ABSTRACT

PANNEERSELVAM, ANUSHADEVI. Ozonolysis – A Novel Pretreatment Technology For Energy Grasses. (Under the direction of Ratna R Sharma- Shivappa).

Ozonolysis has been used to pretreat various lignocellulosic feedstocks as it is highly effective against lignin removal. This study investigates the effect of ozone on Miscanthus × giganteus, M. sinensis ‘Gracillimus’, Saccharum arundinaceum and Saccharum ravennae, collectively termed as “energy grasses”. The overall goal of this study was to optimize ozonolysis and subsequent enzymatic hydrolysis of the pretreated solids. Experiments were carried out to optimize ozonolysis at three different ozone concentrations (40, 50 and 58 mg/l) with two different reactor configurations (uniflow and flip). Ozone pretreatment conditions were optimized based on lignin and glucan content in the pretreated solids. Hydrolysis of unwashed pretreated solids with 0.06g/g biomass of Cellic® CTec2 indicated enzyme inhibition by lignin degraded products.

Optimization of enzymatic hydrolysis of pretreated solids was performed using both washed and unwashed solids pretreated at optimal conditions (AIL and GLU) at three different enzyme concentrations (0.1, 0.2 and 0.3 g/g biomass) of Cellic® CTec2. Results indicated that washing the solids enhanced glucan conversion from 34.3% to 100%. Optimal process condition was identified as the one in which energy grasses were pretreated for maximum delignification followed by hydrolysis of washed solids at 0.1g/g Cellic® CTec2.

A comparative study of alkali pretreatment of energy grasses with 1% NaOH at 121°C for 60 min indicated that M. × giganteus and S. ravennae had higher glucan conversion (p<0.05) when ozonized while no significant difference was observed for M. sinensis ‘Gracillimus’ and S. arundinaceum.
A preliminary study on reaction kinetics of ozone with lignin was done using 1g of energy cane samples at conditions optimized for maximum lignin removal (AIL). Limited delignification and increase in glucan content associated with complete ozone consumption was observed for all the different varieties. It was hypothesized that the hydroxyl radicals produced during ozone lignin interactions depolymerized cellulose and produced by-products such as gluconic acid. Moreover, initial compounds formed during ozone-lignin interactions, could have produced consumed ozone further resulting in additional by-products. However, this aspect needs significant investigation to better understand ozonolysis kinetics during pretreatment of lignocelluloses specifically energy grasses.
Ozonolysis – A Novel Oxidative Pretreatment Technology For Energy Grasses

by
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DEDICATION

To mom and dad.
BIOGRAPHY

Anushadevi Panneerselvam was born in Sivakasi, Tamilnadu, India to Yasotharadevi and Panneerselvam. She was raised in Trichy and Chennai, Tamilnadu for most part of her life in India. After high school, she did her undergraduate program in pharmaceutical engineering and technology at Bharathidasan University, Trichy, India. After her bachelor’s, she moved to Oklahoma, USA to pursue her master’s program in Biosystems Engineering. During her master’s program she was greatly intrigued by research in the field of biofuels and this led her to pursue a PhD program after graduating from her masters.
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CHAPTER 1: LITERATURE REVIEW

1. INTRODUCTION:
Depleting oil and gas resources coupled with soaring energy demand worldwide and environmental issues associated with fossil fuels has increased the interest in alternative feedstocks for production of fuels and chemicals. High oil prices in the past few years have further increased the significance of finding a substitute that is cheaper, efficient and eco-friendly (Henstra et al. 2007). In this respect, plant biomass has been identified as the only sustainable source of organic carbon while bio-fuels derived from them are the only sustainable source of liquid fuels (Huber et al. 2006b). Combustion of fossil fuel produces about 98% of the present carbon emissions; this can be efficiently reduced by replacing fossil fuels by renewable fuels. It has been estimated that by the year 2040, approximately half of the global energy supply will come from renewable resources and the electricity produced using these resources will be more than 80% of the total electricity production. It is also expected that the global oil/ petroleum production will begin to lower by a huge percent around the year 2030 (Demirbas 2009).

2. BIOMASS and BIOETHANOL:
Biomass refers to organic materials that can be derived from plants, animals and microorganisms. It includes materials such as sugar rich plants, starchy crops, wood, herbaceous species, municipal solid waste, bagasse, algae, and animal wastes. Biomass can be used to produce liquid biofuels, heat, or electricity depending on the feedstock and
Figure 1.1: Main biomass conversion process, adapted from Demirbas (2009)
conversion process used (Demirbas 2009). Figure 1.1 shows the main biomass to biofuel/biochemical conversion pathways. Depending on the type of biomass used, biofuels produced from biomass can be classified as:

- **First generation biofuels** – refers to biofuels produced from sugars and starch using conventional technology.
- **Second generation biofuels** – refers to biofuels derived from non-food crops such as miscanthus and switchgrass.
- **Third generation biofuels** – fuels produced from algae.
- **Fourth generation biofuels** – fuels produced from the conversion of vegetable oil and biodiesel into biogasoline.

Use of biomass as a feedstock for biofuel and bioenergy production offers many advantages such as a) renewability, b) reduction in green house gas emissions and c) significant economic potential owing to increasing global oil prices (Demirbas 2009). Consequently, biofuels, which hold the key to sustainable production of liquid transportation fuels without compromising the needs of future generations, can help alleviate problems associated with conventional energy sources. They can be produced from easily available feedstocks, are environmentally friendly, and offer biodegradability and sustainability (Puppan 2002). Of the various biofuels being investigated, bioethanol has gained significant footage due to its ability to be blended with existing transportation fuels (gasoline), without the requirement to modify the engine. Feedstocks that can be used for bioethanol production are classified into three types (Demirbas 2009):
• Sucrose based: eg: Sugarcane, sugar beets, sweet sorghum.
• Starch based: eg: Corn, potato.
• Lignocellulosic: eg: Corn stover, miscanthus, switchgrass, and wheat straw.

While significant work has already been done on conversion of sucrose and starch based feedstocks to biofuels like bioethanol and the processes are well established in many cases, conversion of lignocellulosic biomass to bioethanol still remains a challenge. There is a continual need to develop processes for efficient conversion of these non-food based feedstocks like miscanthus and switchgrass which contains cellulose, hemicellulose, lignin, extractives, lipids, proteins, simple sugars, water, ash and other compounds. The components that are of significant value are cellulose, hemicelluloses and lignin. Cellulose consists of D-glucose units that are linked by β-1, 4 glycosidic bonds and exists in both crystalline and amorphous form in plants. Hemicellulose is a complex carbohydrate made of both hexose and pentose sugars. Hemicellulose acts as a connection between the lignin and the cellulose fibers. Lignin essentially consists of non sugar macromolecules (Demirbas 2009). It is an amorphous heteropolymer made of three different phenyl propane units: p-coumaryl, coniferyl and sinapyl alcohol. It acts as the plants structural support, and gives resistance against microbial attack and oxidative stress (Hendriks and Zeeman 2009).

Conversion of lignocellulosic biomass to bioethanol, can be achieved by two methods (Huber et al. 2006a), (Clausen and Gaddy 1996b):

• Hydrolysis/ Fermentation: Conversion of biomass to sugars by acid/enzymatic hydrolysis, followed by fermentation. There is however a need for preprocessing the
biomass for enhancing hydrolytic efficiency. Figure 1.2 shows a typical process flow diagram for biological conversion of lignocellulosic biomass into ethanol (da Costa Sousa et al. 2009)

- Gasification/ Fermentation: Conversion of biomass to syngas by gasification, followed by either catalytic conversion or fermentation.

Biological conversion of lignocellulosic biomass to bioethanol has been investigated for a variety of feedstocks such as sugar cane, miscanthus, switchgrass and corn stover. Apart from process efficiency, the key to maximizing ethanol yields with this method require that the feedstocks have high biomass yields, contain high levels of carbohydrates and low levels of lignin to allow for generation of high amounts of fermentable sugars.
Figure 1.2: Process flow diagram of unit operations for biological conversion of lignocellulosic biomass into ethanol, adapted from da Costa Sousa et al. (2009)
3. Miscanthus:

Lignocellulosic feedstocks such as switchgrass, miscanthus, waste paper and municipal solid wastes are considered as better alternative to already existing starch based feedstocks such as sugarcane, wheat, corn and sorghum as they do not compete with food crops and are less expensive (Pejo et al. 2008).

*Miscanthus* is a perennial rhizomatous grass capable of growing to a height of 3.5 m (approximately) and uses the C4 photosynthesis pathway (Carroll and Somerville 2009). It belongs to the Poaceae family and originates from the tropics and subtropics. However, different species are nowadays found at various climatic regions in East Asia, Europe and America (Villaverde et al. 2009). *Miscanthus* is known to hybridize with other genera especially *Saccharum* and they are both closely related to each other; the only difference being the presence of spikelets and the fragility of rachis (Hodkinson et al. 2002; Scally et al. 2001). *Miscanthus* has been used as a forage crop in Japan and as an ornamental crop in U.S (Jessup 2009). It is known to have one of the highest energy intensity per hectare land in Europe (Hastings et al. 2008). It has been observed that perennial miscanthus has a energy yield per hectare of the order of 204 GJ/ha (Sims et al. 2006). Some of the varieties of miscanthus that are known to have biomass potential are *Miscanthus sinensis*, *M. sacchariflorus*, and the hybrid *Miscanthus ×giganteus* (Villaverde et al. 2009). The varieties used in this study included *M. ×giganteus*, *M. sinensis* ‘Gracillimus’, *Saccharum arundinaceum* (*Erianthus arundinaceus*) and *Saccharum ravennae* (*Erianthus ravennae*). Miscanthus has holocellulose (cellulose + hemicellulose) content of the order of 64-71%.
There is approximately 25-27% lignin (klason), 1.2% acid-insoluble lignin, 40% α-cellulose, 33% xylose, 0.1% mannose, 0.6% galactose, 2.8% arabinose, and 1.5-4.5% ash content (Brosse et al. 2009; Scurlock 1999; Ververis et al. 2004). A comparative study between miscanthus and switchgrass performed at Illinois, USA reported that miscanthus had a maximum yield of 61 t/ha while switchgrass produced a yield of 10 t/ha (Heaton et al. 2008). This yield was consistent regardless of rainfall, nitrogen fertilizer or growing degree days. A comparative study between miscanthus and maize conducted concluded that miscanthus produced 59% more biomass than maize (Dohleman and Long 2009).

