EVALUATING THE POTENTIAL FOR IMPROVING ANAEROBIC DIGESTION OF CELLULOSIC WASTE VIA ROUTINE BIOAUGMENTATION AND ALKALINE PRETREATMENT

BY

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THESIS

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ABSTRACT

In this study, two methods for improving anaerobic digestion processes were investigated. The first method was routine bioaugmentation and the second was alkaline pretreatment. Both of these methods were applied to a two-phase anaerobic digestion process for treating the residuals from sweet corn processing, which have a significant lignocellulosic fraction as well as some starch from the base of the kernels. The two-phase anaerobic digestion process was proposed as one component of a larger integrated anaerobic/aerobic waste treatment process in which four co-products would be generated namely, methane-rich biogas, fertilizer, single cell protein, and algal biomass.

The first objective of this study was to determine whether bioaugmentation with a cellulolytic bioculture would result in increased methane production compared to a non-bioaugmented control condition. Batch tests were conducted to compare the biogas potential of sweet corn processing residues with and without bioaugmentation using a proprietary cellulolytic bioculture. The results indicated that bioaugmentation was beneficial to digestion performance, increasing the average methane production by 34% compared to non-bioaugmented controls (265 versus 199 ml/g VS_{added}). The average rate of methane production was also increased in the bioaugmented condition compared to non-bioaugmented controls. However, the observed total methane production was relatively low in comparison to the maximum theoretical production (415 ml CH_4/g VS_{added}), suggesting there to be room for further improving digestion efficiency.

The second objective of this study was to verify whether routine bioaugmentation with cellulolytic microorganisms benefited substrate hydrolysis and subsequent methane production compared to one-time bioaugmentation. It was hypothesized that through routine bioaugmentation with cellulolytic microorganisms, a microbial population better suited for degradation of lignocellulosic material could be achieved and maintained,

thereby increasing the rate of hydrolysis and ultimately increasing the rate of methane production. Pursuant to this objective, a two-phase sequencing/semi batch experiment was conducted in which routine bioaugmentation with two sources of cellulolytic microorganisms was compared to one-time bioaugmented and non-bioaugmented conditions. Neutral detergent fiber (NDF) analysis and net soluble chemical oxygen demand (sCOD) generation suggested that routine bioaugmentation improved substrate hydrolysis by 22-25% in comparison to one-time bioaugmentation after 14 days of operation. Methane yields from routine bioaugmented conditions using a proprietary cellulolytic bioculture also showed 15% higher methane production was achieved in comparison to one-time bioaugmentation after 36 days of digestion. In this experiment, bioaugmentation with a proprietary cellulolytic bioculture was compared to bioaugmentation with dairy cattle rumen fluid. The rumen bioaugmentation culture produced higher methane yields than the proprietary bioculture (16-34%). However, both were below theoretical yields, suggesting that further optimization of the bioculture could improve process efficiency.

After evaluating the relative benefits of routine and one-time bioaugmentation, it was apparent that although bioaugmentation improved digester performance, there was still a significant fraction of un-hydrolyzed material. Thus, a third objective was added to determine the benefit of alkaline pretreatment on substrate solubilization and the digestibility of the resulting hydrolysate. Two long-term pretreatment batch tests (29 and 68 days) were conducted to determine the extent and rate of substrate hydrolysis under elevated pH conditions. It was found that through alkaline pretreatment up to pH 12, volatile solids solubilization was increased 2-4 fold compared to non-pretreated controls. Rates of solubilization were dependent on the pH consistency, which fluctuated during the batch tests due to the production of amino acids and fatty acids and intermittent addition of base to re-establish the target pH of 12. A subsequent anaerobic digestion

batch test using the resulting hydrolysate indicated that 50% more methane production could be achieved in the case of alkaline pretreatment compared to a non-pretreated control.

In summary, results from this study indicated that both routine bioaugmentation with a cellulolytic bioculture and alkaline pretreatment were significantly beneficial (34-50% improvement) for the anaerobic digestion of sweet corn processing residues, by contributing to higher rates of substrate hydrolysis and subsequent methane production.

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CHAPTER 1: INTRODUCTION

As a result of global economic development, population growth, and urbanization, solid waste generation is a growing social and environmental concern. At the same time, the organic fraction of solid waste, which consists primarily of lignocellulosic material, represents an enormous source of renewable biomass with potential for bioenergy production and nutrient reuse. Therefore, in order to reduce the costs, health risks, resource loss, and environmental impacts associated with solid waste, methods for sustainably managing solid waste are increasingly important.

Anaerobic digestion is a proven technology that offers significant environmental benefits, and has been considered as one of the most viable options for managing solid organic waste (Khalid, Arshad et al. 2011). Through anaerobic digestion, organic matter is degraded by microorganisms to produce a methane rich biogas that can be used as an alternative to natural gas. In addition, the resulting nutrient rich effluent can be utilized as fertilizer or for the production of other valuable byproducts. Anaerobic digestion conversion efficiencies of solid organic waste, however, are limited due to the recalcitrant nature of lignocellulosic material. These materials typically require long retention times to degrade, on the order of months, which results in higher capital costs for larger reactor volumes, i.e. lower economic value. For anaerobic digestion of high solids content waste streams, hydrolysis of lignocellulosic biomass has been widely recognized as the major rate limiting step (Park, Lee et al. 2005, Mumme, Linke et al. 2010). Therefore, methods for improving the hydrolysis of lignocellulosic biomass are an attractive strategy for improving the process efficiency and economic viability of anaerobic digestion technology as applied to lignocellulosic feedstocks.

Alkaline pretreatment and bioaugmentation are two processes that have been shown to improve digestion efficiencies of lignocellulosic biomass. Alkaline pretreatment is a

commonly known and cost effective method for delignifying lignocellulosic biomass, which has been proven to significantly improve substrate digestibility (Kumar, Wyman 2009, Playne 1984, Shinners, Binversie et al. 2007). Other benefits of alkaline pretreatment include the fact that it can be applied in combination with wet-storage and under ambient temperatures and pressures. Bioaugmentation with hydrolytic bacteria, has also been shown to improve anaerobic digestion of lignocellulosic biomass. Studies have demonstrated increases in methane yields and production rates from anaerobic digestion of lignocellulosic biomass through bioaugmentation with various cellulolytic bacteria (Angelidaki, Ahring 2000, Mladenovska, Ishoy et al. 2001, Nielsen, Mladenovska et al. 2007, Weiss, Tauber et al. 2010). However, in these studies the bioaugmented microorganisms were applied only once, and in several cases increased methane production was not sustained over time as the bioaugmented bacteria were most likely washed-out or otherwise out-competed by indigenous microorganisms over longer time periods (Mladenovska, Ishoy et al. 2001, Nielsen, Mladenovska et al. 2007). Therefore, methods for ensuring the survival of the bioaugmented microorganisms are needed.

In this study, the potential for improving anaerobic digestion of sweet corn processing residues through routine bioaugmentation with a cellulolytic bioculture and long-term alkaline pretreatment was investigated. The sweet corn processing residues used in this study are a unique lignocellulosic substrate consisting of corn husks, corn cobs, and some pieces of corn kernel. The initial motivation for the work came from the Del Monte Foods Company's interest in alternative disposal/reuse options for sweet corn residues generated from their sweet corn processing facility in Mendota, IL. The Del Monte Mendota facility produces approximately 70,000 tons (wet weight) of sweet corn residues annually, within a 1-2 month time period. Their current disposal method is land application, a service for which they pay a tipping fee of approximately $4 per ton for transport and disposal. With opportunity for cogeneration at their sweet corn processing

facility, Del Monte Foods was interested in the methane production potential of the sweet corn residue material.

With that, the initial approach that was investigated for improving methane production from Del Monte Foods sweet corn processing residues was a two-phase anaerobic digestion process applying routine bioaugmentation with a proprietary cellulolytic bioculture in the first phase. The hypothesis was that through continual, routine additions of cellulolytic bacteria, a bacterial population better suited for hydrolysis of cellulosic material could be achieved and maintained, thereby increasing rates of hydrolysis and subsequent methane production. The first objective of this study was to determine whether bioaugmentation with a proprietary cellulolytic bioculture would improve methane production from the unique sweet corn residues substrate compared to non-bioaugmented control conditions. The second objective was to verify whether routine bioaugmentation with cellulolytic microorganisms would increase substrate hydrolysis and subsequent methane production over one-time bioaugmentation. Finally, the addition of a wet-storage alkaline pretreatment stage was proposed as a method for further improving hydrolysis rates and methane production from the sweet corn processing residues. With that, the third objective of this study was to investigate the effects of long-term alkaline pretreatment on substrate solubilization and the digestibility of the resulting hydrolysate.

Following this chapter, a literature review is presented in Chapter 2 providing background on solid waste generation, current solid waste management practices, the anaerobic digestion process, conversion of lignocellulosic material, and previous studies relating to alkaline pretreatment and bioaugmentation. Chapter 3 describes the broader context of this study, which is an integrated waste treatment system combining two-phase anaerobic digestion with an aerobic post-treatment process. Also provided in Chapter 3

are the specific research objectives for this study which focus on improving two-phase anaerobic digestion of lignocellulosic material. In Chapter 4, the experimental methods and materials that were used in this study are described, followed by a presentation and discussion of the experimental results in Chapter 5. Finally, Chapter 6 summarizes key conclusions drawn from results and outlines some recommendations for future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Solid Organic Waste Generation and Current Management Techniques

Global solid organic waste generation is increasing, creating growing risk towards human health, the environment, and the availability of natural resources. At the same time, solid organic waste including the organic fraction of municipal solid waste, industrial waste, and agricultural residues represents an enormous renewable biomass resource with potential for energy production and material reuse. In general, current solid waste management techniques including landfilling, incineration, and composting do not take full advantage of the energy and nutrient content of solid organic waste, and have been associated with several negative environmental impacts including greenhouse gas emissions, water pollution and odor. Therefore, there is a need for the development and implementation of sustainable solid waste management systems.

Solid Organic Waste Generation

Billions of tons of solid waste are generated worldwide, with the majority consisting of organic, lignocellulosic material. Currently, global municipal solid waste generation is approximately 2 billion tons per year and is expected to increase to 3 billion tons by 2025 (Charles, Walker et al. 2009). In the United States, more than 250 million tons of municipal solid wastes (MSW) were generated in 2010, resulting in a 65% increase in MSW generation per capita since 1960 (U.S. EPA 2011). This is shown in Figure 1a. Of the organic fraction of MSW, more that 60% consists of lignocellulosic material including paper, food scraps, yard trimmings and wood, as can be seen in Figure 1b. The energy content of MSW was estimated to be close to 11.7 million Btu per ton in 2005 (U.S. EIA 2007).

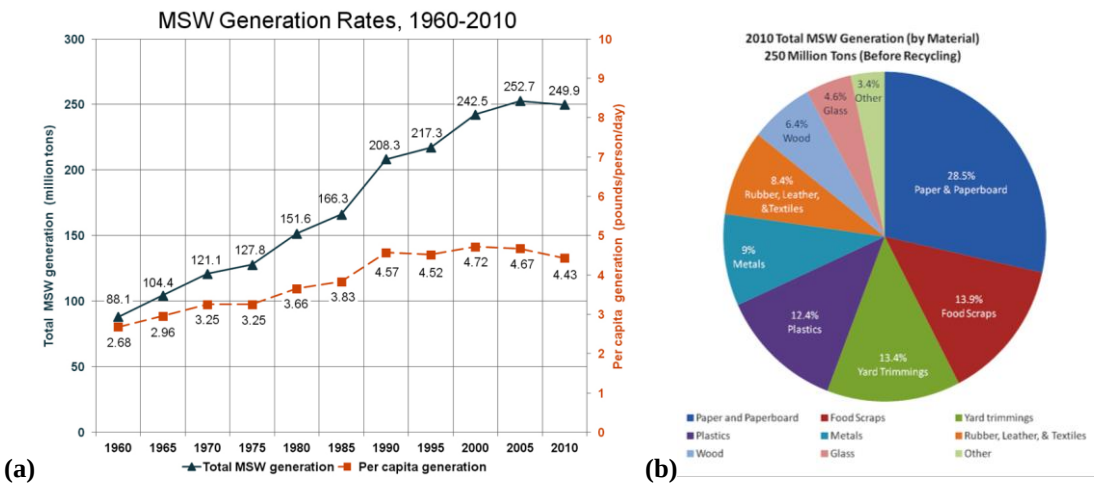


Figure 1: Municipal solid waste generation in the U.S. (a) generation rates from 1960-2010 (b) Total generation by material, 2010 (U.S. EPA 2011)

In addition to MSW, agricultural waste, which consists primarily of crop residues and livestock manure, is also increasing and represents a significant source of lignocellulosic biomass. Worldwide, more than 140 billion tons of waste agricultural biomass is generated annually, with an energy content equivalent to approximately 50 billion tons of oil (United Nations Environment Programme 2009). In the United States, waste crop residues, which make up the majority of agricultural waste, exceeded 350 million dry tons per year, with corn stover making-up about 70% of this total. Since crop residue generation is directly related to crop yields, these numbers are expected to increase. The UDSA projects yields for corn and wheat to increase by approximately 9.5% and 5.2% respectively, over the next ten years. According to the 2012 Billion Ton Update, the total amount of sustainably harvestable agricultural wastes will exceed 150 million dry tons in 2012 and is expected to increase over the next 20 years. This is shown in Figure 2 (U.S. Department of Energy 2011).

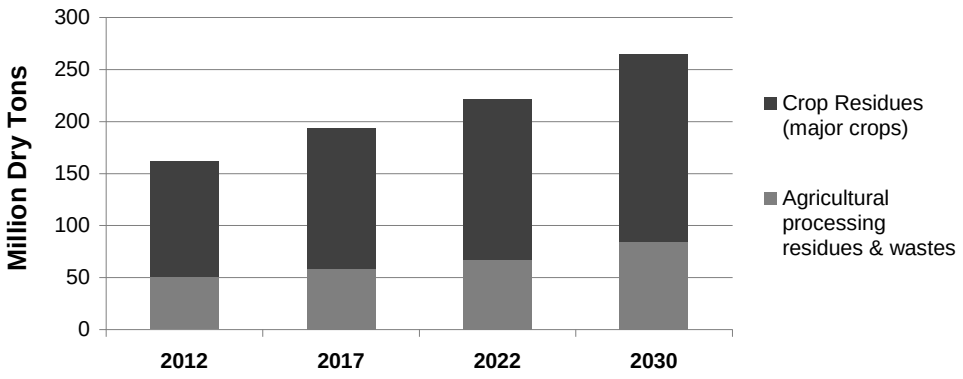


Figure 2: Predicted total annual quantity of sustainably harvestable agricultural residues and waste (<$60 per dry ton) for 2012 to 2030 (U.S. Department of Energy 2011)

Solid organic waste generation from industry, including the food and wood processing industries, is also significant and again consists in large part of lignocellulosic material. In the food processing industry, about 55 million tons of food waste is generated annually in the United States (Gustavsson, Cederberg et al. 2011). In addition, the forest and wood processing industries are major generators of lignocellulosic waste. According to the 2012 Billion Ton Update, approximately 97 million dry tons of forest and wood waste resources will be generated in the United States in 2012, with an expected increase to 102 million dry tons by 2030. In short, lignocellulosic material makes-up a majority of solid organic waste, and as waste generation rates increase methods for effectively managing organic, lignocellulosic waste are critical.

Current Solid Waste Management Practices

Current solid waste management practices have been linked to several negative environmental impacts. Landfilling, which is the primary method for waste disposal in most countries, has been identified as the largest contributor to greenhouse gas emissions (GHG) within the waste sector (United Nations Environment Programme 2010). Of the total MSW generated in the United States, 54.2 percent was disposed of in landfills, generating approximately 16.2 percent of total U.S. anthropogenic methane emissions in

2010, the third largest contribution of any methane source in the United States (U.S. EPA 2010). Landfilling also contributes to water pollution as a result of landfill run-off and leaching, decreased land value surrounding landfill sites, and odor. Landfills with gas capture systems can significantly reduce GHG emissions from landfills if the methane is used to displace fossil fuel-derived energy. However, gas capture systems are surprisingly underutilized and do not capture all of the methane produced. In the United States, there are over 3,000 active landfills with just 500 having gas capture systems (Kelleher 2007), with typical capture efficiencies ranging from 50-80% (United Nations Environment Programme 2010). Incineration is the second largest contributor to GHG emissions from the waste sector contributing an estimated 40 Mt CO_2 equivalent globally compared to the 700 Mt CO_2 equivalent estimated to be generated from landfilling (United Nations Environment Programme 2010). Part of the savings in GHG emissions from incineration is a result of the displacement of fossil fuel-derived energy with the energy harvested from the waste. However, incineration is relatively expensive and it has been found that for wastes that are readily biodegradable, the GHG savings from anaerobic digestion with energy recovery outweighs that of incineration with energy recovery as well as other thermal processing methods (United Nations Environment Programme 2010). In general, biological treatment of the organic fraction of solid waste (i.e. composting or anaerobic digestion) can significantly reduce GHG emissions and provide a method for nutrient recycling. For example, according to Brown et al, a facility that composts an equal mixture of manure, newsprint, and food waste could conserve the equivalent of 3.1 ton CO_2 equivalent per ton of dry feedstock compared to landfilling without gas capture. A drawback to composting, however, is the lack of energy recovery. In the UK, Europe, and Australia, anaerobic digestion is replacing landfilling as a treatment method for MSW. This is largely a result of legislations that have been put in place to limit landfilling, as well as limited space availability and increasing cost.

Anaerobic digestion, a biological process which converts organic matter into a methane rich biogas, offers many benefits as a solid waste treatment method, including reduced GHG emissions, production of a renewable energy source, and generation of a nutrient rich effluent which can be utilized as fertilizer and/or for the production of other valuable byproducts. However, in the United States where landfilling is still the cheapest waste disposal option, improvements in anaerobic digestion process efficiencies are necessary in order for anaerobic digestion to become a more economically viable option.

Current Status of Anaerobic Digestion for Solid Waste Management

Anaerobic digestion is a proven waste treatment technology that is used for various applications around the world. In many developing countries, simple anaerobic digestion systems are used to produce energy for cooking, heating, and lighting. In Europe, largely due to legislations limiting landfilling in order to reduce GHG emissions, more than 9,000 anaerobic digesters are in operation treating agricultural, industrial and MSWs (Center for Climate and Energy Solutions 2011). In the United States, where landfilling is still the cheapest solid waste management option, the majority of anaerobic digesters, approximately 1,500, are found in the wastewater sector (American Biogas Council 2012). Outside of wastewater treatment, anaerobic digestion is most commonly used for livestock manure management. An example of a typical anaerobic digestion scheme for treating livestock manure is shown in Figure 3.

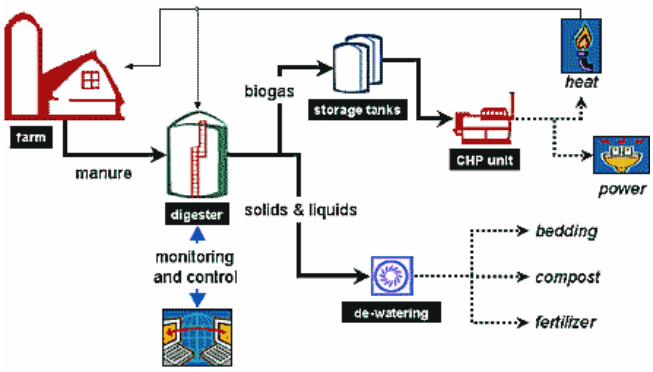


Figure 3: Typical Anaerobic Digestion Process Scheme (Renewable Energy Institute 2003)

Currently, there are at least 176 anaerobic digesters in operation for treating livestock manure in United States, with approximately 16 new digesters coming online each year (Figure 4a) (U.S. EPA 2011). In 2011, these digesters produced the equivalent of approximately 541 million kilowatt-hours (kWh) of useable energy, directly reducing GHG by 1.2 million metric tons CO_2 equivalent and avoiding 301,000 metric tons of CO_2 equivalent by displacing fossil fuels with captured methane (Figure 4b). The U.S. EPA estimates that anaerobic digestion is feasible on over 8,000 farms in the United States (U.S. EPA 2011). Therefore, the benefits from anaerobic digestion of livestock manure that have been achieved thus far only represent a fraction of the potential GHG savings and energy production that can be achieved through utilization of anaerobic digestion technology for solid waste management.

