Investigating the use of anaerobic fungi to enhance the hydrolysis of lignocellulose in lab scale biodigesters

by

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Abstract

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This thesis investigated hydrolysis of lignocellulosic biomass using bioaugmentation with rumen isolated anaerobic fungi (AF). Hydrolysis of corn silage was compared with hydrolysis of common reed using three different genera of AF. Based on production of H₂ and CO₂, accumulation of volatile fatty acids and pH change, low levels of hydrolytic activity were observed in all treatments, possibly due to poor survival of AF in experimental bioreactors. The presence of background microflora during hydrolysis was a confounding factor in assessing the efficacy of AF. In a follow up study, AF were inoculated into sterilized corn silage to remove background microbial activity. In addition to the chemical parameters measured as markers of hydrolysis, hydrolytic enzyme activity was also assessed. However, no significant improvement in hydrolysis was observed regardless of the fungal genera used, again suggesting poor survival of AF in bioreactors.
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<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>AD</td>
<td>Anaerobic digestion</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid detergent fiber</td>
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<tr>
<td>ADL</td>
<td>Acid detergent lignin</td>
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<td>AF</td>
<td>Anaerobic fungi</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BMP</td>
<td>Bio methane potential</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EBA</td>
<td>European biogas association</td>
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<tr>
<td>GHG</td>
<td>Greenhouse gas emissions</td>
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<tr>
<td>H₂</td>
<td>Hydrogen</td>
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<tr>
<td>IPCC</td>
<td>Intergovernmental panel on climate change</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fibre</td>
</tr>
<tr>
<td>NGS</td>
<td>Next generation sequencing</td>
</tr>
<tr>
<td>SSO</td>
<td>Source separated organic waste</td>
</tr>
<tr>
<td>TS</td>
<td>Total solids</td>
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<tr>
<td>TWh</td>
<td>Terawatt hour</td>
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<tr>
<td>VFA</td>
<td>Volatile fatty acids</td>
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<td>VS</td>
<td>Volatile solids</td>
</tr>
<tr>
<td>WBA</td>
<td>World bioenergy association</td>
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1 Chapter - Literature Review - Potential of anaerobic fungi to enhance the hydrolysis of lignocellulose in lab scale biodigesters

1.1 Introduction

Fossil fuels are not renewable and will be depleted if their use continues. They were formed millions of years ago when organic matter was buried under the Earth’s surface. The current global energy system is dominated by fossil fuels and the emissions from them could lead to a further increase in atmospheric greenhouse gas emissions to 600 ppm CO₂ (Edenhofer et al., 2011). A study conducted in 2010, found a shift in the polar vortex that is leading to ice loss in the Arctic ocean due to changes in climatic conditions (Zhang and Zhao, 2010). According to IPCC report by Edenhofer et al. (2011), due to a very rapid economic growth there is an increase in global population and this has led to a rapid change in economic structure. The above factors suggest the need for a shift to renewable sources of energy for future global energy production. Renewable energy refers to energy that is produced without using coal, natural gas or oil.

Renewable energy is clean and reduces our dependence on fossil fuels. The primary renewable energy sources that are available today are solar, wind and hydroelectric power. Biogas produced by biological degradation of organic matter is a viable fourth option (World energy resources, 2016). According to International gas union report (2015), the global substrate potential for biogas production is up to 10,000 TWh and the total production of biogas in the world is estimated to be about 300 and 400 TWh (WBA fact sheet, 2013).
1.2 Importance of biogas

Biogas is produced by anaerobic digestion (AD) by utilizing organic wastes, which has been evaluated as one of the best bioenergy production system for being energy efficient and beneficial to the environment (Merlin Christy et al., 2014). The bacteria occurring inside the AD drives this production. Organic wastes contain complex polymers like carbohydrates, fats and protein. They are in turn broken down into sugars, shorter chain lipids and amino acids by microorganisms and converted to biogas (Weiland, 2010). Biogas is comprised of methane, carbon dioxide and trace amounts of other gases such as oxygen, nitrogen, hydrogen, hydrogen sulphide and carbon monoxide (Rasi et al., 2009). Methane is renewable and can be used as a fuel. However, the methane concentration varies depending on the type of substrate used during the AD process. Apart from providing energy, some anaerobic digestion methods help reduce zoonotic pathogens in livestock (Massé et al., 2011). The effluent from anaerobic digesters can also be used as an organic fertilizer as it contains all essential nutrients (nitrogen (N), phosphorous (P) and potassium (K)) required for the growth of plants (Alfa et al., 2014).

1.3 Overview of Biogas production steps

Biogas fermentation takes place in four steps:

1.3.1 Hydrolysis

This process starts when complex molecules such as proteins, fats and carbohydrates are broken into smaller molecules such as amino acids, fatty acids and simple sugars through the action of extracellular enzymes produced by hydrolytic bacteria such as *Cellulomonas*. These enzymes include cellulosases, hemicellulloses, amylases, lipases and protease (Vavilin
et al., 2011). Hydrolysis of complex substrates can be a rate limiting step in the overall AD process (Pavlostathis et al., 1991).

1.3.2 Acidogenesis

In the second step, simple sugars, amino acids and lipids are further broken down through the action of acidogenic bacteria, such as *Pseudomonas* and *Bacillus*, into short chain organic acids such as butyric acid, propionic acid, acetic acid, formic acid and valeric acid along with alcohols and trace amounts of hydrogen, ammonia and carbon dioxide (Bajpai, 2017).

1.3.3 Acetogenesis

Acetogenic bacteria such as *Syntrophomonas* and *Syntrophobacter* convert the short chain organic acids that are produced in the previous step to acetic acid and hydrogen. It is almost always difficult to distinguish between acidogenesis and acetogenesis reactions as they occur simultaneously. The hydrogen that is produced in this step and those above serve as the substrate for methanogenesis (Bajpai, 2017).

1.3.4 Methanogenesis

The final step takes place due to methanogens. They belong to a special group called Archaea. They are very sensitive to parameters such as pH, toxicity due to ammonia and even small amounts of oxygen. Methanogens have the longest generation times (about 2-25 days), hence they are often the rate-limiting step for easily hydrolyzed substrates (Teghammar, 2013). Methane producing archae are subdivided into three groups: Acetoclastic methanogens that split acetate to make methane and carbon dioxide. Hydrogenotrophic methanogens that combine hydrogen and carbon dioxide to make
methane. The third group, methylotrophic methanogens are able to make methane from methanol (Gerardi, 2003; Paul and Liu 2012).

1.4 Anaerobic digestion substrates

Biogas can be produced from a variety of feedstocks provided they contain biodegradable organic matter. The methane yield depends on the type of feedstock used, digester system and the retention time. The common feedstock used for AD is manure from livestock. The energy content in manure is low compared to other substrates because the animal has already predigested the substrate material (Kopysova et al., 2013). Due to their high buffering capacity, neutral pH, wide range of nutrients and micronutrients, manures are usually used to co-digest with energy crops to improve biogas production (Weiland, 2010). By using manure as a feedstock in anaerobic digesters in rural areas, the nutrients in the manure are preserved, pathogens are reduced, and emissions of greenhouse gases are reduced. The importance of manure has hence increased awareness in proper storage and utilization of livestock manure.

Energy crops co-digested with livestock manure are commonly used for biogas production. The most common energy crop used in biodigesters is corn (Amon et al., 2007). There are also other potential energy crops available for AD, but they are not widely used in biodigesters. However, they have low fertility requirements and low energy costs for planting and harvesting. Some examples include sorghum, napier grass (Wilkie et al., 1986). Corn is harvested, chopped and usually ensiled to preserve it prior to placement in an anaerobic reactor for producing biogas. In a conventional reactor, substrate is fed depending on the type, operating conditions as well as the system design (Gerardi, 2003).
The retention time of the reactors again depends on the substrate itself. Some digesters that feed highly lignocellulosic material have a longer retention time because the material takes a longer time to break down. Reactors that use lignocellulosic material as substrate almost always try to shorten the retention time by performing a pretreatment process so that more substrate can be fed to the reactors and hence more biogas produced.

1.5 Lignocellulose composition and structure

Lignocellulose is the major component of biomass. It makes up half of the matter produced by photosynthesis and in nature it is derived from wood, grass, agricultural residues, forestry wastes and municipal solid wastes (Peréz et al., 2002). It is estimated that the world's annual production of lignocellulosic biomass is around 1x10^{10} million tonnes (Zhang and Zhao, 2010; Sanchez et al., 2008), making it the most abundant renewable biomass resource and the major constituent of solid organic waste.

According to a Canadian Biogas study, December 2013 (http://www.biogasassociation.ca/bioExp/images/uploads/documents/2013/resources/Canadian_Biogas_Study_Summary.pdf), agricultural sources represent 68% of the total biogas opportunity in Canada (Fig. 1.1).
Due to the structure of lignocellulosic substrates, they exhibit low degradability (40–60%) in anaerobic digesters. Lignocellulose is primarily made up of long cellulose fibers reinforced by hemicellulose and covered by lignin. Among them, cellulose is the main component and its structure is formed by a beta (1-4) linked chain of glucose molecules. Hemicellulose, the second component of lignocellulose, is composed of various 5- and 6-carbon sugars, which include arabinose, glucose, galactose, mannose and xylose. The third component, lignin is made up of three major phenolic components: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fliegerova et al., 2012). The complex structure of
lignocellulose plays a major role in the efficiency of the degradation process and the conversion of biomass into biogas.

