genera *Clostridium* and *Ruminococcus*, which synthesize H₂ and acetate or butyrate as fermentation end-products.

Zhao et al., (2012) observed that bacteria in the genus Dorea represented 0.8% of the total microbial population identified using 454-pyrosequencieng of biofocculants during a dry fermentation process with rice straw, in which acetic and butyric acids were the dominant VFAs. This finding evident that the increase in the population of *Dorea* genus could also responsible for the high acetate production. Bacteria in the genus *Sporanaerobacter* are homoacetogens that can produce acetate by conversion of H₂ and CO₂ (Ziganshin, 2014).
Figure 4.3 Volatile fatty acids (VFAs) produced by DMD grown on α-cellulose (Treatment 4).
4.3.3. Comparison of two different enriched microbial consortia grown on \(\alpha\)-cellulose

4.3.3.1. Partial Least Square-Discriminant Analysis

We hypothesized that if an enriched culture of DMD grown with cellulose substrate was mixed with a freshly inoculated culture at a ratio of 1:1 (50% enriched DMD + 50% fresh pretreated DMD), we would observe improved \(H_2\) productivity or yield. Metagenomic analysis was conducted with both the microbial population of DMD grown without a carbon source and with the microbial community selected in the E-DMD experiment.

A PLS-DA loading plot was used to differentiate the microbial communities in the seed inoculum, with \(R^2 = 0.93\) and \(Q^2 = 0.42\) (Figure 4.4). The OTUs in the DMD and E-DMD seed consist of OTUs in the phyla Bacteroidetes, Firmicutes, Proteobacteria, and Synergistetes and these phyla followed the same pattern described in Section 4.4.1. In Figure 4.4, the left side of the plot contained OTUs from DMD grown on \(\alpha\)-cellulose (Treatment 4), while OTUs enriched in the E-DMD experiment associated with the right side of the figure (Treatment 5). When E-DMD seed is compared with DMD grown on \(\alpha\)-cellulose, very few OTUs were significantly correlated specifically to the E-DMD seed. OTUs in the family Clostridiaceae (6.49%), Caulobacteraeceae (3.2%), and the genera *Sporolactobacillus* (1%) and *Lactobacillus* (0.03%) were enriched in Treatment 5. Other microorganisms in the Treatment 5 were enriched (i.e. represented an increased percentage of the total population) compared to Treatment 4.
Figure 4.4 Comparisons of enriched microbial population in DMD grown on α-cellulose (Treatment 4) and E-DMD grown on α-cellulose (Treatment 5). Bacterial taxa were plotted according to their correlations to Treatment 4 and Treatment 5. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 5. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
4.3.3.2. Statistical Analysis

The significant and dominant microflora was identified by the MIXED procedure using SAS program. The statistical analysis and PLA-DA confirmed certain OTUs were dominant in Treatment 5 and Treatment 4. Least square mean values of the dominant microbes were used to calculate the fold-enrichment of microorganisms between the Treatments (Table 4.3). Most notable was a bacterium in the genus *Sporolactobacillus*, which was enriched 4,365-times in the microbial community of Treatment 5 compared to that of Treatment 4, while OTUs in the family *Clostridiaceae* and the genera *Pseudomonas, Ruminococcus*, were enriched 2-fold in Treatment 5 as compared with Treatment 4.

4.3.4. Comparison of end-products produced by two different enriched microbial communities selected by growth on α-cellulose

Hydrogen production by DMD (Treatment 4) and E-DMD (Treatment 5) during fermentation of α-cellulose is displayed in the Figure 4.5. The H$_2$-production by both cultures followed a similar diphasic growth pattern, which produced 5.25 mL and 19.0 mL at 72 h pi. The volume of H$_2$ produced decreased significantly between 72 and 192 h pi in the DMD-cellulose cultures (Treatment 4) and between 96 and 192 h pi in the E-DMD-cellulose cultures (Treatment 5). Hydrogen production then increased significantly after 192 h pi, generating a maximum volume of H$_2$ of 78 mL in the DMD-cellulose cultures and 95 mL in the E-DMD cultures.
Table 4.3 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of DMD (Treatment 4) and E-DMD (Treatment 5) with α-cellulose. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 4 and Treatment 5.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 4 (DMD)</th>
<th>Treatment 5 (E-DMD)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sporolactobacillus (G)</em></td>
<td>0.00048</td>
<td>2.09</td>
<td>4,365</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>4.87</td>
<td>8.12</td>
<td>2</td>
</tr>
<tr>
<td><em>Pseudomonas (G)</em></td>
<td>0.71</td>
<td>1.52</td>
<td>2</td>
</tr>
<tr>
<td>Ruminococcus (G)</td>
<td>35.05</td>
<td>66.27</td>
<td>2</td>
</tr>
</tbody>
</table>

F = Family; G = Genus
VFA analysis of the aqueous phase of the DMD-cellulose cultures (Treatment 4) and E-DMD-cellulose cultures (Treatment 5) are shown in Figure 4.6. Acetate, butyrate, and valerate were produced by the microbial communities selected in DMD-cellulose cultures; whereas the microorganisms selected in the E-DMD cellulose cultures produced acetate, butyrate, lactate, valerate, formate, isobutyrate, and isovalerate. Acetate, butyrate, and valerate were the dominant VFAs in the E-DMD cellulose cultures, with acetate detected at 6.48 ± 0.1 g/L, butyrate at 2.75 ± 0.41 g/L and valerate at 0.08 ± 0.01 g/L. However, lactate, valerate, formate, isobutyrate, and isovalerate were detected at concentrations of < 0.1 g/L, and therefore are not included in Figure 4.6.