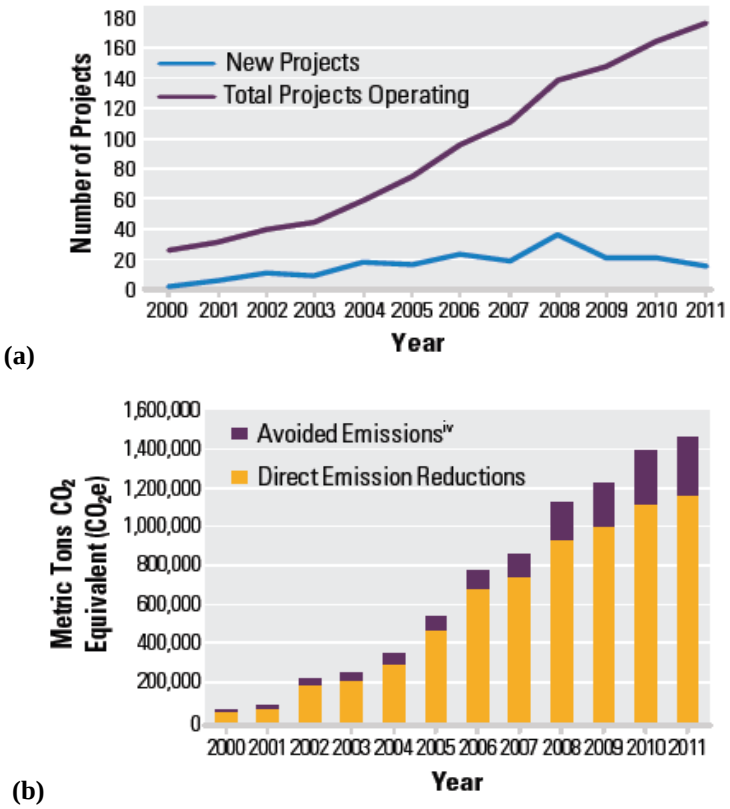


Figure 4: (a) Number of anaerobic digesters in the U.S. (b) Trends in greenhouse gas emissions reductions from anaerobic digestion in U.S. (U.S. EPA 2011)

Bottlenecks in Anaerobic Digestion

While anaerobic digestion presents a sustainable, attractive waste treatment option, there are certain bottlenecks that must be overcome in order to improve its efficiency and economic viability. High capital costs make anaerobic digestion economically unattractive and therefore, methods for increasing digestion rates and reducing reactor volumes are necessary to decrease capital costs. The major bottleneck in anaerobic digestion is conversion of lignocellulosic wastes. Due to its complex biochemical structure, lignocellulosic biomass is extremely slow to degrade, and it has been well recognized that for anaerobic digestion, hydrolysis of lignocellulosic material is typically the rate-limiting step (Mumme, Linke et al. 2010, Park, Lee et al. 2005). Thus, methods for increasing rates of hydrolysis are necessary in order to improve process efficiency. Other bottlenecks associated with anaerobic digestion include long reactor start-up times and process instability due to the slow growth rates and environmental sensitivity of the anaerobic microorganisms involved in the process. Therefore, methods for achieving and maintaining effective, robust microbial communities are needed to ensure stable performance with high process efficiencies.

2.2 The Anaerobic Digestion Process

Anaerobic digestion is a process in which organic matter is degraded by a consortium of microorganisms in the absence of oxygen to produce a mixture of methane and carbon dioxide called biogas. In this process, complex particulate organic material is broken down into simpler soluble compounds which are taken up by microbial cells and ultimately converted into methane and carbon dioxide. Volatile fatty acids (VFAs), alcohols and hydrogen are generated as intermediate products. The process consists of four subsequent phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Figure 5 outlines the four phases of anaerobic digestion and the pathways by which

particulate organic matter is converted to methane and carbon dioxide. Percent conversion of the volatile solids fraction of the substrate is also shown. Each phase is carried out by a different group of microorganisms, which exist in syntrophy with one another, but differ in terms of their growth kinetics and optimal environment requirements. Two-phase anaerobic digestion offers a method for optimizing reactor conditions to accommodate the different groups of microorganisms, by physically separating the process into two reactors. In this case, the four phases are broadly grouped into two phases namely the acid-phase and the methane-phase. Each of the four phases of anaerobic digestion, as well as important operating parameters associated with the process, and the concept of two-phase anaerobic digestion is described further in the following sections.

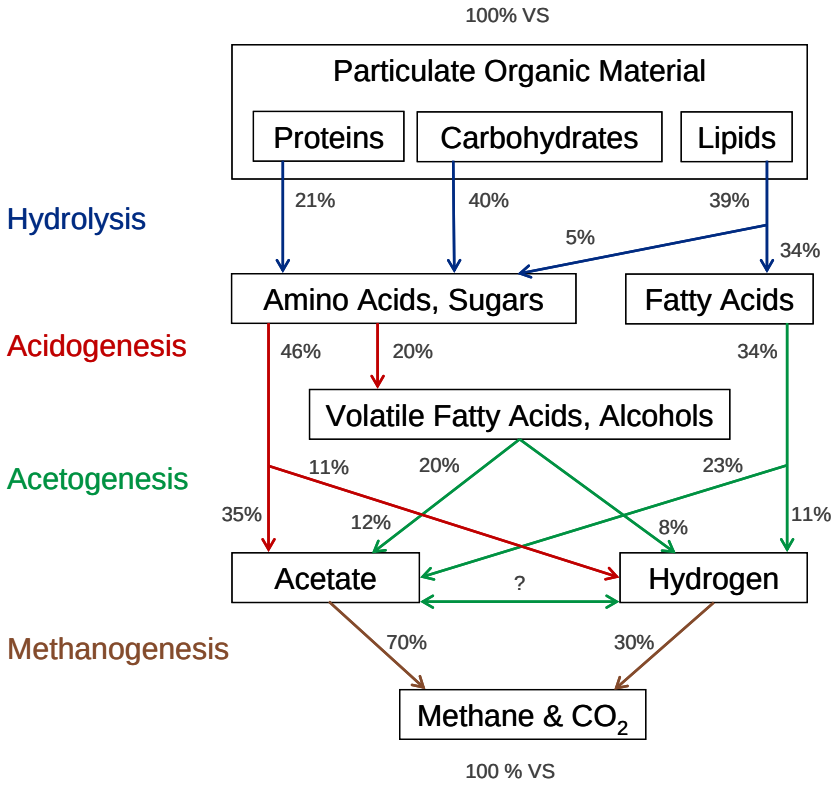


Figure 5: Phases and conversion pathways in anaerobic digestion (Gujer, Zehnder 1983)

Phases of Anaerobic Digestion

Hydrolysis

The first phase of anaerobic digestion is hydrolysis. In this phase, complex particulate organic matter is broken down into smaller water soluble compounds, which can be taken up by microbial cells. Complex macromolecules including carbohydrates, proteins, and fats, are converted into sugars, amino acids, and fatty acids respectively. This occurs via enzymatic hydrolysis, in which various facultative and/or obligate anaerobic hydrolytic bacteria excrete exoenzymes which facilitate the splitting of covalent bonds within the substrate in a chemical reaction with water (Chandra, Takeuchi et al. 2012). The enzymes involved in hydrolysis are called hydrolases. Different hydrolases are produced by specific species of hydrolytic bacteria are required for degrading different macromolecules. For example cellulolytic bacteria produce cellulases for the hydrolysis of cellulose, while lipolytic bacteria produce lipases for the hydrolysis of lipid molecules. Hydrolysis of non-structural carbohydrates occurs relatively quickly, on the order of a few hours, while hydrolysis of proteins and lipids can take up to a few days. Structural carbohydrates, including cellulose and hemicellulose are the most difficult to hydrolyze, and conversion of these molecules tends to be extremely slow and incomplete (Chandra, Takeuchi et al. 2012). As a result, hydrolysis of lignocellulosic material represents a significant rate-limiting step in anaerobic digestion.

Acidogenesis

The second phase of anaerobic digestion is acidogenesis. In this phase, sugars, amino acids, and fatty acids produced in the hydrolysis phase are taken up by various acid forming bacteria (acidogens) and converted into VFAs (e.g. butyric acid, propionic acid, acetate and acetic acid), as well as alcohols, hydrogen, and carbon dioxide. The products

formed in this phase will vary depending on the bacteria present and environmental conditions. The acidogenic bacterial community may include facultative and/or obligate anaerobic bacteria. Examples include *Bacteriodes, Bifidobacterium, Clostridium, Lactobacillus,* and *Streptococcus*. In general acidogens are relatively fast growing microorganisms. This can present a potential problem if acidogens are able to grow and generate VFAs faster than they can be converted to methane. Acidic conditions are toxic to methanogens, therefore accumulation of VFAs will likely cause inhibition of methanogenesis and potentially lead to reactor failure.

Acetogenesis

The third phase of anaerobic digestion is acetogenesis. In this phase, VFAs, alcohols, and hydrogen produced in the acidogenesis phase are converted to acetate via acetogenic bacteria. Two groups of acetogenic bacteria play a role in anaerobic digestion. Under heterotrophic growth, both groups consume VFAs and alcohols, generating acetate and hydrogen as end products (Chandra, Takeuchi et al. 2012). Homoacetogenic bacteria are capable of both heterotrophic and autotrophic growth. Under autotrophic growth, these acetogenic bacteria consume hydrogen and carbon dioxide to produce acetate (Ryan, Forbes et al. 2010). Acetogenic bacteria are obligate anaerobes that can tolerate a wide range of environmental conditions (Zaher, Cheong et al. 2007). Potential rate limiting steps associated with acetogenesis include competition between acetogens and sulfate reducing bacteria for hydrogen, and insufficient generation of acetate due to low populations of acetogenic bacteria.

Methanogenesis

The last phase of anaerobic digestion is methanogenesis. In this phase, methane is formed under strictly anaerobic conditions via various species of methanogenic archea.

There are two major pathways for methane formation in anaerobic digestion. The primary route is conversion of acetate to methane and carbon dioxide. This is carried out by acetoclastic methanogens. The second route is carried out by hydrogenophilic methanogens in which hydrogen is used to reduce carbon dioxide to methane (Zaher, Cheong et al. 2007). Approximately 72% of available volatile solids are converted to methane via acetoclastic methanogenesis, while the remaining 28% is converted via hydrogenotrophic methanogenesis. Methanogenesis can be rate limiting due to the slow growth rates of methanogenic archea. Slow growth rates make methanogens prone to wash-out. In addition, as mentioned in the discussion of acidogenesis, if methanogens are not able to keep pace with VFA generation, acid accumulation can lead to process inhibition and potential reactor failure. Accumulation of other toxic compounds such as ammonia, or hydrogen sulfide can also inhibit methanogenesis. Table 1 below summarizes the phases of anaerobic digestion.

Table 1: Summary of phases of anaerobic digestion (partially adapted from Hunt, MS Thesis)

Phase	Description	Microorganism	Potential Limitations	Two-phase classification
Hydrolysis	Particulate organic material hydrolyzed to soluble compounds	Hydrolytic bacteria	Hydrolysis of lignocellulose	Acid-phase
Acidogenesis	Soluble compounds converted to VFAs, alcohols, H_2, and CO_2	Acidogenic bacteria	Acid accumulation	
Acetogenesis	Conversion of fatty acids and H_2 and CO_2 to acetate	Acetogenic bacteria	Competition with sulfate reducers, low generation of acetate	Methane-phase
Methanogenesis	Conversion of acetate and H_2 and CO_2 to methane	Methanogenic archea	Slow growth rates, wash-out, inhibition due to toxin build-up	

Operating Parameters

The complete process of anaerobic digestion requires the complex interactions of various groups of microorganisms, which must be properly balanced in order to maintain stable reactor performance. With that, there are several operating parameters that can effect microbial activity and potentially disturb reactor equilibrium, resulting in process inhibition and possible reactor failure. Effective management of the following operating parameters is essential for achieving optimum digester performance.

Retention Time

Retention time is a measure of the time that the substrate spends in the reactor and is a significant parameter in terms of conversion efficiency. In typical continuous stirred tank anaerobic digestion systems the solids retention time (SRT) is equal to the hydraulic retention time (HRT). In this case, the extent to which volatile solids in the substrate are converted to biogas is ultimately controlled by HRT. Longer HRTs allow for higher total volatile solids reduction and thereby higher biogas yields. However, shorter retention times are desired in order to reduce system costs and increase process efficiency. As shown in Equation 1 below, HRT is directly related to reactor volume, therefore, for a given influent flow rate, shorter HRTs can allow for smaller reactor volumes, thereby reducing capital cost. In the same respect, for a given substrate and reactor volume, if digestion rates can be increased such that a high degree of conversion to methane can be achieved at shorter HRTs, greater amounts of substrate can be processed in a given period of time, increasing process efficiency.

$$HRT = (V)/(Q)$$

Equation 1

where V is reactor volume and Q is influent flow rate

HRT will vary depending on substrate characteristics. Substrates containing high amounts of lignocellulose will require relatively long HRTs. Studies have indicated digestion times as long as 60-90 days are required in order to achieve nearly complete digestion of lignocellulosic substrates (Rivard, Bordeaux et al. 1988). Conventional anaerobic digestion processes operate at an HRT in the range of 15-30 days (USDA 2009).

While shorter HRTs are desirable, HRT is limited to some extent by microbial regeneration rates. Methanogens are relatively slow growers and require an HRT of at least 10-15 days in order to avoid wash-out from the reactor. A strategy to overcome this limitation is the addition of attached growth media, which can provide additional surface area within the reactor for attached microbial growth, as well as a mechanism for keeping the microbial biomass in the system. This allows for a shortening of the HRT while maintaining the longer solids retention time (SRT) required to avoid wash-out. A second strategy is the use of membranes to filter the effluent as it exits the reactor, allowing only soluble compounds to leave the system. Finally, due to the slow regeneration time of methanogens, longer HRTs are typically required during reactor start-up in order to allow the inoculum sludge enough time to reach a steady-state population (Chandra, Takeuchi et al. 2012).

pH

Due to the formation of different intermediates, pH will vary within each phase of anaerobic digestion. At the same time, the different microbial groups involved in each phase require different pH conditions for optimum growth. In general, hydrolytic and acidogenic bacteria prefer slightly acidic conditions near pH 6. Optimal pH for acidogens has been reported in the ranges of pH 5.5 to 6.5 (Khalid, Arshad et al. 2011) and 5.8 to 6.2 (Zoetemeyer, Vandenheuvel et al. 1982) . In contrast, acidic conditions are toxic to

methanogenic bacteria, which prefer neutral conditions in the range of pH 6.5 to 8.2 (Khalid, Arshad et al. 2011). As a result, acid accumulation is one of the biggest potentials for anaerobic digester failure. In a properly balanced reactor, pH is buffered through the generation of bicarbonate by methanogens (Zaher, Cheong et al. 2007) . Providing excess alkalinity or implementing pH control can safe guard against excess acid accumulation.

Temperature

Anaerobic digestion can occur under a variety of temperatures depending on the species of microorganisms employed. In general, microorganisms are divided into the following three groups depending on their optimal growth temperature: psychrophilic (10-20°C), mesophilic (30-40°C) and thermophilic (50-60°C). Most conventional anaerobic digestion processes occur under mesophilic temperatures. Operation under mesophilic conditions is more stable and requires less energy input compared to operation under thermophilic conditions, and results in a higher degree of digestion compared to operation under psychrophilic conditions (Khalid, Arshad et al. 2011, Chandra, Takeuchi et al. 2012) . Within each temperature range, fluctuations in temperature by even a few degrees can affect microbial activity. Chae et al (2008) found that a fluctuation from 35 to 30˚C caused a significant reduction in biogas production rates. Therefore is important that temperature is maintained constant and uniform throughout the digestion process.

Carbon to Nitrogen Ratio

The carbon to nitrogen ratio (C/N) refers to the relative amounts of elemental carbon and nitrogen present in the substrate. In general, a C/N ratio of 20-30 is considered optimal for anaerobic digestion (Chandra, Takeuchi et al. 2012, Zaher, Cheong et al.

2007). Substrates with high C/N ratios, such as paper and most crop residues will be deficient in nitrogen, which is an essential nutrient for microbial cell growth. Thus, anaerobic digestion of very high C/N ratios may be limited by nitrogen availability. In the case of substrates with low C/N ratios, such as some animal manure, toxic ammonia build-up may become a problem. To overcome deficiencies in either carbon or nitrogen, co-digestion of low C/N materials with high C/N materials has been proven an effective solution (Hartmann, Ahring 2005).

Organic Loading Rate

Organic loading rate (OLR) is defined as the amount of volatile solids or chemical oxygen demand fed to the system per unit volume per time. Higher OLRs can allow for smaller reactor volumes thereby reducing the associated capital cost. However, at high OLRs there is a danger in overloading the reactor, especially during reactor start-up. Also, at high OLRs, retention times must be long enough such that the microorganisms have enough time to sufficiently degrade the material. Thus, there is a balance between OLR and HRT that must be determined in order to optimize digestion efficiency and reactor volume.

Mixing

In order to achieve biological degradation, enzymes and microorganisms must come in contact with the substrate. Therefore, proper mixing is important in order to achieve efficient mass transfer between substrate and microorganisms in the reactor. Mixing is also important in terms of heat transfer and temperature control. Effective mixing can be achieved through a variety of methods including the use of mechanical mixers, recirculation of digester contents, or recirculation of biogas. In general, results from existing anaerobic digestion systems have shown that some level of mixing is necessary

to maintain process stability within the reactor (Zaher, Cheong et al. 2007). At the same time, over-mixing or excessive mixing can disrupt the anaerobic microbes, and therefore consideration must be taken in terms of intensity and duration of mixing. Some research has shown that gentle or slow mixing may improve anaerobic digester performance (Vavilin 2004, Chen, Chynoweth et al. 1990).

Two-Phase Anaerobic Digestion

In conventional anaerobic digestion, a single reactor is used in which all four phases of the process take place. In this situation, because the hydrolytic and acid forming bacteria differ from the methane-forming bacteria in terms of their nutritional needs, environmental conditions, growth kinetics and sensitivity, a delicate balance must be maintained within the reactor in order to in avoid system failure. With that, conventional single-phase operation can be prone to up-sets. Problems with stability and control in single-phase digestion have motivated research in the area of two-phase anaerobic digestion.

Two-phase anaerobic digestion offers a method for optimizing the operating conditions for the various groups of microorganisms involved in the digestion process. In two-phase digestion, the process is physically separated into two reactors. The first reactor is operated under optimal conditions for hydrolysis and acidogenesis and is referred to as the acid-phase reactor, while the second reactor is operated under optimal conditions for methanogenesis and is referred to as the methane-phase reactor. In this case, pH and temperature conditions can be maintained at appropriate levels in either reactor. Two-phase digestion can also increase process stability by optimizing the HRT for either phase of the process. Typically, HRT is shorter in the acid-phase and longer in the methane-phase to accommodate for the variation in growth rate between the rapidly regenerating acidogens and slow growing methanogens. This can help prevent organic

overloading or toxic acid build-up in the methane-phase (Demirer, Chen 2005). Shorter HRTs in the acid-phase also allow for a smaller reactor volume which can reduce capital costs. Finally, two-phase operation allows for the selection and enrichment of different bacteria in each phase (Demirer, Chen 2005).

Several studies have demonstrated the advantages of two-phase digestion over single-phase digestion. In general, two-phase digestion has been successful in treating a wide range of substrates including, but not limited to, domestic and industrial wastewaters (Van Lier, Rebac et al. 1997, Ghosh 1985, Ng 1985, Yushina, Hasegawa 1994, Gharsallah 1994, Massey 1978), municipal solid sludge (Bhattacharya 1996, Ghosh 1987, Kugel, Zingler et al. 1992), food processing wastes (Cohen et al, Lee et al, Raynal et al) the organic fraction of municipal solid waste (Cecchi et al, Hooper and Li, Pavan et al), forest residues (Hooper and Li) and wood hydrolysate (Chakrabarti, et al). Zhang et al compared single- and two-phase processes in terms of bacterial population levels and observed the number of acetate-utilizing methanogens was 2-10 times higher in the two-phase system than in the single-phase system. In two-phase digestion of soft-drink waste, Ghosh et al were able to achieve higher methane production and COD removal at lower HRT and higher loadings compared to conventional single-phase digestion. Similarly, Yeoh observed a threefold increase in methane yield from two-phase digestion of cane-molasses alcohol stillage compared to single-phase digestion. In general, Ghosh reported that two-phase anaerobic digestion of municipal solid sludge resulted in higher efficiencies and rates compared to conventional single-stage digestion at both mesophilic and thermophilic temperatures as well as at a variety of HRTs, loading rates, and feed concentrations. Improvement of cellulose hydrolysis and conversion efficiency as a result of two-phase digestion has been demonstrated by (Khan, Miller et al. 1986, Bavvay, Hashimoto 1984)Khan et al., 1983, Baccay and Hashimoto, 1984, and Koster 1984.

It should be noted that two-phase anaerobic digestion is not the same as two-stage anaerobic digestion. In the case of two-stage anaerobic digestion, two separate reactors are utilized, but there is no physical separation of the acid or methane phases. All four phases of the anaerobic digestion process occur in both reactors, and the system is essentially two single-phase reactors in series. Two-stage anaerobic digestion can be operated such that the first reactor is maintained at thermophilic temperatures and the second at mesophilic temperatures. This is referred to as temperature-phase anaerobic digestion. Schmit et al compared temperature-phase digestion to two-phase digestion in treating a mixture of primary wastewater sludge (PS) and the organic fraction of municipal solid waste (OFMSW). The authors observed an increase in methane production from temperature-phase digestion compared to two-phase digestion at OFMSW to PS concentrations of 0:100, 20:80, and 40:60. However, at OFMSW to PS concentrations of 60:40, and 80:20, the two processes produced comparable results. Thus, the benefits of two-phase compared to temperature-phase anaerobic digestion may depend on substrate characteristics. In either case, two-phase or temperature-stage anaerobic digestion have their pros and cons. Both treatments have been shown to significantly improve volatile solids degradation over conventional single-stage digestion due to increased rates of hydrolysis in the first phase/stage (Schmit, Ellis 2001). The major drawback to these processes is the added costs associated with the addition of a second reactor, including capital cost and energy costs for heating and mixing. Therefore the gains in process efficiency as a result of adding the second phase/stage must justify the added cost.