Hence, because of its high biomass yield and preferred carbohydrate content, miscanthus has been identified as a suitable candidate for ethanol production. It has the potential to produce 133x10⁹ L of ethanol, equivalent to one fifth of the nation’s gasoline usage, from 12 million hectare of land which is significantly high compared to maize which can produce 49x10⁹ L of ethanol (Heaton et al. 2008). According to Heaton et al. (2008), miscanthus is also reported to be more than twice as productive as switchgrass, showing higher rates of photosynthesis and requiring less water and nitrogen than switchgrass.

Apart for use in the production of bioethanol, miscanthus can also be used for the production of building materials, medium density fiber board, and as a thatching material. Use of miscanthus as a bioremediation and composting agent and the use of its ash as fertilizer are also under investigation (Visser and Pignatelli 2001)
4. CONVERSION OF LIGNOCELLULOSIC BIOMASS TO ETHANOL

Lignocellulosic biomass refers to the materials that constitute the plant cell wall, which is primarily composed of cellulose (30-50%), hemicellulose (15-35%) and lignin (10-30%) (da Costa Sousa et al. 2009). The structure and organization of these polymers makes the plant cell, biologically resistant to various external factors (Himmel et al. 2007). The complex network of cellulose, hemicellulose and lignin is therefore a major barrier to the production of chemicals and fuels from lignocellulosic biomass such as miscanthus and switchgrass.

4.1 PRETREATMENT:

The most crucial step in ethanol production from lignocellulosic biomass is the hydrolysis of cellulose and hemicellulose to monomeric sugars. Hydrolysis is best carried out by enzymes such as cellulase; however, efficient hydrolysis can only be performed if cellulose and hemicellulose are easily accessible to the enzymes (Galbe and Zacchi 2007; Mosier et al. 2005). However, lignin acts as a shield and prevents the accessibility of substrates for enzymatic hydrolysis thus limiting the rate of enzymatic hydrolysis (Chang and Holtzapple 2000). Conversion of biomass to ethanol requires a pretreatment step to solubilize lignin and hemicellulose for enhancing hydrolysis, by up to 10 folds by improving accessibility of enzymes to cellulose and hemicellulose (Galbe and Zacchi 2007; Mosier et al. 2005).

Pretreatment is essential as enzymatic hydrolysis of cellulose yields 20% less glucose than the potential (Wright 1988). Figure 1.3 shows the action of pretreatment on lignocellulosic biomass (Hsu et al. 1980).

Pretreatment accounts for 16-19% of the total capital investment and is the second largest
Figure 1.3: Action of pretreatment on lignocellulosic biomass – adapted from Hsu et al. (1980)
expense for a lignocellulosic biorefinery after power plant generator costs (Aden et al. 2002; Wooley et al. 1999). Pretreatment accounted to 30 ¢/gallon ethanol produced through lignocellulosic conversion of biomass (Mosier et al. 2005). Pretreatment prior to hydrolysis, helps in breaking the lignin structure and disrupts cellulose crystallinity making cellulose more accessible to enzymes (cellulases) that convert the carbohydrates into fermentable sugars (Viikari et al. 2012). Without pretreatment, enzymatic hydrolysis of cellulose yields 20% less glucose than its maximum potential (Wright 1988).

In order to be effective, a pretreatment should a) avoid the need for size reduction of biomass, b) minimize sugar loss, c) preserve the hemicellulose fraction, d) maximize enzymatic convertibility/ hydrolysis, e) limit the formation of degradation products that might inhibit the enzyme or the microorganisms used, f) minimize energy demand and g) be scalable to industrial size (Jorgensen et al. 2007; Mosier et al. 2005).

4.2 PRETREATMENT TECHNIQUES:

Lignocellulosic pretreatment techniques can be categorized as physical, chemical, biological and physio-chemical (Pejo et al. 2008; Taherzadeh and Karimi 2008). Physical pretreatment methods include comminution, irradiation, steam explosion, and hydrothermolysis, whereas chemical pretreatments use acids, bases, and ammonia (Hsu 1996).

Physical pretreatments

Physical pretreatment techniques such as milling (ball milling, hammer milling, colloid milling) and irradiation (gamma rays, ultrasound, microwaves or electron beam) increase
surface area by reducing the particle size (Palmowski and Muller 2000; Taherzadeh and Karimi 2008). They also cause shearing and reduce the degree of polymerization of cellulose, which can increase hydrolysis yield by 5-25% while reducing digestion time by 23-59% (Delgenes et al. 2002). Although no product inhibitors are produced during physical pretreatment, this method has a very high energy requirement which makes it economically unsuitable (Hendriks and Zeeman 2009).

Ultrasonication in another physical pretreatment technique that uses sound waves to disintegrate cell components (Nitayavardhana et al. 2008). The non-thermal effect of ultrasound, known as cavitation, produces bubbles that oscillate in a regular fashion which generates a negative pressure that result in the formation of microbubbles. These microbubbles on implosion produce strong hydrodynamic shear forces in the liquid phase which causes a shear stress on cells resulting in their disintegration. Disintegration of cells increases their surface area to enzymes during enzymatic hydrolysis (Nitayavardhana et al. 2008; Sinisterra 1992).

**Chemical pretreatments**

Chemical pretreatment techniques include the use of acid, alkali, organosolvents, peroxides and ozone among others (Keshwani and Cheng 2009). In dilute acid pretreatment, biomass is mixed with an aqueous acid mixture at acid concentrations ranging from 0.2 to 1.5%. The mixture is heated to temperature above 140°C and held for periods ranging from seconds to minutes. The acid can be added to the biomass mixture by spraying onto the heated solids,
direct steam injection, or agitated with the biomass in a reactor (Mosier et al. 2005; Saha et al. 2008; Sorensen et al. 2008; Sun and Cheng 2005). Dilute acid pretreatment results in solublization of hemicellulose to mono- and oligo- saccharides and removal of hemicellulose increases the accessibility of enzymes to cellulose (Jorgensen et al. 2007). Sulfuric acid is the most widely used acid as it is inexpensive and effectively removes hemicellulose (Galbe and Zacchi 2007).

During alkali treatment, alkaline solutions of NaOH, Ca(OH)\textsubscript{2} or NH\textsubscript{3} are applied to cause swelling of the plant biomass pores thereby increasing the internal surface area and decreasing the degree of polymerization and crystallinity. Alkaline pretreatment results in the breakage of bonds between lignin and carbohydrates leading to disruption of the lignin structure while retaining cellulose and a significant portion of hemicellulose in the recovered solids (Galbe and Zacchi 2007; Hendriks and Zeeman 2009; Jorgensen et al. 2007). Sodium hydroxide pretreatment of coastal Bermuda grass reduced lignin content by up to 86\% and increased glucan and xylan conversions to 90.4 and 65.1 \%, respectively (Wang et al. 2010) while lime pretreatment of switchgrass with reduced lignin content by 35.5\% (Xu et al. 2010). Another chemical approach for pretreating lignocellulosic biomass is the organosolv method which uses an organic aqueous solvent along with an inorganic acid catalyst. Interaction between the 2 chemicals causes bond breakage between lignin and carbohydrate (da Costa Sousa et al. 2009; Sun and Cheng 2002). The hydrolyzed lignin is recovered from the organophillic phase. Operating temperatures during organosolv treatments ranges between 90 – 120\degrees\textdegree\textsuperscript{C} for grasses and 220\degrees\textdegree\textsuperscript{C} for wood with residence time ranging between 25-
100 min. Catalyst concentration varies between 0.83-1.67%, while alcohol concentration ranges between 25-74%. Both catalyst and alcohol concentrations depends on type of biomass (da Costa Sousa et al. 2009). Methanol, ethanol, acetone, phenol are some of the organic solvents that have been reported as being used along with either sulfuric acid or hydrochloric acid (Galbe and Zacchi 2007; Taherzadeh and Karimi 2008) during organosolv treatments. Majority of the hemicellulose can be extracted using this method and the lignin obtained is of high quality which can potentially add extra income to a biorefinery (Jorgensen et al. 2007).

Ozonolysis is a pretreatment method in which the lignocellulosic materials are treated with ozone to degrade lignin. This method is known to effectively remove lignin and hemicellulose (Taherzadeh and Karimi 2008). Ozone is a very strong oxidizing agent that can be produced by passing oxygen through an electrical discharge where the oxygen molecules dissociate to form ozone (Eckert and Singh 1975). Ozone has been used to degrade lignin in feedstocks such as bagasse, green hay, pine (Neely 1984), wheat, rye straw (Garcia-Cubero et al. 2009) and poplar sawdust (Vidal and Molinier 1988).

**Physio-chemical pretreatments**

Physio-chemical treatment encompasses steam explosion, hydrothermolysis, and ammonia fiber explosion (AFEX). Steam explosion pretreatment involves exposing the biomass to high pressure (6-34 bar) saturated steam at temperature ranging from 160-240°C. These conditions are held for a time ranging from seconds to a few minutes (Galbe and Zacchi 2007; Sun and Cheng 2002). During this treatment, hemicellulose is solublized into
oligomeric or monomeric sugars and released in to the liquid phase which in turn increases the accessibility of cellulose found in the solid phase to enzyme activity. It has been reported that the efficiency of steam pretreatment can be increased by using an acid catalyst such as H$_2$SO$_4$ or SO$_2$ (Galbe and Zacchi 2007). A disadvantage of this process is the degradation of xylan leading to the subsequent formation of inhibitory compounds for microbial populations (Mueller 2009).

In hydrothermolysis, water maintained at high pressure is used to penetrate the biomass causing removal of hemicellulose and lignin along with hydration of cellulose. It is reported that no inhibitory components are produced during the process (da Costa Sousa et al. 2009; Taherzadeh and Karimi 2008). A major advantage of this process is of the fact that it does not need other chemicals, making it economical (Suryawati et al. 2009).

Ammonia fiber explosion (AFEX) is a pretreatment process similar to steam explosion with regards to the use of high temperature and pressure but requires the addition of ammonia. This process does not produce any inhibitory compounds. However, the ammonia used needs to be recycled owing to environmental issues (Mueller 2009). Moreover, AFEX treated biomass requires enzymes that can hydrolyze the hemicelluloses component in addition to cellulases to produce fermentable sugars (Jorgensen et al. 2007).

**Biological pretreatments**

Biological pretreatments are performed by employing lignin degrading microorganisms like white-, brown- and soft- rot fungi. White- rot fungi are the most effective microorganism for lignocellulosic materials. These organisms secrete extracellular enzymes such as lignin
peroxidases and laccases that remove lignin from biomass (Christian et al. 2005). The advantage of this method is that it is environmentally friendly and energy saving as it requires low temperature and no chemicals for processing. However, the rate of reaction is very slow and loss of sugars is reported as most microorganisms consume sugars made available by digestion of carbohydrates by the microbes themselves (Galbe and Zacchi 2007; Taherzadeh and Karimi 2008).