Greater concentrations of acetate were observed in the DMD cellulose cultures whereas butyrate concentrations were greater in the E-DMD cellulose cultures. OTUs in the family Clostridiaceae and the genera *Pseudomonas* and *Ruminococcus* were dominant in the E-DMD cellulose cultures. Cheng and Cheng (2011) studied the hydrolysis of lignocellulosic feedstock by cellulose enzymes produced by a *Pseudomonas* sp. isolated from forest soil. Enzyme assays and Zymography analysis confirmed the presence of the cellulase enzymes endo-β-1-4-D-glucanase, exo-β-1-4-D-glucanase, and β-1-4-D-glucidase. Our data are consistent with this report, as we observed a two-fold increase in a *Pseudomonas* sp. in the E-DMD cellulose experiments. The huge enrichment of OTUs in the genus *Sporolactobacillus* in the E-DMD cellulose culture compared with the DMD cellulose culture is also consistent with the observed synthesis of lactate. Bacteria in the genus *Sporolactobacillus* are known to be lactic acid producers, and a *Sporolactobacillus* sp. yielded 0.5 ± 0.03 g/L lactate from glucose derived from hydrolysis of α-cellulose (Zhao et al., 2010).
Figure 4.5 Cumulative H$_2$ production by enriched microbial populations from DMD (Treatment 4) and E-DMD (Treatment 5) grown on $\alpha$-cellulose.
Figure 4.6 Comparison of volatile fatty acids (VFAs) produced by enriched microbial population from DMD and E-DMD grown on α-cellulose.
4.4 Conclusions

The objective of the studies reported in this Chapter was to determine the relationship between microbial community structure and fermentation end-product synthesis patterns of microbial communities selected by growth on cellulose. Growth of bacteria derived from dairy manure digestate (DMD) on $\alpha$-cellulose generated microbial communities that were enriched for specific operational taxonomic units (OTUs) and the fermentation end-products synthesized reflected the microbial community structure and diversity.
Chapter 5

Influence of substrate type on microbial community structure and fermentation end-product synthesis patterns III: Raw-wheat straw

5.1 Microbial diversity and community structure selected by growth on wheat straw

Agricultural crops and their residues are a major renewable resource all over the world. Bioconversion of these carbon-rich organic waste materials to biogas is an attractive process, which can contribute to the economic development of agricultural-based industries through bioenergy production. Lignocellulosic biomasses such as wheat straw, woodchips, grass residues, etc… are composed of complex carbohydrates (cellulose, hemicellulose, lignin, pectin) and are promising feedstock for biogas (biomethane, biohydrogen) production (Levin et al., 2007; Saratale et al., 2008).

Biohydrogen production from complex substrates using diverse microorganisms is an environmentally friendly and energy saving process than traditional hydrogen-production process by physical and chemical methods (Fan 2004). Wei et al., (2011) studied the effect of two different mixed microflora obtained from anaerobic digested activated sludge (AS) and anaerobic digested dairy manure (DM) on wheat straw stalks at 35 °C and pH 6.5. The DM inoculant produced more H₂ (37.0 mL/gVS) than the AM inoculated system. Further, X-ray diffraction showed that the wheat straw stalks in the DM fermentation were more disrupted than the wheat straw stalks in the AS fermentation. Zhang et al., (2009) investigated the microbial ecology of electricity-producing microbial communities
developed in two-chamber microbial fuel cells (MFCs), which contain pretreated wheat straw, at 20 °C. PCR-DGGE and DNA sequence analysis of 16S rDNA amplicons extracted from DGGE gels revealed the presence of OTUs in the phyla Bacterioidetes, Alphaproteobacteria, Deltaproteobacteria, and Gammaproteobacteria. However, Kongjan et al., (2010) evaluated H₂-production from wheat straw hydrolysates in batch and CSTR reactors at 70 °C. PCR-DGGE analysis revealed a number of known H₂-synthesizing bacteria, including *Caldanaerobacter subteraneus*, *Thermoanaerobacter subteraneus*, and *Thermoanaerobacterium thermosaccharolyticum*.

In the previous chapters, we found that growth of pretreated dairy manure digestate (DMD) with simple carbon sources like glucose and glycerol selected for subsets of the microbial community within the DMD seed, and that a greater diversity of bacteria was selected when the DMD seed was cultured with crude-glycerol compared with D-glucose (Chapter 3). Growth of pretreated DMD with a complex carbon source, α-cellulose, selected for a more specialized, but less diverse, subset of microorganisms that could utilize cellulose as a carbon source, or could use sugars generated by hydrolysis of cellulose by the cellulolytic bacteria, or could use fermentation end-products (H₂, CO₂, and/or organic acids) for growth (Chapter 4).

α-cellulose is “pure” (100%) cellulose with a significant amount of amorphic regions, which makes it relatively easy for cellulolytic bacteria to hydrolyze (Agbor et al., 2011 Agbor et al., 2014). Agricultural residues like wheat straw have truly complex structures. Raw untreated wheat straw consists of 54.0%
cellulose, 34.5% hemicellulose, and 11.5% lignin. Thus, one might expect to see selection of a slightly different subset of microorganisms when pretreated DMD is grown with wheat straw, compared to those selected by growth on “pure cellulose. Thus, the objective of Chapter 5 was to determine the microbial diversity selected by growth on raw-wheat straw and the resulting impact on fermentation end-product synthesis patterns.

5.2. Experimental design

The differences in the microbial diversity generated by growth of pretreated DMD without a carbon source (Treatment 1) or growth of the pretreated DMD with raw wheat straw (Treatment 6) were examined in batch experiments conducted using the respirometer system described in Chapter 2. To eliminate methanogens, the DMD was pre-treated at 100 °C for 30 minutes, as described in Chapter 2, Section 2.1. Anaerobic batch experiments were conducted as described in Chapter 2, Sections 2.2 and 2.3. Based on the substrate/inoculum ratio 6g COD/g VSseed, the amount of DMD seed was calculated for 100 g of seed, and a substrate:inoculum ratio of 6g COD/g VSseed was maintained in each bottle. The TCOD of raw-wheat straw in the 400 mL working volume was calculated to be 8.5 g COD. The bottles were purged with nitrogen gas for 8 minutes to create anaerobic condition. The bottles were incubated in respirometer water bath at 37 °C for 15–20 days.

The fermentation end-products (H₂, CO₂, and organic acids) synthesized by the microbial communities selected by Treatment 6 were also analyzed. Total gas production in each bottle was monitored each day (i.e. every 24 hours), and the gas composition (mol% H₂ and CO₂) in each experimental condition was evaluated for
every 48 hours, as described in Chapter 2, Section 2.4. Concentrations of volatile fatty acids (VFAs) were determined at the end of the fermentation reactions (day 21), also as described in Chapter 2, Section 2.4.

To evaluate the effect of a “pre-selected” microbial community, a second batch experiment was conducted by mixing DMD cultured on wheat straw for 22 days at 37°C with fresh pre-treated DMD at a ratio of 1:1 (v/v) as the source of inoculum (E-DMD; Treatment 7). The extended DMD (E-DMD) experiment was carried out for 22 days at 37°C and at pH 6.9, instead of 7.2, due to the addition of 50 g of the previously fermented DMD (which had a pH 6.3).

