A CLOSED-LOOP BIOREFINING SYSTEM TO CONVERT ORGANIC RESIDUES INTO FUELS

By

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ABSTRACT

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This project delivers an energy positive and water neutral, closed-loop biorefining system that converts organic wastes into renewable energy and reduces the overall impacts on the environment. The research consisted of three major stages:

The first stage of this project was conducted in an anaerobic co-digestion system. Effects of the ratio of dairy manure-to-food waste as well as operating temperature were tested on the performance of the co-digestion system. Results illustrated an increase in biogas productivity with the increase of supplemental food waste; fiber analysis revealed similar chemical composition (cellulose, hemicellulose and lignin) of final solid digestate regardless their different initial feedstock blends and digestion conditions. The molecular genetic analyses demonstrated that anaerobic methanogenic microorganisms were able to adjust their community assemblage to maximize biogas production and produce homogenized solid digestate.

The second stage utilized electrocoagulation (EC) pretreated liquid digestate from previous stage to culture freshwater algae. Kinetics study showed a similar maximum growth rate (0.201-0.207 g TS day^{-1}) in both 2× and 5× dilutions of EC solution; however, the algal growth was inhibited in original EC solution (1×), possibly due to the high ammonia-to-phosphate ratio. Algal community assemblage changed drastically in different dilutions of EC solution after a 9-day culture. The following semi-continuous culture in 2×

and 5× EC media established steady biomass productivities and nitrogen removal rates; in addition, both conditions illustrated a phenomenon of phosphorus luxury uptake. Biomass composition analyses showed that algae cultured in medium containing higher nitrogen (2× EC medium) accumulated more protein but less carbohydrate and lipid than the 5× EC medium.

The last stage involved hydrolyzing the algal biomass cultured in anaerobic digestion effluent and analyzing the effects of the neutralized algal hydrolysate on the performance of enzymatic hydrolysis of acid and alkali pretreated lignocelluosic substrates (poplar, corn stover, switchgrass, and solid fiber from anaerobic digestion). Results found that algal hydrolysate significantly improved the efficiency of enzymatic hydrolysis of lignin-rich, structurally recalcitrant biomass such as poplar and solid fiber from anaerobic digestion. This discovery broadened the potential application of algal biomass besides direct use for biofuel production.

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Last but not least, I would like to dedicate my success to my parents, my husband, Jason, and people I love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 : Introduction

1. Literature review

With the global gross domestic product rising by an average of 3.6% per year, world energy use is projected to grow from 524 quadrillion Btu in 2010 to 820 quadrillion Btu in 2040 (International Energy Outlook, 2013). Consequently, the global climate change will be intensified due to the accumulation of greenhouse gases (GHG) tightly associated with the increasing energy consumption. High energy prices and environmental concerns of GHG emission have driven many countries to provide incentives to support the development of alternative energy sources, which makes renewables the world's fastest-growing energy sources (Annual Energy Outlook, 2012). In the United States, the comprehensive New Energy for America plan supported by Obama administration proposed to have 80 percent of America's electricity generated from clean energy sources by 2035 (Obama, 2011). Among various clean energy sources, not only biofuels can reduce global warming and acidification emissions in comparison with petroleum fuels, but they also possess advantages such as diversity, consistency, and potential of generating valuable by-products. While most conventional biofuels manufacturers are still using food crops (i.e. soybean, canola, corn) and animal fat as feedstock, more and more attention and investments are flowing towards new technologies that utilize non-food crops (i.e. algae, poplar, switchgrass) and organic wastes (i.e. corn stover, animal manure, municipal wastewater).

In addition to the energy consumption, many human activities also generate excess wastes which severely harm the environment. For instance, approximately 54% of

municipal solid waste in the U.S. (typically containing 50-70% organic material) is directly disposed in the landfills (U.S. EPA, 2008), and about 72 million tons of farm animal manure is generated and disposed in the open-air compost piles or lagoons every year nationwide (Obour, 2015); the decomposition process of these organic materials can contribute significant amount of GHG (such as methane, carbon dioxide, and nitrous oxide) to the atmosphere. Another example would be inappropriate treatment of waste water and sludge; these waste streams contain high organic matter (i.e. animal manure and food waste) which can cause eutrophication to freshwater bodies and their watersheds. Moreover, odor and pathogens carried and spread by these wastes also endanger the entire community that relies on such ecosystem. In order to minimize these environmental impacts, anaerobic digestion (AD) provides full containment of aforementioned potential pollutants and has become a renewed global interest. Historically, AD is one of the oldest processing technologies practiced by mankind. During the digestion, a complex anaerobic microbial consortium also converts high concentration of organic matter into biogas, a carbon neutral and renewable energy source, while odor emission and pathogen count are both well-controlled.

Although AD shows its advantages in significantly decomposing organic materials and generating clean energy, the process does not reduce or recover nutrients such as phosphorus and nitrogen. Therefore, the liquid AD effluent is required to be further treated in order to alleviate its environmental impacts. Several methods have been frequently applied by the municipal wastewater treatment plants to remove nutrients and reclaim water, which include sedimentation, active carbon adsorption, coagulation, and flocculation (Dean, 1991; Tyagi et al., 2010). However, due to the high chemical and

energy consumptions, these treatments become less efficient for large-scale facilities. Recent studies have proposed combining sustainable biological techniques and anaerobic digestion might bring wastewater treatment process benefits such as no secondary pollutant generated, possible in situ bioremediation, and economic viability (Vijayaraghavan and Yun, 2008; Pacheco et al., 2015). Therefore, algal cultivation has emerged as an option for treating wastewater containing high nutrients content, toxins and heavy metals (Mulbry and Wilkie, 2001; McHenry, 2009; Zhang et al., 2012). Since most algae strains used in nutrient removal are autotrophic and excess insoluble solids in wastewater might weaken the light availability, several recent studies reported more efficient nutrient removal using pretreated wastewater, such as effluent from primary decantation, oxidation ditch, and electrocoagulation (Chen et al., 2012a, Ruiz-Martinez et al., 2012; Liu et al., 2015). Integrating AD process with algae culture can also reduce the GHG emission from biogas combustion and accumulate algal biomass for potential biofuel and value-added bio-based chemicals.

1.1. Fundamentals of anaerobic digestion (AD)

1.1.1. The biochemistry and microbiology of AD

AD is a fermentation process using organic matter in an oxygen free environment to produce biogas. It is a highly cooperative process carried out by series of facultative and obligate anaerobic microorganisms with distinct responsibilities (Bryant et al., 1967), and the overall process can be described as:

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4.$$

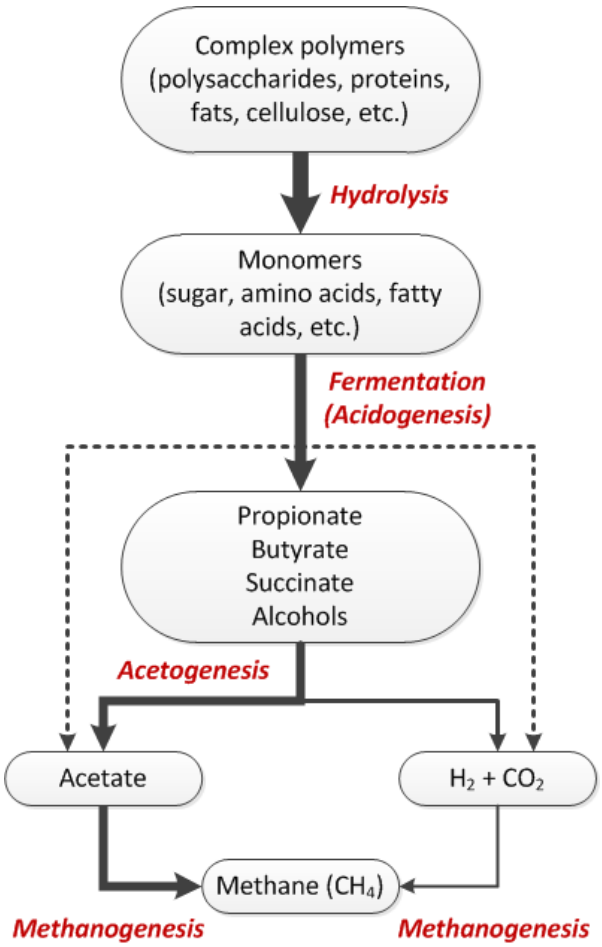


Figure 1.1. The key process stages of anaerobic digestion

There are four key stages of anaerobic digestion process: hydrolysis, fermentation (acidogenesis), acetogenesis, and methanogenesis (Figure 1.1). Undigested biomass is usually made of large organic polymers such as carbohydrates, fats and proteins. In order for microbes to carry out the anaerobic digestion and produce biogas, these large polymers must be broken down into smaller constituent monomers first. Some of these monomers (i.e. simple sugars, fatty acids and amino acids) are directly converted into acetate and hydrogen, which will be utilized by methanogenic archaea; the rest monomers need to go

through several fermentative stages to further break down the intermediate volatile fatty acids (VFAs) (i.e. propionate, butyrate, succinate, and alcohols) into acetate and hydrogen. And eventually, acetoclastic and hydrogenotrophic methanogenic archaea produce methane, carbon dioxide, and water from acetate and hydrogen, respectively.

Because of four different stages in AD process, the anaerobic microorganisms can also be categorized into four groups: hydrolytic bacteria, fermentative bacteria, acetic acid-forming bacteria, and methane-forming archaea. Genera such as *Clostridium* and *Bacillus* were isolated and found to be able to produce extracellular enzymes that facilitate the polymer hydrolysis stage (O'Sullivan et al., 2005; Yang et al., 1990; Zverlov et al., 2010). Phyla *Firmicutes, Bacteroidetes, Chloroflexi, Thermotogae,* and *Proteobacteria* all contain species that are known for fermentation/acidogenesis (Balk et al., 2002; Ueki et al., 2006; Dong et al., 2000; Yamada et al., 2005). Some typical acetogenesis microorganisms such as genera *Syntrophobacter* and *Syntrophomonas* can convert VFAs (i.e. propionate and butyrate) to acetate (Boone and Bryant, 1980; Zhang, 2004), while other acetogens such as *Clostridium* and *Acetobacterium* go through a unique Wood–Ljungdahl pathway to utilize carbon dioxide as electron acceptor and hydrogen gas as electron donor to produce acetate, with the acetyl-CoA synthase as the key enzyme (Balch et al., 1977; Müller, 2003; Müller and Frerichs, 2013). As for methanogens, they can be conceptually divided into three classes based on their phenotypic and phylogenetic similarities (Anderson et al., 2009): Class I methanogens include orders *Methanococcales, Methanobacteriales* and *Methanpyrales*; Class II only includes order *Methanomicrobiales* and Class III only includes *Methanosarcinales*. Even though hydrogenotrophic Class I and II methanogens are important in maintaining low hydrogen partial pressure in the digester by using

hydrogen/carbon dioxide to produce biogas, the majority of methane is produced from acetate by Class III, the acetoclastic methanogens (Mackie and Bryant, 1981).

Although AD process heavily relies on the microbial activity, certain criteria can be controlled or regulated by scientists and engineers in order to maintain a healthy and productive system. The operating temperature directly influences the microbial community and organic decomposition: while thermophilic process (49-57 °C) generally has higher biogas productivity and is able to eliminate pathogens, mesophilic process (30-38 °C) is more resistant to environmental variations and requires less energy and maintenance cost (Chynoweth and Pullammanappallil , 1996; Monnet, 2003). The pH of the digestate also affects the performance of digestion: high acidity can inhibit the activity of both acidogens and methanogens, but high alkalinity due to the excess accumulation of unionized ammonia is also toxic to the microbial community (Mata-Alvarez et al., 2000). Consequently, a balanced carbon-to-nitrogen ratio is required to maintain a robust pH buffering capacity and good gas production, which can be achieved by co-digesting more than one type of feedstock (such as animal manure, food waste, and sewage) (Monnet, 2003). Hydraulic retention time (HRT) indicates the average time that liquid digestate remains in the AD reactor and is determined by the reactor design and feedstock, but in general a lower HRT (faster digestion rate) is preferred when evaluating an AD reactor (Mata-Alvarez et al., 2000).

1.1.2. Configuration and current technologies of AD

There is a wide variety of designs for AD reactor based on cost, feeding plan, solid content and digestion stage. The simplest AD reactor can be constructed in-ground with a heavy-duty synthetic liner and a gas collecting furrow around. It requires minimum

investment; however, this simplified design does not provide steady and controlled operating temperature, which means it will have low efficiency when implemented in regions with long and cold seasons. Most modern designs of AD reactors incorporate element to recycle the residual heat from biogas combustion to maintain its operating temperature.

A plug-flow reactor utilizes the force from a pump during feeding and the motion of biogas when escaping from the digestate to achieve mixing, it is effective and inexpensive when the feedstock has solid content of 10-14%. More rigorous mixing provides a more homogenized condition in relatively low solid feedstock (3-10% TS), which can be performed in a continuously stirred tank reactor, or CSTR: it uses recirculating pumps, propellers, or draft tubes to continuously mix the digestate in order to prevent solid buildup (Demirer and Chen, 2005; Wilkie, 2005).

A microbial activity specific design – multi-stage anaerobic digestion system – has emerged and divides the AD process into several sequential stages. Each stage operates in a relatively small reactor with the environment tailored to its dominant microorganisms. It provides a much better refined configuration and an improved volatile solids reduction comparing with previously mentioned designs, but it also requires higher capital and operational costs (Chenowyth, 1987; Kelleher, 2007).

Feedstock for conventional AD process used to be one form of organic waste due to specific waste source from different applications; for instance, AD on a cattle farm uses manure as the feedstock, while AD in a wastewater treatment plant deals with sewage sludge. However, recent studies have shown that co-digestion of two or more substrates could significantly enhance the biogas and methane production (Mata-Alvarez et al., 2014;

Astals et al. 2014, 2015): animal manure and sewage sludge are commonly good sources for concentrated anaerobic microorganisms and provide relatively robust pH buffering capacity, although they contain either more recalcitrant organic molecules or higher moisture content; other feedstock such as food waste and pulp/paper waste might come with less methanogenic microorganisms, but they provide more high soluble organic matters (i.e. starch, grease, amorphous cellulose) which can be rapidly converted to volatile fatty acids (Cho et al., 1995; Zhai et al., 2015). The combination of these substrates could provide higher organic loading, wider range of biodiversity, balanced pH and nutrients, and synergistic relationship between substrates, all of which contribute to the improvement of AD performance.

1.1.3. Molecular genetic analyses of microbial community in AD system

Most molecular methods for identifying and classifying bacterial and archaeal use the 16S ribosomal RNA (16S rRNA) genes (sometimes referred as 16S rDNA) as a bio-marker, because (*a*) this gene is only about 1542 base pairs (bp) long, it can be quickly and cheaply copied and sequenced, and (*b*) this gene is universally present in all bacteria and archaea. In addition, it evolves slowly and has conserved and essential function for each distinct species, which means the slight changes that have occurred provide clues as to how various organisms are closely related. Many analytic approaches have been established and applied to the microbial studies of AD systems since methods for extracting and analyzing DNA from highly diverse microbial communities have improved recently. A variety of methods have been applied in the present study, including terminal restriction fragment length polymorphism (T-RFLP), clone library, and 454 pyrosequencing. Each of these analyses has its own advantage and disadvantages, which will be briefly discussed.

1.1.3.1. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a community profiling method which measures the size polymorphism of terminal restriction fragments from a polymerase chain reaction (PCR) amplified marker. In detail, near full length of 16S rRNA gene sequences are first amplified from a mixed community DNA sample using either one or two primers that are fluorescently tagged. This mixed PCR product is then digested using one or several restriction enzymes, the length and quantity of the fragments with fluorescent label are determined by capillary gel electrophoresis. The resulting profiles of all fragments can be compared within and between samples to determine relative abundance and diversity; these profiles can also be compared to a database of fragments generated from known species to tentatively identify these unknown fragments (Marsh, 1999; Abdo et al., 2006). The primary limitation of T-RFLP method is that it cannot accurately identify a fragment peak of interest to a known species because the fragment itself cannot be directly sequenced. It is common that several species can be assigned to one fragment since the comparison is solely based on the presence and location of a restriction site. Moreover, noise or false peaks can also make T-RFLP profile interpretation difficult. That is why it is common to construct a clone library in parallel to the T-RFLP analysis in order to assess and interpret the T-RFLP profile. By comparing the T-RFLP profile to a clone library, it is possible to validate each of the peaks as genuine as well as to assess the relative abundance of each variant in the library (Liu et al., 1997; Moeseneder et al, 2001). For instance, a study using T-RFLP in complement with small clone libraries examined changes in the microbial diversity over a three and a half year period of an AD system operated at psychrophilic condition (4-15 °C) (McKeown et al., 2009).

1.1.3.2. Clone library

A clone library is a gene bank containing all DNA sequences (clones) from a single organism with accompanying information. With the help of these banks, unknown DNA sequences can be identified. Construction of a clone library involves creating many recombinant DNA molecules. A mixed community DNA extractant is first amplified using a 16S rRNA gene specific primer, and then the amplicons of the 16S rRNA gene fragments are ligated/inserted into cloning vectors (i.e. plasmids); thirdly, these recombined vectors are transformed/introduced into competent cells (i.e. *E. coli*) which will create a DNA library during growth and reproduction; and lastly, a screening process of these host cells using a culture plate with selective reagent is followed in order to select the library of interest. After a library is created, the cloned 16S construct can be sequenced using the Sanger di-deoxy chain termination method to identify the composition of microorganisms in the original mixed community.

Although clone libraries of 16S rRNA genes is currently one of the most widely used methods to simultaneously evaluate the composition and diversity of a microbial community, some rare and/or unculturable species can be often left out; in addition, the high biodiversity in a given AD system usually requires a very large number of clone sequences in order to adequately describe the entire community, which is always labor-intensive and expensive. For instance, Rivière et al. (2009) examined nearly ten thousand sequences using clone libraries generated from the AD systems of seven wastewater treatment plants around the world; although, they only discovered six universally dominant operational taxonomic units (OTUs) in all samples. To the contrary, the next-generation high-throughput sequencing technologies such as 454 pyrosequencing can parallelize the

sequencing process and offer the ability to achieve massive levels of sequence coverage compared to the traditional cloning and sequencing methods.

1.1.3.3. Next Generation Sequencing (NSG) method: 454 pyrosequencing

Different from classic Sanger sequencing method, pyrosequencing relies on the detection of pyrophosphate released on nucleotide incorporation instead of chain termination with dideoxy-nucleotides (King and Scott-Horton, 2008; Wheeler et al., 2008). This method is capable of sequencing about 400 to 600 Mega bases of DNA in only 10 hour running time (Voelkerding et al., 2009); and because of that, studies have been able to evaluate microbial diversity with a finer resolution in a wider range of environments compared to its predecessors. The number "454" is simply derived from the name of the company, 454 Life Sciences (originally known as "454 Corporation"), who first developed and was awarded for this technology. The name 454 was the code name which the project used to be referred and has no special meaning (Pollack, 2003). To prepare for the sequencing, amplified 16S rRNA gene is first fractionated into fragments of 300-800 bp with blunt ends where short adaptors are ligated onto. The fragments are then individually attached to a DNA-capture bead and amplified by emulsion PCR in an oil-water solution. Finally the beads with amplicons are captured in a PicoTiterPlate on a fabricated substrate and sequenced using GS FLX System (Fakruddin et al, 2012).

But as with any other molecular sequencing method, pyrosequencing also has its limitations. For instance, this method detects long repeated nucleotides (also known as homopolymers) which can result in accumulation of sequencing errors; and since most of pyrosequences contain none or only a few errors, accumulated errors may be interpreted as a rare or pseudo OTU, which changes the estimation of community diversity and richness.

Another concern of pyrosequencing is the short length of reads generated, especially when identifying bacterial species. Clarridge (2004) reported that short sequence reads provide less phylogenetic information than those near full length of the 16S rRNA gene; although it has been commonly accepted that the information from the first 500-bp region of 16S rRNA gene is sufficient to distinguish various bacteria that are associated with human (Tang et al., 1998; Patel et al., 2000; Kattar et al., 2001; Hall et al., 2003).

In comparison, another commonly used modern benchtop high-throughput sequencing system-MiSeq from Illumina® has much higher throughput of data per run; that is 1.5-1.6 Giga bases of data at the speed of 60 Mb per hour. Additionally, MiSeq is considered a more accurate sequencing approach; it has a lower substitution error rate of only 1 substitution per 1000 base, and its indel (insertion or deletion of bases) production is very infrequently at the rate of less than 1 per 100,000 bases. However, MiSeq falls short when it comes to the length of reading (Metzker, 2010; Glenn, 2011; Loman et al., 2012).

1.2. Fundamentals of algae

1.2.1. Algae: an up-and-coming star in chemical and environmental engineering

The word "algae" refers to a diverse group of simple and mostly photoautotrophic organisms typically found in aquatic or moist environment. They vary from single-cell forms (i.e. *Chlorella* is used as dietary supplement, *Microcystis* causes harmful algal bloom) to complex multicellular forms (i.e. kelp for culinary use, decorative seaweed for aquarium). Like many terrestrial plants, algae are able to convert light energy to chemical energy (i.e. sugar, starch) that is useful to all life forms as food and energy sources. On the other hand, many distinct advantages of algae make them stand out from the rest of

photosynthetic organisms: the simple cellular structure and lack of developed reproductive organ ensure the majority of carbon, nutrient and energy they utilize contribute to biomass growth and storage. In addition, since algal biomass is not a substantial food source in many cultures, applying it as feedstock in biofuel and/or bio-based chemical industries does not raise concerns to the "food vs. fuel" debate, which is commonly mentioned in biofuel development using crop-based biomass such as soybean and corn (Pimentel and Patzek, 2005; Chisti, 2007). From environmental point of view, it has been proved that nearly 50% of global carbon dioxide is removed by algae annually, and in return they produce more than 45% of oxygen into the atmosphere (Field, 1998). Moreover, due to the biodiversity and relatively better robustness to changes in the environment, technologies using algae in the treatment of domestic and industrial wastewater has developed rapidly in the past few years. During the process, algae consume nutrients from the wastewater and carbon dioxide from the aerobic bacterial respiration; meanwhile, aerobic bacteria utilize oxygen produced by algae to decompose the organic materials. Economically speaking, this alternative method can reduce more than 50% of the aeration cost in conventional aerobic wastewater treatment (Pacheco et al., 2015).

Last but not least, by varying its culture condition, algal biomass can adapt to accumulate high concentration of carbohydrate, lipid, protein, vitamins and/or minerals, which makes it a versatile feedstock in production of renewable fuels (i.e. ethanol, biodiesel, hydrogen, methane), food and cosmetic additives (i.e. agar, carrageenan, dietary fibers, food coloring, alginate), medicines (i.e. natural iodine, niacin, CoQ10, calcium and magnesium sulfate), and fertilizers (Shifrin and Chisholm, 1980; Chelf, 1990; Kay and Barton, 1991; Li, 2012).

1.2.2. Algal cultivation in pretreated AD effluent

As briefly introduced in the previous sections, AD process cannot reduce or recover nutrients such as ammonia-nitrogen and phosphate, which are both considered as major pollutants to freshwater bodies. Therefore, AD effluent must be treated before discharged to the environment. Among various processes for nutrient management, algal cultivation represents one of the best biological treatments with the advantages of faster nutrients uptake, year-round production, and higher photosynthetic efficiency (Kebede-Westhead et al., 2004). The United States Department of Energy's Aquatic Species Program recommended that an integrated approach that combines wastewater treatment with algal biofuel production should be researched and developed (Sheehan et al., 1998; Mulbry et al., 2008). Unfortunately, due to the high turbidity and viscosity of the AD effluent, it is not an optimal culture medium for photosynthetic algae (Hamdani et al., 2004); a pretreatment step to reduce the total solids (TS) content is highly recommended prior to applying to algal cultivation (Barnet et al., 1994).

In wastewater treatment industry, processes such as coagulation aggregates suspended solids into larger bodies to facilitate physical separation of liquid and solid (Global Health and Education Foundation, 2007). Various types of coagulation are being used to condition water before sedimentation and filtration. Conventionally used chemical coagulants like alum, lime, ferric chloride and ferrous sulfate (Sivaramakrishnan, 2008) might increase the capital cost of the entire process and introduce additional wastes to the environment. Instead, by applying electrical current to a waste stream, the suspended particles will change their surface charge and form an agglomeration which can be easily separated. This process is called electrocoagulation (EC). While EC method is good in

removing suspended particles, heavy metals and compounds that cause biological/chemical oxygen demand (BOD/COD) in a highly conductive wastewater stream, it cannot recover some soluble organic and ammoniacal compounds (i.e. VFAs, ammonia) (Global Advantech, 2011). Fortunately, these residual compounds can be further removed or reduced in an open-pond algae cultivation system: ammoniacal compounds provide essential nitrogen source for algae, while VFAs can be consumed and converted into CO_2 by some bacteria (dissolved CO_2 is also carbon source for photosynthetic algae).

1.3. Fundamentals of lignocellulose biorefining

Lignocellulose refers to plant biomass that consists of carbohydrate polymers (cellulose and hemicellulose) and a class of complex aromatic polymer (lignin). It is the most abundantly available raw material and an appropriate resource for producing biofuels and value-added chemicals (Carroll and Somerville, 2009). However, its heterogeneity and recalcitrance in both structure and composition make it economically difficult to be directly converted to sustainable biofuel (mainly bioethanol). Therefore, numerous research efforts are dedicated to understand the changes of lignocellulose in the process of chemical/physical/biological conversion and the effect of different treatments on these changes.

The process of converting lignocellulosic biomass into ethanol consists of three major steps: pretreatment, enzymatic hydrolysis, and fermentation. Due to the recalcitrant nature of lignocellulose, pretreatment that involves additional acid or alkali under elevated temperature and pressure can facilitate to break the long crystalline structure of cellulose into shorter amorphous fragments and expose more surface area to enzymes. In contrast, hemicellulose is a branched polymer which consists of shorter chains of sugar units; during

the pretreatment of lignocellulosic biomass, most hemicellulose can be hydrolyzed into fermentable pentose and hexose sugar monomers such as glucose, xylose, mannose and galactose. However, as the severity of pretreatment increases, degradation of hemicellulose can lead to the formation of aliphatic acids (i.e. acetic acid, formic acid, levulinic acid) and furan aldehydes (i.e. HMF, furfural), while lignin can be degraded to phenolics and other aromatic compounds; all these aforementioned unfermentable pretreatment by-products can pose inhibition to ethanol producing yeasts. Therefore, a conditioning step such as washing and dewatering pretreated biomass is recommended to alleviate the inhibition issue (Jönsson et al., 2013; Chen, 2014 & 2015; Ruan et al., 2013).

Enzymatic hydrolysis of biomass is a critical stage in biorefining process, in which enzymes cleave bonds in pretreated complex cellulose and hemicellulose and release simple sugar molecules (i.e. glucose, xylose) for further fermentation. However, the efficiency of enzymatic hydrolysis of lignocellulosic biomass is usually hindered by the high lignin content (Tatsumoto et al., 1988), because lignin can irreversibly adsorb enzyme proteins (i.e. cellulase, xylanase, and beta-glucosidase) and reduce their availability during the hydrolysis of cellulose. Therefore, high enzyme loading is one of the reasons that encumber the industrialization of lignocellulosic biofuel. Recent studies demonstrated that dosing exogenous surfactants such as Tween 20 or bovine serum albumin (BSA) prior to cellulase addition could significantly improve the efficiency of lignocellulose hydrolysis (Tengborg et al., 2001; Yang and Wyman, 2006); it is believed that these surfactants are able to deactivate the enzyme-binding sites on lignin and consequently increase the enzyme availability to the hydrolysis. Although the chemical composition of algae varies with species and culture conditions (Becker, 1994); generally, soft algae (exclude diatoms)

tend to accumulate more protein and chlorophyll when the nitrogen and carbon source in the culture medium are sufficient (Piorreck et al., 1984; Becker, 1994; Fleurence, 1999), which makes them an ideal candidate as feedstock and protein-rich catalyst in a lignocellulose biorefinery.

2. Goal, scope and objectives

The long term goal of this study is to develop an integrated and closed-loop biorefining system that reduces global water and carbon footprint while converting organic wastes into renewable energy.