While microbes can degrade cellulose and hemicellulose, lignin prevents the hydrolysis of polysaccharide fibers and cannot itself be decomposed by common anaerobic microorganisms (Cirne et al., 2005; Lyna et al., 2002). The lignocellulolytic structure of plant tissues decreases the effectiveness of the hydrolytic phase of biogas production, making it the rate-limiting step in biogas production. However, the efficiency of natural AD processes can be higher than those observed in anaerobic digesters. For example, AD systems are only 20% efficient at degrading organic matter in comparison to the rumen of ruminant animals (Nair et al., 2005).

1.6 Rumen versus AD system

Ruminants rely on the rumen for digestion because they eat plants, which are made up of lignocellulose. The rumen environment can be considered a natural anaerobic digester containing a complex microbial community of bacteria, protozoa and fungi. The microorganisms in the rumen co-exist in a symbiotic relationship that helps degrade complex plant components. The rumen environment is a better and unique system compared to an anaerobic digester because substrate fermentation takes place by combining both homogenization and stratification. The rumen contains a complex microbial consortia consisting of active cellulosytic bacteria such as *Fibrobacter succinogenes*, which have a positive impact on plant degradation (Shinkai et al., 2009). Other microbes, like rumen protozoa such as *Dasytricha, Diplodinium* are responsible for about 30-40% fiber digestion. Fungi can initially colonize substrate using rhizoids, leading to disintegration of the plant cell wall. The main function of the rumen is to generate a
constant supply of VFA that are absorbed across the rumen wall into the blood stream walls (Bayané and Guiot, 2011).

The efficiency in degrading the plant substrates is almost three times higher in rumen than in AD. This increase in efficiency is because the digestive system rumen results from an organized action of various enzymes such as esterases, lignanses, cellulases combined with physical and chemical actions (Bayané and Guiot, 2011). Further there is a lack of constant removal of VFA, which can lead to an increased accumulation of acids and thus a decrease in pH that can inhibit the AD process (Dehority et al., 2002).

1.7 Substrate pretreatments for AD systems

To improve the efficiency of anaerobic digestion, different pretreatment methods are available. By increasing the accessible surface area and pore size of the substrate, microbes are able to reach the dense carbon complex and thereby results in more effective digestion. Pretreatment is done through physical, chemical, physiochemical or biological means, with the processes applied individually or in combination. These pretreatment methods have been described in detail by others (Hendricks and Taherzadeh and Karimi, 2008; Zeeman, 2009) with each method having advantages and disadvantages.

The main aim of an ideal pretreatment is to cause effective delignification and minimize energy demands and cost requirements on the operational process of biodigestion (Sindhu et al., 2016). Firstly, physical pretreatment uses various methods like milling and irradiation such as gamma ray, electron beam and microwave irradiation that help increase the surface area, pore size and thereby reducing the crystallinity of cellulose. However, the drawback of this pretreatment method is that it requires increased amounts of energy and not necessarily would be able to remove lignin. Secondly, both chemical and
physiochemical pretreatments such as steam explosion, sodium hydroxide, sulphuric acid are commonly used for industrial applications and could improve accessible surface area that can lead to partial to complete lignification. However, their main disadvantage is that they require harsh chemicals and disposing of these chemicals after the treatment process can be expensive (Taherzadeh and Karimi, 2008). Finally, biological pretreatment processes are carried out using microorganisms and their enzymes. The process help cause delignification as well as improve the accessible surface area leading to partial hydrolysis of the plant substrate. The treatments require low energy consumption and cause minimal environmental impact; however, they have a low treatment rate compared to physiochemical methods. Dhiman et al. (2015) were able to pretreat willow and rice straw using a cocktail of hydrolytic and oxidizing enzymes from a fungal consortium, eliminating the need to use hazardous chemicals. Similarly, more and more microorganisms such as white and soft-rot fungi have been tested for pretreatment processes and to find a better solution with less environmental impact and lower energy demands for operational requirements.

1.7.1 Advantages of using biological pretreatment

The hydrolysis step is the rate-limiting step in the production of biogas. There are two ways to perform biological pretreatment. First method is by extracting enzymes from different microbes and adding them to the anaerobic system. A study conducted by adding enzymes such as cellulase and β-glycosidase prior to AD to mixed sludge has shown to improve the methane production by 15% (Parawira et al., 2012). The second method is by pre-treating the substrates with cellulolytic or lignin degrading microbes either prior to or in the digester. Various studies have been conducted on the latter. One example is pre-treating
rice straw with white rot and brown rot fungi. In this process, 1.2 – 1.4 mg (dry weight) of mycelia mat was added to an anaerobic digester of 5-liter capacity. This helped improve methane production by 46% for rice straw treated with white rot fungi and 31% for brown rot fungi (Teghammar, 2013). Another example was by batch digesting and bio-augmenting the whole seed culture of *Clostridium cellulyticum* with wheat straw. The process gave a BMP of 326.3 mL g\(^{-1}\) VS, an increase of 7.6% compared to no-bioaugmented BMP which is 303.3 mL g\(^{-1}\) VS (Peng et al., 2014).

### 1.7.2 Limitations in performing biological pretreatment with available microbes

The disadvantage with current biological pretreatment processes is that they require an aerobic pretreatment with the available microbes. This increases the cost of biogas production. Therefore, introducing an anaerobic microbe such as the anaerobic fungi (AF) from the ruminant animals would theoretically reduce costs as compared to an aerobic pretreatment (Gruninger et al., 2014).

### 1.8 Anaerobic fungi and their important function in the rumen

#### 1.8.1 The discovery

AF belongs to the phylum *Neocallimastigomycota*: class Neocallimastigomycetes; order Neocallimastigales assigned under the kingdom fungi. They are closely related to the chytrids. The phylum currently is comprised of eight genera; *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces, Piromyces*, and two newly formed phyla *Oontomyces and Buwchfawromyces* (Callaghan et al., 2015; Dagar et al., 2015). They are distinguished from each other based on their morphological features such as

a. Thallus morphology, the vegetative part of the fungus whether bulbous like as in *Caecomyces* sp. or filamentous as in *Piromyces* sp;
b. Zoospore flagellation (the flagellum is an external appendage that aids in the movement of the fungal zoospores) - They can be divided into uniflagellate or polyflagellate like the Neocallimastix.

c. Based on the number of sporangia; monocentric i.e., single reproductive body like Piromyces or polycentric like Anaeromyces (Gruninger et al., 2014).

Earlier in taxonomical classification there were disagreements on the status of the former six genera and their sub generic classification due to the difficulties associated with the long-term maintenance of fungal cultures. With the advent of molecular taxonomy, DNA sequence comparison and phylogenetic reconstruction increased the ability to differentiate among various taxa. Initial phylogenetic studies of AF were based on sequencing the 18S region, a highly conserved region of rRNA. However, this conserved region contains little phylogenetic information for subgenera classification (Sahare, 2013). Later, the internal transcribed spacer region (ITS) was used to distinguish between closely related taxa. The ITS regions are basically a piece of non-functional RNA situated between structural rRNA (Sahare, 2013). The various regions of a rRNA gene is illustrated in Fig. 1.2.
Fig. 1.2 Internal transcribed spacer region (Adapted from Sahare, 2013)

The ITS regions are widely used for classifying AF. The advantages of using ITS primers are to study the closely related AF taxa and for knowing their ecological behavior. Unfortunately, the problem associated with the ITS region is the high diversity of sequences within individual isolates. Consequently, this has hindered the widespread use of ITS sequencing for taxonomic studies (Gruninger et al., 2014). Using this technique, Koetschan et al. (2014) were able to revise the whole AF taxonomy by proposing a common secondary structure of the ITS1 region specific to AF. Also, this technique will help in discovering many new genera of AF.
1.8.2 Life cycle

Anaerobic fungi reproduces through asexual reproduction. In the rumen, zoospores are released from anaerobic fungal sporangia in response to the induction signal released from ingested plant material. The sporangium is filled with uninucleated zoospores, which are formed through cytokinesis. When the sporangial wall ruptures and the zoospores are released, they show chemotaxis towards several carbohydrates including glucose, sucrose and move across the plant surface prior to encysting. After release they encyst, and a germ tube is formed from which rhizoids emerge. Until this step, the life cycle is the same for both monocentric and polycentric genera (Fig. 4). The next step differs, in monocentric fungi where the nucleus remains within the cyst that enlarges to form sporangium. As a result, these rhizoids remain anucleated in contrast to polycentric fungi where nuclei migrate into the rhizoid system and form multiple sporangia on thallus (Gruninger et al., 2014).
Fig. 1.3 Life cycle of anaerobic fungi (Gruninger et al., 2014)
1.8.3 Ecology

Anaerobic fungi have been reported in all geographic regions around the world. They are present in animals that have a dedicated digestive chamber with long resident times and having closer to neutral pH environment. However, they are very common among the foregut fermenters and ruminants such as cattle and marsupials including kangaroos. These fungi have also been isolated from the fecal samples of horses, which are hindgut fermenters. In the rumen ecosystem the amount of AF represents $10^3$-$10^5$ zoospores/mL (Kamra., 2005). Interestingly, they are absent in strict herbivorous mammals lacking foregut and hindgut fermentation chambers such as the panda due to the simplicity of their alimentary tract. By using pyro sequencing technique (a DNA sequencing method) it was found that animal host phylogeny played an important role in determining the composition of the anaerobic fungal community (Gruninger et al., 2014)

1.8.4 Anaerobic fungal culturing

Anaerobic fungi can be isolated from rumen liquid and feces from ruminants. The AF are cultured using the Hungate roll tube technique and there are many anaerobic media available to culture them with the most important ingredient in the medium being cell–free rumen fluid, vitamins, volatile fatty acids, micro and macro nutrients (Lowe et al., 1985). A reducing agent such as L-cysteine and resazurin is also added while preparing the medium that acts as an indicator for the presence of oxygen and turns pink when exposed to air. This indicator helps us observe whether the media that we are working with is anaerobic and favorable for the growth of AF. An anoxic gas supply such as CO$_2$ or H$_2$ is required while culturing. During the culturing process gas is passed through a heated copper column that is connected to a gassing nozzle. The copper column is not required if
high purity gas is used. Through the stainless steel nozzle the gas is injected directly into the medium in order to eliminate the dissolved oxygen. Fig. 1.4 illustrates two stainless steel nozzles; one is injected into pure cultures and other to media. A pasteur pipet can be used to transfer a portion of the anaerobic fungal biomass into fresh medium. Finally, it is sealed using rubber stopper and incubated at 39 °C.

