Utah State University

DigitalCommons@USU

All Graduate Plan B and other Reports

Graduate Studies

5-2013

Strategies to Enhance Conversion of Lignocellulosic Biomass to Fermentable Sugars and to Enhance Anaerobic Digestion of Algal Biomass for Biogas Production

Kristen M. Sims

Follow this and additional works at: https://digitalcommons.usu.edu/gradreports

UtahState University
MERRILL-CAZIER LIBRARY

STRATEGIES TO ENHANCE CONVERSION OF LIGNOCELLULOSIC BIOMASS TO FERMENTABLE
SUGARS AND TO ENHANCE ANAEROBIC DIGESTION OF ALGAL BIOMASS FOR BIOGAS
PRODUCTION

By

Kirsten M. Sims

A report submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In

Biological Engineering

Approved:

Charles Miller	Issa Hamud
Committee Chair	Committee Member

Byard Wood
Committee Member

UTAH STATE UNIVERSITY
Logan, Utah
2012

ABSTRACT

Strategies to Enhance Conversion of Lignocellulosic Biomass to Fermentable Sugars

and to Enhance Anaerobic Digestion of Algal Biomass for Biogas Production

By

Kirsten M.Sims, Master of Science

Utah State University, 2012

Major Professor: Dr. Charles Miller
Department: Biological Engineering

For economical conversion of cellulosic biomass to biofuel sources, such as ethanol and biogas, high concentrations of biomass must be processed to minimize downstream costs associated with dilute solutions. Further, the fundamental processes that drive biomass transformation must be understood in order to optimize conversion processes and to efficiently overcome the technological and economic barriers of biofuel production. One such barrier includes the recalcitrance of biomass to degradation. In order to overcome the recalcitrance of biomass, this study focuses on pretreatment strategies to break down the cell wall components and to make the biomass more susceptible to enzymatic action.

Specifically, this study focuses on the use of alkaline peroxide pretreatment under varying conditions for processing lignocellulosic biomass. Optimal conditions for alkaline peroxide pretreatment were identified for increasing subsequent enzymatic saccharification to fermentable sugars.

This study also evaluated the use of alkaline pretreatment for algal biomass that is present in the Logan Lagoon Wastewater System in order to enhance the anaerobic digestibility of the algae. Different bacterial seed sludges were also evaluated for increasing gas production from anaerobic digestion of algal biomass.

ACKNOWLEDGEMENTS

FORWARD

This report investigates potential strategies for enhancing the biological transformation of biological feed stocks to intermediate compounds that can be further utilized to produce biofuels. Specifically, this report focuses on the development of pretreatment processes that include the use of environmentally benign compounds and the reduction of energy input requirements to enhance the conversion of biomass to biofuel. The two feed stocks that were investigated were lignocellulosic biomass and algal biomass.

The first portion of this report focuses on the transformation of lignocellulosic biomass to fermentable sugars that may be subsequently converted to ethanol using enzymatic saccharification processes. This research was carried out under the direction of Dr. Sridhar Viamajala, a USTAR professor in the Biological Engineering Department at Utah State University. After one year of research, Dr. Viamajala took a position at the University of Toledo.

After Dr. Viamajala's departure from USU, I transitioned to work under Dr. Charles Miller, an Assistant Professor in the Biological Engineering Department at USU. Under the guidance of Dr. Miller, I focused on the transformation of algal biomass to methane gas through anaerobic digestion, which may subsequently be used as compressed natural gas. The anaerobic digestion of algal biomass is the focus of the second portion of this report.

<p style="text-align: center;">CONTENTS</p>

LIST OF FIGURES

Introduction

Life on Earth is driven by the photosynthetic conversion of sunlight to chemical energy, wherein photosynthesis results in the production of plant biomass with cellulose as the major component of plant cell walls. Cellulose is the most abundant polysaccharide in the biosphere.

Cellulose-utilizing microorganisms, present in the soil and in the guts of animals, are primarily responsible for major conversion of cellulosic materials in the biosphere. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of carbon flux on micro and macro scales. Microbial cellulose utilization is also an integral component of widely used processes such as anaerobic digestion and composting.

Cellulosic materials are particularly attractive as a sustainable source of fuels and other bioproducts. Important distinguishing features of cellulosic biomass compared to other potential feedstocks for biological processing include low purchase price, potential for supply on a large scale, recalcitrance to reaction, and heterogeneous composition.

For production of biologically derived fuel, as well as other bulk chemicals, a feedstock having a low cost and wide availability is required. The economics of cost-competitive commodity processes are dominated by feedstock cost, and thus require high product yields (Hinman, N.D. et al., 1992; Lynd, L.R. 1999; Lynd, et al.,

1999; Wright, J.D. 1988). A primary technological impediment to the large-scale development and use of cellulosic material as a source of fuel is the general absence of low-cost technology and processing methods for overcoming the recalcitrance of cellulosic biomass.

Specifically, lignocellulosic biomass, which includes agricultural residues, energy crops, wood residues and municipal paper waste, represents a relatively unused source for biogas and ethanol production. Lignocellulose is plant biomass that is composed of cellulose, hemicellulose, and lignin. Many factors, however, are shown to limit the digestibility of the hemicellulose and cellulose present in lignocellulosic biomass. These factors include lignin content, crystallinity of cellulose, and particle size.

Lignocellulosic Biomass Structural Components

Cellulose, the most abundant component of plant biomass, is found in nature almost exclusively in plant cell walls. Cellulose is also known to be produced by some animals and a few bacterial species. Cellulose has been shown to comprise approximately 35-50% of plant dry weight (Lynd, 1999). In most cases, cellulose fibers are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 25 and 5 to 30% of plant dry weight (Lynd, 1999; Marchessault et al., 1957; and Van Soest, 1994), respectively.

<u>Cellulose</u>

In nature, cellulose is synthesized as individual chains of glucosyl residues. Cellulose exists of D-glucose subunits, linked by B-1,4 glycosidic bonds. The linear cellulose chains undergo self-assembly at the site of biosynthesis (Brown et al., 1977). There is evidence that associated hemicelluloses may regulate this aggregation process (Atalla, et al., 1993). Approximately 30 individual cellulose molecules are assembled into larger units, referred to as protofibrils. The protofibrils may be packed into even larger units, known as microfibrils. Microfibrils are in turn assembled into cellulose fibers.

The chains are strengthened by both interchain and intrachain hydrogen bonds. In cellulose type I, the most abundant form of cellulose in nature, sheets of substantially parallel cellulose chains lie adjacent to one another, and overlie one another. These sheets are held together by weak intersheet van der Waals forces. Despite the relative weakness of individual van der Waal forces, their cumulative effect of their many residues in the protofibrils is considerable (Hatfield et al., 1999).

This well-defined organization of cellulose molecules yields a crystalline structure, implying a structural order in which all of the atoms are fixed in discrete positions relative to one another. The component molecules of the resulting crystalline structure microfibrils are packed together tightly such that they sufficiently prevent penetration not only by degradative enzymes, and but also by small molecules such as water (Hatfield et al., 1999)

Although cellulose forms a distinct crystalline structure, cellulose fibers in nature are not purely crystalline, having cellulose fibers that are more loosely associated and can in fact be completely amorphous, as well as all degrees of order in between (Marchessault et al., 1957). In addition to crystalline and amorphous regions, cellulose fibers may contain various structural irregularities, such as voids or twists in the fibers that provide significant additional surface area. These irregularities may be shaped to allow penetration by relatively large molecules, including some cellulolytic enzymes. (Blouin et al., 1970; Cowling, 1975; Fan et al., 1980). The heterogeneity may also allow for portions of the fiber to by hydrated by water when immersed in aqueous media.

Several types of purified celluloses have been identified for use in studies pertaining to hydrolysis. Holocelluloses are produced by delignification of wood or other biomass materials, and may retain substantial amounts of various hemicelluloses. Microcrystalline celluloses are nearly pure cellulose, and the dilute-acid treatment used in their preparation removes both hemicelluloses and some amorphous regions of the cellulose fibers. Commercial microcrystalline celluloses differ in particle size distribution, which has significant implications for the rate of hydrolysis and utilization. Several species of bacteria are known to produce cellulolytic materials, such as that produced by *Acetobacter sylinum.* This bacterial cellulose has been identified as a useful model system for studying cellulose biosynthesis, but has rarely been used for studies in cellulose utilization.

The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to a wide use for investigative studies and for industrial processing of another cellulosic material, carboxymethylcellulose (CMC). Carboxymethylcellulose is a highly soluble and pure cellulose ether.

Utilization of cellulosic biomass is more complex than is that of pure cellulose, however. The added complexity is attributed to the presence of hemicelluloses and lignin, as well as the diverse architecture of plant cells in nature. (Fan et al., 1980).

Hemicellulose

Hemicellulose is a complex carbohydrate structure that consists of different polymers like pentoses (xylose and arabinose), hexoses (mannose, glucose and galactose), and sugar acids. The dominant component of hemicellulose from hardwood and agricultural plants, like grasses and straw, is xylan (Fengel and Wegener, 1984). Hemicellulose has a lower molecular weight than cellulose, and includes branches with short, lateral chains that consist of different sugars. The short branches are easily hydrolyzed polymers (Fengel and Wegener, 1984). Hemicellulose serves to link the lignin and the cellulose fibers, thus increasing the cellulose-hemicellulose-lignin rigidity (Hatfield, et al., 1999).

Lignin

After cellulose and hemicellulose, lignin is one of the most abundant polymers in nature. Lignin is an amorphous heteropolymer consisting of the

following three different phenylpropane units: p-coumaryl, coniferyl, and sinapyl alcohol. These phenylpropane units are held together by various types of linkages.

Lignin serves primarily to give structural support to plants as well as to increase impermeability and resistance against microbial attack and oxidative stress. Lignin is also non-water soluble and optically inactive. Therefore, lignin has been shown to be highly resistant to degradation (Fengel and Wegener, 1984).

Lignin and hemicellulose have been shown to dissolve in water at around 180 degrees C under neutral conditions (Bobleter, 1994). Solubility of lignin in acid, neutral, or alkaline environments is dependent on the precursor molecules and their associations with one another in the lignin structure (Grabber, 2005).

Cellulolytic Organisms and Enzymatic Systems

There also exists in nature a broad distribution of cellulolytic capabilities. It has been suggested that cellulolytic abilities evolved alongside the emergence of algae and land plants, when presumably there existed the selective pressure of abundant cellulose availability following the development of cellulose biosynthesis. Fungi in particular have well-documented cellulolytic capabilities (Lee et al., 2002).

A number of species of the most primitive group of fungi, the anaerobic Chytridomycetes, are well-known for their ability to degrade cellulose in the gastrointestinal tracts of ruminant animals. Cellulolytic capability is also well represented among aerobic fungi. Particularly, fungal subdivisions Ascomycetes, Basidiomycetes, and Deuteromycetes, contain large numbers of cellulolytic species.

Referring to Table 1, members of genera that have received considerable

investigations with respect to their cellulolytic enzymes and/or wood degrading

capability are described (Lee et al., 2002)

Table 1. Yeast genera that have known cellulolytic enzymatic activities

Ascomycetes	Basidiomycetes	Deuteromycetes
Bulgaria	*Coriolus*	*Aspergillus*
Chaeetomium	*Phanerochaete*	*Sladosporium*
Helotium	*Poria*	*Fusarium*
	Schizophyllum	*Geotrichum*
	Serpula	*Myrothecium*
		Paecilomyces
		Penicillium
		Trichoderma

A variety of cellulase enzyme systems produced by cellulolytic organisms

have been identified. Components of cellulase systems were first classified based on

their mode of catalytic action. More recently, the structure of the different

components of cellulolytic systems have been elucidated.

The following three major types of enzymatic activities are known:

i)   Endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4)

ii)   Exoglucanases, including 1,4-β-D-glucan glucanohydrolases

(cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases

(cellobiohydrolases) (EC 3.2.1.91)

iii)   β-glucosidases or β-glucoside glucohydrolases (3.2.1.21)

Endoglucanases act to cut the cellulose polysaccharide chains at random intervals at internal amorphous sites. This generates oligosaccharides of various lengths, and consequently new chain ends that are available to exoglucanases. Exoglucanases act on the reducing or nonreducing ends of cellulose polysaccharide chains in a processive manner, moving along the length of the chain. This action releases either glucose (glucanohydrolases) or cellobiose (cellobiohydrolases) as major products.

Cellulases are unique in their ability to hydrolyze β-1,4-glucosidic bonds between glucosyl residues. The enzymatic breakage of the β-1,4-glucosidic bonds in cellulose proceeds through an acid hydrolysis mechanism. A general feature common to most cellulases is a modular structure often including both a catalytic module and a carbohydrate-binding module (CBM). The CBM contains binding sites for engaging the cellulose surface, and presumably acts to facilitate cellulose hydrolysis by positioning the catalytic domain in close proximity to the substrate. It has been demonstrated that the presence of CBMs is particularly integral to the initiation and processivity of exoglucanases (Teeri et al., 1998).

Cellulase systems exhibit synergy, or a higher collective enzymatic activity than the sum of the activities of individual enzymes. The following four types of synergy have been identified in cellulolytic systems:

i)   endo-exo synergy between endoglucanases and exoglucanases

ii)   exo-exo synergy between exoglucanases processing from the reducing

        and non-reducing ends of cellulose chins

iii)   synergy between exoglucanases and β-glucosidases and remove

        cellobiose (and cellodextrins) as end products of the first two enzymes

iv)   intramolecular synergy between catalytic domains and CBMs (Teeri,

        1997; Aoyagi et al., 1995)

Cellulases from aerobic fungi have received more investigation than have those of other physiological groups. Fungal cellulase systems are of particular interest due to their wide spread use in industrial ethanol-producing applications. *Trichoderma reesei* has been the primary focus of much of the research for at least 50 years (Mandels and Weber, 1957; Reese, 1956; Reese and Mandels, 1971; and Reese et al., 1950). *T. reesei* produces at least two exoglucanases, five endoglucanases and two β-glucosidases.

The two exoglucanases, referred to as Cellobiohyrolase I and II (CBH I and CBH II) are the principal components of the *T. reesei* cellulase system, representing 60 and 20% respectively (Wood, 1992). Both include a CBM to facilitate binding and drive processive degradation along the length of the cellulose chains.

Crystallography has elucidated the three-dimensional structure of CBH I and CBH II. Both enzymes have been identified to contain a tunnel-like structural feature. CBHI has been shown to include four surface loops that comprise a tunnel, with a length of approximately 50 Angstroms.   CBHII contains two surface loops that comprise a tunnel of approximately 20 Angstroms. The tunnels are essential for

the processive cleavage of the cellulose chains from the reducing or non-reducing ends. It has been demonstrated that as the enzymes move along the length of the chain, the chain is passed through at least a portion of the tunnel structures, where cleavage of the bonds occur.

A 3-D structure of CBHI confirmed that cellobiose is the major hydrolytic product as the cellulose chain is passed through the tunnel. Both CBHI and CBHII are indicated to be relatively slow to decrease the degree of polymerization.

The five endoglucanases, EGI, EG II, EGIII, EGIV and EGV are thought to be primarily responsible for decreasing the degree of polymerization of cellulose by internal cleaving of cellulose chains at relatively amorphous regions, thereby generating new cellulose chain ends available for action by the cellobiohydrolases (Teeri et al., 1998).

The structure of EGI has also been resolved using crystallography (Kleywegt et al., 1997) to reveal a plurality of sorter loops that comprise a groove rather than a tunnel. The groove presumably acts to allow the entry of the cellulose chain and facilitates cleavage of the chain. A similar groove is seen to exist in EGIII, although EGII lacks a CBM.

T. reesei produces β-glucosidases at low levels compared to other fungi species (Reczey et al., 1998). The production of at least two β-glucosidases by *T. reesei* facilitates by hydrolysis of cellobiose and small oligosaccharides to glucose (Reczey et al., 1998; Usami et al., 1990) The β-glucosidases in *T. reesi* cellulase

cohorts have also been shown to be subject to product inhibition, primarily glucose inhibition (Chen et al., 1992; Gong et al., 1977; Maguire, 1977). This inhibition effect is thought to be exacerbated when the solids concentration during enzymatic action is increased (Sridhar Viamajala, personal communication, June, 2008), presumably due to the overall increase in glucose production.

Rate Limiting Factors for Cellulolytic Action

For enzymatic hydrolysis of natural celluloses, several hydrolysis rate-determining factors have been proposed, including crystallinity, degree of polymerization, particle size, pore volume, and accessible surface area (Fan et al., 1980).

Within any given cellulose sample, there is a great degree of variability in the size and shape of individual particles (Walker et al., 1990; Weimer et al., 1990). Measurements of fine-structure features, including particle size, crystallinity, or surface area yield average values for that particular population. Thus, experiments to evaluate the quantitative effect of these rate-limiting factors on hydrolysis are restricted to comparing measurements of hydrolysis or utilization among populations of particles and their average structural characteristics.

Even within a given population of cellulose fibers, structure-utilization relationships are further complicated by various interrelationships among various structural features. For example, structural discontinuities that contribute to increased pore volume also act to lower the average degree of crystallinity. Therefore, it is difficult to alter a first fine-structure feature without simultaneously,

19

and often inversely, altering at least a second fine-structure feature to determine the effect of the first structure feature on utilization and rate of hydrolysis.

Crystallinity is widely considered a major determinant of cellulose hydrolysis at both enzymatic and microbial levels. Several pretreatment process options have been identified to decrease the degree of crystallinity. Studies with pure celluloses indicate that amorphous celluloses are degraded five to ten times more rapidly than are high crystalline celluloses by fungal enzymes (Gama et al., 1994). Pretreatment strategies will be discussed in further detail later in this review.

Cellulose utilization efficiency has also been shown to be subject to physical and chemical conditions, such as pH and redox potential in the surrounding environment. Cellulosic substrates occurring in nature and those resulting from pretreatment processes typically contain lignin, to which many cellulase components bind. Naturally occurring cellulosic substrates and some pretreated substrates also contain hemicellulose, which impedes access of cellulase components to 1,4-β-glucosidic bonds.

Cellulose materials that include hemicellulose may require distinct hydrolysis enzymatic activities from those involved in cellulose degradation. Kinetic properties, such as adsorption capacity and affinity may vary over the course of hydrolysis as the structure of the cellulose material changes. Chemical properties, such as fractional composition of different components (for example, the overall ratio of cellulose to hemicellulose) and physical properties such as size, shape, density, and rigidity, may also vary over the course of hydrolysis. Given the

enzymatic, substrate and organismal properties, as well as the interactions among these properties, cellulose hydrolysis is recognized as an exceedingly complex phenomenon. Many kinetic models that have been developed represent simplifications of the real situation.

Pretreatment

Incubation of naturally occurring cellulosic material in the presence of either pure cultures of cellulolytic microorganisms or cell-free cellulase enzyme preparations have generally resulted in cellulose hydrolysis yields that are less than 20% of theoretical hydrolysis values (Gama et al., 1994). Consequently, the need for a pretreatment process before hydrolysis is nearly always required to bring the hydrolysis value closer to theoretical values, thus increasing the economic feasibility of cellulosic ethanol production. "Pretreatment" of cellulosic materials generally refers to an engineering process step in which the recalcitrance of lignocellulosic biomass to enzyme systems is reduced, increasing the availability of the biomass to enzymatic hydrolysis.

β-glucosidic bonds in naturally occurring lignocellulosic materials are inaccessible to cellulase enzymes due to small pore sizes and enzyme-associated inaccessibility. The structural complexity, including association of hemicellulose and other structural polysaccharides, further hinders enzyme activity on the cellulosic material. Overcoming these physical and chemical barriers require a pretreatment step to render lignocellulosic materials amenable to enzymatic hydrolysis. Pretreated lignocellulosic materials are generally characterized by increased surface

area that is accessible to cellulase enzymes and solubilization, specifically increased porosity and/or redistribution of lignin. Increasing porosity, and therefore surface area, requires hemicellulose solubilization, lignin solubilization and lignin redistribution.

Pretreatment Methods

The goal of pretreatment is to improve the digestibility of lignocellulosic biomass. Each pretreatment has a unique effect on the cellulose, hemicellulose, and lignin.