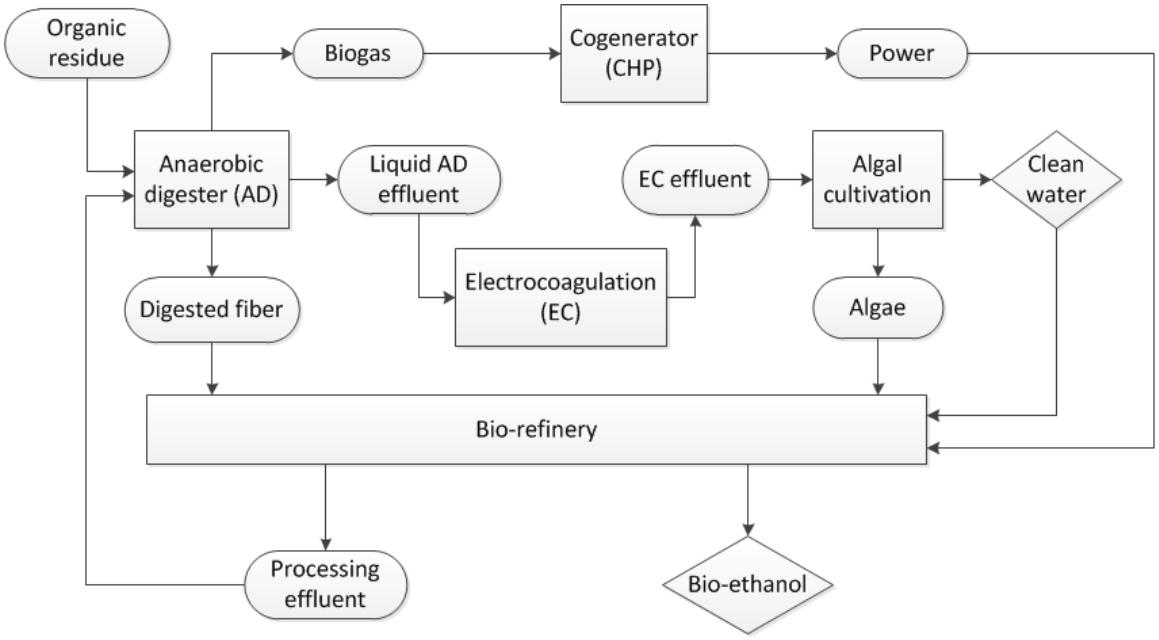


Figure 1.2: Flow chart of proposed closed-loop biorefining system

The scope of the entire study is shown in Figure 1.2. Organic wastes (livestock manure, food waste, etc.) are collected and diluted to desired organic loading rate (%) before feeding to the anaerobic digester (CSTR design). Three types of output in different

phases are produced from the AD process: biogas, solid digestate (AD fiber) and liquid digestate (AD effluent). The biogas is purified and combusted in a combined heat and power (CHP) cogenerator, from which power is supplied to the following processes such as EC and biorefining; and the residual heat is utilized to maintain the AD system at a certain temperature (meso- or thermo-philic). AD fiber has similar composition and structure as many secondary energy crops and residues, therefore can be used as feedstock for biofuel (bio-ethanol) production (Teater et al., 2011; Yue et al., 2010 & 2011; Chen et al., 2012b). Liquid AD effluent is first treated using EC process to remove large organic particles which affect the turbidity and viscosity; treated effluent is then applied to an algae cultivation system as medium. The water coming out of the algae pond contains very low concentration of nitrogen and phosphorus, and it can be either directly used for irrigation or further treated for potable water using simple techniques such as reverse osmosis. And the algal biomass harvested can perform as a co-feedstock as well as a catalyst in the following biorefining process. Moreover, the processing effluent from the biorefinery containing diluted alkali and small organic molecules (such as amino acids and VFAs) can be recycled as a nutritious pH buffer to dilute the original organic wastes for the AD.

The specific objectives of this study are: (1) developing an optimized condition for anaerobic digestion of dairy manure-food waste mixture, and assessing the microbial communities among various treatments; (2) culturing freshwater algae on EC treated AD effluent for nutrient removal and biomass production; and (3) using algae (algal hydrolysate) to improve enzymatic hydrolysis of lignocellulosic materials.

Chapter 2 : Responses of Anaerobic Microorganisms to Different Culture Conditions and Corresponding Effects on Biogas Production and Solid Digestate Quality

Abstract

Microbial communities of anaerobic digestion have been intensively investigated in thepast decades. Majority of these studies focused on correlating microbial diversity with biogas production. The relationship between microbial communities and compositional changes of the solid digestate (AD fiber) has not been comprehensively studied to date. Therefore, the objective of this study was to understand the responses of microbial communities to different operational conditions of anaerobic co-digestion and their influences on biogas production and solid digestate quality. Two temperatures and three manure-to-food waste ratios were investigated by a completely randomized design. Molecular analyses demonstrate that both temperature and manure-to-food waste ratio greatly influenced the bacterial communities, while archaeal communities were mainly influenced by temperature. The digestion performance showed that biogas productivity increased with the increase of supplemental food wastes, and there were no significant differences on carbohydrate contents among different digestions. The statistical analyses conclude that microbes changed their community configuration under various conditions to enhance digestion performance for biogas and homogenized solid digestate production.

1. Introduction

Anaerobic digestion (AD) is one of the oldest biotechnologies that mankind has practiced to treat organic wastes for several centuries. A complex anaerobic microbial consortium converts organic matter in the wastes into methane biogas - a carbon neutral and renewable energy source, and correspondingly alleviates the odor and pathogen problems. The classic AD systems often used animal manure or sewage sludge as feedstock to function as both source of nutrients and inoculum of anaerobic microorganisms (Humenik, et al., 2004). However, due to the structural and nutritional limitation of manure and sludge, single-sourced AD systems have been described as "not energy efficient nor cost effective" (Loehr, 1974). Co-digestion of more than one type of feedstock was hence introduced to enhance AD performance of biogas production and total solids (TS) reduction (Gou, et al., 2014, Liu, et al., 2009, Mata-Alvarez, et al., 2014). On the other hand, the overall performance of an AD system depends on not only the composition of feedstock, but also operational parameters such as temperature (Safferman, et al., 2012). The most conventional operational temperature levels are mesophilic (30-38 °C) and thermophilic (49-57 °C), and it has been proven that operational temperature is one of the most important determinants of the microbial community structure in an AD system (Safferman, et al., 2012, Song, et al., 2004).

Numerous studies have been conducted on the microbiology of anaerobic co-digestion system to correlate biogas production with microbial diversity (Dearman, et al., 2006, Lee, et al., 2009, Martin-Gonzalez, et al., 2011, Yu, et al., 2014, Zhang, et al., 2011). However, the relationship between microbial communities and compositional changes of the solid digestate (AD fiber) have yet been widely reported (Yue, et al., 2013). Several

recent studies have discovered that solid digestate has a similar cellulose conversion potential with other energy crops and residues such as switchgrass and corn stover, and it can be used as a potential cellulosic feedstock for biorefining of fuel and chemical production (Chen, et al., 2014, 2012, Teater, et al., 2011, Yue, et al., 2010, 2011). Therefore, a clear understanding on the relationship between mixed feedstock, microbial communities, biogas production, and solid digestate quality should be achieved in order to advance AD technology into a pretreatment unit operation for the next-generation fuel and chemical biorefining.

The objective of this study was to delineate the responses of microbial communities to changes in substrate composition and reaction temperature of anaerobic co-digestion. Dairy manure was mixed with food waste as the substrates to feed anaerobic digesters. The 16S rRNA gene-based 454 pyrosequencing, Terminal Restriction Fragment Length Polymorphism (T-RFLP) and clone library were used to investigate the communities. Microbial assembly was also correlated with performance parameters such as daily biogas accumulation, TS reduction, biogas production, and solid digestate quality (cellulose, xylan, and lignin).

2. Materials and methods

2.1. Feedstock

Fresh dairy manure was collected from the Michigan State University dairy farm (42°41’53.80”N, 84°29’8.63”W), and stored at -20 °C prior to use. Dairy cows were fed on an alfalfa and corn silage blend diet formulated according to the standard Total Mixed Rations (TMRs) (Nutrition, et al., 2001). Food waste collected from cafeterias on campus

was homogenized using a commercial immersion blender (Waring WSB70, Waring, Stamford, CT) and stored at 4 °C prior to use.

2.2. Anaerobic digestion systems

A continuously stirred tank reactor (CSTR) was used as the anaerobic digester in this study. Three different weight ratios of dairy manure to food waste were used as feeds for the anaerobic digesters: 100:0, 90:10 and 80:20 (based on dry weight). Each digester contained 5% TS. Two culture temperatures of 35 and 50 °C were tested. The hydraulic retention time (HRT) was 20 days. A completely randomized design (CRD) was applied on both factors of manure-to-food waste ratio and temperature. Six treatments with replicates were cultured on New Brunswick shakers (Eppendorf, Enfield, CT) at 150 rpm for 4 full HRTs (80 days). All digesters had a working volume of 0.50 L with 0.25 L headspace. The digesters were first purged with nitrogen gas for 30 second and then sealed with rubber septum caps. Daily biogas accumulation was measured using a water displacement system. Biogas sample from the digesters was collected for gas composition analysis. All digesters were fed every other day with 50 mL of aforementioned feed. Fresh feed was prepared every 14 days and stored at 4 °C. Before feeding, an equal volume (50 mL) of digestate was removed from the digesters as the digestate samples. 40 mL of the digestate samples were stored at -20 °C for TS, cellulose, xylan, and lignin analyses. 10 mL of the digestate samples were stored at -80 °C for microbial community analysis. The pH of all digesters was controlled above 6.70 by dosing 20% sodium hydroxide (NaOH). The operations of sampling, feeding, and pH adjustment were carried on using a Simplicity 888 automatic anaerobic chamber (PLAS Lab, Lansing, MI) purged with a medical grade specialty gas (85% nitrogen, 10% hydrogen and 5% carbon dioxide).

2.3. Analytical methods

Methane and carbon dioxide content were quantified using a SRI 8610c gas chromatograph (Torrance, CA). The system was equipped with a thermal conductivity detector. The detector was kept at 150 °C during the analysis. Hydrogen and helium were carrier gases, and maintained at 21 psi. The biogas sample volume was 100 µL, and the syringe was purged three times before sample injection. Fiber composition of the digestate was analyzed according to the National Renewable Energy Laboratory (NREL) Analytical Procedure (LAP) (Sluiter, et al., 2008) (APPENDIX A). The free sugars and starch was analyzed using a commercial starch assay kit (Catalog No. SA20. Sigma-Aldrich Co. LLC, St. Louis, MO).

2.4. Bacterial community analysis

A Power-Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA) was utilized to extract community genomic DNA from digestate samples (APPENDIX B), and a NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) was applied to quantify the DNA extractions. Polymerase Chain Reactions (PCR) were conducted to amplify the bacterial 16S rRNA gene sequences using 357F (5'-CCTACGGGAGGCAGCAG-3') and 926R (5'-CCGTCAATTCMTTTRAGT-3') as the forward and reverse primers, respectively (Brinkman, et al., 2011, Haas, et al., 2011, Preidis, et al., 2012) which targeted on the hypervariable V3-V5 region of rRNA genes (APPENDIX C). A 454 "A" adapter and unique barcode sequences were incorporated in the reverse primer, and a "B" adapter was incorporated in the forward primer. A 75 µL reaction solution contained 0.2 µM of each primer, 0.6 µL of high fidelity Taq polymerase (5 U μL^{-1}) (Life Technologies™, Grand Island, NY), 7.5 µL 10× AccuPrime PCR Buffer

II, and 20-25 ng DNA template. The reaction solution was mixed with DNase and RNase free water for PCR reaction. The amplification included an initial denaturing step at 95 °C for 5 minutes, followed by 30 cycles of 3 temperature steps (denaturing at 95 °C for 45 seconds, annealing at 50 °C for 45 seconds, and elongation at 72 °C for 90 seconds), and a final extension at 72 °C for 5 minutes. The PCR products were purified using QiaQuick PCR Product Purification kit (Qiagen, Valencia, CA). Purified amplicons were diluted to 0.5 ng dsDNA μL^{-1} and sequenced using a Roche 454 GSFLX Titanium Sequencer at the Research Technology Support Facility of Michigan State University. All bacterial 16S rRNA amplicon sequences were trimmed, screened and analyzed using Ribosomal Database Project (RDP) Pyrosequencing Pipeline Initial Process tools (Cole, et al., 2014) with a minimum sequence length of 300 bp and no ambiguous bases. Chimeras were identified using USEARCH implemented UCHIME algorithm in reference mode with Silva Gold Alignment database (Edgar, et al., 2011). Sequences were assigned with genus names at 80% confidence level by RDP Multi-Classifier and clustered at 97% similarity by Complete Linkage Clustering (Yue, et al., 2013).

2.5. Archaeal community analysis

DNA extractions from the previous step were also used for archaeal community analysis. The archaeal communities were examined using 16S rRNA gene-based Terminal Restriction Fragment Length Polymorphism (T-RFLP). The 16S rRNA gene was amplified (APPENDIX D) with archaeal domain-specific primers 344aF-FAM (FAM-5'-CGGGGYGCASCAGGCGCGAA-3') and 1119aR (5’-GGYRSGGGTCTCGCTCGTT-3’) (Koch, et al., 2006, Yue, et al., 2013). A 100 µL reaction solution containing 20 to 40 ng DNA template, 90 µL Platinum® PCR SuperMix (Invitrogen, Life Technologies

Corporation, Carlsbad, CA), 0.25 µM forward primer, 0.2 µM reverse primer, and 0.1 mg mL^{-1} bovine serum albumin (BSA) was prepared for PCR reaction. The amplification included an initial denaturing step at 94 °C for 5 minutes, followed by 25 cycles of 3 temperature steps (denaturing at 94 °C for 1 minute, annealing at 50 °C for 45 seconds, and elongation at 71 °C for 100 seconds), and a final extension at 72 °C for 5 minutes. The PCR products were then purified using QiaQuick PCR Product Purification kit (Qiagen, Valencia, CA). The purified PCR products were subjected to restriction enzyme digestion with MspI (New England Biolabs Inc., Ipswich, MA). A 15 mL digestion mixture contained 300-400 ng of purified PCR product, 1.5 µL 10X enzyme buffer, 0.5 µL enzyme (20 U $µL^{-1}$), and 0.1 mg mL^{-1} BSA. The digestion mixture was incubated at 37 °C for 3 hours and deactivated at 65 °C for 10 minutes. The digested DNA samples (7 µL) were sequenced at the Research Technology Support Facility at Michigan State University.

In order to construct archaeal clone libraries, the 16S rRNA genes of four representative samples were amplified with archaea domain-specific primers of 344aF and 1119aR. Unlike the forward primer 344aF-FAM used in previous T-RFLP experiment, 344aF does not contain any fluorescent label (FAM). TOPO TA cloning kit (Invitrogen, Life Technologies Corporation, Carlsbad, CA) with One Shot® TOP10 Chemically Competent *Escherichia coli* was used for cloning (APPENDIX E). A total of 192 clones were picked and screened for each sample, and 96 clones with correct inserts were sequenced at the Research Technology Support Facility of Michigan State University.

Archaeal 16S rRNA gene sequences obtained from clone libraries were processed using RDP. Phylogenetic affiliations were analyzed using the RDP Classifier at an 80% confidence threshold at genus level.

2.6. Statistical analysis

Two-way analysis of variance (ANOVA) and pair-wise comparison using Statistical Analysis System program 9.0 (SAS Institute Inc., Cary, NC) were conducted on biogas production, TS reduction, and AD fiber composition to compare the AD characteristics and performance among six experimental treatments (APPENDIX F).

Statistical software R with package Vegan was used to perform the operational taxonomic unit (OTU)- and phylotype-based analyses of both bacterial and archaeal communities. Specifically, non-metric multidimensional scaling analysis (NMDS) was used to correlate the dissimilarities between samples and the variations in phylotype abundance. The R package Vegan was also applied to estimate the diversity index (Shannon's *H*), community evenness (Pielou's *J*) and rarefaction curve for each sample based on clustered sequences. The sampling coverage (**C**) of each bacterial sample was calculated based on Good's method, $\mathbf{C}=1-n_1/N$, where n_1 represents number of OTUs, and *N* is the total number of sequences in the sample (Esty, 1986).

Peak Scanner™ Software v1.0 (Applied Biosystems®, Life Technologies Corporation, Carlsbad, CA) was applied to perform DNA fragment analysis; peaks below 50 fluorescence units were filtered out to eliminate the background noise. Comparisons of T-RFLP results among samples were conducted by T-Align (Smith, et al., 2005).

3. Results and discussion

3.1. Characteristics of different feedstock

Table 2.1 presents the characteristics of dairy manure and food waste, which includes total carbon (C), total nitrogen (N), free sugars and starch, structural carbohydrates (cellulose and xylan), as well as lignin. It is noticeable that even though food waste contained a similar amount of total carbon (47.8%) with dairy manure (43.7%), it contained significantly more available carbon ($p = 0.001$) but less cellulose ($p = 0.05$) and xylan ($p = 0.007$). This is due to the difference in diets between ruminant and human: dairy cows were fed on alfalfa and corn silage, both of which are fibrous lignocelluloses; while food waste from the university cafeterias contained less fiber but more free sugars and starch (34.6%, Table 2.1).

Table 2.1: Characteristics of feedstock *

	Dairy manure	**Food waste**	**Comparison**
Total carbon (wt%)	43.7	47.8	$p = 0.061$
Total available carbon (wt%)	24.8	39.9	$p = 0.001$
Free sugars and starch (wt%)	Not detectable	34.6	$p < 0.001$
Cellulose (wt%)	22.7	16.5	$p = 0.005$
Xylan (wt%)	13.9	4.9	$p = 0.007$
Lignin (wt%)	28.4	11.9	$p = 0.009$
Total nitrogen (wt%)	2.1	5.3	$p = 0.005$
C:N ratio, total C	20.6	9.0	-
C:N ratio, available C **	11.8	7.5	-

*: Data listed represent the average of two biological replicates.

**: Available carbon excludes organic matters (i.e. lignin) that do not participate in AD.

In addition, high total nitrogen content in food waste also attributes to human's high protein diet (meat and dairy). C/N ratio in the organic material plays a crucial role in the anaerobic digestion. A high C/N ratio indicates a rapid consumption of nitrogen by the microbes, which could result in a reduction of biogas productivity (Verma, 2002). While, a lower C/N ratio leads to carbon deficiency, ammonia accumulation and pH increase, all of which inhibit reproduction and metabolism of the methanogens (Chen, et al., 2008, Monnet, 2003). Therefore, mixing food waste with dairy manure could provide a balanced C/N for a healthy and efficient AD process (Khalid, et al., 2011, Sievers and Brune, 1978, Weiland, 2006).

3.2. Effects of feedstock composition and culture temperature on AD performance

Figure 2.1 and Table 2.2 illustrate the performance of digesters under six different conditions. In general, methane content in biogas ranged from 58.3 to 67.6% without significant difference among all experimental runs ($p = 0.117$). Daily biogas production and carbon removed from the feed were significantly correlated to supplemental food waste ($p = 0.001$ and 0.004, respectively) and reaction temperature ($p = 0.001$ and 0.005, respectively). The interactions between these two factors also had significant impacts ($p = 0.001$ and 0.041, respectively) on the daily biogas production and carbon removal from the feed. With the increase of the food waste percentage, both mean biogas production and carbon removal were significantly improved. On the contrary, fiber analysis demonstrated that neither reaction temperature nor supplemental food waste had impacts on cellulose ($p = 0.632$ and 0.522, respectively), xylan ($p = 0.478$ and 0.253, respectively), and lignin ($p = 0.998$ and 0.165, respectively) contents in the solid digestate. In other words, this study demonstrated that within a certain range of variation in feedstock composition (mainly

controlled by available C/N ratio), AD system could adjust itself in order to maximize carbon utilization for biogas production and generate homogenized solid digestate with similar carbohydrate content. Considering the potential application of solid digestate as a feedstock for biorefining of biofuel and chemical production (Chen, et al., 2014, 2012, Teater, et al., 2011, Yue, et al., 2010, 2011), the AD function of homogenizing carbohydrate components in the solid digestate from different feeds might provide a solution to address the compositional diversity the issue of different lignocellulosic feedstock that lignocellulosic biorefining processes encounter. Tang et al. (2011) found similar functions among microorganisms belonging to different families and even different phyla. Their co-existence and functional redundancy could ensure a more effective and economic digestion to maximize carbon utilization and methane production. O'Sullivan et al. (2005) also reported such adjustment of microbial community structure to enhance methane production and generate solid digestate with minimum composition changes.

In order to further evaluate the AD performance between different combination of temperature and feed ratio, daily biogas productivity was introduced to conduct the comparison. The daily biogas productivity [mL g^{-1} TS reduction L^{-1} digestion] was calculated by dividing the daily biogas accumulation [mL L^{-1} digestion day^{-1}] by daily TS reduction [g TS reduction day^{-1}]. The biogas productivity data (Figure 2.2) present that within the boundary of the experimental conditions temperature had no significant influence ($p = 0.206$) on biogas production. While, increasing the percentage of food waste in the feed significantly increased the biogas productivity ($p = 0.002$). It is apparent that easy-hydrolyzing carbon sources of free sugars and starch in food waste enhanced the AD performance to produce biogas, homogenized fiber quality of the solid digestate.

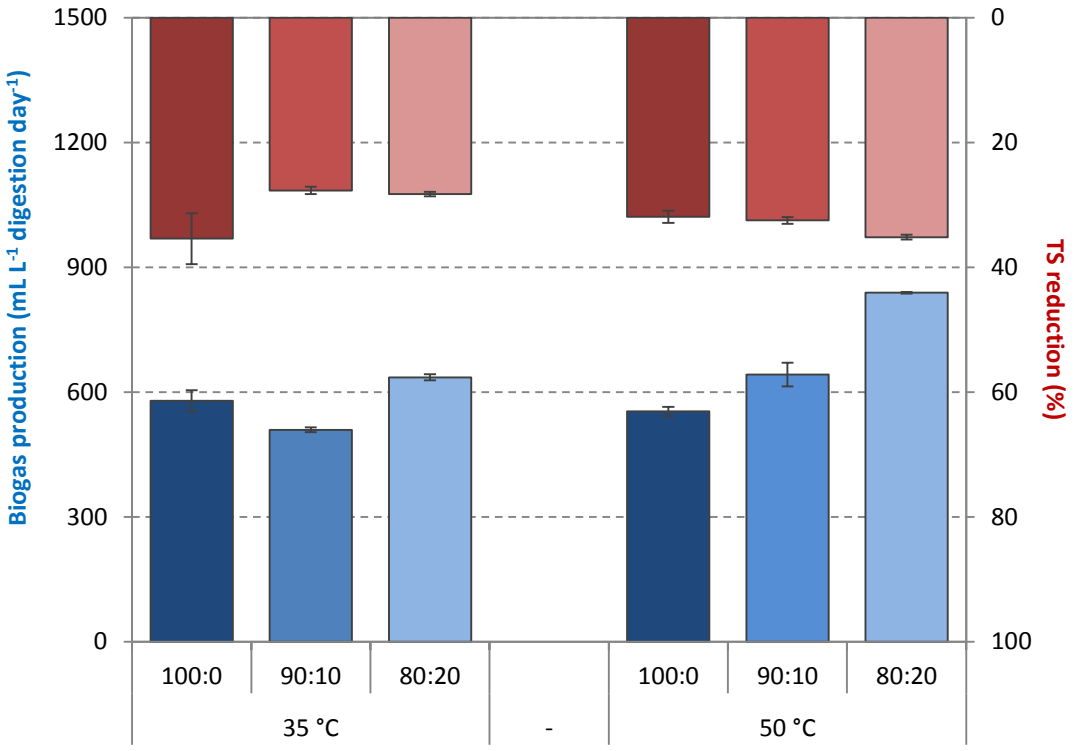


Figure 2.1: Effects of feedstock composition and culture temperature on AD performance

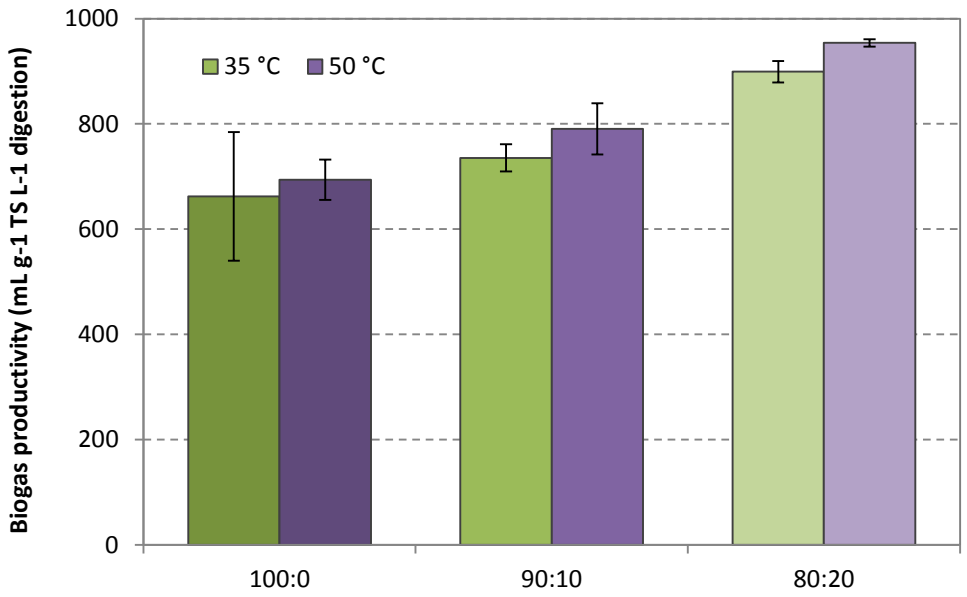


Figure 2.2: Daily biogas productivity (daily biogas accumulation vs TS reduction)

Table 2.2: Performance of anaerobic digestion*

		Daily biogas ($mL\ L^{-1}$ AD day^{-1})	CH_4 (%)	Carbon removal from the feed** (%)	Cellulose in residue (%)	Xylan in residue (%)	Lignin in residue (%)
35 °C	100/0	558.9	58.3	27.4	23.1	14.3	36.4
	90/10	499.1	65.2	24.2	22.3	13.5	36.4
	80/20	626.5	60.1	30.2	23.9	13.6	34.4
50 °C	100/0	554.4	59.0	27.2	25.3	14.8	38.0
	90/10	642.1	58.6	31.2	22.4	13.8	35.0
	80/20	848.8	67.6	40.9	23.4	13.7	34.3

*: Data listed represent the average of two biological replicates.

**: Carbon removed from the feed means that the percentage of carbon in the feed has been consumed for biogas production.

3.3. Effects of feedstock composition and culture temperature on anaerobic microbes

The aforementioned performance results demonstrate that AD can efficiently adjust itself to adapt into different nutrient conditions to maintain the performance of digestion. Considering that anaerobic microbes are the powerhouse of AD, the relationship between microbial communities, digestion conditions, biogas production, and digestate composition should be delineated in order to better understand microbial responses to digestion conditions, and enable engineering of anaerobic microbial communities to fulfill both biogas production and lignocellulose pretreatment. Metagenomic analysis was carried on to elucidate such relationship. The bacterial and archaeal communities were separately discussed in this section.

3.3.1. Anaerobic bacterial community

The pyrosequencing results demonstrate that the total bacterial 16S rRNA gene sequences in a sample ranged from 1,594 to 30,295 among 12 digestate samples (Table 2.3). Although rarefaction curves (Figure 2.3) demonstrate a great unsampled diversity across all 12 digesters, especially in those who had fewer sequences, the Good's coverage (**C**) ranged from 89.0% to 98.8% with an average of 94.5%. Allers et al. (Allers, et al., 2008) used Good's numbers to indicate the diversity of an environmental microbial community and they found that most of the gammaproteobacteria were covered with the Good's numbers fell between 70% and 80%. Therefore, the Good's numbers from all 12 AD samples indicated a high sampling coverage of bacterial community. Additionally, a recent study by McMurdie and Holmes (McMurdie and Holmes, 2014) argued that if the total numbers of sequences are vastly different among samples, commonly used rarefaction (or individual-based taxon resampling) technique is inadequate when comparing the relative proportions of each microbial strain across the entire community, rarefied counts might overlook the over-dispersion among biological replicates and suffer from a loss of power. Given the numbers of sequences in this study varied immensely among treatments and biological replicates, the normalization/rarefaction of the sequence numbers was not applied.