2.3 Conversion of Lignocellulosic Biomass

With an estimated annual production of $1x10^{10}$ million tons (Sanchez, Cardona 2008) lignocellulose is the world's most abundant renewable biomass resource and the major constituent of solid organic waste. The three major components of lignocellulosic biomass include cellulose, hemicellulose, and lignin. Water is also present, as well as other minor components such as pectin, protein, non-structural carbohydrates, waxes and minerals (Chandra, Takeuchi et al. 2012, Harmsen, Wouter et al. 2010). In general, the conversion of lignocellulosic material to biogas requires the release of cellulose from the complex lignocellulosic matrix, and subsequent enzymatic hydrolysis of the long-chain polysaccharides into their component 5- and 6-carbon sugars. This process represents a significant challenge due to the inherent recalcitrant nature of the lignocellulosic matrix. As a result, pretreatment to disrupt the lignin and/or hemicellulose bonds with cellulose is a common practice in order to make cellulose more accessible for microbial and enzymatic attack. The structure of lignocellulosic biomass, common pretreatment methods, and microbial enzymatic hydrolysis are discussed in the following sections.

Structure of Lignocellulosic Biomass

Cellulose is the major structural component of plant cell walls typically making up 35 to 50 percent (Takara, Khanal 2012). Cellulose is a linear polysaccharide polymer of glucose molecule linked together via β-1,4 glucosidic bonds. The nature of the β-1,4 glucosidic bond allows the polymer to exist in long straight chains. The degree of polymerization of cellulose, which refers to the number of glucose units making up one polymer molecule, can range from 800-10,000 units (Harmsen, Wouter et al. 2010). Cellulose exists in both an unorganized amorphous form and organized crystalline form. Crystalline cellulose is the most abundant form of cellulose within the cell wall, and less susceptible to enzymatic degradation than amorphous cellulose (Chandra, Takeuchi et al.

2012). In crystalline cellulose, several cellulose polymers are linked together in parallel via hydrogen bonds to form polymer chains (Figure 6), which coalesce to form cellulose microfibrils.

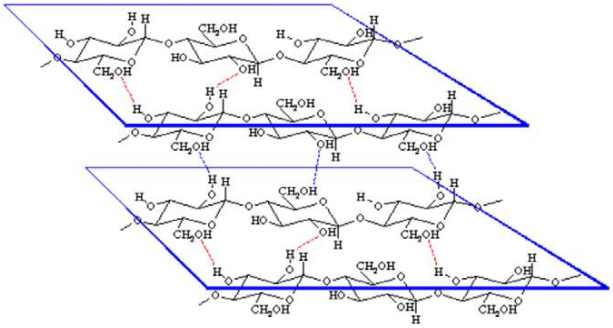


Figure 6: Illustration of bonding between cellulose polymers to for crystalline cellulose (Harmsen, Wouter et al. 2010)

Hemicellulose is the second most abundant structural component of plant cell walls making up 20 to 35 percent (Takara, Khanal 2012). Several cellulose microfibrils are linked together via hydrogen bonds with hemicellulose to form cellulose macrofibrils (fibers). Hemicellulose exists as amorphous polysaccharides of various 5- and 6- carbon sugars, including arabion-xylans, gluco-mannans, galactans, and others. The family of polysaccharides most common to hemicellulose is xylan, which is composed primarily of the 5-carbon sugar xylose and some 6-carbon sugars such as glucose. The structure of xylan hemicellulose is show in Figure 7 below. The degree of polymerization of hemicellulose is much less than that seen for cellulose, and typically does not exceed 200 units (Harmsen, Wouter et al. 2010).

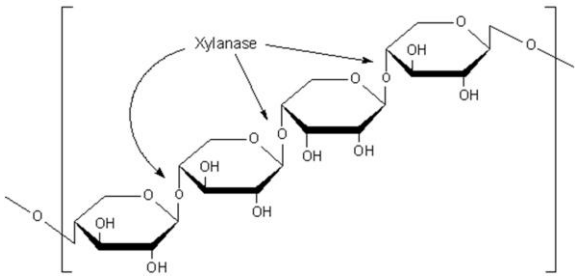


Figure 7: Xylan Hemicellulose (Sigma-Aldrich 2011)

Lignin is the third structural component of plant cell walls making up 10 to 25 percent (Takara, Khanal 2012). It exists as a complex, three-dimensional amorphous polymer composed of various phenolic monomer units (Chandra, Takeuchi et al. 2012, Harmsen, Wouter et al. 2010). Lignin is essentially the glue within the lignocellulosic matrix, binding with cellulose and hemicellulose via a variety of chemical bonds to provide structural support as well as a protective barrier making the plant resistant to chemical and biological degradation. As a result, removal of lignin via pretreatment prior to hydrolysis is necessary in order for cellulose and hemicellulose to be accessible to the microorganisms and enzymes. Figure 8 below illustrates the interactions of cellulose, hemicellulose, and lignin.

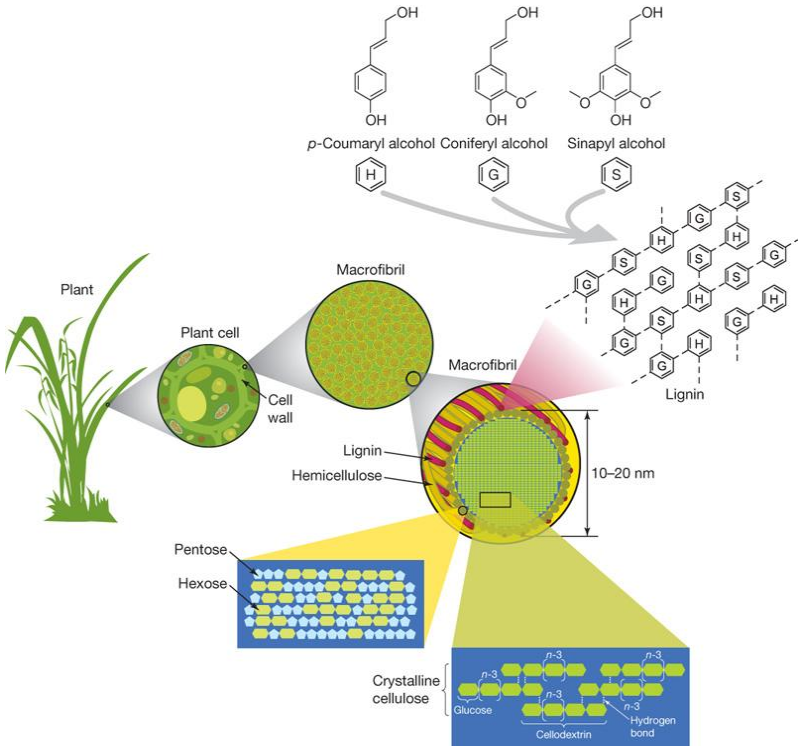


Figure 8: Schematic illustration of the interactions of cellulose, hemicellulose, and lignin in lignocellulosic material (Rubin 2008)

Pretreatment Methods

The goal of pretreatment is to overcome various substrate-related factors that limit the availability of cellulose to enzymatic hydrolysis. In general, possible aims of pretreatment should be disruption of interpolymer linkages (i.e. lignin-cellulose, lignin-hemicellulose, hemicellulose-cellulose), reduction of the degree of polymerization and crystallinity of cellulose, and increasing of surface area and porosity. In this manner, cellulose can be more easily accessible for enzymatic hydrolysis, allowing for increased rates and extent of conversion. In addition, an effective pretreatment method should avoid the degradation or loss of carbohydrate, avoid the formation of toxic by-products, and be cost-effective. Figure 9 below illustrates the basic role of pretreatment.

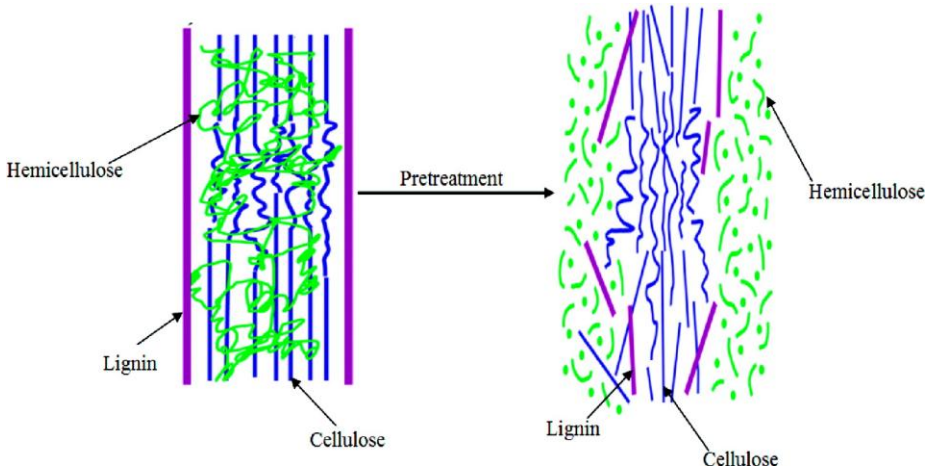


Figure 9: Schematic illustration of the role of pretreatment on lignocellulosic biomass (Kumar, Barrett et al. 2009)

In general, pretreatment methods are separated into three categories including physical, chemical, and biological pretreatment. A description of several common pretreatment methods is given in Table 2 below. Each method has some advantages and disadvantages which must be considered in order to optimize process efficiency. One disadvantage that is associated with several pretreatment methods is the formation of non-carbohydrate compounds such as lignin polymers/oligomers and other lignin derivatives that are toxic to the fermentation microbes involved in ethanol production. In

general, these compounds are left in the hydrolysate after pretreatment, which is then discarded due to its toxicity in ethanol fermentation. However, in the context of anaerobic digestion, it has been shown that these compounds can be successfully converted to biomethane (Barakat, Monlau et al. 2012). In which case, both the solid and liquid fractions resulting from pretreatment can be utilized for biomethane production via anaerobic digestion. This could potentially result in higher energy recovery from anaerobic digestion compared to ethanol production as many pretreatment processes result in a loss of hemicellulose, which is solubilized to xylose and removed with the hydrolysate after pretreatment. Thus, through anaerobic digestion, the energy content of xylose- and lignin- derived compounds can be recovered in addition to that of cellulose. Studies that have shown the anaerobic digestibility of pretreatment hydrolysate and byproducts include (Barakat, Monlau et al. 2012, Fox, Noike et al. 2003, Fedorak, Hrudey 1984). Figure 10 compares the paths for conversion of lignocellulosic biomass to biomethane versus bioethanol after pretreatment.

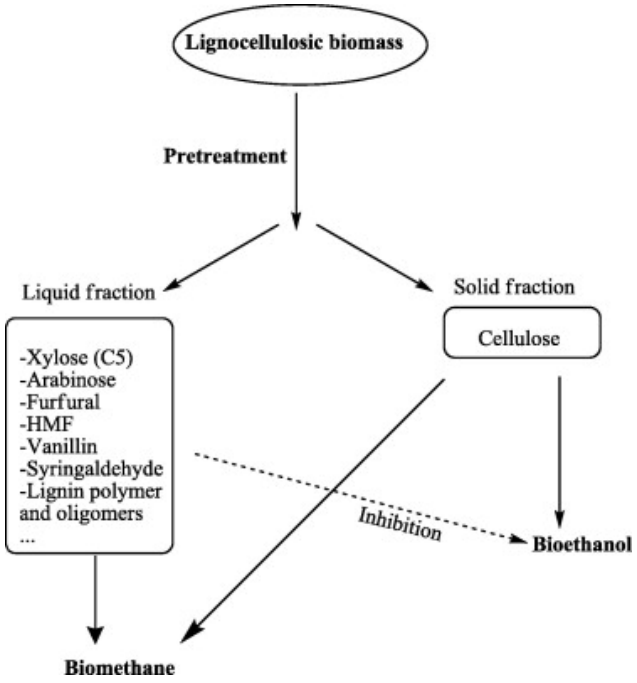


Figure 10: Conversion routes for pretreated lignocellulosic biomass (Barakat, Monlau et al. 2012)

Table 2: Some common pretreatment methods for lignocellulosic biomass

Pretreatment Method	Description	Advantages	Disadvantages	Ref.
Physical:				
Mechanical comminution	Physical reduction in substrate particle size (i.e. grinding, milling, etc)	- Reduced cellulose crystallinity and degree of polymerization - Increased surface area	- Usually negative energy balance	1 2 3 4
Irradiation	Biomass undergoes high energy radiation (i.e. γ-ray, ultrasound, electron beam, pulsed electrical field, UV, microwave heating)	Results in one or more changes to biomass: - Increased surface area - Reduced cellulose crystallinity and polymerization - Partial depolymerization of lignin	- Slow - Energy intensive - Prohibitively expensive	2 3 4
Steam explosion	Substrate particles rapidly heated by high-pressure saturated steam. Explosive decompression caused by quick release of pressure Acids released aid in hemicellulose hydrolysis	- Causes hemicellulose solubilization and lignin transformation - Cost effective	- Destruction of a portion of the xylan fraction - Generation of toxic compounds	1 2 4
Hydrothermal	Substrate is subject to high-temperature/high pressure water	- Hemicellulose solubilization - Partial delignification	- High water and energy demand	1 2 3 4
Chemical:				
Alkaline	Addition of base causes swelling, increasing internal surface of cellulose which provokes lignin structure disruption (NaOH, KOH, Lime, $Mg(OH)_2$, NH_4OH)	- Lignin solubilization - Reduced cellulose crystallinity and degree of polymerization - Increased surface area - Can be done at ambient temperature - Relatively inexpensive	- Relatively long residence times required - Irrecoverable salts formed and incorporated into biomass	1 2 3 4

Table 2: (cont.)

Acid	Addition of dilute or concentrated acid solutions results in hemicellulose hydrolysis (H_2SO_4, HCl, HNO_3, H_3PO_4)	- Hemicellulose hydrolysis and conversion to fermentable sugars - Alters lignin structure - With high acid concentrations can be done at room temp.	- Relatively expensive - Corrosive - High operational and maintenance costs - Some inhibitory compounds formed	1 2 3 4
Catalyzed steam explosion	Similar to steam explosion with addition of acid catalyst (SO_2, H_2SO_4, CO_2, oxalic acid)	- Hemicellulose solubilization	- Some inhibitory compounds formed - Portion of xylan fraction lost - Incomplete disruption of lignin-carbohydrate matrix	2 3 4
Ammonia fiber explosion (AFEX)	Substrate is exposed to hot liquid ammonia under high pressure. Pressure is released suddenly breaking open biomass structure	- Delignification - Increases surface area - Reduced cellulose crystallinity - Low formation of inhibitors	- Hemicellulose not significantly removed - Very high pressure requirements - Expensive	2 3 4
Wet Oxidation	Dissolved oxygen oxidizes substrate	- Efficient removal of lignin - Low formation of inhibitors - Exothermic	- High cost of oxygen and alkaline catalyst - High temps and pressures	3 4
Organo-solvent extraction	Organic solvents are applied, with or without addition of an acid or alkali catalyst to degrade internal lignin and hemicellulose bonds	- Delignification - Some hemicellulose solubilization - Recovery of relatively pure lignin as by-product	- Solvent removal is necessary - Relatively expensive	2 3 4
Biological:				
Fungi and Actimycetes	Microorganisms degrade/alter biomass structure (white-, brown-, soft-rot fungi, & bacteria)	- Degrades lignin and hemicellulose - Low energy consumption	- Low rate of hydrolysis	1 2 3

References: (1) Takara, et al 2012 (2) Zheng, et al 2009 (3) Khalid, et al, 2011 (4) Alvira, et al 2010

Enzymatic Hydrolysis and Cellulolytic Bacteria

In general, particulate organic matter including polymeric carbohydrates, lipids and proteins cannot be taken up by microbial cells. Therefore, microorganisms produce hydrolytic enzymes that catalyze the breakdown of these molecules into soluble monomers which can be transported through the cell membrane (Parawira 2012). This process is known as enzymatic hydrolysis.

In terms of lignocellulosic biomass, after a pretreatment method has been applied, to make cellulose more accessible, cellulolytic microorganisms produce an enzyme system or group of several enzymes called cellulases, which act synergistically to bind and cleave cellulose chains to produce glucose (Lynd, Weimer et al. 2002). Three major groups of cellulases are involved. First, endoglucanases cut at random amorphous regions within the cellulose chain, generating oligosaccharides of various lengths, and thereby creating new free chain-ends. Next, exoglucanases act on the free chain-ends, cutting in a progressive manner to remove either glucose or cellobiose as major products. Exoglucanases can also degrade microcrystalline cellulose, by peeling cellulose chains from the microcrystalline structure. Finally, β-glucosidases hydrolyze soluble cellobiose to glucose (Sun, Cheng 2002, Lynd, Weimer et al. 2002). Figure 11 provides an outlines of the process by which enzymatic hydrolysis of cellulose occurs.

Hemicellulose can also be degraded via enzymatic hydrolysis. Several different enzymes are involved in this process including glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannanase and glucomannanase (Sun, Cheng 2002). Many cellulolytic bacteria are able to produce at least some hemicellulases (Lynd, Weimer et al. 2002), and in general hemicellulose degradation and utilization varies depending on species and strain (Coen et al, 1970).

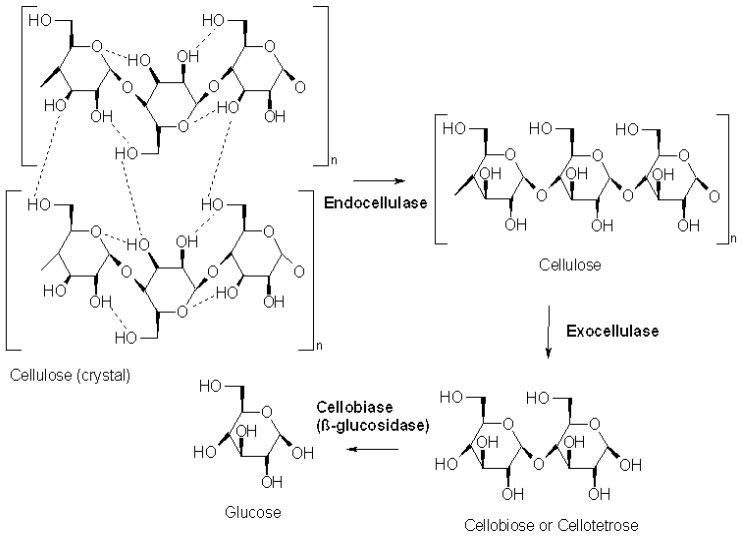


Figure 11: Enzymatic hydrolysis of crystalline cellulose (eNotes 2012)

Within the domain *Bacteria*, the ability to digest cellulose has been identified in a wide range of bacterial genera and species as well as different physiological groups including aerobic and anaerobic bacteria. Besides oxygen tolerance, a wide variation in temperature, pH, and salinity requirements for cellulolytic bacteria has also been observed, highlighting the wide distribution of cellulose in nature. Anaerobic cellulolytic bacteria can be found in a variety of habitats such as soil, sewage, hot springs and the intestines of ruminants and termites. Table 3 lists some of the predominantly known facultative and anaerobic cellulolytic genera. In some cases, only a few species within the listed genera may be actively cellulolytic. Some representative species are listed in the table. To this date, *Cellulomonas* is the only known genus to contain facultative anaerobic cellulolytic bacteria (Lynd, Weimer et al. 2002).

Table 3: Predominantly known Facultative and Anaerobic Cellulolytic Bacterial Genera (Adapted from Schwarz, 2003 and Lynd et al, 2002)

Genus	Example species	Oxygen Tolerance	Growth Temperature	Habitats
Acetivibrio	*D. cellulolyticus*	Anaerobic	Mesophilic	Sewage
Anaerocellum	*D. thermophilum*	Anaerobic	Thermophilic	
Butyrivibrio	*B. fibrisolvens*	Anaerobic	Mesophilic	Rumen
Caldicellulo-siruptor	*C. saccharolyticum*	Anaerobic	Thermophilic	Hot springs
Cellulomonas	*C. flavigena, C. uda*	Facultative	Thermophilic	Soil, sewage
Clostridium	*C. thermocellum, C. cellulolyticum*	Anaerobic	Thermophilic, Mesophilic	Soil, compost, rumen, sewage, manure
Eubacterium	*E. cellulosolvens*	Anaerobic	Mesophilic	Rumen
Fibrobacter	*F. succinogenes*	Anaerobic	Mesophilic	Rumen
Halocella	*H. cellulolytica*	Anaerobic	Mesophilic	
Ruminococcus	*R. albus, R. flavefaciens*	Anaerobic	Mesophilic	Rumen
Spirochaeta	*S. thermophila*	Anaerobic	Thermophilic	

Between aerobic and anaerobic bacteria there is a distinct difference in the strategies by which these two groups hydrolyze cellulose. Aerobic bacteria degrade cellulose by producing a significant amount of extracellular cellulases which are absorbed on to the cellulose. Therefore, for aerobic bacteria physical content is not necessary, and in fact, Kauri et al showed that separation of aerobic cellulolytic microbes from cellulose via an agar membrane enhanced cellulose utilization (Lynd, Weimer et al. 2002). In contrast, most anaerobic cellulolytic bacteria do not excrete measureable amounts of extracellular cellulases, and instead degrade cellulose via large cellulase complexes known as cellulosomes, which are attached on the outer side of their cell walls (Lynd, Weimer et al. 2002). As a result, adhesion to the substrate is generally required for optimum growth of anaerobic cellulolytic bacteria, and in some cases is obligate.