Lignin redistribution is the primary result of dilute acid and steam explosion pretreatment methods, although lignin is not significantly removed (Converse, 1993). It is proposed that during these procedures, lignin "melts", and then coalesces upon cooling, substantially altering its properties (Brownell and Saddler, 1987; Converse, 1993; Donaldson et al., 1988; Tanahashi et al., 1983).

Additional proposed pretreatment processes that have been identified as promising include dilute acid, steam explosion at high solids concentration, "hydrothermal" process, "organosolv" processes involving organic solvents in an aqueous medium, ammonia fiber explosion (AFEX), and strong alkali processes using a strong base such as NaOH, or lime. Each of these processes have been shown to produce pretreated fiber derived from herbaceous and/or hardwood-derived feedstocks that retains nearly all of the cellulose present in the original material and allows close to theoretical yields of sugars upon enzymatic hydrolysis, under

appropriate conditions. These processes, similar processes and combinations of these processes, are discussed in further detail below.

Thermal pretreatment processes include heating the lignocellulosic biomass. Hemicelluloses and lignin will begin to solubilize as the temperature approaches 150-180 degrees C. Composition of the hemicellulose backbone, as well as the arrangement of branching groups determine the thermal, acid and alkali stability of the hemicellulose. Xylans are slightly less thermally stable than glucomannans. Above 180 degrees C, an exothermal reaction of hemicellulose begins (Beall and Eickner, 1970; Domansky and Rendos, 1962).

During thermal processes, a part of the hemicellulose is hydrolyzed, forming various acids. These acids are assumed to further catalyze hydrolysis of hemicellulose (Gregg and Saddler, 1996). Compounds that are released during lignin solubilization as temperature increases are usually phenolic and have been shown to have an inhibitory effect of cellulosic bacteria, yeast and methanogens/archae (Gossett et al., 1982). The phenolics are also highly reactive and unless they are removed quickly will re-condense on the biomass (Liu and Wyman, 2003), presenting challenges to thermal pretreatment in the presence of lignin.

When solids concentrations exceeded 3% with temperatures of 220 degrees C during a two-minute pretreatment process, ethanol production was essentially completely inhibited, due to formation of furfural and other compounds (Laser et al., 2002). Temperatures, however, cannot exceed 250 degrees C to avoid inducing pyrolysis reactions (Brownell et al., 1986). Some studies have also shown that

23

thermal pretreatment can also cause an increase in crystallinity (Weimer et al., 1995).

Thermal processes include steam pretreatment/steam explosion (ST/SE). The objective of a steam pretreatment/steam explosion is to solubilize the hemicellulose to make the cellulose more accessible for enzymatic hydrolysis, and to avoid the formation of inhibitory bi-products. During steam pretreatment processes, the biomass is put in a large vessel and exposed to steam at very high temperatures and pressure (up to 240 degrees C) for a short duration (2-3 minutes). The steam is then suddenly released and the biomass is quickly cooled. The quick depressurization and cooling differentiates "steam preatreatment" and "steam explosion". The depressurization causes the water in the biomass to "explode", although the advantageous effect of the explosion is somewhat doubted (Brownell et al., 1986).

During steam pretreatment, *in situ* formed acids help to catalyze (auto-cleave) the process itself. The acids most likely catalyze the hydrolysis of the soluble hemicellulose oligomers (Bobleter et al., 1991; Mok and Antal, 1992). Low-pressure steam pretreatment (2 bars, 120 degrees C, and pretreatment times up to 300 minutes) did not have a large effect on the composition and structure of pretreated wheat straw according to Lawther et al. (1996).

Liquid hot water (LHW) is another example of thermal pretreatment, in which liquid hot water is used instead of steam. The objective of LHW is primarily to solubilize hemicellulose, thus making the cellulose more accessible, and to avoid the

formation of inhibitors. To avoid the formation of inhibitory compounds, the pH is kept between 4 and 7 during pretreatment (Kohlmann et al., 1995; Mosier et al. 2005; Weil et al., 1997).

In LHW, the amount of solubilized products is higher than in steam pretreatment, while the concentration of these products is lower than in steam pretreatment (Bobleter, 1994). This most likely results from dilution due to higher water input in LHW. The yield of solubilized xylan is generally also higher for LHW pretreatment, though this trend does not hold when solid concentration increases.

Another study has shown that flow-through systems removed more hemicellulose and lignin from corn-stover during pretreatment than batch systems did, under the same pH and temperature conditions. Additionally, the addition of an external acid during a flow through process caused higher hemicellulose and lignin removal, while in batch processes, the addition of an external acid caused less lignin removal. Presumably, the inhibitory factors are captured in the effluent for removal during flow-through process. Other studies, however, have shown that acid additions have no substantial effect or are not the only factor affecting the solubilization of hemicellulose and lignin.

Methane producing bacteria have been shown to be able to adapt to the inhibitory compounds that may result from steam pretreatment/steam explosion, at least to certain concentrations. The adaption has been observed in various studies, wherein up to a six-fold increase in enzymatic digestibility has been reported after steam pretreatment (Benjamin et al., 1984; Fox et al., 2003).

The use of LHW is advantageous, as the solubilized hemicellulose and lignin products are present in lower concentrations when compared to steam pretreatment. Weil et al. (1998) showed a 2- to 5- fold increase in enzymatic hydrolysis of their substrate after LHW pretreatment.

Pretreatment of lignocellulose material is sometimes performed with acids to enhance the anaerobic digestibility. The objective is to solubilize the hemicellulose, making the cellose better accessible. Acid pretreatment is done with either dilute or strong acids.

Xylan is particularly affected, while glucomannan is relatively acid stable. Solubilized oligomers can be subjected to hydrolytic reactions producing monomers, furfural, HMF and other volatile products in acidic environments (Fengel and Wegener, 1984; Ramos, 2003). During acid pretreatment, solubilized lignin will quickly condense and re-precipitate in acidic environments (Liu and Wyman, 2003; Shevchenko et al., 1999). Solubilization of hemicellulose and precipitation of solubilized lignin is more pronounced in strong acid pretreatment.

The risk of formation of volatile degradation products during acid pretreatment may lead to lost carbon for the conversion to ethanol. These volatile products may be converted to methane, however. Strong acid pretreatment for ethanol production has been determined to be unfeasible due to the high amounts of inhibiting compounds that are likely to be formed. Dilute acid pretreatment, however, has been identified to be one of the most promising pretreatment methods.

Acid pretreatment is particularly attractive for methane production, because methanogens have been shown to be able to handle compounds such as furfural and HMF at certain concentrations after an acclimatization period. For both ethanol and methane production, soluble lignin compounds are often inhibiting. Methanogens, however, are more capable of adapting. (Benjamin et al., 1984; Xiao and Clarkson, 1997). The use of sulfuric or nitric acids in pretreatment, however, may reduce methane production as a result of reduction of sulfate and nitrate to H_2S and N_2.

Another example of a pretreatment method includes the use of ammonia. This pretreatment method, commonly referred to as AFEX pretreatment, is conducted with ammonia loadings around 1:1 (kg ammonia/kg dw biomass) at temperatures ranging from ambient temperature with a duration between 10 and 60 days, to temperatures of 120 degrees C with a duration of only several minutes (Alizadeh et al., 2005; Kim and Lee, 2005). A six-fold increase in enzymatic hydrolysis yield and a 2.5 fold ethanol yield after AFEX pretreatment has been reported (Alizadeh et al., 2005). Significant swelling of the cellulose and increased delignification have been identified as factors responsible for the increased yield.

Alkaline pretreatment causes biomass to swell via solvation and saphonication. This causes the biomass to be more accessible to enzyme action. At "strong" alkali concentrations, end groups are peeled and alkaline hydrolysis occurs. This peeling is an advantage for later conversion, although carbon dioxide production also occurs, leading to the loss of carbon for conversion processes.

Xylan can be targeted for removal with aqueous potassium hydroxide and low temperature. Low temperature prevents peeling (Hon and Shiraishi, 2001). During alkaline pretreatment processes, the biomass consumes a portion of the alkali itself. Alkali extraction can also cause solubilization, redistribution and condensation of lignin as well as modifications in the crystalline state of the cellulose. These effects can lower, or counteract the positive effects of lignin removal and cellulose swelling (Gregg and Saddler, 1996). Additionally, during alkaline pretreatment, the cellulose structure is changed to a form that is denser and thermodynamically more stable than the native cellulose.

Gosset et al. (1982) concluded that lignin-containing biomass containing lignin concentrations of over 1g/l that is subjected to alkaline heat pretreatment yielded a major inhibitory effect to methanogenic microorganisms. This inhibition is most likely caused by the inhibitory products that are released from lignin during the alkaline heat pretreatment. The loss of fermentable sugars and the production of inhibitory compounds makes the alkaline pretreatment less attractive for high-lignin biomass for enzymatic saccharification and subsequent ethanol production. The production of inhibitors is less severe for methanogens as compared to yeasts for ethanol production.

Oxidative pretreatment is yet another pretreatment method. The process consists of the addition of an oxidizing compound, such as hydrogen peroxide or peracetic acid to the biomass that has been suspended in water. The objective of oxidative pretreatment is to remove the hemicellulose and lignin, thereby increasing

28

the accessibility of the cellulose. Several reactions target the various linkages in the lignin structure, such as electrophilic substitution, displacement of side chains, cleavage of alkyl aryl ether linkages or oxidative cleavage of aromatic nuclei.

The oxidant is generally not selective. Therefore, loss of hemicellulose and cellulose may occur. Formation of inhibitory compounds is also a significant risk, as lignin is oxidized and soluble aromatic compounds are formed. Peracetic acid, however is very lignin selective, and no significant carbohydrate loss has been observed. The enzymatic hydrolysis of the cellulose increased from 6.8% in the untreated biomass to a maximum hydrolysis of about 98% on the pretreated biomass at a 21% peracetic acid level in a study performed by Teixeira et al. (1999). The same study also showed similar results for digestibility using a mixture of NaOH and peracetic acid. These experiments were performed at ambient temperatures. Experiments that were conducted at higher temperatures did not show as significant increases in hydrolysis rates, however.

Gould (1984) demonstrated the use of H_2O_2 for delignification with a maximum amount of delignification at pH 11.5 to 11.6. Delignification most likely results from the action of the hydroxyl ion, which is generated as a degradation product of hydrogen peroxide with a maximum production occuring at pH 11.5-11.6. Approximately half of the total lignin content was solubilized when the reaction was carried out at around 25 degrees C and for a duration of 18-24 hours. Delignification is significantly reduced as the pH is lowered, with a minimum occurring below a pH of 10.0. Also, hydrogen peroxide is shown to have no real

effect on the enzymatic digestibility at a pH 12.5 or higher. Gould (1984) found that the hydrogen peroxide concentration should be at least 1%, and the weight ratio between H_2O_2 and biomass should be 0.25 for sufficient delignification.

The pretreatment methods that have been described may be combined for enhanced results. For example, a way to improve the effect of thermal steam or LHW pretreatment is the addition of an external acid. The acid addition catalyzes the solubilization of the hemicellulose, lowers the optimal pretreatment temperature, and gives a better enzymatic hydrolysable substrate (Brownell et al. 1986; Gregg and Saddler, 1996). In this process, the lignocellulose may be impregnated with SO_2, which is converted to H_2SO_4 quickly during steam pretreatment. The H_2SO_4 subsequently catalyzes hydrolysation of the hemicellulose.

Another example of a pretreatment combination includes thermal pretreatment in combination with alkaline pretreatment. Lime is most commonly used as an external source of alkalinity. Lime additions of approximately 0.1 g Ca(OH) g substratae^{-1} to biomass that has been heated to 100-150 degrees C (Chang et al., 2001). The lime pretreatment acts to loosen up the lignin, making the substrate more accessible to hydrolysis (Chang and Holtzapple, 2000). According to Kaar and Holtzapple (2000), lime pretreatment with heated biomass sufficiently increases the digestibility of biomass with low lignin content, but not for high lignin biomass. Lime pretreatment of switchgrass and corn stover does not seem to inhibit the enzymatic saccharification and fermentation. Pretreated softwood, however, needs to be washed before enzymatic saccharification and fermentation to prevent

inhibition by the relatively large amount of solubilized lignin, adding an additional process step, thus lowering the economic feasibility. Lime, however, is relatively inexpensive and safe (Gandi et al., 1997) and the calcium can be regained as insoluble calcium carbonate by reacting the leachate with carbon dioxide. The calcium carbonate can then be reconverted to lime again using a lime kiln. One study reported an improvement in methane production with by a factor of 3 to 4.5 after pretreating newspaper with alkaline heat pretreatment.

Thermal pretreatment can also be combined with an oxidative pretreatment method, such as peracetic acid, or with an alkaline oxidative pretreatment. As previously noted, the effectiveness of lime pretreatment is severely reduced with high-lignin biomass. The addition of oxygen increased the digestibility of the treated biomass by 13 fold, although the pretreated biomass had to be washed to remove any inhibiting soluble lignin compounds (Chang et al. 2001). Approximately 21% of the added lime could be recovered after carbon dioxide carbonation.

Carbon dioxide may also be used at high pressure and high temperatures as a carbon-dioxide explosive steam pretreatment method. This may cause the liquid in which the biomass is suspended to become acidic, leading to the hydrolysis of the hemicellulose (Puri and Mamers, 1983). The high energy-input requirements however, make this pretreatment method less economically feasible.

In addition to the pretreatment processes presented above, mechanical pretreatment processes are also contemplated. Mechanical pretreatment generally involves milling, or reducing the lignocellulosic biomass into smaller pieces. The

objective is to reduce particle size and crystallinity. The reduction in particle size

increases the available specific surface area and reduces the degree of

polymerization (DP) (Palmowski and Muller, 1999). Milling using a particle

reduction size below 40 mesh has been shown to increase the shear on the biomass

and to increase the available specific surface area. This results in an increase of the

hydrolysis yield of lignocellulose up to 24%, and a reduction of the required

digestion time by 23-59%, thus increasing the hydrolysis rate (Delgenes et al. 2002;

Hartmann et al., 1999).

Milling has shown to increase methane production if the pretreated

lignocellulosic biomass is anaerobically digested. Methane production increases of

5-25% have been reported (Delgenes et al., 2002). No production of inhibitors, such

as furfural and hydroxymethylfurfural (HMF) are produced during milling, making it

suitable for subsequent methane and ethanol production. However, milling requires

a high energy input (Cowling and Kirk, 1976; Ramos, 2003) and therefore was

determined not to be economically feasible as a pretreatment method for large scale

operations (Fan et al., 1987).

<u>Enzymatic Digestion of Pretreated Biomass</u>

The following characteristics have been identified as key desirable traits for

cellulolytic systems for processing cellulosic biomass that has undergone a

pretreatment process:

1)   The cellulolytic enzymes system should be effective against crystalline

cellulose. Cellulose crystallinity does not decrease as a result of pretreatment of

biomass by dilute acid (Grethelein 1985; Knappert et al., 1981; Thompson et al., 1992.), seam explosion (Puls et al., 1985), or lime (Chang and Holtzapple, 2000) under conditions that result in high hydrolysis yields.

2)   The cellulolytic system should remain metabolically active in the presence of inhibitory compounds generated during pretreatment with minimal cost for detoxification methods. Such compounds result from hydrolytic release of compounds present in unpretreated biomass, such as organic acids, extractives, and phenolics. Other inhibitory compounds are the result of reactions of carbohydrates and other solubilized components to form degadation products, such as furfural and hydroxymethyl furfural. Still other inhibitory compounds arise from corrosion, resulting in the release of inorganic ions. The amounts of inhibitors produced depend greatly on process conditions and configuration.

Process Engineering

Converting cellulosic biomass into fuels and chemicals comprises the following process steps:

1)   Cellulase system production

2)   Hydrolysis of cellulose and other present insoluble polysaccharides

3)   Fermentation of soluble cellulose hydrolysis products

4)   Fermentation of soluble hemicellulose hydrolysis products

The above listed processes may be condensed into fewer individual steps. For example, simultaneous saccharification and cofermentation (SSCF) involves two process steps: cellulase production and a second step in which cellulose hydrolysis and fermentation of both cellulose and hemicellulose hydrolysis products occurs. In

33

another example, consolidated bioprocessing (CBP), cellulase production, hydrolysis and fermentation of products of both cellulose and hemicellulose hydrolysis are accomplished in a single process step.

Summary and Problem Statement

In order to achieve low cost biochemical conversion of lignocellulosic biomass to liquid biofuel, high initial concentrations of biomass must be used in order to reduce high downstream processing costs associated with dilute solutions. Further, the recalcitrance of the biomass must be overcome by developing pretreatment processes that are minimally energy intensive and that use reagents that are both environmentally safe and of relatively low cost.

Economic analyses performed at the National Renewable Energy Laboratory (NREL) suggest that increasing total solids concentration in enzymatic digestion step of the biochemical lignocellulose conversion process from 20% to 30% would result in a 10% decrease in the ethanol production cost. By starting with a high concentration of solid substrate, a higher concentration of sugars can be achieved after enzymatic digestion. Subsequent fermentation of these concentrated sugar streams would produce higher ethanol concentrations in the process stream that would ultimately result in improved process economics due to lower product purification costs.

The cost of downstream processing is a significant cost sink of the lignocellulose conversion process. Other cost sinks include the high cost of equipment and energy input costs. Higher substrate concentrations imply lower process equipment volumes and will therefore lead to the reduction of equipment and energy input costs.

A wealth of research has focused on the characterization of cellulase activity in the hydrolysis of cellulose to monomeric sugars. However, most of these studies have been performed at low solid concentrations to avoid confounding mass transfer or product inhibition effects. While these studies provide valuable and fundamental insights into the biochemical mechanisms of cellulase systems under ideal reaction conditions, they provide only limited understanding of lignocellulose degradation under process-relevant conditions. Only a few studies have reported enzymatic digestibility results at high solid concentrations (up to 40% w/w); however, none has sought to characterize the kinetics of the process to develop predictive mathematical models.

<u>Project Goal</u>

The goal of the current project is to develop an understanding of the effect of high solids concentrations on enzymatic digestion of lignocellulosic biomass material. Further, the goal of this work is to elucidate optimal pretreatment conditions for lignocellulosic biomass using an alkaline-peroxide pretreatment strategies. Specifically, this study focused on identifying optimal pretreatment levels of H_2O_2, optimal pretreatment duration, and optimal pretreatment temperatures. Once optimal pretreatment conditions had been identified, the effect of lignocellulose biomass composition on pretreatment efficacy was determined.

Research Design and Methods:

1. Development of Sugar Standards Using a DNS Assay

Sugar standards were determined using the Dinitrosalicylic Acid Reagent Assay (DNS), as developed by Miller (1959). The standard curves generated using the DNS assay were used to determine the amount of reducing sugars liberated in reaction mixtures. Sugar standards were made for Glucose, Xylose and Arabinose. This assay was used to determine the concentration of reducing sugars that were released from lignocellulosic biomass after pretreatment and subsequent enzymatic saccharification in the following experiments.

2. Effect of High Solids Concentration on Enzymatic Digestibility

Inhibition of enzymatic digestion rates and yields were quantified in the presence of added background sugars. This was done in order to account for potential compounding inhibition and rate determining factors, such as a change in physical substrate characteristics during treatment.

Kinetic experiments were performed at solid concentrations between 2-40% (w/w) using freeze-dried, dilute-acid pretreated corn stover. The corn-stover was obtained from the National Renewable Energy Laboratory (NREL). A commercial

cellulase mix, Spezyme CP was used as the source of enzymes for enzymatic digestion of the pretreated biomass. The Spezyme CP, which is sold by Genecor International (Copenhagen, Denmark), was supplemented by Novo188, B-glucosidase, sold by Novozymes Ltd. (Basvaerd, Germany).

Digestions using the commercial enzymes were performed in sealed 100mL bottles containing a total mass of 30g (solid substrate + liquid enzyme solution). The following two enzyme loadings were used: 1) A low-loading of 25mg/g cellulose, as recommended by the standard NREL protocol (Brown and Torget, 1996), and 2) a high loading of 100mg/g cellulose to provide enough cellulase to enable more complete digestion. A glucosidase concentration of 90mg/g cellulose was used in both cases (Selig et al., 2007). Tetracyline and cyclohexamide was added to inhibit microbial growth and maintain sterile conditions. Enzyme solutions were prepared in 200mM citrate buffer at pH 5 and reactions were incubated at 60 degrees C.