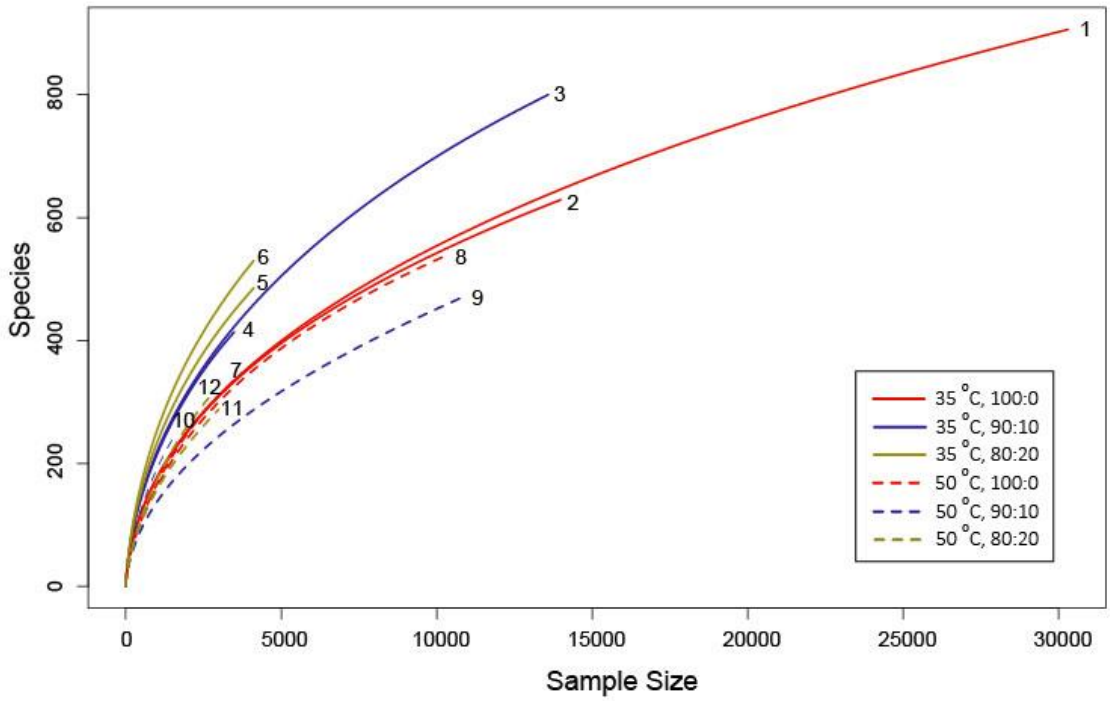


Figure 2.3: Rarefaction curves for 12 samples obtained by 454 pyrosequencing. The curves demonstrate a great unsampled diversity across all 12 digesters.

Table 2.3: The diversity and evenness of bacterial and archaeal communities calculated based on their 16S rRNA gene targeted sequencing.

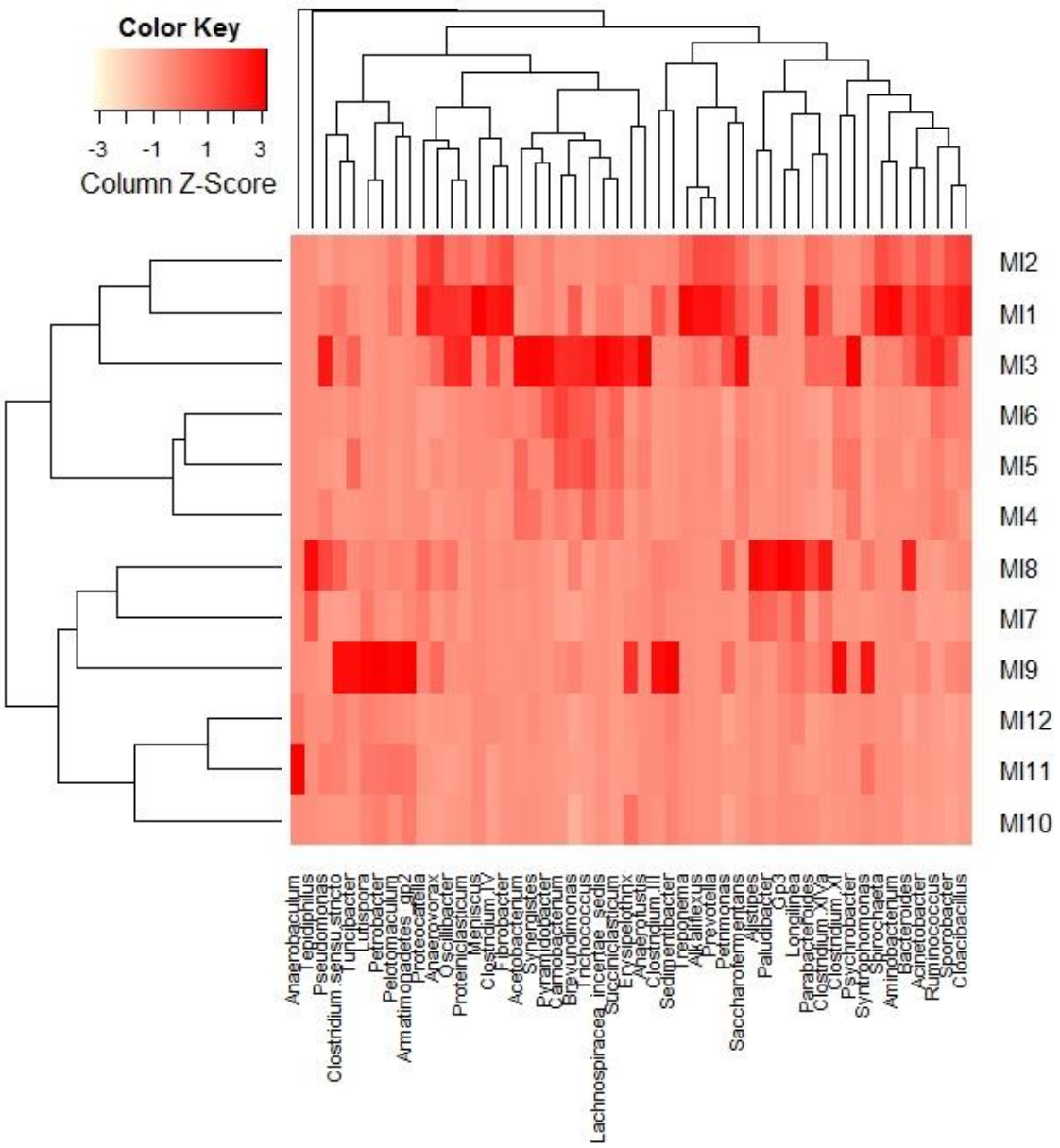
Temp	Ratio	ID	Bacteria					Archaea	
			N_{bact} [a]	OTU_{obs} [b]	**C** (%) [c]	H_{bact} [d]	J_{bact} [e]	H_{arc} [d]	J_{arch} [e]
35°C	100:0	**MI1**	30295	368	98.79	2.67	0.55	1.67	0.80
		MI2	13987	227	98.38	2.78	0.59	1.85	0.77
	90:10	**MI3**	13589	224	98.35	3.08	0.64	2.06	0.83
		MI4	3492	282	91.92	3.07	0.71	1.98	0.77
	80:20	**MI5**	4126	277	93.29	3.25	0.73	1.84	0.77
		MI6	4116	301	92.69	3.46	0.75	1.74	0.76
50°C	100:0	**MI7**	3770	328	91.30	2.52	0.58	1.80	0.72
		MI8	10416	220	97.89	2.73	0.58	1.52	0.85
	90:10	**MI9**	10849	213	98.04	2.23	0.43	1.59	0.82
		MI10	1594	176	88.96	2.81	0.66	1.66	0.80
	80:20	**MI11**	2988	247	91.73	2.65	0.62	1.63	0.84
		MI12	2689	204	92.41	2.62	0.59	1.60	0.82

Note:

a. N_{bact} is the total bacterial 16S rRNA gene sequences in the sample.
b. OTU_{obs} is the number of observed OTUs for an OTU definition.
c. **C** is the sample/Good's coverage for an OTU definition.
d. *H* is the Shannon's index which indicates the diversity of the microbial community; the subscript bact represents bacteria and arch represents archaea.
e. *J* is the Pielou's index which indicates the evenness of the microbial community; the subscript bact represents bacteria and arch represents archaea.

Based on bacterial 16S rRNA gene targeted sequencing, bacterial diversities (H_{bact}) of mesophilic digesters were much higher than thermophilic ($p = 0.006$). Several previous studies on anaerobic digestive microbial community also revealed similar trend (Sekiguchi, et al., 1998, Tiago, et al., 2004, vanLier, 1996), which could be the reason why mesophilic AD is more robust to environmental changes than thermophilic process. However, the Pielou's evenness (J_{bact}) indices did not show any significant difference among treatments ($p \geq 0.084$), which means that different bacterial communities had similar variations

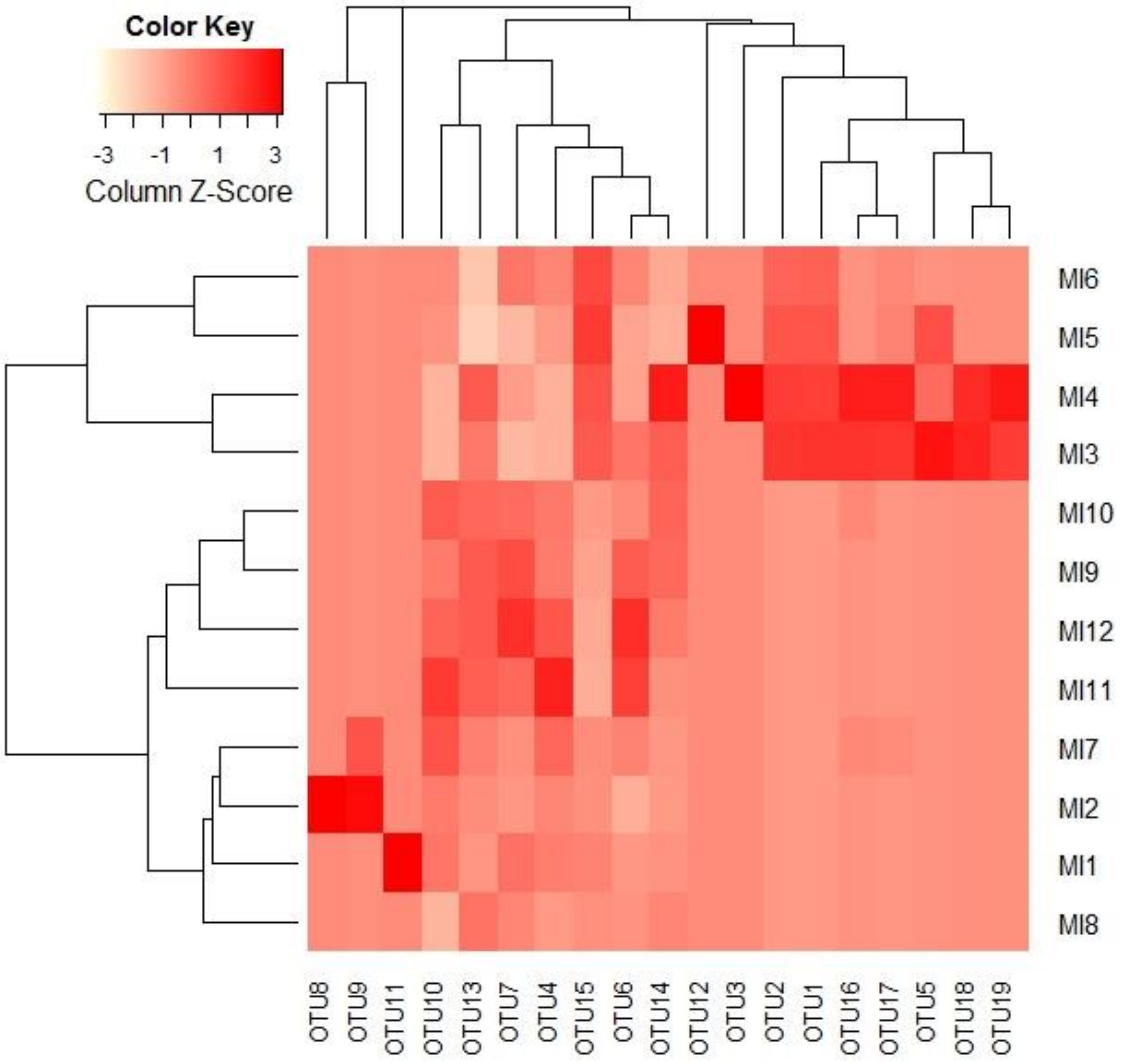
between OTUs. In addition, a combined dendrogram and heat map was generated to demonstrate the similarity of bacterial communities across all samples (Figure 2.4-a). Starting from the top of the dendrogram (left-side of the figure), the first separation of clades shows a community shift caused by reaction temperature. The cluster with samples MI7-MI12 is thermophilic digesters (50 °C) and the one with samples MI1-MI6 is mesophilic digesters (35 °C). At both temperatures, the digesters fed on dairy manure only (MI1 & 2 at 35 °C, and MI7 & 8 at 50 °C) were significantly differentiated from the ones fed on the mixture feed with 80:20 ratio (MI5 & 6 at 35 °C, and MI11 & 12 at 50 °C). Meanwhile, the bacterial communities in 90:10 digesters (MI3 & 4 at 35 °C, and MI9 & 10 at 50 °C) behaved like an intermediate state between the other two feed ratios, and their replicates illustrated closeness to either 100% dairy manure or 80:20 ratio digesters. This result indicated that bacterial community of an AD system gradually shifted its structure with the change of the feedstock. Yue et al. (2013) also observed a bacterial community shift by supplementing corn stover into a dairy manure AD system. A heat map of the most abundant bacterial genera in 12 samples (Figure 2.4-a) demonstrates a higher microbial density and diversity appeared in mesophilic digesters (MI1-6); moreover, digesters had higher manure content in the feedstock generally had higher microbial density.



a. Dendrogram and heat map of bacterial community (based on the most common genera from 454 pyrosequencing) in 12 samples

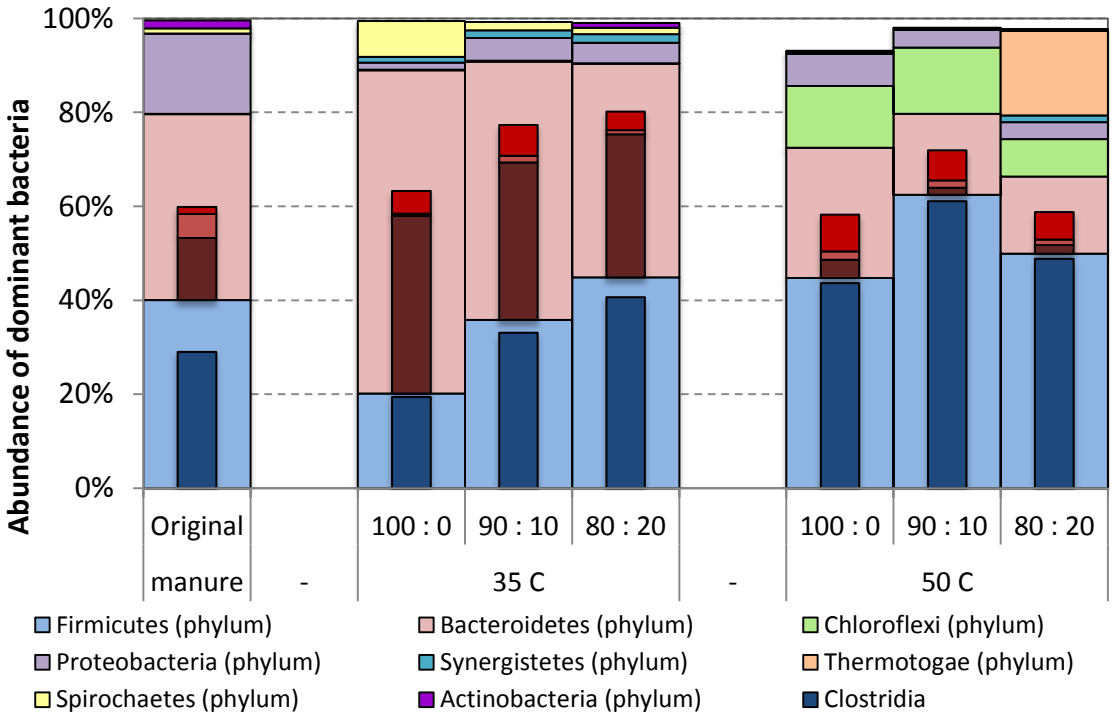
Figure 2.4: Dendrograms and heat map of the microbial community.

Figure 2.4 (cont'd)

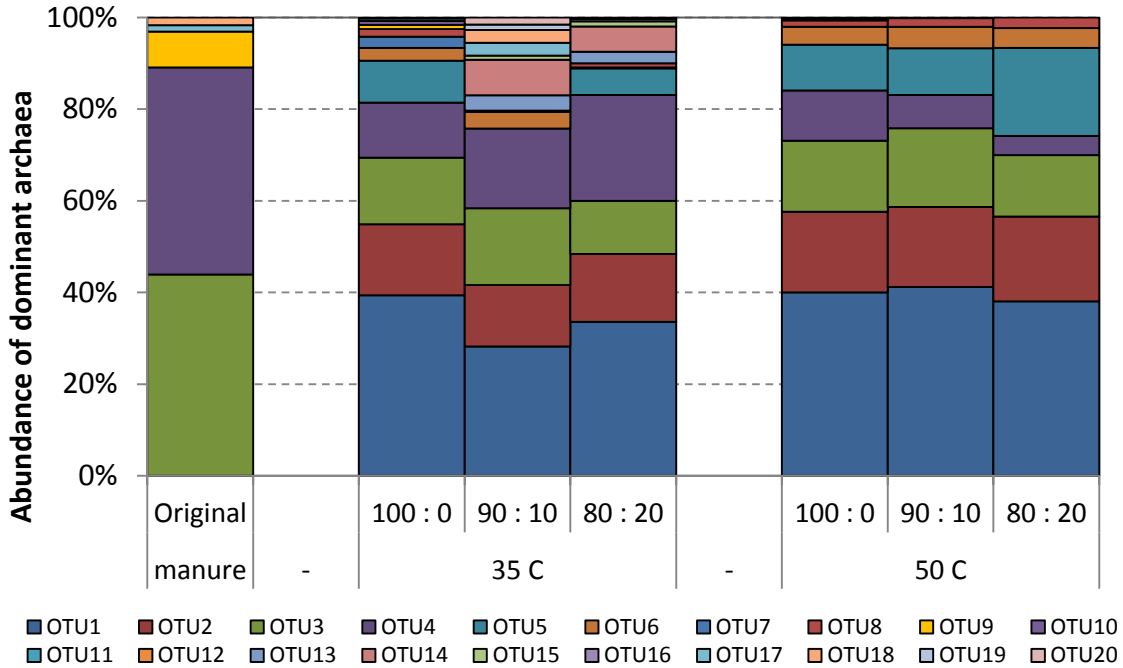


b. Dendrogram and heat map of archaeal community (based on T-RFLP results) in 12 samples

Ribosomal Database Project's Multi-Classifier with a minimal bootstrap value of 80 was used to determine the bacterial taxa. A total of 23 phyla were assigned and overall 8.6% of total sequence was categorized as unclassified bacteria. At genus level, a total of 363 bacterial groups (275 classified and 88 unclassified) were identified. *Bacteroidetes* (46-69% at 35 °C, 16-28% at 50 °C), *Firmicutes* (20-45% at 35 °C, 45-62% at 50 °C), *Proteobacteria* (2-5% at 35 °C, 4-7% at 50 °C) and *Spirochaetes* (1-8% at 35 °C) were the most abundant phyla in all 6 treatments (12 digesters) (Figure 2.5-a). In addition, *Thermotogae* (18%) was only observed in thermophilic digesters with the 80:20 ratio. *Synergistetes* (1-2%) in mesophilic digesters and *Chloroflexi* (8-14%) in thermophilic digesters were also major components of their microbial communities (Figure 2.5-a, wide columns). Within these phyla, *Clostridia* (19-41% at 35 °C, 44-61% at 50 °C), unclassified *Bacteroidetes* (30-38% at 35 °C, 2-4% at 50 °C), *Petrimonas* (4-7% at 35 °C, 6-8% at 50 °C) and *Bacteroides* (1% at 35 °C, 1-2% at 50 °C) were highly abundant (Figure 2.5-a, thin columns). Thermophilic digesters tended to accumulate more *Firmicutes* while mesophilic ones had significantly more *Bacteroidetes*. Class *Clostridia* comprised 91-98% of the phylum *Firmicutes* across 6 runs (12 digesters). Within phylum *Bacteroidetes*, unclassified *Bacteroidetes* was a significant component ($p < 0.001$) in mesophilic digesters. In addition, the fractions of *Petrimonas* in all 6 runs were similar (5-8%), but total amount of *Bacteroides* in the AD treatments was significantly lower than that in original dairy manure.



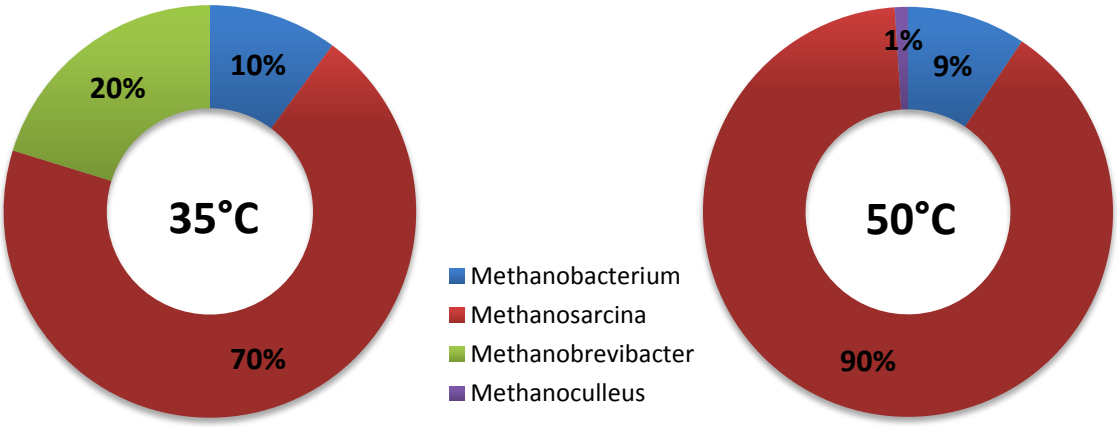
a. Assembly of dominant bacteria, where wide columns indicate dominant bacterial phyla and thin columns indicate dominant bacterial classes.



b. Assembly of dominant archaea, based on T-RFLP results

Figure 2.5: Abundance of bacterial and archaeal communities.

Figure 2.5 (cont'd)



c. Clone library of archaeal communities at different temperature settings

Phylum *Bacteroidetes* as one of the major bacterial groups in AD include several strains such as *Flavobacterium johnsoniae*, *Sporocytophaga myxococcoides*, and *Cytophaga sp.* that have been repeatedly reported as degraders of structural carbohydrates of plants (Coughlan and Mayer, 1992, Lednicka, et al., 2000, Mullings and Parish, 1984). A recent study on bacterial community in anaerobic digesters (Yang, et al., 2014) also showed that unclassified *Bacteroidetes* was one of dominant taxa in lignocellulose-rich co-digestion systems. Besides cellulose/hemicellulose degradation, it has also been reported that many members of *Bacteroidetes* are proteolytic bacteria which can degrade protein and convert amino acids to acetate (Riviere, et al., 2009, Zehnder, 1988).

Class *Clostridia* was another major bacterial group in anaerobic digestion. As saprophytic bacteria, they commonly show high cellulolytic activity as well as capability of degrading volatile fatty acids such as butyrate and its analog compounds, which indicates these bacteria play an important role in cellulose degradation during the AD

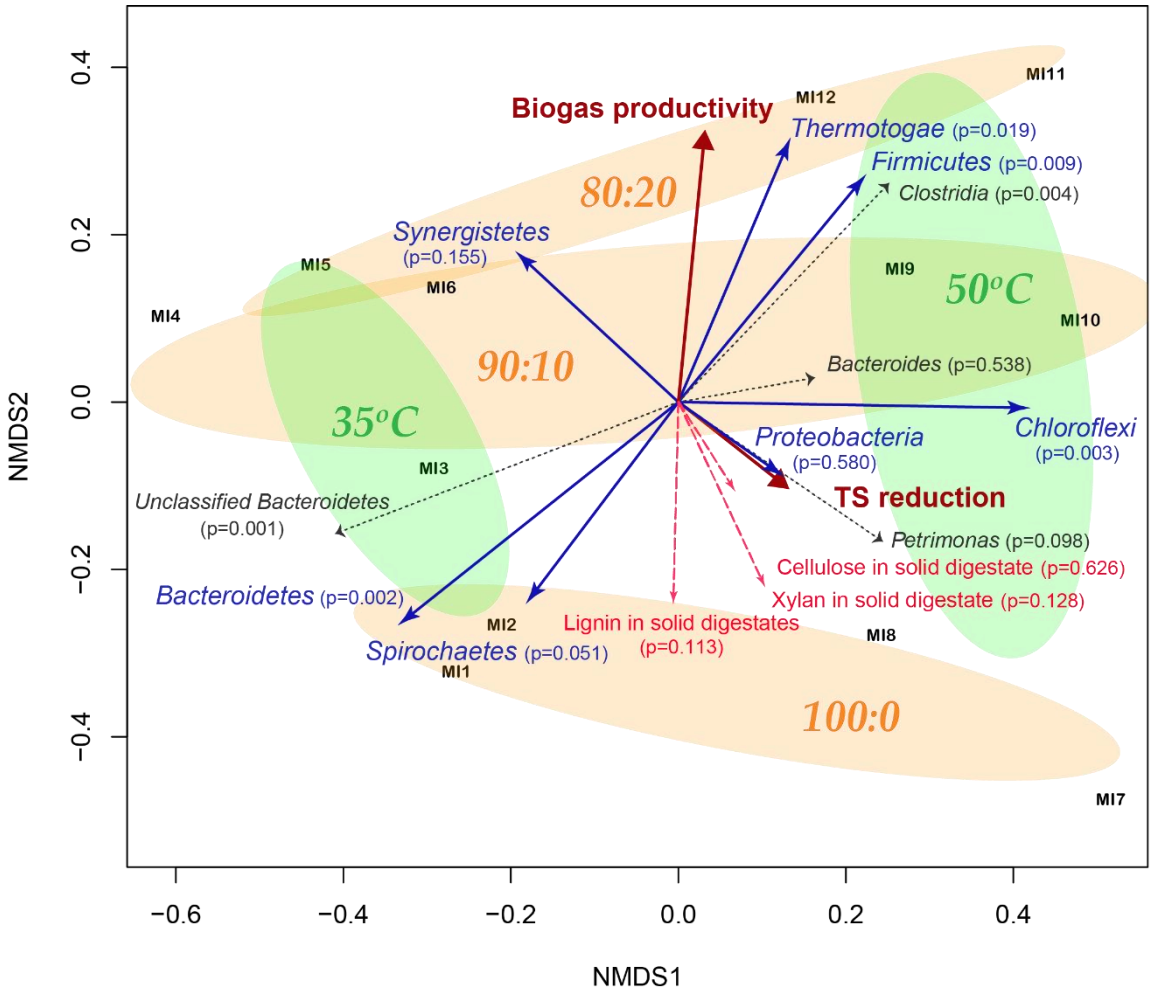
(Riviere, et al., 2009; Wirth, et al., 2012; Tang et al., 2004; Chouari et al., 2005; Goberna et al., 2009; Sasaki et al., 2007; Weiss et al., 2009; Sasaki et al., 2011; Wiegel et al., 2005; Goberna et al., 2009; Sasaki et al., 2011; Tang et al., 2011). Moreover, some strains of *Clostridia* can also utilize cellobiose and glucose generated from carbohydrate degradation to produce proton and hydrogen gas (Yang, et al., 2014).

Chlorflexi, Synergistetes, Spirochaetes and *Thermotogae* are other phyla that have been detected in the digesters. It has been reported that *Chloroflexi* have potential to treat wastes in anaerobic environment, such as thriving in naturally anaerobic dechlorination (Chandler, et al., 1998), wastewater treatment processes (Bjornsson, et al., 2002), and degrading carbohydrates (Riviere, et al., 2009, Sekiguchi, et al., 2001). *Synergistetes* are able to consume amino acids and produce short chain fatty acids as well as sulphate for methanogenic archaea and sulphate-reducing bacteria (Vartoukian, et al., 2007). They prefer mesophilic environment (Ganesan, et al., 2008) as was shown in this study. *Spirochaetes* can break down cellulose and other plant polysaccharides, and their optimum living temperature is also mesophilic (Lee, et al., 2013). Noticeably, *Thermotogae* only appeared in thermophilic digesters with 80:20 ratio that had the highest biogas productivity among all treatments, which may be related to their capability of degrading different complex-carbohydrates and producing acetic acid, carbon dioxide and hydrogen gas (Conners, et al., 2006).