Although a number of pretreatment techniques have shown potential in overcoming challenges related to hydrolysis of lignocellulosic biomass, high energy requirements, need for handling toxic waste streams has highlighted the need for investigating pretreatments that are not energy intensive and are environmentally friendly. Of the various pretreatments mentioned ozonolysis is a potential candidate because it has various advantages such as a) absence of toxic residues in treated material, b) suitability for onsite generation, thus avoiding the problem of chemical supply c) ozonation reaction takes place at ambient temperature and pressure, d) effective degradation/solubilization of lignin and hemicellulose without significant effect on cellulose, e) biodegradability of degradation fragments and potential for ozonolysis products like formic acid and acetic acid to be metabolized by animals (Neely 1984; Vidal and Molinier 1988).

4.2.1 OZONOLYSIS AS A PRETREATMENT TECHNIQUE

Ozonolysis is a pretreatment method in which the lignocellulosic material is treated with ozone that’s generated on site. This method is known to effectively remove lignin and hemicellulose (Taherzadeh and Karimi 2008). Ozone is a very strong oxidizing agent that
can be produced by passing oxygen through an electrical discharge where the oxygen molecules dissociate to form ozone (Eckert and Singh 1975). In a non–aqueous media, ozone reacts with organic substrates by cleaving the olefinic and activated aromatic bonds. However, in aqueous media, ozone abstracts an electron from easily oxidized organic substrates such as phenolates. This electron transfer produces either hydroxyl radicals or superoxide as shown by equations 1.1 and 1.2, respectively. It is hard to decide which of the radical species get formed initially as they can be easily transformed into one another. Figure 1.4 shows the reaction mediated by superoxide radicals in the presence of oxygen and an organic substrate.

\[ RH + O_3 \rightarrow R + HO^\cdot + O_2 \]  
(1.1)  
\[ RH + O_3 \rightarrow RO + H^+ + O_2^- \]  
(1.2)  

It is believed that ozone has a high reactivity towards compounds having double bonds and functional groups with high electron densities. In lignocellulosic compounds the moiety that is most likely to be oxidized is lignin, owing to its high carbon- carbon double bonds (Garcia-Cubero et al. 2009).
Figure 1.4: Chain reaction of ozone decomposition in the presence of oxygen and organic substrate such as phenol. Adapted from Ragnar et al. 1999.
Ozonolysis of wheat straw removed 60% of lignin and increased the rate of enzymatic hydrolysis by a factor of five (Binder and Scharf 1980). Treatment in poplar sawdust decreased the lignin content from 29% to 8% which led to an increase in hydrolysis yield from 0% to 57% in poplar sawdust (Vidal and Molinier 1988). Garcia – Cubero et al. (2009) observed that ozonolysis increased enzymatic hydrolysis yield from 29% and 16% to 88.6% in wheat straw and to 57% in rye straw. Parameters that are of significance for efficient ozonolysis according to Neely (1984) are water content of biomass (25-35%) and ozone concentration (2-6%). According to Neely, the biomass need not be reduced to smaller size, this is of importance as biomass preparation for pretreatment holds a significant portion of the process cost, he also observed that a minimum time of 10 minutes produces a useful degree of pretreatment while maximum pretreatment efficiency could be produced by treating for 30 minutes.

4.3 HYDROLYSIS:

The major chemical components of various biomass/feedstocks are cellulose, hemicellulose and lignin (Clausen and Gaddy 1996b). During hydrolysis, the polysaccharides cellulose and hemicellulose are hydrolyzed to glucose and a mixture of 5- and 6- carbon sugars, respectively, by using a weak acid (sulfuric acid, hydrochloric acid) solution or cellulolytic enzymes. The hydrolytic conversion of cellulose to glucose molecules is shown in Equation 1.6. Glucose produced during hydrolysis can be further converted to ethanol by fermentation (Huber et al. 2006c) as shown in Equation 1.7.

\[(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \]  

(1.6)
nC₆H₁₂O₆ → 2C₂H₅OH + 2CO₂  \hspace{1cm} (1.7)

One method of hydrolyzing cellulose into glucose for fermentation is by using extracellular enzymes produced by certain fungi. The cellulase enzyme system obtained from *Trichoderma* species is the most commonly used system. It consists of three classes of enzymes:

- **Cellobiohydrolases or exo-1, 4-β-D-glucanases** – move along the cellulose chain and cleave cellobiose units from the end.
- **Endo-1,4-β-D-gluconses** – hydrolyse internal glucosidic bonds on the cellulose chains.
- **1, 4 – β-D-glucosidases** - hydrolyze cellobiose to glucose and also cleave glucose units from oligosaccharides.

To begin reducing the polymerization of cellulose, endoglucanases hydrolyze random glucosidic bonds in cellulose and open the crystalline structure into long chains.

Exocellulases then move along these long chains and release cellobiose units. The released cellobiose units get split into individual glucose monomers by the action of β-D-glucosidases. All the enzymes work synergistically to hydrolyze cellulose by creating new sites for each other, removing obstacles and relieving product inhibition (Demirbas 2005; Jorgensen et al. 2007). Figure 1.5 shows the overview of cellulase action on lignocellulosic biomass. Apart for cellulose hydrolyzing enzymes, some feedstocks (depending on composition and/or pretreatment) may require the use of enzymes for hemicelluloses.
hydrolysis. Some of the enzymes that are capable of hydrolyzing hemicellulose include glucuronidase, acetylene, xylan, \( \beta \) – xylosidase and glucomannanase (Duff and Murray 1996).

Optimum temperature required for cellulose hydrolysis by enzymes from *Trichoderma* species is between 30-50°C (Faga 2009; Zhang and Lynd 2004). The presence of cellobiose and glucose decreases the rate of cellulose and cellobiose hydrolysis by product inhibition of cellulase and \( \beta \)-glucosidase enzymes, respectively (Philippidis et al. 1993). Varying the ratio of endocellulase, exocellulase, and \( \beta \)-glucosidase from that found in natural systems can increase the effectiveness of the cellulase systems (Zhou et al. 2009). Increasing the effectiveness of cellulase enzymes by pretreatment and optimization is significant as the cost and cellulolytic efficiency of enzymes are major economic factors that are slowing the commercialization of cellulosic ethanol process (Faga 2009).

The process of enzymatic hydrolysis has several operational problems. It suffers from conversion inefficiencies due to the lignin fraction of the biomass which cannot be converted to ethanol and also pentose sugars produced from hemicellulose cannot be fermented to ethanol with proven technology (Clausen and Gaddy 1996b; Yang et al. 2011). Moreover, efficiency of this process depends on the nature of the substrate. The substrate must be clean, well milled and pretreated to increase the yield (Cheremisinoff et al. 1980; Lin and Tanaka 2006).

In a study on organosolv pretreatment of miscanthus (presoaked with dilute sulfuric acid) using aqueous ethanol with sulfuric acid as catalyst it was observed that 98% cellulose to
glucose conversion could be obtained up on hydrolysis with cellulase supplemented with β-glucosidase (Brosse et al. 2009). In another study, hydrolysis of acid (H$_2$SO$_4$) presoaked miscanthus pretreated by wet explosion method resulted in a yield of 94.9% xylose and 61.3% glucose when atmospheric air was used as an oxidizing agent (Sorensen et al. 2008).
Figure 1.5: Overview of cellulase enzyme hydrolysis, adapted from Jorgensen et al. (2007)
4.4 FERMENTATION:

Fermentable sugars obtained from hydrolysis can be fermented into ethanol by ethanol producing microorganisms, which may be naturally occurring or genetically engineered. Hexoses such as glucose, galactose and mannose are readily fermented to ethanol by various microorganisms however pentose sugars such as xylose, arabinose are fermented only by few native strains that typically have very low yields. In order to make the economics of cellulosic ethanol production possible it is essential to utilize the pentose sugars. Recent advances have been made to develop various recombinant strains of bacteria and yeasts that are capable of co-fermenting pentoses and hexoses into ethanol at high yields (Zheng et al. 2009). As an estimate, theoretical yield of ethanol is 0.511 on the basis of mass of glucose metabolized.

Saccharomyces cerevisiae is the most widely used industrial fermentation microorganism. It has high ethanol productivity and tolerance. One of the main disadvantages observed with S. cerevisiae is its inability to ferment pentoses. However recombinant strains with genes encoded for xylose degradation has made it possible for it to ferment xylose (Pejo et al. 2008). S. cerevisiae has been reported to yield higher ethanol at a pH ranging between 5-5.5. Weak organic acids such as acetic, malic, lactic and malonic acids are known to inhibit the growth of S. cerevisiae as they lower the pH. S. cerevisiae is known to work best at 30ºC, higher temperature could stress the yeast and make them susceptible to other stresses such as low pH and ethanol (Wilkins et al. 2007). S. cerevisiae follows the Embden – Meyerhof-Parnas (EMP) pathway through which it metabolizes glucose and produces ethanol under
anaerobic conditions with the release of carbon dioxide (CO$_2$). Ethanol production by \textit{S. cerevisiae} is coupled with cell growth as metabolism of glucose yields two ATPs that are used for cell biosynthesis. Failure to consume the ATPs leads to interruption of glucose metabolism (Bai et al. 2008). According to Bai et al. (2008), yeast cells suffer various environmental and metabolic stresses during ethanol fermentation that could affect the viability of the cell and lower ethanol yield. Figure 1.6 shows the various stresses that a yeast cell is subjected, during fermentation.

\textit{Zymomonas mobilis}- an anaerobic, gram negative bacterium is capable of producing ethanol from glucose. It utilizes the Entner-Doudoroff (ED) pathway to ferment glucose to produce ethanol. \textit{Z.mobilis} is considered a better fermentation organism than \textit{S. cerevisiae} as it produces less biomass and funnels more carbon towards ethanol fermentation. Theoretical yield could be as high as 97% with \textit{Z. mobilis} while only 90-93% could be achieved with \textit{S.cerevisiae}. However, \textit{Z.mobilis} has a very specific substrate spectrum which includes only three sugars: D-glucose, D-fructose and sucrose. Its growth on sucrose produces fructose oligomers and sorbitol which reduces ethanol yield, making it unsuitable for fermenting molasses and starch materials, thus favoring \textit{S.cerevisiae} for industrial ethanol production (Bai et al. 2008).

Since pentoses constitute a significant portion of the fermentable sugars that can be produced from lignocellulosic biomass, efforts have been focused towards utilization of these sugars for ethanol production. Although some organisms have been identified as being capable of fermenting xylose, \textit{Pichia stipitis}, \textit{Candida shehatae} and \textit{Pachysolen tannophilus}, they have
Figure 1.6: Factors affecting growth and metabolism of *S. cerevisiae* (Ingledew 1999).
less tolerance to ethanol concentration, require microaerophilic conditions and are highly sensitive to inhibitors and pH changes. Some other alternatives like *Escherichia coli*, *Klebsiella oxytoca* and *Kluveromyces marxianus* (Pejo et al. 2008) also have limitations and need further investigation.