Soluble sugars were separated via HPLC and analyzed using a refractive index detector. The HPLC was run using a Shodex SP0810 sugar column, with an injection volume of 20 uL, at a flow rate of 0.6 mL/min and at 80 degrees C for 35 minutes. Triplicate experiments were performed in each case. Standard deviations and 95% confidence intervals of mean measured values were calculated.

3. Pretreatment Optimization Experiments

a. Optimal H_2O_2

Milled wheat straw was slurried in water (8.6% w/v, unless otherwise indicated) with varied amounts of H_2O_2 in 15 mL centrifuge tubes. The following levels of H_2O_2 were tested: 0%, 1.0%, 2.0%, 3.0%, 5.0%, 7.0%, and 9.0% (v/v). The pH was then adjusted to 11.5 using NaOH. The samples were placed in a shaking incubator at 250 rpm at 35 degrees C for 24 hours to allow extensive pretreatment. After pretreatment, the pH was adjusted to 5.0 using HCl. Enzymatic saccharification was then performed at 45 degrees C at 4ml/g biomass for 120 hours. The resulting soluble sugars were separated via HPLC and analyzed using a refractive index detector.

b. Optimal Pretreatment Duration

Milled wheat straw was slurried in water (8.6% w/v) containing H_2O_2 (3% v/v) and adjusted to pH 11.5 using NaOH and shaken in an incubator at 250 rpm at 35 degrees C for the following time intervals: 3 hours, 6 hours, 9 hours, 12 hours, 15 hours, 18 hours and 24 hours. After pretreatment, the pH was adjusted to 5.0 using HCl. Enzyme saccharification was then performed at 45 degrees C at 4ml/g biomass for 120 hours. The resulting soluble sugars were separated via HPLC and analyzed using a refractive index detector.

c. Optimal Pretreatment Temperature

Milled wheat straw was slurried in water (8.6% w/v) containing H_2O_2 (3% v/v) and adjusted to pH 11.5 using NaOH and shaken in an incubator at 250 rpm for 18 hours at the following temperatures: 18 degrees C, 25 degrees C, 35 degrees C and 40 degrees C. After pretreatment, the pH was adjusted to 5.0 using HCl. Enzyme

saccharification was then performed at 45 degrees C at 4 ml/g biomass for 120 hours. The resulting soluble sugars were separated via HPLC and analyzed using a refractive index detector.

4. Determination of Structural Carbohydrates in Three Types of Biomass

The structural composition of three types of biomass was determined using sections 10.1 and 10.4 the NREL standard Laboratory Analytical Procedure (LAP), titled "Determination of Structural Carbohydrates and Lignin in Biomass", issued April 25, 2008. The following types of lignocellulosic biomass were analyzed: Tall Wheat Grass, Intermediate Wheat Grass and Basin Wild Rye. The samples that were analyzed were obtained from Dr. Sridhar Viamajala. The samples were collected in the early summer months.

Tall wheatgrass is difficult to cultivate in saline environments due to high concentrations of salts and heavy metals, as well as potentially high emission levels of nitrous and sulfuric oxides. It is a tall, long- lived perennial bunchgrass reaching 1 to 3 m tall. If is found throughout all western states of the U.S and most Canadian provinces (USDA Conservation Plant Report, 2011).

Intermediate Wheatgrass is a cool-season perennial sod forming grass. It grows to a height of 23-47 inches. Typical growth season for intermediate wheatgrass extends between mid-April through September. It is projected to

perform well in the Central Great Plains, Upper Midwest and parts of the Intermountain West (USDA Conservation Plant Report, 2011).

Basin wildrye is found throughout the western United States and Canada. It is a large, coarse and robust perennial bunchgrass. It is a long-lived, cool-season native. Clumps of Basin wildrye may stand 3 to 6 feet all (USDA Conservation Plant Report, 2011).

5. Determination of Structural Composition of Biomass on Pretreatment Efficacy

The three types of biomass that were analyzed for structural composition were subjected to alkaline peroxide pretreatment under the conditions that were determined to be "optimal" for milled wheat straw. Each biomass was slurried in water containing H_2O_2 (4%) for 18 hours at 35 degrees C in 15 mL centrifuge tubes. During pretreatment, the pH was adjusted to 11.5 using NaOH. After pretreatment, the pH was adjusted to 5.0 using HCL. Enzyme saccharification was then performed at 45 degrees C at 4mL/g biomass for 120 hours. The resulting soluble sugars were separated via HPLC and analyzed using a refractive index detector.

Results

1.   *Effect of High Solids Concentration on Enzymatic Digestibility*

Referring to Figures 1a and 1b, a five-day digestibility study to elucidate the relationship between solids concentration and digestibility of pretreated corn-stover indicates that as solids concentration is increased, overall digestibility of the biomass decreases. Figure 1a indicates that as the solids concentration, represented

as the insoluble solid fraction increases from 215% to 40%, the amount of biomass

converted decreases substantially. Further, as indicated by the results shown in

Figure 1b, the amount of glucose released per unit of biomass plateaus after a solids

concentration of 30%.

Figure 1: 5-day enzymatic digestibility data for pretreated corn stover showing (a) decrease in conversion with increasing solids concentration and (b) Plateau in glucose release after a solids concentration of 30%.
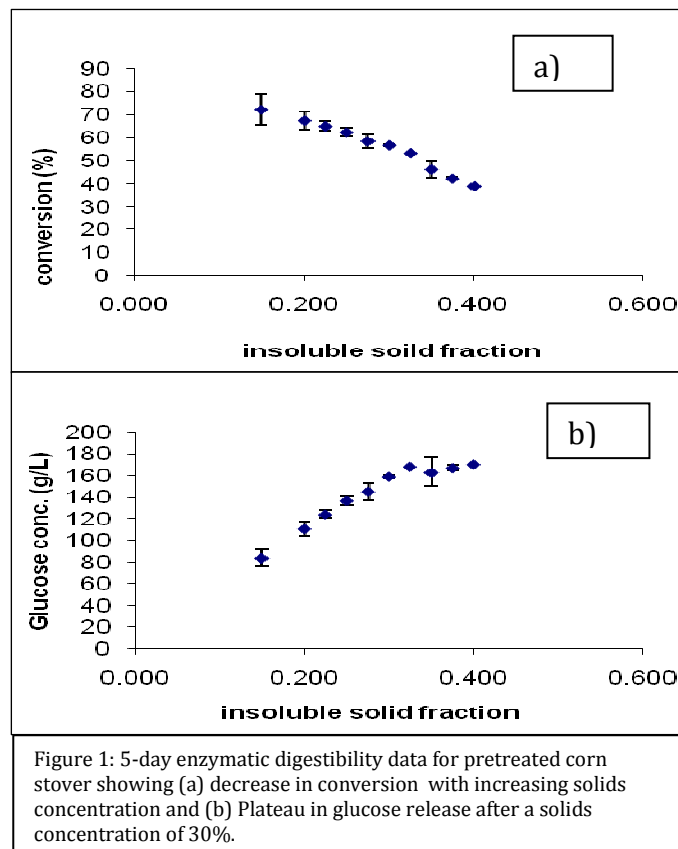
2.   *Pretreatment Optimization Experiments*

      a.   *Optimal H_2O_2*

Referring to Figure 2, the effect of H_2O_2 level (0-10%, v/v) for the pretreatment of milled wheat straw is shown. An optimal level of 3% H_2O_2 (v/v) was demonstrated, as indicated by the maximal sugar release that was demonstrated after enzymatic hydrolysis of the pretreated biomass. Sugar release did not increase significantly after 3% H_2O_2 (v/v), and in fact, showed a slight decrease, indicating perhaps increased toxicity or release of inhibitory compounds that effect the cellulolytic enzymes at higher levels of H_2O_2 pretreatment.
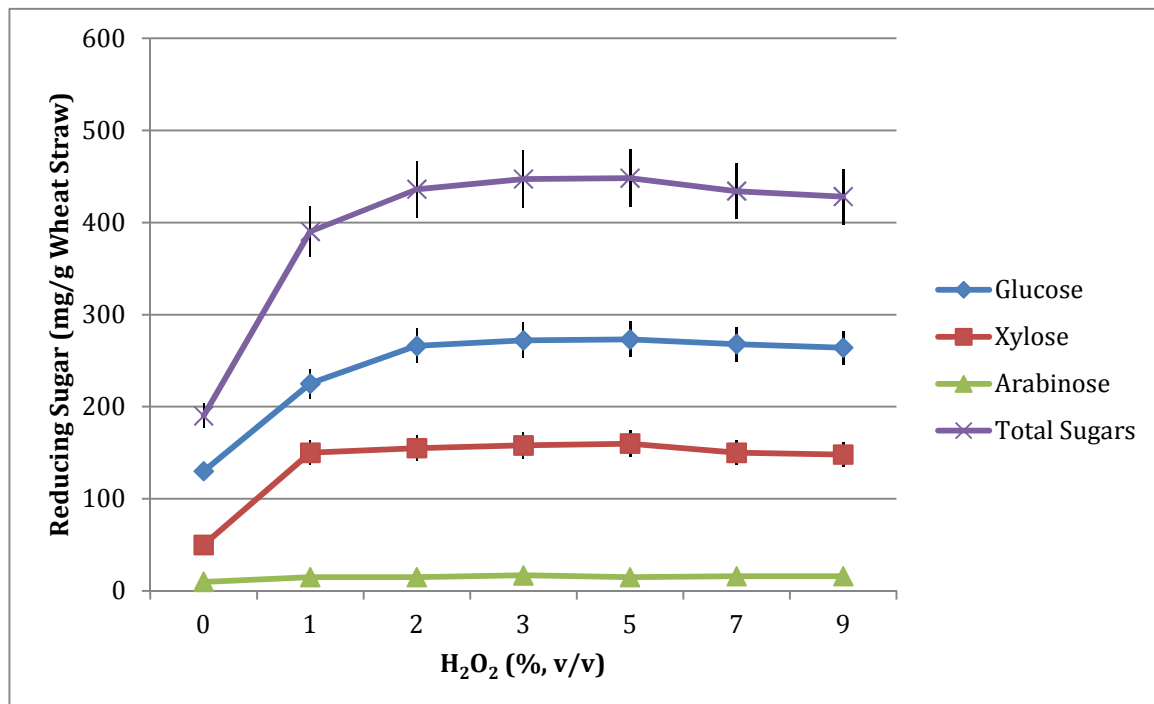
Figure 2. Effect of H_2O_2 level on digestibility of corn-stover. Sugar production did not increase significantly after an H202 level of 3.0%.

b. *Optimal Pretreatment Duration*

AAs shown in Figure 3, an increase in formation of total sugars of 29 ± 4 mg/g of straw was observed by increasing the pretreatment time from 3 to 24 hours at 3% H_2O_2. It has been reported that the reaction is sufficiently completed within 24 hours, however, some studies have reported that sugar yields increase slightly up to 120 hours, after which there is no more increase in sugar concentration. This indicates that there is a long incubation time is required for obtaining maximum sugar yield under the conditions used. Table 2 provides the released sugar values and statistical range.
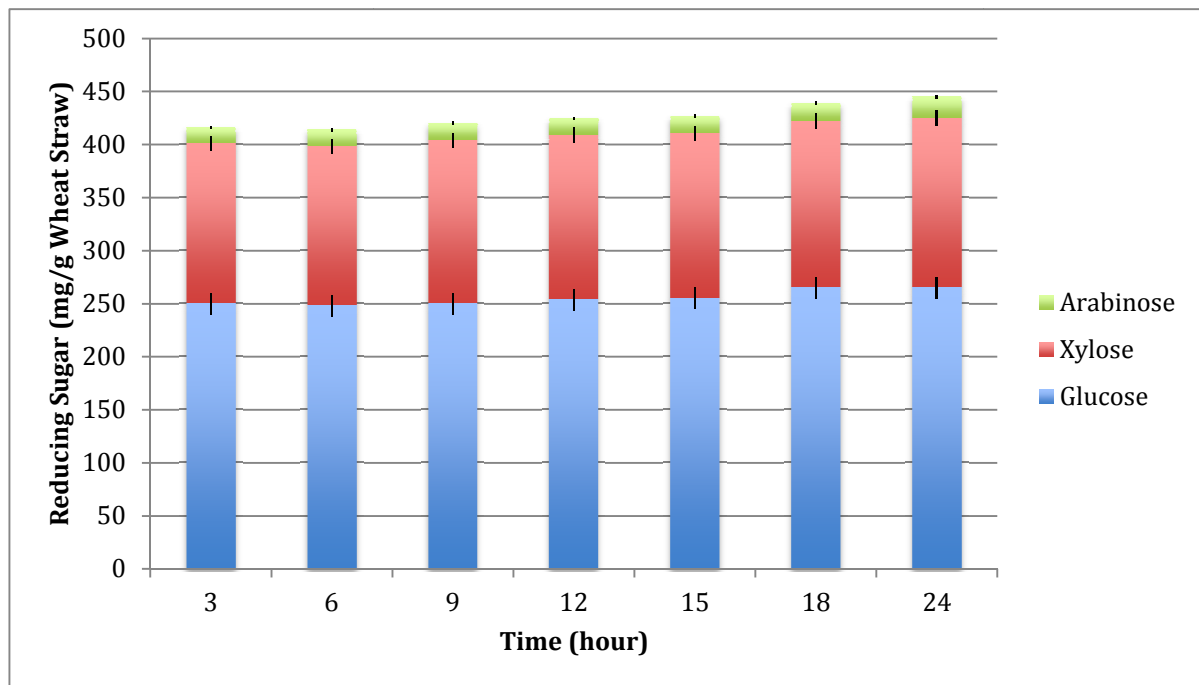
Figure 3. Effect of pretreatment duration on digestibility of corn-stover. The amount of sugar released increased substantially between 3 and 24 hours. Studies have indicated that sugar release continues to occur up to 120 hours of pretreatment. Significant increases in sugar production, however, did not occur between 18 hours and 24 hours.

Table 2. Amount of reducing sugars released after different pretreatment durations

Time	Glucose	Xylose	Arabinose
3	250 ± 8.5	151 ± 4.9	15 ± 0.9
6	248 ± 8.4	150 ± 5.1	16 ± 0.8
9	250 ± 8.4	154 ± 3.4	16 ± 0.9
12	254 ± 8.9	155 ± 6.2	16 ± 1.0
15	255 ± 8.4	155 ± 5.2	17 ± 0.8
18	265 ± 6.1	157 ± 5.2	18 ± 1.3
24	265 ± 6.8	160 ± 5.1	20 ± 1.5

c. *Optimal Pretreatment Temperature*

Referring to Figure 4, an increase in soluble sugar release of 34 mg/g of

wheat straw was observed in samples digested at 18 degrees C and samples

digested at 40 degrees C for 18 hours at 3% H_2O_2. There was not a significant

difference, however, between sugar yields at 35 degrees C and 40 degrees C.
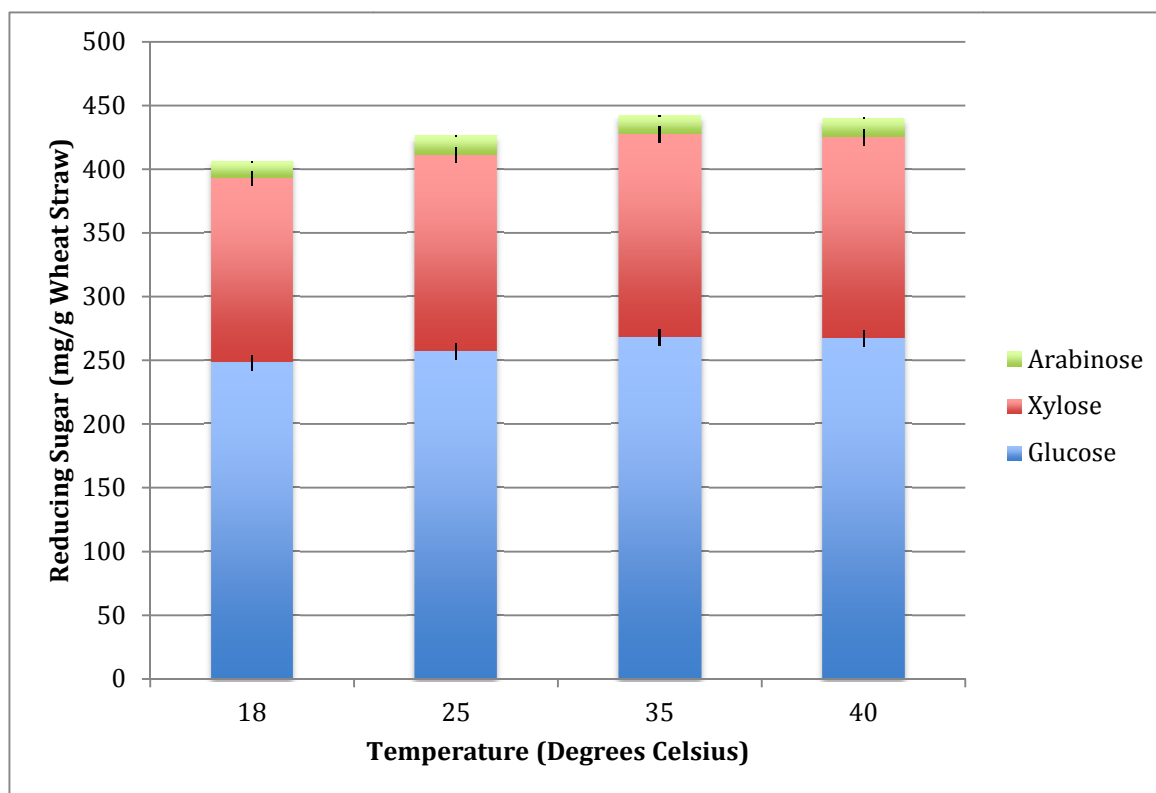
Figure 4. Effect of temperature during alkaline-peroxide on digestibility of corn-stover. No significant increase in sugar production was observed between 35 and 40 degrees C. A substantial increase was seen, however, between 18 degrees C and 35 degrees C.

Table 3. Reducing sugar release after pretreatment at various temperatures, after enzymatic saccharification.

Temp (Degrees C)	Glucose	Xylose	Arabinose
18	248 ± 5.7	145 ± 5.8	13 ± 1
25	257 ± 5.2	154 ± 3.1	15 ± 1
35	268 ± 4.3	159 ± 2.1	15 ± 1
40	267 ± 4.4	158 ± 2.3	15 ± 1

3. *Determination of Structural Carbohydrates in Three Types of Biomass*

Referring to Table 4, the structural composition of three different biomass samples was determined. Biomass composition, particularly of perennial grasses that are found in the plain and northern states in the US, is known to change in response to seasonal changes (Nyren et al. 2008). Generally, perennial grasses become higher in cellulose during late summer and fall months. Surrounding ecology (i.e. various plant and environmental interactions) is also known to change the structural composition of different perennial grasses.

Of the samples that were analyzed, Tall Wheat Grass exhibited the highest percentage of cellulose, wherein the Basin Wild Rye exhibited the lowest percentage of cellulose.

Table 4. Structural Composition of 3 perennial grasses

Biomass	*Cellulose %*	*Hemicellulose %*
Tall Wheat Grass	*35.2 ±3.4*	*22.1 ±2.7*
Intermediate Wheat Grass	*31.5 ±3.2*	*26.7 ±2.5*
Basin Wild Rye	*25.2 ±2.9*	*16.8 ±1.8*

4. *Determination of Structural Composition of Biomass on Alkaline Peroxide*

Pretreatment Efficacy

After determining the structural composition of the three different perennial grasses, presented in Table 4, the three different grasses were pretreated and subsequently subjected to enzymatic saccharification. The samples were slurried in water containing H_2O_2 (4%) for 18 hours at 35 degrees C in 15 mL centrifuge tubes. During pretreatment, the pH was adjusted to 11.5 using NaOH. After pretreatment, the pH was adjusted to 5.0 using HCL. Enzyme saccharification was then performed at 45 degrees C at 4mL/g biomass for 120 hours. The resulting soluble sugars were separated via HPLC and analyzed using a refractive index detector.