Fig. 1.4 Anaerobic fungi culturing set-up, picture taken while culturing AF in the rumen microbiology laboratory at the Agriculture and Agri-Food Canada, Lethbridge Research Centre.

1.8.5 Importance in fiber digestion

The role of AF in degradation of plant fiber has been studied extensively. These fungi are better at penetrating plant tissue than bacteria or protozoa (Orpin et al., 1994). Such penetration leads to more complete degradation of plant fiber that enters the rumen, however, not necessarily faster due to the slow growth rate of AF. Solubilization of lignin
in the plant cell walls is an important characteristic of AF. Zoospores of many species appear to colonize lignin containing tissues and penetrate through the cuticle barrier, a process that other rumen microbes are incapable of performing. Hence, a greater ability of AF to weaken forage fiber may be important in enhancing forage utilization of the host animal (Akin et al., 1983).

1.8.6 Hydrogenosome

When considering aerobic members of the kingdom fungi they have mitochondria that help in their cellular energy production. Although AF do not have any mitochondria, they do possess hydrogenosomes. Hydrogenosomes are amorphous globules consisting of an electron dense matrix enclosed by a double membrane. They typically do not have a fixed shape except occasionally finger like projections are noted (Müller et al., 1993). When such projections are present they are oriented towards the kinetosomes, which are the basal body associated with the formation of flagella. Since hydrogenosomes are in close proximity to the flagella, their primary function is thought to provide energy to support motility in the form of Adenosine triphosphate (ATP). The hydrogenosome contains the enzyme hydrogenase to help produce hydrogen, CO₂, formate and acetate as metabolic waste products. These along with lactate and ethanol are the main fermentation end products produced by fungal degradation and fermentation of plant cell wall polysaccharides.

1.8.7 Hydrolytic enzymes

Anaerobic fungi are one of the initial colonizers of lignocellulose in the rumen and play an important role in plant digestion with their rhizoids, exposing regions of the plant cell wall that can be exploited by other microbes such as bacteria and protozoa. They produce a wide range of hydrolytic enzymes. Among these enzymes, feruloyl and p-coumaroyl esterases
have a major role in cleaving ferulic and coumaric acid from lignin/ hemicellulose complexes. Consequently, they have considerable significance in the dissolution of plant cell walls. These enzymes are not capable of degrading lignin; however, it has been suggested that lignin may be partly solubilized by these organisms (Sánchez et al., 2009). Anaerobic fungi are the only known members of the kingdom fungi possessing cellulosomes. Cellulosomes are a multi-enzyme cellulolytic complex that is present on the surface of AF. These enzymes play a major role when AF come in contact with a plant substrate by binding themselves to the cellulose and hemi-cellulose and thereby enabling them to hydrolyze these substrates. They can also be described as the nature’s most detailed nano machine that helps deconstruct the plant cell via carbohydrate binding molecule (Fontes and Gilbert, 2010).

1.8.8 Anaerobic fungi interaction with rumen microbes

Interaction among rumen microbes can be positive, negative or neutral depending on the microbe and the feed type. Anaerobic fungi produce considerable amounts of hydrogen, with methanogens being the principal utilizers of hydrogen in the rumen. Co-culturing of AF and methanogens results in increased fungal biomass and cellulose degradation in vitro (Fonty and Joblin, 1991). However, on the other hand co-culturing of AF with rumen bacteria such as Ruminococci produces a thermo liable protein that inhibits the growth of fungi. Also, co-incubation of AF with protozoa decreases the fungal population. Small sized zoospores are preyed upon by protozoa that are capable of ingesting and digesting fungi (Nagpal et al., 2010).
1.9 Potential Advantages of bioaugmenting AF in AD system as a pretreatment step

Anaerobic fungi are well equipped with enzymes for fermentation. Hence many studies are being conducted to attempt to increase biogas yield using AF. A study conducted in 2012 used three different reactors; batch, fed batch and semi-continuous reactors, different substrates; cellulose, maize and grass silage and inoculated with different genera of AF; *Anaeromyces* sp., *Orpinomyces* sp., and *Piromyces* sp. All experiments showed an increase in biogas production by 4-22%, depending on the substrate and AF used (Prochàzka et al., 2012). Another experiment conducted in 2015, in two stage AD system used two different substrates, cattail and corn silage, bioaugmented with *Piromyces rhizinflata* YM600 and found an initial increase of methane and hydrogen yield (Nkemka et al., 2015). However, both these studies showed that AF die off after 10 days of incubation and this leads to a decrease in enzymatic activity required to perform the hydrolytic step. Therefore, it is necessary to perform a pre-hydrolysis stage separately in lab-scale biodigesters using AF before being implemented into conventional biogas reactors. In this study the same was attempted.

1.10 Research hypothesis

**Hypothesis 1**: Experiment 1 was based on the hypothesis: Bio-augmenting AF into batch reactors using two different types of agricultural substrates will promote the degradation of lignocellulosic components.

Objectives:

1. To observe the effect of hydrolysis on corn silage and common reed by bioaugmenting with three different AF using batch digestion.
2. To analyze the gas concentration and composition that arises from the fermentation of these substrates during hydrolysis.

3. To compare the various chemical changes that the substrates have undergone during hydrolysis.

**Hypothesis 2:** Experiment 2 was based on the knowledge gaps from experiment 1: Bioaugmenting AF with heat-sterilized corn silage will help us understand whether hydrolysis had occurred due to AF or due to background micro flora within corn silage.

Objectives:

1. To study the effect of bioaugmentation with two different rumen fungal species on the hydrolysis of heat-sterilized corn silage using batch digesters.

2. To study the enzymatic activity of the AF during hydrolysis. This in turn would help us understand their activity inside lab-scale biodigesters after bioaugmentation.

3. To analyze the gas concentration and composition that arises as result of substrate hydrolysis.

4. To compare the various chemical changes in heat-sterilized corn silage as a result of hydrolysis that have occurred using AF.
2 Chapter - Effect of bioaugmentation with anaerobic fungi isolated from ruminants on the hydrolysis of corn silage and common reed

2.1 Introduction

Anaerobic digestion is a microbial driven process that results in the conversion of organic feedstocks to methane rich biogas, which can be used as a source of renewable energy. Different types of feedstocks can be used in AD, ranging from agricultural residues and purpose grown crops to various organic waste streams (Torquati et al., 2014). Anaerobic digestion of agricultural biomass has both environmental and economic benefits for farmers.

Corn (Zea mays L.) silage constitutes a major feedstock for biogas production in many parts of the world. For example, in Europe more than 17,000 biogas plants utilize corn silage as the main feedstock (Hutňan, 2016) out of 17, 376 biogas plants (EBA, 2017). During the ensiling process, organic acid (i.e. lactic acid) production from microbial activity reduces silage pH below 4.5, resulting in preservation and mild hydrolytic pretreatment of the biomass. Estimated biogas yield from corn silage based on the experience in Europe is 180 m$^3$ t$^{-1}$ fresh material (OMAFRA, 2016). While corn silage is the most widely used energy crop for biogas production, there is interest in using other sources of plant biomass for environmental, economic, and societal reasons (Fletcher et al., 2011). For example, Phragmites australis (Cav.) Trin. ex Steud. (common reed) is a perennial invasive wetland plant species in North America that produces substantial quantities of biomass of up to 30 t ha$^{-1}$ y$^{-1}$ (Baute et al., 2016). Although abundant, common reed currently has no economic value in North America and control efforts to mitigate its
invasion of wetlands, waterways, and roadsides are expensive. Utilization of common reed biomass for biogas production may present a way to both help control spread of the plant and to produce renewable energy. While all parts of the common reed can be used for both biogas and biofuel production (Wichmann and Wichtmann, 2009), the estimated biogas yields reported in the literature are only 150 L kg\(^{-1}\) volatile solids (VS) of fresh material compared to grass and pig manure that yield more than 280 and 340 L kg\(^{-1}\) VS, respectively (Akula, 2013; Baute et al., 2016). The issues of low degradability and poor conversion to biogas are also applicable to other potential energy crops, such as Miscanthus and Arundo donax L (Brosse et al., 2012; Lemons e silva et al., 2015). Extensive research has been conducted on various physical, chemical, and biological pretreatments that could be used to increase biogas yield from lignocellulosic sources (Taherzadeh and Karimi, 2008).