5. CONCLUSION:
The U.S produced 7.96 billion gallons of ethanol in 2011 (EIA 2012), most of which was produced from corn. However, with the ongoing food vs. fuel debate and the increasing demand for energy around the world, it becomes essential to look for alternative resources. Lignocellulosic feedstocks are a potential substitute for sugar based feedstocks and are capable of replacing 40% of gasoline in U.S (Sun and Cheng 2002). Use of lignocellulosic materials can also reduce the cost of ethanol as the biomass required is of low cost. However, though bioethanol production from lignocellulosic biomass has advanced, there are still challenges that need to be addressed. Developing more efficient, environmental friendly pretreatment technologies for lignocellulosic biomass is a sector that needs to be looked upon as it has direct influence on the economics of ethanol production.

With this background, this study was undertaken to investigate the potential of ozonolysis as a pretreatment method for *Miscanthus* and *Saccharum* varieties. The objectives of this research were:

- Optimization of ozone pretreatment of energy grasses.
- Optimization of enzymatic hydrolysis of the ozone pretreated biomass.
• Comparison of alkali and ozone pretreated energy grasses based on glucan conversions.

• To understand the kinetics of the gaseous ozone on the biomass.
REFERENCES


EIA. 2012. How much ethanol is produced, imported, and consumed in the U.S.?


CHAPTER 2:
POTENTIAL OF OZONOLYSIS AS A PRETREATMENT FOR ENERGY GRASSES

ABSTRACT:
Ozonolysis as a pretreatment has been attempted on a variety of lignocellulosic feedstocks as it is highly effective in lignin removal and is reported to be less inhibitory compared to conventional chemical pretreatments. This study investigated the effect of ozonolysis on Miscanthus × giganteus, M. sinensis ‘Gracillimus’, Saccharum arundinaceum and S. ravennae, collectively termed as “energy grasses”. Studies were conducted at three different ozone concentrations (40, 50 and 58 mg/L) using two reactor configurations – uniflow and flip. Ozone pretreatment conditions for each variety were optimized based on lignin content and glucan recovery in the pretreated solids. Effect of pretreatment on cellulolytic enzyme efficiency was evaluated by conducting enzymatic hydrolysis with 0.06g/ g raw biomass of Cellic® CTec2. Results showed that ozonolysis was an effective pretreatment method that removed up to 59.9% lignin without cellulose degradation. However, hydrolysis of pretreated solids had lower glucan conversion than untreated samples suggesting potential enzyme inhibition by lignin by-products formed during ozonolysis. Future studies investigating hydrolysis efficiency of washed pretreated solids with higher enzyme loadings are warranted to improve the hydrolysis process and make it functionally feasible.

Keywords:
Miscanthus, saccharum, optimization, reactor configuration, enzymatic hydrolysis.
1. INTRODUCTION:

Production of biofuels from lignocellulosic materials such as wood and agricultural residues has gained immense significance worldwide with an increasing number of countries aspiring to attain a secure and sustainable energy supply (Galbe and Zacchi 2007). In this respect, bioethanol has been identified as the predominant renewable liquid fuel due to the ease of blending it with existing fuels as a gasoline blend. Bioethanol can be produced from lignocellulosic materials by hydrolyzing the biomass to sugars with acid or enzymes (Chang and Holtzapple 2000; Clausen and Gaddy 1996a). However, direct enzymatic hydrolysis is not economically favorable as lignocellulosic feedstocks require pretreatment to remove lignin and facilitate cellulose access to the enzymes during hydrolysis (Himmel et al. 2007; Zheng et al. 2009). Although, significant research has been conducted on physical, chemical and physio-chemical pretreatment techniques for solublizing lignin and/or hemicelluloses in plant biomass, drawbacks such as need for strong chemicals, high energy inputs, generation of toxic waste streams, and partial degradation of the carbohydrate component during pretreatment have limited wide scale application of these methods.

Gaseous ozone is a strong oxidizing agent with very high reactivity towards compounds having double bonds and functional groups with high densities. In the case of lignocellulosic materials the moiety with a high carbon-carbon double bond is lignin which is responsible for the materials recalcitrant nature (Garcia-Cubero et al. 2009). Due to ozone’s reactivity towards lignin and its ability to delignify feedstocks, ozonolysis has the potential to be used as a pretreatment technique. Ozone is reported to have various advantages such as (a) on site
generation—thus avoiding problems associated with chemical supply and storage, (b) reaction at ambient temperature and pressure, (c) ability to effectively degrade/solublize lignin, (d) biodegradability of degradation fragments and (e) ozonolysis products like formic acid and acetic acid can be metabolized by animals (Neely 1984; Vidal and Molinier 1988).

Parameters that are of significance for efficient ozonolysis are moisture content and ozone concentration (Neely 1984; Vidal and Molinier 1988). Neely (1984) suggested that biomass moisture content of 25-35% and ozone concentration of 2-6% (w/w) was suitable for enhancing ozonolysis. Ozonolysis of wheat straw removed 60% of lignin and increased the rate of enzymatic hydrolysis by a factor of five (Binder and Scharf 1980). Treatment of poplar sawdust decreased its lignin content from 29 to 8% leading to an increase in hydrolysis yield from 0 to 57% (Vidal and Molinier 1988). Garcia–Cubero et al. (2009) also observed that ozonolysis increased enzymatic hydrolysis yield from 29 and 16% to 88.6 and 57% in wheat straw and rye straw, respectively.

Gauging the potential of ozonolysis, 4 energy grass varieties (M. × giganteus, M. sinensis ‘Gracillimus’, S. arundinaceum and S. ravennae), representing lignocellulosic biomass, were treated with gaseous ozone in this study. The term “energy grass” represents these feedstocks, due to their potential as lignocellulosic energy sources. Miscanthus is closely related to sugarcanes (S. spp.), sorghum (S. bicolor) and maize (Z. mays) (Dohleman and Long 2009) and has been found to generate more biomass per unit area (13.2 tons/acre) than corn (7.8 tons/acre) and switchgrass (4.6 tons/acre) (Heaton et al. 2008; Somerville et al. 2010). Along with saccharum varieties,
miscanthus has very high sugar content and is expected to have a high potential for use as an energy crop.

Ozonolysis of the above mentioned four energy grass varieties was carried out in a packed bed reactor at 3 ozone concentrations to develop a process for maximized hydrolytic sugar production. Two reactor configurations were investigated to study the impact of mass transfer conditions in the sample bed. Effectiveness of pretreatment was evaluated by determining the extent of delignification and carbohydrate (cellulose and hemicellulose) recovery in pretreated samples. Optimal pretreatment conditions for each of the energy grasses were obtained on the basis of highest delignification and glucan content. Samples were also hydrolyzed to determine the effect of ozone on cellulolytic enzyme efficiency and fermentable sugar production.

2 MATERIALS AND METHODS:

2.1 Biomass Preparation:

Energy grasses (M. ×giganteus, M. sinensis ‘Gracillimus’, S. arundinaceum and S. ravennae) from the Mountain Horticultural Crops Research and Extension Center (Mills River, NC) harvested in December 2009 and dried at 45°C for 72h were ground to pass a 2mm sieve using a Wiley mill (Model: 4, Thomas, Philadelphia, PA, USA). Ground samples were stored in ziplock bags at room temperature until further use.

2.2 Ozone Pretreatment:

Samples from each of the 4 energy grass varieties were prepared for ozonolysis by adding
30% moisture (dry basis) to 5g of ground, oven dried –moisture free biomass and allowed to equilibrate for an hour. The sample was placed in a vertically aligned glass column reactor tube of 5cm diameter and 50 cm height (Product no: 5813 – 26, Ace glassware, NJ, USA) for ozonolysis. One end of the reactor was plugged with glass wool to support the biomass inside the reactor while ozone gas was introduced from the other end. Three ozone concentrations – 40, 50 and 58mg/L, were tested for each energy grass variety in a factorial design (4 varieties x 3 concentrations x 2 reactor configurations). Ozone was produced on-site by an ozone generator (Model: OL80 A, Ozone lab instrument, Canada) supplied with industrial grade oxygen (Airgas National Welders, Raleigh, NC). The oxygen flow rate of 0.25 L/min was maintained throughout the 2 hour treatment using a mass flow controller (Model no: FMA5516, Omega, CT, USA).

Effect of change in reactor configuration was studied by changing the direction of gas flowing into the reactor. One batch of samples was pretreated with ozone flow in one direction (uniflow) and another batch was pretreated by reversing the flow of gas by flipping the reactor tube after 1 hour (flip) to overcome gas-solid mass transfer limitations. Pretreated samples were manually stirred and stored in ziplock bags until further analysis for determination of solid recovery, acid insoluble lignin, acid soluble lignin and carbohydrate (cellulose and hemicellulose as represented by glucose, xylose and arabinose measurements) contents.

2.3 Enzymatic Hydrolysis:

Enzymatic hydrolysis was performed on ozone pretreated samples at 8% solid loading (dry
basis) using Cellic® Ctec2 provided by Novozymes North America, Inc. (Franklinton, North Carolina) added at 0.06 g enzyme protein/ g dry biomass (FPU= 136/ml). The total volume was made up to 20 ml in 50ml tubes with 0.5 M citrate buffer (pH 4.8) containing 40µg/ml tetracycline hydrochloride (ICN Biomedicals, Inc. CA, USA). Hydrolysis was conducted in a water bath shaker (Model: 89032-226, VWR International, PA, USA) at 50 ºC, 150 rpm for 72 h. After hydrolysis, the samples were centrifuged at 4000 rpm and 4ºC for 10 min and the supernatant was filtered through 0.2µm syringe filters and stored at -80ºC for subsequent high performance liquid chromatography (HPLC) analysis for fermentable sugar determination. Untreated samples were also hydrolyzed to serve as control.

2.4 Analytical Methods:

Total solids, acid insoluble lignin (AIL), acid soluble lignin (ASL) and ash content of untreated samples were determined using the procedure described by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2006; Sluiter et al. 2005a; Sluiter et al. 2005b). Monomeric sugars (glucose, xylose and arabinose) in the untreated biomass, pretreated solids recovered and hydrolyzate samples were determined using HPLC (Model: UltiMate 3000, Dionex Corporation, CA, USA) with a Refractive Index (RI) detector. An Aminex HPX-87H column was used at 65 ºC with the eluent, 5mM sulfuric acid, flowing through it at 0.6 ml/min.