As expected, the sample that had the highest cellulose content had the highest sugar release. After pretreatment and subsequent enzyme saccharification, Tall Wheatgrass released a total of 418 mg sugars/g grass. The sample that had the lowest cellulose content had the lowest sugar release. After pretreatment and subsequent enzyme saccharification, Basin Wildrye released 370 mg sugars/g grass.
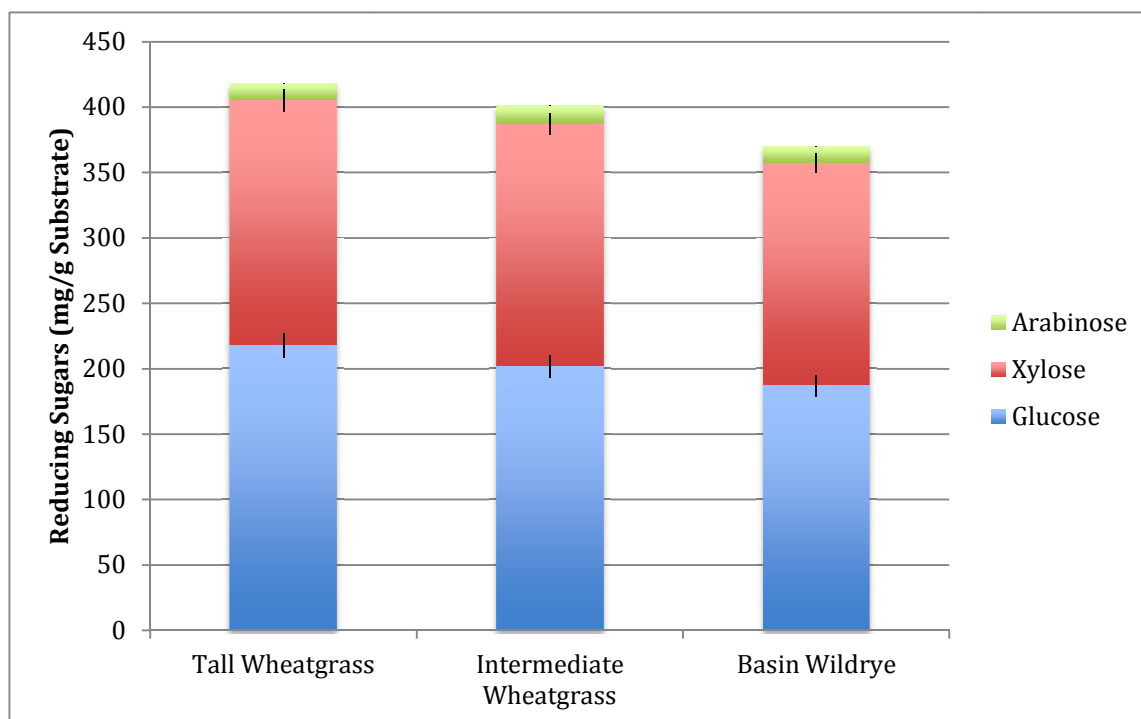
Figure 5. Three perennial grasses were subjected to alkaline peroxide pretreatment under the pretreatment conditions that were determined to be optimal in the optimization experiments. As expected, Tall Wheatgrass, which has the highest composition of cellulose, had the highest level of digestibility as indicated by the highest level of sugar release.

Table 5. Reducing Sugars released from various biomass types after pretreatment and enzymatic saccharification

Biomass Type	Glucose	Xylose	Arabinose
Tall Wheatgrass	218 ±9.3	187 ±6.5	13 ±1
Intermediate Wheatgrass	202 ±7.9	185 ±6.3	14 ±1
Basin Wild Rye	187 ±8.04	170 ±5.9	13 ±1

Discussion and Conclusion

   The observed decrease of cellulose conversion to glucose as solids concentration
is increased suggests inhibition effects at higher concentrations. Further, the plateau
in sugar concentrations supports this hypothesis, suggesting that digestibilities are
lower as a result of highly accumulated sugar levels. Since this data was generated
by a single measurement at 5 days, it is possible that the digestions at lower solid
concentrations were more near completion while at higher solid concentrations,
digestions were farther from completion due to slower kinetics.

   Further, it is not known if the observed inhibition was due to products directly
inhibiting cellulases by interacting with the active site, or if secondary effects such
as lowering of water activity or increase in ionic strength caused inhibition.

   Lack of "available" water can alter enzyme activity and/or binding due to altered
hydration of enzymes leading to poor digestion (Fervais et al., 1988; Yang et al.,
2004). High ionic strength of the solution from sugar accumulation can alter protein
structure and/or function, again resulting in lower enzyme activity (Kim and Hong,
2000). Mass transfer limitations may arise due to challenging slurry rheology, i.e.
insufficient free water can limit transport of products from the site of enzymatic
action, leading to localized accumulation of product sugars that can exacerbate any
product inhibition effects.

   The digestibility of lignocellulosic material may be enhanced by pretreatment
methods that reduce or remove lignin from biomass surfaces, thus exposing the
more easily digestible cellulose and hemicellulose components to enzymatic action.

Pretreatment optimization experiments indicated that for corn-stover feedstock, optimal pretreatment occurs at 35 degrees C and at 3% H_2O_2. Subsequent pretreatment studies were carried out for 18 hours, even though sugar release has been shown to occur up to 120 hours. Sugar release greatly slowed down at 18 hours. Thus to increase process efficiency and to reduce process costs, a shortened pretreatment duration is desirable.

Alternative lignocellulosic biomass feedstocks were also studied. Specifically, perennial grasses were considered as alternative to traditional corn-stover in order to mitigate the conflict between food and fuel sources. Tall Wheatgrass, Intermediate Wheatgrass and Basin Wild Rye were selected, and were analyzed for structural composition. Specifically, the grasses were analyzed for their cellulose and hemicellulose content. Tall Wheatgrass was shown to have the highest percentage of cellulose, while Basin Wild Rye was shown to have the lowest percentage of cellulose. When these grasses were subjected to alkaline peroxide pretreatment and subsequent enzyme saccharification, the sample with the highest cellulose content yielded the most reducing sugars, as expected. None of the perennial grass samples, however, yielded the amount of sugar that was released by corn-stover after alkaline peroxide pretreatment and saccharification.

<u>Future Work</u>

Kinetic and yield data should be developed for enzymatic cellulose digestions at high solids digestion concentration. The following additional control experiments should also be performed:

1) Digestions of commercially available purified microcrystalline cellulose (avicel, Sigma Aldrich, St. Louis, MO) to determine the digestibility of cellulose in the absence of lignin.

2) Enzyme and substrate-free reactions

3) Reactions supplemented with initial glucose at concentrations of 50, 100, 150 and 200 mg/L to independently observe inhibition of reaction rate in the presence of glucose.

Additionally, studies on enzyme activities of a purified exo-glucanase (CBH1) should also be performed at high solid and product concentrations to elucidate enzyme-specific inhibition. Water activity and ionic strength should be monitored during digestion studies to quantify changes in these parameters.

The pretreatment optimization experiments for alkaline peroxide pretreatment should be repeated using a variety of biomass feedstocks in order to determine a relationship between feedstock composition and pretreatment condition optimization.

Biomass composition of perennial wheatgrasses should be conducted on samples that were collected over a range of seasons and in a variety of environments. Further quality control experiments should include the development of calibration verification standards, and lignin structural composition should be determined using the NREL LAP entitled "Determination of Structural Carbohydrates and Lignin in Biomass", sections 10.2 and 10.3.

Literature Review

Alizadeh, H., Teymouri, F., Gilbert, T.I., Dale, B.E. 2005. Pretreatment of switchgrass by ammonia fiber explosion (AFEX). Appl. Biochem. Biotechnol., 1133-1141.

Aoyagi, H., Uemura, O., Takebe, H., Tanaka, H. 1995. Estimation of microbial cell concentration in suspension culture by the osmotic pressure measurement of culture broth. Biotechnol. Tech. **9:** 429-434.

Atalla, R.H., Hackney, J.M., Uhlin, I., Thompson, N.S. 1993. Hemicelluloses as structure regulators in the aggregation of native cellulose. Int. J. Biol. Macromol. **15:** 109-112.

Beall, F.C., Eickner, H.W. 1970. Thermal degradation of wood components: a review of the Literature. U.S.D.A Forest service, research paper FPL 130.

Benjamin, M.M., Woods, S.L., Ferguson, J.F. 1984. Anaerobic toxicity and biodegradability of pulp mill waste consituents. Water Res. **18:** 601-607.

Blouin, F.A., Martin, L.F., Rowland, S.P. 1970. Gel permeation properties of cellulose. III. Measurement of pore structure of unmodified and of mercerized cottons in fibrous form. Textile Re. J. **40:** 809-813.

Bobleter, O. 1994. Hydrothermal degradation of polymers derived from plants. Prog. Polym. Sci. **19**: 797-841.

Bobleter, O., Bonn, G., Prutsch, W. 1991. Steam explosion-hydrothermolysis-organolsolv. A comparison. *In* Focher, B., Marzetti, A., Crerscenzi, V. (Eds.) Steam explosion techniques. Gordon and Breach, Philadelphia, pp. 59-82.

Brownell, H.H., Saddler, J.N. 1987. Steam pretreatment of lignocellulosic material for enhanced enzymatic hydrolysis. Biotechnol. Bioeng. **29:** 228-235.

Brownell, H.H., Yu, E.K.C., Saddler, J.N. 1986. Steam=explosion pretreatment of wood: effect of chip size, acid, moisture content and pressure drop. Biotechnol. Bioeng. **28:** 792-801.

Brown, D.E., and M.A. Zainudeen. 1977. Growth kinetics and cellulase biosynthesis in continuous culture of *Trichoderma viride.* Biotechnol. Bio-eng. **14:** 941-958.

Brown, L., Torget, R. 1996. Enzymatic Saccharification of Lignocellulosic Biomass. Laboratory Analytical Procedure #009. National Renewable Energy Laboratory, Golden, CO.

Chang, V.S., Holtzapple, M.T. 2000. Fundamental factors affecting enzymatic reactivity. Appl. Biochem. Biotechnol., 5-37.

Chang, V.S., Kaaar, W.E., Burr, B., Holtzapple, M.T. 2001. Simultaneous saccharification and fermentation of lime-treated biomass. Biotechnol. Lett. **23:** 1327-1333.

Chen, H.Z., Hayn, M., Esterbauer, H. 1992. Purification and characterization of two extracellular B-glucosidases from *Trichoderma reesei.* Biochem. Biophys. Acta **1121:** 54-60.

Converse, A.O. 1993. Substrate factors limiting enzymatic hydrolysis, p. 93-106. *In* J.N. Saddler (ed.) Bioconversion of forest and agricultural plant residues. CAB International, Wallinford, Conn.

Cowling, E.B. 1975. Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. Biotechnol. Bioeng. Symp. **5:** 163-181.

Cowling, E.B., Kirk, T.K. 1976. Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion process. Biotechnol. Bioeng. Symp. **6:** 95-123.

Delgenes, J.P., Penaud, V., Moletta, R. 2002. Pretreatments for the enhancement of anaerotic digestion of solid wastes Chapter 8. *In* Biomethanization of the Organic Fraction of Municipal Solid Wastes. IWA Publishing, pp. 201-228.

Domansky, R., Rendos, F. 1962. On the pyrolysis of wood and its components. Holz Roh Werkst. **29:** 473-476.

Donaldson, L.A., Wong, K.K.Y., Mackie, K.L. 1988. Ultrastrucutre of steam-exploded wood. Wood Sci. Technol. **22:** 103-114.

Fan, L.T., Gharpuray, M.M., Lee, Y.H. 1987. Cellulose hydrolysis. Springer-Verlag, Berlin, Germany.

Fan, L.T., Lee, Y.G., and Beardmore, D.H. 1980. Mechanism of the enzymatic hydrolysis of cellulse: effects of major structural features of cellulse on enzymatic hydrolysis. Biotecnol. Bioegn. **22:** 177-199.

Fan, L.T., Lee, Y.H., Beardmore, D.H. 1980. Major chemical and physical features of cellulose materials as substrates for enzymatic hydrolysis. Adv. Biochem. Eng. **14:** 101-117.

Fengel, D., Wegener, G. 1984. Chemical composition and analysis of wood. *In* Wood: Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin. 26-65.

Fox, M.H., Noike, T., Ohki, T. 2003. Alkaline subcritical-water treatment and alkaline heat treatment for the increase in biodegradability of newsprint waste. Water Sci. Technol. **48:** 77-84.

Gama, F.M., Teixeira, J.A., Mota, M. 1994. Cellulose morphology and enzymatic reactivity: a modified solute exclusion technique. Biotechnol. Bioeng. **43:** 381-387.

Gandi, J., Holtzapple, M.T., Ferrer, A., Byers, F.M., Turner, N.D., Nagwani, M., Change, S. 1997. Lime treatment of agricultural residues to improve rumen digestibility. Anim. Feed Sci. Technol. **68**: 195-211.

Gervais, P. Bensoussan, M., GrajckW. 1988. Water activity and water content: comparative effects on the growth of *Peniculium roqueforti* on solid substrate. Applied Microbiology and Biotechnology. **27:** 389-392

Gong, C.S., Ladisch, M.R., Tsao, G.T. 1977. Cellobiase from *Trichoderma viride:* purification, properties, kinetics, and mechanism. Biotechnol. Bioeng. **19:** 959-981.

Gossett, J.M., Stuckey, D.C., Owen, W.F., McCarty, P.L. 1982. Heat treatment and anaerobic digestion of refuse. J. Environ. Eng. Div. **108:** 437-454.

Gould, D., Saddler, J.N. 1984. Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Biotechnol. Bioeng. **26:** 46-52.

Grabber, J.H. 2005. How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci. **45:** 820-831.

Gregg, D., Saddler, J.N. 1996. A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. Appl. Biochem. Biotechnol. 711-727.

Grethelein, H.E. 1985. The effect of pore size distribution on the rate of enzymatic hydrolysis of cellulosic substrates. Bio/Technology **2:** 155-160.

Hatfield, R.D., Ralph, J., Grabber, J.H. 1999. Cell wall structural foundation: molecular basis for improving forage digestibility. Crop Sci. **29:** 27-37.

Hartmann, H., Angelidaki, I., Ahring, B.K. 1999. Increase of anaerobic degradation of particulate organic matter in full-scale biogas plants by mechanical maceration. *In* Mata-Alvarez, J., Tilche, A., Cecchi, F. (Eds). Proceedings of the Second International Symposium on Anaerobic Digestion of Solid Wastes. Barcelona vol. 1, pp 129-136.

Hinman, N.D., J Schell, C.J. Riley, P.W Bergeron, and P.J. Walter. 1992. Preliminary estimate of the cost of ethanol production for SSF technology. Appl. Biochem. Biotechnol. **34-35:** 639-649.

Hon, D.N.S., Shiraishi, N. 2001. Wood and Cellulosic Chemistry, second ed. Dekker, New York.

Kaar, W.E., Holtzapple, M.T. 2000. Using lime pretreatment to facilitate the enzymatic hydrolysis of corn stover. Biomass Bioenergy **18:** 189-199.

Kim, T.H., Lee, Y.Y. 2005. Pretreatment of corn stover by soakin in aqueous ammonia. Appl. Biochem. Biotechnol., 1119-1132.

Kleywegt, G.J., Zou, J.Y., Civne, C., Davies, G.J., Sinning, I., Stahlberg, J., Reinikainen, T., Srisodsuck, M., Teeri, T.T., Jones, T.A. 1997. The crystal

structure of the catalytic core domain of endoglucanases I from *Trichoderma reesei* at 3.6 A resolution, and a comparison with related enzymes. J. Mol. Biol. **272:** 383-397.

Knappert, D.R., Grethlein, H.E., Converse, A.O. 1981. Partial acid hydrolysis of poplar wood as a pretreatment for enzymatic hydrolysis. Biotechnol. Bioeng. Symp. Ser. **11**: 66-77.

Kohlmann, K.L., Westgate, P.J., Sarikaya, A., Velayudhan, A., Weil, J., Hendrickson, R., Ladisch, M.R. 1995. Enhanced enzyme activities on hydrated lignocellulosic substrates. BTEC paper 127. *In* 207th American Chemical Society National Meeting, ACS Symposium series No. 618. Enzymatic Degradation of Insoluble Carbohydrates, pp. 237-255.

Laser, M., Schulman, D., Allen, S.G., Lichwa, J., Antal Jr, M.J., Lynd, L.R. 2002. A comparison of liquid hot water and steam pretreatments of sugar cane bagasse for bioconversion to ethanol. Bioresour. Technol. **81:** 33-44.

Lawther, J.M., Sun, R., Banks, W.B. 1996. Effect of steam treatment on the chemical composition of wheat straw. Holzforschung **50:** 365-371.

Lee, Lynd R., Weimer, Paul J., van Zyl, William H., Preorius, Isak S. 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews. **3:** 506-577.

Liu, C., Wyman, C.E. 2003. The effect of flow rate of compressed hot water on xylan, lignin and total mass removal from corn stover. Ind. Eng. Chem. Res. **42:** 5409-5416.

Lynd, L.R. 1989. Ethanol production from lignoce3llulosic substrates using thermophilic bacteria: critical evaluation of potential and review, Adv. Biochem. Eng. Biotechnol. **38:** 1-52.

Lynd, L.R., C.E. Wyman, and T.U. Gerngross. 1999. Biocommodity engineering. Biotechnol. Prog. **15:** 777-7693.

Maguire, R.J. 1977. Kinetics of the hydrolysis of cellobiose and p-nitro-phenyl-B-D-glucoside by cellobiase of *Trichoderma viride.* Can. J. Biochem. **55:** 19-26.

Mandels, M., Weber, J. 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. J. Bacteriol. **73:** 269-278.

Marchessault, R.H., and P.R. Sundararajan. 1957. Experimental evaluation of the lateral order distribution in cellulose. Text. Res. J. **27:** 30-41.

Mok, W.S.L., Antal, M.J.A. 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. Ind. Eng. Chem. Res. **31:** 1157-1161.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R. 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour. Technol. **96**: 1986-1993.

Palmowski, L., Muller, J. 1999. Influence of the size reduction of organic waste on their anaerobic digestion. *In* II International Symposium on Anaerobic Digestion of Solid Waste. Barcelona 15-17 June, pp. 137-144.

Puls, J., Poutanen, K., Korner, H.U., Viikari, L. 1985. Biotechnoligcal utilization of wood carbohydrates after steamin pretreatment. Appl. Microbiol. Biotechnol. **22:** 416-423.

Puri, V.P., Mamers, H., 1983. Explosive pretreatment of lignocellulosic residues with high-pressure carbon dioxide for the production of fermentation substrates. Biotechnol. Bioeng. **25:** 3149-3161.

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Quim. Nova. **26:** 863-871.

Reczey, L., Brumbauer, A., Bollok, M., Szengyel, Z., Zacchi, G. 1998. Use of hemicellulose hydrolysate for B-glucosidase fermentation. Appl. Biochem. Biotechnol. **70-72:** 225-235.

Reese, E.T. 1956. A microbiological process report: enzymatic hydrolysis of cellulose. Appl. Microbiol. **4:** 39-45.

Reese, E.T., Mandels, M. 1971. Enzymatic degradation. P. 1079-1094 *In* N.M. Bikales and L. Segal (ed.), Cellulose and cellulose derivatives. Wiley Interscience, New York, N.Y.

Reese, E.T., Sui, R.G.H., Levnison, H.S. 1950. The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. J. Bacteriol. **59:** 485-497.

Selig, M.M., Viamajala, S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2007. Deposition of Lignin Droplets Produced During Dilute Acid Pretreatment of Maize Stems Retards Enzymatic Hydrolysis of Cellulose. Biotechnology Progress. **23:** 1333-1339.

Shevchenko, S.M., Beatson, R.P., Saddler, J.N. 1999. The nature of lignin from steam explosion/enzymatic hydrolysis of softwood. Appl. Biochem. Biotechnol. 867-876.

Tanahashi, M., Takada, S., Aoki, T., Goto, T., Higuchi, T., Hanai, S. 1983. Characterization of explosion wood. 1. Structure and physical properties. Wood Res. **69:** 36-51.

Teeri, T. T. 1997. Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. **15:**160-178.

Teeri, T.A., Koivula, A., Linder, M., Wohlfarht, G., Divne, C., and Jones, T.A. 1998. *Trichoderma reesei* cellobiohydrolases: why so efficient on crystalline cellulose? Biochem. Soc. Trans. **26:** 173-178.

Teixeira, L.C., Linden, J.C., Schroeder, H.A. 1999. Alkaline and peracetic acid pretreatments of biomass for ethanol production. Appl. Biochem. Biotechnol., 19-34.