In nature, one of the most efficient systems for unlocking the energy found in lignocellulosic substrates is the rumen of animals such as cattle and sheep. While the stepwise fermentation process (hydrolysis, acidification, acetogenesis, methanogenesis) that occurs during AD shares digestive process in common with the rumen. However, no bio digester system that has been developed so far has reached to that of the rumen digestive performance (Bayané and Guiot, 2011). One reason for this reduced efficiency of AD likely lies in differences in the microbial populations between these two fermentation systems. The anaerobic digestive system of the rumen has been extensively studied and AF are known to be involved in the digestion of the most recalcitrant lignocellulose within the rumen (Bauchop, 1979). Anaerobic fungi use rhizoids to physically penetrate and disrupt the lignin layer of lignocellulose, while also enzymatically degrading plant cell walls using a diverse suite of extracellular hydrolytic enzymes, including cellulases, hemicellulases,
pectinases, and phenolic acid esterases (Wei et al., 2016). Some of the extracellular hydrolytic enzymes produced by these organisms are freely released into the milieu; others are bound to the cellular surface as components of multi-enzyme cellulosomes (Dollhofer et al., 2015). Using feruloyl esterase activity, AF cleaves the bonds between hemicellulose and lignin increasing the access of microbial enzyme to hemicelluloses. Although AF are known to degrade lignin, they do not utilize the lignin themselves (Akin and Benner, 1988). While AF are known to play an essential role within the rumen, their presence, abundance, and activity level in AD is currently unknown.

Bioaugmentation involves adding specific microorganisms into a system or process in order to improve its efficiency (Vogel, 1996). Bioaugmentation has been successfully used in agriculture, wastewater treatment, and soil remediation (El Fantroussi and Agathos, 2005). Several studies have been conducted using bioaugmentation with bacteria or fungi as a pretreatment for the hydrolysis of lignocellulosic substrates prior to anaerobic digestion (Dollhofer et al., 2015). In one study, the addition of thermophilic Geobacillus sp. strain AT1 to a biogas reactor using sewage sludge as substrate resulted in a 210% increase in biogas production due to the protease activity of the microbe (Miah et al., 2004). In another study, 22 isolates of white rot fungi were used individually to pretreat wheat straw, with the greatest lignin degradation and subsequent increase in biogas yield (from 0.293 L g⁻¹ to 0.343 L g⁻¹) obtained from an isolate of Pleurotus florida (Müller and Trösch, 1986).

Recently, studies utilizing AF to improve biogas production and speed up substrate degradation have been reported. To date isolates of the genera Anaeromyces and Piromyces have been added to AD systems in an effort to improve lignocellulose degradation and
ultimately improve methane yield (Procházka et al., 2012; Nkemka et al., 2015). The study conducted by Prochazka et al. (2012) demonstrated increased biogas yields from different substrates, such as maize silage, anaerobic sludge, and microcrystalline cellulose, with bioaugmentation of AF in fed batch semi-continuous digesters. In their study, addition of 8 mg dry mycelium of *Anaeromyces* sp. (strains KF8 or JF1) or mixed cultures of 1.9 mg dry mycelium of *Anaeromyces* sp. KF8 and *Piromyces* sp. KF9 increased biogas yield by up to 22%. Although the study demonstrated an increase in biogas yield with AF, the researchers did not determine if the increase in biogas occurred as a result of the addition of AF or the anaerobic microbes that were already present in sludge. Recently in a two-stage reactor bioaugmented with *Piromyces rhizinflata* by Nkemka et al. (2015), using corn silage and cattail as substrates resulted in an initial increase of H₂ and CH₄ production but with no overall increase in biogas production. They proposed that this response occurred as a result of rapid wash out of AF from the anaerobic digester systems. There may also have been additional challenges with integration of AF into the microbial populations within the AD. A recent study by Dollhofer et al. (2017) surveyed 10 agricultural biogas plants for the presence and transcriptional activity of AF, concluding that survival and activity were impeded by the process conditions prevalent in biogas systems.

Based on the challenges outlined in previous studies attempting bioaugmentation of AF directly into AD systems and maintain their viability throughout the fermentation process, this study was designed to evaluate the efficacy of AF as a hydrolytic pretreatment for lignocellulosic biomass. We evaluated the activity of three different fungal species (*Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27 and *Piromyces rhizinflatus* YM600) available at the microbial collection lab were utilized during hydrolysis of corn
silage (with background microflora activity) or common reed (without background microflora).

2.2 Materials and Methods

2.2.1 Feedstock

Corn silage (*Zea mays* L.) and common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) were used as substrates for fungal hydrolysis. Corn silage from the 2014 fall harvest was obtained from a commercial beef cattle feedlot in Lethbridge County. Common reed was obtained from Ridgetown, Ontario from the July harvest of 2014.

2.2.2 Anaerobic fungal strains, media and culturing conditions

Pure cultures of three AF were obtained from the microbial collection lab at the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre: *Anaeromyces mucronatus* YE505 (elk isolate), *Neocallimastix frontalis* 27 (cow isolate) and *Piromyces rhizinflatus* YM600 (moose isolate). Inocula of the fungal cultures were maintained anaerobically at 39 °C in modified semi-defined Lowe’s medium B (Lowe et al., 1985) with barley straw (ground <1 mm) as the sole carbon source. The ground barley comprised 5% of the mass (0.05 g) of the anaerobic media (about 5 mL) in the test tube and then autoclaved for 20 minutes at 120 °C with 103.4 kPa pressure. After autoclaving, the media was cooled down and fungal cultivation was carried out using the Hungate technique (Hungate, 1950); tubes were inoculated by transferring fungal biomass from already existing culture tubes using a Pasteur pipet under anaerobic conditions. After inoculation, tubes were incubated at 39 °C in an incubator for 4 days to allow for fungal growth and then the AF with spent medium was transferred to Erlenmeyer flasks at the start of the hydrolysis experiment.
2.2.3 Hydrolysis experiment

The hydrolysis experiment was conducted in 0.5 L Erlenmeyer flasks. The total solids (TS) content of all flasks was set at 7.9 % (w/w) as mentioned in APHA (1998) protocol. A single lot of anaerobic sludge was obtained in January 2015 from a commercial scale biogas facility (Lethbridge Biogas LP) that co-digests livestock manures with industrial food processing waste. Anaerobic sludge was autoclaved for 20 minutes at 120° C with 103.4kPa pressure to inactivate background microbial activity and then used as a buffering solution in each flask. Triplicate samples of autoclaved sludge were analyzed and used to determine the chemical and physical properties. The sludge had a pH of 7.88, total bicarbonate alkalinity of 16.66 g L\(^{-1}\) and TS of 1.66%.

A total of 36 flasks were used for this hydrolysis experiment. Flasks containing either corn silage or common reed were individually inoculated with each of the three AF in triplicate. Each corn silage flask contained 200 mL of anaerobic sludge, 80 mL fungal inoculum (comprising 20% of the total working volume), 92.8 g of corn silage and 100 mL of distilled water. Each common reed flask contained 200 mL of anaerobic sludge, 80 mL fungal inoculum, 57.6 g common reed, and 140 mL of distilled water. Control flasks were also set up in triplicate in a manner identical to those described above, except that the fungal inocula were first killed by autoclaving prior to addition to the flasks. Inoculated flasks were then flushed with nitrogen for 1-2 min to ensure anaerobic conditions and sealed with butyl rubber stoppers connected to aluminum gas tight bags (Multi-layer transofoil, Flextrus AD, Sweden) as described in Nkemka and Murto (2013). Flasks were equipped with sampling ports for gas and liquid sample extraction. The experiment was conducted under mesophilic
conditions (40 °C ± 1 °C) by placing flasks in a water bath (2870; Thermo Fisher Scientific, USA) and manually agitated at least three times a day.

2.3 Analytical methods

2.3.1 Gas analysis

Gas samples (10 mL) were taken daily from the headspace of each flask and transferred to 5.9 mL evacuated glass vials (Exetainer; Labco Limited, Lampeter, UK) prior to analysis using gas chromatography (GC). Gas samples were analyzed for CO₂ and CH₄ concentrations using a two-channel micro GC (Varian 4900, Palo Alto, USA) equipped with a thermal conductivity detector. Operational parameters of the GC were as follows: Channel A (H₂ analysis) injector 110 °C, column oven 40 °C, argon carrier gas at 150 kPa; Channel B (CH₄, CO₂ analysis) injector 80 °C, column oven 40 °C, helium carrier gas at 100 kPa. Total gas volume from each flask was captured in individual gas-tight bags and quantified using a 0.1 L glass syringe (Perfektum™ Jumbo Glass Syringes, Cadence Science™, USA).

2.3.2 Liquid analysis

Liquid samples were extracted from a sampling port on each flask every 48 h using a 10 mL syringe and divided into aliquots for further analysis as described below. The TS and VS of liquid samples were measured following a standard protocol (APHA, 1998).

To estimate the extent of lignocellulose hydrolysis and the amount of remaining dissolved organic matter, soluble chemical oxygen demand (COD) was determined according to the manufacturer’s protocol (Dr. Lange test kit HR mercury free, 20-1500 mg L⁻¹, Mississauga, Canada). Samples used for COD analysis were first syringe filtered through 0.45 µm
nylon filter (Chromatographic Specialties Inc., Brockville, Canada) and then digested using a digital reactor block (HACH DRB200, London, Canada) at 150 °C for 2 h. After digestion, absorbance of the sample was measured using a spectrophotometer (DR900, HACH, Mississauga, Canada).