Cellulosomes consist of a large, non-catalytic scaffoldin protein which acts to organize the various cellulolytic enzyme subunits. Cohesion modules on the scaffoldin interact with dockerin modules on the enzymes to produce a stable enzyme complex that is firm yet flexible, allowing for a tight bond to both the bacterial cell wall and cellulose (Lynd, Weimer et al. 2002). Cellulosomes are described as fist-like structures that open upon attachment to cellulose, distributing local catalytic domains over the substrate. Cellulosomes are thought to enable optimum synergism among cellulases as well as localized and concerted enzyme activity. This keeps the resulting hydrolysis products in closer proximity to the cell allowing for efficient uptake and prevention of diffusion into the environment (Lynd, Weimer et al. 2002). Figure 12 below shows a schematic of the hydrolysis of cellulose by both cell-free extracellular enzyme excretion and cell-bound cellulosome systems.

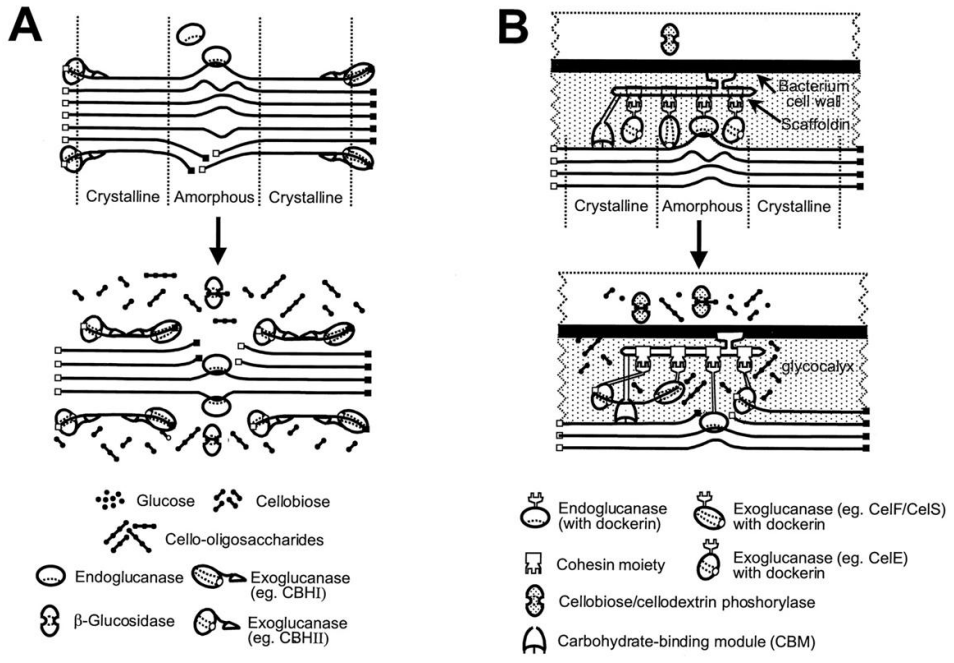


Figure 12: Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by A) cell-free extracellular enzyme excretion and B) cell-bound cellulosome systems (Lynd, Weimer et al. 2002)

2.4 Alkaline Pretreatment

Of the pretreatment methods listed in Table 3, alkaline pretreatment is one of the most commonly used and cost-effective options. Alkaline pretreatment is essentially a delignification process, in which a significant amount of hemicellulose may be solubilized as well (Yi 2009). Alkaline pretreatment affects the biomass by inducing swelling, causing an increase in the internal surface area of cellulose and disruption of the lignin structure. This process also results in a decrease in the degree of polymerization and crystallinity of cellulose (Alvira, Tomas Pejo et al. 2010, Kumar, Barrett et al. 2009, Yi 2009).

In comparison with other pretreatment technologies, alkaline pretreatment offers a less expensive option. In general, alkaline pretreatment requires lower temperatures and pressures than other pretreatment methods, and can even be applied effectively at ambient conditions, reducing the required energy input and operation costs. Application under mild conditions also offers the benefit of preventing lignin condensation which can result in low lignin solubility (Harmsen, Wouter et al. 2010). Under ambient conditions, alkaline pretreatment generally requires longer reaction times compared to other pretreatment methods, on the order of hours to days (Cui, Shi et al. 2012, Chandra, Takeuchi et al. 2012, Kumar, Barrett et al. 2009). Compared to other chemical pretreatments, alkaline pretreatment with lime is significantly less expensive in terms of chemical costs. Alkaline pretreatment has also been shown to be more effective for lignin solubilization, compared to acid and hydrothermal pretreatment, and results in less sugar loss than acid pretreatment (Carvalheiro, Duarte et al. 2008, Kumar, Barrett et al. 2009). A disadvantage to alkaline pretreatment is the generation of irrecoverable salts and/or the incorporation of salts into the biomass during pretreatment reactions (Yi 2009, Harmsen, Wouter et al. 2010).

Sodium, potassium, ammonium and calcium hydroxide are common alkaline pretreatment chemicals. NaOH has been reported to increase hardwood digestibility from 14-55%, through reduction of lignin content from 24-55% to 20% (Kumar, Barrett et al. 2009). In terms of the benefit to biogas production, He at al reported solid state pretreatment of rice straw with NaOH increased biogas production up to 64.5% compared to un-pretreated. Similarly, Pang et al reported a 48.5% increase in biogas yield from corn stover pretreated with 6% NaOH at 65 g/L organic loading rate. In general, pretreatment with lime has been shown to be less expensive than other bases per kilogram of hydroxide with less safety requirements compared to NaOH or KOH (Kumar, Barrett et al. 2009). In addition, lime can be easily recovered from the hydrolysate by reacting with CO_2, and can be regenerated using established lime kiln technology (Alvira, Tomas Pejo et al. 2010, Kumar, Barrett et al. 2009). Using lime, Playne et al found that pretreatment at ambient conditions for up to 192 hours enhanced enzyme digestibility of sugarcane bagasse from 20% to 72%. Under more extreme temperature conditions, 120°C for 4 hours, increased enzymatic hydrolysis of corn stover by factor of 9 compared to a non-pretreated condition. Corn stover pretreated with excess lime, in oxidative and non-oxidative conditions, at temperatures from 25-55C experienced 90% removal of acetyl groups within in approximately 1 week (Kim, Holtzapple 2006). The authors also found that maximum lignin removal from corn stover of 87.5% obtained at 55C after four weeks of lime pretreatment. In short, alkaline pretreatment is a well-established pretreatment method that can significantly improve the enzymatic digestibility of lignocellulosic material.

Often times, large amounts of lignocellulosic biomass are produced at one time as a result of crop harvest, and must be stored for a period of time before conversion to bioenergy. Wet-storage of lignocellulosic biomass has been shown to be more effective in preserving biomass carbohydrates, reducing dry matter losses by 1-5% compared to

dry-storage, while increasing biomass digestibility (Shinners, Binversie et al. 2007). In addition, alkaline pretreatment can be applied in combination with wet-storage to providing a cost-effective method for producing a homogenous delignified feedstock for bioenergy production (Cui, Shi et al. 2012). In this case, the fact that alkaline pretreatment requires longer reaction times than other pretreatment methods is not a problem, as biomass is typically stored for months at a time. Cui et al reported a 2-3 fold increase in enzymatic degradability of corn stover by 2-3 fold after 90 days of wet storage and alkaline conditions. NaOH, NH_3, and lime have all been successfully applied in combination wet-storage alkaline pretreatment (Digman, Shinners et al. 2010, Digman, Shinners et al. 2010, Zhu, Gikas et al. 2009, Felix, Diarra 1993).

2.5 Bioaugmentation

Bioaugmentation is the addition of specific microorganisms to a system in order to correct or enhance a desired process or activity (Ritmann, Whiteman 1994, Schauer Gimenez, Zitomer et al. 2010). Typically, the microorganisms that serve as the bioaugment are added one time to the system. Bioaugmentation has been used for a variety of reasons in several applications including soil and groundwater bioremediation, wastewater treatment, and anaerobic digestion of agricultural, industrial, and municipal solid wastes. In wastewater treatment, bioaugmentation has been applied most frequently in aerobic systems (Schauer Gimenez, Zitomer et al. 2010). In these cases it has been used to improve flocculation and degradation of specific substrates (Van Limbergen, Top et al. 1998), as well as to increase the population of nitrifying bacteria after systems upsets resulting from pH or temperature fluctuations, uncontrolled biomass loss, or toxic events (Ritmann, Whiteman 1994, Abeysinghe, De Silva et al. 2002, Satoh, Okabe et al. 2003, Head, Oleszkiewicz 2005). In anaerobic digestion applications, bioaugmentation has been investigated for its benefits in overcoming shock loading or toxic events (Lynch,

Daniels et al. 1987, Schauer Gimenez, Zitomer et al. 2010), improving reactor start-up (Saravanane, Murthy et al. 2001), odor reduction (Duran, Tepe et al. 2006), and degradation of specific compounds or substrates (Charest, Bisaillon et al. 1999, Hajji, Lepine et al. 2000, Guiot, Tawfiki Hajji et al. 2000, Guiot, Tartakovsky et al. 2002, Tartakovsky, Levesque et al. 1999, Ahring 1992, Horber, Christiansen et al. 1998, Cirne, Bjornsson et al. 2006, Angelidaki, Ahring 2000, Mladenovska, Ishoy et al. 2001, Nielsen, Mladenovska et al. 2007, Neumann, Scherer 2011) Table 4 summarizes some previous studies that have investigated bioaugmentation in anaerobic digestion processes.

Focusing on hydrolysis, bioaugmentation with hydrolytic bacteria has proven to be beneficial in several cases, resulting in increased methane yields and rates of production. Cirne et al investigated the effects of bioaugmentation with an anaerobic lipolytic bacterium, isolated from bovine rumen, on the anaerobic digestion of lipid-rich restaurant waste. The authors founds that bioaugmentation increased the rate of methane production, and were able to achieve 80% of methane yield in 30% less time in than in the non-bioaugmented control. Weiss et al investigated bioaugmentation with mesophilic hemicellulolytic bacteria immobilized on activated zeolite as a method for enhancing biogas production from hemicellulose-rich substrates. The hemicellulolytic cultures were obtained through enrichment of a common anaerobic digester consortium with xylose powder. Subsequent batch tests using xylose power as the substrate resulted in a 53% increase in methane production compared to a non-bioaugmented control. Last, Costa et al investigated the benefits of bioaugmentation with three different cellulolytic bacterial strains on the hydrolysis and methane production from poultry litter. Of the three strains investigated bioaugmentation with *C. cellulolyticum* showed a significant positive effect on biogas production resulting in a 15% increase in cumulative methane production compared to a non-bioaugmented control. VFA concentrations were also increased, lending to the conclusion that bioaugmentation with *C. cellulolyticum* enhanced

hydrolysis and subsequent acidogenesis of the substrate. Batch tests indicated a solids concentration of 1% TS provided the best scenario in terms of methane production. Higher solids concentrations resulted in inhibitory effects most likely related to VFA, alcohols and COD accumulation. Bioaugmentation with the other two cultures, *C. thermocellum,* and *C. saccharlyticus,* did not result in a significant increase in methane production, but did cause a significant increase in substrate solubilization. The authors concluded that in these cases methanogenesis was the rate-limiting step in conversion of the substrate. They believe this could be attributed to the fact the temperature was maintained at thermophilic conditions (55 and 65˚C) which was optimal for the growth of the bioaugmented species, but which may have negatively influenced the mesophilic methanogenic inoculum. From this study, the authors believed that separation of the hydrolysis from the subsequent phases may be necessary for maximizing process efficiency.

The benefit of bioaugmentation with cellulolytic bacteria on the degradation of agricultural residues and biofibers has been investigated by several authors. In a two-stage continuous reactor set-up, operating under thermophilic conditions, Nielsen et al achieved up to a 93% increase in methane production from cattle manure through bioaugmentation with the cellulose degrading bacterium *Caldicellulosiruptor lactoaceticus*. Similarly, Mladenovska et al observed an increase in methane production from cattle manure, through bioaugmentation with xylanolytic and cellulolytic bacteria. Angelidaki et al investigated both bioaugmentation as well as the addition of cellulase enzymes as separated methods for improving the methane potential of cattle manure biofibers. The authors found that treatment with hemicellulolytic and cellulolytic enzymes did not result in any significant increase in methane production compared to control conditions. In contrast, bioaugmentation with hemicellulose degrading bacterium B4 resulted in a 30% increase in methane production compared to non-bioaugmented

controls. Romano et al, also found that the addition of cellulase enzymes had no significant improvement on methane yield or solids reduction for anaerobic digestion of Jose Tall Wheat Grass (Romano, Zhang et al. 2009).

The latter two studies highlight the potential of bioaugmentation as an effective alternative to enzyme addition as a method for improving hydrolysis. While many studies have demonstrated benefits from the addition of hydrolytic enzymes in terms of increased methane production and solids reduction (Wawrzynczyk 2003, Davidsson, Wawrzynczyk et al. 2007, Roman, Burgess et al. 2006, Parmar, Singh et al. 2001), there are several drawbacks associated with enzyme application that make bioaugmentation a more attractive option. One of the major drawbacks is the high cost associated with commercial enzyme production. Other concerns are uneven distribution of enzymes or loss of enzyme activity due to entrapment within the solid waste matrix, thermal denaturation, active site inactivation, loss of cofactors or prosthetic groups, and inhibition (Ahuja, Ferreira et al. 2004, Aitken 1993, Gianfreda, Rao 2004). Also, in contrast to microorganisms, enzymes are not able to adapt to environments outside of their optimal range, and because they are soluble and unstable they can only be used once in solutions (Parawira 2012). Parawira stated that, "Bioaugmentation offers the possibility of enzyme production over a longer period of time provided that the microorganism added is able to compete with the other microbes present in the reactor". Therefore, bioaugmentation offers a promising alterative to enzyme addition.

In that light, there are several factors that can influence the survival and productivity of bioaugmented microorganisms with in the reactor. These include substrate variability, predation and/or competition among indigenous microorganisms, and wash-out (El Fantroussi, Agathos 2005). Examples of this include the previously mentioned studies by Nielsen et al and Mladenovska et al. In both cases, the authors were able to achieve

significant increases in methane production as a result of bioaugmentation but, this increase was not sustained over time. In both cases, the suspected cause for the decline in methane production was wash-out due to an inability to adapt and compete within the indigenous microbial community. With that, methods for ensuring stable growth and the persistence of bioaugmented microorganisms within anaerobic digesters are necessary in order to achieve maximum process efficiency.

Table 4: Previous studies investigating bioaugmentation in anaerobic digestion processes

Purpose for Bioaugmentation	Substrate	Bioaugment/ Microorganism	Reactor Configuration	Benefits	Reference
Improve recovery from toxic exposure to oxygen	Synthetic municipal wastewater solids	H2-utilizing culture	Single-phase semi-continuous in serum bottle, mesophilic	25-60% increase in CH_4 production	Schauer-Gimenez *et al* 2010
Improve reactor start-up	Pharma-ceutical effluent	Anaerobic sludge collected from plant treating antibiotic effluent	Fluidized-bed reactor	Decrease in reactor start-up time and increase in COD removal	Sarvanane *et at*, 2001
Odor control	Anaerobic biosolids	Commercial product containing selected strains of *Bacillus*, *Pseudomonas*, & *Actinomycetes*	One-stage bench-scale continuous, mesophilic	29% increase in CH4 production, reduced generation of organic sulfide compounds	Duran and Tepe, *et al* 2006
Improve digestion efficiency	Fodder beet silage	Compost: hydrogenotrophic methanogens	One-stage bench-scale continuous, mesophilic	2-4 fold shorter HRTs and 6% increase in biogas production	Neumann *et al*, 2011

Table 4: (cont.)

Improve hydrolysis of lipids	Lipid-rich restaurant waste	Lipolytic bacterium: *Clostridium lundense*	Single-phase & two-phase batch test, mesophilic	Increased CH4 production rates	Cirne *et al*, 2006
Improve hydrolysis of poultry litter	Poultry litter	Cellulolytic bacteria: *C. cellulolyticum*, *C. thermocellum*, *C. saccharlyticus*	Batch tests, mesophilic and thermophilic	Up to 74% increase in substrate solubilization, 15% increase in CH4 production	Costa *et al*, 2012
Degradation of biofibers	Cattle manure fibers	Hemicellulose degrading bacterium B4	Batch test, thermophilic (70˚C)	30% increase in CH4 production	Angelidaki *et al*, 2000
Degradation of biofibers	Cattle manure	Xylanolytic & cellulolytic bacteria	Bench-scale continuous, mesophilic	Increased CH4 production rates	Mladenovska *et al*, 2001
Degradation of biofibers	Cattle manure	Cellulolytic bacteria: *Caldicellusiruptor* & *Dictyoglomus*	Two-stage (68˚C/55˚C) batch test and bench-scale continuous, thermophilic	Increased CH4 yields	Nielsen *et al*, 2007
Degradation of xylose	Xylose powder	Hemicellulolytic bacteria	Batch test, mesophilic	53% increase in CH4 production	Weiss *et at*, 2010

CHAPTER 3: PROPOSED SYSTEM DESIGN AND RESEARCH OBJECTIVES

The main purpose of this study was to investigate the potential for improving anaerobic digestion of cellulosic waste, in terms of solids reduction and methane production. The waste used in this study was Del Monte Foods Company's sweet corn processing residues, and the initial approach to improve anaerobic digestion was to use routine bioaugmentation with a proprietary cellulolytic bioculture in a two-phase anaerobic digestion system. The project was a collaboration between the University of Illinois at Urbana-Champaign (UIUC), Del Monte Foods Company, and Phylein Inc. who provided the proprietary bioculture. The two-phase anaerobic digestion process with routine bioaugmentation was originally proposed as the first component of a novel two-stage, anaerobic/aerobic waste treatment scheme. From this process, there is the potential for generating four co-products: methane, fertilizer, single-cell protein, and algal biomass. The addition of a pretreatment phase was proposed as a method to further improving the digestibility of the sweet corn processing residues, adding a third stage to the proposed waste treatment system. Figure 13 is a schematic representation of the proposed three-stage waste treatment process. Each stage of the process and the specific objectives for this research is discussed in the following sections.

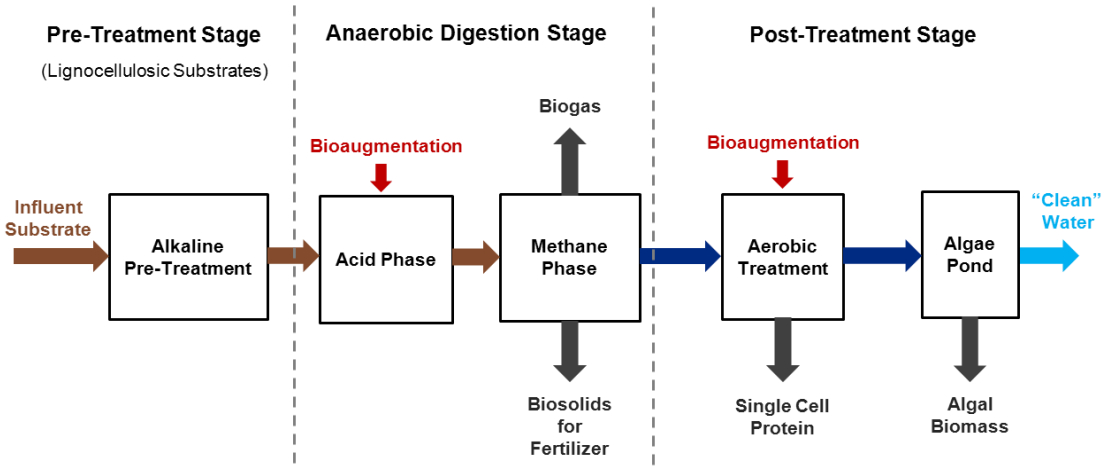


Figure 13: Schematic of proposed anaerobic/aerobic waste treatment scheme with additional alkaline pretreatment phase

3.1 Proposed System Design

Pretreatment Stage

With the whole 70,000 tons of Del Monte sweet corn processing residues being generated within a 1-2 month time period, storage of the material prior to anaerobic digestion would be necessary. Therefore, wet-storage with alkaline pretreatment was proposed as a pretreatment stage. Agri-bags, which are essentially large plastic bags that are commonly used in agricultural practice for applications such as ensiling, were proposed as the method for containment during the pretreatment-storage stage. Figure 14 below is an image of filled agri-bags. A similar set-up was observed at the UIUC beef farm, in which corn stover was stored in agri-bags at pH12 and later mixed with ethanol co-products (DDGS) to provide a corn feed replacement.

Figure 14: Corn silage filled agri-bags (UIUC Dairy Farm)

Anaerobic Digestion Stage

In this stage, pretreated material would be fed to a two-phase anaerobic digestion system with routine bioaugmentation (2PAD-BA). A cellulolytic bioculture would be applied routinely to the first phase of the 2PAD-BA process. The hypothesis behind the 2PAD-BA process is that through routine bioaugmentation the microbial population will shift and a community better suited for hydrolysis of cellulose will be maintained, thereby improving process efficiency through increased hydrolysis and subsequent methane production. The aim is to be able to operate at lower than conventional anaerobic digestion hydraulic retention times, which typically range from 20 to 30 days. The target HRT for the novel process would be 0.5-2 days HRT in the acid phase and 10-15 days in the methane phase. The system will operate under mesophilic conditions (37- 40˚C). Figure 15 is a schematic representation of the proposed 2PAD-BA system.