Thompson, D.N., Chen, H.C., Grethlein, H.E. 1992. Comparison of pretreatment methods on the basis of available surface area. Biores. Technol. **39:** 155-163.

Usami, S., Kirimura, K., Imura, M., Morikawa, S. 1990. Cellular localization of the constitutive B-glucosidase in *Trichoderma viride.* J. Ferment. Bioeng. **70:** 185-187.

Van Soest, P.J. 1994. Nutritional ecology of the ruminant, 2nd ed. Cornell University Press, Ithaca, N.Y.

Walker, L.P., Wilson, D.B., Irwin, D.C. 1990. Measuring fragmentation of cellulose by *Thermomonospora fusca* cellulase. Enzyme Microb. Technol. **12:** 378-386.

Weil, J.R., Brewer, M., Hendrickson, R., Sarikaya, A., Ladisch, M.R. 1997. Continuous pH monitoring during pretreatment of yellow poplar wood sawdust by pressure cooking in water. Appl. Biochem. Biotechnol. **68:** 21-40.

Weimer, P.J., Hacnkey, J.M., French, A.D. 1995. Effects of chemical treatments and heating on the crystallinity of celluloses and their implications for evaluating the effect of crystallinity on cellulose biodegradation. Biotechnol. Bioeng. **48:** 169-178.

Weimer, P.J., Lopez-Guisa, J.M., French, A.D. 1990. Effect of cellulose fine structure on kinetics of its digestion by mixed ruminal microorganisms invitro. Appl. Environ. Microbiol. **56:** 2421-2429.

Wood, T.T. 1992. Fungal cellulases. Biochem. Soc. Trans. **20:** 46-53.

Wright, J.D. 1988. Ethanol from biomass by enzymatic hydrolysis. Chem. Eng. Pog. **84:** 62-74.

Xiia, W., Clarkson, W.W. 1997. Acid solubilization of lignin and bioconversion of treated newsprint to methane. Biodegradation **8:** 61-66.

Yang, K., Want, Y-J., and Juo, M.I. 2004. Effects of Substrate Pretreatment and Water Activity on Lipase-Catalyzed Cellulose Acetylation in Organic Media. Biotechnology Progress. 20: 1053-1061

<u>Introduction</u>

Anaerobic digestion has been the process most often used to stabilize wastewaters and biosolids since the early 20th century. This process has also been used to treat a wide variety of other types of biological wastes, such as brewery wastewater, food waste, household waste, agricultural waste, petrochemical wastewaters, and the organic portion of municipal solid wastes (Sheatley et al., 1997; Bouallagui et al., 2005; Krzystek et al.,2001; Badawi et al., 1992; Britz et al., 1988; and Bolzonella et al., 2005). Recently, the potential of microalgae as a source of biofuels and as a feedstock for anaerobic digestion has been subjected to intense academic and industrial research (Afi et al., 1996; Asinari et al., 1982; Chen 1987).

In systems that include the anaerobic digestion of algal biomass, solar energy is transformed into cellular energy, which can subsequently be converted to the chemical energy of methane through the anaerobic fermentation of algae by bacteria. The resultant methane can either be burned in a gas-turbine-generator system to produce electricity or, through catalytic processes, be converted to hydrocarbon fuel.

Methanogenic anaerobic digestion of organic material in wastewater is advantageous over aerobic active sludge systems because of its high organic removal rates, low energy-input requirement, energy production (methane) and low sludge production (Angenent *et al.*).

Additional fundamental benefits of anaerobic treatment include the following (Thauer, 1998):

-     Low cost technology

- Easy implementation

- High space loading rates

- Low space requirements of anaerobic digesters

- Low volume of excess sludge produced in anaerobic treatment

- High dewatering capacity

- Well stabilized excess sludge

- Useful byproducts such as ammonia can be recovered from the process

There are, however, a number of challenges associated with anaerobic digestion that need to be addressed in order to develop a strategy to successfully utilize the technology as a method for treating municipal and industrial wastewater (Visser et al., 1993; Stewart et al., 1984). These challenges include, but are not limited to the following:

- Relatively high susceptibility of methanogens and acetogens to a variety of xenobiotic compounds

- Presumed low stability of anaerobic treatment (susceptibility to digester upsets)

- Slow "start-up" time of digester process

- Inaccessibility of plant material due to the recalcitrance of the cell wall

These challenges are most likely due to a general lack of understanding concerning the fundamental microbial processes that drive anaerobic digestion. Therefore, by investigating the properties of the microbial cohort that are responsible for the transformation of biomass to methane through synergistic

processes, we can successfully address these challenges, making anaerobic digestion of biosolids a promising and easily implemented technology for advanced waste-water treatment.
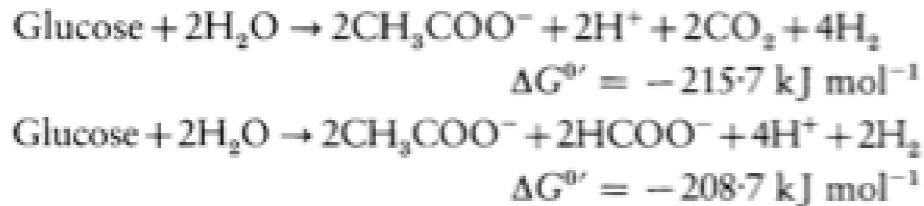
Many studies have been conducted to develop possible methods to minimize or eliminate the above-mentioned problems. Complexities within the microbiological processes that occur during anaerobic digestion have been particularly investigated (Zeikus, 1980; Richards et al., 1991; Pavlostathis et al., 1985).

Microbiological Processes of Anaerobic Digestion

The relative abundance of bacteria within a typical anaerobic digester is often greater than 10^{16} cells/ml. Bacterial populations within an anaerobic digester consists of saccharolytic bacteria, proteolytic bacteria, lipolytic bacteria, and methanogens (Gerardi, 2003). The anaerobic bioconversion of complex organic material to methane requires four major steps and five physiologically distinct groups of microorganisms (Gerardi, 2003). The four major steps include hydrolysis, acidogenesis, acetogenesis and methanogenesis. (Ahring, 2003; Bitton, 2005). Anaerobic digestion occurs in the absence of free molecular oxygen, and therefore relies on the ability of organisms to respire using an alternative electron acceptor such as nitrate or sulfate. The absence of molecular oxygen suggests that the process must occur in a highly reducing environment (i.e., an oxidation-reduction environment of -200 to -400 mV).

The purpose of the first three microbial processes (hydrolysis, acidogenesis, acetogenesis) is to ferment complex organic molecules to acetate, CO_2 and H_2, or to acetate, formate and H_2, (Equation 1):

Equation 1:

$$Glucose + 2H_2O \rightarrow 2CH_3COO^- + 2H^+ + 2CO_2 + 4H_2$$
$$\Delta G^{0\prime} = -215 \cdot 7 \text{ kJ mol}^{-1}$$
$$Glucose + 2H_2O \rightarrow 2CH_3COO^- + 2HCOO^- + 4H^+ + 2H_2$$
$$\Delta G^{0\prime} = -208 \cdot 7 \text{ kJ mol}^{-1}$$

1. Hydrolysis

In the first stage, large insoluble compounds (most likely cellulose, hemicellulose, lipids and proteins) are hydrolyzed by bacterial enzymes, causing the smaller organic subunits to become soluble and bio-available to the organisms involved in subsequent stages of digestion (Kelleher et al., 2000). Hydrolysis is typically carried out by extracellular enzymes. The kinetics of hydrolysis is dependent on several different parameters, such as particle size, pH, enzyme production, and diffusion and adsorption of enzymes to particles. Equation 2 shows an example of a hydrolysis reaction in which organic waste is broken down into a simple sugar, in this case, glucose during hydrolysis (Ostrem, 2004).

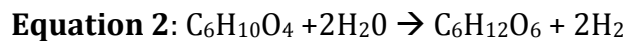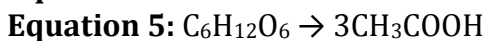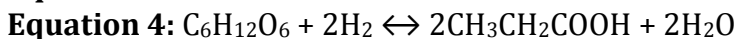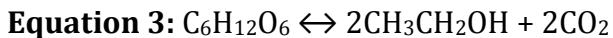**Equation 2**: $C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2$

Table 1 presents typical hydrolysis rates of various substrates during this phase of anaerobic digestion.

Table. 1 Typical hydrolysis rates for various substrates during AD

| Substrate	Hydrolysis Rate, d^{-1}	Reference
Carbohydrates	0.025-0.200	Christ et al., 2000
Cellulose	0.040-0.130	Gujer and Zehnder, 1983
Proteins	0.015-0.075	Christ et al., 2000
Lipids	0.005-0.010	Christ et al., 2000

2. Acidogenesis

During acidogenesis, which is also referred to as the fermentative step of anaerobic digestion, dissolved organic matter is transformed mainly to volatile fatty acids (VFAs) ketones, alcohols, hydrogen and carbon dioxide by a heterogenous microbial population. The principal acidogenesis stage products are propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$), acetic acid (CH_3COOH), formic acid ($HCOOH$), lactic acid ($C_3H_6O_3$), ethanol (C_2H_5OH) and methanol (CH_3OH). The hydrogen, carbon dioxide and acetic acid will be utilized directly by the methanogenic bacterial in the final stage of anaerobic digestion, while the other organic products will be subsequently subjected to the acetogenesis phase. Equations 3-5 illustrated three typical acidogenesis reactions whereby glucose is converted to ethanol, propionate and acetic acid, respectively:

Equation 3: $C_6H_{12}O_6 \leftrightarrow 2CH_3CH_2OH + 2CO_2$
Equation 4: $C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$
**Equation 5:** $C_6H_{12}O_6 \rightarrow 3CH_3COOH$

Factors that have been shown to affect the fermentation process in an anaerobic digester include interspecies hydrogen transfer, pH, hydraulic retention time, and previous acclimation of the anaerobic culture (Gunaseelan, 1997).

3. Acetogenesis

During the third stage of anaerobic digestion, the residual products of acidogenesis (i.e. the propionic acid, butyric acid and alcohols) are transformed by acetogenic bacteria into hydrogen, carbon dioxide and acetic acid, which will be subsequently utilized by the methanogenic bacteria. Hydrogen plays an important intermediary role in this process. The reactions have been shown to occur only if the partial pressure of hydrogen is low enough to thermodynamically allow the conversion of all of the acids. The partial pressure is lowered due to hydrogen-scavenging bacteria. Thus, the hydrogen concentration in a digester can be used as an indicator of the health and stability of the digester conditions (Mata-Alvarez, 2003). Equation 6 illustrates the conversion of propionate to acetate, which is only achievable at low hydrogen pressure.

Equation 6: $CH_3CH_2COO^- + 3H_2O \leftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$
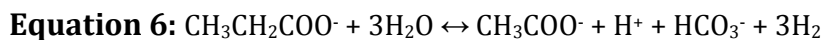
Equation 7 shows the tranformation of ethanol to acetate during the third stage of fermenations (Ostrem, 2004).

**Equation 7:** $CH_3CH_2OH + 2H_2O \leftrightarrow CH_3COO^- + 2H_2 + H^+$
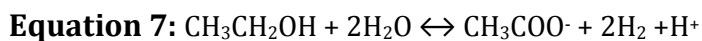
4. Methanogenesis

Methanoarchaea include the diverse group of organisms that facilitate the reduction of the metabolites that were generated in the previous three steps (hydrolysis, acidogenesis, acetogenesis) to methane. It is important to understand the kinetics specifically of the methanogenic bacteria. Due to the low energy yields of their metabolic processes, they are generally extremely slow growing and require a high level of substrate. Optimal operational conditions must then be maintained for satisfactory rates of solids conversion and methane production.

A. Methanogenic Pathway

The energy metabolism of methanogens can be addressed in two parts: an "oxidative" set of reactions, in which coenzyme M (H-S-CoM, 2-thioethanesulfonate) and coenzyme B (H-S-CoB, 7-thioheptanoylthreonine-phosphate) are oxidized to a heterodisulfide, and a "reductive" set of reactions, in which the heterodisulfide is re-reduced:
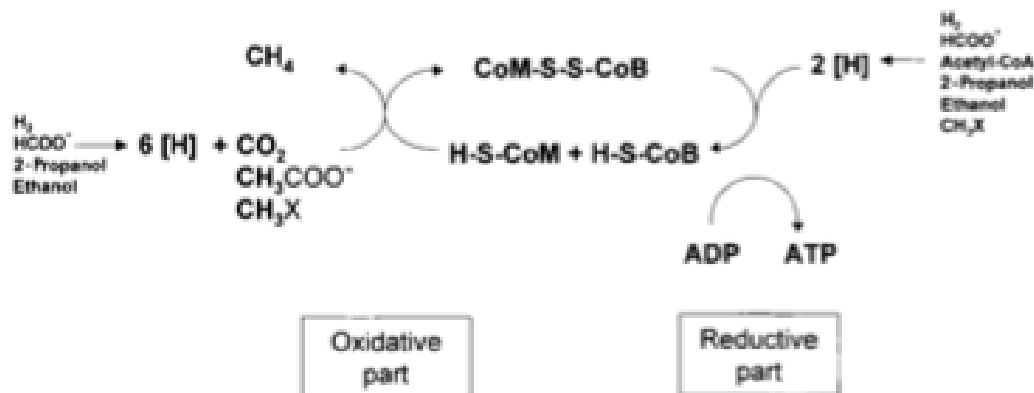
The specific enzymes and reactions involved in the transformation of the three primary substrates for methanogenesis are listed in the tables 2-4.

Table 2: Reactions and enzymes known to be involved in methane formation from acetate in the Methanocarcinales (Adapted from Thauer, 1998; Yang and Okos, 1987).

Reaction	Enzyme
Acetate + CoA → Acetyl CoA + H2O	Acetate Kinase and phosphotransacetylase
Acetyl-CoA + H_4SPT → CH3 H_4SPT + CO_2 + CoA + 2H	Carbon-monoxide dehydrogenase/acetyl CoA synthase
CH3-H4SPT + H-S-CoM → CH3-S-CoM + CH4	Methyl-H4SPT: Coenzyme M Mehtyltransferase
CH3-S-CoM + H-S-CoB→CoM-S-S-CoB + CH4	Methyl-coenzyme M reductase
CoM-S-S-CoB + 2H→ H-S-CoM + H-S-CoB	Heterodisulphide reductase

Table 3: Reactions and enzymes known to be involved in methane formation from CO2 (Adapted from Thauer, 1998).

Reaction	Enzyme
CO, + MFR + 2[H] → formyl-MFR	Formylmethanofuran dehydorgenase
Formyl-MFR + H,MPT → formyl-H,MPT + MFR	Formethylmethanofuran: H4MPT formyltransferase
Formyl-H,MPT + H+ → methenyl-H,MPT+ + H,O	Methenyl-H4MPT cyclohydrolase
Methenyl-H,MPT+ + F,,,H, → methylene-H,MPT + F420 + H+	F420-dependent methylene-H4MPT dehyrogenase
Methenyl-H,MPT+ + H,→ methylene-H,MPT + H+	H2-forming methylene-HrMPT dehydrogenase
Methylene-H,MPT + F420H→ CH,-H,MPT + F,20	F420-dependent methylene-H4MPT reductase
CH,-H,MPT + H-S-COM+→ CH,-S-CoM + H,MPT	Methyl-H4MPT: coenzyme M methyl-trasferase
CH,-S-CoM + H-S-COB→ COM-S-S-COB + CH,	Methyl coenzyme M reductase
COM-S-S-COB + 2[H]→ H-S-COM + H-S-COB	Heterodisulphide reductase

Table 4: Reactions and enzymes known to be involved in methane and CO2 formation from methanol in Methanosarcina **(**Adapted from Thauer, 1998).

Reaction	Enzyme
1. Methane Formation:	
$H30H + H\text{-}S\text{-}CoM \rightarrow CH3\text{-}S\text{-}CoM + H2O$	Methanol: coenzyme M methyltransferase
$CH3\text{-}S\text{-}CoM + H\text{-}S\text{-}CoB \rightarrow CoM\text{-}S\text{-}S\text{-}CoB + CH4$	Methyl-coenzyme M reductase
$CoM\text{-}S\text{-}S\text{-}CoB + 2H \rightarrow H\text{-}S\text{-}CoM + H\text{-}S\ CoB$	Heterodisulphide reductase
2. CO_2 Formation:	
$CH3\text{-}OH + H\text{-}S\text{-}CoM \rightarrow CH3\text{-}S\text{-}CoM + H2O$	Methanol: coenzyme M methyltransferase
$CH3\text{-}S\text{-}CoM \_ H4SPT \rightarrow H\text{-}S\text{-}CoM + CH3\text{-}H4SPT$	Methyl-H4SPT: coenzyme M methyltransferase
$CH3OH + H4SPT \rightarrow CH3\text{-}H\$SPT + H2O$	Enzyme not yet identified
$CH3\text{-} H_4SPT + F420 \rightarrow$ methylene- $H_4SPT + F420H2$	F420 dependent methylene-H4SPT reductase
$Methylene\text{-}H4 + F420 + H+ \rightarrow$ methenyl- $H_4SPT + F420H2$	F420-dependent methylene-H4SPT dehydrogenase
$Methenyl\text{-} H_4SPT + H2O \rightarrow$ formyl- $H_4SPT + H+$	Methenyl-H4SPT cyclohydrolase
$Formyl\text{-} H_4SPT + MFR \rightarrow$ formyl-MFR + H_4SPT	Formylmethanofuran H4SPT formyltransferase
$Formyl\text{-}MFR \rightarrow CO_2 + MFR + 2H$	Formylmethanofuran dehydrogenase

In the pathways described in Tables 2-4, a methyl group is transferred to coenzyme M in the oxidative part of the energy metabolism. This process is coupled with the build-up of an electrochemical sodium gradient. In turn, the reduction of the heterodisulfide bond during the reduction process is coupled with electrogenic hydrogen (H^+) translocation. Methanogens have been shown to contain a Na^+/H^+ antiporter capable of interconversion of the electrochemical and H^+ and Na^+ potentials.

The stoichiometry of coupling methanogenesis with ADP phosphorylation has appeared to be inconsistent and has been shown to be dependent on H_2, concentrations.

Methanogens, like many anaerobic bacteria and archaea, can use sulfur as a terminal electron acceptor, but unlike other sulfur reducers, they can act independent of an external sulfur source as electron acceptor since they can reoxidize the "reduced sulfur" with CO_2 or one of the other methanogenic carbon substrates, which themselves are concomitantly reduced to methane.

B) Kinetics of Methanogenesis

Methane formation has been found to be associated with cell growth (Yang and Okos, 1987). Kinetic models have been developed based on a number of different initial substrates. A study by Borja et. al. resulted in a Michaelis-Menten equation type for methane production (Equation 8):

$$\frac{dS_M}{dt} = \left[\frac{k_6 S_{TVA}}{k_7 + S_{TVA}}\right]$$

Equation 8:

The following kinetic constants were obtained:

- Maximum rate of total volatile acids consumption: k_6= 3.6 +/- 0.2 g TVA-Cod/l-day

- Saturation constant: k_7= 3.1 +/- 0.2 g TVA-COD/liter.

A study by Yang et al. (1987) showed nearly equimolar methane production on acetate during methanogenic growth, and about 1.94 g of cells were formed from each mole of acetate consumed. They found a maximum specific growth rate of 0.022 hr $^{-1}$ at acetic acid concentrations around 7 g/L. Substantial substrate inhibition was found when the acetate concentration was higher than 0.12 M. Evidence is available that during growth of methanogens on H_2, and CO_2, the growth yield increases with decreasing H_2, concentrations, indicating that at low H_2, concentrations, coupling of heterodisulfide reduction with ADP phosphorylation is tighter than at high H_2, concentrations (Thauer, 1998).