All experiments were conducted with six biological replicates, and the data were plotted by taking average of three best replicate bottles in the batch experiments. The microbial community structure and diversity resulting from growth on wheat straw was determined as described in Chapter 2, Section 2.5.

5.3. Results and Discussion

5.3.1. Metagenomic analysis of bacteria grown on raw-wheat straw

5.3.1.1 Partial least square analysis

Partial Least Square-Discriminant Analysis (PLS-DA) was used to differentiate the OTUs enriched by growth of pretreated DMD with wheat straw from the total OTUs of microorganisms present in DMD. The OTUs selected by growth of DMD without a carbon source (Treatment 1) and growth of DMD with wheat straw (Treatment 6) are plotted in Figure 5.1, with two components: $R^2_Y = 0.99$ and $Q^2_X = 0.99$, respectively. The Y-variable represents the OTUs and X-variable represents the two Treatments.
A total of 63 OTUs were distributed among phyla Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, OP9, Proteobacteria, Spirochaetes, Synergistetes, TM7, Tenericutes, and WS-6. Of these, phyla Chloroflexi, OP9, Spirochaetes, TM7, and WS-6 were represented by only OTUs in 1 family and 1 order, respectively. The phyla Bacteroidetes, Proteobacteria and Synergistetes each contributed 5 OTUs to the total population, the phylum Actinobacteria contributed 2 OTUs to the total population, and the phylum Firmicutes contributed 5 orders, 10 families, and 24 genera from the total OTUs represented in the microbial community. The OTUs of microbes grown without substrates (Treatment 1) clustered on the left side of Figure 5.1, while the OTUs correlated to growth of pretreated DMD on wheat straw (Treatment 6) clustered on the right side of the Figure 5.1.

The dominant microbes selected by Treatment 1 were bacteria in the phylum Bacteroides, and were represented by OTUs in the families Porphyromonadaceae (5.6%) and Bacteroidaceae (2.8%), and the order Bacteroidales (3.6%). Bacteria in the phylum Firmicutes were also dominant, and were represented by OTUs in the families Planococcaceae (4.2%) and Ruminococcaceae (2.57%), the orders BSA2B08 (5.48%) and MBA08 (2.1%), and the genera Coprococcus (2.3%) and Butyribirio (1.5%) respectively. OTUs in other phyla contributed less than 2% of total population microbial population grown without any substrates (Treatment 1).
Figure 5.1 Partial Least Square-Discriminate Analysis of microbial communities in the pretreated DMD grown without substrate (Treatment 1) or grown with wheat straw (Treatment 6). Bacterial taxa were plotted according to their correlations to Treatment 1 and Treatment 6. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 6. Some sequences could only be affiliated to Phylum (P), Order (O), or Family (F) levels.
The dominant bacteria in Treatment 6 were identified as OTUs in the family Ruminococcaceae (10.6% of the total population) and the genus *Coprococcus* (11.2% of the total population). Other significantly correlated OTUs were bacteria in the order Thermoanaerobacterales, the family Enterococcaceae, and the genera *Ruminococcus*, *Oxobacter*, and *Paenibacillus*, with each constituting an average of 0.5% of the total population. OTUs in the family Clostridiaceae (5%) and the genus *Clostridium* (4.63%), were also present in the microbial community selected by growth of the pretreated DMD with wheat straw.

### 5.3.1.2. Statistical analysis

The Normality of the distribution of OTUs was analyzed by the “Mixed procedure” in the SAS statistical analysis program. This analysis revealed the importance of each OTU in each Treatment. The mean values of OTUs were used to calculate the fold-enrichment of taxa present in each Treatment. Table 5.1 lists the taxa enriched by growth of pretreated DMD with wheat straw (Treatment 6) compared with the taxa present in the pretreated DMD grown without carbon source. Firmicutes was the most dominant phylum supported by wheat straw, particularly OTUs in the family Enterococcaceae, which were enriched 48-fold, and OTUs in the genera *Ruminococcus* and *Oxobacter*, which were enriched 24-fold and 12-fold, respectively.

OTUs in the order Thermoanaerobacterales, and in the genera *Paenibacillus*, and *Coprococcus* were enriched 5 to 6-fold (p < 0.05) in the microbial community selected by growth on wheat straw. Unique bacteria in the family Enterobacteriaceae (phylum Proteobacteria) were also enhanced 52-fold in
Treatment 6. In contrast, OTUs in the family Clostridiaceae increased only 1-fold in Treatment 6 as compared to Treatment 1.

5.3.2 Microbial diversity and end-product distribution patterns as a consequence of growth on wheat straw

5.3.2.1. Hydrogen and carbon dioxide synthesis by the selected microbial communities

Nucleotide sequence analysis of 16S rDNA amplified from bacteria selected by Treatment 6 DMD determined that OTUs in the order Thermoanaerobacterales, the families Enterobacteriaceae, Enterococcaceae, Clostridiacea, and Ruminococcaceae, and the genera of Oxobacter, Ruminococcus, and Paenibacillus were enriched in cultures grown on wheat straw. The selected microbial community produced a total cumulative biogas production of 310 mL, with a yield of 5 mL H₂ / g wheat straw, after 288 hours of incubation at 37 °C (Figure 5.2).