2.5 Statistical Analysis:

All experiments in this study were performed in triplicate. Statistical analysis was done using SAS® 9.2 (SAS Institute, Cary, NC) to test the significance of different pretreatment
conditions. Main and interaction effects between the reactor configuration (uniflow and flip) and ozone concentrations for each variety were examined by the Tukey-Kramer test at 95% confidence level was used. The optimal treatment conditions for each variety were chosen based on minimum lignin content and highest glucan recovery after pretreatment.

3 RESULTS AND DISCUSSION:

3.1 Composition of energy grasses:

Table 2.1 shows the composition of the four energy grass varieties (*M. × giganteus, M. sinensis ‘Gracillimus’, S. arundinaceum and S. ravennae*) used in this study. Total lignin content (AIL+ASL) of the varieties ranged between 23.3 - 24.7%, while the total carbohydrate content (glucan+ xylan+ arabinan) ranged between 53.5 - 59.1%. *M. × giganteus* had the highest lignin and carbohydrate content while *S. arundinaceum* had the highest xylan content (18.6%). Moisture content of the energy grasses ranged between 4.07 – 5.70%.
Table 2.1: Initial composition of energy grasses (dry basis).

<table>
<thead>
<tr>
<th></th>
<th>M. ×giganteus</th>
<th>M. sinensis ‘Gracillimus’</th>
<th>S. arundinaceum</th>
<th>S. ravennae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash (%)</td>
<td>1.0±0.00</td>
<td>0.9±0.01</td>
<td>0.9±0.02</td>
<td>1.0±0.02</td>
</tr>
<tr>
<td>AIL (%)</td>
<td>23.3±0.56</td>
<td>21.8±0.1</td>
<td>20.8±0.05</td>
<td>22.5±0.25</td>
</tr>
<tr>
<td>ASL (%)</td>
<td>1.4±0.2</td>
<td>2.0±0.2</td>
<td>2.5±0.04</td>
<td>2.0± 0.07</td>
</tr>
<tr>
<td>Glucan (%)</td>
<td>40.4±4.06</td>
<td>34.2±4.31</td>
<td>34.4±3.14</td>
<td>35.4±3.29</td>
</tr>
<tr>
<td>Xylan (%)</td>
<td>16.9±1.72</td>
<td>17.1±2.3</td>
<td>18.5±1.74</td>
<td>18.2±1.67</td>
</tr>
<tr>
<td>Arabinan (%)</td>
<td>1.7±0.31</td>
<td>2.1±0.52</td>
<td>2.5±0.28</td>
<td>2.3±0.25</td>
</tr>
<tr>
<td>Extractives (%)</td>
<td>1.1±0.03</td>
<td>1.1±0.11</td>
<td>n/a</td>
<td>8.3±0.94</td>
</tr>
</tbody>
</table>
3.2 Effect of ozonolysis on composition of energy grasses

Gaseous ozone is a strong oxidizing agent and has been shown to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Garcia-Cubero et al. 2010), bagasse, peanut, pine (Neely 1984) and poplar sawdust (Vidal and Molinier 1988) with minimal effect on cellulose. Changes in lignin content (AIL) of energy grasses, ozone pretreated using the 2 reactor configurations – flip and uniflow, at the various ozone concentrations used in this study, are shown in Figure 2.1. It was observed that net reduction in AIL ranged between 20.7-59.9% for uniflow and between 27.6-50.4% for the flip study. Lowest lignin was observed in *S. ravennae* (flip) and *S.arundinaceum* (uniflow), indicating higher recalcitrance in miscanthus varieties. Similar results have been observed, for wheat, rye, barley and oats, ozonated in a packed bed reactor, resulting in delignification of 38, 50, 41 and 40.3%, respectively (Garcia-Cubero et al. 2010). An overall increase in delignification (p< 0.05) was observed in *M. sinensis* ‘Gracillimus’ and *S. arundinaceum* samples ozonated in the flip reactor indicating a plausible solid- gas mass transfer limitation in the uniflow configuration. No significant difference in delignification (p> 0.05) of *M. ×giganteus* and *S. ravennae* was observed relative to the two reactor configurations. The effect of ozonolysis on acid soluble lignin (ASL) in the energy grasses is presented in Table 2.2. ASL content increased (p<0.05) after ozonolysis, potentially due to the accumulation of lignin degradation products from AIL (Garcia-Cubero et al. 2010).

Tables 2.3 and 2.4 show the glucan, xylan and arabinan present in energy grass samples after
pretreatment with uniflow and flip reactor configurations, respectively. Statistical inference on comparison of glucan concentration in untreated and pretreated solids showed that there was no significant difference (p >0.05) between them. This indicates that ozone as a pretreatment method did not have a detrimental effect on carbohydrate recovery and has the potential to be used for treating energy grasses. Conversely, significant solubilization of xylan occurred in most samples after pretreatment and xylan content after pretreatment was significantly lower than that of the untreated (p<0.05). This trend suggests that hemicellulose is more susceptible to ozonolysis than cellulose.
Figure 2.1: Acid insoluble lignin content of energy grasses pretreated with ozone using a) uniflow and b) flip reactor configurations.
Table 2.2: Acid soluble lignin (%) content of energy grasses pretreated with ozone using uniflow and flip reactor configurations.

<table>
<thead>
<tr>
<th>Reactor configuration/ Ozone concentration (mg/L)</th>
<th>$M. \times$ giganteus</th>
<th>$M. \ sinensis$ ‘Gracillimus’</th>
<th>$S. \ arundinaceum$</th>
<th>$S. \ ravennae$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.41±0.09</td>
<td>2.03±0.11</td>
<td>2.51±0.04</td>
<td>2.01±0.07</td>
</tr>
<tr>
<td><strong>Uniflow</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>2.88±0.11</td>
<td>4.22±0.37</td>
<td>4.95±0.71</td>
<td>3.88±0.05</td>
</tr>
<tr>
<td>50</td>
<td>3.14±0.36</td>
<td>3.59±0.17</td>
<td>5.22±0.14</td>
<td>4.16±0.32</td>
</tr>
<tr>
<td>58</td>
<td>4.11±0.13</td>
<td>3.80±0.42</td>
<td>4.37±0.24</td>
<td>4.13±0.78</td>
</tr>
<tr>
<td><strong>Flip</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>3.19±0.27</td>
<td>3.60±0.39</td>
<td>3.91±0.22</td>
<td>3.93±0.32</td>
</tr>
<tr>
<td>50</td>
<td>2.83±0.09</td>
<td>3.01±0.36</td>
<td>3.75±0.16</td>
<td>3.62±0.08</td>
</tr>
<tr>
<td>58</td>
<td>3.28±0.31</td>
<td>3.66±0.14</td>
<td>4.14±0.46</td>
<td>5.47±0.98</td>
</tr>
</tbody>
</table>
Table 2.3: Carbohydrate content of pretreated solids recovered from uniflow reactor configuration.

<table>
<thead>
<tr>
<th>Variety/Concentration</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. × giganteus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>40.4±4.07</td>
<td>16.9±1.72</td>
<td>1.7±0.31</td>
</tr>
<tr>
<td>40mg/l O₃</td>
<td>24.2±10.76</td>
<td>9.1±3.91</td>
<td>0.9±0.43</td>
</tr>
<tr>
<td>50 mg/l O₃</td>
<td>22.5±5.30</td>
<td>8.2±2.18</td>
<td>0.9±0.26</td>
</tr>
<tr>
<td>58mg/l O₃</td>
<td>34.1±7.15</td>
<td>11.3±2.11</td>
<td>1.5±0.31</td>
</tr>
<tr>
<td><strong>M. sinensis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Gracillimus’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>34.2±4.32</td>
<td>17.2±2.34</td>
<td>2.1±0.53</td>
</tr>
<tr>
<td>40mg/l O₃</td>
<td>30.0±7.40</td>
<td>13.6±3.71</td>
<td>1.8±0.55</td>
</tr>
<tr>
<td>50 mg/l O₃</td>
<td>27.8±0.93</td>
<td>12.4±0.67</td>
<td>1.6±0.12</td>
</tr>
<tr>
<td>58mg/l O₃</td>
<td>27.2±1.16</td>
<td>11.8±1.13</td>
<td>2.3±1.27</td>
</tr>
<tr>
<td><strong>S. arundinaceum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>34.3±3.15</td>
<td>18.5±1.75</td>
<td>2.5±0.28</td>
</tr>
<tr>
<td>40mg/l O₃</td>
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<td>50 mg/l O₃</td>
<td>32.1±7.81</td>
<td>15.2±3.63</td>
<td>2.4±0.55</td>
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<td>58mg/l O₃</td>
<td>29.7±1.44</td>
<td>14.6±0.97</td>
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<tr>
<td><strong>S. ravennae</strong></td>
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</tr>
<tr>
<td>Untreated</td>
<td>35.4±3.30</td>
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<td>58mg/l O₃</td>
<td>30.5±2.91</td>
<td>14.1±1.55</td>
<td>2.0±0.31</td>
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Table 2.4: Carbohydrate content of pretreated solids recovered from flip reactor configuration.