C) Thermodynamics of Methanogenesis

In anaerobic freshwater sediments, plant material such as glucose from cellulose in algae is completely decomposed to CO_2 and CH_4 (Equation 9):

Equation 9: Glucose → 3 CO_2 + 3CH_4 $\qquad\qquad$ ΔG°= -418.1 kJ/mol

This reaction is the result of syntrophic associations of micro-organisms who ferment the glucose first to i) acetate, CO_2 and H_2,, or to ii) acetate, formate and H_2, (Equations 10-11):

Equation 10: Glucose + $2H_2O$ → $2CH_3COO^-$ + $2H^+$ + $2 CO_2$ + $4 H_2$, $\Delta G°$= -215.7 kJ/mol
Equation 11: Glucose + $2H_2O$ → $2CH_3COO^-$ + $2HCOO^-$ + $4H^+$ + $2 H_2$, $\Delta G°$= -208.7 kJ/mol
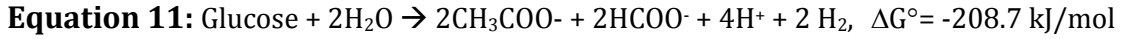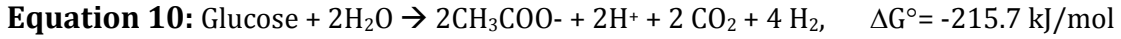
The intermediates (acetate, formate, H_2, and CO_2) are then converted by methanogens to CH_4, CO_2, and water (Equations 12-14):
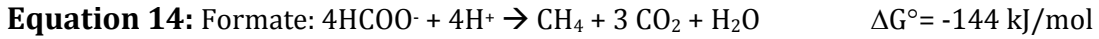
Equation 12: Acetate: $CH3COO^-$ + H^+ → CO_2 + CH_4 $\Delta G°$= -36 kJ/mol
Equation 13: H_2, and CO_2: $4 H_2$, + CO_2 → CH_4 + H_2O $\Delta G°$= -131 kJ/mol
Equation 14: Formate: $4HCOO^-$ + $4H^+$ → CH_4 + $3 CO_2$ + H_2O $\Delta G°$= -144 kJ/mol
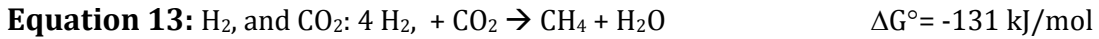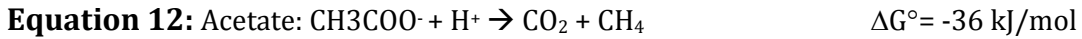
Tables 5-6 give the thermodynamic values associated with methane production from acetate and CO_2.

Table 5: Thermodynamic Values for methane formation from acetate

Reaction	Thermodynamic Value
Acetate + CoA → Acetyl CoA + H2O	$\Delta G°$= + 35.7 kJ/mol
Acetyl-CoA + H4SPT → CH3H4SPT + CO2 + CoA + 2H	$\Delta G°$= 41.3 kJ/mol E'o= - 200mV
CH3-H4SPT + H-S-CoM → CH3-S-CoM + CH4	$\Delta G°$= - 30 kJ/mol
CH3-S-CoM + H-S-CoB→CoM-S-S-CoB + CH4	$\Delta G°$= -45 kJ/mol
CoM-S-S-CoB + 2H→ H-S-CoM + H-S-CoB	$\Delta G°$= - 40 kJ/mol E'o= - 200mV

Table 6: Thermodynamic values for methane generation from CO2

Reaction	Thermodynamic Value
CO, + MFR + 2[H] → formyl-MFR	$\Delta G°= + 16$ kJ/mol E'o= - 530 mV
Formyl-MFR + H,MPT → formyl-H,MPT + MFR	$\Delta G°= - 4.4$ kJ/mol
Formyl-H,MPT + H+ → methenyl-H,MPT+ + H,O	$\Delta G°= - 4.6$ kJ/mol
Methenyl-H,MPT+ + F,,,H, → methylene-H,MPT + F420 + H+	$\Delta G°= + 5.5$ kJ/mol
Methenyl-H,MPT+ + H,→ methylene-H,MPT + H+	$\Delta G°= -5.5$ kJ/mol
Methylene-H,MPT + F420H→ CH,-H,MPT + F,20	$\Delta G°= -6.2$ kJ/mol
CH,-H,MPT + H-S-COM+→ CH,-S-CoM + H,MPT	$\Delta G°= -30$kJ/mol
CH,-S-CoM + H-S-COB→ COM-S-S-COB + **CH,**	$\Delta G°= -45$ kJ/mol
COM-S-S-COB + 2[H]→ H-S-COM + H-S-COB	$\Delta G°= -40$ kJ/mol E'o= -200 mV

Thermodynamics of a) Oxidative Reactions and b) Reductive Reactions

a)   Oxidative Reactions: Methyl coenzyme M, formed from coenzyme M and acetate, CO_2 or reduced C1 compounds is the central intermediate. It is subsequently reduced with coenzyme B to methane with concomitant formation of heterodisulfide of coenzyme M and coenzyme B (Equation 15):

Equation 15: CH_3-S-CoM + H-S-CoB → CH_4 + CoM-S-S-CoB $\Delta G°= -45$ kJ/mol
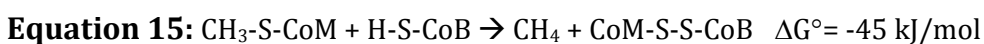
b)　　　Reductive Reactions: Energy for growth of methanogens is generated during the reduction of heterodisulfide bond that were generated in the oxidative reactions. The reduction of the heterodisulfide is coupled with phosphorylation of ADP via a chemiosmotic mechanism as described in Table 7.

Table 7: Free energy change associated with heterodisulfide reduction (adapted from Borja et al., 2005).

Compound	Free Energy Change
H_2	$\Delta G° = -40$ kJ/mol
Formate	$\Delta G° = = 43.5$ kJ/mol
Ethanol (acetate)	$\Delta G° = -35$ kJ/mol
Methanol (CO_2)	$\Delta G° = -34$ kJ/mol

Each of these is sufficient to drive the phosphorylation of 1 mol ADP: $\Delta G° = +31.8$ kJ/mol.

D) *Relevant Organisms involved in Methanogenesis*

Methanogenic organisms were originally thought to be eubacteria, but are now recognized as belonging to a separate phylogenetic domain, the *Archaea.* They are highly specialized, as they can only use acetate, H_2 and CO_2, methylthiols and methylamines as energy substrates. Despite the high degree of specialization, not all methanogens are phylogenetically closely related. Taxonomically all belong to the archaeal kingdom of *Euryarchaeota* and are classified in the following five orders: *Methanobacteriales, Methanococcales, Methanomicroiales, Methanopyrales* and *Methosarcinales* (Table 8).

Table 8: Characteristics of 4 methanogenic orders

Order	Morphology	Physiology
Methanobacteriales	Rods or filaments	Hydrogenotrophic, mesophilic or thermophilic
Methanococcales	Irregular cocci	Hydrogenotrophic, mesophilic or thermophilic
Methanomicrobiales	Small rods, irregular cocci, flat oval shaped cells	Hydrogenotrophic, mesophilic
Methanosarchinales	Rods or filaments, irregular cocci	Strict aceticlastic, aceticlastic or hydrogenotrophic, mesophilic or thermophilic

E.   *Effect of Environmental Conditions on Methanogenesis*

Methane-forming bacteria are strict anaerobes and are extremely sensitive to environmental changes in alkalinity (buffering capacity), pH and temperature. The optimal temperature for methanogenesis is dependent on the microbial community present, but is generally in the range of 55 - 65°C. Small, sudden shifts in temperature will significantly disrupt the microbial activity (Zinder et al., 1984). Methanogenesis occurs at fairly neutral pH values (6.75-7.4), and is usually inhibited almost completely at pH values greater than 8 (Visser et al., 1993). The process of methanogens occurs in the absence of free molecular oxygen, suggesting that the process must occur in a highly reducing environment.

F) *Nutrient Requirement:*

A study conducted at the University of Illinois by Bryant (1971) showed that strains of methanogens that are contained in the rumens of animals and humans that are grown in H_2 and with CO_2 as the energy source require NH_4^+ as the main source of cell nitrogen and have little ability to incorporate or metabolize organic nitrogen compounds such as amino acids or peptides (Bryant, 1971). Therefore, a sufficient amount of ammonia is required for methanogenic growth. Other nutrient requirements of methanogens include trace metals, branched chain fatty acids and coenzyme M, as well as low partial pressures of H_2 to keep methanogenesis thermodynamically favorable.

Anaerobic Digestion Process Engineering Parameters

Methane-forming bacteria are strict anaerobes, and are extremely sensitive to environmental changes in alkalinity (buffering capacity), pH (optimum = 6.8 – 7.2) and temperature (optimum = ~35°C). Digester operational conditions must be closely monitored to determine how sensitive our system is to changes in these parameters.

Most anaerobic digestion takes place at mesophilic temperatures, between 30-38 degrees C. Digesters can also be operated at higher thermophilic temperatures, typically 50-60 degrees C. Thermophilic digestion has been shown to have several advantages, including human pathogen reduction and improved sludge dewatering characteristics (Parkin and Owen, 1986). Higher energy requirements,

poor supernatant quality, and less process stability have also been indentified as disadvantages of thermophilic digestion.

Other parameters that must be monitored include gas composition, hydraulic retention time (HRT), oxidation-reduction potential and solids retention time (SRT). Changes in the SRT have been shown to be more influential on the performance of the digester than changes in the HRT because the generation time of methane-forming bacteria is slow. If the SRT is too short, the organisms will wash out of the digester before being able to reproduce. An SRT of less than 10 days would cause a system wash-out.

Once the reactor has reached steady state, factors that may be responsible for upsets and causing the reactor to become unstable should be monitored. These conditions are usually related, and include hydraulic overload, organic overload, pH changes, temperature fluctuations, toxicity, a large withdrawl of algal sludge and air contamination. Ammonia build up in the digester leads to significant toxicity, which is closely linked to changes in pH. As the pH drops below 7.2 (the pKa of ammonia) NH_4^+ is favored over NH_3 leading to increased toxicity levels in the digester. Ammonia toxicity can be avoided if the pH of the digester is maintained within the optimum ranges of 6.8 to 7.2. A decrease in methane composition of the gas is a reliable indicator that the digester may have become unstable, because methane production represents final degradation of organic compounds. Methane production and alkalinity may be correlated, and this may also be used as an indication of an unstable digester system.

Recommended solids loading for anaerobic digesters that are mixed and heated are 200 - 450 lb volatile solids/1000 ft^3/day (Malina and Pohland, 1992). This is dependent, however, on the concentration of the algal sludge. Another valuable optimization is the investigation of the effects of solids loading of algal biomass on the performance of the system that would contribute to the development of a kinetic-based model of the response of the organisms to increased solid loading.

Anaerobic Digestion Processes and Designs

A)   Continually Fed Digesters

Continually fed digesters are typical digesters that are used as a conventional single stage digestion process. In these digesters, the rate of feeding should be continuous for maximum efficiency, however, for practical reasons, the digesters are usually fed intermittently. In a continuously stirred tank reactor (CSTR), an influent substrate concentration of 3-8% total solids (TS) is added daily, and an equal amount of effluent is withdrawn. The digester is maintained constantly at mesophilic or thermophilic temperatures. The addition of large amounts of water requires large reactor volume and high post-treatment costs for the digester residue.

B)   High solids anaerobic digestion takes place at a TS concentration of more than 25% and is also referred to as "dry anaerobic fermentation". Most high solids anaerobic digestions studies have been performed using municipal solid waste (MSW) (Shulze, 1958; Cecchi, et al 1988).

Anaerobic Digester Reactors

Digesters involved in anaerobic digesters can be placed into two different types: those that retain biomass, and those that allow biomass to pass through.

In a reactor that retains biomass, the SRT is decoupled from the HRT, thus allowing for a higher concentration of "active mass" within the reactor and faster reactions. These types of reactors are also able to adapt more quickly, provided that the feed is consistent. Cultures will adapt to self optimize for the influent conditions.

Different seed cultures will contain different organisms in varying concentrations. Therefore, if the seed culture is adapted to a feed that is different from the feed to be treated, it could require additional time for the culture to adapt before a stable community can develop.

1) Conventional Single Stage Digestion

a) Continually fed digesters are typical digesters that are used as a conventional single stage digestion process. In these digesters, the rate of feeding should be continuous for maximum efficiency; however, for practical reason, the digesters are usually fed intermittently. In a continuously stirred tank reactor (CSTR) an influent substrate concentration of 3-8% total solids (TS) is added daily, and an equal amount of effluent is withdrawn. The digester is maintained constantly at mesophilic or thermophilic temperatures. The addition of large amounts of water requires large reactor volume and high post-treatment costs for the digester residue.

c) In a plug-flow digester, also referred to as a tubular plug-flow digester, a volume of the medium with a suitable inocculum enters at one end of the tubular digester. If the rate of passage of the medium is correct, by the time the medium reaches the other end of the reactor, the digestion is completed. For continuous operation, some of the digested effluent flowing from the end of the tube is separated and returned to the influent substrate. One type of plug-flow reactor is the Upflow Anaerobic Sludge Blanket (UASB) reactor, developed by Dr. Gatze Lettinga (Lettinga and Hulschoff Pol, 1991, Lettinga et al., 1980). Lettinga et al. observed that under certain conditions, anaerobic consortia form granular particles, which settle easily in aqueous solutions, forming relatively dense beds in the bottoms of vertical upflow bioreactors.

In these reactors, the influent enters from the bottom of the reactor via a diffuser to provide equal distribution through the sludge blanket. The substrate is passed through the sludge blanket vertically, converting the Chemical Oxygen Demand (COD) of the substrate to biogas, and some biomass. Biogas is directed via deflector to a separator, while the treated liquid effluent flows to a reactor outlet.

The principle that drives UASB is the separation of SRT and HRT. As the microbial consortia required for digestion are slow to reproduce, they can be retained in a reactor while the fluid that carries dissolved carbon substrates that the microbes feed on passes freely through at a rate that is optimized to the metabolic capabilities of the active solids (Lettinga et al., 1980). Therefore, the volumetric efficiency of the reactor is maximized. The active biomass, therefore, acts effectively as a filter, metabolizing available carbon to methane and carbon dioxide. The UASB

is capable of treating both high and low strength wastes effectively, although the substrates must be relatively clean, and free of non-volatile suspended solids that may displace of interfere with active solids (Lettinga et al., 1980)

The Induced Bed Reactor (IBR) was developed at Utah State University to apply high-rate anaerobic digestion techniques to high solids content substrates (Dustin, 2010). The IBR includes an upflow design, similar to UASB. The IBR has been optimized to treat complex high solids and wastewaters such as dairy manures.

In order to accommodate the high solids content and large maximum particle sizes anticipated in the influent, a relatively large inlet is required to permit the solids to pass without plugging the feed line. The IBR is generally taller than the typical UASB designs (10 m vs 5-8 m) (Lettinga and Hulschoff, Pol, 1991), to provide additional separation time for active solids to settle. Operationally, the IBR is typically operated at HRTs of 3-7 days, with waste strengths of 25-85 kg/m^3 COD, results in 30-60% reduction in COD. A typical UASB is operated at 4-14 hours HRT, with waste strengths of 1-18 kg/m3 COD, and results in 90-95% reduction of COD.

Major advantages of the IBR include a reduced need for solids separation and the ability to treat all of the material in the feed stream. Additionally, the HRT is significantly less than a similarly performing plug or CSTR digester with a 20-day HRT, therefore requiring significantly less operational volume.

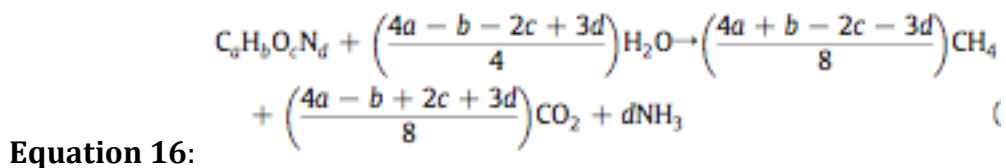Algal Biomass as Feedstock for Anaerobic Digestion

Microalgae have been identified as having potential for large-scale production of biofuel. Microalgae require large quantities of nitrogen and phosphorus for large-scale production, making environmental and economic sustainability difficult to maintain. A process that includes the recycling of nitrogen and phosphorous is therefore required in order to reduce the use of added nutrients or fertilizers and to increase the economic feasibility (Olguin, 2003)

Furthermore, to reach an economical balance, the process of anaerobic digestion of algal biomass may be integrated into a comprehensive system to harness and transform the photosynthestic energy captured in algae. For example, alagl lipids may be extracted to develop several bioproducts, such as bioplastics or biodiesel. The remaining biomass, primarily the compounds that comprise the algal cell walls may then be digested to methane to balance the process economically. Chisti (2007) has shown the algal biomass that remains after lipid extraction can be transformed into methane via anaerobic digestion.

Anaerobic degradation of algal cells occurs naturally in aquatic environments as algal material sinks toward the anoxic and aphotic zones after cell death. Ammonium and phosphate release occurs as algal cells are degraded naturally, which serves to sustain growth of phytoplanktonic communities in the surrounding environment. Golueke et al. (1957) published the first study on engineered anaerobic digestion of an algal biomass. In 1960, they proposed an integrated process associating the production of microalgae in an open pond for the treatment of sewage water and the energetic recovery of the algal biomass by anaerobic digestion (Oswald and Golueke 1960; Golueke 1963).

Kinetics of anaerobic degradation of algae is highly dependent upon the resistance of the cell wall (Chen, 1987; Afi et al., 1996; Chen and Oswald, 1998). Determining the composition of microalgae has allowed researchers to determine digestion potential. The average composition of microalgae has been determined as $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Grobbelaar, 2004). Microalgae also contains oligo nutrients such as iron, zinc and colbalt, which are known to stimulate methanogenesis (Speece, 1996).

The composition with respect to cellular structural components has also been determined for various types of microalgae. Cell composition is strongly species-dependent, and is also affected significantly by environmental conditions (Leadbeater, 2006; and Droop, 1983). Two methods can be used to determine the theoretical amount of methane produced by each of the individual species. One method to evaluate the theoretical methane yield from anaerobic digestion of these species is to use the following formula adapted from Symons and Buswell (1933) (Equation 16):

$$C_a H_b O_c N_d + \left(\frac{4a - b - 2c + 3d}{4}\right) H_2O \rightarrow \left(\frac{4a + b - 2c - 3d}{8}\right) CH_4$$
$$+ \left(\frac{4a - b + 2c + 3d}{8}\right) CO_2 + dNH_3$$

Equation 16:

The second method includes using half-reactions for the individual components, combined with the reduction reaction of CO_2 to CH_4, and using the resulting stoichiometry to elucidate theoretical methane potential. Ideally, these two

methods should yield similar results for methane potential. These calculations are provided in Appendix 1.

Using this equation, the theoretical CH_4 yields from various cellular components have been estimated and presented in Table 9.

Table 9. Estimated cellular composition calculated using Eq. 15 and using half-reactions

Component	Composition	Methane Yield Using Eq. 15	Methane Yield Using Half-Reactions
Proteins	$C_{16}H_{24}O_5N_4$	0.524 L CH_4/ g VS	0.54 L CH_4/ g VS
Lipids	$C_8H_{16}O$	1.01 L CH_4/ g VS	1.01 L CH_4/ g VS
Carbohydrates	$(C_6H_{10}O_5)_n$	0.373 L CH_4/ g VS	0.373 L CH_4/ g VS

The following percentage values have been identified as typical ranges for structural components in microalgae (Brown et al., 1997):

Proteins: 6-52%
Lipids: 7-23%
Carbohydrates: 5-25%

Anaerobic Digestion of Algal Material from the Logan Lagoons

Conditions exist in the Logan Lagoon system such that it is desirable to remove algae from the lagoon effluent after they have taken up sufficient levels of phosphorus. Under these circumstances, a readily applicable method of processing the algae to generate a sustainable source of energy as biomethane is that of

controlled anaerobic digestion. Studies performed by researchers at Utah State

University have indicated that two of the primary algae species present in the Logan

Lagoon system are *Chlorella* and *Scenedesmus* (Christenson and Sims 2012).

For the two species that have been identified to exist in the Logan Lagoons,

Chlorella and *Scenedesmus*, the relative composition has been determined. From this

information, we can calculate the theoretical methane values that would result from

anaerobic digestion of the individual species, which are presented in Table 10.