These results are consistent with previous studies reported in the literature. Collins et al., (1994) sequenced 16S rRNA genes of Clostridium tetani strain (NCTC279), Clostridium rectum from the Deutsche Sammlung von Mikroorganismen and Zelkulturen (DSMZ). The analysis identified relationships among many Clostridium species, and proposed several new genera, such as Oxobacter and Thermoanerobacter that putatively correlate with the OTUs identified in the microbial community selected by culturing pretreated DMD with wheat straw. Many of these bacteria are known to synthesize H₂, CO₂, and organic acids.
Table 5.1 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of pretreated DMD seed with wheat straw. The OTUs are arranged in the order of highest to lowest fold-enrichment values in Treatment 6.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 1 (Blank)</th>
<th>Treatment 6 (Wheat Straw)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacteriaceae (F)</td>
<td>0.085</td>
<td>4.44</td>
<td>52</td>
</tr>
<tr>
<td>Enterococcaceae (F)</td>
<td>0.12</td>
<td>5.96</td>
<td>48</td>
</tr>
<tr>
<td>Ruminococcus (G)</td>
<td>0.40</td>
<td>9.42</td>
<td>24</td>
</tr>
<tr>
<td>Oxobacter (G)</td>
<td>0.049</td>
<td>0.63</td>
<td>12</td>
</tr>
<tr>
<td>Coprococcus (G)</td>
<td>3.70</td>
<td>21.68</td>
<td>6</td>
</tr>
<tr>
<td>Thermoanaerobacterales (O)</td>
<td>0.14</td>
<td>0.92</td>
<td>6</td>
</tr>
<tr>
<td>Paenibacillus (G)</td>
<td>0.11</td>
<td>0.56</td>
<td>5</td>
</tr>
<tr>
<td>Ruminococcaceae (F)</td>
<td>5.22</td>
<td>16.97</td>
<td>3</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td>4.02</td>
<td>4.57</td>
<td>1</td>
</tr>
</tbody>
</table>

F = Family; G = Genus
Figure 5.2 Cumulative H₂ and CO₂ production by DMD grown on wheat straw (Treatment 6).
Fan et al., (2006) reported that the \( \text{H}_2 \) yield from wheat straw waste by cow dung compost was 0.5 mL/g TVS of wheat straw (10-times less that observed in Treatment 6). Wei et al. (2011) reported that the digested dairy manure (DM) yielded maximum hydrogen compared to anaerobic digested active sludge (AS) from wheat stalks. Our observations of increase in hydrogen production from wheat straw using dairy manure digestate are consistent with Wei et al. (2011).

Moore and Holdeman (1974) studied the bacterial communities from the gastrointestinal tract and feces of humans. The isolated colonies were analyzed for their morphological characteristics, biochemical reactions, fermentation products, and the G+C content of the DNA. The six strains were identified as members of the genus *Ruminococcus*. The major fermentation end-products were \( \text{H}_2 \), \( \text{CO}_2 \), acetate, formate, and in anaerobic fermentation reactions with glucose or fructose as the carbon source.

*Enterobacter aerogenes* (family Enterobacteriaceae) is a known \( \text{H}_2 \)-producing bacterium (Palazzi et al., 2000), which is consistent with the observed enrichment of OTUs in the family Enterobacteriaceae in pretreated DMD grown on wheat straw. Xing et al., (2006) isolated bacteria from anaerobic activated sludge of molasses wastewater and studied the phylogenetic relationships among the isolated species. Phylogenetic analyses revealed a close association of *Ruminococcus* and *Clostridium* species, and that both clades contained numerous \( \text{H}_2 \) synthesizing species.
5.3.2.2. Volatile Fatty acids

The soluble end-products detected in the aqueous phase of the pretreated DMD wheat straw fermentation reactions (Treatment 6) consisted of acetate (1.0 ± 0.54 g/L), butyrate (0.46 ± 0.06 g/L), and propionate (0.05 ± 0.02 g/L) (Figure 5.3). The pH of medium decreased from 7.2 to 6.7 over the 288 hour fermentation period. The higher concentrations of acetate and butyrate, and low concentrations of propionate, suggested that members of the microbial community selected by growth on wheat straw utilized the acetic-butyric acid pathway (Mizuno et al., 2000).

Our data are consistent with those of Fan et al., (2006) who observed higher concentrations of acetate and butyrate, and lower concentrations of propionate, along with H₂ and CO₂, in fermentation reactions containing cow dung compost grown with wheat straw waste. Zhao et al., (2012) studied the microbial diversity associated with bioflocculants in rice straw liquor. Acetate and butyrate acid were the dominant aqueous phase fermentation end-products and 16S rDNA phylotyping identified OTUs in the families of Ruminococcaceae, Enterococcaceae, Clostridiaceae, Enterobacteriaceae, and in the genera Ruminococcus, Oxobacter, and Paenibacillus. These data show that there are a common set of microorganisms that are associated with synthesis of H₂, CO₂, and the volatile fatty acids (acetate, butyrate, and propionate).
Figure 5.3 Volatile fatty acids (VFAs) produced by DMD grown on wheat straw (Treatment 6).
5.3.3. Comparison of two different enriched microbial consortia grown on wheat straw

5.3.3.1 Partial Least Square-Discriminant Analysis

We hypothesized that if an enriched culture of DMD grown with cellulose was mixed with a freshly inoculated culture at a ratio of 1:1 (50% enriched DMD + 50% fresh pretreated DMD), we would expect to observe improved H₂ productivity or yield. To test this hypothesis, a batch anaerobic experiment was conducted with digested DMD grown on wheat straw, and then mixed 1:1 (v/v) with fresh DMD as the source of inoculum (E-DMD; Treatment 7). The experiment was carried out for 22 days at 37 °C and pH was changed to 6.9 instead of 7.2 due to the addition of 50 g digested digestate (which had a pH of 6.3).

Metagenomic analysis was conducted with both the microbial population of DMD grown on wheat straw (Treatment 6) and with the microbial community selected in the E-DMD experiment (Treatment 7). A PLS-DA loading plot was constructed to distinguish the OTUs within the microbial community enriched in DMD by growth on wheat straw (Treatment 6) and the OTUs enriched in the E-DMD microbial community (Treatment 7), with R²Y = 0.99 and Q²X = 0.97 (Figure 5.4). OTUs enriched by Treatment 6 associated with the left side of the figure, while OTUs enriched by Treatment 7 associated with the right side of the figure.

OTUs present in the microbial communities enriched by both Treatment 6 and Treatment 7 include bacteria in the phyla Firmicutes, Proteobacteria, Synergistetes, OP9, and WS6. Of these OTUs in the phylum Firmicutes are
Figure 5.4 Comparisons of enriched microbial populations in DMD grown on wheat straw source (Treatment 6), with the E-DMD grown culture (Treatment 7). Bacterial taxa were plotted according to their correlations to Treatment 6 and Treatment 7. Taxa are represented by colored circles. The size of the circle corresponds to the relative abundance of the taxa in the total population (% of total OTUs detected). (+) indicates taxa with a positive correlation to Treatment 7. Some sequences could only be identified to Phylum (P), Order (O), or Family (F) levels.
represented by 6 Orders, 6 Families, and 34 Genera. The second most dominant set of bacteria were in the phylum Proteobacteria, and were represented by 3 families and 2 genera. The phyla Syneristetes, OP9, and WS6 each contributed OTUs in 1 order and 2 genera to the microbial communities from both Treatments.