The pH and total bicarbonate alkalinity were measured using a BIOGAS titration Manager (R41T114, HACH, Vésenaz, Switzerland). Liquid samples were also analyzed for volatile fatty acids (VFA; acetate acid, n-butyrate, iso-butyrate, propionate, n-valerate, iso-valerate and caproate) by GC (Agilent 6890 N, Agilent, Mississauga, Canada). The samples were prepared by first filtering using 0.45 µm nylon filter Chromatographic Specialties Inc., Brockville, Canada) then 25% meta phosphoric acid was added to the filtered sample in the ratio of 5:1 sample to acid. The gas chromatograph was equipped with a flame ionization detector maintained at 250 °C, and a fused silica capillary column (ZB-FFAP, 30 m x 0.32 mm x 1.0 µm; Phenomenex, Torrance, USA). The equipment was set at split mode and the split injection ratio was 5:1. Helium was used as the carrier gas and the analytical steps were performed according to the procedures outlined in Gilroyed et al. (2015).

Concentration of soluble ions (Na⁺, K⁺, Ca²⁺, Mg²⁺) and ammonium ion were determined after filtration through 0.45 µm filter paper using ion chromatography (ICS-1000 and DX-600, Dionex, Sunnyvale, USA). To determine the ratio of total carbon to total nitrogen in samples, a subsample (5 mg) was freeze dried for 1 week and ground to a size < 0.15 mm using a Cyclone sample mill (UDY Corporation, USA) and then analyzed using a CNS analyzer (NA-1500, Carlo Erba, Rodano, Italy) linked via a continuous flow interface to an Optima isotope ratio mass spectrometer (Micromass, Manchester, UK).
2.3.3 Fiber analysis

Fiber analysis was performed in order to characterize the composition (cellulose, hemicellulose and lignin content) of corn silage and common reed before hydrolysis. Before taking samples for analysis, corn silage and common reed were thoroughly mixed in the containers that they stored to get a uniform and unbiased sample for analysis. Triplicate samples of each feedstock were air dried for 1 week and then ground through a screen of 1 mm mesh size in a tabletop mill grinder (Wiley mill standard model 4; Arthur H. Thomas Co., Philadelphia, USA). The contents of lignin, hemicellulose and cellulose were analyzed according to a modified method of Van Soest et al. (1991) with thermal stable amylase (Termamyl® 120, Sigma-Aldrich Co. LLC., St. Louis, USA) and sodium sulfite (S430-3 sodium sulfite anhydrous, Fisher Scientific Int., Inc., Pittsburgh, USA) included in the NDF procedure (Mertens et al., 2002).

2.4 Statistical analysis

One-way ANOVA and repeated measures ANOVA tests were performed for statistical analysis using IBM SPSS version 24.0. The different treatments (Anaeromyces mucronatus YE505, Neocallimastix frontalis 27 and Piromyces rhizinflatus YM600) were kept as independent variables and the different analytical tests such as average cumulative hydrogen and CO₂ gas production, changes in COD, pH, VFA were considered as dependent variables.
2.5 Results and Discussion

2.5.1 Feedstock characteristics

The TS of corn silage and common reed were 33.5% ± 0.6 and 54.7% ± 0.8, of which 96.3% ± 0.6 and 94.9% ± 0.4 were VS, respectively (Table 2.1). The C: N ratio of corn silage and common reed was 31.2% ±0.1 and 26.3% ±0.7, respectively; both values were almost within the optimum range of 20 to 30 for AD (Teghammar, 2013). In terms of fiber composition, corn silage had about two times lower (p<0.05) concentrations of cellulose, hemicellulose, and lignin than common reed (Table 1).

2.5.2 Gas production and composition

Cumulative methane production was <1 mL g\(^{-1}\) VS in all treatments for both corn silage and common reed substrates. Since only hydrolysis was conducted in this study, minimal methane volume was expected. Anaerobic fungi are known to produce H\(_2\) and CO\(_2\) during substrate hydrolysis (Gruninger et al., 2014). Nkemka et al. (2015) demonstrated that bioaugmentation with anaerobic AF into a two-stage AD can increase H\(_2\) production within the system in the days following inoculation. In our study, an initial increase in hydrogen (Fig. 2.1) and CO\(_2\) (Fig. 2.2) production were observed with all fungal species that were added to digesters containing corn silage. Over the course of the 11-day hydrolysis experiment, all three fungal treatments produced similar cumulative volumes of H\(_2\), in the range of 46-60 mL g\(^{-1}\) VS (p>0.05). Similar trends were observed for CO\(_2\) gas production, with cumulative CO\(_2\) production for all three treatments of corn silage in the range of 78-93 mL g\(^{-1}\) VS (p>0.05). The initial increase in gas production observed for corn silage may have been due to the fact that the material had already undergone ensiling, which is in itself a pretreatment and may have contributed to the reduced NDF and ADF concentrations in
corn silage versus common reed (Table 2.1). Additionally, background microbes such as H$_2$-producing *Clostridia* are known to be present in corn silage and were likely actively contributing to the gas production observed (Muck, 2010).

During hydrolysis of common reed, smaller volumes of H$_2$ (<1 mL g$^{-1}$ VS) (Fig. 2.1) and CO$_2$ (<15.5 mL g$^{-1}$ VS) (Fig. 2.2) gas were evolved from all three treatments than was observed with corn silage (p>0.05). Further studies are required to either eliminate the contribution of background microflora present on the substrate, or to account for the magnitude of their activity within the overall microbial consortia present during substrate hydrolysis.

### 2.5.3 Chemical changes during hydrolysis

Chemical oxygen demand was measured over the course of the hydrolysis experiment to examine the amount of soluble COD released because of hydrolysis and for further AD (Fig. 2.3). Soluble COD concentrations for corn silage treatments trended upwards over time but did not significantly differ pre- and post-hydrolysis. Similarly, soluble COD concentration did not increase in common reed regardless of treatment (p>0.05) and was approximately half the value compared to corn silage. The higher initial COD concentration in corn silage compared to common reed was likely due to conversion of some corn biomass to soluble fermentation products during the ensiling process.

The main VFA produced during hydrolysis of corn silage were acetic, propionic, and butyric acids. Total VFA concentration trended upward over the course of the hydrolysis experiment for corn silage in all treatments; however, these increases were not statistically significant (Fig. 2.4). In comparison, VFA production during hydrolysis of common reed
was limited, with no significant difference in concentration observed over the course of the experiment for any treatments. It couldn’t have been possible to assume that the VFA might have been consumed by other microorganisms that were present in the digester flask and making it appear as there were no accumulation of VFA. The little gas volume that was collected overtime from the reactor flasks and also the autoclaved anaerobic sludge that wouldn’t have contained any necessaryacetogenic bacteria to consume the VFA to convert to acetic acid and hydrogen can support the above statement. Therefore, this suggests that no fermentation or digestion had occurred.

The pH of the digesta in corn silage flasks decreased from approximately 7.0 on Day 1 to 5.6-5.8 on Day 11 for all treatments (Fig. 2.5). The pH of digesta in common reed flasks ranged between 7.2-8.0 on Day 1, which was not statistically different from corn silage (p>0.05). At the end of hydrolysis, the pH values of common reed decreased to 6.1-6.9, which again was not statistically different from corn silage (p>0.05). The optimum pH range for the growth of AF is between 6.0 and 7.0 (Magan, 2007), so reduction of pH <6.5 due to VFA accumulation may have contributed to conditions unfavorable for anaerobic fungal activity. Also it is important to realize that the optimum pH for plant cell wall hydrolysis is pH 5.5-6.5 while for methanogenesis it is 6.8-7.6 (Jha et al., 2011). In the rumen, there is both a constant supply of buffering capacity as well as organic acid removal through the production of saliva and the symbiotic activities of the host animal and microbial consortium, respectively. The complexity of the rumen system is difficult to mimic in vitro in the laboratory, but a better approximation of the conditions which are favorable for AF to survive and be active will be essential for future success in this area of research.
Ammonia is known to inhibit to hydrolysis during AD at >200 mg L⁻¹ (Chen et al., 2008), but that threshold was not exceeded in our study (Table 2.2) (Chen et al., 2008). Similarly, metals can inhibit biological hydrolysis processes when present in sufficient concentration. Basically, alkali metals such as Na⁺, Mg²⁺, K⁺ and Ca²⁺ up to a range of 400 mg/L helps maintain alkalinity and pH in AD. However, higher concentrations would cause toxicity and inhibit AD processes (Gerardi, 2003). In our study, Na⁺ and Mg²⁺ concentrations were very low (< 1 mg L⁻¹) in all treatments and well below reported inhibitory levels, i.e., <750 mg L⁻¹ for Mg²⁺ (Romero-Grüiza et al., 2016) and 3500-5500 mg L⁻¹ for Na⁺ (McCarty, 1964). Concentration of K⁺ was 1-3 mg L⁻¹ for all treatments, again below the inhibitory concentration of 400 mg L⁻¹ (Chen et al., 2008). Similarly, for Ca²⁺ the values were <1 mg L⁻¹ for all treatments and below inhibitory concentrations (>7000 mg L⁻¹). Based on this, the low degree of hydrolysis observed in all treatments was not likely caused by inhibition from ammonia or metals. The other reason would be the lack of favourable conditions for the activity of AF microbes inside lab-scale batch reactors.