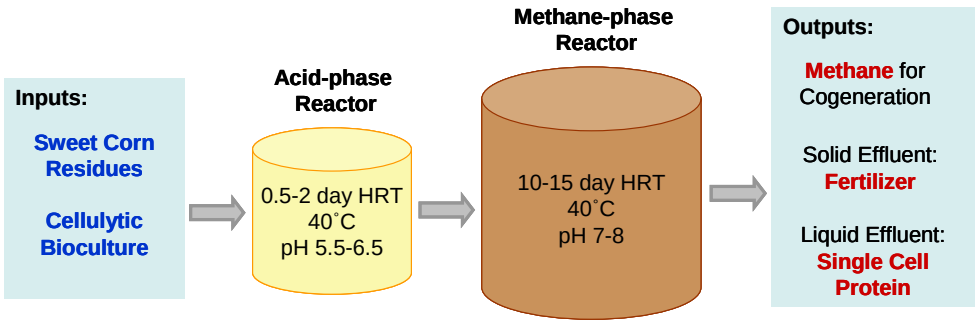


Figure 15: Schematic of proposed two-phase anaerobic digestion system with routine bioaugmentation

From the anaerobic digestion phase, methane from the biogas can be utilized for combined heat and power generation. The solid effluent, consisting of microbial biomass generated during the process as well as any un-digested substrate, can be used for fertilizer production, as is common practice. The liquid fraction, which will contain soluble organics and nutrients, will be further processed in the following post-treatment stage.

Post-treatment Stage

The final stage of the proposed system is an aerobic post-treatment stage for processing the soluble organics and nutrients remaining in the liquid effluent coming from the anaerobic digestion stage. In this stage, the liquid effluent is sent to an aerobic treatment tank which is seeded and regularly bioaugmented with a single-cell protein bioculture. The term "single-cell protein" refers to the dried cells of microorganisms such as algae, bacteria, yeasts, etc., which are grown in large-scale culture systems for use as protein sources in human foods or animal feeds (Litchfield 1977). In this system, bacterial single cell protein would be produced. After aerobic treatment, effluent will be sent to an algae pond where the remaining nutrients will be utilized for the production of algal biomass. This biomass can then be utilized for methane production by sending it back to the anaerobic digester, or for other products such as animal feed.

3.2 Research Objectives and Hypotheses

The scope of work presented in this thesis was focused on the anaerobic digestion and pretreatment components of the proposed three-stage system. The specific objectives for the presented research were as follow:

1. To determine the biogas potential of sweet corn processing residues from two-phase anaerobic digestion with and without bioaugmentation with a cellulolytic bioculture in the first phase.

2. To assess the effect of routine bioaugmentation versus one-time bioaugmentation with a cellulolytic bioculture on the hydrolysis and subsequent methane production of sweet corn processing residues.

3. To evaluate the benefit of long-term alkaline pretreatment on solubilization of sweet corn processing residues and the digestibility of the resulting hydrolysate.

Objective 1

As described in section 2.5 of the literature review, previous studies have shown an increase in methane production from anaerobic digestion of lignocellulosic materials as a result of bioaugmentation with cellulolytic microorganisms. Therefore, the first objective of this study sought to answer the question of whether bioaugmentation with a cellulolytic bioculture could similarly improve methane production from anaerobic digestion of the unique sweet corn processing residues substrate. A batch experiment comparing methane production from bioaugmented versus non-bioaugmented conditions, using a proprietary cellulolytic bioculture, was conducted in order to achieve this objective. It was hypothesized that the bioaugmented conditions would result in a higher methane production rate and total methane yield in comparison to the non-bioaugmented control conditions.

Objective 2

In several studies described in section 2.5 of the literature review, the benefit of bioaugmentation with cellulolytic microorganisms, specifically increased methane production, was not sustained over time due to wash-out of the bioaugmented microorganisms. In these cases, the bioaugmented microorganisms had been applied to the system only once. Therefore, the second objective of this study sought to determine whether routine bioaugmentation with a cellulolytic bioculture would improve substrate hydrolysis and subsequent methane production compared to one-time bioaugmentation. A two-phase sequencing/semi batch experiment was conducted to achieve this objective, in which routine bioaugmentation with two sources of cellulolytic microorganisms was compared to one-time bioaugmentation in terms of neutral detergent fiber removal, net soluble COD generation, and methane production. It was hypothesized that routinely bioaugmented conditions would show evidence of increased hydrolysis as well as increased methane production compared one-time bioaugmented conditions.

Objective 3

As alkaline pretreatment has proven to be an effective pretreatment method for lignocellulosic materials, this last objective sought to determine whether alkaline pretreatment would be an effective pretreatment method for the unique sweet corn residue substrate prior to anaerobic digestion. Pretreatment batch experiments were conducted to measure the extent and rate of substrate solubilization as a result of long-term alkaline pretreatment, and were followed by an anaerobic batch experiment to determine the digestibility of the resulting hydrolysate. It was hypothesized that alkaline pretreated conditions would result in significantly higher rates of solubilization compared to non-pretreated controls, and that digestion of the resulting hydrolysate would result in comparable or higher rates of methane production than hydrolysate from non-pretreated conditions.

CHAPTER 4: MATERIALS AND METHODS

4.1 Materials

Del Monte Sweet Corn Residues

The substrate for this study was sweet corn residues provided by Del Monte Foods Company. The residues were produced from Del Monte Foods' sweet corn processing facility in Mendota, IL and consisted primarily of corn husks, corn cobs, and some pieces of the sweet corn kernels. The material was transported and contained in 5-gallon, plastic buckets, which were sealed and stored at 4ºC until use. A picture of the sweet corn residues is shown in Figure 16.

Substrate Drying and Grinding

Due to the relatively small opening on the serum bottles that were used for batch experiments, as well as for pump-ability within a semi-continuous reactor set-up, it was necessary to reduce the size of the substrate. With that, the sweet corn residual material was dried at 160°F for 24 hours and ground to approximately 850 micrometers (0.0331 in) using a using a hammer mill (Arthur Thomas Co.).

Substrate Characterization

Characterization of the dried ground substrate including ash, moisture, crude protein, crude fat, non-structural carbohydrate, acid and neutral detergent fiber, and lignin analysis was conducted by Midwest Laboratories in Omaha, NE. Values are listed in Table 5. Elemental carbon, hydrogen, and nitrogen (CHN) analysis was conducted by the University of Illinois at Urbana-Champaign (UIUC) Microanalysis Lab using a CHN analyzer (Exeter Analytical, Inc. CE-440). Percent oxygen was assumed to be 100% minus the combined C, H, and N percentages. Values are listed in Table 5. Moisture

content, volatile solids (VS) percent and pH for the substrate were measured according to Standard Methods for the Examination of Water and Wastewater (APHA 1998).

Table 5: Characteristics of Del Monte sweet corn processing residues

Component	Value	Std. Dev.
pH	3.16	0.33
Moisture	78%	
Volatile Solids	96% *dry wt.*	0.02
	% (dry wt.)	
Fiber:	62.76	2.45
Cellulose	26.53	0.95
Hemicellulose	31.49	2.14
Lignin	4.74	0.64
Non-Structural Carbs	14.8	2.55
Crude Protein	11.9	0.71
Crude Fat	5.98	0.47
Ash	4.54	0.13
Elemental make-up:		
Carbon	46	0.002
Hydrogen	6	< 0.001
Nitrogen	2	0.001
Oxygen (calculated)	46	0.001

Proprietary Bioculture

A proprietary cellulolytic bioculture mixture, provided by Phylein Inc., was used as the primary bioaugmentation source in this study. The bioculture consisted of inactivated bacteria attached to a cornmeal medium. A picture of the bioculture is shown in Figure 17. 16s-rRNA sequencing of the bioculture was carried out by Dr. Brian White's laboratory in the Department of Animal Science at UIUC. 16s-rRNA sequencing indicated *Clostridium* to be the predominant genus within the bioculture mixture. The bioculture was stored in a sealed zip-lock bag at 4ºC until use.

(a)  (b) 

Figure 16: Del Monte Food's sweet corn residue: (a) fresh (b) dried and ground

Figure 17: Proprietary cellulolytic bioculture

4.2 Batch Tests

Equipment Set-up

In this study, three different batch experiments were performed. Details specific to each batch experiment are described below. In general, each experiment consisted of at least two-phases: (1) an acid or pretreatment phase followed by (2) a methane production phase. In all cases, the methane phase consisted of a sealed, serum bottle either 250 or 150 ml (Wheaton Brand), seeded with anaerobic sludge collected from the municipal wastewater treatment facility in Urbana, IL (UCSD, Urbana-Champaign Sanitary District). In all cases, a condition consisting of an equal volume of anaerobic sludge was set-up as a biogas control. Upon loading, each serum bottle was sparged with nitrogen for approximately 60 seconds to remove oxygen from the headspace. Two replicates were run for all conditions, in both phases, for all experiments. Any material transfer between

phases was done via syringe, using a 16 gauge needle. All bottles were shaken continuously at 100 RPM by means of an orbital shaker (Lab-line Orbit Shaker, Model No. 3520), and kept under mesophilic temperatures (37- 40ºC) within a temperature controlled warm room.

Biogas Analysis

In all cases, biogas production was measured using a water displacement column. Biogas samples were collected for quality analysis via syringe with an 18 gauge needle and Vacutainer sample vials (BD Vacutainer, 8020128). Biogas quality was measured by gas chromatography (Varian, Model 3800).

Batch Test 1: Bioaugmentation versus No Bioaugmentation

In this batch test, methane production from two experimental conditions: (1) with bioaugmentation (2) without bioaugmentation, was investigated. A single 250 ml serum bottle was used for both phases of this experiment. Dried, ground sweet corn residues were loaded into the serum bottles at approximately a 1% total solids (TS) concentration. In the non-bioaugmented condition, 1.2 gram of substrate was added to 120 ml of deionized water. In the bioaugmented condition, 1.2 gram of substrate was added to 60 ml of deioinized water and 60 ml of liquid bioculture. The liquid bioculture was liquid effluent collected from a 1 liter, continuously operated acid phase reactor treating the same dry ground substrate. The continuously operated acid phase reactor was fed with new substrate and bioaugmented using the proprietary bioculture daily, at a 10:1 ratio of substrate to bioculture. Further description of the continuously operated acid phase reactor is provided in Section 2 of the Appendix. In the bioaugmentation batch test, after loading the serum bottles, 20 ml was sampled from all conditions for VS measurement, bringing the final working volume to 100 ml. The serum bottles were then sealed, and left for 24 hours.

After 24 hours of acid phase treatment, the serum bottles were opened. At this point, half the liquid and solid volume (50 ml) was removed from all conditions, and replaced with an equal volume of anaerobic sludge. All conditions were neutralized to pH 7 (using 2M NaOH) prior to the addition of anaerobic sludge. The serum bottles were then resealed. Digestion in the methane phase proceeded for 35 days, with regular measurement of biogas production and biogas quality. After 35 days, the serum bottles were opened and sampled for VS analysis to determine percent VS reduction.

Batch Test 2: Routine Bioaugmentation

Five experimental conditions were investigated in this batch test: (1) non-bioaugmented control (2) one-time bioaugmented with proprietary bioculture (3) routinely bioaugmented with proprietary bioculture (4) one-time bioaugmented with rumen fluid (5) routinely bioaugmented with rumen fluid. In the routinely bioaugmented conditions bioaugmentation was applied daily. Rumen fluid used in this experiment was collected from a fistulated dairy cow at the UIUC dairy farm, with the assistance of Travis Michaels, and stored at 4˚C.

Due to the cornmeal medium within the proprietary bioculture, the proprietary bioculture contained a greater amount of particulate VS compared to the rumen fluid. Therefore, prior to experimental start-up, a dosage concentration for the proprietary bioculture that had an equivalent particulate VS percent to the rumen fluid was determined. To achieve this, various concentrations of bioculture in deionized water were soaked for 24 hours at 4˚C then swirled vigorously in an attempt to bring bacteria attached to the cornmeal media into the liquid fraction. The liquid fraction was then quickly sampled via syringe and applied as the bioaugment. A concentration of 5 g of proprietary bioculture in 100 ml deionized water was determined to provide an equivalent fraction of particulate VS as that of the rumen fluid (0.8% particulate VS in the

proprietary bioculture and 0.7% particulate VS in the rumen fluid). Total VS concentration in both biocultures was measured to be 1.1% ± 0.5. The consistency of this sampling technique was verified by obtaining a 0-0.2% variation in measured particulate VS from 8 sampling attempts.

Dried, ground sweet corn residues were used as the substrate for this batch test. A 500 mL media bottle was used for the acid phase. 5 g of substrate was loaded into the media bottles with 500 ml of corresponding bioculture to achieve an approximate 1:1 VS ratio of substrate to bioculture. In the control condition, 5 g of substrate was added to 500 ml deionized water. Upon loading, pH was neutralized to 7 in all conditions. The acid phase was operated as a sequencing batch for 14 days. 100 ml liquid effluent from the acid phase was removed daily and replaced with 2.5 g fresh substrate in 100 ml deionized water. In the case of the routinely bioaugmented conditions, 25 of the 100 ml of deionized water was replaced with 25 ml of the appropriate bioculture. Thus, the routine dosage ratio for the routinely bioaugmented conditions was 1:10 g of bioculture per g of substrate.

A sealed 250 mL serum bottle, seeded with 25 mL anaerobic sludge, was used for the second, methane production phase. 10 ml of liquid effluent from the acid phase conditions was added to corresponding methane phase serum bottles daily. The methane phase was operated as a semi batch for 14 days. After 14 days, no additional material was added, and thus the methane phase was operated as a batch for the remainder of the experiment. Digestion proceeded for 36 days, with regular measurement of biogas production and biogas quality.

Batch Test 3: Long-Term Alkaline Pretreatment

Raw sweet corn residues (wet and un-ground) were used as the substrate for two long-term alkaline pretreatment experiments: Experiment 1 (29 days), and Experiment 2 (68 days). In both cases, 200 mL media bottles were used for the pretreatment phase. Raw sweet corn residues were loaded into the media bottles at a 6% VS concentration with deionized water. In Experiment 1, 15 g of wet, raw sweet corn residues was loaded into the serum bottles with 100 ml deionized water. In Experiment 2, 30 g of wet, raw sweet corn residues was loaded into the serum bottles with 200 ml deionized water. Alkaline pretreated conditions were adjusted to pH 12 using a 4 M KOH solution. 2.5 ml 2 M KOH were added in Experiment 1, and 4 ml 4 M KOH were added in Experiment 2. Non-pretreated control conditions were left unadjusted at approximately pH 3. Routine pH measurements and adjustments were made to maintain the alkaline pretreatment conditions at pH 12. In Experiment 1, pH measurements/adjustments were made relatively frequently, (every day for the first 3 days, and approximately every 3 days after that), pH adjusted was made using 0.5-1 ml 4 M KOH. In Experiment 2, pH measurements/adjustments were made every 5 days for the first 35 days, pH adjustments were made using 2-1 ml 4M KOH. Hydrolysate samples were collected at each pH measurement/adjustment time point via syringe. Total VS of the hydrolysate was measured to determine percent VS solubilization of the substrate. In Experiment 2, hydrolysate generated after 68 days was added to an equal volume of anaerobic sludge in 150 mL serum bottles. Prior to loading the serum bottles, hydrolysate pH was neutralized to pH 7 (Using 2M KOH). Digestion proceeded for 56 days, with regular measurement of biogas production and biogas quality.

4.3 Analytical Methods

Moisture Content, Volatile Solids, Chemical Oxygen Demand, pH

Moisture content, total and particulate volatile solids, soluble chemical oxygen demand and pH were measured according to Standard Methods for the Examination of Water and Wastewater (APHA 1998).

Neutral Detergent Fiber Analysis

Solids from the acid phase of the routine bioaugmentation batch experiment were dried at 160°C for 24 hours, sealed in zip-lock bags, and mailed to Midwest Laboratories, in Omaha, Nebraska for neutral detergent fiber analysis.

Volatile Fatty Acid Analysis

Samples for volatile fatty acid analysis were prepared according to Supelco Bulletin 856B, which involved acidifying with 25% metaphosphoric acid and centrifugation at 4000 rpm for 25 minutes. Prepared samples were stored at 4˚C prior to analysis by Dr. Alex Ulanov at the UIUC Metabolomics Laboratory.

4.4 Statistical Methods

In all experiments, two replicates were run for every condition. An average and standard deviation was then calculated for each condition based on results from each replicate. Results between conditions were considered to be significantly different if the average values plus or minus one standard deviation did not coincide.

CHAPTER 5: RESULTS AND DISCUSSION

5.1 Bioaugmentation versus Non-Bioaugmentation

The effect of bioaugmentation with a proprietary cellulolytic bioculture on the methane production of Del Monte Foods' sweet corn processing residues was first investigated in a two-phase batch experiment. Cumulative methane production per gram of VS added is shown in Figure 18. Results indicated that a significant benefit to methane production was observed as a result of bioaugmentation. After 35 days of digestion, average cumulative methane production per gram of VS_{added} was 34% higher in the bioaugmented conditions compared to non-bioaugmented controls. In addition to higher methane yield, the rate of methane production was also increased in the bioaugmented conditions. Within 3 days, the bioaugmented batches had already achieved 35% higher methane production compared to non-bioaugmented conditions (138 versus 102 ml/g VS_{added}). By day 35, when it appeared methane production had reached near a plateau value, methane yields in the test and control conditions were 265 and 199 ml/g VS_{added} respectively (0.265 and 0.199 m^3/kg VS_{added}). These values are comparable to literature values for maximum methane yields from similar substrates, although non-bioaugmented batches were somewhat lower than previous reports. Lane, et al reported a methane production potential of 0.267 m^3/kg VS_{added} from corn cobs. Deublin and Steinhauser reported methane production from maize straw in the range of 0.22-0.55 m^3/kg VS_{added}, with a biogas methane content of approximately 55%. Other authors have reported methane yields from corn stover ranging from 0.300 to 0.360 m^3/kg VS_{added} (Richards et al, Tong et al). In this batch test, average methane content in the biogas ranged from 55 to 68% with an average of 60% in both conditions, which is slightly higher than reported values with other similar substrates as noted above.

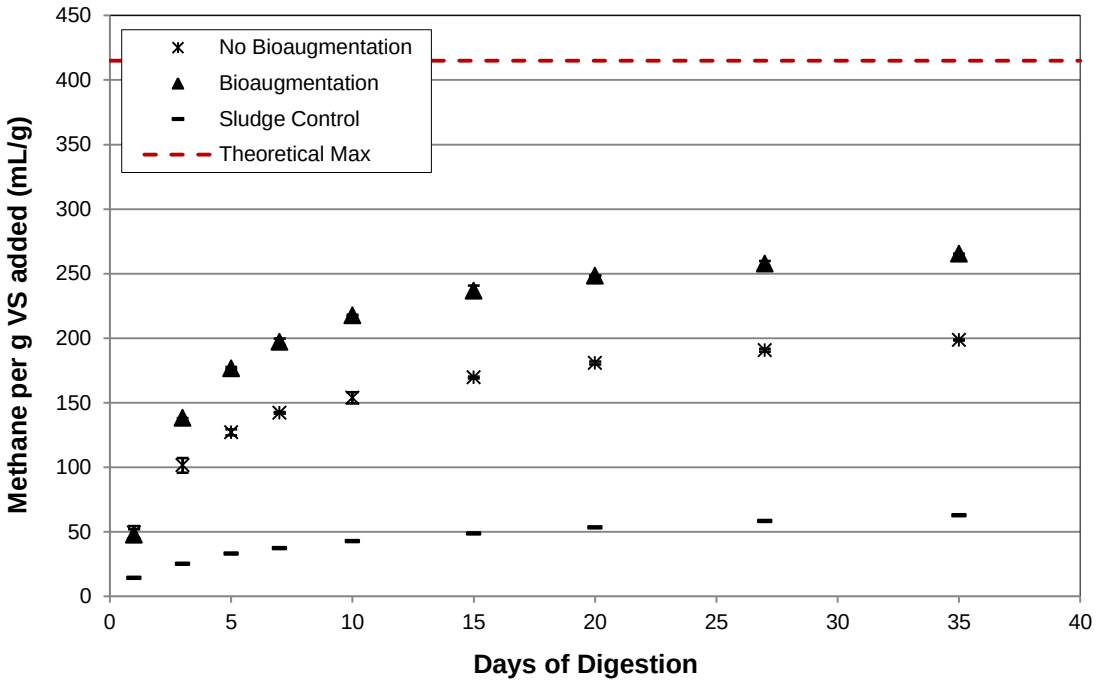


Figure 18: Cumulative methane production results from methane phase of two-phase batch test comparing anaerobic digestion of sweet corn residues with and without bioaugmentation with liquid effluent from a routinely bioaugmented acid phase reactor

A theoretical maximum methane yield was calculated for the sweet corn residues based on elemental make-up and using Equation 2 from Klimiuk et al (2010) as shown below.

$$C_aH_bO_cN_d + (4a-b-2c+3d)/4\ H_2O = (4a+b-2c-3d)/8\ CH_4 + (4a-b+2c+3d)/8\ CO_2 + dNH_3$$

Equation 2: Theoretical conversion of biomass to biogas based on elemental composition (Klimiuk, et al, 2010)

The theoretical maximum methane yield for the sweet corn residues was determined to be 415 ml/g VS, reflecting the high percentage of carbon in the substrate. Comparing batch test methane yields to the calculated theoretical maximum, it can be seen that while bioaugmentation did improve methane production, methane yield was still significantly lower than the theoretical maximum. This comparison indicates that there was still room

for improving the organic solids degradation and anaerobic digestion efficiency of the sweet corn residues. A volatile solid reduction of 58% in the bioaugmented condition and 53% in the control condition was achieved after 35 days. With that, results from this batch test motivated the investigation of methods for further improving volatile solids degradation such that higher digestion efficiency could be achieved.