Table 10. Relative cellular components in Logan Lagoon Algae species and theoretical methane production

Species	Proteins (%)	Lipids (%)	Carbohydrate (%)	L CH$_4$/ g VS Using Eq. 16	L CH$_4$/ g VS Using Half Reactions
Chlorella	56	12	19	1.12	1.124
Scenedesmus	50-56	13	17	0.5	0.5

It has been documented that both *Scenedesmus* and *Chlorella* have

particularly recalcitrant cellulosic cell walls (Okuda, 2002). Another study,

performed by Takeda and Hirokawa (1978) demonstrated that the cell wall of

Chlorella ellipsoidea was composed to two major constituents: alkali-soluble

hemicellulose and alkali-insoluble rigid wall.

Pretreatment can be successfully applied in order to lower the recalcitrant

organic fraction, and thus increase the methane conversion. Chen and Oswald

(1998) studied different pretreatments for an algal biomass produced in sewage

treatment ponds. The temperature appeared to have the most important effect. The

optimal pretreatment consisted of heating at 100 degrees C for 8 hours, which resulted in a 33% improvement in methane production. While the energy input is high for this pretreatment option, the improvement in methane yield can energetically balance the thermal pretreatment. Ultrasonic pretreatment has been shown to enhance the crude protein digestibility, as shown with experiments on the digestibility of *C. vulgaris* in rats (Janczyk et al., 2007). An additional pretreatment option that should be considered is the use of alkaline peroxide, which relies on radical oxygen chemistry to break down cellulose in the algal cell wall into simple monomers.

Another potential strategy for improving methane yield is to alter the cellular composition by "stressing" the organisms. When algae are deprived of nitrogen, their metabolism is altered toward enhanced lipid production. A study performed by Illman et al. (2000), showed that when algae are subjected to low nitrogen conditions, their lipid content nearly doubles. Lipids yield more methane per gram than carbohydrates. Thus, by increasing lipid content, more theoretical methane can be obtained. Also, the study showed a decrease in ammonia release as well, leading to less toxicity to the anaerobic organisms.

Summary and Problem Statement

A comprehensive understanding of the process fundamentals involved in anaerobic digestion is necessary to ensure efficient and reliable operation. The current conditions in the Logan City Wastewater Lagoon system require a nutrient removal solution in order to meet anticipated permit requirements.

A proposed integrated nutrient removal system allows for the uptake of nutrients by algae that exist naturally in the Logan Lagoons. The algae may subsequently be removed and processed to yield bioproducts, including bioplastics, biodiesel, and biomethane. The algal material may be processed immediately after removal in an anaerobic digester to produce methane. In order to maximize the anaerobic transformation and biogas production, this study sought to understand the effect of digester inoculum on the digestion of algae collected from the Logan Lagoons, as well as to understand the effect of several chemical pretreatments on algal digestibility.

The Logan Wastewater Lagoons have been an isolated system for 40 years. Therefore, through selective evolutionary pressures, the system has likely become optimized for digestion (both aerobic and anaerobic) of algae species native to the Lagoons, as the native bacteria may have become optimized to degrade the algal species common to the system. A study performed at Utah State University (Ellis et al., 2012) has shown that the Logan Lagoons contain a unique consortium of methanogenic bacteria. Therefore, by inoculating anaerobic digesters with sludge/biomass that has been collected from the bottom of the Logan Lagoons at a depth of five feet, we predict that algal methane production may be enhanced due to optimized bacterial cultures.

The cell walls of both *Chlorella* and *Scenedesmus* have both been demonstrated to be extremely recalcitrant and resistant to anaerobic digestion. (Afi et al., 1996). This study sought to determine if the pretreatment of algal species found in the Logan Lagoons is successful in reducing the recalcitrance of the cell

85

wall, thus making the cellular components available to methanogenic bacteria, as discussed previously in this report. Two pretreatment strategies were investigated- dilute acid pretreatment and alkaline pretreatment.

By determining the effect of the inoculum and of pretreatment on the anaerobic digestion of algae collected from the Logan Lagoons, we may reduce or eliminate some of the process and economic barriers to algal transformation to biofuels and other bioproducts that are identified and discussed in this review, and enhance the efficiency of algae digestion in order to increase bioproduct production.

Materials and Methods

A. Conversion Efficiency Based on Digester Inoculum

Mixed culture algae grab samples from the Logan Lagoons Wastewater System were provided by Dr. Daniel Dye (Department of Biological Engineering, Utah State University). Pure cultures of *Chlorella vulgaris* and *Scenedesmus obliquus* were obtained from the American Type Culture Collection and were grown in Algal proteose medium. These species were chosen for evaluation because previous researchers at Utah State University have shown that these algal species are the most dominant in the Logan Lagoons (Bare et al., 1975).

Anaerobic digester sludge was provided by Dr. Shaun Dustin (Energy Dynamics Laboratory, Utah State University Research Foundation). The sludge obtained from Dr. Dustin had been obtained from a sampling port of a continuously operating dairy waste Induced Bed Reactor anaerobic digester. Lagoon-bed sludge was obtained from the bottom of the "A1" pond of the Logan Lagoon Wastewater Pond System. The sludge was collected from a depth of 5 feet. The sludges collected from the Logan Lagoons and from the IBR digester were used as bacterial seed for anaerobic digestion studies.

The relative digestibility of the different algal cultures was analyzed using the Biochemical Methane Potential (BMP) Assay. The BMP Assays were performed according to the procedure described in Owen et al. (1979). The Assays were performed by placing 116 mL deionized water, 13 mL prepared bacterial seed, 1.0 gram dried algal substrate, and 1.04 grams of sodium biocarbonate into 500 mL

Erlenmeyer Flasks. The flasks were flushed with argon gas and were stored at 37

degrees C for 35 days.

Gas samples were extracted using a 60 mL syringe at day 20, 25 and 33 of the

experiment. Total volumetric gas production was determined, and relative gas

composition were determined using a gas chromatograph.

Figure 1 illustrates a flow-diagram of the experimental design used to

evaluate the relative digestibilities of three difference algal cultures when they are

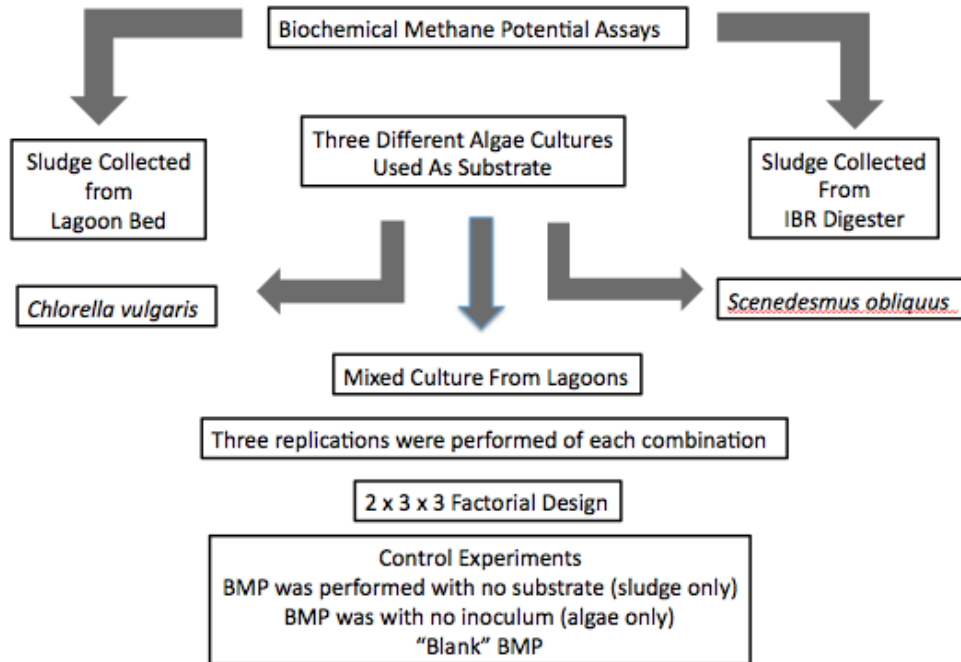digested anaerobically with two different inoculum sludge types.

Figure 1. Experimental design for digestion efficiency based on inoculum source.

Three replications of each combination of algae type and inoculum source

were performed. A standard deviation was then determined from the three

replications to represent the statistical relationship between the combinations.

B. *Effect of Pretreatment on Anaerobic Digestion of Algae*

Mixed culture algae from the Logan Lagoons were dried for 1 hour at 105 degrees C to remove available water prior to pretreatment. Dilute acid pretreatment was performed at 10% solids concentration, using four different concentrations of sulfuric acid: 0.5%, 1.0%, 2.0% and 4.0%. The dilute acid pretreatment procedure was performed at two different temperatures: 50 degrees C and 80 degrees C. Pretreatment duration was 24 hours.

Alkaline pretreatment was performed at 10% solids concentration, using four different levels of NaOH base: 0.25 M, 0.5 M, 1.0 M, and 2.0 M. The alkaline procedure was performed at two different temperatures: 50 degrees C and 80 degrees C. Pretreatment duration was 24 hours. A control experiment was performed in which non-pretreated algae from the Logan Lagoons was placed into pH 7 H_2O for 24 hours (no alkaline or acid) for 24 hours at 30 degrees C and at 50 degrees C.

After pretreatment, the algae were digested in BMP assays to evaluate the effect of pretreatment on the digestibility of algal biomass. The BMP assays were performed using the Logan Lagoon Bed Sludge as the prepared bacterial seed.

Volumetric gas production was measured each day for 35 days using a 60 mL syringe. Total volumetric gas production over the period of the study was determined.

Results/Discussion

The theoretical methane production from both *Chlorella* and *Scenedesmus* were determined using the stoichiometric relationships presented previously in order to evaluate the efficiency of the algae digestion processes. These values are presented in Table 1.

Species	Protein (%)	Lipid (%)	Carbohydrate (%)	Theoretical Methane L CH$_4$/g VS
Chlorella	56	12	19	0.50
Scenedesmus	50-56	13	21	0.48

Table 1. Theoretical methane values based on stoichiometrical calculations. For example calculations see Appendix 1.

A. Conversion Efficiency Based on Digester Inoculum

Figure 2 presents the total measured biogas production of pure algae strains (*Chlorella vulgaris* and *Scenedesmus obliquus*) that were digested using the two different inoculum sludges containing the anaerobic micriobiological consortium.
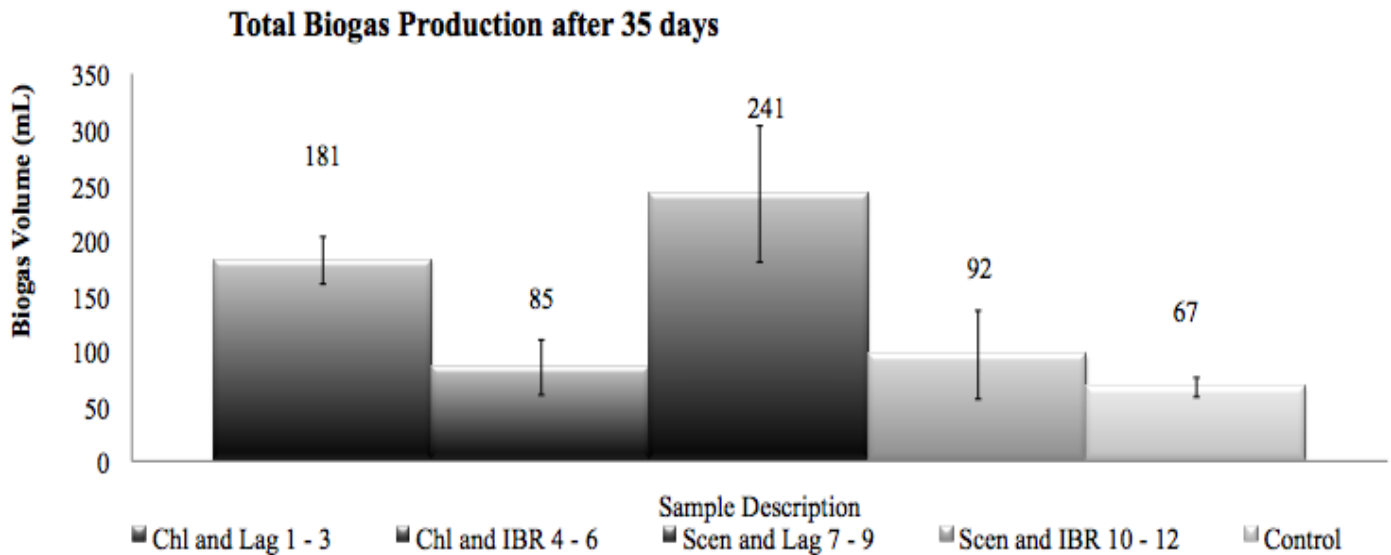
Figure 2. Total biogas production after 35 days of pure algae strains (*Chlorella vulgaris* and *Scenedesmus obliquus)* that were anaerobically digested using two different bacterial seed/inoculum (Lagoon Sludge and IBR Sludge). Numbers (1-3), (4-6), (7-9) and (10-12) designate the individual replication numbers.

After 35 days, algae samples that had been digested in BMP assays using bacteria seed sludge that was collected from the bed of the lagoons produced statistically significantly more gas than algae samples that had been digested using bacteria seed sludge that had been collected from an operating IBR reactor. Further, both the *Chlorella* and *Scenedesmus* algae samples that were digested with the IBR sludge produced only slightly more gas than algae that was incubated with no bacterial seed/inoculum. The sludge that was collected from the operating dairy waste digester had most likely been acclimated to digest substrates other than algae, and thus was unable to efficiently convert the algal biomass to methane gas.

Samples of *Scenedesmus obliquus* produced substantially more biogas when digested with Lagoon sludge than samples of *Chlorella vulgaris* that was digested with Lagoon sludge. There was no significant difference, however, between gas

production by samples of *Scenedesmus obliquus* that were digested with IBR sludge and samples of *Chlorella vulgaris* that were digested with IBR sludge.

Scenedesmus obliquus has been shown, to have a lower average lipid content than *Chlorella vulgaris* (Becker, 1994). Therefore, it would be expected that *Scenedesmus obliquus* would result in less total methane production. Possible explanations for the higher methane production by *Scenedesmus obliquus* may include differentiations in the chemistry of the cell wall.

Figure 3 illustrates the total biogas production of mixed culture algae from the Logan Lagoons that was inoculated with the two different sludges (Lagoon sludge and IBR sludge).
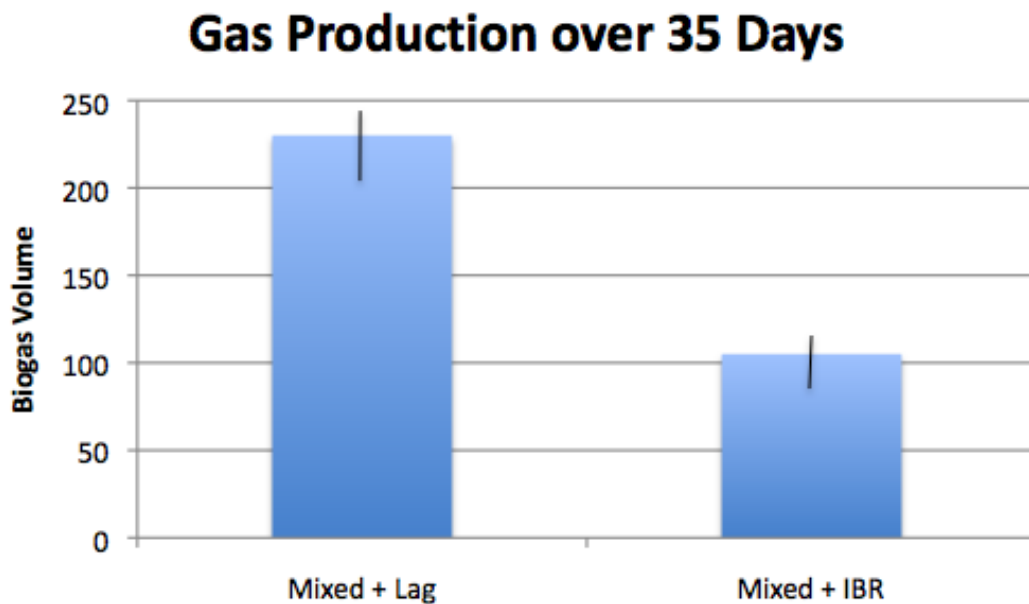

Figure 3. Total biogas production after 35 days of a mixed algae sample from the Logan Lagoons after being anaerobically digested using two different bacterial seed/inoculum (Lagoon Sludge and IBR Sludge).

When mixed culture algae that was collected from the Logan Lagoons was digested using the different bacterial seed/inoculum (Lagoon sludge or IBR sludge),

the total gas released after the 35 day digestion BMP assay with Lagoon sludge was

statistically equivalent to the total gas released from *Scenedesmus obliquus*. Similar

to the pure strain assays, when mixed algae from the Logan Lagoons was digested

with IBR sludge, a large reduction in biogas production was observed, suggesting

the IBR sludge is not as well adapted to algal digestion as is sludge from the

Lagoons.

       Table 2 presents the conversion efficiencies based on the assumption the

biogas produced was approximately 70% methane and 30% carbon dioxide (Sialve

et al., 2009). This proportion of methane in the biogas produced is in a similar range

(69-70%) has been demonstrated in numerous studies (Golueke et al., 1957; Chen,

1987; Asinari Di San Marzano et al., 1982; Samson and LeDuy 1986) regardless of

species and operating conditions for anaerobic digestion of algal biomass.

Table 2. Percentage of methane gas measured compared to theoretical methane gas production by
pure algae strains (*Chlorella vulgaris* and *Scenedesmus obliquus)* that were anaerobically digested
using two different bacterial seed/inoculum.

	Scenedesmus obliquus	*Chlorella vulgaris*
Lagoon Sludge	$35.1 \pm 7.2\%$	$25.3 \pm 2.66\ \%$
IBR Sludge	$13.4 \pm 3.7\%$	$11.9 \pm 43.36\%$

       When compared to the theoretical methane production potential,

Scenedesmus obliquus digested with lagoon sludge had an average digestion

efficiency of 35%, whereas when *Scenedesmus obliquus* was digested with IBR

sludge, the average conversion efficiency was only 13.4%. Samples of *Chlorella*

vulgaris also saw a large increase in conversion efficiency when digested with

sludge that was collected from the Lagoons. When digested with Lagoon sludge, *Chlorella vulgaris* had an average conversion efficiency of 25.3%, whereas when digested with IBR sludge, the average conversion efficiency was 11.9%. This drop in efficiency is likely due to the fact that the IBR sludge had a bacterium consortia that is acclimated to the digestion of dairy waste organics, whereas the Lagoon sludge bacterium consortia is acclimated to the degradation of algal material in the cell wall.