Non-metric multidimentional scaling (NMDS) analysis was performed based on the complete linkage clustering of 16S rRNA gene sequences of all 12 digesters (6 treatments with duplicates) (Figure 2.6-a). The differences of bacterial communities between two reaction temperatures were significant ($p = 0.001$), though, the supplemental food waste

did not have significant impact on the community shift ($p = 0.148$). The biogas productivities were significantly different among treatments ($p = 0.012$), and the direction of its arrow indicates that digesters with 80:20 feed ratio had the highest biogas productivity (Figure 2.6-a). Similarly, the arrow of TS reduction shows an improved performance trend with elevated temperature and no-supplemental food wastes, even though the difference was not significant ($p = 0.453$). Fitting the dominant bacetrial taxa to the community distances reveals that phyla *Bacteroidetes* ($p < 0.001$), *Synergistetes* ($p = 0.038$) and *Spirochaetes* ($p = 0.028$) preferred mesophilic AD condition, while phyla *Chloroflexi* ($p < 0.001$), *Thermotogae* ($p = 0.019$) and *Firmicutes* ($p = 0.004$) tended to acccumulate more at thermophilic condition (50 °C). In addition, *Firmicutes* ($p = 0.035$) prefered the increased amount of supplemental food waste, while *Bacteroidetes* ($p = 0.003$) had higher abundance in 100% dairy manure digesters. Although both phyla *Firmicutes* (especially class *Clostridia*) and *Bacteroidetes* were reported to be able to degrade crystalline fiber into organic acids (Flint, et al., 2008, Wan, et al., 2013, Yue, et al., 2013), Sundberg et al. (2013) reported that *Bacteroidetes* were more susceptible to the environmental change caused by additional food waste, such as ammonia accumulation and pH fluctuation. The correlation between AD performance and bacterial community change also becomes obvious on this NMDS diagram. Bacterial communities tended to adapt themselves into different culture conditions and maximize their capability to convert all available carbon sources (free sugar, starch, protein, fat, hemicellulose, and easy-degradable cellulose) into biogas (Figure 2.1 and Table 2.2). As a result, differences in compositional cellulose ($p = 0.626$), xylan ($p = 0.128$) and lignin ($p = 0.113$) of the solid digestates among all six treatments were not significant, which means the bacterial

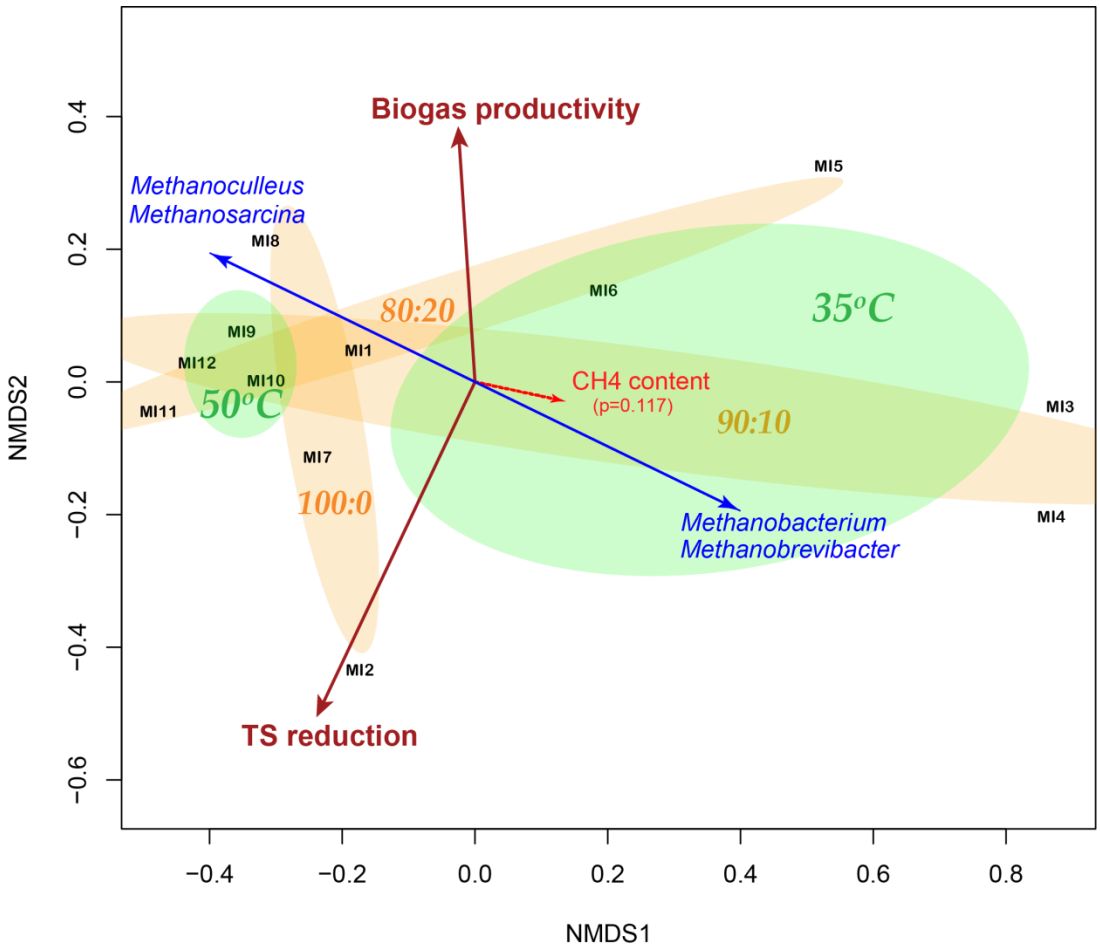
metabolism reached an equilibrium for each treatment and relatively homogenized carbohydrate composition in the solid digestate.



a. NMDS diagram of bacteria

Figure 2.6: Nonmetric Multidimensional Scaling (NMDS) of bacteria and archaea. The blue solid arrows demonstrate dominant phyla; the blue dashed arrows demonstrate dominant classes or genera; the ellipses demonstrate the dispersion of each factor using standard error of the weighted average scores.

Figure 2.6 (cont'd)



b. NMDS diagram of archaea

3.3.2. Anaerobic archaeal community

The Shannon's diversity indices of archaea (H_{arch}) calculated from the aligned and clustered (0.03% cutoff) sequences (Table 2.3) were relatively low, which indicated a relatively low diversity within archaeal communities of all treatments; the Pielou's evenness indices (J_{arch}) of archaea also showed that archaeal communities had less variation. In Figure 2.4-b, the archaeal dendrogram demonstrates community similarity across all treatments, and the heat map shows that several archaeal OTUs had higher density within mesophilic digesters. When temperature increased, archaea in the co-

digestion systems also shifted accordingly, though, the ones in 100% manure digesters were relatively consistent regardless of temperature change. Statistically, reaction temperature ($p = 0.001$) had significant impacts on the change of archaeal community while the amount of supplemental food waste did not ($p = 0.441$). The community abundance of archaea based on T-RFLP test (Figure 2.5-b) showed a relatively uniform assembly across all treatments. However, they were all significantly different from the archaea community in the original dairy manure. Moreover, similar to bacteria, archaeal communities in mesophilic digesters were more diverse than thermophilic digesters. Further phylogenetic affiliations based on clone library illustrated four genera of methanogenic archaea were detected in the digesters (Figure 2.5-c). In details, the abundance of *Methanosarcina* increased from 70% to 90% when reaction temperature was raised from 35 °C to 50 °C, while *Methanobrevibacter* reduced from 20% to non-detectable. Results also demonstrate a higher hydrogentrophic methanogen assembly (i.e. *Methanobrevibacter*, *Methanobacterium* and *Methanoculleus*) in mesophilic digeseters. The abundance change of *Methanobrevibacter* due to temperature was expected since the optimum temperature for both genera was 37-38 °C (Miller and Lin, 2002, Zellner, et al., 1987). *Methanocarsina* is a genus that uses aceticlastic pathway to generate methane (Yue, et al., 2013); therefore, its dominance in the clone libraries illustrates that aceticlastic reactions of methanogensis were the dominant route to methane in all digesters.

The NMDS analysis of archaeal community (Figure 2.6-b) shows the methane content in biogas was similar among all treatments ($p = 0.117$). The direction of the arrow illustrates that digesters at 35 °C had relatively higher methane content. Similar observation was reported previously (Gallert and Winter, 1997, Hashimoto, et al., 1981,

Mackie and Bryant, 1995). The biogas productivities and TS reduction were discussed in previous bacterial NMDS section. Fitting the dominant archaeal genera to the community distances demonstrated that increasing the reaction temperature had significant impact on *Methanosarcina* ($p = 0.001$) positively, but negatively on *Methanobrevibacter* ($p = 0.031$).

4. Conclusion

A variety of molecular and statistical approaches were applied to examine the responses of microbial communities to the changes of digestion conditions and their impacts on biogas production and solid digestate quality. The biogas productivity increased significantly with the increase of supplemental food waste. Reaction temperature did not show any significant effect within the experimental conditions. There were no significant differences on carbohydrate contents of solid digestate among six treatments. Both *Firmicutes* and *Bacteriodetes* were dominant phyla found in all treatments; however, more *Firmicutes* were observed at higher digestion temperature and higher food waste content of the feedstock, while *Bacteroidetes* were prevailing in the mesophilic digesters with higher manure content. The similarity of methane content among all six treatments and the analysis of archaea community both proved that methanogen community was lack of variation and it was only affected by reaction temperature. The co-existence of functionally similar/redundant microorganisms (both bacteria and archaea) guaranteed rapid and effective utilization of organic matters for biogas production, which could also explain the relatively homogenized composition of the solid fiber after AD regardless the deviation of community assembly. In-depth studies on the AD function of homogenizing solid digestate are urgently needed in order to develop an AD-based pretreatment method for lignocellulosic biorefining of biofuel and chemical production.

Chapter 3 : Using an environment-friendly system combining electrocoagulation process and algal cultivation to treat high strength wastewater

Abstract

This study investigated an alternative treatment approach using electrocoagulation (EC) and algae culture to reduce excess nutrients and turbidity in the liquid anaerobic digestion effluent as well as to accumulate algal biomass for potential chemicals and/or biofuels production. Batch culture demonstrates similar maximum growth rate (0.201-0.207 g TS L-1 day-1) from two dilutions (2× and 5×) of the EC solution. Excess ammonia was one possible growth inhibition factor for the culture in 1× EC medium, and high nitrogen-to-phosphorus ratio might also have limited the growth of algal biomass. In addition, community assemblage of the fresh water algae changed significantly in different dilutions of EC medium after 9-day cultivation. Semi-continuous culture established steady biomass productivities and nitrogen removal in 2× and 5× EC media. However, both conditions exhibited an increase of phosphorus removal rate which could be explained by the luxury uptake theory. Biomass composition analysis proved that algae cultured in medium with higher nitrogen concentration accumulated more proteins but less carbohydrates and lipids.

1. Introduction

Technologically sound and cost effective waste management is crucial to both municipal and agricultural development and corresponding well-being. While many conventional waste treatment plants are still using either landfill or relatively expensive and harsh chemicals to handle organic wastes, more and more modern operations have adopted inexpensive and environment-conscious approaches to reach the same goal. Anaerobic digestion (AD) is a sustainable technique which has been served for waste treatment and biofuel generation for centuries (Mata-Alvarez et al., 2000): the process uses microorganisms to convert biodegradable materials to combustible biogas in the absence of oxygen (Chen et al., 2015; Yue et al., 2010) . Although AD process significantly confines organic wastes and reduces the number of pathogens (Teater et al., 2011; Yue et al., 2013; Yue et al., 2010; Yue et al., 2011), nutrients such as ammonium, nitrate and phosphate are remained and concentrated in the liquid digestate (Chen et al., 2012a; Chen et al., 2012b; Liu and Vyverman, 2015; Liu et al., 2015). The most common application of this nutritious digestate is to directly apply in the field as liquid fertilizer (Chen et al., 2015); however, since arable soil tends to retain less nutrients from liquid fertilizer than its solid form, such practice must be well regulated and excess liquid digestate needs to be further processed to reduce its eutrophication potential (Chen et al., 2012a; Chen et al., 2015; Macias-Corral et al., 2008; Smith et al., 2007).

In order to realize an eco-friendly manner to treat liquid AD digestate, studies and practices such as systematic algal culture in wastewater have emerged (Chen et al., 2012a; Karns et al., 1998; Mulbry et al., 2008; Mulbry and Wilkie, 2001; Pizarro et al., 2002). These bioremediation processes combine nutrient uptake, dissolved oxygen enrichment,

and pH buffering to provide safe and effective ways to treat liquid AD digestate and other wastewater. Meanwhile, algal biomass collected from these processes also has potential to produce alternative fuel and bio-derived chemicals. Nevertheless, due to the relatively high turbidity in the effluent, most of these commercial scale algae ponds have to have shallow bed to ensure sufficient access to light (Adey et al., 2011; Chen et al., 2012a; Mulbry et al., 2008; Mulbry and Wilkie, 2001; Pacheco et al., 2015), which could raise issues such as excess land use and water evaporation. Therefore, an additional step which can further reduce turbidity in the effluent was recommended prior to algal cultivation.

Among various modern wastewater treatment techniques, electrocoagulation (EC) stands out as an electron driven coagulation method that eliminates chemical additives, reduces pathogens, and is able to handle high strength wastewater (Liu et al., 2013; Liu et al., 2015; Mollah et al., 2004). Industries such as paper, metal and mining all have stable and successful experience with EC treating their waste streams (Bellebia et al., 2012; Mollah et al., 2001; Parga et al., 2009); several recent studies on AD liquid digestate also reported that EC could significantly reduce chemical oxygen demand (COD), phosphate, and solution turbidity (Liu and Liu, 2015; Liu et al., 2013; Liu et al., 2015).

This study focused on integrating electrochemical technology and algal cultivation to develop a sustainable high-strength wastewater (i.e. liquid AD digestate) treatment system. The objectives of this study were to: (1) demonstrate the impact of EC treated liquid AD digestate (EC medium) on the fresh water algal assemblage; (2) determine an appropriate dilution of EC medium to achieve maximum nutrient removal and algal growth; and (3) analyze chemical composition of algal biomass cultured in the EC medium.

2. Material and methods

2.1. EC treatment of liquid AD digestate

Liquid digestate was collected from the commercial anaerobic digester at Michigan State University (N42°41'55", W84°29'18"). The digester is a continuously stirred tank reactor (CSTR) and has an effective volume of 1800 m^3. Feedstock of the digester consisted of roughly 60% dairy manure and 40% food waste (wet mass): dairy cows were fed on an alfalfa and corn silage blend diet according to the standard Total Mixed Rations (TMRs) for dairy cattle by Natural Research Council (NRC, 2001), and food waste was mainly collected from campus cafeterias. The digester was operated at 35 °C with a hydraulic retention time of 25 days, and the digestate was separated into liquid and solid portions using a screw press with 2 mm screen. The liquid digestate containing 4.8% total solids (TS, w/w), 3.1 g L^{-1} total nitrogen (TN), 1.5 g L^{-1} total phosphorus (TP), 21.5 g L^{-1} chemical oxygen demand (COD), and a pH of 8.0, was used as the solution for the EC treatment.

The EC treatment of the liquid digestate was carried out according to previous studies (Liu and Liu, 2015; Liu et al., 2015) with minor modifications. Briefly, the original liquid digestate was diluted 4 times using tap water and treated in a 50 L column EC reactor with anode surface area/volume ratio as 0.124 cm^{-1} (Figure 3.1). A DC power supply (XPOWER^{TM} 30 V, 5 A) was used to power the reaction, the current was maintained at 5 A, and the retention time was 4 hrs. After EC treatment, the effluent was centrifuged at 3500 rpm for 10 min and the supernatant (EC medium) was collected for algal culture. The original EC medium contained 0.03% TS (w/w), 350 mg L^{-1} TN, 25.4 mg L^{-1} TP, 907 mg L^{-1} COD, 5.41 mg L^{-1} total iron (Fe), and pH of 8.5.

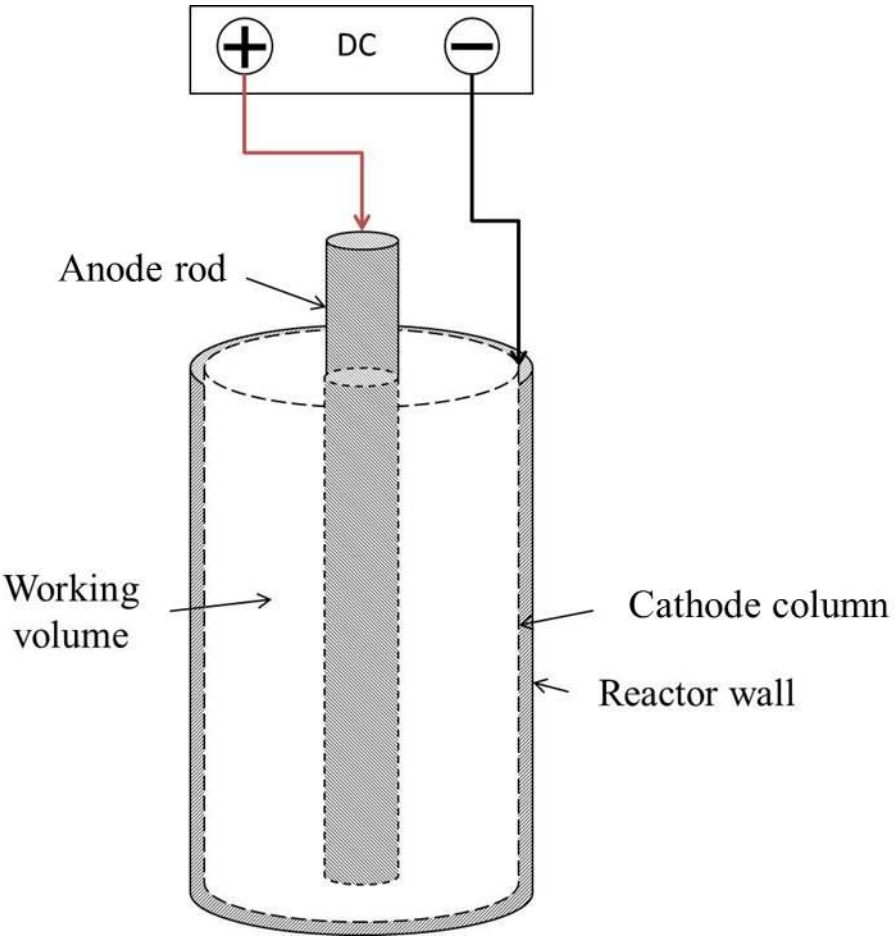


Figure 3.1: Sketch of column EC reactor (Liu and Liu, 2015).

2.2. Preparation of algal inoculum

A freshwater algal sample was collected from a pond located near the dairy farm at Michigan State University (N42°41’54”, W84°29’17”). The pond water was initially poured through a sheet of one-layer cheesecloth four times to screen out stones, debris, invertebrate larva (i.e. mayfly larva), and aquatic plants (i.e. duckweeds). The screened pond water was then centrifuged at 3750 rpm for 10 min to concentrate algal biomass. The final algae concentrate was stored at 4 °C briefly before being applied as the inoculum for

following kinetics study and semi-continuous cultures; the inoculum contained 0.34 g L^{-1} algal biomass (dry weight) and the community composition of the original algal assemblage is shown in Figure 3.4.

2.3. Kinetics study of algal culture in EC medium

The effects of three EC medium concentrations: original (1×), twice dilution (2×) and five-time dilution (5×), were investigated in the algal kinetics study with a set of two biological replicates. A 3 mL algal inoculum and 50 mL EC medium of each concentration were added to a 125 mL Erlenmeyer flask; a total of 12 flasks of each EC medium concentration were prepared and two were randomly sampled on day 1, 2, 3, 5, 7, and 9. The culture was conducted on orbital shakers (2.33 Hz, 150 rpm) at 22 ± 2 °C under continuous illumination using fluorescent lamps (100 μE m^{-2} s^{-1}), and all culture media had a slightly alkaline pH (8.1 ± 0.4) due to the nature of AD effluent and EC process. A culture using only deionized water and the same algal inoculum was applied as blank for comparison, and a set of EC media in three concentrations without algal inoculation was also prepared as controls to eliminate nutrient loss due to the non-biological processes (i.e. volatilization) during culture (APPENDIX G, Figure AP.1 – AP.4).

An aliquot of 1 mL algal culture solution was collected to determine the optical density (OD_{750nm}) of biomass, cell count, standardized biovolume, and algal community. An aliquot of 50 mL was centrifuged at 8000 rpm for 15 min to separate algal biomass from medium; biomass was dried overnight at 78 ± 3 °C, and the liquid medium was collected for measurements of TN, TP, Fe, and turbidity (OD_{600nm}) (Sloof et al., 1995; Chen et al., 2012a).

In order to determine growth rate, polynomial curve fitting based on the coefficient of determination (R^2) was applied to TS data. The concavity of fitted polynomial equation was measured by its second derivative, and the slope at inflection point of the polynomial was used to estimate the maximum growth rate.

2.4. Semi-continuous algal culture in EC medium

The optimal EC medium dilution based on the kinetics study was chosen for semi-continuous culture. Two 2-L Erlenmeyer flasks were used to prepare biological replicates; each flask contained 60 mL algal inoculum and 1 L EC medium at the beginning of the culture. The culture was conducted on orbital shakers (2.33 Hz, 150 rpm) at 22 ± 2 °C under continuous illumination using fluorescent lamps (100 μE m^{-2} s^{-1}). The lag and log growth phases were also determined by kinetics result; when algal growth reached to its maximum rate, an aliquot of 100 mL algal culture from each flask was sampled daily, and same amount of fresh diluted EC medium was added back into the flasks. Biomass optical density (OD_{750nm}), cell count, community assembly, dry biomass weight, TN, TP, Fe and turbidity (OD_{600nm}) were tested on the 100 mL daily culture sample using the same methods stated in previous section. Dry biomass collected at the end of the semi-continuous culture was ground using mortar and pestle for chemical composition analysis.

2.5. Analytical methods

Cell density (cell per mL culture) was determined using a compound microscope (Nikon Eclipse 50i, 40× objective, 400× total system magnification) and a microscopic hemocytometer. The average biovolume (mL biomass per cell) of algal cells were measured using imaging software NIS-Elements D 3.00 (Nikon Instruments Inc., Melville,

NY). Standardized biovolume (mL biomass per mL culture) was calculated as the product of cell density and average cell biovolume. TN, TP, and Fe were analyzed using HACH testing reagent sets (HACH, Loveland, CO. Product #: 2714100, 2767245, 2415915, and TNT858, respectively). Carbohydrates in algal biomass were determined based on the analytical procedure by National Renewable Energy Laboratory (Van Wychen and Laurens, 2013) (APPENDIX H). Protein content was measured using a bicinchoninic acid assay kit (BCA1, Sigma-Aldrich, St. Louis, MO) (APPENDIX I). Crude lipid was measured using chloroform-methanol extraction method (Bligh and Dyer, 1959) (APPENDIX J).

3. Results and Discussion

3.1. Kinetics study

Algal growth in EC medium with different dilutions were recorded based on dry weight, biomass density, and standardized biovolume (Figure 3.2, APPENDIX K). There was no significant growth for the culture on the 1× EC medium (Figure 3.2-a). It has been reported that the optimal algal culture medium made by the chemical treated AD effluent had a OD_{600nm} of 0.92 (Chen et al., 2012a), in comparison, OD_{600nm} of the 1× EC medium in this study was 0.48, which indicates light penetration might not be a significant factor of the slow growth in the 1× EC medium. On the other hand, the ammonia (NH_3) concentration in the 1× EC medium was relatively high, which could have the inhibitory effect on the algal growth (Azov and Goldman, 1982; Ohmori et al., 1977; Syrett, 1962). Compared to the culture on the 1× EC medium, the cell growth on both 5× EC and 2× EC media showed much better growth (Figure 3.2-a). The cell growth in the 5× EC medium had the shorter lag phase (less than 1 day) than the 2× EC medium (2-3 days), which

suggests that the freshwater algae preferred relatively balanced and mild nutrient concentration.

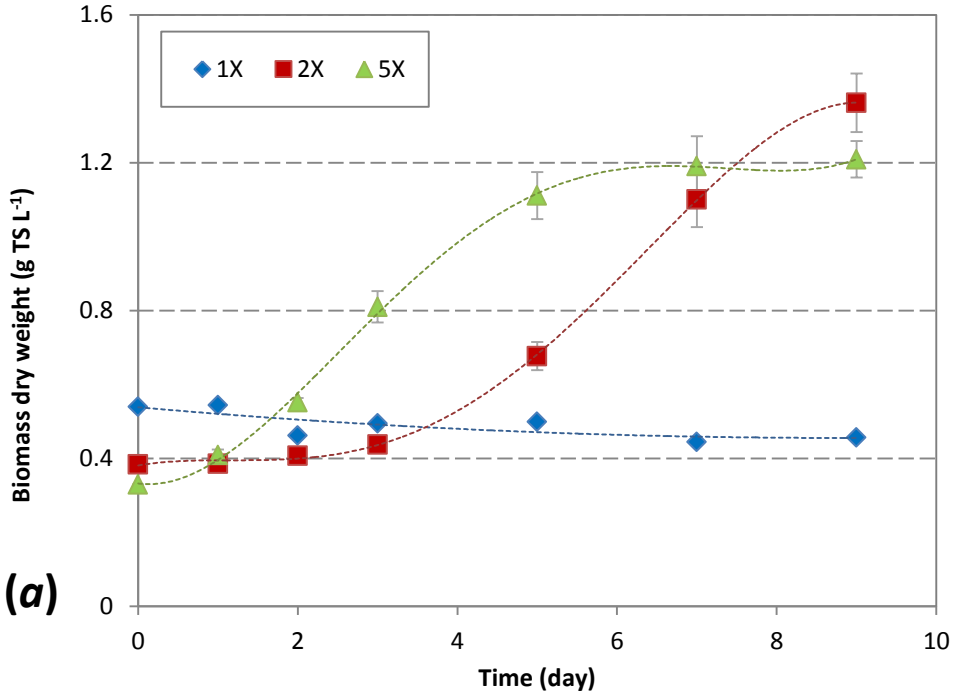
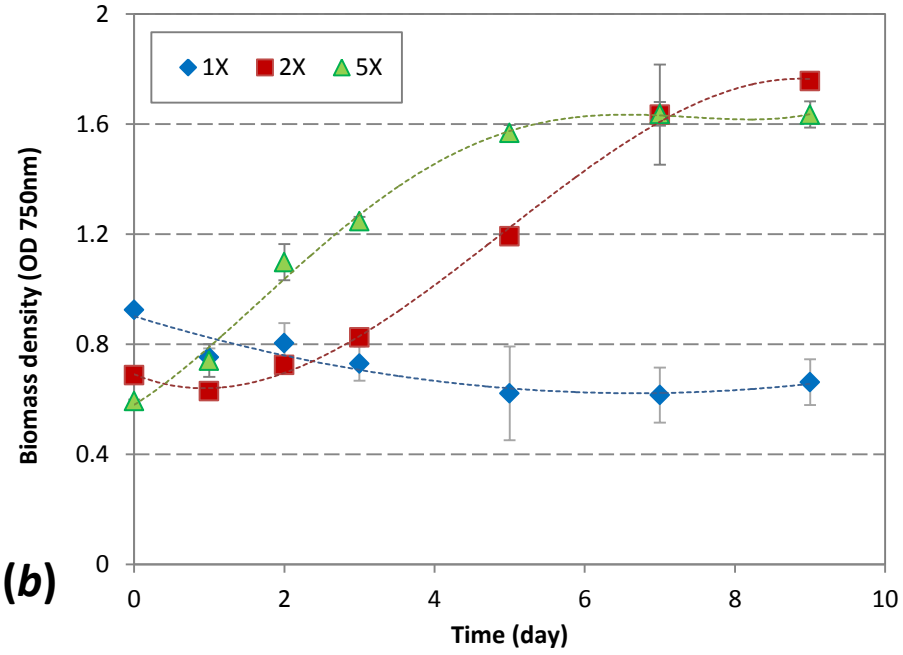


Figure 3.2: Algal growth kinetics in diluted EC media based on (a) biomass dry weight (g TS L^{-1}), (b) biomass density (OD_{750nm}), and (c) standardized biovolume (mL biomass per mL culture). Specific growth rate was calculated based on the dry weight regression models*.

*The biomass dry weight (g TS L^{-1}) regression models and corresponding R^2 values are:
$f_{TS}(N_{5\times}) = 0.001x^4 - 0.0197x^3 + 0.1106x^2 - 0.0384x + 0.3324$ ($R^2 = 0.9987$);
$f_{TS}(N_{2\times}) = -0.0008x^4 + 0.0115x^3 - 0.0339x^2 + 0.0571x + 0.3812$ ($R^2 = 0.9998$);
$f_{TS}(N_{1\times}) = 0.0011x^2 - 0.0188x + 0.5386$ ($R^2 = 0.6395$).