DMD grown on wheat straw (Treatment 6) resulted in a microbial community that was enriched for OTUs in the genera *Coprococcus*, *Oscillospra*, *Syntrophomonas*, *Oxobacter*, and *Clostridium* that represented 11.08%, 8.16%, 3.0%, 2.5%, and 2.46% of the total population, respectively. Additionally OTUs in the order Thermoanaerobacterales, the family Lachnospirraceae, and the genera *Enterococcus* and *Paenibacillus* constituted 0.5 to 1.0% of the total population. The microbial community selected in the E-DMD experiment (Treatment 7) consisted of OTUs in the order Natranaerobiales (1.8%), the families Ruminococcaceae (2.89%) and Rhodopirillaceae (0.15%), and the genera *Pelotomaculum* (1.7%) and *Pseudomonas* (0.3%).

### 5.3.3.2 Statistical analysis

Comparisons of the fold-enrichment of unique microorganisms grown from E-DMD on cellulose (Treatment 5) were listed in Chapter 4, Table 4.2. When we compared the growth of microorganisms between Treatment 6 and Treatment 7, OTUs in the genus Ruminococcus and in the order *Natranaerobiales* increased 606-times in E-DMD grown on wheat straw. The fold enrichment of other OTUs enriched by Treatment 7 as compared to Treatment 6 is listed below (Table 5.2).
5.3.4. Comparison of end-products produced by two different enriched microbial communities selected by growth on wheat straw

Metagenomic analysis revealed that OTUs in the order Thermoanaerobacterales, the families Enterobacteriaceae Enterococcaceae, Clostridiaceae, and Ruminococcaceae, and the genera Oxobacter, and Paenibacillus were dominant in the microbial community selected by growth of pretreated DMD on wheat straw (Treatment 6). In contrast, OTUs in the order Natranaerobiales, the families Caulobacteraceae and Ruminococcaceae, and the genera Pelotomaculum, Pseudomonas, Ruminococcus, and Sporanaerobacter were enriched many folds in the E-DMD experiment grown on wheat straw (Treatment 7).

Figure 5.5 show that H₂ synthesis by the E-DMD microbial community followed a diphasic pattern. It is well established that H₂ synthesis by anaerobic bacteria in the genus Clostridium follows closely the increase in cell numbers, i.e. cell growth (Islam et al., 2006; Islam et al., 2009; Levin et al., 2006; Ramachandran et al., 2008). Thus, the diphasic production of H₂ is assumed to be a consequence of diphasic growth of bacteria in the microbial community. In the first phase of H₂ synthesis (cell growth), microorganisms produced 37 mL of H₂ by 144 h pi. A second phase of H₂ synthesis (cell growth) was observed between 288 and 384 h pi. A significant drop in H₂ and CO₂ was observed between 144 and 288 h pi. This could represent consumption of H₂ and CO₂ by the enriched population of homoacetogenic bacteria in the genus Sporanaerobacter (Hernandez-Eugenio et al., 2002).
Table 5.2 Fold-enrichment of selected OTUs identified in microbial communities selected by growth of DMD with wheat straw (Treatment 6) and E-DMD with wheat straw (Treatment 7). The OTUs are arranged in the order of highest to lowest fold-enrichment values in the treatment 7 and Treatment 8.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Treatment 7 (Wheat Straw)</th>
<th>Treatment 8 (E-DMD)</th>
<th>Fold-Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ruminococcus</em> (G)</td>
<td>0.015</td>
<td>9.39</td>
<td>606</td>
</tr>
<tr>
<td>Natranaerobiales (O)</td>
<td>0.13</td>
<td>3.60</td>
<td>28</td>
</tr>
<tr>
<td><em>Sporanaerobacter</em> (G)</td>
<td>0.46</td>
<td>5.40</td>
<td>12</td>
</tr>
<tr>
<td><em>Pelotomaculum</em> (G)</td>
<td>0.32</td>
<td>3.09</td>
<td>9</td>
</tr>
<tr>
<td>Caulobacteraceae (F)</td>
<td>0.31</td>
<td>2.34</td>
<td>8</td>
</tr>
<tr>
<td><em>Pseudomonas</em> (G)</td>
<td>0.092</td>
<td>0.67</td>
<td>7</td>
</tr>
<tr>
<td>Ruminococcaceae (F)</td>
<td>0.77</td>
<td>5.01</td>
<td>6</td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus
Figure 5.5 Comparison of gas (H\textsubscript{2} and CO\textsubscript{2}) production by enriched microbial population from DMD (Treatment 6) and E-DMD (Treatment 7) grown on raw-wheat straw.
Figure 5.6 Comparison of volatile fatty acids (VFAs) produced by enriched microbial populations from DMD and E-DMD grown on raw-wheat straw.
The VFA concentrations produced by the microbial communities were measured at the end of the DMD (Treatment 6) and E-DMD (Treatment 7) fermentation grown on wheat straw (Figure 5.6). The dominant VFAs produced by both cultures were acetate, butyrate, and propionate. The microbial community selected in the E-DMD wheat straw fermentation reaction (Treatment 7) produced more acetate (and slightly more butyrate) than the DMD wheat straw fermentation reaction (Treatment 6), while both fermentations produced low concentrations of propionate).

5.4 Conclusions

The objective of the studies reported in this Chapter was to determine the relationship between microbial community structure and fermentation end-product synthesis patterns in microbial communities selected by growth on wheat straw. Wheat straw has a structure that is much more complex than δ-cellulose, and we hypothesized that the microbial communities selected by growth on wheat straw would be different from those selected by growth on δ-cellulose. This hypothesis was confirmed. Moreover, extended growth of a microbial community “pre-selected” by growth on wheat straw lead to further enrichment of selected bacteria as they adapted to the complex substrate. Finally, as previously observed, the end-product synthesis pattern reflected the microbial community structure selected by the growth conditions and substrate type.
Chapter 6

Conclusions

6.1. Analysis of microbial community diversity: Alpha-diversity

The microbial communities enriched by simple and complex substrates were compared with each other to understand how the carbon source influenced the microbial populations selected using Alpha-diversity and PERAMANOVA analyses. Microbial community analysis and the end-products (H₂, CO₂, and VFAs) synthesized by bacteria in dairy manure digestate (DMD) grown on simple substrates, such as D-glucose and crude-glycerol were discussed in detail in Chapter 3. Microbial community analysis and the end-products (H₂, CO₂, and VFAs) synthesized by bacteria in dairy manure digestate (DMD) grown on complex substrates, such as α-cellulose and wheat straw were discussed in detail in Chapters 4 and 5, respectively.