<table>
<thead>
<tr>
<th>Variety/Concentration</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
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<tr>
<td><strong>M. ×giganteus</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>40 mg/l O₃</td>
<td>47.2±5.75</td>
<td>18.3±0.78</td>
<td>2.1±0.70</td>
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<tr>
<td>50 mg/l O₃</td>
<td>42.5±6.78</td>
<td>14.7±2.42</td>
<td>1.7±0.51</td>
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<tr>
<td>58 mg/l O₃</td>
<td>48.3±0.75</td>
<td>16.8±0.28</td>
<td>1.6±0.18</td>
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</tbody>
</table>

| **M. sinensis**       |            |           |              |
| **‘Gracillimus’**     |            |           |              |
| 40 mg/l O₃           | 37.1±12.53 | 16.4±5.97 | 0.2±0.10     |
| 50 mg/l O₃           | 46.3±1.38  | 20.0±0.80 | 0.2±0.05     |
| 58 mg/l O₃           | 42.9±2.38  | 18.4±1.06 | 2.7±0.16     |

| **S. arundinaceum**   |            |           |              |
| 40 mg/l O₃           | 34.3±1.51  | 16.5±0.89 | 2.4±0.40     |
| 50 mg/l O₃           | 39.1±1.33  | 19.5±0.59 | 3.1±0.15     |
| 58 mg/l O₃           | 35.8±1.92  | 17.1±0.43 | 2.7±0.09     |

| **S. ravennae**       |            |           |              |
| 40 mg/l O₃           | 39.6±1.51  | 17.4±1.58 | 2.3±0.25     |
| 50 mg/l O₃           | 43.3±1.19  | 20.1±0.27 | 3.1±0.25     |
| 58 mg/l O₃           | 42.6±1.92  | 18.0±2.22 | 2.6±0.73     |
3.3 Effect of ozonolysis on enzymatic hydrolysis

Figure 2.3 details the glucose yield after hydrolysis of energy grass samples pretreated under uniflow and flip configurations. It was observed that, glucose yield from untreated samples was significantly higher (p < 0.05) than the pretreated solids at the following conditions- *M. ×giganteus* (40, 50 and 58mg/l at uniflow), *M. sinensis ‘Gracillimus’* (40, 50 and 58mg/l at uniflow), and *S. ravennae* (Uniflow at 40 and 50 mg/l; flip at 40, 50 and 58 mg/L). However, in the case of *S. arundinaceum*, solids pretreated at 40mg/l (flip) produced significantly more (p < 0.05) glucose than untreated solids. At the remaining conditions there was no significant difference (p > 0.05) between the glucose concentration produced by the untreated and pretreated solids. Glucan conversion followed the same trend as glucose yield (Table 2.5). Higher or comparable yield observed in the untreated samples indicates potential enzyme inhibition in the pretreated samples, attributed to lignin degradation products. Since, the pretreated samples were not washed prior to hydrolysis, the inhibitors potentially interfered with the enzyme binding site on the biomass. The wash step after pretreatment was eliminated on the basis of reports in prior studies suggesting that ozone does not generate toxic byproducts (Neely 1984; Vidal and Molinier 1988). It has been estimated that the water requirement for ethanol production from corn stover using dilute acid pretreatment is 5.4 gal/gal ethanol (Humbird et al. 2011) and eliminating the wash step could provide significant cost savings and make the overall process economical. Some degradation products observed by GC/MS from the ozonolysis of corn stalk include glycolic, oxalic, malonic, glyoxalic, glyceral, p-hydroxybenzoic and malic acid. Vanillin and p-hydroxybenzaldehyde have also
been identified (Quesada et al. 1999). Ozonation of poplar sawdust at 45% moisture content yielded oxalic, formic, glycolic, glyoxalic, succinic, glyceric, malonic, p-hydroxybenzoic, fumaric and propanoic acids (Euphrosine-Moy et al. 1991; Lasry et al. 1990) while ozonation of coastal Bermuda grass and Kentucky 31 tall fescue at 50% moisture content produced caproic, levulinic, p-hydroxybenzoic, vanillic, azelaic and malonic acids along with p-hydroxybenzaldehyde, vanillin and hydroquinone (Morrison and Akin 1990). It was observed that lignin degradation products due to ozonolysis differ based on substrate and their moisture content. Preliminary analysis of lignin degradation products generated in this study on ozonolysis of energy grasses indicated that syringaldehyde and vanillin were present in the wash water.

It was observed that xylose yield (Table 2.6) was higher (p<0.05) in pretreated samples compared to untreated samples of *S. arundinaceum* and *S. ravennae* from both reactor configurations. Both miscanthus varieties yielded significantly higher (p<0.05) xylose for samples from flip reactor configuration. However, xylose yield of pretreated *M. × giganteus* at all concentrations and *M. sinensis ‘Gracillimus’* at 50 and 58mg/L with uniflow configuration were not significantly different (p>0.05) from the untreated samples. The maximum xylose yield of 77.5 mg/g raw biomass was obtained from *S. arundinaceum* at flip configuration. Xylan conversion followed the same trend as xylose yield (Table 2.6). Increase in xylan conversion or insignificant differences may be due to the targeted impact of pretreatment by-products towards glucan conversion and not xylan/ arabinan conversion reactions.
3.4 Selection of optimal pretreatment conditions:

Statistical analysis was performed to determine optimal pretreatment conditions relative to ozone concentration and reactor configuration for each variety based on AIL content and glucan availability after pretreatment. Table 2.7 summarizes the pretreatment conditions identified as optimal for the different varieties. It was observed that 40mg/l of ozone at 0.25 l/min resulted in least lignin concentration (AIL) across the varieties. In the case of *M. ×giganteus* and *S. ravennae*—uniflow configuration, was chosen as there was no significant difference (p< 0.05) in delignification between the uniflow and flip configurations and uniflow reactor configuration has easier operation requiring reduced effort compared to flip. With regards to optimization based on maximum glucan content in the biomass after pretreatment, all varieties except *S. arundinaceum* had significantly higher glucan content under flip conditions.
Figure 2.2: Glucose produced per gram of untreated biomass after hydrolysis using a) uniflow and b) flip reactor configurations.
<table>
<thead>
<tr>
<th>Variety/Concentration</th>
<th>Uniflow Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
<th>Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
<th>Flip Glucan (%)</th>
<th>Xylan (%)</th>
<th>Arabinan (%)</th>
</tr>
</thead>
<tbody>
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<tr>
<td><em>M. × giganteus</em></td>
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<tr>
<td>Untreated</td>
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<td>18.5±3.78</td>
<td>6.1±0.43</td>
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<tr>
<td>40mg/l O₃</td>
<td>12.9±0.58</td>
<td>9.3±0.47</td>
<td>74.9±4.08</td>
<td>15.1±0.97</td>
<td>18.7±6.37</td>
<td>60.5±2.88</td>
<td>15.1±0.97</td>
<td>18.7±6.37</td>
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<td>50 mg/l O₃</td>
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Table 2.6: Hemicellulose yield (mg) per gram of untreated biomass after hydrolysis.

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<tr>
<th>Variety/Concentration</th>
<th>Uniflow Xylose</th>
<th>Uniflow Arabinose</th>
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<td>1.2±0.00</td>
<td>12.5±0.88</td>
<td>1.2±0.00</td>
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<td>40mg/l O₃</td>
<td>14.5±2.53</td>
<td>15.0±0.63</td>
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<td>50 mg/l O₃</td>
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<td>11.8±2.86</td>
<td>39.1±3.44</td>
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<td>19.1±0.95</td>
<td>14.1±3.66</td>
<td>38.3±13.03</td>
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<td><strong>M. sinensis</strong></td>
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<tr>
<td>'Gracillimus'</td>
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</tr>
<tr>
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<td>10.2±4.69</td>
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<td><strong>S. arundinaceum</strong></td>
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<tr>
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<td>2.9±0.72</td>
<td>76.0±3.55</td>
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<td><strong>S. ravennae</strong></td>
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<tr>
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<td>25.2±1.30</td>
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Table 2.7: Optimal pretreatment conditions for ozonolysis of energy grasses

<table>
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<th>Variety</th>
<th>Acid Insoluble Lignin</th>
<th>Glucan</th>
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</thead>
<tbody>
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<td>Reactor Configuration</td>
<td>Concentration (mg/L)</td>
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<td>Uniflow</td>
<td>40</td>
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<tr>
<td><em>M. sinensis</em></td>
<td>Flip</td>
<td>40</td>
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<tr>
<td>‘Gracillimus’</td>
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<tr>
<td><em>S. arundinaceum</em></td>
<td>Flip</td>
<td>40</td>
</tr>
<tr>
<td><em>S. ravennae</em></td>
<td>Uniflow</td>
<td>40</td>
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</table>

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4. Conclusion:

Pretreatment of *M. ×giganteus*, *M. sinensis* ‘Gracillus’, *S. arundinaceum* and *S. ravennae* with ozone in a packed bed reactor did not impact the cellulose content significantly and was effective in removing lignin. Pretreatment optimization for ozone concentration and reactor configuration, for each energy grass variety, was performed on the basis of minimum lignin content and highest glucan recovery after pretreatment. However, ozone pretreated samples did not provide enhanced sugar yields and carbohydrate conversion during enzymatic hydrolysis, potentially due to the presence of lignin degradation products and/or inadequate enzyme concentration. Lower glucan conversion in the pretreated materials, observed throughout the study was speculated to be the result of enzyme inhibition caused by lignin degradation products. Although, ozone pretreatment is widely reported as not producing any inhibitory/toxic products (Neely 1984), it is known to degrade lignin into various compounds and the nature of the degraded products to a great extent depends on the substrate. Cellulolytic enzymes may thus have been inhibited by the product(s) formed over the course of the reaction. Washing the pretreated samples after pretreatment may help in overcoming the hurdle and thus needs to be investigated. Hence future studies should focus on pretreating feedstocks at conditions identified as optimal and investigating the impact of washing pretreated solids to remove lignin degradation products. Enzyme loading should also be optimized to make the process economically and functionally feasible.
Acknowledgements:

The authors would like to thank Dr. Dhanalekshmi Savithri for her consistent help with sugar analysis during the course of this study. We appreciate Dr. Kurt Creamer’s assistance in obtaining enzymes from Novozymes N.A., Franklinton for this study. This material is based upon works partially supported by the Department of Energy under Award Number GO88053 and the Center for Biomass Research and Development, IUCRC-NSF.

Disclaimer:

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REFERENCES


CHAPTER 3:
HYDROLYSIS OF OZONE PRETREATED ENERGY GRASSES FOR OPTIMAL FERMENTABLE SUGAR PRODUCTION

ABSTRACT:

Pretreatment is an important step in the production of biofuels from lignocellulosic materials. In this study 4 energy cane varieties were ozonated and enzymatically hydrolyzed using Cellic® CTec2 at three different enzyme loadings. Conditions for ozonolysis were selected on the basis of maximum delignification (AIL) and glucan retention (GLU) after pretreatment. In order to study the effect, on cellulolytic enzymes, of removing lignin degradation products generated during ozonolysis, hydrolysis was carried out for both washed and unwashed pretreated solids. The results indicated that washing the solids significantly (p<0.05) enhanced glucan conversion from 34.3 to 100% while resulting in glucose yields of 146.2-431.9 mg/g biomass. Overall, the optimal process for highest fermentable sugar generation was identified as the one in which energy grasses were ozonated for maximum delignification followed by enzymatic hydrolysis of washed solids using 0.1 g/g Cellic® CTec2. A comparative study on alkali pretreatment of energy grasses with 1% NaOH for 60 min suggested that S.arundinaceum had the highest glucan conversion with maximum sugar production of 467.9 mg/g. Although ozonolysis was found to be an effective treatment, a better understanding of the degradation products formed during pretreatment is required to further enhance glucan conversion during enzymatic hydrolysis.
1. INTRODUCTION:

Meeting the ever-growing energy requirements for transportation, heating and industrial processes while substantially reducing greenhouse gas emissions is a major challenge facing the 21st century (Hahn-Hagerdal et al. 2006). More than 96% of the United States transportation sector is dependent on oil (Wyman et al. 2009) and the demand for liquid fuels is expected to increase by 4.7 quadrillion Btu from 2009 to 2035. Low cost processing of cellulosic biomass to liquid fuels with high yields is thus a necessity (Wyman et al. 2009). However, the complex network of cellulose, hemicellulose and lignin is a major barrier to the production of fuels from lignocellulosic biomass. Pretreatment of lignocellulosic materials can aid in overcoming the barrier by breaking down lignin and facilitating enzymatic hydrolysis of polysaccharides into soluble monomeric sugars which can be fermented into fuels and chemicals (Mosier et al. 2005). In spite of significant advances though, pretreatment is the most expensive unit operation involved in the production of biofuels from lignocellulosic materials followed by cost of enzymes (Wyman 2007). Hence in addition to developing functionally and economically feasible pretreatment techniques, optimization of enzymatic hydrolysis has been an evolving process. Ozonolysis is a pretreatment technique which has proven to be highly effective in delignifying feedstocks such as wheat straw (Binder and Scharf 1980), poplar sawdust and rye straw (Garcia-Cubero et al. 2010) and also improve sugar yield after hydrolysis. It is advantageous because (a) it allows for on-site generation of ozone gas thus eliminating the need for storing toxic chemicals, (b) ozonolysis takes place at room temperature and pressure,
and (c) degradation fragments are biodegradable (Neely 1984; Vidal and Molinier 1988). In our study on optimization of ozonolysis of energy grasses we observed that ozonolysis delignified energy grasses by up to 59.9% (chapter 2).