Figure 3.2 (cont'd)



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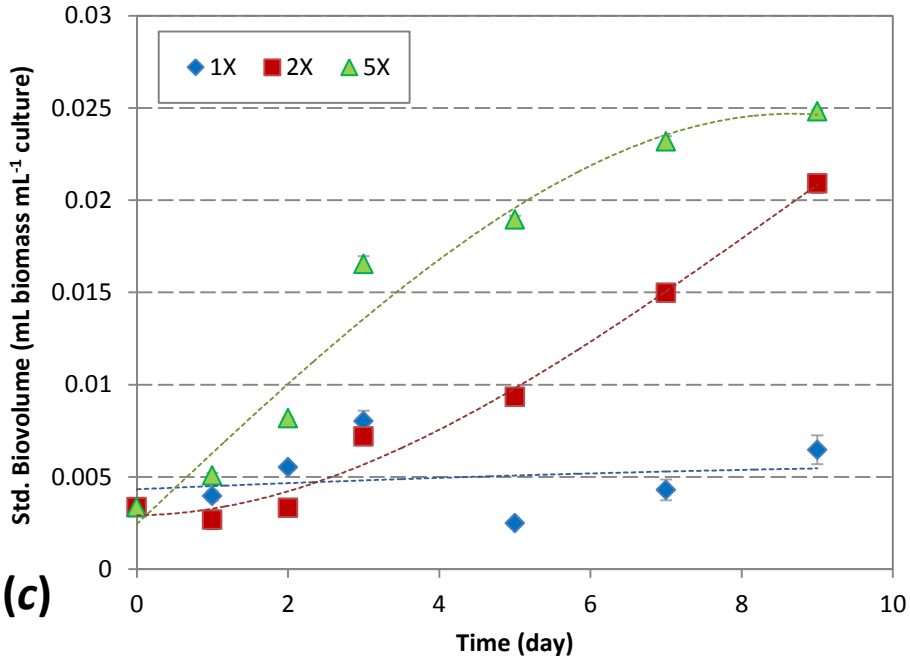

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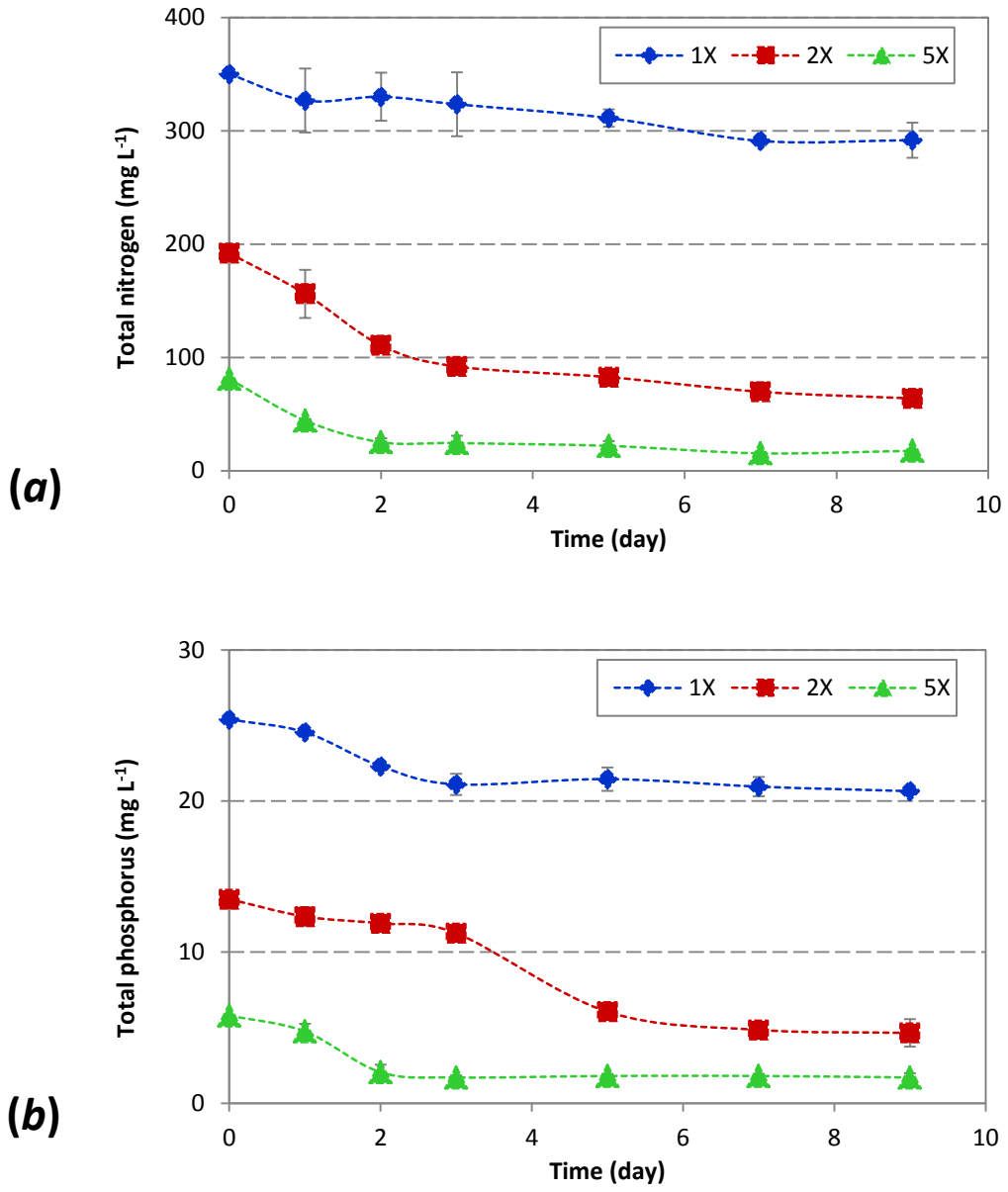
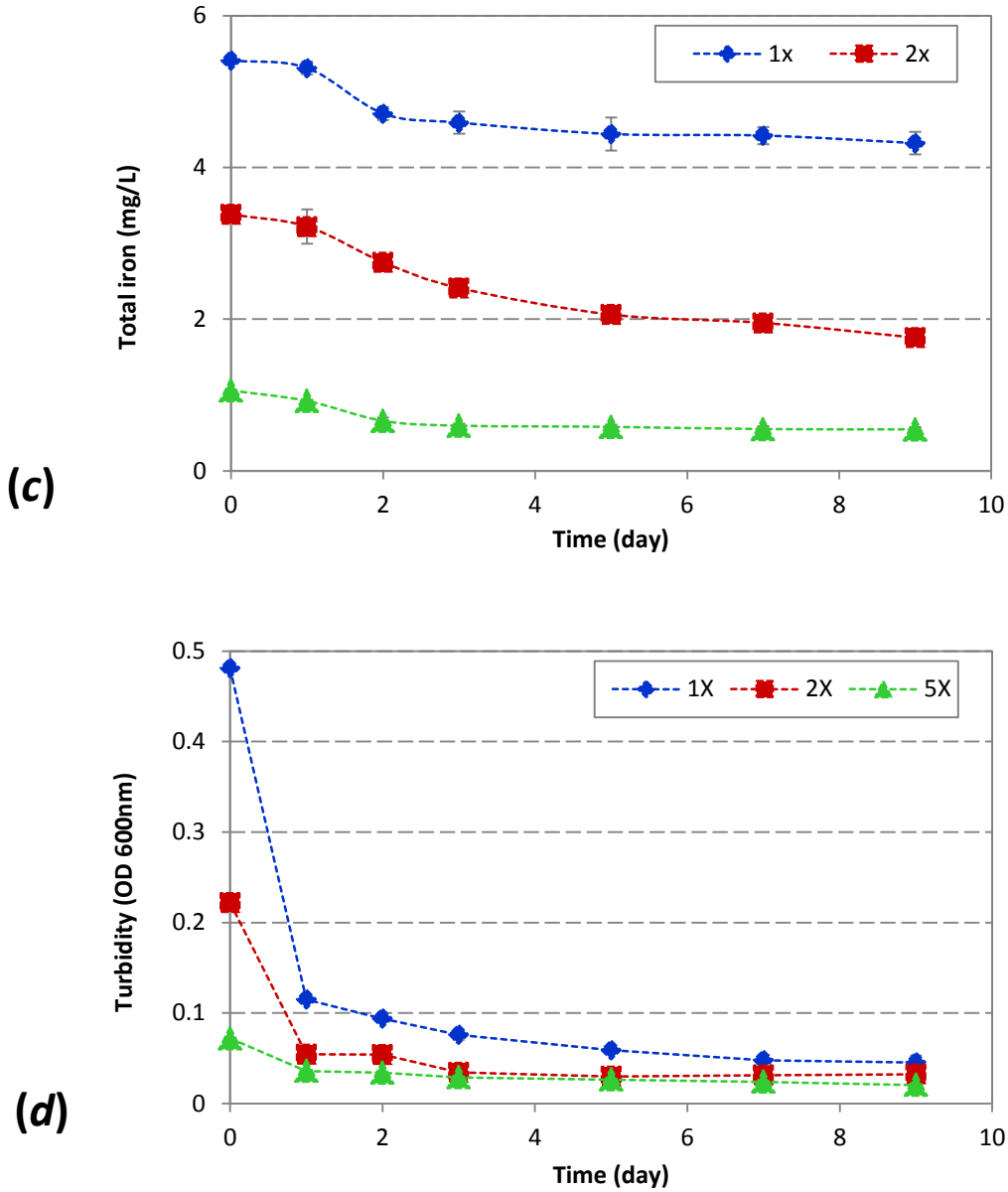


Figure 3.3: Nutrients including (a) TN, (b) TP, (c) Fe, and (d) turbidity removal by algal growth in various EC media throughout 9-day culture.

Figure 3.3 (cont'd)



Polynomial regression models ($f_{TS}(N)$) based on biomass dry weight (g TS L^{-1}) was used to estimate growth rate and to compare growth kinetics among different treatments. Setting the second derivative of a continuous regression model to zero ($f_{TS}"(N) = 0$) was used to locate the inflection point, or in this case, the maximum growth

rate (K). The results show that algae in the 5× EC medium reached to their maximum growth rate ($K_{5\times}$) of 0.207 g TS L^{-1} day^{-1} at 2.51 day; in the 2× EC medium, it was $K_{2\times}$ = 0.201 g TS L^{-1} day^{-1} at 6.01 day; and in the 1× EC medium, the growth rate was negative and not used for comparison. The relatively similar $K_{5\times}$ and $K_{2\times}$ values indicate that the algal assemblage could adjust themselves in medium with elevated nutrient concentrations. Same trend was also observed in biomass density (OD_{750nm}) and standardized biovolume (Figure 3.2-b and c).

TN diagram (Figure 3.3-a) demonstrates that algae in the 5× EC medium had the highest nitrogen reduction (78.3%); it was 66.8% for the 2× EC medium, and only 16.7% for the 1× EC medium. As for the absolute reduction of TN, algae in the 2× EC medium consumed the highest amount of nitrogen (128.2 mg L^{-1} TN), the 5× EC medium reduced 63.4 mg L-1, and the 1× EC medium reduced 56.2 mg L^{-1}. TP diagram (Figure 3.3-b) shows that most available phosphorus in the 5× EC medium was depleted in the first two days of culture (70.1%), whereas in the 2× EC medium it was on the fifth day of culture (66.5%), and only 18.7% was reduced in the 1× EC medium in 9 days. It is noticeable that algal biomass in the 2× EC medium started to outgrow the culture in the 5× EC medium after Day 7, it was possibly due to the completion of phosphorus in the 5× EC medium (1.7 mg L^{-1} on Day 9). Choi and Lee (Choi and Lee, 2015) conducted a study to delineate the relationship between biomass productivity and N/P ratio, and concluded that TP removal rate greatly depended on N/P ratio and algal growth, but no strong correlation was observed between TN removal and biomass productivity.

Most iron (Figure 3.3-c) in the EC medium was introduced by the anode during EC reaction. The iron removal during algal cultivation was caused by both biological (algal

uptake and adsorption) and non-biological (participation) activities. The highest biological iron removal was approximately 50% from algal culture in the 2× and 5× EC media. While, algae in 1× EC medium were only able to consume about 18% of iron, and mostly within the first 2 to 3 days of culture. Algae in the 2× EC medium were able to utilize the ferric ion throughout the 9-day culture, but the consumption rate in the 5× EC medium slowed down after the third day. This is another indication that growth of algae in the 5× EC medium was limited, and the consumption of one element might also rely on the other available nutrients. Many classic studies demonstrated the positive correlation between iron and algal growth (Aparicio et al., 1971; Rueler and Ades, 1987; Roche et al., 1996). Specifically, Hopkins and Wann (1927) as well as Walker (1954) discovered that *Chlorella* requires a high minimum iron concentration to grow, and only ionized iron can be biologically available. Therefore, various uptake rates of the nutrients in different dilution of the culture media could also explain the algal community changes in this study. Meanwhile, the non-biological activities also removed a significant amount of iron in the media (APPENDIX G). It has been suggested by both freshwater and seawater studies that most ferric ions (Fe (III)) exist in a form of soluble chelates or suspended colloid ; the physical property of ferric chelates and colloid (mostly due to the electrostatic interactions among the molecules) makes them difficult to sink by gravity or centrifugation (Gunnars et al., 2002). However, constant agitation and oxidization could change the form of ferric ions in the medium and made them unavailable to microorganisms. For instance, ferric ions can easily react with carbon dioxide dissolved in the medium and form insoluble ferric carbonate ($Fe_2(CO_3)_3$). Interestingly, several studies using electron microscopic examination showed that more iron colloids were observed when a water body also

contained relatively high phosphorus content (Bernard et al., 1989; Tipping and Ohnstad, 1984); moreover, removal of phosphorus directly led to precipitation of ferric ions (Buffle et al., 1989; van Leeuwen et al., 2012). Therefore, the non-biological iron reduction in this study was possibly due to the ferric ion precipitation caused by the disturbance of the colloidal electric charge during culture, insoluble chemical formation, as well as rapid phosphorus uptake by algae.

3.2. Effects of EC media on algal assemblage

Figure 3.4 illustrates the algal communities cultured on different EC media at Day 9 in comparison with the inoculum. The most dominant algal strain in the inoculum was *Scenedesmus* sp., the total amount of other two strains, *Chlorella* sp. and *Pseudophormidium* sp., was less than 10% in the inoculum. After nine days of culture, *Scenedesmus* in the control of the water medium still remained dominant, but *Chlorella* increased to about 20% of the entire community, and *Pseudophormidium* was disappeared in the water medium; the algal biomass collected from the culture on the water medium was significantly lower than 2× and 5× cultures due to the lack of nutrients. The culture on the 1× EC medium had a similar community composition on day 9 as the culture on the water medium; its biomass amount was also significantly lower than the cultures on the 2× and 5× EC media. It is mainly because of less algal growth on the 1× EC medium, so that the algal community maintained as it was in the inoculum. The most significant community turnover was appeared in the 2× EC medium, where *Chlorella* became dominant strain (90%) and *Scenedesmus* reduced to less than 10%. This result indicates that *Chlorella* could tolerate higher nutrient concentration (Chen et al., 2012a; Parravicini et al., 2008; Roelke et al., 1999; Syrett, 1962), while *Scenedesmus* consumed nutrients

faster when the growth was not restrained. In the 5× EC medium, dominant *Scenedesmus* took up to 80% of the entire algae assembly, while filamentous cyanobacteria *Pseudophormidium* and unicellular green algae *Chlorella* each counted for about 10%. Azov et al. (Azov and Goldman, 1982) observed the inhibition of excess NH_3 to algal growth, and proposed that converting NH_3 to non-toxic ammonium (NH_4^+) by adjusting the culture pH to neutral to slightly acidic could prevent NH_3 toxicity in algal wastewater treatment system, which could be a solution to further enhance the algal growth on the EC medium (the pH of EC media are on alkaline side).

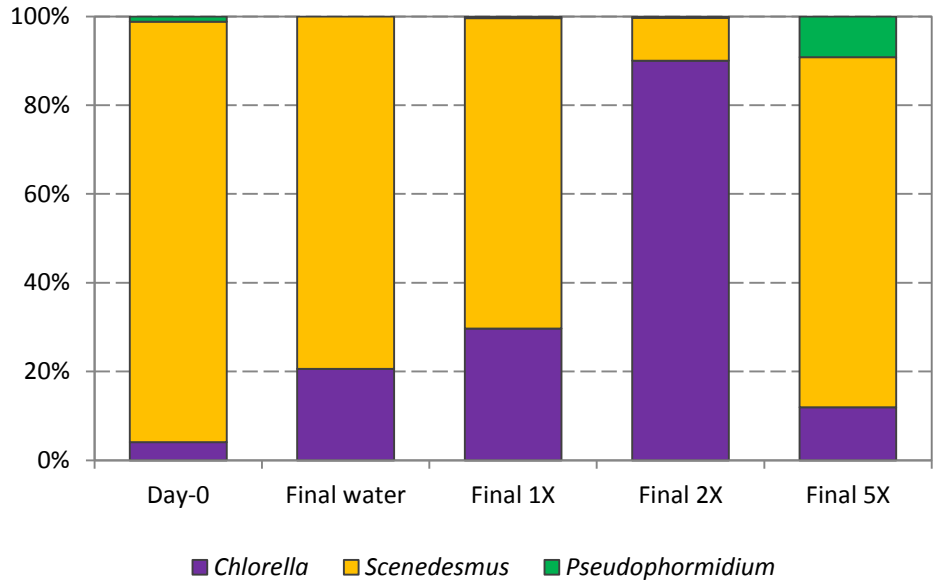


Figure 3.4: Algae community assembly on day-0 and day-9 within various culture media.

3.3.Semi-continuous algal culture

Given the similar maximum growth rates in both 2× and 5× EC media, these two conditions were carried out using 1-L semi-continuous algal culture units. According to the maximum growth rates concluded from the kinetics study, the feeding was scheduled to

start on day 3 for the culture on the 5× EC medium, and day 7 for the culture on the 2× EC medium. Figure 3.5 demonstrates the biomass productivity from day 7 to day 19 (total of 13 days culture). The productivity in both media kept at steady states: the culture on the 2× EC medium managed to produce 0.077 ± 0.004 g dried algae per liter per day (g TS L^{-1} day^{-1}), which was 47% higher than the productivity from the culture on the 5× EC medium (0.052 ± 0.005 g TS L^{-1} day^{-1}). For nutrient uptake, TN concentrations (Table 3.1) in the 2× EC and 5× EC media maintained steady at 84 and 22 mg L^{-1}, respectively; the TN daily consumption rates were 19.2 and 8.0 mg L^{-1} day^{-1}, respectively. However, the algal TP consumption rates in both cultures increased throughout the experiment: from 1.35 to 3.28 mg L^{-1} day-1 in 2× EC medium and from 0.58 to 2.01 mg L^{-1} day^{-1} in 5× EC medium. Previous studies has reported this phenomenon as luxury uptake of phosphorus in algae, it occurs when the algae consume more phosphorus than required for growth without going through a prior starvation stage (Eixler et al., 2006). Powell et al. (Powell et al., 2009) discovered that the polyphosphate accumulation of algae in high nutrient concentrations was mainly caused by luxury uptake. It was also reported that this mechanism has no effect on the algal growth (Azad and Borchardt, 1970), which explains the biomass productivity in this study was kept steady, while the phosphorus uptake rate gradually increased in the cultures on both 2× and 5× EC media. Figure 3.3-d and Figure 3.6 shows that algal culture facilitated clarify and de-color the EC treated AD effluents (both 2× and 5×), and algal biomass cultured in the 2× EC medium had higher biomass density than that in 5× EC medium. These results clearly demonstrate that using the combined EC-algae treatment system can significantly reduce the environmental impact of the AD liquid digestate.

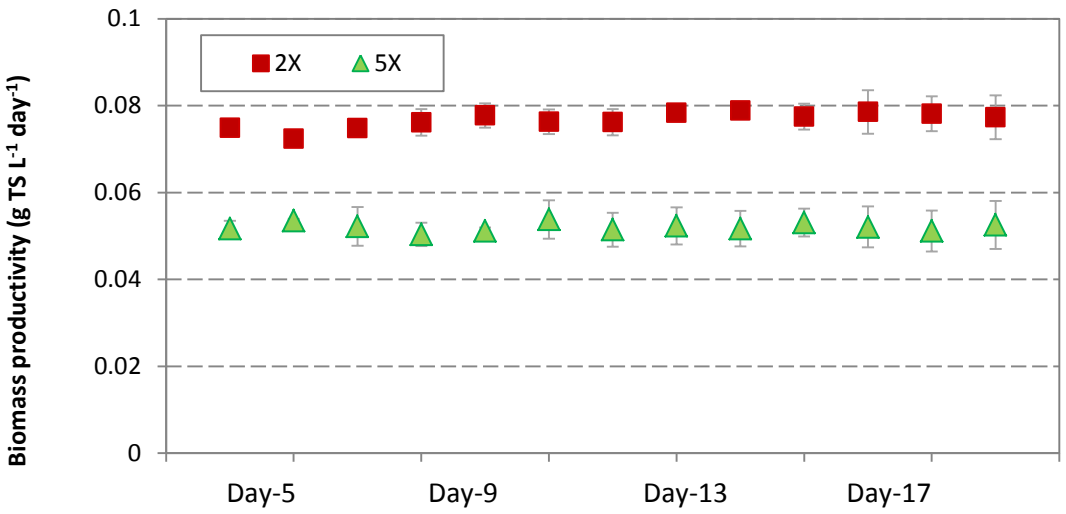


Figure 3.5: Algal biomass productivity from two different EC media in semi-continuous culture during steady growth phase.

In addition, chemical analysis of algal biomass derived from the cultures on both 2× and 5× EC media (Table 3.2) demonstrated that 53.4% VS of the biomass from the 2× EC medium was protein, which was 13% higher than that from the 5× EC medium. It was possibly because of the significantly higher TN concentration in the 2× EC medium. On the other hand, algae from the 5× EC medium tended to accumulate more carbohydrates (36.6% VS, versus 27.4% VS in the 2× EC medium) and lipid (10.6% VS, versus 7.5% VS in the 2× EC medium). This pattern has been previous described in several studies of algal cultivation on nitrogen-rich wastewater streams (Garnier et al., 2014; Song et al., 2013; Yang et al., 2013), and the conclusions were similar: excess inorganic nitrogen in cultural media could lead freshwater algae to accumulating more protein but less carbohydrates and lipids accumulation. However, Liu et al. (Liu and Liu, 2015) conducted a study using pure algal strains to culture in EC treated AD digestate, in which the algal biomass was able to accumulate a relatively large amount of lipids. Liu's culture was also with pH control (pH

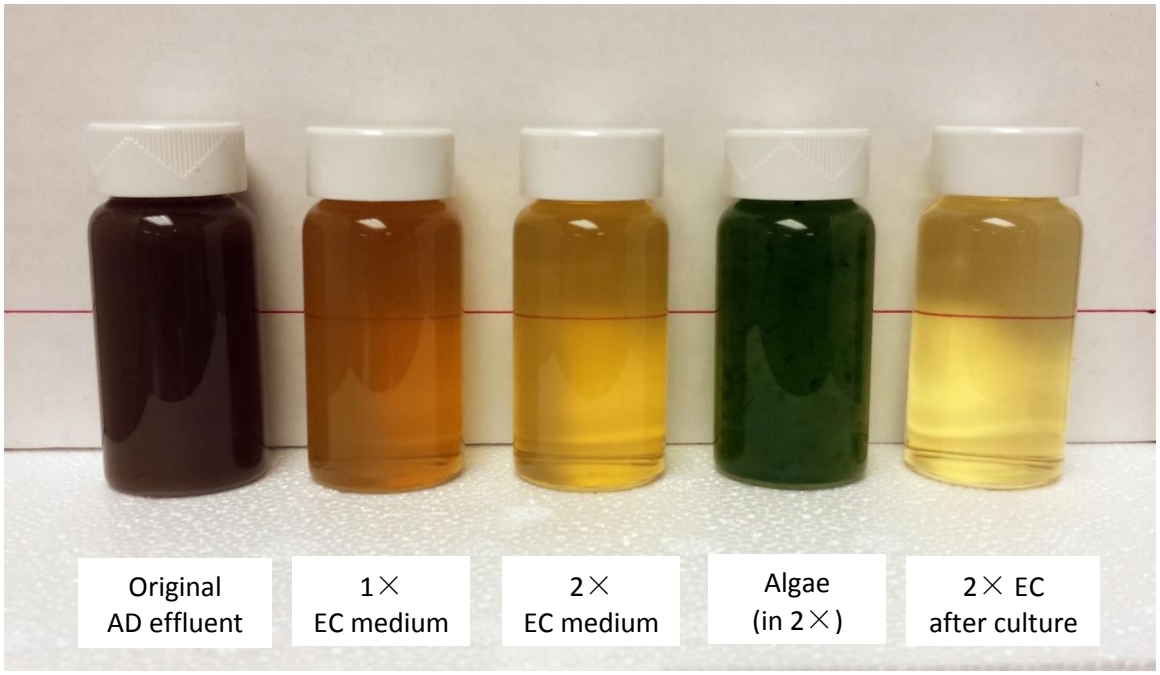
6-7) and CO_2 feeding (5% CO_2 in the mixed gas. In contrast, the culture in this study utilized a mixed wild algal inoculum without pH control or CO_2 supplement. It has been reported that CO_2 feeding can significantly increase the lipid content during the culture in the pH range of 6-7 (Widjaja et al., 2009), which might be the main reason that Liu's study had higher lipid content in the algal biomass. This presented study minimized the chemicals supplements to the system, and consequently reduced the operational cost for wastewater treatment facility. In addition, using mixed wild algal strains as inoculum could also provide a more robust and adaptive biome to the environmental variations.

Table 3.1: Chemical analysis of culture media before and after semi-continuous culture.

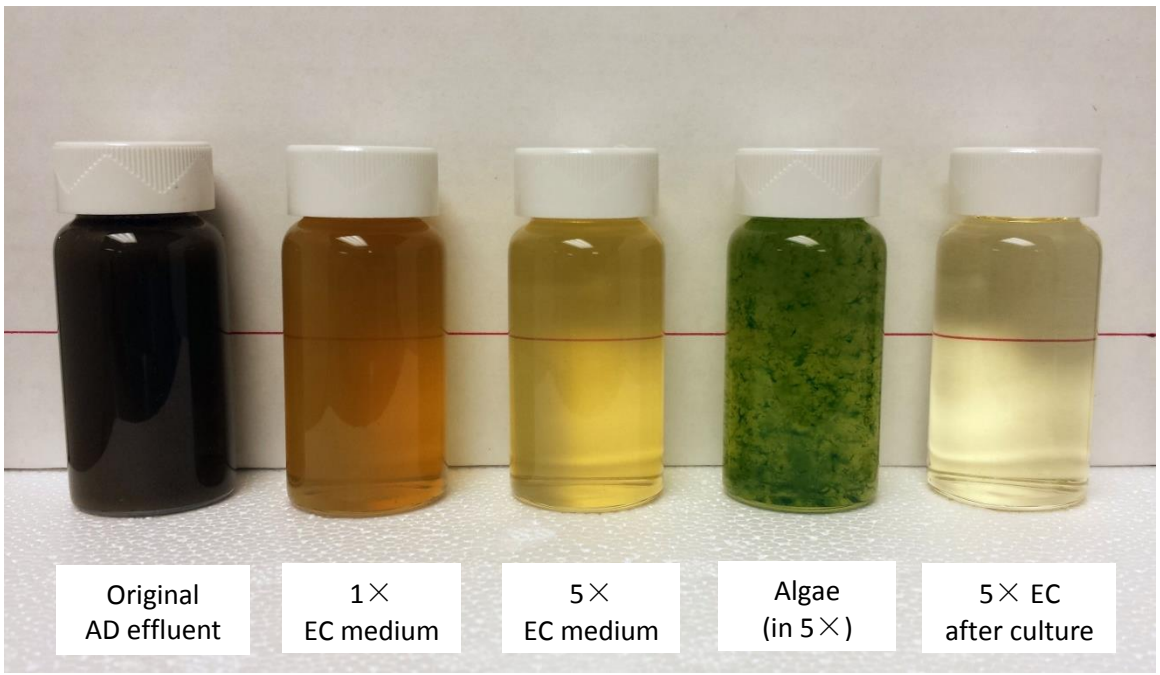
	TN (mg L^{-1})		TP (mg L^{-1})		Turbidity (OD_{600nm})		Iron (mg L^{-1})	
	Before	After	Before	After	Before	After	Before	After
2× EC medium	84.0	83.5	10.7	2.6	0.040	0.038	1.675	1.670
5× EC medium	21.5	22.0	14.5	8.3	0.028	0.029	0.843	0.856

Table 3.2: Algal biomass chemical composition.

	Protein	Carbohydrate	Lipid	Ash
	% VS			% TS
2× EC medium	53.4 ± 0.5	27.4 ± 1.4	7.5 ± 1.9	14.2 ± 1.5
5× EC medium	47.3 ± 0.9	36.6 ± 0.8	10.6 ± 1.8	8.4 ± 1.0



(a) 2× EC medium



(b) 5× EC medium

Figure 3.6: Comparing color and clarity of various fluids referred in this study.