Alpha-diversity analysis reveals the mean diversity of microorganisms in a particular habitat. In the case of this work, the mean diversity refers to the OTUs selected by growth of the DMD inoculant with the different substrates in the anaerobic respirometer system. The richness of species selected by growth of DMD seed (and in the extended E-DMD experiments) with simple and complex substrates is compared in Figure 6.1. The rarefaction curves reveal amount of species diversity by comparing the number of new OTUs detected per 5000 sequences. In microbial communities with high diversity, new OTUs continue to be detected as the number of sequences sampled increase. In contrast, in microbial communities with low diversity, few or no new OTUs are detected as the number of sequences sampled increase. The rarefaction curves revealed that the DMD microbes grown without a carbon source (Blank) had greatest number of OTUs compared to other
microbial communities generated by growth on the four substrates tested. The second highest diversity of microbes was observed in communities selected by growth of the DMD seed on crude-glycerol and wheat straw. In contrast, less diversity was observed in microbial communities selected by growth on glucose and \( \alpha \)-cellulose. Comparison of the microbial community structure selected by growth on pure substrates (D-glucose and \( \alpha \)-cellulose) versus impure substrates (crude-glycerol and wheat straw) revealed that impure substrates (complex or a mixture of two or three carbon sources) had greater diversity than pure substrates. The diversity of OTUs detected after extended incubation of microbes “pre-selected” by growth on \( \alpha \)-cellulose or wheat straw (the E-DMD Treatments) was similar to the diversity of OTUs selected by the initial growth of the DMD on \( \alpha \)-cellulose.

The Alpha-diversity curves suggest that the changes in substrate resulted in great variation in bacterial richness and diversity when compared to the diversity present in the DMS seed alone. It also confirms that certain microorganisms can only grow on specific substrates at 37 °C and pH 7.2, irrespective of the diversity of microbial population present in the seed inoculum.

DMD grown on simple substrates, such as D-glucose and \( \alpha \)-cellulose, yielded high cumulative \( \text{H}_2 \) concentration (2905 mL and 77 mL) compared to complex substrates, such as crude-glycerol and wheat straw (142 mL and 32 mL), respectively. The result suggests that pure carbon sources were metabolized more efficiently by hydrogen synthesizing bacteria in the microbial communities selected by growth on these substrates. The comparison of hydrogen production by enriched cultures (E-DMD) and DMD grown on \( \alpha \)-cellulose and wheat straw revealed that enriched culture produced more cumulative \( \text{H}_2 \) (94 mL and 36 mL) than DMD. The study found that there was high diversity observed in
crude-glycerol and wheat straw but there is no significant increase in the hydrogen production.

**6.2 Analysis of microbial community diversity: PERAMANOVA analysis**

Comparisons of similarities and diversity between the communities supported by growth on simple (glucose, crude-glycerol) and complex substrates (α-cellulose; wheat straw) were performed using PERAMANOVA software to obtain statistical significance and p-values. The PERAMANOVA was used to compare the diversity of OTUs in the DMD seed grown without substrates (Blank) to the diversity of OTUs enriched in the DMD seed grown with simple or complex substrates.

PERAMANOVA analysis clearly showed that the microbial diversity in the DMD seed grown without a carbon source (Blank) was significantly different, statistically (p-value < 0.05), from the diversity generated by growth on glucose or glycerol. The microbial diversity generated by growth on α-cellulose or wheat straw was also unique and distinct from DMD grown without these substrates. However, the comparison of DMD seed and E-DMD seed grown on complex substrates were not statistically different from each other (P-value > 0.05). PERAMANOVA analyses confirmed that a difference in the diversity of OTUs in the microbial communities was associated with the substrate used to grow the culture. It was also evident that DMD grown without substrate (Blank) has large endogenous diversity from which all the different microbial communities were selected as they grew on the different substrates.
Figure 6.1 Alpha-diversity (Rarefaction) analysis of microbial communities generated by growth of DMD and E-DMD on different substrates in the anaerobic respirometer batch system. 1) Treatment 1, DMD grown without substrates; 2) Treatment 2, DMD grown on glucose for 9 days; 3) Treatment 3, DMD grown on glycerol for 13 days; 4) Treatment 4, DMD grown on α-cellulose; 5) Treatment 5, DMD grown on wheat straw for 12 days; 6) Treatment 6, E-DMD grown on α-cellulose for 22 days; and 7) Treatment 7, E-DMD grown on wheat straw for 22 days.
6.3 Analyses of richness and diversity indices in the microbial communities selected by growth of DMD seed on simple and complex substrates

Microbial communities generated by growth of DMD on D-glucose, glycerol, α-cellulose, and wheat straw compared with DMD grown without substrate using the Simpson and Shannon indices of species richness and diversity (Table 6.1). The Simpson and Shannon indices for Treatments 2, 3, 4 and 6 were significantly correlated with Treatment 1 (p < 0.05). In contrast the percent of coverage and average number of trimmed sequences of all OTUs detected in the four Treatments was not significantly correlated with the DMD sample. Additionally, the phylogenetic diversity (pd) whole tree and observed species of Treatment 3 were not significantly correlated with Treatment 1 (p > 0.05), which indicates that crude-glycerol substrate supported more or less same microorganisms present in the DMD.