The scale up of cellulosic ethanol production using enzymes first received support by the US department of energy (DOE) after the energy crisis of 1970’s. Despite various advantage of enzymatic hydrolysis of cellulose to liquid fuels (like higher yield, milder operating conditions, and lower energy costs), the technology was considered high risk from an industrial perspective at that time (Wyman 2001). The emergence of biotechnology and advancement of knowledge about cellulolytic enzymes has helped in the reduction of ethanol production cost over the years. Enzymes with features such as higher specific activity with balanced synergistic characteristics, thermal stability and improved resistance towards inhibitors enabling higher yield of sugars at lower costs have been developed (Yang et al. 2011). However, requirement of higher enzyme doses owing to the complexity involved in releasing sugars from recalcitrant lignocellulosic materials has put constraints on significant reduction in bioethanol production costs (Himmel et al. 1999; Wingren et al. 2005; Wyman 2007).

Efficiency of enzymatic hydrolysis of lignocellulosic material is limited by a number of factors including (a) higher substrate concentrations, (b) presence of lignin and (c) need for removal of end products (Jorgensen et al. 2007). Optimization of enzymatic hydrolysis is therefore of immense significance to make the process economically viable for application to a variety of pretreatments and feedstocks since typically, commercial enzyme products are
optimized for one specific substrate and not tailored for a broader spectrum of lignocellulosic materials (Zhang et al. 2006).

Hence as a follow on to our study on optimization of ozonolysis of energy grasses, this study was undertaken to optimize enzymatic hydrolysis of energy grasses on the basis of enzyme loading required for maximum fermentable sugar production. Four energy cane varieties, *Miscanthus ×giganteus*, *Miscanthus sinensis* ‘Gracillimus’, *Saccharum arundinaceum* and *Saccharum ravennae*, pretreated with gaseous ozone at conditions determined to be optimal in a previous study were hydrolyzed at three enzyme concentrations (0.1-0.3 g enzyme protein/ g biomass). Effect of washing the pretreated solids, prior to enzymatic hydrolysis, on sugar yields and carbohydrate conversion was also investigated. The energy cane varieties were pretreated with sodium hydroxide to establish a comparison with a conventional pretreatment technique.

2. MATERIAls AND METHODS:

2.1 Biomass Preparation:

Energy grasses (*M. ×giganteus*, *M. sinensis* ‘Gracillimus’, *S. arundinaceum* and *S. ravennae*) from the Mountain Horticultural Crops Research and Extension Center (Mills River, NC) were harvested, dried at 45°C for 72 hours and ground to pass a 2mm sieve using a Wiley mill (Model: 4, Thomas, Philadelphia, PA, USA). Ground samples were stored in ziplock bags at room temperature. Energy grasses used for ozone pretreatment were harvested in December 2009 and those for alkaline pretreatment were harvested in December 2011.
2.2 Pretreatment:

Energy grasses were pretreated with ozone at conditions identified as optimal on the basis of minimum lignin (AIL) and maximum glucan (GLU) contents as concluded in chapter 2 (Tables 2.7/3.1). Alkali pretreatment with sodium hydroxide was performed to provide a baseline comparison of ozonolysis relative to a conventional pretreatment method.

2.2.1 Ozone Pretreatment:

Energy grass samples from each of the 4 varieties were prepared for ozonolysis by adding 30% moisture to 5 g of ground biomass (dry basis) and allowed to equilibrate for an hour. Ozonolysis was performed for 2 h in a glass column reactor tube (Product no: 5813 – 26, Ace glassware, NJ, USA) with 5 cm diameter and 50 cm length. One end of the reactor was plugged with glasswool to support the biomass. Ozone was produced on-site by an ozone generator (Model: OL80 A Ozone lab instrument, Canada) supplied with industrial grade oxygen (Airgas National Welders, Raleigh, NC) at a flow rate of 0.25 L/min maintained using a mass flow controller (Model no: FMA5516, Omega, CT, USA). Initial moisture content and time of reaction were based on the work of Neely (1984).

Ozone concentrations (40 – 58 mg/L) and reactor configurations (flip or uniflow) for each variety were chosen on the basis of previous studies (Chapter 2) performed to optimize ozone pretreatment. Each variety was subjected to two different ozonolysis treatments – one based on maximum delignification and another based on maximum glucan recovery. In the uniflow reactor configuration, direction of ozone was maintained for the entire 2 h reaction period while in flip reactor configuration, direction of ozone flow was reversed after 1 h. Pretreated
samples were stored in ziplock bags at room temperature until further use for composition analysis and enzymatic hydrolysis. 10 g sub samples drawn from the pretreated solids were washed by suspending in 100 ml deionized water followed by centrifugation and filtration to remove potential inhibitors prior to hydrolysis. Unwashed and washed samples with minimum acid insoluble lignin (AIL) content after ozonolysis were identified as AIL-U and AIL-W, respectively. Similarly, unwashed and washed samples with maximum glucan recovery (GLU) after ozonolysis were identified as GLU-U and GLU-W, respectively.

2.2.2 Alkali Pretreatment:

Ten gram of biomass from each energy grass variety was mixed with 100 ml of 1% (w/v) sodium hydroxide (NaOH) solution in serum bottles and the bottles were crimp sealed. Pretreatment was carried out by autoclaving the bottles at 121°C for 1h (Xu et al. 2012). Pretreated biomass was recovered by filtering and washing the solids with 100 ml water in a Buchner funnel-vacuum pump assembly to remove excess alkali and/or sugar degradation products that may affect hydrolysis. All pretreatments were performed in triplicate and residual biomass was analyzed for solid recovery, carbohydrate availability (in terms of glucan, xylan and arabinan contents determined using respective monomeric sugar measurements divided by a conversion factor of 1.1) and acid insoluble and acid soluble lignin contents using standard methods described later.
Table 3.1: Optimal pretreatment conditions for ozonolysis of energy grasses.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Acid Insoluble Lignin</th>
<th>Glucan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reactor Configuration</td>
<td>Concentration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg/L)</td>
</tr>
<tr>
<td><em>M. × giganteus</em></td>
<td>Uniflow</td>
<td>40</td>
</tr>
<tr>
<td><em>M. sinensis</em></td>
<td>Flip</td>
<td>40</td>
</tr>
<tr>
<td>‘Gracillimus’</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. arundinaceum</em></td>
<td>Flip</td>
<td>40</td>
</tr>
<tr>
<td><em>S. ravennae</em></td>
<td>Uniflow</td>
<td>40</td>
</tr>
</tbody>
</table>
2.3 Enzymatic Hydrolysis:

Enzymatic hydrolysis was performed on ozone and alkali pretreated samples at 8% solid loading (dry basis) using Cellic® CTec2 provided by Novozymes North America, Inc. (Franklinton, NC). Unwashed and washed samples from ozone pretreatments were hydrolyzed at enzyme loadings of 0.0, 0.1, 0.2 and 0.3 g enzyme protein/ g dry biomass. The activity of Cellic CTec2 was 138 FPU (filter paper unit/ml). Alkali pretreated samples were hydrolyzed, each, with 0.1 g of enzyme protein/ g dry biomass of Cellic® CTec2 and Alternafuel 200L (Dyadic, Jupiter, FL). The total volume was made up to 20 ml in 50 ml centrifuge tubes by adding tetracycline (40 µg/ml) for preventing microbial contamination, enzyme suspension and 0.05M sodium citrate buffer (pH: 4.8). The tubes were incubated in a water bath shaker (Model: 89032-226, VWR International, PA.) at 50 °C, 150 rpm for 72 h. After hydrolysis, the samples were centrifuged at 4000 rpm and 4 °C for 10 min and the supernatant was filtered through a 0.2 µm syringe filter and stored at -80°C for subsequent high performance liquid chromatography (HPLC) analysis for fermentable sugar determination. Untreated samples and samples without biomass were also hydrolyzed as control.

2.4 Analytical Methods:

Total solids, acid insoluble lignin (AIL), acid soluble lignin (ASL) and ash were determined using the procedure described by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2006; Sluiter et al. 2005a; Sluiter et al. 2005b). Monomeric sugars including glucose, xylose and arabinose in the untreated and pretreated biomass and enzyme hydrolyzate were
determined using a HPLC (Model: UltiMate 3000, Dionex Corporation, Sunnyvale, CA) with a refractive index detector. An Aminex HPX-87H column fitted with a guard column was used at 65 °C with 0.005 M sulfuric acid flowing at 0.6ml/min as eluent.

2.5 Statistical Analysis:
Statistical analysis was done using SAS® 9.2 (SAS Institute, Cary, NC) to test the significance of different enzyme loadings and washing. Interaction effects between different pretreatment conditions and enzyme loadings for each variety were performed by the Tukey-Kramer test at 95% confidence level using PROC MIXED. The best treatment conditions for each variety were chosen based on maximum glucan conversion.

3. RESULTS AND DISCUSSION:
The four energy grasses (M. ×giganteus, M. sinensis ‘Gracillimus’, S. arundinaceum and S. ravennae) harvested in December 2009 for use in the study on ozonolysis had a total carbohydrate content ranging between 53.5 - 59.1%. Table 3.2 summarizes the ash and carbohydrate content of untreated energy grasses used for the ozone study. The acid insoluble and acid soluble lignin contents for untreated and ozone pretreated energy grasses are presented in Figures 3.1 a and b. The total lignin content (AIL + ASL) ranged between 23.3 - 24.74%. Overall, M. ×giganteus contained the highest lignin and carbohydrate contents and S. arundinaceum contained the highest xylan content (18.6%).
Table 3.2: Carbohydrate and ash content of energy grasses used in ozonolysis.