4. Conclusion

A study on the effect of EC treated AD liquid digestate on growth, nutrient uptake, and community assembly of freshwater algal assemblage was conducted. Kinetics revealed that algae cultured in two different dilutions of the EC media (2× and 5×) shared similar maximum growth rate (0.201-0.207 g TS L^{-1} day^{-1}), and the culture on the 2× EC medium had longer lag phase possibly due to the inhibition of excess ammonia. After 9 days of culture, *Scenedesmus* sp. remained as the most dominant taxon in the culture on the 5× EC medium, but *Cholorella* sp. in the 2× EC medium outgrew other taxa and took over 90% of the algal community. A semi-continuous culture further demonstrated that the culture on the 2× EC medium had significantly higher productivity (0.077 g TS L^{-1} day^{-1}) than that the 5× EC medium (0.052 g TS L^{-1} day^{-1}), though, both conditions showed significant advantage in nitrogen/phosphorus reduction and water clarification. It is recommended that combining EC treatment and algal cultivation could be an effective approach to be incorporated in wastewater treatment process to deal with high-strength wastewaters such as animal manure and food wastes.

Chapter 4 : Effects of algal hydrolysate as reaction medium on enzymatic hydrolysis of lignocelluloses

Abstract

Effects of an algal hydrolysate on the enzymatic hydrolysis of lignocelluloses were examined using four bioenergy substrates (poplar, corn stover, switchgrass, and anaerobically digested manure fiber). Substrates were pretreated using dilute acid or alkali prior to hydrolysis. Hydrolysis reactions were conducted using the neutralized algal hydrolysate, citrate buffer, or deioinized water as reaction media. Results demonstrated that algal hydrolysate significantly improved the efficiency of enzymatic hydrolysis of lignin-rich or structurally recalcitrant biomass such as poplar and anaerobically digested manure fiber. This study showed that algal biomass can be used as not only a biofuel feedstock for direct diesel and ethanol production, but also a supplemental feedstock to enhance the performance of lignocellulosic biorefining.

*** Note:** the original article was published as

Chen R, Thomas BD, Liu Y, Mulbry W, Liao W. 2014. Effects of algal hydrolyzate as reaction medium on enzymatic hydrolysis of lignocelluloses. Biomass and Bioenergy, 67:72-78.

Minor formatting changes were made to satisfy the requirement of the graduate school at Michigan State University.

1. Introduction

As aquatic photosynthesizing organisms, algae are considered as one of the most promising alternative bio-resources that could gradually replace fossil-based transportation fuels (Greenwell, et al., 2010, Sheehan, et al., 1998). Compared to terrestrial energy crops, algae have major advantages such as using marginal land, faster growth, higher photosynthetic efficiency, year-around production, efficient uptake of nutrients in waste streams, and alleviating the global aquatic eutrophication potential (Groom, et al., 2008, Kothari, et al., 2012, Mulbry and Wilkie, 2001, Zhang, et al., 2012). Tremendous efforts have been made in the past decades to develop economic algal biofuel production (McHenry, 2009). However, due to the complex composition of algal biomass, conversion processes that focus only on a single component, such as algal lipid, jeopardize the economic viability of algal biorefining. Full utilization of all algal components is critical to realizing the potential of algal biofuel and chemical products at commercial scale.

Lignocellulosic materials such as agricultural and forestry residues, as well as herbaceous and short rotation woody crops, have also been extensively examined as feedstock for biofuel production. At present, the major challenge of biofuel production from lignocellulose is to increase the productivity while minimizing the use of expensive chemicals and enzymes (Harmsen, et al., 2010, Singh and Trivedi, 2013, Wyman, 1996). Altering the lignin/cellulosic structure (Taherzadeh and Karimi, 2008) and introducing lignin-binding surfactants (Eriksson, et al., 2002) are two feasible solutions to expose more cellulose and hemicellulose in lignocellulosic materials to enzymatic attack. A recent study on converting algal biomass to bioethanol showed that a hydrolysate from algae grown in dairy manure improves the efficiency of enzymatic hydrolysis of a recalcitrant

lignocellulosic material (anaerobically digested manure fiber) by 50-80% (Chen, et al., 2012). This result suggests that, in addition to direct production of biofuels using algae, components such as proteins, carbohydrates, and lipids from algal biomass can also be utilized to enhance lignocellulose conversion of biofuel production.

The objective of this study was to extend our understanding of the effect of algal hydrolysate on the enzymatic hydrolysis of other lignocellulosic materials. In this study, we determined the hydrolytic efficiencies of four different lignocellulosic substrates: poplar, corn stover, switchgrass, and anaerobically digested manure fiber (AD fiber). These results will be useful for developing efficient and integrated biorefining processes of using lignocellulosic materials and algal biomass.

2. Methods and Materials

2.1. Lignocellulosic feedstock

Corn stover was harvested and collected in 2009 from a private farm in Muir, MI. Switchgrass was collected from the Michigan State University Crop and Soil Science Teaching and Research Field Facility in 2010. Poplar hybrids were planted in 1998 using a uniform spacing of 8x8 feet and harvested in fall of 2009. All of above substrates were dried and ground using an electric mill (Willey Mill, Standard Model No. 3; Arthur H. Thomas, Philadelphia, PA) with a sieve size of 2 mm. AD fiber was collected from the Scenic View Freeport Dairy Farm (Freeport, MI). The farm's dairy cows were fed alfalfa and corn silage blended according to standard total mixed rations by the Natural Research Council (2001). The farm's anaerobic digester was a completely stirred tank reactor (CSTR) operated at temperature of 35°C with a hydraulic retention time of 30 days. AD

fiber was separated from the liquid digestate using a 5.5 kW FAN screw press with 2 mm screen. A sample of AD fiber was dried overnight at 75°C prior to use in these experiments.

Compositional analyses of the glucan, xylan and lignin content of substrates were conducted according to the analytical procedures for determination of structural carbohydrates and lignin in biomass provided by National Renewable Energy Laboratory (NREL) (Sluiter, et al., 2008) (APPENDIX A).

2.2. Algae

Algal biomass was grown using dilute AD dairy manure liquid effluent (USDA Dairy Research Unit, Beltsville, MD) recirculated in pilot-scale algal turf scrubber (ATS) raceways (Mulbry, et al., 2008). Dominant species of the filamentous green algae assembly included *Rhizoclonium hieroglyphicum* (C.A. Agardh), *Microspora willeana* Lagerh., *Ulothrix ozonata* (Weber and Mohr) Kütz, *R. hieroglyphicum* (C.A. Agardh) Kütz and *Oedogonium* sp. (Chen, et al., 2012). Wet algal biomass was first dewatered using 2 mm mesh nylon netting and then air dried to approximately 90% of total solids. Dry algae was milled to pass a 3 mm sieve and stored at room temperature (Mulbry, et al., 2006). The carbohydrate profile of algal biomass was analyzed using a concentrated acid hydrolysis method described by Chen et al. (2012). Briefly, dry algal biomass was mixed with 75% (wt) sulfuric acid to a 3:5 sample-to-acid ratio (wt). The mixture reacted at room temperature for 30 min and was then heated at 130°C for 10 min in an autoclave. Total protein and total fatty acid contents were determined by the Experiment Station Chemical Laboratories at the University of Missouri.

2.3. Pretreatment of feedstock and algal biomass

The four substrates (AD fiber, switchgrass, poplar, and corn stover) were pretreated using either dilute sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) solutions under the optimal conditions determined in previous studies (Ruan, et al., 2013, Teater, et al., 2011, Ucar, 1990, Yue, et al., 2011) (Table 4.1). Pretreated sample was neutralized to pH 5 using 30% NaOH or 20% H_2SO_4 solutions and washed five times each with 500 mL deionized water until the supernatant was transparent. Pretreated sample was dewatered using eight-layer cheese cloth and then oven-dried overnight at 75°C. Glucan, xylan and lignin contents of the substrates were determined before and after pre-treatment.

Table 4.1: Pretreatment conditions for each feedstock (all concentrations are in wt%)

Feedstock	**Acid pretreatment condition**	**Alkali pretreatment condition**
AD fiber	3% H_2SO_4, 130°C, 2 h (Yue et al., 2011)	2% NaOH, 130°C, 3 h (Yue et al., 2011)
Poplar	5% H_2SO_4, 130°C, 2 h (unpublished data)	2.5% NaOH, 100°C, 2 h (Ucar, 1990)
Corn stover	2% H_2SO_4, 130°C, 1 h (Ruan et al., 2013)	1% NaOH, 130°C, 2 h (Teater et al., 2011)
Switchgrass	2% H_2SO_4, 130°C, 2 h (Ruan et al., 2013)	1% NaOH, 130°C, 2 h (Teater et al., 2011)

Algal biomass was hydrolyzed using a 5% TS loading using 4% (wt) H_2SO_4 at 116°C for 30 min (Chen, et al., 2012). The hydrolyzed mixture was neutralized to pH 5 using calcium carbonate ($CaCO_3$). Residual solid material was completely removed by centrifugation (2846 ×*g*, 10 min). The liquid hydrolysate was saved and its carbohydrate profile was determined using high performance liquid chromatography (HPLC) and its protein content was measured using a bicinchoninic acid protein assay kit (BCA1 and B9643, Sigma-Aldrich, St. Louis, MO),.

2.4. Enzymatic hydrolysis

In order to determine the effect of the reaction medium on the enzymatic hydrolysis of the four substrates, separate hydrolysis reactions were conducted using pretreated substrates and de-ionized water, sodium citrate buffer (50 mM, pH 4.8) and neutralized algal hydrolysate. Aliquots (2 g DW) of each pretreated feedstock were combined with 20 g of each reaction medium and 17 g of de-ionized water. The resulting mixtures were sterilized using an autoclave (15 min, 120 C) and cooled to room temperature prior to the addition of cellulase (Accelerase 1500®, Genencor, Rochester, NY) to a final concentration of 25 FPU g^{-1} dry feedstock. Samples were mixed using a shaker table orbiting at 150 rpm at 50°C for 72 h. Samples (1 ml) of hydrolysates were taken at hour 0, 24, 48 and 72. Hydrolysate samples were boiled for 5 min and filtered using Millex-GS 0.22 μm syringe filters prior to analysis of monosaccharide content. The net sugar concentrations during the enzymatic hydrolysis were calculated by subtracting the concentration of detected sugars in the algal hydrolysate medium from the total concentration of detected sugars in the hydrolysate samples.

Overall glucan/xylan conversion of each raw feedstock was used to determine the effects of pretreatment and of the reaction medium on the entire saccharification process. The overall glucan conversion is defined as the percentage of net glucose production over the glucose equivalent amount in the raw feedstock. The overall xylan conversion is defined as the percentage of net xylose production over the xylose equivalent amount in the raw feedstock.

Enzymatic glucan/xylan conversion of pretreated feedstock was used to demonstrate the effects of different treatments on the enzymatic hydrolysis. The enzymatic glucan

conversion is defined as the percentage of net glucose production over the glucose equivalent amount in the pretreated feedstock. The enzymatic xylose conversion is defined as the percentage of net xylose production over the xylose equivalent amount in the pretreated feedstock.

Improvement of glucan conversion was calculated using net enzymatic glucan conversions in algal hydrolysate or buffer to divide net enzymatic glucan conversion in water for individual substrate. The improvement of glucan conversion was used to compare the effects of different pretreatments, substrates and reaction media on hydrolysis.

2.5. Analytical methods

A Shimadzu 2010 HPLC system equipped with Bio-rad Aminex® HPX-87P analytical column (300×7.8 mm) and a refractive index detector was used for determination of monosaccharide profiles. The mobile phase was ultrapure water (Synergy Ultrapure Water Purification System, MiliporeTM, Billerica, MA), the flow rate was 0.6 mL min^{-1}, and the column temperature was 80°C. High purity standards including glucose, xylose, galactose, arabinose, and mannose were purchased from Sigma-Aldrich (St. Louis, MO).

2.6. Statistical analysis

A mixed linear model using the Statistical Analysis System program 9.2 (SAS Institute Inc., NC) was used to perform one-way analysis of variance (ANOVA), Tukey's pair-wise comparison and simple main effects analysis (slicing) (APPENDIX L).

3. Results and Discussion

3.1. Characteristics of fibers and algae

Compositional analyses of four raw lignocellulosic feedstock show that glucan is the most abundant component in all substrates (Table 4.2). Poplar contained the highest glucan content (45%) which makes it a good potential feedstock for bio-conversion to glucose. However, poplar also had the highest lignin content (23%) among the four substrates, which suggests that it would require more energy, and chemical/enzymatic inputs to process the same amount of poplar glucan compared to other substrates. Corn stover and switchgrass are both commonly used as energy biomass substrates and they contain similar levels of glucan (40% and 37%, respectively) and lignin (19% and 21%, respectively), but different levels of xylan (30% in corn stover compared to 22% in switchgrass). AD fiber contains relatively less glucan (30%) and xylan (12%) compared to the other three substrates, though it has been recently discovered that AD fiber also has overall glucose conversion similar to many energy crops and residuals, which also makes it a potential feedstock for biofuel production (Yue, et al., 2011). The compositions of each feedstock after acid or alkali pretreatments are shown in Table 4.3. Acid pretreatment significantly reduced the xylan content in all four substrates. This is likely because the random and amorphous structure of hemicellulose is easily hydrolyzed by dilute acid solutions. Wyman et al. (2005, 2009) concluded that dilute sulfuric acid pretreatment can significantly recover most of the hemicellulose from lignocellulosic biomass. This pretreatment also exposes cellulose to enzymes and facilitates high sugar conversion from subsequent biorefining processes. Alkali pretreatment removed more lignin from the substrates, which also increases the accessibility of cellulose to enzymatic attack

(Taherzadeh and Karimi, 2008). Both fiber structure and chemical composition varied among different pretreated feedstock, and would influence the efficiency of following enzymatic hydrolysis.

Table 4.2: Structural carbohydrate and lignin content of the raw lignocellulosic biomass

Biomass	Glucan (wt%)	Xylan (wt %)	Lignin (wt %)
AD fiber	30.3±1.9	11.6±1.1	14.2±0.8
Poplar	44.8±0.5	20.5±0.5	22.6±0.5
Corn stover	39.7±0.7	29.9±0.5	18.6±0.7
Switchgrass	37.4±0.9	22.1±0.2	20.5±0.2

Table 4.3: Structural carbohydrate and lignin of pretreated lignocellulosic biomass

Biomass	Pretreatment	Glucan (wt%)	Xylan (wt %)	Lignin (wt %)
AD fiber	Acid	35.7±0.2	5.2±0.2	59.5±0.9
	Alkali	51.2±0.5	16.3±0.3	31.6±0.7
Poplar	Acid	62.1±1.2	0.0±0.2	32.3±0.1
	Alkali	53.4±0.7	11.9±0.2	26.5±0.7
Corn stover	Acid	63.1±0.6	11.3±0.4	28.6±0.5
	Alkali	63.6±0.3	29.5±0.1	8.1±0.3
Switchgrass	Acid	63.4±0.6	8.3±0.2	31.0±1.3
	Alkali	58.7±0.1	28.9±0.1	15.4±0.2

Table 4.4: Composition of Algal Biomass

Carbohydrate (wt %)	Primary components of carbohydrate (wt%)					Protein (wt %)	Fatty acid (wt %)
	Glucose	Xylose	Galactose	Arabinose	Mannose		
27.50±0.6	10.98±0.5	3.68±0.1	6.61±1.3	1.75±0.7	2.89±0.5	30.65±1.1	4.06±0.3

The algal biomass predominantly consisted of filamentous green algae. The algae were cultured in nutrient-rich AD effluent. Consequently, the algal biomass contained relatively more protein (30%) and less fatty acid (4%) (Table 4.4) than green algae grown in natural waters (Becker, 1994). Total carbohydrates comprised 27% of the algal biomass, and its major components were: glucose (11% of the total biomass dry matter), galactose (6.6%), xylose (3.7%), mannose (2.9%), and arabinose (1.8%). It is difficult to refine biodiesel from the algal biomass due to the low fatty acids content. However, its high protein and sugar composition makes it a superior feedstock or supplemental feedstock to produce bio-alcohol and other value-added products such as fertilizer and fuel additives.

Table 4.5: Characteristics of algal hydrolysate*

Protein ($g\ L^{-1}$)	Glucose ($g\ L^{-1}$)	Xylose ($g\ L^{-1}$)	Galactose ($g\ L^{-1}$)	Arabinose ($g\ L^{-1}$)	Mannose ($g\ L^{-1}$)
5.31±0.50	3.44±0.16	1.28±0.33	1.86±0.42	0.56±0.10	0.99±0.15

*: The data are the average of two replicates with standard errors.

3.2. Enzymatic hydrolysis of lignocellulosic substrates using algal hydrolysate as reaction medium

The algal hydrolysate contained 8.3 $g\ L^{-1}$ of sugar (Table 4.5), of which glucose (3.4 $g\ L^{-1}$), galactose (1.9 $g\ L^{-1}$), and xylose (1.3 $g\ L^{-1}$) were the most abundant components. The hydrolysate contained 5.3 $g\ L^{-1}$ of total protein. Chen et al. (2012) reported that an algal hydrolysate with similar protein content increased the enzymatic glucan conversion from alkali pretreated AD fiber by nearly 50% compared to reactions performed in water or buffer.

Table 4.6: Sugar concentrations and overall glucan and xylan conversions of different lignocellulosic feedstock [a, b, c, d]

Pretreat-ment	Feedstock	Reaction Medium	Glucose (g L^{-1})	Xylose (g L^{-1})	Galactose (g L^{-1})	Arabinose (g L^{-1})	Mannose (g L^{-1})	Overall Glucan conversion (%)	Overall Xylan conversion (%)
Acid	AD fiber	Water	2.86±0.0	0.17±0.1	0.05±0.0	ND	0.07±0.0	9.6±0.0	1.5±0.6
		Buffer	3.69±0.1	0.20±0.0	0.05±0.0	ND	0.07±0.0	12.3±0.4	1.7±0.1
		Algae	4.44±0.1	0.53±0.2	0.47±0.0	0.22±0.1	0.23±0.2	14.8±0.3	4.6±1.0
	Corn stover	Water	14.96±0.4	1.17±1.0	ND	0.01±0.0	0.07±0.0	33.5±1.2	3.5±1.7
		Buffer	18.23±0.5	1.40±0.3	0.07±0.0	ND	0.09±0.0	40.8±1.2	4.2±0.9
		Algae	18.99±0.2	1.94±0.7	1.15±0.1	0.30±0.0	0.69±0.1	42.5±0.5	5.8±1.8
	Poplar	Water	13.72±0.4	0.16±0.1	0.03±0.0	ND	ND	32.4±1.0	0.8±0.4
		Buffer	16.60±0.2	0.15±0.0	0.14±0.0	0.09±0.0	0.07±0.0	39.2±0.5	0.8±0.1
		Algae	22.99±0.8	0.25±0.1	ND	ND	0.04±0.0	54.3±1.8	1.3±0.5
	Switchgrass	Water	20.02±0.2	0.42±0.1	0.02±0.0	ND	0.08±0.0	53.0±0.5	1.3±0.4
		Buffer	22.82±0.4	0.56±0.0	0.06±0.0	0.06±0.0	0.10±0.1	60.4±1.1	1.7±0.0
		Algae	25.75±1.0	1.45±0.1	1.73±0.5	0.45±0.1	1.10±0.6	68.2±2.6	4.4±0.3
Alkali	AD fiber	Water	12.30±0.0	4.79±0.8	0.32±0.1	0.44±0.3	0.44±0.1	42.9±0.1	43.6±6.7
		Buffer	12.54±0.3	4.41±0.2	0.34±0.1	0.90±0.2	0.55±0.0	43.7±1.1	40.2±1.6
		Algae	18.11±1.4	6.02±0.5	1.75±0.1	1.61±1.1	1.38±0.4	63.2±4.1	54.8±4.2
	Corn stover	Water	24.15±1.1	7.05±0.2	ND	0.52±0.1	0.07±0.0	55.3±2.3	21.4±0.6
		Buffer	25.84±0.8	6.78±0.2	ND	0.38±0.0	0.08±0.0	59.2±1.6	20.6±0.6
		Algae	26.28±2.2	7.83±0.6	0.59±0.3	0.95±0.4	0.19±0.0	60.2±4.5	23.8±1.4
	Poplar	Water	12.38±0.2	3.42±0.0	ND	ND	0.38±0.0	29.1±0.5	18.1±0.1
		Buffer	13.15±0.3	3.53±0.6	ND	ND	0.42±0.2	33.9±0.7	21.9±3.0
		Algae	12.63±0.3	3.48±0.7	0.37±0.1	0.14±0.1	0.34±0.2	32.4±0.8	18.7±3.3
	Switchgrass	Water	19.08±1.4	5.62±0.2	ND	0.48±0.1	0.06±0.0	53.7±4.0	18.0±0.7
		Buffer	19.56±1.0	5.33±0.6	ND	0.34±0.0	0.06±0.0	55.1±2.7	17.1±1.7
		Algae	20.11±0.5	6.47±1.4	0.55±0.3	0.93±0.5	0.15±0.0	56.6±1.3	20.8±3.8

a: The overall conversion was calculated based on the chemical composition of the original raw feedstock. Do not confuse with the enzymatic conversion, which was calculated based on the chemical composition of pretreated feedstock.
b: The data are the average of two replicates with standard errors.
c: The data are from a 72-hour enzymatic hydrolysis.
d: ND represents not detectable.

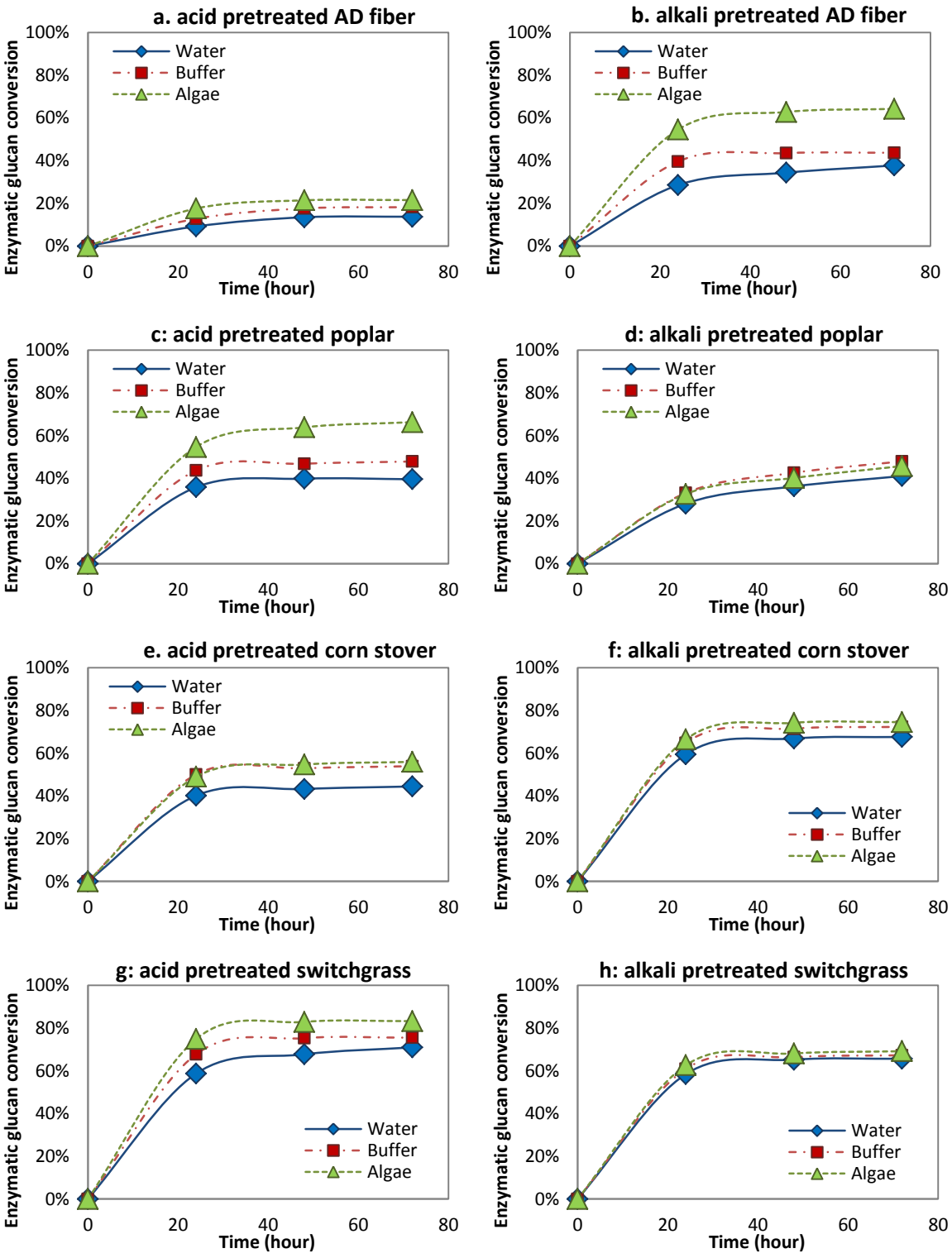


Figure 4.1: Enzymatic glucan conversion of differently treated feedstock using de-ionized water, citrate buffer, and algal hydrolysates as reaction medium.

The overall glucan conversion using different pretreatments and reaction media are presented in Table 6 and the kinetics of enzymatic glucan conversion in Figure 4.1. It is apparent that dilute acid was not an efficient pretreatment for AD fiber (Figure 4.1-a), which had only 18% enzymatic glucan conversion using citrate buffer and 22% conversion using algal hydrolysate as media (corresponding to increases of 29% and 57%, respectively compared to conversions using the control medium of water). Alkali-pretreated AD fiber (Figure 4.1-b) had a greater glucose conversion, 44% in citrate buffer and 64% in algal hydrolysate (corresponding to increases of 16% and 70%, respectively, compared to water). The difference on hydrolysis between acid and alkali treated AD fibers was caused by the high alkalinity of raw AD fiber, which was derived from the anaerobic digestion process (Yue, et al., 2010). The high alkalinity of the AD fiber neutralized sulfuric acid during the dilute acid pretreatment and led to a decreased efficiency of the pretreatment process. The differences in pretreatment efficiencies were also related to the recalcitrant nature of AD fiber and the fiber structure after different pretreatment. AD fiber contains less easily digestible cellulose and hemicellulose branches than other agricultural residuals, and alkali is better than acid in terms of facilitating lignin removal and fiber deconstruction. Hydrolyses in algal hydrolysate media for both acid and alkali treated AD fiber had better enzymatic glucan conversion than water and citrate buffer media. As it has been reported in a previous study (Chen, et al., 2012), the protein and other small molecules in algal hydrolysate played as surfactants to bind the protein-active sites on lignin before introducing the enzymes, increase available active enzyme in the solution, and consequently enhance the performance of enzymatic hydrolysis. According to Table 4.3, pretreated AD fiber contained more lignin than other pretreated feedstock, which explained

the improvement of enzymatic efficiency by using algal hydrolysate (compared to citrate buffer) was more significant ($p < 0.05$) on AD fiber than other feedstock.

For poplar, pretreatments did not have significant effects on the enzymatic glucan conversion using water and citrate buffer media (Figure 4.1-c & d). However, acid-pretreated poplar in algal hydrolysate medium had a significantly higher ($p < 0.0001$) enzymatic glucan conversion (66%) than the alkali-pretreated poplar (47%) using the same medium. This was possibly due to the aforementioned function of algal hydrolysate that the algal protein and other molecules played as a surfactant to bind lignin (acid pretreated poplar had much higher lignin content than alkali pretreated poplar), and improved the hydrolysis performance of the acid pretreated poplar.