5.2 Routine Bioaugmentation versus One-time Bioaugmentation

Two verify whether routine bioaugmentation was beneficial in terms of increasing substrate hydrolysis a two-phase sequencing batch experiment was conducted comparing routine bioaugmentation with one-time bioaugmentation. In this experiment, bioaugmentation using the proprietary cellulolytic bioculture was also compared with bioaugmentation with dairy cow rumen fluid in order to assess the relative effectiveness of the two sources of cellulolytic microbes.

Neutral detergent fiber (NDF) analysis on the solids remaining in the acid phase reactors at the end of the 14 day experiment indicated that a greater percent (5-20%) of NDF was removed after 14 days in the bioaugmented conditions compared to the non-bioaugmented control conditions. These results are shown in Figure 19. In addition, both routinely bioaugmented conditions showed greater NDF removal (9-15%) than either of the one-time bioaugmented conditions. These results suggested that routine bioaugmentation resulted in greater fiber degradation compared to one-time bioaugmentation.

Soluble COD generation in the acid phase reactors was also determined in order to assess the relative effects on substrate hydrolysis among each of the bioaugmented conditions. Soluble COD (sCOD) concentrations in the acid phase reactor of each condition were measured from liquid effluent samples collected daily. The measured values and resulting trend in sCOD concentrations are shown in Figure 20. From Figure

20 it can be seen that initial sCOD concentrations were higher in the bioaugmented conditions due to the sCOD within the inoculum itself. Rumen bioaugmented conditions had the highest initial sCOD concentration followed by the proprietary bioculture conditions and finally the non-bioaugmented control.

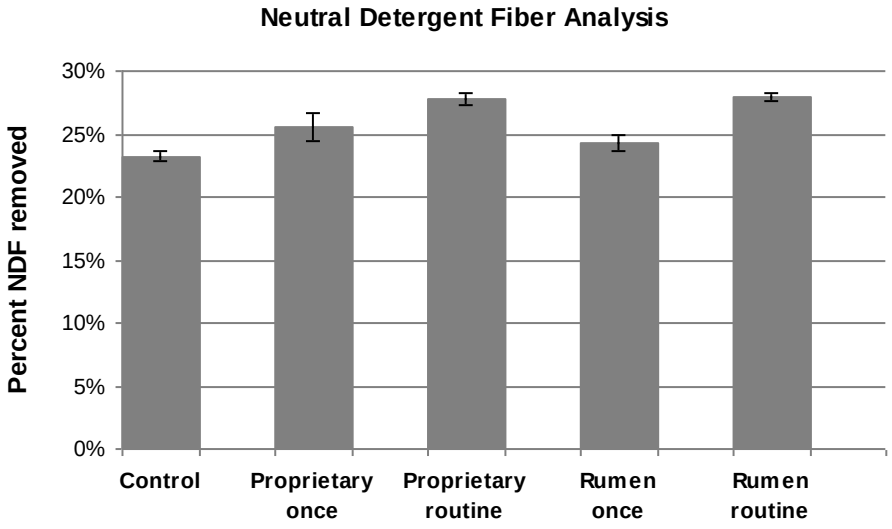


Figure 19: Neutral detergent fiber analysis results for routine bioaugmentation batch test showing percent of NDF removed from total NDF added to the acid phase reactor after 14 days

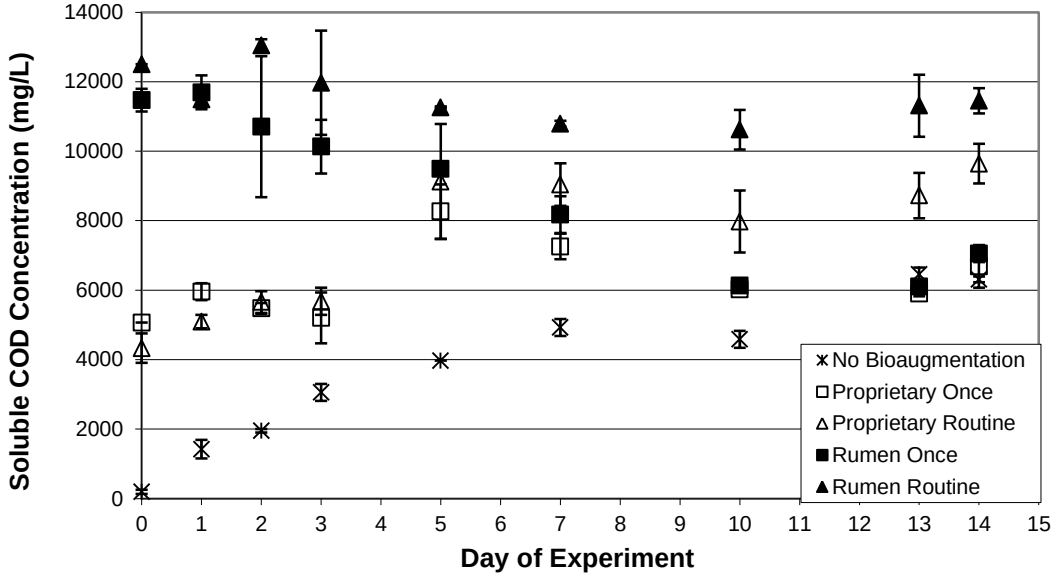


Figure 20: Measured daily soluble COD concentrations for various bioaugmentation conditions in the acid phase of the two-phase routine bioaugmentation sequencing batch experiment

In this experiment, if there had been no generation of sCOD in the acid phase, a decrease in daily measured sCOD concentration in the bioaugmented conditions could have been expected for the following reason. On a daily basis, 100 ml of influent material containing one fourth the sCOD concentration of the original 500 ml of starting material was added to the reactors, while 100 ml of whatever sCOD had been left in the reactor the day before, plus any new amount of sCOD that had been generated as a result of substrate hydrolysis, was removed. Therefore, because the initial sCOD concentration was much higher than that being added a net decrease could be expected had no significant amount of sCOD been generated. In this case, sCOD generation for a given day was calculated as follows:

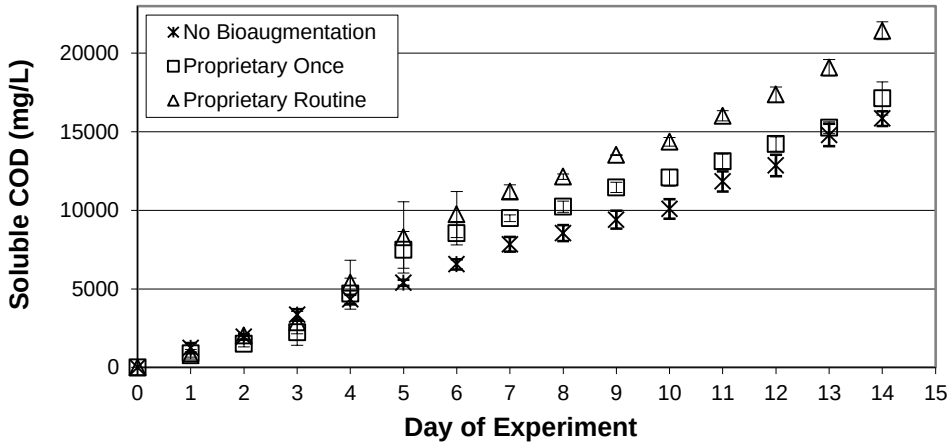
$$sCOD_{generated} = sCOD_{measured} - sCOD_{left\ in\ reactor}$$

and

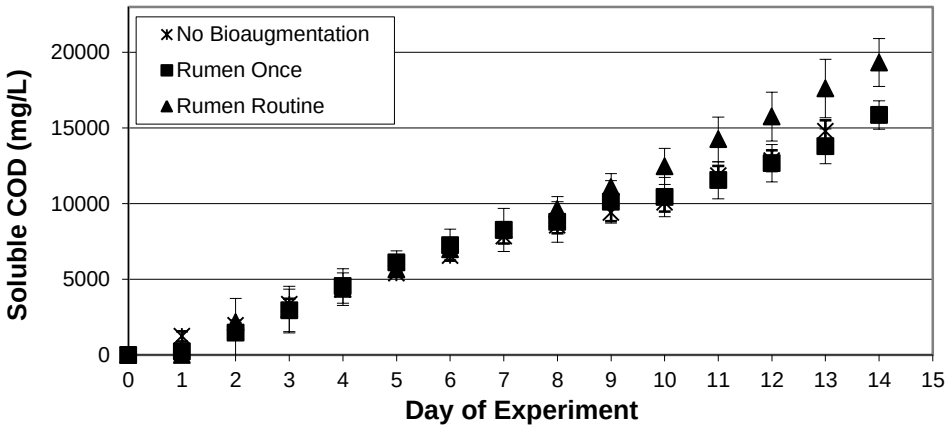
$$sCOD_{left\ in\ reactor} = [sCOD_{previous}]*500ml - [sCOD_{previous}]*100ml + [sCOD_{added}]*100ml$$

where $sCOD_{measured}$ is the measured amount of sCOD in the reactor that day before feeding, $sCOD_{left\ in\ reactor}$ is the amount of sCOD remaining in the reactor after feeding the day before, $sCOD_{previous}$ is the measured sCOD concentration in the reactor from the previous day before feeding, and $sCOD_{added}$ is the amount of sCOD added as a result of feeding the previous day.

Daily sCOD measurements as shown in Figure 20, were used to calculate cumulative net sCOD generation in each condition, which is shown in Figure 21 a-c. For the days when sCOD was not measured, the average measured sCOD of time points before and after was used in the calculation of cumulative net sCOD generation.



(a)



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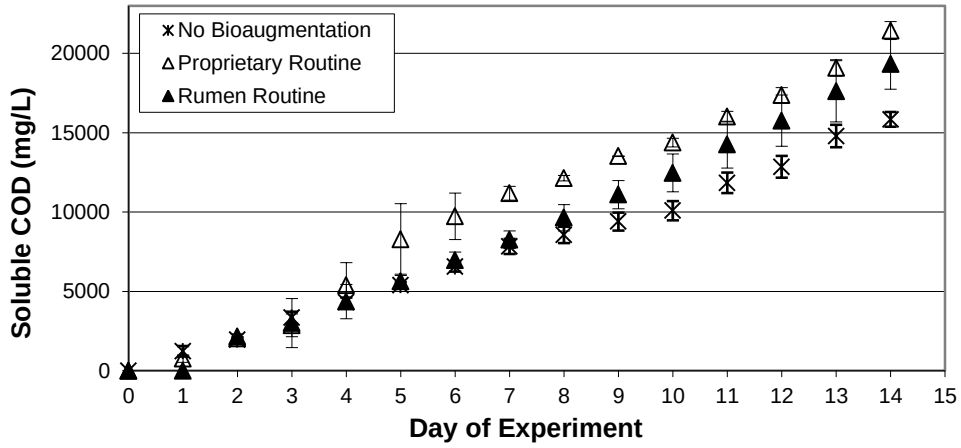


(c)

Figure 21: Cumulative net soluble COD generation for various bioaugmentation conditions in the acid phase of the two-phase routine bioaugmentation sequencing batch experiment (a) proprietary bioculture conditions, (b) rumen conditions, and (c) routinely bioaugmented conditions

Results for cumulative net sCOD generation indicated that routinely bioaugmented conditions had greater net sCOD generation (22-25%) compared to one-time and non-bioaugmented conditions. In the proprietary bioculture conditions, a significant increase in sCOD generation was seen in the routinely bioaugmented condition compared to the one-time and non-bioaugmented conditions after 7 days. The routinely bioaugmented condition showed a 17% and 25% percent increase in sCOD generation after 7 and 14 days respectively compared to the one-time bioaugmented condition (Figure 21a). It is also worth noting that in the case of the one-time bioaugmented, an increase in net sCOD generation compared to the non-bioaugmented condition, was seen after 5 days. However, this increase diminished after 11 days, and by the end of 14 days there was no significant difference between the one-time bioaugmented condition and the non-bioaugmented control.

In the rumen fluid bioaugmented conditions, the routinely bioaugmented condition resulted in significantly greater net sCOD generation (22%) compared to the one-time bioaugmented condition after 14 days (Figure 21b). Comparing the two routinely bioaugmented conditions, the proprietary bioculture condition resulted in higher net sCOD generation between days 6 and 10, however, there was no significant difference between the two routinely bioaugmented conditions by day 14 (Figure 21c).

The benefit of routine versus one-time bioaugmentation on subsequent methane production was evaluated through semi-batch/batch digestion of acid phase liquid effluent. Results for cumulative methane production are shown in Figure 22. From Figure 22 it can be seen that after 21 days of digestion all bioaugmented conditions had produced significantly more methane, approximately 48%, than the non-bioaugmented control condition. After 28 days of digestion, the rumen one-time bioaugmented condition had produced 21% more methane than the proprietary bioculture bioaugmented

conditions. Finally, by day 36, a significant difference between the routinely bioaugmented and one-time bioaugmented proprietary conditions was seen, with the routinely bioaugmented condition having produced 15% more methane than the one-time bioaugmented condition. It should be noted that methane production results from the routinely bioaugmented rumen condition were compromised due to leakage, and therefore the routinely bioaugmented rumen condition was removed from the methane phase of this experiment.

The fact that the rumen one-time bioaugmented condition resulted in higher methane production than either of the proprietary bioculture conditions, but did not show evidence of higher substrate solubilization, could be a result of the rumen culture having converted the available VS into a more beneficial, readily convertible product for methanogenesis (i.e. acetic acid versus longer chain fatty acids). VFA analysis from acid phase effluent of both routinely bioaugmented conditions indicated that the rumen condition did have a higher acetic acid concentration than the proprietary and non-bioaugmented conditions (Figure 23).

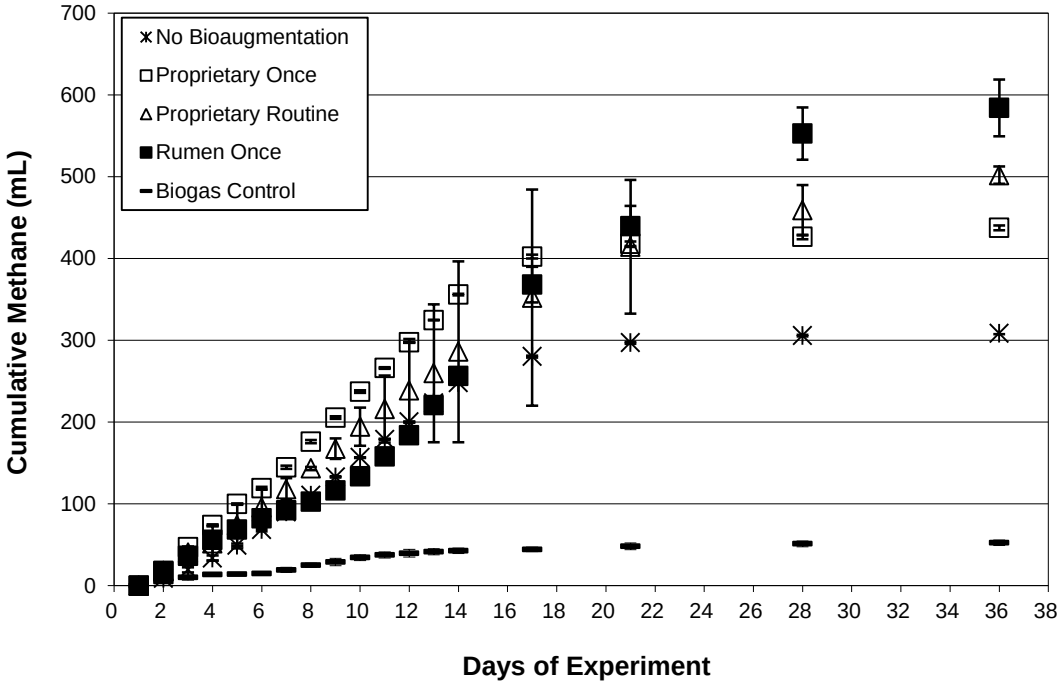
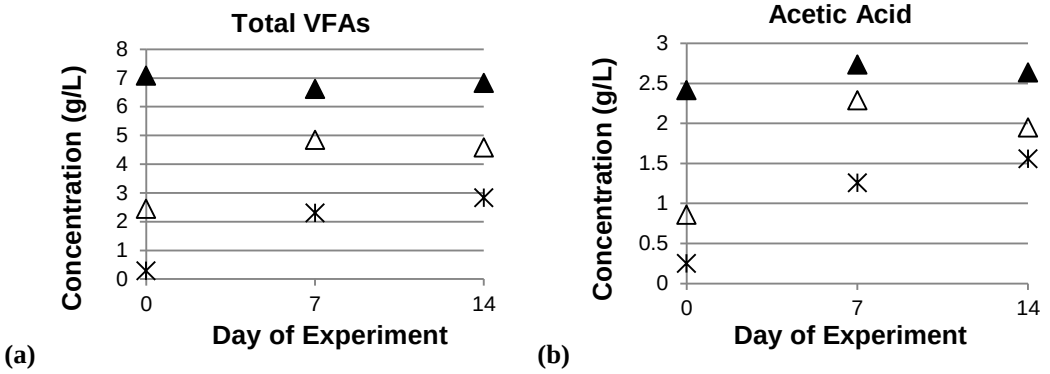


Figure 22: Cumulative methane production per gram of VS added in routine bioaugmentation sequencing/semi batch test



(a) **(b)**

Figure 23: VFA concentrations for ▲ rumen routine-, ∆ proprietary routine-, and X non-bioaugmented conditions on days 0, 7, and 14 of routine versus one time bioaugmentation batch experiment: (a) shows total VFA concentration (including acetic, propanoic, 2-methylpropanoic, butanoic, isopentanoic, pentanoic, & hexanoic acid), while (b) shows concentration of acetic acid only

In short, results from the routine versus one-time bioaugmentation batch experiment provided evidence that routine bioaugmentation was beneficial over one-time bioaugmentation, resulting in greater substrate hydrolysis and subsequent methane production. This suggests that routine bioaugmentation may provide a method that is more effective than conventional one-time bioaugmentation in achieving and maintaining a microbial population better suited for hydrolysis of lignocellulosic substrates, and overcoming the problem of wash-out of the bioaugmented microorganism as was seen in various studies in which conventional one-time bioaugmentation was applied. However, in this experiment there was no transfer of solid material out of the acid phase reactor, and as a result there would have been very little chance for wash-out of the bioaugmented microorganisms. Therefore, to further assess the benefits of routine bioaugmentation in terms of maintaining an effective microbial community, this process should be applied in a continuous reactor system with solids transfer.

It should be noted that investigation of the routine bioaugmentation process in a bench scale two-phase semi-continuous system was initiated during the time of this study. However, up to this point biogas production from operation of the semi-continuous system was somewhat erratic and lower than expected, warranting further investigation. Information regarding set-up and operation as well as daily biogas production results for the semi-continuous reactor system is presented and discussed in Appendix B.

5.3 Long-term Alkaline Pretreatment

After observing that bioaugmentation with cellulolytic microorganisms was beneficial in terms of increasing substrate hydrolysis and methane production, but that overall methane production was still lower than theoretical maximum, alkaline pretreatment was investigated as a method for further improving substrate digestibility.

Two long-term pretreatment batch experiments (29 and 68 days) were conducted to determine the extent of substrate solubilization under alkaline conditions (pH 12). Results, which are shown in Figure 24, indicated that up to a 4 fold increase in VS solubilization could be achieved through alkaline pretreatment. In this case, VS solubilization was measured as the amount of VS generated in the hydrolysate divided by the amount of VS in the starting material. These results also suggested that frequency of pH re-adjustment had an effect on the rate of VS solubilization. This can be seen from the difference in % VS solubilization between Experiments 1 and 2 shown in Figure 24. Experiment 1, which was re-adjusted to pH 12 more frequently, showed a 400% increase in VS solubilization after 29 days of pretreatment compared to a non-pretreated control, whereas less than a 150% increase in VS solubilization was achieved after 68 days of pretreatment in Experiment 2.