The highly dominant OTUs of all the substrates are listed separately for each substrate (Table 6.2). Known hydrogen producers in the family Clostridiaceae and genera *Clostridium* and *Ruminococcus* were detected in all substrates and show that these bacteria are able to grow on wide-range of substrates at 37°C and pH 7.2. The other dominant OTUs in Table 6.3 are known to carry-out mixed acid fermentation and convert organic substrates to H₂, CO₂, acetate, butyrate, and/or propionate.
Table 6.1 Indices of richness and diversity calculated for microbial communities selected by growth of dairy manure digestate (DMD) seed on simple and complex substrates.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment 2 (glucose)</th>
<th>Treatment 3 (glycerol)</th>
<th>Treatment 4 (α-cellulose)</th>
<th>Treatment 6 (wheat straw)</th>
<th>p(B-2)</th>
<th>p(B-3)</th>
<th>p(B-4)</th>
<th>p(B-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimmed sequences per sample</td>
<td>9980</td>
<td>9980</td>
<td>10645</td>
<td>9980</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pd whole tree</td>
<td>30.27</td>
<td>61.30</td>
<td>27.45</td>
<td>45.11</td>
<td>0.0059</td>
<td>0.75</td>
<td>0.003</td>
<td>&lt;.0002</td>
</tr>
<tr>
<td>Richness chao1</td>
<td>842.17</td>
<td>1804.54</td>
<td>788.21</td>
<td>1577.89</td>
<td>0.016</td>
<td>0.013</td>
<td>&lt;.0001</td>
<td>0.0014</td>
</tr>
<tr>
<td>Percent Coverage</td>
<td>0.96</td>
<td>0.91</td>
<td>0.96</td>
<td>0.92</td>
<td>0.29</td>
<td>0.94</td>
<td>0.24</td>
<td>0.81</td>
</tr>
<tr>
<td>Observed species</td>
<td>418.92</td>
<td>950.10</td>
<td>402.20</td>
<td>787.08</td>
<td>0.0003</td>
<td>0.27</td>
<td>0.0002</td>
<td>0.053</td>
</tr>
<tr>
<td>Shannon Index</td>
<td>3.96</td>
<td>6.42</td>
<td>3.43</td>
<td>5.93</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Simpson Index</td>
<td>0.81</td>
<td>0.94</td>
<td>0.68</td>
<td>0.94</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

p(B-2), p-values of diversity indices of DMD grown on D-glucose (Treatment 2) compared to DMD without substrates (Treatment 1); p(B-3), p-values of diversity indices of DMD grown on glycerol (Treatment 3) compared to DMD without substrates (Treatment 1); p(B-4), p-values of diversity indices of DMD grown on α-cellulose (Treatment 4) compared to DMD without substrates (Treatment 1); p(B-6), p-values of diversity indices of DMD grown on wheat straw (Treatment 6) compared to DMD without substrates (Treatment 1).
Table 6.2 Dominant OTUs detected in microbial communities selected by growth of dairy manure digestate (DMD) seed on simple and complex substrates.

<table>
<thead>
<tr>
<th>Treatment 2 (Glucose)</th>
<th>Treatment 3 (Glycerol)</th>
<th>Treatment 4 (α-cellulose)</th>
<th>Treatment 6 (Wheat straw)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sporolactobacillus</em> (G)</td>
<td>Caulobacteraceae (F)</td>
<td>Natranaerobiales (O)</td>
<td>Enterobacteriaceae (F)</td>
</tr>
<tr>
<td>Enterobacteriaceae (F)</td>
<td><em>Ruminococcus</em> (G)</td>
<td><em>Ruminococcus</em> (G)</td>
<td>Enterococcaceae (F)</td>
</tr>
<tr>
<td>Clostridiaceae (F)</td>
<td><em>Syntrophomonas</em> (G)</td>
<td><em>Sporanaerobacter</em> (G)</td>
<td><em>Ruminococcus</em> (G)</td>
</tr>
<tr>
<td><em>Ruminococcus</em> (G)</td>
<td>Clostridiaceae (F)</td>
<td><em>Clostridium</em> (G)</td>
<td><em>Oxobacter</em> (G)</td>
</tr>
<tr>
<td><em>Clostridium</em> (G)</td>
<td><em>Clostridium</em> (G)</td>
<td>Ruminococcaceae (F)</td>
<td><em>Coprococcus</em> (G)</td>
</tr>
<tr>
<td>Bacteroidaceae (F)</td>
<td>Bacteroidales (O)</td>
<td>Clostridiaceae (F)</td>
<td>Thermoanaerobacterales (O)</td>
</tr>
<tr>
<td>Bacteroidales (O)</td>
<td>Thermoanaerobacterales (O)</td>
<td>Thermoanaerobacteriales (O)</td>
<td><em>Paenibacillus</em> (G)</td>
</tr>
</tbody>
</table>

OTUs were listed based on high relative abundance (%) of total population. OTUs presented in Order of abundance; Treatment 2) DMD grown on D-glucose; Treatment 3) DMD grown on glycerol; Treatment 4) DMD grown on α-cellulose; Treatment 6) DMD grown on wheat straw. O = Order; F = Family; G = Genus.
6.4. Comparison of richness and diversity indices calculated from microbial communities generated from growth of two different seed cultures on D-glucose and α-cellulose

Differences in the microbial communities in anaerobic digestate sludge (ADS) collected from a Wastewater Treatment plant (Gupta et al., 2014) and dairy manure digestate (DMD) collected from a dairy manure reactor were investigated using 16S rDNA illumina sequencing. Both the seed inocula were grown on D-glucose or α-cellulose substrates at 37°C and pH 6.5 and 7.2, in batch experiments. Using the 16S rDNA sequencing results, the bacterial richness and diversity indices of two different seed cultures grown glucose and α-cellulose were calculated (Table 6.4). The phylogenetic diversity (pd) trees generated using OTUs identified in the microbial communities selected by growth of ADS and DMD on D-glucose or α-cellulose substrates were significantly different from each other (p < 0.05), with significant variations in the branch length of OTUs of microorganisms present in the ADS and DMD seed. All diversity indices of two seed cultures grown on glucose showed significant correlations except the percent of coverage (P = 0.2294). However, the Simpson and Shannon diversity indices from cultures grown on α-cellulose revealed significant difference between the two seed cultures.

Partial Least Square-Discriminant Analysis was conducted to find the dominant OTUs in the ADS and DMD seed grown without substrate, or grown with glucose or α-cellulose (Table 6.4). DMD seed had a greater number of dominant OTUs compared to the ADS seed. OTUs in the Phylum Firmicutes were dominant in the ADS seed, whereas OTUs of Families Bacteroidaceae and Porphyromonadaceae were dominant in the DMD seed (Table 6.3).
Table 6.3 Indices of richness and diversity calculated for microbial communities selected by growth of ADS and DMD seed on glucose or α-cellulose.