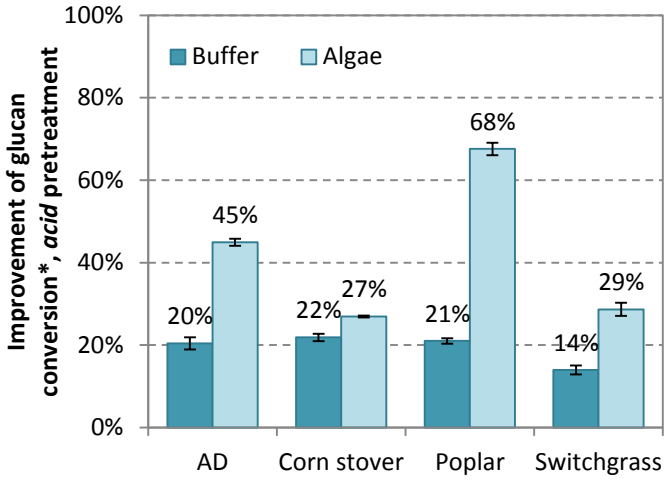
For corn stover (Figure 4.1-e & f), alkali pretreated fiber released more glucose (68-74%) than acid-pretreated (44-56% of enzymatic glucan conversion) in all three media, but the difference between citrate buffer and algal hydrolysate media was not significant in corn stover ($p = 0.0598$). For switchgrass (Figure 4.1-g & h), acid-pretreatment showed better performance on enzymatic glucan conversion (71-83%) than alkali-pretreatment (66-69%), while the effect of different reaction media on conversion was not significant under alkali-pretreatment ($p = 0.5247$). Corn stover and switchgrass are both agricultural residuals that have great potential for biofuels production because of their relatively amendable fiber structure compared to poplar and AD fiber (Wyman, et al., 2009). It is possible the majority of glucan from these two fibers became hydrolytically available after the chemical pretreatments so that additional organic and/or inorganic chemicals from citrate buffer and algal hydrolysate did not show any enhancement as they did on structurally recalcitrant poplar and AD fiber.

3.3. Combined effect of pretreatment and reaction medium on the improvement of enzymatic hydrolysis

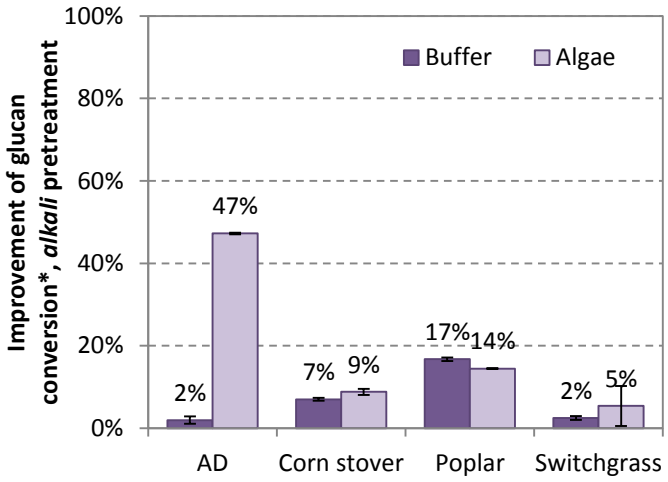
In order to demonstrate the combined effect of pretreatment and reaction medium on the improvement of enzymatic hydrolysis of different feedstock, the enzymatic glucan conversion at 72-hour (Figure 4.1) were compared against the conversion of control group using water as the medium (Figure 4.2). Overall, acid pretreated feedstock in both algal hydrolysate and citrate buffer had significantly better improvement of glucan conversion than alkali pretreated feedstock (Figure 4.2-a & b). The improvement results along with the actual conversion (Table 4.3) indicated that acid- treated substrates were more recalcitrant than alkali-treated feedstock, and required pH buffer and surfactants to further facilitate the enzymatic hydrolysis in order to obtain better conversion.

As for the algal hydrolysate medium, all four substrates under acid pretreatment had significant improvement ($p = 0.0004$, 0.0008, 0.0001, 0.005 for AD fiber, corn stover, poplar and switchgrass, respectively) than water and citrate buffer. However, under alkali pretreatment, only AD fiber in algal hydrolysate showed a significant improvement ($p < 0.0001$) than other media (Figure 4.2-b). According to the chemical composition of pretreated feedstock (Table 4.3), all the pretreated feedstock with higher lignin content had significant improvement of glucan conversion. Previous studies have reported that lignin can reduce the enzyme accessibility of cellulose and hemicellulose during hydrolysis of lignocellulosic biomass, which negatively affects the conversion (Tatsumoto, et al., 1988). These results suggest that organic compounds such as proteins and/or other small molecules in the algal hydrolysate may block the enzyme-absorption sites of lignin, which led to increased enzyme accessibility for the hydrolysis. It is also possible that the algal

hydrolysate could also provide a more chemical- and pH-balanced environment than the other reaction media.



a. Acid pretreatment



b. Alkali pretreatment

Figure 4.2: Effects of pretreatment and reaction medium on the improvement of enzymatic glucan conversion from different feedstock*

*: all compared to the control group using water as reaction medium.

4. Conclusion

Algal hydrolysate as a reaction medium significantly enhanced the enzymatic hydrolysis of alkali pretreated AD fiber as well as acid-pretreated poplar. In contrast, the algal hydrolysate did not show a significant effect on the enzymatic hydrolysis of pretreated corn stover or switchgrass. This difference is likely caused by the fiber structure and lignin content of the pretreated lignocelluloses. Applying algal hydrolysate as reaction medium not only enhances enzymatic hydrolysis of recalcitrant lignocelluloses, but also eliminates use of pH buffer solution, which could make a significant contribution to lignocellulosic biorefining.

Summary

Booming human population and expanding industrialization in the last few decades have had drastic and irreversible damage to the environment. In order to protect our limited natural resources and redefine a sustainable society to future generations, environment-awareness must be implemented in all human activities. This study took a deep look at current issues in waste management, water recycle, and renewable bioenergy; it analyzed pros and cons of each individual practice and developed an integrated solution to maximize each practice's potential and minimize their shortcomings. This closed-loop system managed to reduce the overall environmental impacts from organic wastes and convert them into renewable energy and value-added by-products.

Chapter 1 of this dissertation conducted a thorough literature review to describe the fundamentals, significance and current technical status of the study.

Chapter 2 applied molecular and statistical approaches to test the response of anaerobic microbial community to the change of anaerobic digestion conditions, and their consequent influence on biogas productivity and solid digestate quality. It was discovered that even though the performance of digestion was enhanced with the increase of supplemental food waste, this variation in feedstock did not have significant impact on the chemical composition of final solid digestate (AD fiber). In addition, the anaerobic microbial community demonstrated that they were able to adopt their configuration in order to maximize the conversion of available carbon to biogas.

Chapter 3 utilized the liquid digestate (AD effluent) from previous step in an electrocoagulation-algae combined treatment system to reduce its turbidity and nutrients

(i.e. ammonia-N, phosphate) concentration. In the meantime, accumulated algal biomass in this system became a potential substrate in biofuel and chemical production. Results showed algae cultured in both 2× and 5× dilution of EC medium had reached similar maximum growth rate (0.201-0.207 g TS L^{-1} day^{-1}), even though they experienced different lag phase. Inhibition of algal growth in the original EC medium (1×) was possibly due to the high unionized ammonia concentration. Semi-continuous algal culture based on the kinetics study illustrated higher biomass productivity in 2× than in 5× EC medium because of its more sufficient nutrients (i.e. phosphate) supply. Both cultures maintained steady growth and nitrogen consumption rates throughout the period of study, but their phosphorus consumption rates kept increasing possibly because of algae's luxury phosphorus uptake mechanism. The combined EC-algae treatment proved to be able to significantly and sustainably reduce the turbidity and eutrophication-causing nutrients in AD effluent.

Chapter 4 explored a new application of algal biomass cultured in nitrogen-rich media. Acid hydrolysate from algal biomass was neutralized and applied to the enzymatic hydrolysis (EH) process on lignocellulosic substrates as buffer solution. Results showed significant enhancement in EH efficiency on substrates containing higher lignin and structurally more recalcitrant (i.e. poplar and AD fiber) using algal hydrolysate in comparison with controlled treatment using either water or sodium citrate buffer. It was proposed that protein remained in the algal hydrolysate facilitated the EC process by serving as a lignin-binding surfactant and increase the availability of enzymes.

The integrated system and its corresponding preliminaries as described previously provide a general concept of a new and green way for treating waste biomass. Further

research is required to explore more possible substrates and mixing ratios for anaerobic co-digestion, optimizing the EC condition to reduce energy consumption and to provide efficient nutrients for algal culture, exploring other algal strains to increase the nutrient uptake rate, and improving the biorefining conditions for both algal biomass and lignocellulosic feedstock. In addition, because of the fast development of molecular genetic technologies, more advanced sequencing methods are also recommended in the future studies of microbial community (i.e. anaerobic bacteria, anaerobic archaea, and interaction of aerobic bacteria and algae).

APPENDICES

APPENDIX A: Procedure for analyzing structural carbohydrates and lignin content of lignocellulosic biomass

1. Weigh 300.0 ± 10.0 mg of the biomass into a tared autoclave-safe bottle.
2. Add 4.92 ± 0.01 g of 72% sulfuric acid. Mix sample and acid with glass rod for 1 min.
3. Place bottles in 30 ± 3 °C incubator for 60 min, stir samples with glass rods every 5-10 min.
4. After 60 min incubation, add 84.00 ± 0.04 mL deionized water to dilute the acid to a 4% concentration. Mix sample in solution gently, and autoclave the solution at 121 °C for 1 hour. Allow the bottles to cool down before removing the caps.
5. Transfer approximately 50 mL autoclaved solution into a clean and dry crucible with vacuum filter; apply pressure with an air pump to facilitate filtration. Measure the filtrate using spectrophotometer at 320 nm for dissolved lignin. Neutralize the rest filtrate with calcium carbonate to pH 5-6. Filter the neutralized solution using 0.22 µm pore size syringe filter for HPLC analysis of sugar composition.
6. Transfer the rest autoclaved solution into the crucible with vacuum filter. Wash the solid particles remained on the vacuum filter with deionized water until the pH of flow through is close to 5-6. Leave the crucible in the 105 °C oven until a constant weight is achieved. Record the dry weight of insoluble solids before placing the crucible in the muffle furnace at 575 °C for 24 hours for ash content.

APPENDIX B: Procedure for DNA extraction

1. If samples are in liquid-sludge form, transfer all the contents in PowerBead Tubes into a sterilized 2 mL Collection Tube provided, add 1.5 to 2.0 mL of liquid sample depending on the viscosity of the sample; centrifuge at 10,000 rpm for 10 min and carefully remove the supernatant; pour the original content from PowerBead Tubes back. If samples are in solid form, add 0.25 g directly into PowerBead Tubes. Vortex to mix.
2. Add 60 µL dissolved Solution C1 (a cell lysis reagent) into each PowerBead Tube and invert several times.
3. Secure PowerBead Tubes on a beadbeater, and leave the beadbeater on for 2 min.
4. Centrifuge PowerBead Tubes at 10,000 rpm for 30 sec at room temperature.
5. Transfer supernatant to a clean 2 mL Collection Tube, add 250 µL Solution C2 (an inhibitor removal reagent) and vortex for 5 sec. Incubate at 4 °C for 5 min.
6. Centrifuge at 10,000 rpm for 1 min at room temperature, carefully transfer 600 µL supernatant to a clean 2 mL Collection Tube. Add 200 µL Solution C3 (another inhibitor/cell debris removal reagent) and vortex briefly. Incubate at 4 °C for 5 min.
7. Centrifuge at 10,000 rpm for 1 min at room temperature, carefully transfer 750 µL supernatant to a clean 2 mL Collection Tube. Add 1.2 mL Solution C4 (high concentration salt solution to facilitate DNA binding on silica filter) and vortex for 5 sec.
8. Load 675 µL onto a Spin Filter and centrifuge at 10,000 rpm for 1 min at room temperature. Discard the flow through. Repeat until all solution is centrifuged.

9. Add 500 µL Solution C5 (an ethanol based solution to clean DNA bound on silica filter) and centrifuge at 10,000 rpm for 30 sec at room temperature. Discard the flow through.
10. Centrifuge at 10,000 rpm for 1 min at room temperature to remove residual ethanol.
11. Carefully transfer the Spin Filter in a clean 2 mL Collection Tube, add 100 µL Solution C6 (DNA elusion buffer) and centrifuge at 10,000 rpm for 30 sec at room temperature. Discard the Spin Filter.
12. Measure DNA concentration using NanoDrop spectrophotometer. Frozen samples for future use if they have more than 25 ng μL^{-1} dsDNA and the A260/A280 ratio (DNA purity test) close to 1.8.
13. If the dsDNA concentration or A260/A280 ratio is low, wash and concentrate DNA using the following steps:
    a. Add 4 µL of 5 M NaCl into each 100 µL DNA solution, invert 3-5 times.
    b. Add 200 µL of 100% (200 proof) cold ethanol, invert 3-5 times.
    c. Centrifuge at 10,000 rpm for 10 min at room temperature.
    d. Carefully decant supernatant; the residual ethanol can be further removed in a ventilated clean hood on ice. Resuspend precipitated DNA in sterile 10 mM Tris.

Note: This procedure was modified based on the procedure from Power Soil DNA Extraction Kit, MOBIO

APPENDIX C: PCR procedure for 454 pyrosequencing of bacterial 16S rDNA

1. Pilot PCR mixture (per sample): 15.7 µL RNAse/DNAse free water, 2 µL 10× AccuPrime PCR Buffer II, 0.16 µL Taq polymerase, 0.4 µL Forward Primer (10 µM), 1.34 µL DNA Template (~ 5 ng μL^{-1}), 0.4 µL Reverse Primer with barcode (10 µM) (one barcode corresponding to only one sample).
2. Regular PCR mixture (per sample): 58.9 µL RNAse/DNAse free water, 7.5 µL 10× AccuPrime PCR Buffer II, 0.6 µL Taq polymerase, 1.5 µL Forward Primer (10 µM), 5 µL DNA Template (~ 5 ng μL^{-1}), 1.5µL Reverse Primer with barcode (10 µM) (one barcode corresponding to only one sample).
3. Vortex each PCR tube to homogenize PCR mixture, then spin briefly (5-6 sec) in a centrifuge.
4. Place PCR tubes in thermos cycler, and the cycle as follows:
    (1) Initial denaturing: 95 °C for 5 min;
    (2) 30 cycles of amplification: denaturing at 95 °C for 45 sec, annealing at 50 °C for 45 sec, elongation at 72 °C for 90 sec;
    (3) Final extension: 72 °C for 5 min;
    (4) Storage: 4 °C for infinity time.
5. Mix 5 µL of PCR product with 1 µL loading dye (6×), and load the dyed PCR product onto a sheet of 1% agarose 1× TAE gel for electrophoresis (100 V) for approximately 20-30 min.
6. Transfer the gel sheet from previous step into EtBr (caution: carcinogen), stain for 5 min. Then transfer stained gel into water, de-stain for 10 min.

7. Observe the existence, position, width, and brightness of bands under UV light. If the band looks ideal in pilot PCR, proceed to actual PCR and submit the product for 454 pyrosequencing.

APPENDIX D: PCR procedure for T-RFLP of archaeal 16S rDNA

1. Pilot PCR mixture (per sample): 13.5 µL Platinum PCR SuperMix, 0.3 µL Forward Primer (344aF, 10 µM), 0.3 µL Reverse Primer (1119aR, 10 µM), 0.15 µL BSA (100× or 10 mg mL^{-1}), 0.75 µL DNA Template (~ 5 to 10 ng μL^{-1}).
2. Regular PCR mixture (per sample): 90 µL Platinum PCR SuperMix, 2.5 µL FAM labelled Forward Primer (344aF-FAM, 10 µM), 2 µL Reverse Primer (1119aR, 10 µM), 1 µL BSA (100× or 10 mg mL^{-1}), 4.5 µL DNA Template (~ 5 to 10 ng μL^{-1}). (Note: FAM labelled forward primer (344aF-FAM) is light sensitive. Conduct the following steps in low light/dark room for actual PCR.)
3. Vortex each PCR tube to homogenize PCR mixture, then spin briefly (5-6 sec) in a centrifuge.
4. Place PCR tubes in thermos cycler, and the cycle as follows:
    (1) Initial denaturing: 94 °C for 5 min;
    (2) 30 cycles of amplification: denaturing at 94 °C for 1 min, annealing at 50 °C for 45 sec, elongation at 71 °C for 100 sec;
    (3) Final extension: 72 °C for 5 min;
    (4) Storage: 4 °C for infinity time.
5. Mix 5 µL of PCR product with 1 µL loading dye (6×), and load the dyed PCR product onto a sheet of 1% agarose 1× TAE gel for electrophoresis (100 V) for approximately 20-30 min.
6. Transfer the gel sheet from previous step into EtBr (caution: carcinogen), stain for 5 min. Then transfer stained gel into water, de-stain for 10 min.

7. Observe the existence, position, width, and brightness of bands under UV light. If the band looks ideal in pilot PCR, proceed to actual PCR and submit the product for T-RFLP analysis.

APPENDIX E: Procedure for archaeal 16S rDNA cloning

1. TOPO Cloning reaction: mix 1 µL salt solution, 1 µL TOPO vector and 2 µL of water with 2 µL of archaeal PCR product (using un-labelled forward primer) for each reaction. Vortex gently and incubate for 20-30 min at room temperature.
2. Add 2 µL of the TOPO Cloning reaction from previous step into a vial of One Shot Chemically Competent *E. coli* and mix gently. (Do not mix using pipette.)
3. Incubate on ice for 10 min, and heat-shock the cell at 42 °C for 30 sec without shaking. Then immediately transfer to ice.
4. Add 250 µL S.O.C. medium at room temperature, cap the tube tightly and shake horizontally on an orbital shaker (200 rpm) at 37 °C for 1 hour.
5. While waiting, spread 40 µL of 40 mg mL^{-1} X-gal solution on each plate and warm up the plates at 37 °C for at least 20 min.
6. Spread 10 µL of the solution from each transformation and 20 µL S.O.C. medium on the pre-warmed selective plate (kanamycin) and incubate upside-down at 37 °C overnight.
7. Pick successfully inserted white colonies using toothpicks and add into growth medium on a 96 well plate. Cover each well tightly to prevent cross-contamination and evaporation. Culture on an orbital shaker (80-100 rpm) at room temperature overnight.
8. A biological replicate of the 96 well plate can be generated on the second day.
9. Sequence the cultures of inserted competent cells using Sanger's method.

Note: This procedure was modified based on the procedure for TOPO Cloning Kit.

APPENDIX F: Statistical analysis for AD performance analysis

Table AP.F.1: Two-way ANOVA: Biogas (mL/L AD) versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	32179	32179.5	114.81	0.001
Ratio	2	73895	36947.6	131.82	0.001
Interaction	2	27483	13741.7	49.03	0.001
Error	6	1682	280.3		
Total	11	135240			

S = 16.74 R-Sq = 98.76% R-Sq(adj) = 97.72%

Table AP.F.2: Two-way ANOVA: TS reduced (%) versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	22.431	22.4307	4.94	0.068
Ratio	2	25.627	12.8137	2.82	0.137
Interaction	2	60.454	30.2271	6.65	0.030
Error	6	27.258	4.5430		
Total	11	135.770			

S = 2.131 R-Sq = 79.92% R-Sq(adj) = 63.19%

Table AP.F.3: Two-way ANOVA: Productivity (TS) versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	6679	6678.9	2.01	0.206
Ratio	2	127444	63721.9	19.17	0.002
Interaction	2	362	181.1	0.05	0.947
Error	6	19946	3324.4		
Total	11	154431			

S = 57.66 R-Sq = 87.08% R-Sq(adj) = 76.32%

Table AP.F.4: Two-way ANOVA: Cellulose in residue versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	0.0001259	0.0001259	0.25	0.632
Ratio	2	0.0007174	0.0003587	0.73	0.522
Interaction	2	0.0004194	0.0002097	0.42	0.672
Error	6	0.0029659	0.0004943		
Total	11	0.0042286			

S = 0.02223 R-Sq = 29.86% R-Sq(adj) = 0.00%

Table AP.F.5: Two-way ANOVA: Xylan in residue versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	0.0000337	0.0000337	0.57	0.478
Ratio	2	0.0002053	0.0001027	1.75	0.253
Interaction	2	0.0000096	0.0000048	0.08	0.922
Error	6	0.0003529	0.0000588		
Total	11	0.0006015			

S = 0.007669 R-Sq = 41.34% R-Sq(adj) = 0.00%

Table AP.F.6: Two-way ANOVA: Lignin in residue versus Temp, Ratio

Source	DF	SS	MS	F	P
Temp	1	0.0000000	0.0000000	0.00	0.998
Ratio	2	0.0015936	0.0007968	2.47	0.165
Interaction	2	0.0004505	0.0002253	0.70	0.534
Error	6	0.0019389	0.0003232		
Total	11	0.0039831			

S = 0.01798 R-Sq = 51.32% R-Sq(adj) = 10.75%

Table AP.F.7: Pairwise comparison: Biogas (mL/L AD)

	35C, 100/0	35C, 90/10	35C, 80/20	50C, 100/0	50C, 90/10	50C, 80/20
35C, 100/0		0.0135	0.0077	0.2411	0.0203	<0.0001
35C, 90/10			0.0002	0.0707	0.0007	<0.0001
35C, 80/20				0.0012	0.6778	<0.0001
50C, 100/0					0.0046	<0.0001
50C, 90/10						<0.0001
50C, 80/20						

Table AP.F.8: Pairwise comparison: TS reduced (%)

	35C, 100/0	35C, 90/10	35C, 80/20	50C, 100/0	50C, 90/10	50C, 80/20
35C, 100/0		0.0249	0.0332	0.3720	0.3037	0.9262
35C, 90/10			0.2860	0.1551	0.0002	<.0001
35C, 80/20				0.2060	0.0001	<.0001
50C, 100/0					0.8325	0.2540
50C, 90/10						0.0016
50C, 80/20						

APPENDIX G: Control tests for algal growth (in DI water) and nutrient reduction (TN, TP, Iron) in batch kinetics study

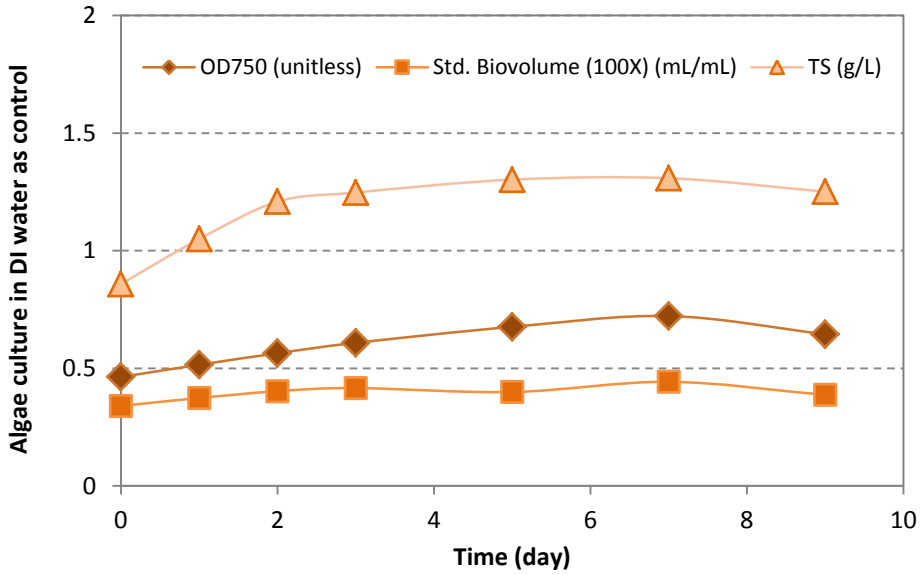


Figure AP.1: Algal growth in DI water based on cell density (OD_{750nm}, unitless), standardized biovolume (100× dilution, unit: mL cell per mL culture), and biomass total solids (unit: g L^{-1}).

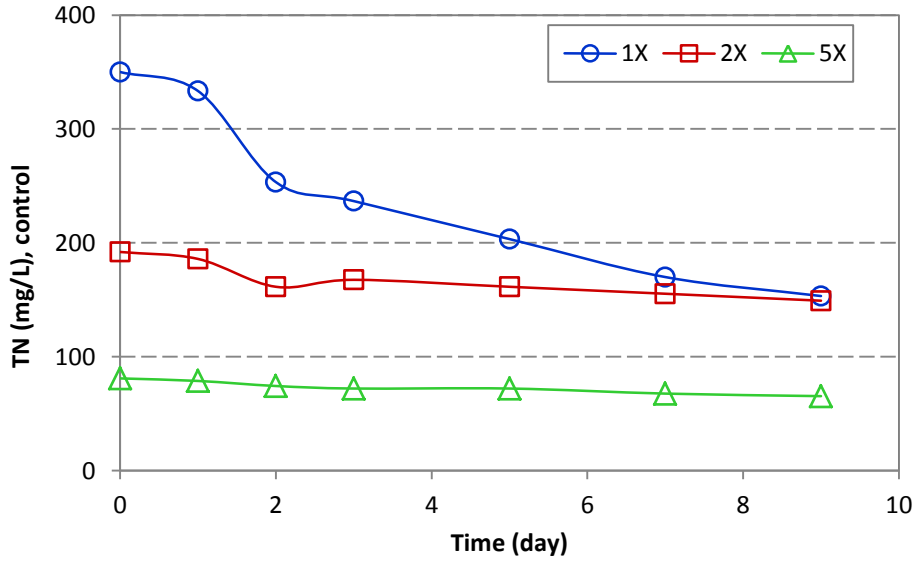


Figure AP.2: Total nitrogen reduction in control culture without algae inoculation.

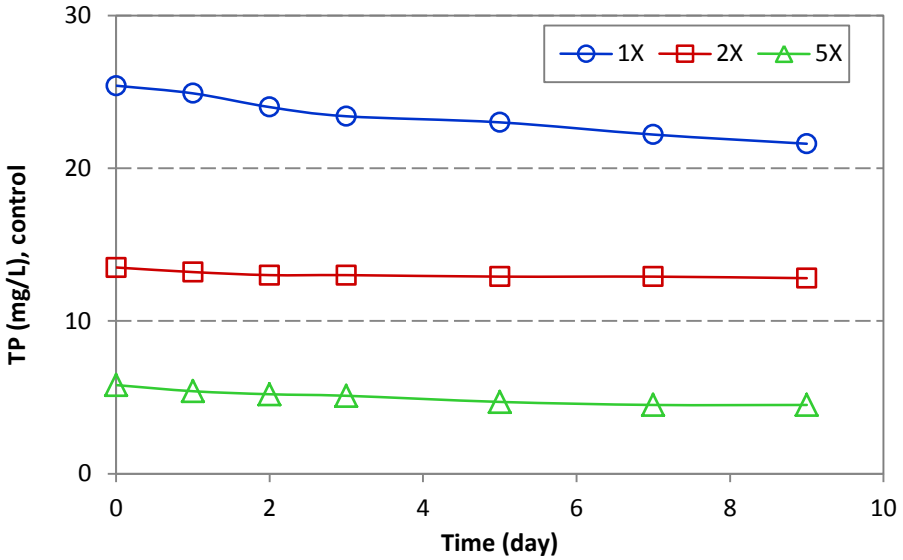


Figure AP.3: Total phosphorus reduction in control culture without algae inoculation.

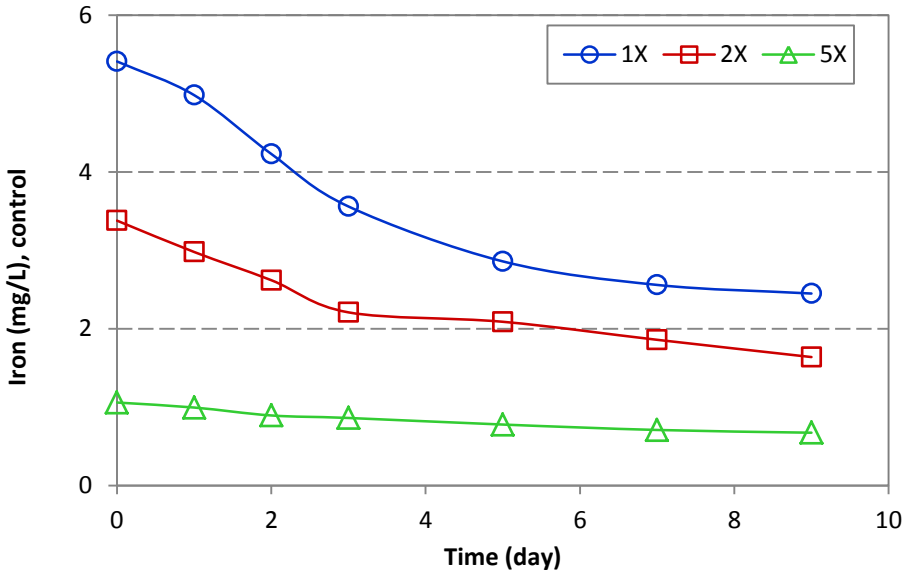


Figure AP.4: Total iron reduction in control culture without algae inoculation.

APPENDIX H: Algal carbohydrate analysis procedure

1. Weigh 25.0 ± 2.5 mg of the algal biomass into a tared autoclave-safe bottle.
2. Add 250 µL of 72% sulfuric acid. Vortex gently for mixing.
3. Place bottles in 30 ± 3 °C incubator for 60 min, vortex gently in every 5-10 min.
4. After 60 min incubation, add 7 mL deionized water to dilute the acid to a 4% concentration. Mix sample in solution gently, and autoclave the solution at 121 °C for 1 hour. Allow the bottles to cool down before removing the caps.
5. Neutralize the autoclaved solution with calcium carbonate to pH 5-6. Filter the neutralized solution using 0.22 µm pore size syringe filter for HPLC analysis of sugar composition.