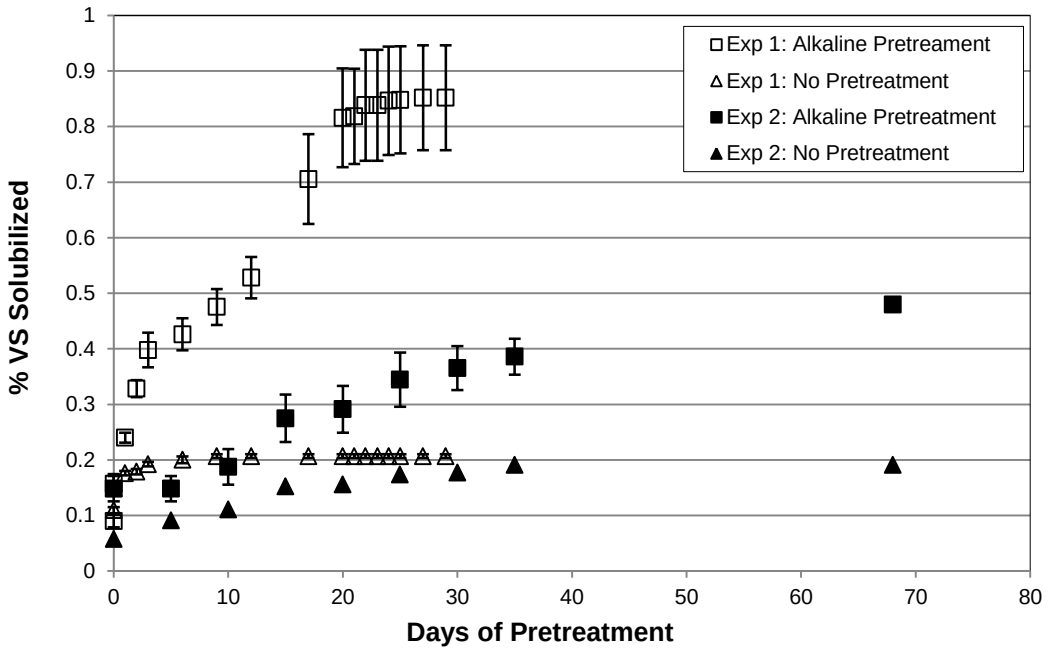
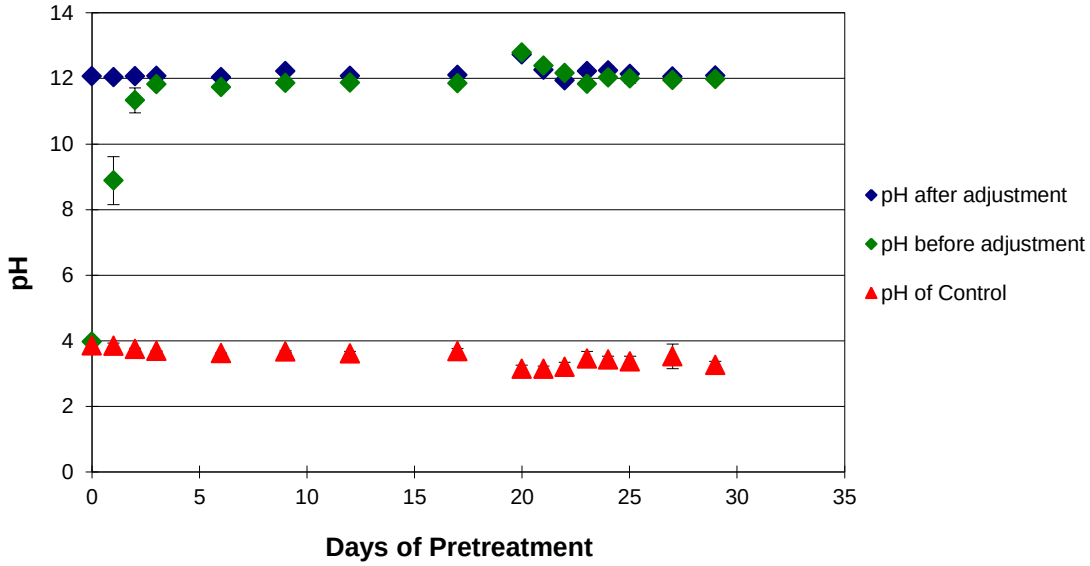


Figure 24: Percent of substrate VS solubilized into hydrolysate during long-term alkaline pretreatment batch experiments, with Experiment 1 having more frequent pH adjustment

Figure 25 shows the pH levels before and after adjustments in both alkaline pretreatment experiments. In both experiments, a drop in pH was observed during the first part of the experiment. This is most likely due to the fact that as the substrate was hydrolyzed, amino acids and fatty acids were generated as a result of hydrolysis of the protein and lipid fractions within the substrate. In general, these compounds, which made up approximately 18% of the substrate, hydrolyze relatively quickly, on the order of hours to days. Therefore, pH readjustment would be expected to be necessary during the earlier part of pretreatment in order to counteract the drop in pH resulting from acid formation, and maintain the substrate under alkaline conditions. After a certain point, pH re-adjustment would become unnecessary. This phenomenon was observed in both experiments, and the point at which pH readjustment became unnecessary was achieved faster in the case of Experiment 1 where more frequent pH adjustment was applied early on (daily for the first 3 days). As a result of tighter pH control at the beginning of the experiment, the substrate in Experiment 1 was effectively maintained near pH 12 for the entire 29 days, and resulted in essentially complete VS solubilization. In Experiment 2, due to less frequent pH re-adjustment during the first 30 days, the substrate was effectively at pH 12 for 44-49 days out of the total 68 days of pretreatment (approx. 68% of the time). Despite having been at pH 12 for a longer number of days, just less than 50% VS solubilization was achieved in Experiment 2. In short, the rate of substrate solubilization was shown to be dependent on maintaining the substrate at pH 12. A picture of the resulting substrate and hydrolysate from both experiments is shown in Figure 26.



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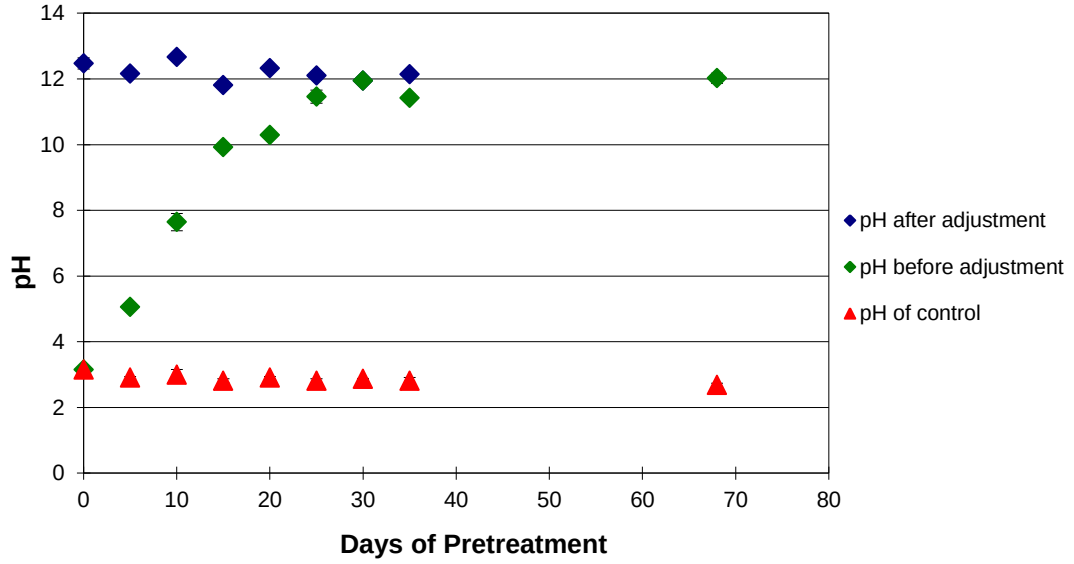


(b)

Figure 25: Trends in pH before and after re-adjustment, in pretreatment phase of long-term alkaline pretreatment experiments: (a) Experiment 1 (b) Experiment 2

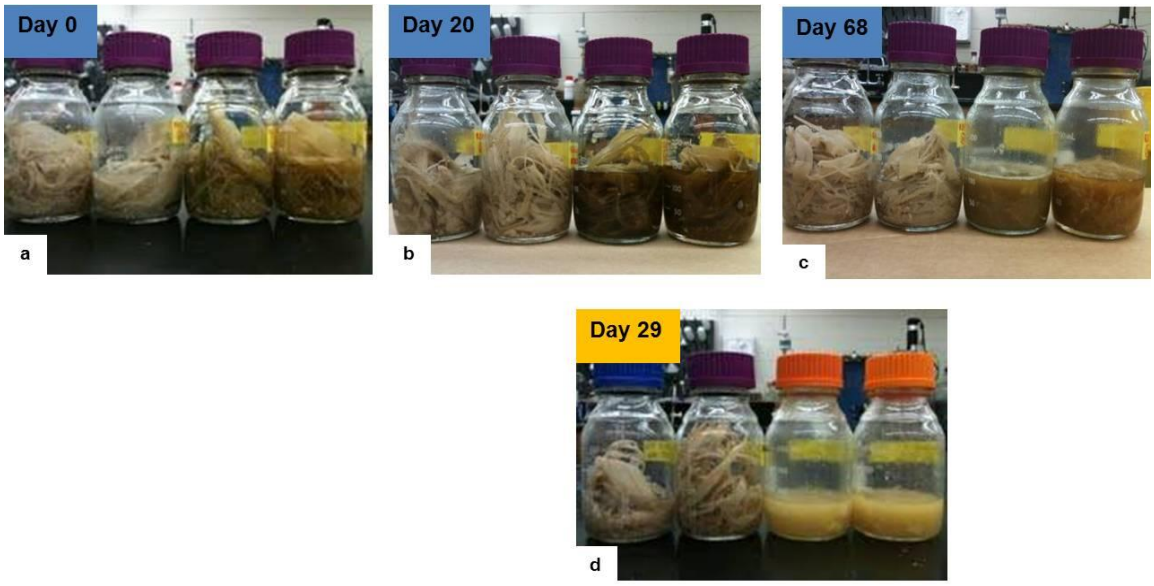
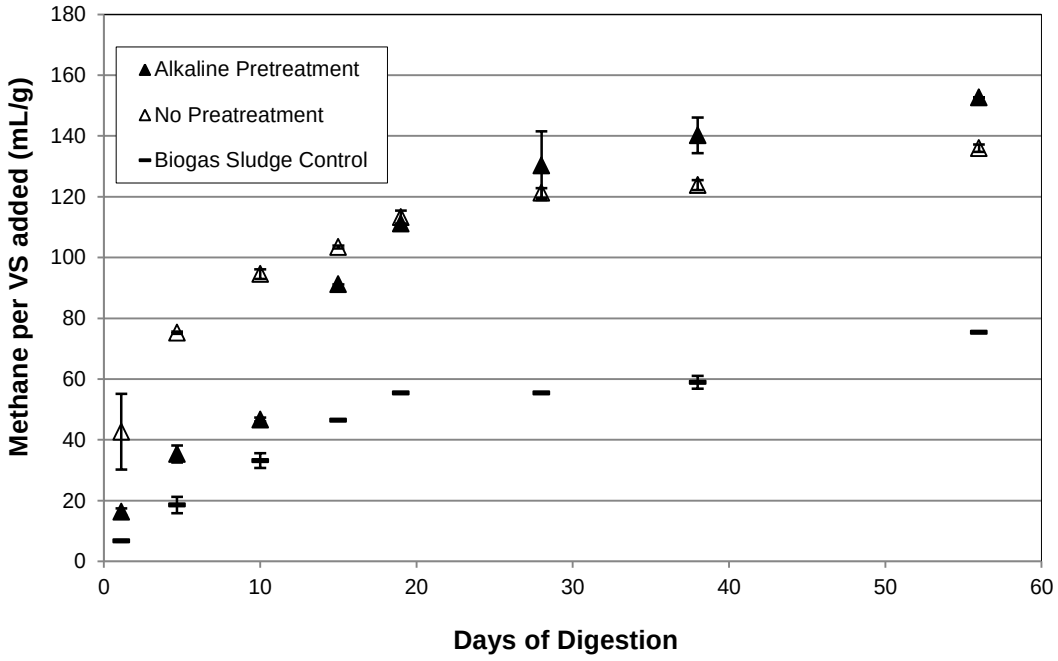
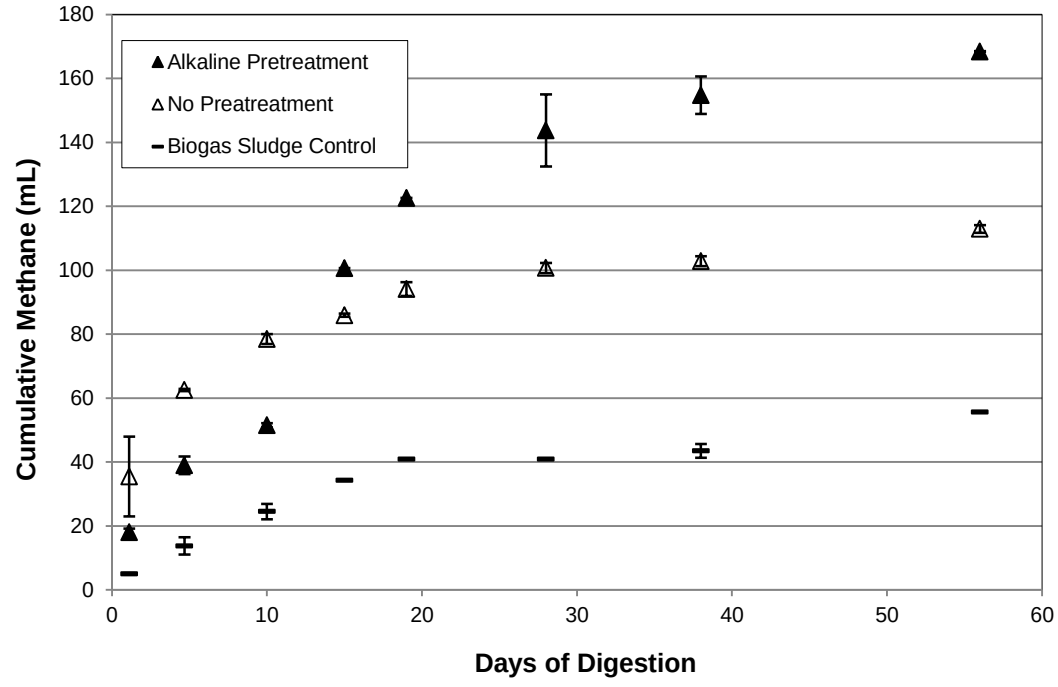


Figure 26: Image of alkaline pretreatment batch experiments after various days of treatment: (a-c) shows days 0, 20, and 68 of Experiment 2 respectively, (d) shows day 29 of Experiment 1. In each figure the two bottles on the left are the non-pretreated control condition, while the two bottles on the right are the alkaline pretreated test condition

To assess the digestibility of the resulting hydrolysate, hydrolysate from day 68 of Experiment 2 was combined with anaerobic sludge in a batch test. In general, the rate of methane production per gram of VS_{added} was similar in both alkaline pretreated and non-pretreated conditions, although alkaline pretreated conditions did result in 12% higher methane production after 56 days of digestion (153 versus 136 mL/g VS_{added}). During the first two weeks of digestion, the rate of methane production per gram of VS_{added} was lower in the pretreated condition than in the non-pretreated control. This could most likely be due to higher salinity in the hydrolysate resulting from the alkaline pretreated condition, and as such, the methanogens may have needed some additional time to acclimate. Results for cumulative methane production per gram of VS added are shown in Figure 27a.



(a)



(b)

Figure 27: Methane production from alkaline pretreatment hydrolysate batch test after 68 days of pretreatment: (a) shows methane production per gram of VS added to the batch, while (b) shows total cumulative methane production

While methane production per gram of VS was increased by just 12%, the total amount of methane produced in the alkaline pretreated condition was almost 50% higher than the control condition (168 versus 113 mL of methane), reflecting the fact that more volatile solids were present in the resulting hydrolysate due to greater substrate solubilization in the alkaline pretreated condition. Total cumulative methane production results are shown in Figure 27b. In this batch test, average methane content in the biogas ranged from 17 to 55% with an average of 44% in the alkaline pretreated condition, and 60 to 74% with an average of 67% in the non-pretreated condition. The relatively low methane percent in the alkaline pretreated condition is again likely due to higher salinity in the hydrolysate of the pretreated condition which may have adversely affected the anaerobic microbial community. Note that pH of the pretreatment batches was adjusted to neutral pH values prior to the addition of anaerobic sludge for digestion to methane. Thus, the lower methane purity in the biogas was not affected by the starting pH of the anaerobic digestion step.

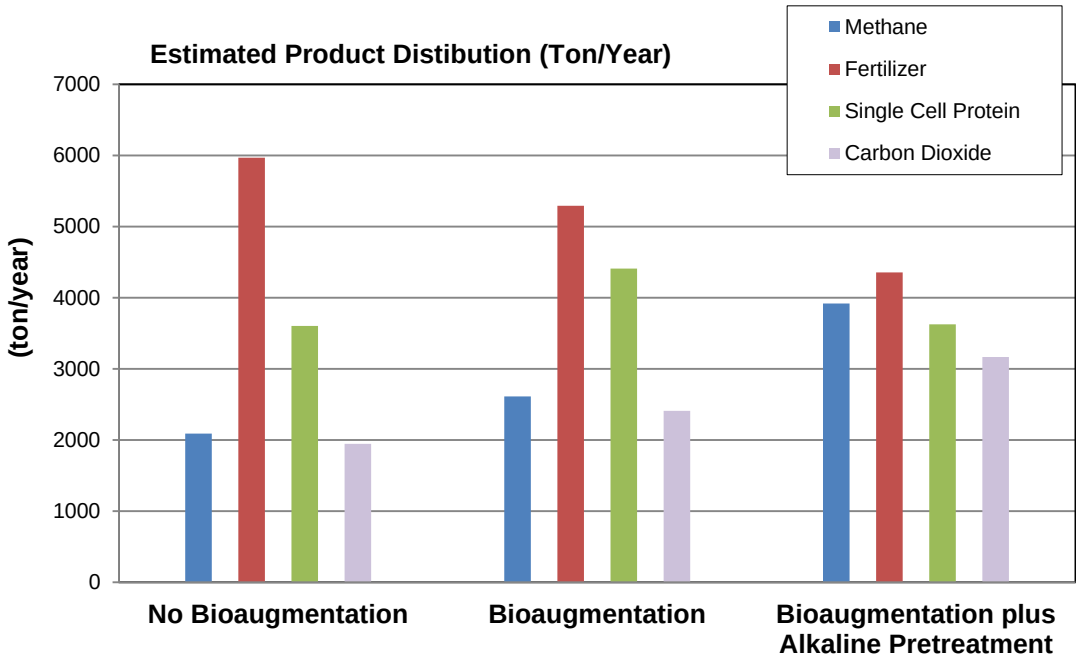
Overall, these results suggest that alkaline pretreatment can be an effective method for increasing substrate solubilization and methane yields. However, not all of the additional solubilized substrate from the alkaline pretreatment was effectively converted into biomethane, and it would appear there is room for improvement in that area. Combining alkaline pretreatment with the proposed two-phase anaerobic digestion process with routine bioaugmentation may further improve process efficiency; however, this combination was not investigated experimentally as part of this study.

5.4 Product Distribution Estimation

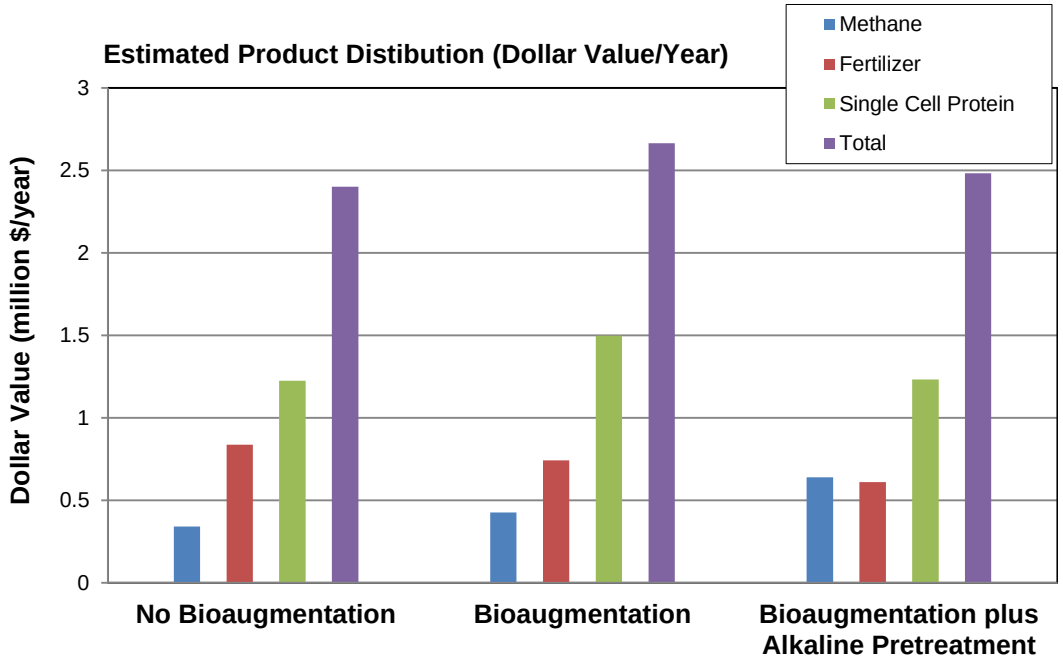
Using results from the described batch experiments, an estimate of the potential product yields for full scale operation of the integrated anaerobic/aerobic waste treatment process (as described in Chapter 3), was determined. In this estimation, three process scenarios were considered including two-phase anaerobic digestion without bioaugmentation, with bioaugmentation, and with bioaugmentation plus alkaline pretreatment. A loading rate of 70,000 tons per year of sweet corn residues, corresponding to approximately 13.2 million kg of volatile solids was assumed for the calculations. Values for potential methane production and volatile solids reduction were assumed to be the maximum methane production and volatile solids reduction achieved in the bioaugmentation versus no-bioaugmentation batch test. It was assumed that 50% more methane could be produced through the addition of alkaline pretreatment. These assumptions thus represented a potential maximum achievable methane production and volatile solids reduction as based on batch test results from this study.