When considering our overall results, we can conclude: 1) that there was very little hydrolytic activity in any of the reactors, regardless of fungal species or substrate type; and 2) the activity that was present was likely due to background microflora including the bacteria that are present on the feedstock and not the AF. The most likely explanation for these results is that the AF were unable to survive, or at least be active, in the environment provided in this study. Due to time constrains we were not able to perform any specific analysis supporting the above statement. However, there have been issues with poor anaerobic fungal survival when applied to non-rumen environments reported by others (Dollhofer et al., 2017; Nkemka et al., 2015; Prochazka et al., 2012).
Anaerobic fungi are known for having a close symbiotic activity and interspecies H\textsubscript{2} transfer with other microbes in the rumen (Bauchop and Mountfort, 1989; Cheng et al., 2009). The absence of these relationships, or the lack of time for such relationships to develop using the experimental design of this study, may also account for the poor hydrolytic activity observed. One suggestion to improve anaerobic fungal viability inside AD systems would be to use a sterilized rumen fluid which contains all the nutrients essential for anaerobic fungal survival but eliminates other microbes present in the rumen fluid. Addition of this to the biodigesters could result in the digesta being more similar in composition to rumen fluid. Another option would be to focus on co-culturing AF with other rumen hydrolytic bacteria such as *Fibrobacter succinogenes*, which could also potentially help enhance the breakdown of lignocellulose from the substrate (Kobayashi et al., 2008). Joblin et al. (2001) inoculated *F. succinogenes* together with methanogenic co-cultures of *Caecomyces/M. smithii* grown on rye grass. They found that there was an increase in stem degradation and attributed this to complementary fibrolytic activities between the two species. By comparison, our study utilized only AF to help degrade the substrate. The heat sterilization used to eliminate background microflora from anaerobic digestate may have limited potential for symbiotic relationships to develop between AF and bacteria. The combination of our results and those by Yildirim et al. (2017) strongly suggest that successful outcomes from the addition of AF to hydrolysis and/or anaerobic digestion may require that they form symbiotic relationships with other microbes.

### 2.6 Conclusion

Hydrolysis of corn silage and common reed were not improved by bioaugmentation with three different species of AF, as evidenced by a lack of significant H\textsubscript{2} production or
substrate degradation compared to controls. The most likely explanation for these results is that AF had low activity and/or survival in the anaerobic fermentation systems used in this study. More research is required to better understand survival of AF in anaerobic digestion processes to determine the feasibility of exploiting these organisms for lignocellulosic degradation.
Table 2.1. Initial substrate characteristics of corn silage and common reed used for hydrolysis experiments. Values shown represent mean (n=3) and standard error.

<table>
<thead>
<tr>
<th></th>
<th>Corn silage</th>
<th>Common reed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TS(^a) (%)</strong></td>
<td>33.5 ± 0.6</td>
<td>54.7 ± 0.8</td>
</tr>
<tr>
<td><strong>VS(^b) (%) TS</strong></td>
<td>96.3 ± 0.6</td>
<td>94.9 ± 0.4</td>
</tr>
<tr>
<td><strong>VS Added (g)</strong></td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Moisture content (%)</strong></td>
<td>66 ± 0.6</td>
<td>44.3 ± 0.8</td>
</tr>
<tr>
<td><strong>Total Carbon: Total Nitrogen ratio</strong></td>
<td>31.2 ± 0.1</td>
<td>26.3 ± 0.7</td>
</tr>
<tr>
<td><strong>Hemicellulose (% of TS)</strong></td>
<td>12 ± 4.3</td>
<td>28.7 ± 0.4</td>
</tr>
<tr>
<td><strong>Cellulose (% of TS)</strong></td>
<td>17.2 ± 1.8</td>
<td>38.7 ± 0.4</td>
</tr>
<tr>
<td><strong>ADL(^c) (% TS)</strong></td>
<td>2.7 ± 0.0</td>
<td>7.9 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) Total solids

\(^b\) Volatile solids

\(^c\) Acid detergent lignin
**Table 2.2.** Initial concentrations of ammonia and soluble ions during hydrolysis. Values shown represent mean (n=3) and standard error.

<table>
<thead>
<tr>
<th>Substrate type</th>
<th>NH$_3^+$ mg/mL</th>
<th>Na$^+$ mg/mL</th>
<th>K$^+$ mg/mL</th>
<th>Mg$^{2+}$ mg/mL</th>
<th>Ca$^{2+}$ mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage - bioaugmented with</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat inactivated <em>Neocallimastix frontalis</em></td>
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<td>0.87 ± 0.18</td>
<td>2.14 ± 0.13</td>
<td>0.14 ± 0.00</td>
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</tr>
<tr>
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<td>0.79 ± 0.08</td>
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</tr>
<tr>
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<td>0.72 ± 0.03</td>
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### Common Reed - bioaugmented with

<table>
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<th></th>
<th>Heat inactivated Neocallimastix frontalis</th>
<th>Live Neocallimastix frontalis</th>
<th>Heat inactivated Anaeromyces mucronatus</th>
<th>Live Anaeromyces mucronatus</th>
<th>Heat inactivated Piromyces rhizinflatus</th>
<th>Live Piromyces rhizinflatus</th>
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<tr>
<td></td>
<td>0.50 ± 0.04</td>
<td>0.57 ± 0.03</td>
<td>1.63 ± 0.02</td>
<td>0.12 ± 0.01</td>
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<tr>
<td></td>
<td>0.47 ± 0.00</td>
<td>0.61 ± 0.02</td>
<td>1.64 ± 0.03</td>
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<td>0.22 ± 0.01</td>
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<td></td>
</tr>
<tr>
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<td>0.51 ± 0.01</td>
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<td>1.59 ± 0.02</td>
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<td>0.21 ± 0.00</td>
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<tr>
<td></td>
<td>0.61 ± 0.67</td>
<td>0.64 ± 0.02</td>
<td>1.58 ± 0.03</td>
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<tr>
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<td>0.59 ± 0.03</td>
<td>1.63 ± 0.07</td>
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<tr>
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<td>0.58 ± 0.03</td>
<td>1.58 ± 0.08</td>
<td>0.10 ± 0.00</td>
<td>0.21 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

**N/A** - not identified (below the detection limit)
Figure 2.1. Average cumulative H\textsubscript{2} yield produced overtime during hydrolysis of corn silage and common reed using either Anaeromyces mucronatus YE505, Neocallimastix frontalis 27 or Piromyces rhizinflatus YM600. In control treatments, heat-sterilized rumen fungal inocula were used. Values represent mean (n=3).
Figure 2.2. Average cumulative CO$_2$ yield produced overtime during hydrolysis of corn silage and common reed using either *Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized rumen fungal inocula were used. Values represent mean (n=3).
Figure 2.3. Changes in chemical oxygen demand (COD) concentration overtime during hydrolysis of corn silage and common reed using either *Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized rumen fungal inocula were used. Values represent mean (n=3).
Figure 2.4. Changes in VFA concentration overtime during hydrolysis of corn silage and common reed using either *Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized rumen fungal inocula were used. Values represent mean (n=3).
Figure 2.5. Change in pH overtime during hydrolysis of corn silage and common reed using either Anaeromyces mucronatus YE505, Neocallimastix frontalis 27 or Piromyces rhizinflatus YM600. In control treatments, heat-sterilized rumen fungal inocula were used. Values represent mean (n=3).
3  Chapter - Effect of bioaugmentation by anaerobic fungi on the hydrolysis of heat-sterilized corn silage

3.1  Introduction

Hydrolysis is typically considered the rate-limiting step in anaerobic digestion, which converts organic matter to biogas through microbial activity (Marín Pérez and Weber, 2013). To solve this problem, a two-stage anaerobic digestion system can be utilized, where a hydrolysis reactor facilitates substrate hydrolysis and acidogenesis upstream of a biogas reactor where acidogenesis and methanogenesis occur. Separating anaerobic digestion into two stages helps the hydrolytic microorganisms that have a high growth rate accelerate the degradation of recalcitrant fiber-rich substrates and release VFA that will be treated in a high rate methane reactor under stable operational conditions to achieve higher methane concentrations (Jha et al., 2011; Nkemka and Murto, 2013).

Several studies have been conducted on the pretreatment of energy crops using various microbes, particularly aerobic fungi, to improve biogas production. Biological pretreatment can involve either the use of whole cultures, or addition of specific enzymes (Taherzadeh and Karimi, 2008). Biological pretreatment technologies provide an advantage over thermal or chemical processes because of their lower energy requirements, fewer safety concerns and environmental impacts, and milder process conditions (Sun and Cheng, 2002). However, most of the pretreatment research conducted to date for use in biogas systems has focused on aerobic fungi, such as white rot fungi (Taniguchi et al., 2005). As such, there is a need to investigate other microbes to find organisms that are both effective and feasible to use industrially.
In Chapter 2, we evaluated the effect of anaerobic fungal strains from three different genera (*Anaeromyces mucronatus* YE505, *Neocallimastix frontalis* 27 and *Piromyces rhizinflatus* YM600) on the hydrolysis of corn silage (with background microflora) and common reed (without background microflora). The key finding in our research was that AF did not provide any significant substrate degradation compared to controls, likely due to low survival in lab-scale bioreactors with background microflora. We did observe some microbial activity in the previous study, as evidenced by hydrogen gas production, but could not discern whether that activity was attributed to bioaugmented fungi or the background community. We also did not know if the presence of a background microbial community reduced the survival of bioaugmented AF inside the bioreactors.

Based on the knowledge gap from our previous experiment, in this study corn silage was autoclaved to eliminate any background microflora and then treated with one of two anaerobic rumen fungal strains, *Neocallimastix frontalis* or *Piromyces rhizinflatus*, to study the hydrolysis progress and their corresponding enzymatic activity. The main reason to choose these strains is that initially when the project was started we had three different fungal strains (as mentioned in chapter 2) but due to the difficulties involved in maintaining and culturing AF, only two strains survived out of the three.