APPENDIX I: Algal protein analysis procedure (bicinchoninic acid assay)

1. Mixing 2 mL bicinchoninic acid solution (Reagent A) with 0.04 mL copper (II) sulfate pentahydrate 4% (w/v) solution (Reagent B) for each testing sample (including standards).
2. For standard curve of protein, prepare 200, 400, 600, 800, and 1000 µg mL^{-1} BSA solutions. Add 0.1 mL of each standard solution into 2 mL working reagent from previous step and vortex thoroughly.
3. For unknown samples, add 0.1 mL of each sample solution into 2 mL working reagent and vortex thoroughly.
4. Incubate mixed standards and samples at 60 °C for 15 min.
5. Transfer incubated standards and samples into cuvettes and measure at 562 nm using a UV spectrophotometer.
6. Create a standard curve using A_{562} readings of the reacted standards. Then curve-fit the unknown samples to acquire protein concentration.

APPENDIX J: Algal crude lipid extraction procedure

1. Weigh 0.5 g dry algal biomass. Add 2.5 mL chloroform and 5 mL methanol to dry algal sample and mix with a handheld homogenizer for 2 min.
2. Add additional 2.5 mL chloroform and homogenize for 30 sec.
3. Add 2.5 mL deionized water to the previous mixture and homogenize for 30 sec.
4. Filter the mixture through Whatman No. 1 filter paper on a Coors No. 3 Buchner funnel with slight suction by an air pump.
5. Transfer filtrate liquid to a glass vial and allow complete separation of water phase (top layer) and chloroform-lipid phase (bottom layer), approximately in 2 min.
6. Carefully remove bottom layer with pipette and place in an aluminum tray. Leave the tray in a semi-covered and ventilated hood until all solvents have evaporated.
7. Weigh the residual (lipid) and calculate crude lipid concentration (%) based on initial weight of algal biomass.

APPENDIX K: Statistical analysis for algal growth and nutrient reduction in kinetics study

Table AP.K.1: Two-way ANOVA: TS versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	0.0017060	0.0008530	200.16	0.000
Time	6	0.0051195	0.0008533	200.22	0.000
Interaction	12	0.0039369	0.0003281	76.98	0.000
Error	21	0.0000895	0.0000043		
Total	41	0.0108519			

S = 0.002064 R-Sq = 99.18% R-Sq(adj) = 98.39%

Table AP.K.2: Two-way ANOVA: OD750 versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	1.73650	0.868249	173.20	0.000
Time	6	2.41273	0.402121	80.22	0.000
Interaction	12	2.59112	0.215927	43.07	0.000
Error	21	0.10527	0.005013		
Total	41	6.84562			

S = 0.07080 R-Sq = 98.46% R-Sq(adj) = 97.00%

Table AP.K.3: Two-way ANOVA: Std. Biovolume versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	221454	110727	1161.18	0.000
Time	6	439473	73245	768.12	0.000
Interaction	12	358790	29899	313.55	0.000
Error	21	2003	95		
Total	41	1021719			

S = 9.765 R-Sq = 99.80% R-Sq(adj) = 99.62%

Table AP.K.4: Two-way ANOVA: TN versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	608483	304241	5364.96	0.000
Time	6	32066	5344	94.24	0.000
Interaction	12	7275	606	10.69	0.000
Error	21	1191	57		
Total	41	649015			

S = 7.531 R-Sq = 99.82% R-Sq(adj) = 99.64%

Table AP.K.5: Two-way ANOVA: TP versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	2778.21	1389.10	7937.73	0.000
Time	6	192.80	32.13	183.62	0.000
Interaction	12	64.40	5.37	30.67	0.000
Error	21	3.67	0.17		
Total	41	3039.08			

S = 0.4183 R-Sq = 99.88% R-Sq(adj) = 99.76%

Table AP.K.6: Two-way ANOVA: Fe versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	55.439	27.7193	2818.79	0.000
Time	6	39.450	6.5750	668.62	0.000
Interaction	12	11.650	0.9708	98.72	0.000
Error	21	0.207	0.0098		
Total	41	106.745			

S = 0.09917 R-Sq = 99.81% R-Sq(adj) = 99.62%

Table AP.K.7: Two-way ANOVA: Turbidity versus Dilution, Time

Source	DF	SS	MS	F	P
Dilution	2	0.068179	0.0340896	3261.42	0.000
Time	6	0.236341	0.0393902	3768.53	0.000
Interaction	12	0.118767	0.0098972	946.89	0.000
Error	21	0.000219	0.0000105		
Total	41	0.423506			

S = 0.003233 R-Sq = 99.95% R-Sq(adj) = 99.90%

APPENDIX L: Statistical analysis for algae enhanced enzymatic hydrolysis of lignocelluloses

Table AP.L.1: Differences of Least Squares Means

Effect	fiber	pretreatment	medium	_fiber	_pretreatment	_medium	Standard Error
fiber*pretrea*medium	AD	H	Buffer	AD	H	Algae	0.05931
fiber*pretrea*medium	AD	H	Buffer	AD	OH	Buffer	0.05931
fiber*pretrea*medium	AD	H	Buffer	AD	OH	Algae	0.05931
fiber*pretrea*medium	AD	H	Buffer	CS	H	Buffer	0.04284
fiber*pretrea*medium	AD	H	Buffer	CS	H	Algae	0.04284
fiber*pretrea*medium	AD	H	Buffer	CS	OH	Buffer	0.04284
fiber*pretrea*medium	AD	H	Buffer	CS	OH	Algae	0.04284
fiber*pretrea*medium	AD	H	Buffer	Poplar	H	Buffer	0.04523
fiber*pretrea*medium	AD	H	Buffer	Poplar	H	Algae	0.04523
fiber*pretrea*medium	AD	H	Buffer	Poplar	OH	Buffer	0.04523
fiber*pretrea*medium	AD	H	Buffer	Poplar	OH	Algae	0.04523
fiber*pretrea*medium	AD	H	Buffer	SG	H	Buffer	0.05136
fiber*pretrea*medium	AD	H	Buffer	SG	H	Algae	0.05136
fiber*pretrea*medium	AD	H	Buffer	SG	OH	Buffer	0.05136
fiber*pretrea*medium	AD	H	Buffer	SG	OH	Algae	0.05136
fiber*pretrea*medium	AD	H	Algae	AD	OH	Buffer	0.05931
fiber*pretrea*medium	AD	H	Algae	AD	OH	Algae	0.05931
fiber*pretrea*medium	AD	H	Algae	CS	H	Buffer	0.04284
fiber*pretrea*medium	AD	H	Algae	CS	H	Algae	0.04284
fiber*pretrea*medium	AD	H	Algae	CS	OH	Buffer	0.04284
fiber*pretrea*medium	AD	H	Algae	CS	OH	Algae	0.04284
fiber*pretrea*medium	AD	H	Algae	Poplar	H	Buffer	0.04523
fiber*pretrea*medium	AD	H	Algae	Poplar	H	Algae	0.04523
fiber*pretrea*medium	AD	H	Algae	Poplar	OH	Buffer	0.04523
fiber*pretrea*medium	AD	H	Algae	Poplar	OH	Algae	0.04523
fiber*pretrea*medium	AD	H	Algae	SG	H	Buffer	0.05136
fiber*pretrea*medium	AD	H	Algae	SG	H	Algae	0.05136
fiber*pretrea*medium	AD	H	Algae	SG	OH	Buffer	0.05136
fiber*pretrea*medium	AD	H	Algae	SG	OH	Algae	0.05136
fiber*pretrea*medium	AD	OH	Buffer	AD	OH	Algae	0.05931
fiber*pretrea*medium	AD	OH	Buffer	CS	H	Buffer	0.04284
fiber*pretrea*medium	AD	OH	Buffer	CS	H	Algae	0.04284
fiber*pretrea*medium	AD	OH	Buffer	CS	OH	Buffer	0.04284
fiber*pretrea*medium	AD	OH	Buffer	CS	OH	Algae	0.04284
fiber*pretrea*medium	AD	OH	Buffer	Poplar	H	Buffer	0.04523
fiber*pretrea*medium	AD	OH	Buffer	Poplar	H	Algae	0.04523
fiber*pretrea*medium	AD	OH	Buffer	Poplar	OH	Buffer	0.04523
fiber*pretrea*medium	AD	OH	Buffer	Poplar	OH	Algae	0.04523
fiber*pretrea*medium	AD	OH	Buffer	SG	H	Buffer	0.05136
fiber*pretrea*medium	AD	OH	Buffer	SG	H	Algae	0.05136
fiber*pretrea*medium	AD	OH	Buffer	SG	OH	Buffer	0.05136
fiber*pretrea*medium	AD	OH	Buffer	SG	OH	Algae	0.05136
fiber*pretrea*medium	AD	OH	Algae	CS	H	Buffer	0.04284
fiber*pretrea*medium	AD	OH	Algae	CS	H	Algae	0.04284
fiber*pretrea*medium	AD	OH	Algae	CS	OH	Buffer	0.04284
fiber*pretrea*medium	AD	OH	Algae	CS	OH	Algae	0.04284
fiber*pretrea*medium	AD	OH	Algae	Poplar	H	Buffer	0.04523
fiber*pretrea*medium	AD	OH	Algae	Poplar	H	Algae	0.04523
fiber*pretrea*medium	AD	OH	Algae	Poplar	OH	Buffer	0.04523
fiber*pretrea*medium	AD	OH	Algae	Poplar	OH	Algae	0.04523
fiber*pretrea*medium	AD	OH	Algae	SG	H	Buffer	0.05136
fiber*pretrea*medium	AD	OH	Algae	SG	H	Algae	0.05136
fiber*pretrea*medium	AD	OH	Algae	SG	OH	Buffer	0.05136
fiber*pretrea*medium	AD	OH	Algae	SG	OH	Algae	0.05136
fiber*pretrea*medium	CS	H	Buffer	CS	H	Algae	0.01237

Table AP.L.1: (cont'd)

fiber*pretrea*medium	CS	H	Buffer	CS	OH	Buffer	0.01237
fiber*pretrea*medium	CS	H	Buffer	CS	OH	Algae	0.01237
fiber*pretrea*medium	CS	H	Buffer	Poplar	H	Buffer	0.01906
fiber*pretrea*medium	CS	H	Buffer	Poplar	H	Algae	0.01906
fiber*pretrea*medium	CS	H	Buffer	Poplar	OH	Buffer	0.01906
fiber*pretrea*medium	CS	H	Buffer	Poplar	OH	Algae	0.01906
fiber*pretrea*medium	CS	H	Buffer	SG	H	Buffer	0.03091
fiber*pretrea*medium	CS	H	Buffer	SG	H	Algae	0.03091
fiber*pretrea*medium	CS	H	Buffer	SG	OH	Buffer	0.03091
fiber*pretrea*medium	CS	H	Buffer	SG	OH	Algae	0.03091
fiber*pretrea*medium	CS	H	Algae	CS	OH	Buffer	0.01237
fiber*pretrea*medium	CS	H	Algae	CS	OH	Algae	0.01237
fiber*pretrea*medium	CS	H	Algae	Poplar	H	Buffer	0.01906
fiber*pretrea*medium	CS	H	Algae	Poplar	H	Algae	0.01906
fiber*pretrea*medium	CS	H	Algae	Poplar	OH	Buffer	0.01906
fiber*pretrea*medium	CS	H	Algae	Poplar	OH	Algae	0.01906
fiber*pretrea*medium	CS	H	Algae	SG	H	Buffer	0.03091
fiber*pretrea*medium	CS	H	Algae	SG	H	Algae	0.03091
fiber*pretrea*medium	CS	H	Algae	SG	OH	Buffer	0.03091
fiber*pretrea*medium	CS	H	Algae	SG	OH	Algae	0.03091
fiber*pretrea*medium	CS	OH	Buffer	CS	OH	Algae	0.01237
fiber*pretrea*medium	CS	OH	Buffer	Poplar	H	Buffer	0.01906
fiber*pretrea*medium	CS	OH	Buffer	Poplar	H	Algae	0.01906
fiber*pretrea*medium	CS	OH	Buffer	Poplar	OH	Buffer	0.01906
fiber*pretrea*medium	CS	OH	Buffer	Poplar	OH	Algae	0.01906
fiber*pretrea*medium	CS	OH	Buffer	SG	H	Buffer	0.03091
fiber*pretrea*medium	CS	OH	Buffer	SG	H	Algae	0.03091
fiber*pretrea*medium	CS	OH	Buffer	SG	OH	Buffer	0.03091
fiber*pretrea*medium	CS	OH	Buffer	SG	OH	Algae	0.03091
fiber*pretrea*medium	CS	OH	Algae	Poplar	H	Buffer	0.01906
fiber*pretrea*medium	CS	OH	Algae	Poplar	H	Algae	0.01906
fiber*pretrea*medium	CS	OH	Algae	Poplar	OH	Buffer	0.01906
fiber*pretrea*medium	CS	OH	Algae	Poplar	OH	Algae	0.01906
fiber*pretrea*medium	CS	OH	Algae	SG	H	Buffer	0.03091
fiber*pretrea*medium	CS	OH	Algae	SG	H	Algae	0.03091
fiber*pretrea*medium	CS	OH	Algae	SG	OH	Buffer	0.03091
fiber*pretrea*medium	CS	OH	Algae	SG	OH	Algae	0.03091
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	H	Algae	0.02396
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	OH	Buffer	0.02396
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	OH	Algae	0.02396
fiber*pretrea*medium	Poplar	H	Buffer	SG	H	Buffer	0.03414
fiber*pretrea*medium	Poplar	H	Buffer	SG	H	Algae	0.03414
fiber*pretrea*medium	Poplar	H	Buffer	SG	OH	Buffer	0.03414
fiber*pretrea*medium	Poplar	H	Buffer	SG	OH	Algae	0.03414
fiber*pretrea*medium	Poplar	H	Algae	Poplar	OH	Buffer	0.02396
fiber*pretrea*medium	Poplar	H	Algae	Poplar	OH	Algae	0.02396
fiber*pretrea*medium	Poplar	H	Algae	SG	H	Buffer	0.03414
fiber*pretrea*medium	Poplar	H	Algae	SG	H	Algae	0.03414
fiber*pretrea*medium	Poplar	H	Algae	SG	OH	Buffer	0.03414
fiber*pretrea*medium	Poplar	H	Algae	SG	OH	Algae	0.03414
fiber*pretrea*medium	Poplar	OH	Buffer	Poplar	OH	Algae	0.02396
fiber*pretrea*medium	Poplar	OH	Buffer	SG	H	Buffer	0.03414
fiber*pretrea*medium	Poplar	OH	Buffer	SG	H	Algae	0.03414
fiber*pretrea*medium	Poplar	OH	Buffer	SG	OH	Buffer	0.03414
fiber*pretrea*medium	Poplar	OH	Buffer	SG	OH	Algae	0.03414
fiber*pretrea*medium	Poplar	OH	Algae	SG	H	Buffer	0.03414
fiber*pretrea*medium	Poplar	OH	Algae	SG	H	Algae	0.03414
fiber*pretrea*medium	Poplar	OH	Algae	SG	OH	Buffer	0.03414
fiber*pretrea*medium	Poplar	OH	Algae	SG	OH	Algae	0.03414
fiber*pretrea*medium	SG	H	Buffer	SG	H	Algae	0.04192
fiber*pretrea*medium	SG	H	Buffer	SG	OH	Buffer	0.04192
fiber*pretrea*medium	SG	H	Buffer	SG	OH	Algae	0.04192
fiber*pretrea*medium	SG	H	Algae	SG	OH	Buffer	0.04192
fiber*pretrea*medium	SG	H	Algae	SG	OH	Algae	0.04192
fiber*pretrea*medium	SG	OH	Buffer	SG	OH	Algae	0.04192

Table AP.L.1: (cont'd)

Effect	fiber	pretreatment	medium	_fiber	_pretreatment	_medium	Adj P
fiber*pretrea*medium	AD	H	Buffer	AD	H	Algae	0.0234
fiber*pretrea*medium	AD	H	Buffer	AD	OH	Buffer	0.0192
fiber*pretrea*medium	AD	H	Buffer	AD	OH	Algae	0.2262
fiber*pretrea*medium	AD	H	Buffer	CS	H	Buffer	0.9397
fiber*pretrea*medium	AD	H	Buffer	CS	H	Algae	1.0000
fiber*pretrea*medium	AD	H	Buffer	CS	OH	Buffer	0.0067
fiber*pretrea*medium	AD	H	Buffer	CS	OH	Algae	0.0148
fiber*pretrea*medium	AD	H	Buffer	Poplar	H	Buffer	0.9105
fiber*pretrea*medium	AD	H	Buffer	Poplar	H	Algae	<.0001
fiber*pretrea*medium	AD	H	Buffer	Poplar	OH	Buffer	0.0080
fiber*pretrea*medium	AD	H	Buffer	Poplar	OH	Algae	0.0014
fiber*pretrea*medium	AD	H	Buffer	SG	H	Buffer	0.3141
fiber*pretrea*medium	AD	H	Buffer	SG	H	Algae	1.0000
fiber*pretrea*medium	AD	H	Buffer	SG	OH	Buffer	0.0063
fiber*pretrea*medium	AD	H	Buffer	SG	OH	Algae	0.0212
fiber*pretrea*medium	AD	H	Algae	AD	OH	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	AD	OH	Algae	0.9891
fiber*pretrea*medium	AD	H	Algae	CS	H	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	CS	H	Algae	0.0005
fiber*pretrea*medium	AD	H	Algae	CS	OH	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	CS	OH	Algae	<.0001
fiber*pretrea*medium	AD	H	Algae	Poplar	H	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	Poplar	H	Algae	0.3666
fiber*pretrea*medium	AD	H	Algae	Poplar	OH	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	AD	H	Algae	SG	H	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	SG	H	Algae	0.0062
fiber*pretrea*medium	AD	H	Algae	SG	OH	Buffer	<.0001
fiber*pretrea*medium	AD	H	Algae	SG	OH	Algae	<.0001
fiber*pretrea*medium	AD	OH	Buffer	AD	OH	Algae	<.0001
fiber*pretrea*medium	AD	OH	Buffer	CS	H	Buffer	0.0155
fiber*pretrea*medium	AD	OH	Buffer	CS	H	Algae	0.0017
fiber*pretrea*medium	AD	OH	Buffer	CS	OH	Buffer	0.9960
fiber*pretrea*medium	AD	OH	Buffer	CS	OH	Algae	0.9461
fiber*pretrea*medium	AD	OH	Buffer	Poplar	H	Buffer	0.0344
fiber*pretrea*medium	AD	OH	Buffer	Poplar	H	Algae	<.0001
fiber*pretrea*medium	AD	OH	Buffer	Poplar	OH	Buffer	0.9996
fiber*pretrea*medium	AD	OH	Buffer	Poplar	OH	Algae	1.0000
fiber*pretrea*medium	AD	OH	Buffer	SG	H	Buffer	0.6049
fiber*pretrea*medium	AD	OH	Buffer	SG	H	Algae	0.0055
fiber*pretrea*medium	AD	OH	Buffer	SG	OH	Buffer	1.0000
fiber*pretrea*medium	AD	OH	Buffer	SG	OH	Algae	1.0000
fiber*pretrea*medium	AD	OH	Algae	CS	H	Buffer	0.0014
fiber*pretrea*medium	AD	OH	Algae	CS	H	Algae	0.0128
fiber*pretrea*medium	AD	OH	Algae	CS	OH	Buffer	<.0001
fiber*pretrea*medium	AD	OH	Algae	CS	OH	Algae	<.0001
fiber*pretrea*medium	AD	OH	Algae	Poplar	H	Buffer	0.0018
fiber*pretrea*medium	AD	OH	Algae	Poplar	H	Algae	0.0206
fiber*pretrea*medium	AD	OH	Algae	Poplar	OH	Buffer	<.0001
fiber*pretrea*medium	AD	OH	Algae	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	AD	OH	Algae	SG	H	Buffer	0.0005
fiber*pretrea*medium	AD	OH	Algae	SG	H	Algae	0.0983
fiber*pretrea*medium	AD	OH	Algae	SG	OH	Buffer	<.0001
fiber*pretrea*medium	AD	OH	Algae	SG	OH	Algae	<.0001
fiber*pretrea*medium	CS	H	Buffer	CS	H	Algae	0.0406
fiber*pretrea*medium	CS	H	Buffer	CS	OH	Buffer	<.0001
fiber*pretrea*medium	CS	H	Buffer	CS	OH	Algae	<.0001
fiber*pretrea*medium	CS	H	Buffer	Poplar	H	Buffer	1.0000
fiber*pretrea*medium	CS	H	Buffer	Poplar	H	Algae	<.0001
fiber*pretrea*medium	CS	H	Buffer	Poplar	OH	Buffer	<.0001
fiber*pretrea*medium	CS	H	Buffer	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	CS	H	Buffer	SG	H	Buffer	0.4885
fiber*pretrea*medium	CS	H	Buffer	SG	H	Algae	0.6833

Table AP.L.1: (cont'd)

fiber*pretrea*medium	CS	H	Buffer	SG	OH	Buffer	0.0008
fiber*pretrea*medium	CS	H	Buffer	SG	OH	Algae	0.0057
fiber*pretrea*medium	CS	H	Algae	CS	OH	Buffer	<.0001
fiber*pretrea*medium	CS	H	Algae	CS	OH	Algae	<.0001
fiber*pretrea*medium	CS	H	Algae	Poplar	H	Buffer	0.2248
fiber*pretrea*medium	CS	H	Algae	Poplar	H	Algae	<.0001
fiber*pretrea*medium	CS	H	Algae	Poplar	OH	Buffer	<.0001
fiber*pretrea*medium	CS	H	Algae	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	CS	H	Algae	SG	H	Buffer	0.0356
fiber*pretrea*medium	CS	H	Algae	SG	H	Algae	1.0000
fiber*pretrea*medium	CS	H	Algae	SG	OH	Buffer	<.0001
fiber*pretrea*medium	CS	H	Algae	SG	OH	Algae	0.0003
fiber*pretrea*medium	CS	OH	Buffer	CS	OH	Algae	0.9710
fiber*pretrea*medium	CS	OH	Buffer	Poplar	H	Buffer	0.0001
fiber*pretrea*medium	CS	OH	Buffer	Poplar	H	Algae	<.0001
fiber*pretrea*medium	CS	OH	Buffer	Poplar	OH	Buffer	1.0000
fiber*pretrea*medium	CS	OH	Buffer	Poplar	OH	Algae	0.4390
fiber*pretrea*medium	CS	OH	Buffer	SG	H	Buffer	0.6519
fiber*pretrea*medium	CS	OH	Buffer	SG	H	Algae	0.0002
fiber*pretrea*medium	CS	OH	Buffer	SG	OH	Buffer	0.9737
fiber*pretrea*medium	CS	OH	Buffer	SG	OH	Algae	1.0000
fiber*pretrea*medium	CS	OH	Algae	Poplar	H	Buffer	0.0006
fiber*pretrea*medium	CS	OH	Algae	Poplar	H	Algae	<.0001
fiber*pretrea*medium	CS	OH	Algae	Poplar	OH	Buffer	0.9835
fiber*pretrea*medium	CS	OH	Algae	Poplar	OH	Algae	0.1041
fiber*pretrea*medium	CS	OH	Algae	SG	H	Buffer	0.9274
fiber*pretrea*medium	CS	OH	Algae	SG	H	Algae	0.0006
fiber*pretrea*medium	CS	OH	Algae	SG	OH	Buffer	0.7739
fiber*pretrea*medium	CS	OH	Algae	SG	OH	Algae	0.9995
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	H	Algae	<.0001
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	OH	Buffer	0.0009
fiber*pretrea*medium	Poplar	H	Buffer	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	Poplar	H	Buffer	SG	H	Buffer	0.7679
fiber*pretrea*medium	Poplar	H	Buffer	SG	H	Algae	0.6615
fiber*pretrea*medium	Poplar	H	Buffer	SG	OH	Buffer	0.0037
fiber*pretrea*medium	Poplar	H	Buffer	SG	OH	Algae	0.0225
fiber*pretrea*medium	Poplar	H	Algae	Poplar	OH	Buffer	<.0001
fiber*pretrea*medium	Poplar	H	Algae	Poplar	OH	Algae	<.0001
fiber*pretrea*medium	Poplar	H	Algae	SG	H	Buffer	<.0001
fiber*pretrea*medium	Poplar	H	Algae	SG	H	Algae	<.0001
fiber*pretrea*medium	Poplar	H	Algae	SG	OH	Buffer	<.0001
fiber*pretrea*medium	Poplar	H	Algae	SG	OH	Algae	<.0001
fiber*pretrea*medium	Poplar	OH	Buffer	Poplar	OH	Algae	0.8986
fiber*pretrea*medium	Poplar	OH	Buffer	SG	H	Buffer	0.6406
fiber*pretrea*medium	Poplar	OH	Buffer	SG	H	Algae	0.0005
fiber*pretrea*medium	Poplar	OH	Buffer	SG	OH	Buffer	0.9982
fiber*pretrea*medium	Poplar	OH	Buffer	SG	OH	Algae	1.0000
fiber*pretrea*medium	Poplar	OH	Algae	SG	H	Buffer	0.1174
fiber*pretrea*medium	Poplar	OH	Algae	SG	H	Algae	<.0001
fiber*pretrea*medium	Poplar	OH	Algae	SG	OH	Buffer	1.0000
fiber*pretrea*medium	Poplar	OH	Algae	SG	OH	Algae	0.9972
fiber*pretrea*medium	SG	H	Buffer	SG	H	Algae	0.1205
fiber*pretrea*medium	SG	H	Buffer	SG	OH	Buffer	0.3836
fiber*pretrea*medium	SG	H	Buffer	SG	OH	Algae	0.8237
fiber*pretrea*medium	SG	H	Algae	SG	OH	Buffer	0.0008
fiber*pretrea*medium	SG	H	Algae	SG	OH	Algae	0.0035
fiber*pretrea*medium	SG	OH	Buffer	SG	OH	Algae	0.9999

A general linear model using the Statistical Analysis System program 9.2 (SAS institute Inc., NC) was applied to perform an analysis of variance (ANOVA) and multiple comparisons of the normalized glucose yield from all eight enzymatic hydrolyses. Tukey's pair-wise comparison and slicing were carried out to determine the simple main effect of each factor (fiber, pretreatment and medium) and their interactions.

Table AP.L.2: Tests of Effect Slices

Effect	fiber	pretreatment	medium	Num DF	Den DF	F Value	Pr > F
fiber*pretrea*medium	AD	H		1	16	19.53	0.0004
fiber*pretrea*medium	AD	OH		1	16	58.33	<.0001
fiber*pretrea*medium	CS	H		1	16	16.96	0.0008
fiber*pretrea*medium	CS	OH		1	16	2.17	0.1604
fiber*pretrea*medium	Poplar	H		1	16	377.77	<.0001
fiber*pretrea*medium	Poplar	OH		1	16	3.30	0.0472
fiber*pretrea*medium	SG	H		1	16	12.26	0.0030
fiber*pretrea*medium	SG	OH		1	16	0.63	0.4378
fiber*pretrea*medium		H	Buffer	3	16	3.23	0.0504
fiber*pretrea*medium		H	Algae	3	16	160.32	<.0001
fiber*pretrea*medium		OH	Buffer	3	16	1.11	0.3745
fiber*pretrea*medium		OH	Algae	3	16	33.72	<.0001
fiber*pretrea*medium	AD		Buffer	1	16	20.49	0.0003
fiber*pretrea*medium	AD		Algae	1	16	1.71	0.2092
fiber*pretrea*medium	CS		Buffer	1	16	144.00	<.0001
fiber*pretrea*medium	CS		Algae	1	16	214.52	<.0001
fiber*pretrea*medium	Poplar		Buffer	1	16	38.17	<.0001
fiber*pretrea*medium	Poplar		Algae	1	16	749.46	<.0001
fiber*pretrea*medium	SG		Buffer	1	16	7.50	0.0146
fiber*pretrea*medium	SG		Algae	1	16	29.64	<.0001
fiber*pretrea*medium	AD			3	16	31.63	<.0001
fiber*pretrea*medium	CS			3	16	124.72	<.0001
fiber*pretrea*medium	Poplar			3	16	314.61	<.0001
fiber*pretrea*medium	SG			3	16	15.46	<.0001
fiber*pretrea*medium		H		7	16	96.63	<.0001
fiber*pretrea*medium		OH		7	16	15.51	<.0001
fiber*pretrea*medium			Buffer	7	16	31.21	<.0001
fiber*pretrea*medium			Algae	7	16	179.29	<.0001

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