Knowing the fraction of soluble organics in the final effluent, yields for fertilizer and single cell protein (SCP) were calculated. It was assumed that 100% of particulate organics in the resulting anaerobic digester effluent would be converted to fertilizer, and 80% of remaining soluble organics would be converted to SCP, based on the experience of other graduate students working in our research group on similar integrated processes. Although algal biomass was presented as a potential co-product in the proposed system design, it was excluded from this estimation do to having two degrees of separation from the anaerobic digestion process, which was the primary focus of this research. Carbon dioxide yields were also estimated in order to provide a complete mass balance of volatile solids conversion. Table 6 in Appendix A provides a list of the assumptions and calculations used for the estimated product distribution.

Results from the estimated product distribution are shown in Figure 28. Estimated product tonnage per year is shown in Figure 28a. From these results it can be seen that in all cases fertilizer is estimated to be the major product. Methane becomes increasingly significant with bioaugmentation and bioaugmentation plus alkaline pretreatment, reflecting the greater reduction in volatile solids achieved in these processes. Comparing the resulting estimated dollar values, as shown in Figure 28b, an increase in estimated total dollar value is seen with the addition of bioaugmentation. The reason that bioaugmentation plus alkaline pretreatment does not further increase the estimated total dollar value compared to bioaugmentation alone, is due to the low price of natural gas relative to the other products. However, it should be noted that the dollar values calculated for fertilizer and SCP in this study are rough estimates, and do not include the production cost involved in converting the raw materials generated from the digestion process into a final marketable product. Such costs include energy costs associated with aeration for single cell protein production, and dewatering/drying for fertilizer production. It is expected that after considering these costs, dollar values will favor methane production, due to the fact that the methane generated from the anaerobic digestion process can be used almost directly for heating or combined heat and power generation, with minimal further processing. In short, a more comprehensive economic analysis taking into account these and other production costs for items such as the bioculture, alkaline chemicals, and the agri-bag system is required in order to better compare the net profit associated with each process scenario. A comprehensive life cycle analysis comparing the GHG reductions and energy savings associated with each process would also be beneficial in order to identify the best option.



(a)



(b)

Figure 28: (a) Estimated distribution and (b) dollar amounts for potential methane, fertilizer, and single cell protein products generated from integrated two-stage anaerobic/aerobic treatment system under three different scenarios: no-bioaugmentation, with bioaugmentation, with bioaugmentation plus alkaline pretreatment

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary and Conclusions

In summary, the main objective of this study was to investigate the potential for improving anaerobic digestion of sweet corn processing residues through routine bioaugmentation with a cellulolytic bioculture and alkaline pretreatment. Results indicated that both routine bioaugmentation and alkaline pretreatment were beneficial in improving anaerobic digestion of sweet corn processing residues in terms of increasing rates of substrate hydrolysis and methane production. These results are significant because higher rates of digestion correspond to reduced reactor volumes and lower capital costs.

Bioaugmentation with a proprietary bioculture increased methane production by 34% compared to a non-bioaugmented control. Routine bioaugmentation with cellulolytic microorganisms showed benefits over one-time bioaugmentation in terms of increasing substrate hydrolysis (22-25%), based on measurement of neutral detergent fiber removal and soluble COD generation in a sequencing/semi batch test. Routine bioaugmentation with a proprietary cellulolytic bioculture also resulted in 15% higher methane production compared to one-time bioaugmentation. These results suggest that routine bioaugmentation may provide a method that is more effective than conventional one-time bioaugmentation in achieving and maintaining a microbial population better suited for hydrolysis of lignocellulosic substrates.

In the same routine bioaugmentation experiment, results indicated that bioaugmentation with dairy cow rumen fluid resulted in 16-34% higher methane production than bioaugmentation with a proprietary cellulolytic bioculture. This suggests that optimization of the existing bioculture or development of a custom bioculture, could potentially further improve the routine bioaugmentation process. This could be pursued

via isolation, identification, and cultivation of more effective strains of cellulolytic bacteria.

From alkaline pretreatment batch experiments, the results indicated that long-term alkaline pretreatment could increase substrate solubilization by 2-4 fold compared to a non-pretreated control, and produce a readily digestible hydrolysate. This suggests that wet-storage with concurrent alkaline pretreatment could be a viable option for a full-scale system treating sweet corn residues and other lignocellulosic wastes. In addition, the combination of alkaline pretreatment and routine bioaugmentation may further improve the efficiency and economic viability of the novel anaerobic/aerobic waste treatment process proposed in this study. However, experimental confirmation as well as a comprehensive economic analysis is needed to verify the benefits of adding wet-storage with alkaline pretreatment to the front end of the two-phase anaerobic digestion process with routine bioaugmentation. With that, a summary of the key conclusions from this study is presented below.

Summary of Key Conclusions

- For sweet corn processing residuals, bioaugmentation with cellulolytic microorganisms, dosed to the first phase of a two-phase anaerobic digestion process, was beneficial and resulted in 34% more methane production than without bioaugmentation.

- Routine bioaugmentation showed benefits over one-time bioaugmentation in terms of increasing substrate hydrolysis (22-25%) and subsequent methane production (15%).

- Bioaugmentation with dairy cattle rumen fluid resulted in (16-34%) higher methane production than bioaugmentation with a proprietary cellulolytic

bioculture suggesting that optimization of the bioculture could further improve process efficiency.

- Long-term alkaline pretreatment increased solubilization rates of sweet corn processing residues by 2-4 fold and produced a readily digestible hydrolysate.

6.2 Recommendations for Future Work

Seeing benefits in terms of increased substrate hydrolysis and methane production, from batch tests as a result of bioaugmentation and alkaline pretreatment, one logical next step is to apply both of these processes in a larger-scale, continuous-flow system in order to identify optimal operating parameters for full-scale application. Investigation of the routine bioaugmentation process in a bench scale two-phase semi-continuous system was initiated during the time of this study. Up to this point, however, biogas production in the semi-continuous system was somewhat erratic and lower than expected, warranting further investigation. Information regarding set-up and operation as well as daily biogas production results for the semi-continuous reactor system is presented and discussed in Appendix B. With that, recommended future work includes achieving steady-state operation in a bench scale two-phase semi-continuous or continuous reactor system, while applying routine bioaugmentation with a cellulolytic bioculture in the first phase. After determining optimal operating parameters for continuous operation (i.e. organic loading rate and HRT), the system should then be operated without bioaugmentation, in order to verify the effectiveness of the routine bioaugmentation process in terms of improving digestion efficiency, as observed from the batch tests conducted in this study. It is expected that once routine bioaugmentation has been stopped in the continuous system, methane production will decline after a period of time, supporting the hypothesis that routine bioaugmentation is an effective method for overcoming wash-out of the

bioaugmented microorganisms and maintaining an effective microbial community compared to conventional one-time bioaugmentation.

The combination of wet-storage with alkaline pretreatment prior to two-phase anaerobic digestion with routine bioaugmentation should also be investigated experimentally, and within a continuous system. With each of the individual processes showing benefits in terms of increasing substrate hydrolysis and methane production, the ability for a combined process to further improve digestion efficiency must be verified. Applying a wet-storage alkaline pretreatment phase to the front of a larger scale, continuous two-phase anaerobic digestion system with routine bioaugmentation would allow for assessment of the benefits of the combined process in improving process efficiency and identification of optimal full-scale operating parameters.

Having determined the optimal operating parameters for the various process scenarios (i.e. no bioaugmentation, bioaugmentation, bioaugmentation plus alkaline pretreatment), under continuous, steady-state operation, a more comprehensive and economic analysis should then be conducted to compare the potential full-scale economic benefits of each scenario. In this analysis, costs associated with producing the final marketable end products of methane, fertilizer, and single cell protein should be considered as well as digester operation costs and the respective costs of bioculture, base, and the agri-bag system for each process scenario. Such an analysis will allow for a more accurate comparison and better assessment of the economic viability of each process. A comprehensive life-cycle analysis should also be conducted, comparing the benefits in terms of GHG emission and energy savings for each process. With knowledge of both the economic and environmental benefits, the best option for full-scale operation can then be identified.

Finally, investigation of microbial community dynamics within the system as a result of routine bioaugmentation may provide further insight into the mechanisms by which routine bioaugmentation benefits anaerobic digestion. With that, monitoring of microbial community populations in the reactor using microbial community analysis techniques such as, automated ribosomal intergenic spacer analysis (ARISA) or fluorescence in-situ hybridization (FISH), as well as 16s-rRNA sequencing may provide insight into how the microbial community is changing as a result of routine bioaugmentation and which species are surviving and/or becoming dominant within the reactor. This information, as well as identification of effective strains of cellulolytic bacteria through literature or cultivation from various sources of cellulolytic bacteria such as rumen fluid, could allow for further optimization/customization of the bioculture, potentially further improving the routine bioaugmentation process. With that, a summary of the recommended future work is presented below.

Summary of Recommended Future Work:

- Apply routine bioaugmentation process in semi-continuous/continuous reactor system to determine optimal operating parameters.
- Investigate the combination of routine bioaugmentation and alkaline pretreatment.
- Conduct a comprehensive economic analysis comparing net profit of various process combinations of bioaugmentation and alkaline pretreatment.
- Conduct a comprehensive life-cycle analysis to compare benefits of various process scenarios in terms of GHG emissions and energy savings.
- Investigate microbial community dynamics and develop a custom bioculture to further improve the bioaugmentation process.

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APPENDIX A: ESTIMATED PRODUCT DISTRIBUTION SPREADSHEET

Table 6: Estimated Product Distribution Spreadsheet

Del Monte Corn Silage		**No Bioaugmentation**	**Bioaugmentation**	**Bioaugmentation plus Alkaline Pretreatment**
Influent characteristics	VS (%)	95.0%	95.0%	95.0%
	Moisture Content (%)	78.0%	78.0%	78.0%
Amount of Material	Q (ton/year) wet weight	70,000.00	70,000.00	70,000.00
	Q (ton/year) dry weight	15,400.00	15,400.00	15,400.00
VS loading	Influent VS loading total (ton/year)	14,630.00	14,630.00	14,630.00
	Influent VS loading total (kg/year)	13,272,043.40	13,272,043.40	13,272,043.40
	Influent VS loading total (lbs/year)	5,972,419.53	5,972,419.53	5,972,419.53
Biogas Production				
(From batch test results. Plus alkaline biogas is 1.5x of bioaugmentation)				
Methane Production	Methane (ml/g VS added)	**200.0**	**250.0**	**375.0**
	Methane production (L/year)	2.7E+09	3.3E+09	5.0E+09
	Methane production (cf/year)	93,737,788.13	117,125,783.01	175,688,674.51
16g/mol	Methane (mol/year)	118,487,798.17	148,109,747.71	222,164,621.57
	Methane production (ton/year)	**2,089.77**	**2,612.21**	**3,918.31**
From engineeringtoolbox.com	Methane heating value (BTU/cf)	910.0	910.0	910.0
Heat and power generated	Methane heating energy (BTU/year)	8.53E+10	1.07E+11	1.60E+11
	Methane heating energy (Therm/yr)	853,013.87	1,065,844.63	1,598,766.94
CH4:CO2 = 1.5, (1.3 in alkaline)	Carbon Dioxide (ml/g VS added)	133.3	166.7	288.5
	CO2 (L/year)	1.8E+09	2.2E+09	3.8E+09
	CO2 prodcution (cf/year)	62,467,084.27	78,083,855.34	135,145,134.24
12g/mol	CO2 (mol/year)	78,991,865.45	98,739,831.81	170,895,862.75
	CO2 prodcution (ton/year)	**1,044.88**	**1,306.10**	**2,260.57**
Fertilizer and SCP Production (and CO2)				
assume alkaline = bioaug.	Cummulative CH4 produced in batch test (ml)	373	554	554
	VS reduction from batch test	53%	58%	58%
	g VS reduced per ml CH4 produced	**0.0014**	**0.0010**	**0.0010**
	VS reduced (%)	28.42%	26.17%	39.26%
	Total VS remaining (kg)	9,500,363.51	9,798,313.63	8,061,448.74
	Percent particulate VS (from batch)	**0.57**	**0.49**	**0.49**
	Percent soluble VS (from batch)	**0.43**	**0.51**	**0.51**
100% of particulate VS to fertilizer	Fertilizer (kg/year)	5,415,207.20	4,801,173.68	3,950,109.88
	Fertilizer (ton/year)	**5,970.46**	**5,293.47**	**4,355.14**
80% of soluble VS to SCP	SCP (kg/year)	3,268,125.05	3,997,711.96	3,289,071.09
	SCP (ton/year)	**3,603.22**	**4,407.62**	**3,626.32**
20% of soluble VS to CO2	CO2 from SCP production (kg/year)	817,031.26	999,427.99	822,267.77
	CO2 from SCP production (ton/year)	900.81	1,101.91	906.58
	Total CO2 (ton/year)	**1,945.69**	**2,408.01**	**3,167.14**
Total Sum of Products (ton/year)				
		13,609.14	14,721.31	15,066.91
Dollar amount				
Source:				
EIA, 2012	Methane ($ per thousand cf)	3.64	3.64	3.64
	Methane (milllion $ per year)	**0.34**	**0.43**	**0.64**
Feeco International, 2011	Fertilizer ($ per ton)	140.00	140.00	140.00
(wholesale price of milorganite)	Fertilizer (million $ per year)	**0.84**	**0.74**	**0.61**
alibada.com, 2012	SCP ($ per ton)	340.00	340.00	340.00
(avg. price of yeast powder)	SCP (million $ per year)	**1.23**	**1.50**	**1.23**
	Total (million $ per year)	**2.40**	**2.67**	**2.48**

APPENDIX B: SEMI-CONTINUOUS REACTOR OPERATION

B.1 Semi-Continuous (aka Sequencing Batch) Reactor Set-up

During the time that the batch experiments presented in this study were being conducted, a bench scale two-phase semi-continuous reactor was also set-up to evaluate the routine bioaugmentation process under steady state conditions. The reactor was operated for approximately 400 days. Inputs included the dry ground sweet corn residues and the proprietary bioculture in a 1:10 g of bioculture per g of substrate ratio. The acid phase reactor consisted of a 3 L bioreactor (Belco Biotechnology, No. 1585-4L), which was operated at a 2 day HRT, with a loading rate of 3% total solids and was maintained at pH 5.0-6.5. Temperature in the acid phase reactor was maintained under mesophilic temperatures (37-40˚C) by means of a hot plate (Corning Hot Plate, No. PC-35). Mixing was provided by a mechanical mixer (Stir-Pack Laboratory Mixer, No. 3-250) which was set to mixing speed 1.

The methane phase reactor consisted of a 14 L New Brunswick Bioflo 115 bioreactor. The Bioflo unit provided mixing, temperature, and pH control. Default settings for these operating parameters were 50 RPM, 40˚C and pH 7.5, respectively; however several variations of these parameters over the 400 days of operation were made in attempts to improve reactor performance. Material was transferred through the system via electric pumps (SCC Pumps Incorporated, Model No. AC-10615). Default HRT in the methane phase reactor was 10 days, but again this varied on occasion, with the reactor being operated as a semi-batch for some period of time in an attempt to improve reactor performance. Biogas production was measured via a water displacement column that was connected to an outlet on the reactor. Sampling of the head space via syringe and a septum port on the top of the reactor was done periodically to determine biogas quality.

Biogas samples were collected in Vacutainer sample vials (BD Vacutainer, 8020128), and measured by gas chromatography (Varian, Model 3800).

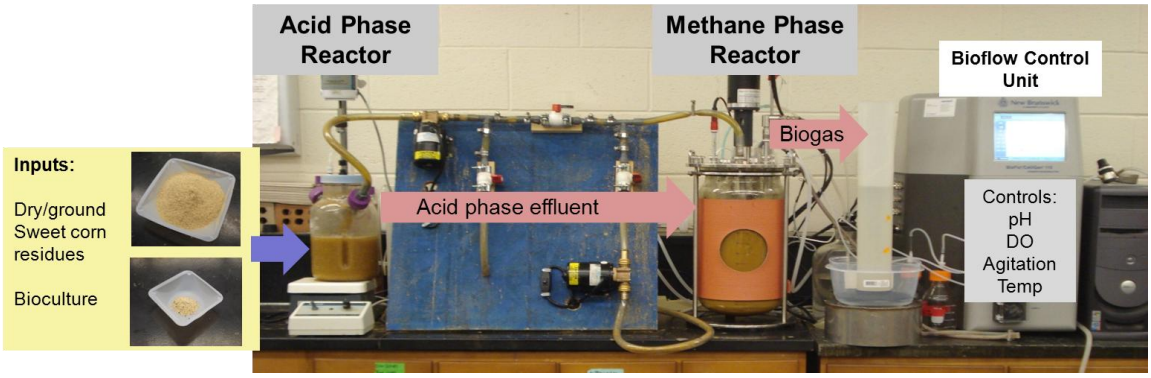

Figure 29: Picture of bench scale two-phase semi-continuous reactor set-up

B.2 Semi-Continuous Reactor Results

The two-phase semi-continuous reactor was operated for approximately 400 days to evaluate the routine bioaugmentation process at a larger scale and determine optimum operating parameters under steady-state continuous operation. However, over the 400 days of operation, biogas production from the semi-continuous reactor system was somewhat erratic and in general lower than expected based on batch test results. The average biogas yield over the 400 days of operation was 50 ml per gram of VS_{added} and ranged from as low as 3 to 161 ml per gram VS_{added}. Average methane content in the biogas was 56%. Daily biogas production results from the semi-continuous reactor are shown in Figure 30.

Speculation of potential causes for the low performance seen in the semi-continuous reactor included leakage, acid accumulation, poor mixing, wash-out, and insufficient HRT. Several actions were taken in an attempt to address each of these potential causes including installation of auto pH adjustment, variation in mixing conditions, reseeding

with fresh methanogens, operation as a semi-batch, and even operation under thermophilic conditions, however little or no improvement was seen.

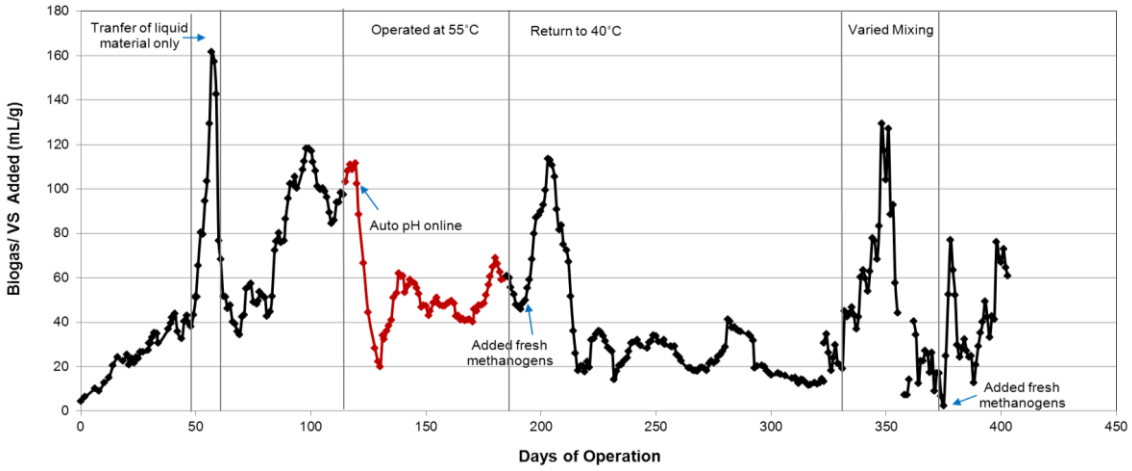

Figure 30: Semi-continuous reactor results: daily biogas production per gram of VS added

At this point, it is the author's opinion that relatively high organic loading and short HRT may be potential causes for the inconsistency in methane production seen between previous batch test results and semi-continuous reactor operation. Results from the bioaugmentation versus no-bioaugmentation batch test presented in this study indicated that a methane yield near 200 ml per gram of VS_{added} was achieved after 10 days of digestion in the bioaugmented condition. Thus, methane production near 200 ml per gram of VS_{added} was expected from the semi-continuous reactor system, as the methane phase was operated at a 10 day HRT. However, the organic loading rate in the semi-continuous reactor was higher than that of the batch experiment (3g per ml versus 1g per ml); therefore a longer retention time may be required in the semi-continuous reactor in order to achieve the same degree of digestion.

Another difference between the batch experiment and semi-continuous reactor system is the fact that in the batch test there is no removal of material from the methane phase, whereas in the semi-continuous reactor material is regularly added and removed

from the methane phase reactor. Therefore, it is also possible that wash-out of methanogens from the methane phase of the semi-continuous system is another potential cause for the low performance. On two occasions, which are indicated on Figure 30, fresh methanogens were added to the methane phase reactor which resulted in an initial increase in biogas production. This increase, however, was not sustained overtime, supporting the hypothesis of methanogen wash-out. It is possible that by lengthening HRT in the methane phase reactor, wash-out of the methanogens may be prevented. Also, because the sweet corn processing residues which served as the substrate in this system do not have a substantial inherent microbial community associated with them, routine bioaugmentation with methanogens or co-digestion with animal manure could provide a regular recharge of methanogens to the system allowing for operation at relatively low HRT.