### 3.2 Materials and methods

#### 3.2.1 Corn silage as substrate

Corn silage (*Zea mays* L.) was used as substrate for the hydrolysis experiment. Corn silage from the 2014 fall harvest was obtained from a commercial beef cattle feedlot in Lethbridge County, Canada. Corn silage (88.5 g) was weighed into Erlenmeyer flasks and autoclaved using the media cycle for 20 min at 120° C, 103.42 kPa of pressure. Sterilization of corn
silage was done to ensure that any potential substrate hydrolysis was due to the activity of bioaugmented AF, not background microflora already present in the corn silage.

3.2.2 Rumen fungal strains, media and culturing conditions

Pure cultures of *Neocallimastix frontalis* 27 (bovine isolate), and *Piromyces rhizinflatus* YM600 (moose isolate) were obtained from the microbial collection lab at Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Station. A semi-defined Lowe’s medium B (Lowe et al., 1985) was prepared under anaerobic conditions to culture fungi at 39 °C using ground barley straw (representing 5% of the total volume and ground to <1 mm) as the carbon substrate. The culturing was carried out by following the Hungate culturing technique (Hungate et al., 1950). The fungal biomass from the previous stock was first disrupted by using a sterile Pasteur pipet and then some mycelia and media were transferred to freshly prepared barley straw media and incubated at 39 °C for 4 days to allow maximum fungal growth. During the start of the hydrolysis experiment the fungi were then transferred to the Erlenmeyer flasks.

3.2.3 Hydrolysis of heat-sterilized corn silage

To ensure sterility throughout the experiment and to avoid cross contamination, all glassware and equipment were sterilized by either autoclaving or by cleaning with ethanol before the start of the experiment. The hydrolysis experiment was conducted in 0.5 L Erlenmeyer flasks. The total solids (TS) content of all flasks was set at 7.9 % (w/w). A single lot of anaerobic sludge was obtained in April 2015 from a commercial scale biogas facility (Lethbridge Biogas LP) that co-digested livestock manures with industrial food processing waste. Anaerobic sludge was autoclaved in media cycle for 20 min at 120° C,
103.42 kPa of pressure for sterility and then used as a buffering solution in each flask. The purpose of the autoclave step was to eliminate background microbial activity that could alter substrate hydrolysis. After autoclaving, the anaerobic sludge had a pH of 8.2, total bicarbonate alkalinity of 18.75 g L$^{-1}$ and TS of 1.66%.

Triplicate flasks containing heat-sterilized corn silage were individually inoculated with either one of the two-rumen fungal strains, or were left uninoculated (control). Each corn silage flask contained 200 mL of anaerobic sludge, 77.69 mL fungal inoculum, 88.46 g of corn silage and 100 mL of sterilized water. Control flasks were also set up with identical composition to those described above, except that the fungal inocula were first killed by autoclaving. Inoculated flasks were then flushed with nitrogen to ensure anaerobic conditions and sealed with butyl rubber stoppers connected to aluminum gas-tight bags (Multi-layer transofoil Flextrus AD, Sweden) as described in Nkemka & Murto (2013). This procedure allowed quantification of gas volume produced. Flasks were equipped with sampling ports for gas and liquid sample extraction. The experiment was conducted under mesophilic conditions (40 °C ± 1 °C) for 13 days by placing flasks in a heated water bath (2870, Thermo Fischer Scientific, USA).

### 3.3 Analytical methods

#### 3.3.1 Gas analysis

Gas samples (10 mL) were taken from the headspace of each flask on days 1, 3, 5, 7, 10 & 13 and transferred to 5.9 mL evacuated glass vials (Exetainer, Labco Limited, Lampeter, UK) prior to compositional analysis using GC. Gas samples were analyzed using a two-channel micro GC (Varian 4900, Varian, USA) equipped with a thermal conductivity detector. The operation parameter of the GC was same as described previously in Chapter
2. For measuring the total gas volume from each flask, individual aluminum bags attached to the top of the flasks were measured and quantified using a 0.1 L glass syringe (Perfektum™Jumbo Glass Syringes, CAdence Science™, USA).

### 3.3.2 Liquid analysis

Liquid samples were extracted from the sampling port on the same days as the gas samples in order to minimize the amount of air entering the reactor.

The TS and VS of liquid samples were measured following the standard protocol as mentioned in APHA (1998). Sample preparation and operational parameters for soluble COD, pH, total bicarbonate alkalinity, VFA, total carbon-to-total nitrogen ratio were conducted as described in detail in Chapter 2.

### 3.3.3 Fiber analysis

The main fiber contents in the corn silage (i.e., cellulose, hemicellulose and lignin) were determined based on the modified methods of Van Soest et al. (1991) and Mertens et al. (2002). A detailed description of the sample preparation and method of fiber analysis is provided in Chapter 2.

### 3.3.4 Enzyme analysis

To perform enzyme analysis liquid samples were drawn from the reactor’s liquid sampling port carefully with sterile syringe and falcon tubes without causing any contamination and stored immediately at -40 °C freezer immediately until analysis. Endoxylanase and endoglucanase activities were assayed based on the Miller method (Miller, 1959; Colombatto and Beauchemin, 2003) and were estimated as follows:
3.3.4.1 Estimation of endoxylanase activity

Oat spelt xylan (1% w/v) prepared in sodium citrate buffer (50 mM, pH 6.0) was used as substrate for the determination of endoxylanase activity (endo 1,4-β-D xylanase, EC 3.2.1.8). A falcon tube was used to mix the reaction mixture that contained equal amounts of test enzyme and test substrate (1 mL) and incubated in a water bath at 50 °C for 5 min. The falcon tube was removed, and the reaction was stopped by adding 3 mL of dinitrosalicylic acid (DNS) followed by boiling the mixture in a water bath set at 100°C for 5 min. The mixture was cooled to room temperature and the color developed was read using a microplate spectrophotometer (GO UV/VIS, Thermo Scientific, USA) at 540 nm. The amount of sugars released was then quantified by comparison to a xylose standard curve that had a concentration range between 0.1 to 0.5 mg.

3.3.4.2 Estimation of endoglucanase activity

Carboxymethyl cellulose (1% w/v), prepared in sodium citrate buffer (50 mM, pH 6.0) was used as substrate for determination of endoglucanase (1, 4-B-D-glucan 4-glucanohydrolase; EC 3.2.1.4, cellulase) activity. The test enzyme and substrate was prepared and digested the same way as done in analyzing endoxylanase activity. The results were then read using a microplate spectrophotometer at 540 nm (instrument mentioned above). The amount of sugars released after analysis was then quantified by comparing with a glucose standard curve that had a concentration range between 0.1 to 0.5 mg.

3.4 Statistical methods

Two different kinds of tests were performed for statistical analysis using IBM SPSS version 24.0. One-way and repeated measures ANOVA were performed (Neocallimastix,
Piromyces and Anaeromyces) when comparing the treatments overtime. The different treatments were kept as independent variables and the different analytical tests such as average cumulative hydrogen and CO₂ gas production, changes in enzyme concentration, changes in COD, pH, VFA were considered as dependent variables.

3.5 Results & Discussion

3.5.1 Feedstock characteristics

The TS% of heat-sterilized corn silage was 34.7% ± 1.0 of which 97.59% ± 2.29 was VS (Table 3.1). The C: N ratio of corn silage was 30, which was the optimum range of 20 to 30 for anaerobic digestion (Teghammar, 2013). Fiber characteristics of corn silage at the beginning of the experiment can be found in Table 3.1.

3.5.2 Enzyme activities

Cellulase and xylanase activities were measured every 48-72 h for 13 days (Figures 3.5 and 3.7) and their values were lower than expected. Cellulase activity of N. frontalis varied from 1.13 mg min⁻¹ at the start of the experiment (Day 0) and declined to 0.19 mg min⁻¹ on Day 1 and then remaining stable after Day 3 at 1.11 mg min⁻¹ of cellulase activity. This interesting pattern of cellulase activity was observed on both treatment and control, respectively. Statistical analysis showed that there was no statistical difference between the treatment and control (p>0.05). Similar pattern was observed with heat-sterilized corn silage bioaugmented with P. rhizinflatus and again no significant difference (p>0.05) was observed between the treatment and control. Xylanase activity of both N. frontalis and P. frontalis declined on Day 1 but increased to about 4 mg min⁻¹ on Day 3 with no difference (p>0.05) observed between treatment and control.
The drop in cellulase and xylanase activities on Day 1 by both AF could be due to the sudden drop in pH to 5.6 on Day 1 (Fig. 3.1), which is outside the desired range for survival and growth of AF (pH of 6.0-7.0) (Magan, 2007). However, as the experiment progressed both the controls and treatments showed a small increase in both cellulase and xylanase activities. The reason for this could be due to incomplete inactivation of endospores attached to the heat-sterilized corn silage such as highly heat resistant spores in corn silage that are able to survive up to 130° C (Scheldeman et al., 2006), whereas in this study corn silage was subjected to heat sterilization only at 120° C. The other probable reason would be there weren’t enough AF available in the system to produce enzymes and conduct the hydrolysis process effectively.