Because it was observed that *Scenedesmus obliquus* had a higher conversion efficiency when digested with Lagoon sludge than *Chlorella vulgaris,* this suggests that the Lagoon sludge may contain a bacterial seed population that is more capable of overcoming the recalcitrance of the call wall of *Scenedesmus obliquus* than of *Chlorella vulgaris.*

Referring to Figure 4, the biogas production over time during the 35 day digestion assay is presented. Representative first order rate constants were determined and presented in Figure 5.
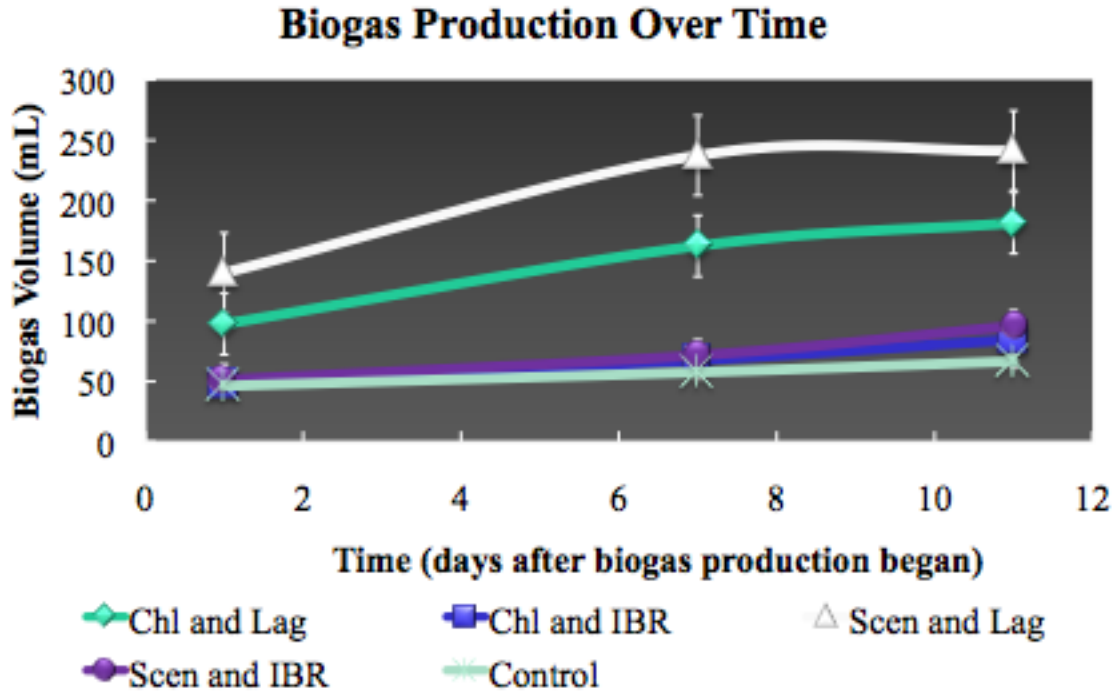
Figure 4. Rate of Biogas Production over 35 days of two different algae strains (*Chlorella vulgaris* and *Scenedesmus obliquus)*

Sample	First Order Rate Constant
Chlorella and Lagoon Sludge	0.051 day $^{-1}$
Chlorella and IBR Sludge	0.035 day $^{-1}$
Scenedesmus and Lagoon Sludge	0.053 day $^{-1}$
Scenedesmus and IBR Sludge	0.033 day $^{-1}$
Control	0.022 day $^{-1}$

Figure 5. First order rate constant for biogas production determined using rate of biogas generation for different algae & inoculum

The decomposition of the biodegradable organic fraction can be adequately described by first-order decay kinetics (Foree and McCarty, 1970). Representative first order rate constants have been reported in the range of 0.011-0.032 day $^{-1}$. The rate constants presented in Figure 3 range from 0.022 to 0.053 day $^{-1}$.

B. *Effect of Pretreatment on Anaerobic Digestion of Algae*

Figure 6 presents the total gas production of mixed culture algae samples that were collected from the Logan Lagoons and then pretreated using either dilute acid (H_2SO_4) or base (NaOH) at two different temperatures (30 degrees C and 50 degrees C)
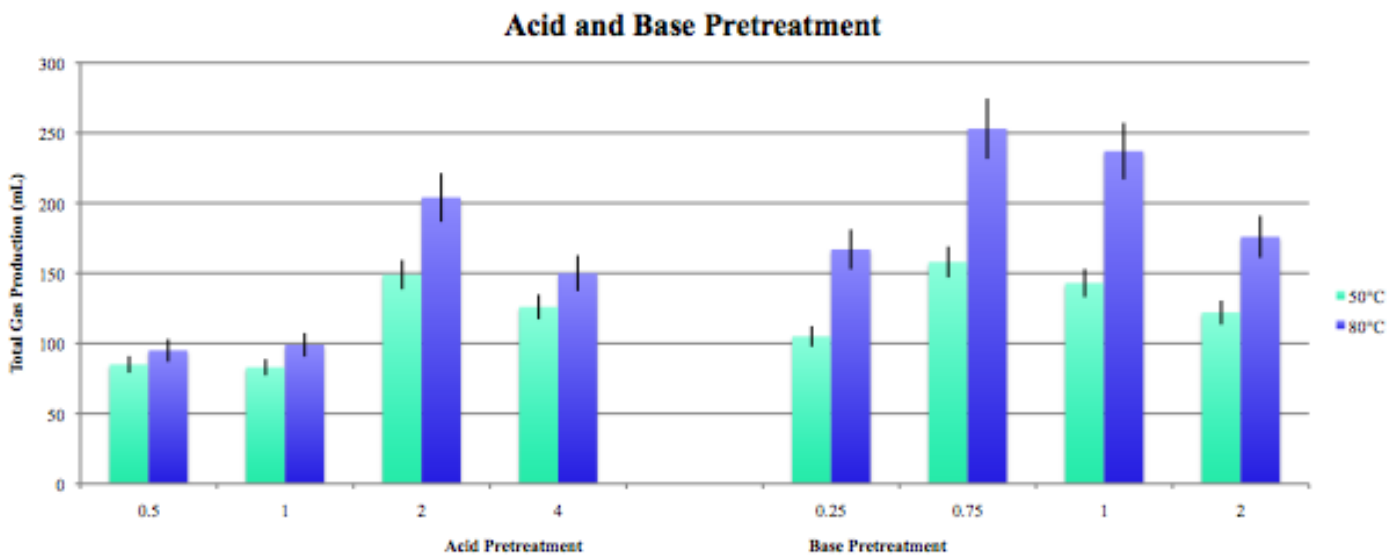

Figure 6. Biogas generated after a 35 day BMP assay of Logan Lagoon Algae following dilute acid and alkaline pretreatment.

Figure 6 shows that alkaline pretreatment is more effective than dilute acid pretreatment at both 30 degrees C and 50 degrees C in overcoming the recalcitrance of the algal cell wall of mixed algae cultures that were collected from the Logan Lagoons, allowing the methanogenic organisms increased access to degradable internal cellular components.

Figure 7 presents the total gas production of mixed culture algae from the Logan Lagoons that were not pretreated using dilute acid or base. These cultures were, however, left in neutral water at two different temperatures (30 degrees C and 50 degrees C) for 24 hours, and were then digested using Lagoon sludge in the BMP assays.
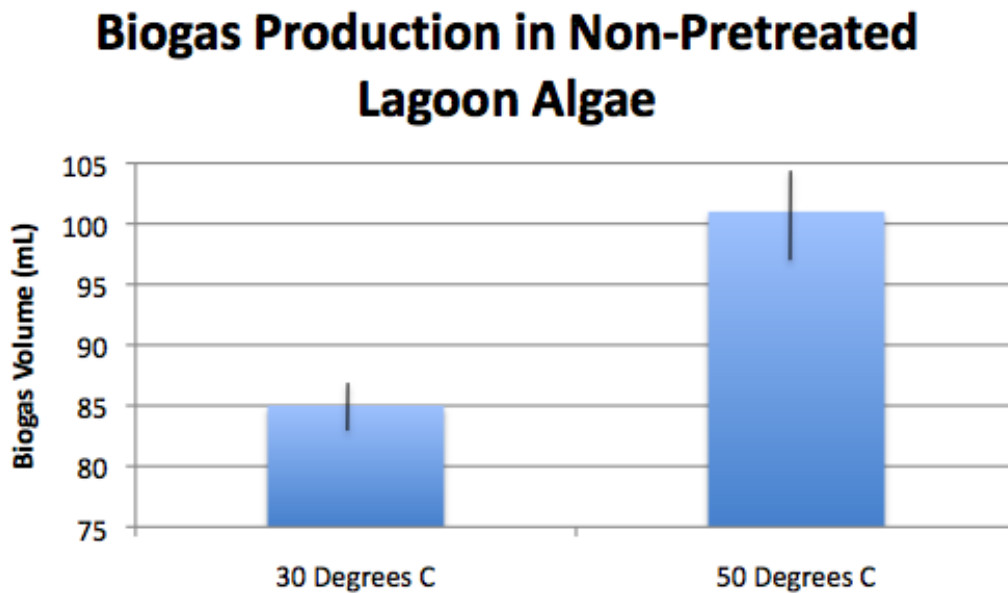

Figure 7. Total biogas produced after 35-day digestion of non-pretreated mixed culture algae from the Logan Lagoons when digested with sludge from the bed of the Logan Lagoons.

At the higher temperature, non-pretreated algae released substantially less gas than all of the alkaline pretreated samples, and also produced less gas than the algae samples that were pretreated with higher levels of acid (2% and 4% H_2SO_4).

At the lower temperature, the total amount of biogas released was also much lower than all of the alkaline pretreated algae samples, and was also lower than the algae samples digested with the higher levels of acid (2% and 4%).

Conclusion

The data presented in Figures 3-5 supports the hypothesis that BMP units inoculated with Lagoon sludge will yield higher amounts of biogas than those inoculated with dairy waste-fed IBR effluent sludge after 35 days of digestion. As expected, when the algae strains that are known to be dominant in the Logan Lagoons (*Scenedesmus obliquus and Chlorella vulgaris)* were anaerobically digested with bacterial seed that was present in sludge collected from the bed of the Logan Lagoons, substantially more biogas was released after the 35 day digestion assay that when the algae strains was digested with sludge from a dairy anaerobic digester. This suggests that the bacteria consortium present in the Logan Lagoons have become acclimated to digest the algal species common to the system.

Further, the pretreatment of mixed algal species from the Logan Lagoons was shown to enhance biogas production, presumably because the pretreatment worked to break down the cellular wall to allow the cellular components to be better accessed by the methanogenic bacteria present in the sludge.

Future Work

Pilot-scale studies should be performed to monitor the gas production and gas quality produced by algal biomass digested in larger scale anaerobic digesters that have been inoculated with sludge from the Logan Lagoons.

The effect of flocculants and coagulants should also be evaluated on the digestibility of mixed algal species that are present in the Logan Lagoons as well as on the production of methane. Processes that require metal-containing flocculants or polymer-containing flocculants should be compared with processes utilizing organic flocculants, such as chitosan or quaternary ammonium compounds (Anthony, 2012). Further, the digestibility of algal biomass that remains after a series of lipid extraction methods to produce biodiesel should be evaluated on both bench and large scale operations.

By understanding the effect of sludge inoculum and pretreatment on the anaerobic digestibility of mixed culture algae from the Logan Lagoons, the process may be optimized and better integrated into a comprehensive system for nutrient removal from the Logan Lagoons and bioproduct development. The conditions within the digester may be optimized to produce the highest quality and quantity of biomass from the algal feedstock. Any processes that occur prior to anaerobic digestion, including steps such as flocculation and coagulation and lipid extraction may be altered as to allow for maximum digestion and gas production from the resulting biomass.

Afi L., Metzger P., Largeau C., Connan, J., Berkaloff C., Rousseau B. 1996. Bacterial degradation of green microalgae: incubation of *Chlorella emersonii* and *Chlorell vulgaris* with *Pseudomonas oleovorans* and *Flavobacterium aquatile.* Proceedings of the 17th International Meeting on Organic Geochemistry. **25:** 117-130.

Amani, T., Nosrati, M., Sreekrishnan, T.R. Anaerobic digestion from the view point of microbiological, chemical and operational aspects. 2010. Environmental Reviews. **18**: 255-278.

Angenent, L.T., Khursheed, K., Marhuanna, A.H., Wrenn, B.A., Domiguez-Espinosa, R. (2004) Production of bioenergy and biochemical's from industrial and agricultural wastewater. Trends in Biotechnology. **22**: 477-485.

Anthony, R., Sathish, A., Ellis, J.T., Rahman, A., Miller, C., Sims, R.C. 2012. Effects of coagulant/flocculants on the production of biodiesel, biosolvents and bioplastic from microalgae. Algal Biomass, Biofuels and Bioproducts 2012 Conference, Sand Diego, CA. June 2011.

Asinari Di San Marzano C.M., Legros A., Naveau H.P., Nyns, E.J. 1982. Biomethanation of the marine algae *Tetraselnis.* Int. J Sustain Energy **1:** 263-272.

Atlas, Ronald M. 1946. "Handbook of Microbiological Media". (3rd ed): 289.

Bare, W.F.R., Jones, N.B., Middlebrooks, E.J. 1975. Algae Removal Using Dissolved Air Flotation. Water Pollution Control Federation, **37:** 153-169.

Becker, E.W. 1994. *In*: "Microalgae: biotechnology and microbiology" Ed. Baddiley, J. et al., 186-187. Cambridge Univ. Press. Cambridge, New York.

Borja, R., Martin, A., Sanchez, E., Rincon, B., Rasposo, F. 2005. Kinetic Modeling of the hydrolysis, acidogenic and methanogenic steps in the anaerobic digestion of two-phase olive pomace. Process Biochemistry. 40: 1841-1847.

Brown, M.R, Jeffrey, S.W., Volkman, J.K., Bunstan, **G.A. 1997** Nutritional Properties of microalgae for mariculture. Aquaculture. **151**:315-331.

Bryant, M P. Nutrient requirements of methanogenic bacteria. *In* "Advances in chemistry series Anaerobic Biological Treatment Processes. **105(1971):** 23-30.

Cecchi, F., Traverson, P.G., Mata-Alverez, J., Clancy J, Zror, C. 1988. State-of-the-art of R&D in the anaerobic digestion process of municipal solid waste in Europe. Biomass. **16**: 257-284.

Chen, P.H. 1987. Factors invluencing methane fermentation of micro-algae. PhD thesis, University of California, Berkely.

Chen, P.H., Oswald, W.J. 1998. Thermochemical treatment for algal fermentation. Environ Int. **24:** 889-897.

Christ O., Wilderer, P.A., Angerhofer, R., Faulstich, M. (2000). Mathetmatical modeling of the hydrolysis of anaerobic processes. Water Sci Technol **41:** 61-65.

Christension, L., Sims R., 2012. Rotating Algal Biofilm reactor and Spool Harvester for Wastewater Treatment with Biofuels By-Products. Biotechnology and Bioengineering. DOI 10 1002.

Dustin, J.S. 2010. Fundamentals of operation of the induced bed reactor (IR) anaerobic digester. Ph.D. dissertation. Utah State University Logan.

Ellis, Joshua T., Tramp, C., Sims, Ronald C., Miller, C. 2012. Metagenome analysis of a methanogenic community within an algae fed anaerobic digester. ISRN Microbiology. Submitted.

Golueke, C.G., Oswald, W.J., Gotaas H.B. 1957. Anaerobic digestion of algae. Appl Micriol. **5:** 47-55.

Golueke, C., Oswald, W. 1963. Power from Solar Energy via Algae-Produced Methane. Solar Energy **7(3):** 86-92.

Gujer, W., Zehnder, A.J.B. (1983). Water Sci Technol **15:** 127-167.

Gunaseelan, V. Nallathambi. 1997. Anaerobic Digestion of Biomass for Methane Production: A Review. Biomass and Bioenergy **13:** 83-114.

Illman, A., Scragg, A., Shales, S. 2000. Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb Technol. **27:** 631-635.

Janczyk, P., Franke, H., Souffrant, W.B. 2007. Nutritional value of *Chlorella vulgaris:* effects of ultrasonication and electroporation on digestibility in rats. Anim Feed Sci Technol. **132:** 163-169.

Karakashev, D., Vatstone, D.J., Angelidaki, I. Influence of Environmental Conditions of Methanogenic Compositions in Anaerobic Biogas Reactors. Applied and Environmental Microbiology. 2005. 71: 1, 331-338.

Leadbeater, B.S.C. 2006. The "Droop-Equation"- Michael Droop and the legacy of the "cell-quota model" of phytoplankton growth. Protist. **157(3**):345-358.

Malina, F.J., Pohland, F.G. 1992. Design of anaerobic process for the treatment of industrial and municipal wastes. Water Quality Management Library. **7.**

Mata-Alverez, J. 2003. Biomethanization of the organic fraction of municipal solid wastes.

Okuda, K. 2002. Structure and phylogeny of cell coverings. J Plant Res. **115:** 283-288.

Olguin, E.J. 2003. Phycoremediation: key issues for cost-effective nutrient removal process. Biotechnol. Adv. **22:** 1-91.

Ostrem, K. 2004. Greening Waste: Anaerobic Digestion for Treating the Organic Fraction of Municipal Solid Wastes. Earth Engineering Center Columbia University.

Oswald, W.J., Golueke, C. 1960. Biological Transformation of Solar Energy. Advances in Applied Microbiology. **2:** 223-262.

Owen, W., Dtucky, D., Healy, J., Young, L., McCarty, P. 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Research. **13:** 485-492.

Pavlostathis, S.G., Gosset, J.M. 1985. Alkaline treatment of wheat straw for increasing anaerobic biodegradability. Biotechnology and Bioengineering. **27:** 334-344.

Parkin, G.F., Owen, W.F. (1986). Fundamentals of Anaerobic Digestion of Wastewater Sludges. ASCE, Journal of Environmental Engineering Division. **122:** 867-920.

Richards, B.K., Cummings, R.J., Jewell, W.J. and Herndon, F.G. 1991. High solids anaerobic methane fermentation of sorghum and cellulose. Biomass and Bioenergy. **1**: 47-53.

Rubio-Loza, L.A., Noyola, A. Two-phase (acidogenic-methanogenic) anaerobic thermophilic/mesophilic digestion system for producing Class A biosolids from municipal sludge. Bioresouce technology. Zs 101 (2): Jan 2010: 576-585.

Samson, R., LeDuy, A. 1986. Detailed study of anaerobic digestion of *Spirulina maxima* algae biomass. Biotechnol Bioeng. **28:** 1014-1023.

Schulze, K.L., 1958. Sludge digestion and methane fermentation, Part 1. Increase solids concept. Sewage and Industrial Wastes. **30**: 28-33.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances. 2009. **27**:409-416.

Stewart, D.J., Bogue, M.J., Badger, B.M. 1984. Biogas production rom crops and organic wastes. 2. Results of continuous digestion tests. New Zealand J. Sci., **27:** 285-294.

Symons, G.E., Buswell, A.M. 1933. The methane fermentation of carbohydrates. J Am Chem Soc. **55:**2028-2036.

Thauer, R.K. Biochemistry of methanogenesis: a tribute to Marjory Stephenson. Microbiology. 1998. 144: 2377-2406.

Visser, A., Gao, Y., Letting A.G. Effects of pH on methanogenesis and sulphate reduction in thermophilic UASB reactors. Bioresource technology. 1993: Vol 44 (2): 113-121

Yang, S.T., Okos, M.R. Kinetic Study and Mathematical Modeling of Methanogenesis of Acetate Using Pure Cultures of Methanogens. Biotechnology and Bioengineering.. 30: 5, 1987: 661-667
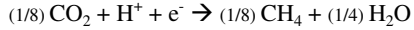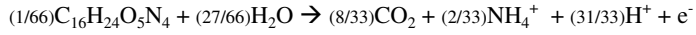
Zeikus, J.G. 1980. Microbial Populations in digesters. *In* "Anaerobic Digestion" (Eds. D.A. Stafford et al.) Applied Science Publishers Ltd. London. 61-87.

Zinder, S.H., Anguish, T., Cardwell, S.C. Effects of Temperature on Methanogenesis in a Thermophilic Anaerobic Digestor. Appl Environ Microbiol. 1984. 47(4): 808-813.

APPENDIX 1: Methane Potential Calculations:
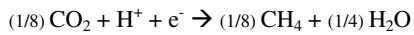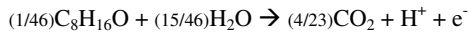
a. Cellular Component Potential

Proteins:

$(1/66)C_{16}H_{24}O_5N_4 + (27/66)H_2O \rightarrow (8/33)CO_2 + (2/33)NH_4^+ + (31/33)H^+ + e^-$

$(1/8) CO_2 + H^+ + e^- \rightarrow (1/8) CH_4 + (1/4) H_2O$

 = 8.25 mol CH$_4$ / mol protein   Molecular Weight = 352 g/mol

Lipids:

$(1/46)C_8H_{16}O + (15/46)H_2O \rightarrow (4/23)CO_2 + H^+ + e^-$

$(1/8) CO_2 + H^+ + e^- \rightarrow (1/8) CH_4 + (1/4) H_2O$

= 5.75 mol CH$_4$ / mol lipid   Molecular Weight = 128 g/mol

Carbohydrates:

$(1/4)CH_2O + (1/4)H_2O \rightarrow (1/4)CO_2 + H^+ + e^-$

$(1/8) CO_2 + H^+ + e^- \rightarrow (1/8) CH_4 + (1/4) H_2O$
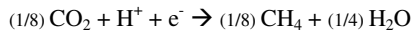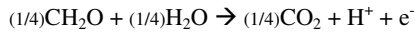
= 0.5 mol CH$_4$ / mol carbohydrate   Molecular Weight = 30 g/mol

b. Methane Yield For *Chlorella*

Proteins = 56%   Lipids= 12 %   Carbohydrates = 19%

c. Methane Yield for *Scenedesmus*

Proteins = 53%   Lipids = 13%   Carbohydrates = 15%