<table>
<thead>
<tr>
<th>Item</th>
<th>ADS Seed</th>
<th>DMD Seed</th>
<th>ADS-Glucose</th>
<th>DMD-Glucose</th>
<th>ADS-Cellulose</th>
<th>DMD-Cellulose</th>
<th>p(ADS-DMD)</th>
<th>p(ADS-DMD) Glucose</th>
<th>p(ADS-DMD) Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trimmed sequences /Sample</td>
<td>10300</td>
<td>9980</td>
<td>10300</td>
<td>9980</td>
<td>10300</td>
<td>10645</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pd whole tree</td>
<td>141.15</td>
<td>83.58</td>
<td>129.96</td>
<td>30.27</td>
<td>59.82</td>
<td>27.45</td>
<td>0.0021</td>
<td>&lt;.0001</td>
<td>0.29</td>
</tr>
<tr>
<td>Richness chao1</td>
<td>3019.15</td>
<td>3017.79</td>
<td>3039.33</td>
<td>842.17</td>
<td>1023.83</td>
<td>788.21</td>
<td>1</td>
<td>&lt;.0001</td>
<td>1</td>
</tr>
<tr>
<td>Percent of Coverage</td>
<td>0.85</td>
<td>0.86</td>
<td>0.85</td>
<td>0.96</td>
<td>0.95</td>
<td>0.9622</td>
<td>1</td>
<td>0.23</td>
<td>1</td>
</tr>
<tr>
<td>Observed species</td>
<td>1678.92</td>
<td>1499.08</td>
<td>1607.48</td>
<td>418.92</td>
<td>621.21</td>
<td>402.20</td>
<td>1</td>
<td>&lt;.0001</td>
<td>0.98</td>
</tr>
<tr>
<td>Shannon index</td>
<td>7.77</td>
<td>7.62</td>
<td>7.66</td>
<td>3.96</td>
<td>4.59</td>
<td>3.43</td>
<td>1</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Simpson index</td>
<td>0.97</td>
<td>0.98</td>
<td>0.97</td>
<td>0.81</td>
<td>0.88</td>
<td>0.68</td>
<td>0.075</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

p(ADS-DMD), p-values of diversity indices of ADS and DMD grown without substrates separately; p(ADS-DMD) cellulose, p-values of diversity indices of ADS compared to DMD grown α-cellulose; p(ADS-DMD) glucose, p-values of diversity indices of ADS compared to DMD grown D-glucose.
Table 6.4 Dominant OTUs in the ADS and DMD seed, and in cultures grown on glucose or cellulose. OTUs were listed based on high relative abundance (%) of total population. OTUs presented in order of abundance.

<table>
<thead>
<tr>
<th>ADS Seed</th>
<th>DMD Seed</th>
<th>ADS-Glucose</th>
<th>DMD-Glucose</th>
<th>ADS-Cellulose</th>
<th>DMD-Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cloaecamonaceae (F)</td>
<td>Clostridiaceae (F)</td>
<td>Ruminococcaceae (F)</td>
<td>Sporolactobacillus (G)</td>
<td>Clostridia (C)</td>
<td>Natranaerobiales (O)</td>
</tr>
<tr>
<td>T78 (G)</td>
<td>Clostridium (G)</td>
<td>Ethanoligenens (G)</td>
<td>Enterobacteriaceae (F)</td>
<td>Clostridium (G)</td>
<td>Ruminococcus (G)</td>
</tr>
<tr>
<td>OP8-1 (C)</td>
<td>Coprococcus (G)</td>
<td>Ruminococcus (G)</td>
<td>Clostridiaceae (F)</td>
<td>Lachnospiraceae (F)</td>
<td>Sporanaerobacter (G)</td>
</tr>
<tr>
<td>Ruminococcaceae (F)</td>
<td>Enterobacteriaceae (F)</td>
<td>OP8-1 (C)</td>
<td>Clostridium (G)</td>
<td>Bacteroides (G)</td>
<td>Clostridium (G)</td>
</tr>
<tr>
<td>Bacteroidaceae (F)</td>
<td>Porphyromonadaceae (F)</td>
<td>T78 (G)</td>
<td>Ethanoligenens (G)</td>
<td>Ruminococcus (G)</td>
<td>Clostridium (G)</td>
</tr>
<tr>
<td>Planococcaceae (F)</td>
<td>Thermoanaerobacterales (O)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

O = Order; F = Family; G = Genus.
Growth of DMD and ADS seed in glucose or α-cellulose generated microbial communities with very different OTUs. The microbial community selected by growth of ADS on glucose contained a greater diversity of dominant OTUs that were different from those selected by growth of DMD seed on glucose. In contrast, the microbial community selected by growth of the DMD seed with α-cellulose contained a greater diversity of OTUs that were different from those selected by the growth of ADS seed on α-cellulose.

6.5. Summary and Conclusions

The objective of the research presented in this thesis was to determine the relationships between microbial community diversity and fermentation end-product synthesis patterns in microbial communities selected by growth of a seed culture on different carbon sources ranging from simple substrates like glucose and glycerol to complex substrates like α-cellulose and wheat straw. The underlying hypothesis of this research was that the structure and diversity microbial communities is determined by the carbon source used for microbial growth, and the fermentation end-products synthesized reflect the microbes in the resulting microbial community. This hypothesis was supported by the data.

Several conclusions can be drawn from this work: 1) Dairy manure digestate (DMD) and Anaerobic Digestate Sludge contained a wide diversity of bacteria; 2) DMD seed contained a greater diversity of bacteria, and different sets of dominant species, compared with ADS seed; 3) growth of the seed cultures on different substrates selected and enriched for subsets of the microbial community within the seed cultures; 4) very different sets of bacteria were enriched in the seed
cultures grown with different carbon sources; and 5) the fermentation end-products (H₂, CO₂, organic acids, and alcohols) synthesized, and their levels of synthesis, were determined by the carbon source used to select the microbial community structure.

6.6 Future Research

The anaerobic batch studies in this thesis have described the influence of carbon source on microbial communities and associated end-products synthesized by these communities in fermentation reactions. However, the growth of microbial communities at various time intervals should be pursued further. Future studies could undertake continuous fermentation reactions with different carbon sources, and the collection of samples for characterization of microbial community structure and diversity at various time points. These experiments will provide further insight into the dynamics of how microbial populations change and establish stable communities over time on different carbon sources, and how these changes impact end-product synthesis patterns.
Chapter 7

References