3.5.3 Gas production and composition

Cumulative methane production was <1 mL g⁻¹ VS in all treatments, which was expected because of the absence of methanogens. The average cumulative gas production of H₂ and CO₂ at different times by all the treatments and control is given in Figures 3.2 and 3.3. When autoclaved corn silage was treated with AF, there was no difference (p>0.05) in the total gas production between the controls and treatments. There was a sudden increase in both H₂ and CO₂ gas production on Day 3, and then the production plateaued for the remainder of the study. The results for gas production in this study are similar to the previous experiment (chapter 2) as the average cumulative volumes for H₂ and CO₂ ranged from 33-55 mL g⁻¹ VS and 67-91 mL g⁻¹ VS, respectively. Regardless of whether corn silage was autoclaved or not, the results look quite similar and collectively suggest that anaerobic fungal survival was poor, resulting in minimal hydrolytic activity.
3.5.4 Chemical changes during hydrolysis

The pH changes for all the treatments are shown in Fig. 3.1. The initial pH of the treatments decreased to 5.7, which could be due to a poorer buffering quality of the anaerobic sludge. This resulted from almost neutral becoming more acidic and not providing an ideal environment for the survival or activity of AF. Looking at the VFA patterns for both controls and treatments, the initial VFA concentration was just 2 g L\(^{-1}\) a level that increased by 4 to 6 times on Day 1, with levels being the highest for *P. rhizinflatus* at 12.65 g L\(^{-1}\) VFA (see Fig. 3.6). Since batch reactors were used in this study, there was no ongoing addition of buffer to the reactors, and as such a neutral environment could not be maintained.

The soluble COD contents of all the treatments when measured on Day 1 were between 31.5 g L\(^{-1}\) and 33.9 g L\(^{-1}\) (Fig. 3.4). On Day 13, there was only a minimal increase in soluble COD concentration to between 34 and 37 g L\(^{-1}\) (p>0.05). The cumulative results from both this study and our previous study suggest that AF have a low ability to survive in lab-scale bioreactors regardless of species or whether substrate was autoclaved or not. This conclusion is supported by limited enzymatic activity and substrate hydrolysis regardless of treatment. Both treatments and controls behaved very similarly, which might be due to incomplete sterilization of corn silage or sludge with a possibility that the enzymes present in them were responsible for the minimal hydrolysis that was observed. Also, compared to the rumen the lab scale biodigestors may not have achieved a sufficient level of anaerobic conditions for the fungi to thrive as well. This fluctuation in the environment could have happened while withdrawing samples and thereby affecting the AF growth. Considering other possibilities such as the experiments not being conducted in a 100% sterile
environment and not using a anaerobic chamber could have led to a certain degree of cross contamination. There is also a possibility that the system had picked up certain bacteria already present in the air leading to the limited hydrolysis that was observed. Based on the above discussion, future research should be focused on extracting enzymes from AF and adding them to the reactors or trying to inoculate live cultures of AF with the substrate under anaerobic conditions and then perform AD. Future bioaugmentation studies could be conducted using AF subjected to these alternative approaches to culture management.

The first would be to try mixing the AF with other microbes such as Actinomycetes. In a study, mixed consortia of Actinomycetes from cow dung have helped improve biogas production between the range 8.4-44% (Merlin Christy et al., 2014). The second approach is to try the hydrolysis experiment by adding a high proportion of animal-based substrate such as manure along with the lignocellulose substrate. Finally, with the advent in genetic engineering; microorganisms and enzymes can be genetically engineered to improve biogas production. For example, recombinant strains of *Saccharomyces cerevisiae* are made to produce extracellular endoglucanase and glucosidase that are able to ferment cellulose, hemicellulose and xylose to ethanol. With the high cost involved in using commercial enzymes for the pretreatment step, recombinant DNA technology will help produce large amounts of recombinant enzymes and thereby cutting down the cost on using commercial enzymes. Similar efforts were carried out by genetic modification of *Saccharomyces cerevisiae* using the isomerases of *Piromyces* and *Orpinomyces*. The attempt was successful and patents have been filed as well (Dollhofer et al., 2015).
3.6 Conclusion

The study conducted in this experiment shows us that the two genera of AF tested, *Neocallimastix frontalis* 27 and *Piromyces rhizinflatus* YM600, to bioaugment and improve biogas generation suggest that there are difficulties for them to hydrolyze and thrive at lab-scale biodigestors.
**Table 3.1.** Initial substrate characteristics of heat-sterilized corn silage used for hydrolysis experiments. Values shown are mean (n=3) and standard error.

<table>
<thead>
<tr>
<th></th>
<th>Corn silage</th>
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<tbody>
<tr>
<td><strong>TS</strong>&lt;sup&gt;a&lt;/sup&gt; (%)</td>
<td>34.75 ± 1.0</td>
</tr>
<tr>
<td><strong>VS</strong>&lt;sup&gt;b&lt;/sup&gt; (% TS)</td>
<td>97.59 ± 2.29</td>
</tr>
<tr>
<td><strong>Moisture content</strong> (%)</td>
<td>65.96 ± 1.01</td>
</tr>
<tr>
<td><strong>Total Carbon:Total Nitrogen ratio</strong></td>
<td>30.26 ± 0.1</td>
</tr>
<tr>
<td><strong>Hemicellulose</strong> (% of TS)</td>
<td>11.02 ± 2.82</td>
</tr>
<tr>
<td><strong>Cellulose</strong> (% of TS)</td>
<td>17.25 ± 1.82</td>
</tr>
<tr>
<td><strong>ADL</strong>&lt;sup&gt;c&lt;/sup&gt; (% TS)</td>
<td>2.80 ± 0.07</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total solids

<sup>b</sup> Volatile solids

<sup>c</sup> Acid detergent lignin
Figure 3.1. Change in substrate pH over time during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
**Figure 3.2.** Average cumulative H₂ produced overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
Figure 3.3. Average cumulative CO₂ produced overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
Figure 3.4. Change in substrate COD overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
Figure 3.5. Change in cellulase activity overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
Figure 3.6. Total volatile fatty acids produced overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
Figure 3.7. Change in xylanase activity overtime during hydrolysis of heat-sterilized corn silage using either *Neocallimastix frontalis* 27 or *Piromyces rhizinflatus* YM600. In control treatments, heat-sterilized fungal inocula were used. Values represent mean (n=3).
4 Chapter - Discussion

4.1 Context

According to the climatic study conducted by NASA, 2016 has been recorded to be the hottest year in history. Their study shows that the biggest culprit is the greenhouse gas emissions (GHG) especially those emitted from the burning of fossil fuels. Educating the industrial sector to use a lot of less energy overall and as well as increasing the availability of renewable energy and their production is the only option left to save humanity from the consequences that would occur due to the impact from climatic change. Though there is much renewable energy available, biogas is one such renewable energy technology that has been rapidly growing especially in Europe from 6000 biogas plants in 2009 to 17000 in 2015 (EBA, 2017). However, one such drawback that every biogas industry faces is the low hydrolysis rate during the anaerobic digestion. Several pretreatment technologies are currently available to improve this step however, most of the pretreatment technologies are not cost effective or environmentally safe (Taherzadeh and Karimi, 2008). Therefore more research is now conducted on the use of microbes for performing the pretreatment steps. Though the microbes used for bioaugmenting purposes are environmentally safe, however they have a low treatment rate (Saritha et al., 2012). Hence, there is a constant search in researching the usage of a new microbe to perform this process. The one group of microbes we have studied with respect to this process are AF from the digestive tract of herbivores. In this work we tested them on two different kinds of lignocellulosic substrates since these microbes are well known to disintegrate the stubborn lignin layer from plant substrates in their natural environment.
4.2 Significance

In this study we investigated AF from three different genera isolated from different ruminant sources were applied as a pretreatment step for two different substrates in lab scale biodigesters. The main significant finding from this study was that AF is unable to adapt inside AD and has a symbiotic relationship with the microbes from the substrates and cause an increase in the lignocellulose degradation unlike rumen. Also, they have very low adaptability when used as a hydrolytic pretreatment step.

4.3 Recommendations for future research

According to our experiment, AF did not improve the hydrolysis phase in lab-scale anaerobic digesters nor help degrade the substrate faster. However, a few changes or improvements need to be made with respect to this process if these microorganisms are to be used as a pretreatment process.

Firstly, AF are obligate anaerobes and therefore there are problems associated in culturing and maintaining them. Anaerobic fungi also requires repetitive batch culturing with frequent transfers thereby making it a time consuming process. Therefore, methods to increase the amount of fungal biomass need to be investigated to speed up this process for industrial purposes in future. Secondly, it would also be better if the extent of hydrolysis was investigated after co-culturing with other microbes such as methanogens as studies in other systems have shown that this has a positive impact on the digestion process. Also, in rumen systems it is found that AF shows an increase in enzyme production and fiber degradation as a result of interspecies H₂ transfer (Orpin and Joblin, 1997).
In our experiment only two substrates were tested but in the future it would be better to test the hydrolysis with other plant substrates and to see how these AF behave with different substrates in artificial systems. Finally, to try making artificial enzymes those mimic some function of the hydrolytic enzymes of AF. The main advantage of such artificial enzymes is that they are completely robust and are extremely difficult to degrade in any system unlike bioaugmenting AD with a microorganism where the tension involves maintaining their viability throughout the process and the tedious process involved in culturing the microorganism before the start of the experiment. Similarly, artificial mini cellulosomes were designed by joining the AF endoglucanase of glycosyl hydrolase family 6 produced by Neocallimastix patricarium with the various endoglucanase of glycosyl hydrolase from Clostridium cellulosolicum. This attempt produced a higher cellulytic activity compared to any other bacterial enzyme assembly (Dollhofer et al., 2015).

Thus based on this project and the previous projects from others that were conducted using AF as a pretreatment step shows that although AF have all the necessary enzymes to help speed up the lignocellulose degradation process in the rumen but as far as using them on AD is concerned more research is to be conducted to maintain their viability. The current and future trends that are being developed should be tested on AF and thereby making them efficiently usable inside AD.
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