<table>
<thead>
<tr>
<th></th>
<th><em>M. × giganteus</em></th>
<th><em>M. sinensis</em></th>
<th><em>S. arundinaceum</em></th>
<th><em>S. ravennae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash (%)</td>
<td>1.0±0.00</td>
<td>0.9±0.01</td>
<td>0.9±0.02</td>
<td>1.0±0.02</td>
</tr>
<tr>
<td>Glucan (%)</td>
<td>40.4±4.06</td>
<td>34.2±4.31</td>
<td>34.4±3.14</td>
<td>35.4±3.29</td>
</tr>
<tr>
<td>Xylan (%)</td>
<td>16.9±1.72</td>
<td>17.1±2.3</td>
<td>18.5±1.74</td>
<td>18.2±1.67</td>
</tr>
<tr>
<td>Arabinan (%)</td>
<td>1.7±0.31</td>
<td>2.1±0.52</td>
<td>2.5±0.28</td>
<td>2.3±0.25</td>
</tr>
<tr>
<td>Extractives (%)</td>
<td>1.1±0.03</td>
<td>1.1±0.11</td>
<td>n/a</td>
<td>8.3±0.94</td>
</tr>
</tbody>
</table>
Figure 3.1: (a) Acid insoluble lignin and (b) Acid soluble lignin content of untreated, unwashed-ozone pretreated and washed-ozone pretreated energy cane samples.
3.1 Effect of ozone pretreatment on lignin in energy grasses:

The presence of lignin in lignocellulosic materials acts as a shield and limits the accessibility of substrates for enzymatic hydrolysis thus reducing the rate of enzymatic hydrolysis (Chang and Holtzapple 2000). Conversion of biomass to ethanol thus requires a pretreatment step to solubilize lignin and hemicellulose for enhancing hydrolysis 3-10 folds by improving accessibility of enzymes to cellulose and hemicellulose (Galbe and Zacchi 2007; Mosier et al. 2005). Ozonolysis, as its primary mechanism, helps in breaking the lignin structure by oxidizing it and thus increases hydrolytic enzyme efficiency. However in a previous study on ozonolysis reactor configuration and ozone concentration an inhibitory effect was observed during preliminary hydrolysis of ozone treated samples (Chapter 2). This inhibition effect may be attributed to oxidation products that may have been produced during pretreatment. Hence, in order to investigate if washing could help resolve the issue of inhibition, pretreated solids were washed with deionized water before hydrolysis.

It was observed that ozonolysis reduced AIL concentration in energy grasses for all pretreated samples. The net reduction in AIL ranged between 29.2 – 42.2% (AIL-U), 16.9-27.8% (AIL-W), 36.1-46.7% (GLU-U) and 24.0-50.9% (GLU-W) (Figure 3.1a). Similar results have been reported for wheat, rye, barley and oats ozonated in a fixed bed reactor, where delignification of 38, 50, 41 and 40%, respectively, were observed (Garcia-Cubero et al. 2010). It was noted that solid recovery ranged between 61.2-89.9% and 58.1-85.6% for AIL-W and GLU-W samples, respectively. For unwashed samples the solid recovery was over 99%.
ASL content of unwashed energy grasses increased by 52.9-145.1% after ozonolysis while it reduced by 23.8-45.1% after washing (Figure 3.1b). Increase in ASL concentration in unwashed samples was potentially due to the accumulation of AIL degradation products. Similar results of increase in ASL content were observed during the ozone pretreatment of wheat, rye, barley and oat (Garcia-Cubero et al. 2010).

3.2 Effect of ozonolysis and prehydrolysis washing on hydrolytic sugar production:

Untreated and ozonated (unwashed and washed) energy grass samples were hydrolyzed at enzyme loadings up to 0.3 g enzyme protein/g biomass. Considerably higher enzyme loadings were considered since ozonolysis as a pretreatment technique and its impact on hydrolysis are relatively less understood. Sugar yields and carbohydrate conversions were observed to be impacted by washing pretreated samples prior to hydrolysis. Washing the ozone pretreated samples with water increased glucose yield and glucan conversion significantly (p<0.05) for all the varieties, indicating presence of inhibitory products in the unwashed ozone pretreated samples (Figure 3.2). It was observed that glucose yield from untreated *M. × giganteus* hydrolyzed with 0.1 g/g Cellic® CTec2, was significantly higher (p<0.05) than the AIL-U samples. In the case of *M. sinensis* ‘Gracillimus’, *S. ravennae* and *S. arundinaceum*, there were no significant difference (p>0.05) in the glucose yield between untreated and unwashed samples. Unlike glucan conversion, xylan conversion (Tables 3.3) was significantly higher (p<0.05) in pretreated samples (AIL-U and GLU-U) than in untreated samples at 0.1 g/g enzyme loading. There were no significant differences (p>0.05) in xylan conversion between AIL-W and GLU-W for *M. × giganteus*, *S. arundinaceum* and
S. ravennae. Xylan conversion was observed to be higher in AIL-W than AIL-U for all the varieties. Similar trend was observed with GLU-W and GLU-U for M. ×giganteus, however no difference was observed for M. sinensis ‘Gracillimus’ and S. ravennae. Data has not been shown for arabinose content due to low concentrations (< 3%).

Increasing the enzyme loading from 0.1 g/g to 0.2 g/g for hydrolyzing the washed samples (AIL –W and GLU-W) had no significant impact (p > 0.05) on glucose yield and the corresponding glucan conversion (Appendix B). Enzymatic hydrolysis of the energy grasses carried out at even higher enzyme loading of 0.3 g/g Cellic® CTec2 resulted in glucan conversions over 100%. Canella et al. (2012) observed that when hydrothermally pretreated wheat straw was hydrolyzed with Cellic® CTec2, oxidative enzymes (GH61) present in Cellic® CTec2 resulted in the formation of gluconic and cellobionic acids. These by-products can result in an overestimation of glucose in the hydrolyzate when using an Aminex HPX-87H column on an HPLC with a RI detector, due to the co-elution of gluconic acid with glucose (Cannella et al. 2012). In summation, hydrolysis of the energy grasses pretreated under various conditions, with and without subsequent washing, suggested that AIL-W samples from all 4 varieties hydrolyzed with 0.1 g/g Cellic® CTec2 resulted in higher glucan conversion (p<0.05). Hence, for future studies investigating reaction kinetics and scale up, these optimal process conditions are recommended.
Figure 3.2: (a) Glucose yield per gram of biomass and (b) Glucan conversion after hydrolysis of energy grasses pretreated with ozone.
Table 3.3: Xylose yield in untreated and ozone pretreated energy grasses after hydrolysis

<table>
<thead>
<tr>
<th>Variety</th>
<th>Enzyme loading (%)</th>
<th>Xylose yield (mg/g untreated biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>AIL-U</td>
</tr>
<tr>
<td><em>M. × giganteus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.0±0.00</td>
<td>1.0±1.79</td>
</tr>
<tr>
<td>10</td>
<td>16.6±0.72</td>
<td>48.0±4.21</td>
</tr>
<tr>
<td><em>M. sinensis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>‘Gracillimus’</td>
<td>0</td>
<td>4.4±0.14</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>19.8±2.11</td>
</tr>
<tr>
<td><em>S. arundinaceum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>49.7±2.84</td>
<td>21.7±3.60</td>
</tr>
<tr>
<td>10</td>
<td>64.5±10.46</td>
<td>81.5±4.68</td>
</tr>
<tr>
<td><em>S. ravennae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>6.1±0.27</td>
<td>10.2±1.16</td>
</tr>
<tr>
<td>10</td>
<td>25.9±2.65</td>
<td>51.6±3.16</td>
</tr>
</tbody>
</table>
Table 3.4: Xylan conversion in untreated and ozone pretreated energy grasses after hydrolysis

<table>
<thead>
<tr>
<th>Variety</th>
<th>Enzyme loading (%)</th>
<th>Xylan conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>AIL-U</td>
</tr>
<tr>
<td>M. × giganteus</td>
<td>0</td>
<td>0.0±0.00</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8.1±0.35</td>
</tr>
<tr>
<td>M. sinensis 'Gracillimus'</td>
<td>0</td>
<td>2.1±0.09</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.5±1.02</td>
</tr>
<tr>
<td>S. arundinaceum</td>
<td>0</td>
<td>22.8±0.49</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>28.6±4.65</td>
</tr>
<tr>
<td>S. ravennae</td>
<td>0</td>
<td>2.8±0.12</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.7±1.20</td>
</tr>
</tbody>
</table>
3.3 Alkaline pretreatment of energy grasses

In order to provide comparative information on the extent of sugar generation from ozone pretreated energy grasses relative to that from a conventional chemical pretreatment, alkaline pretreatment with NaOH was also performed. Table 3.4 summarizes the composition of energy grasses, harvested in December 2011 for use in alkali pretreatment. Total lignin content (AIL + ASL) of the different energy grasses ranged between 25.02 - 32.02% while the total carbohydrates content ranged between 50.6 - 61.4 %. *M. ×giganteus* contained the highest lignin and carbohydrate contents while *M. sinensis ‘Gracillimus’* had the highest xylan content (23.6%).

3.3.1 Effect of alkali pretreatment on lignin in energy grasses

The AIL content of alkali treated samples dropped to 15.5 ± 0.38, 10.4 ± 0.71, 9.4 ± 0.67 and 11.9 ± 0.37% for *M. ×giganteus*, *M. sinensis ‘Gracillimus’*, *S. arundinaceum* and *S. ravennae*, respectively, after alkaline pretreatment. The corresponding ASL content for the 4 varieties was 0.89, 0.70, 0.75 and 0.95%, respectively. Overall, delignification of energy grasses after NaOH pretreatment ranged between 41.7 - 53.6%. Least reduction in AIL was observed with *S. arundinaceum* while *M. ×giganteus* had the highest delignification. Solid recovery of alkali pretreated samples ranged between 58.3 -61.6 %.
Table 3.5: Initial composition of energy grasses used in alkaline pretreatment study

<table>
<thead>
<tr>
<th></th>
<th>M. ×giganteus</th>
<th>M. sinensis ‘Gracillimus’</th>
<th>S. arundinaceum</th>
<th>S. ravennae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash (%)</td>
<td>1.03±0.05</td>
<td>1.03±0.03</td>
<td>1.03±0.01</td>
<td>1.00±0.01</td>
</tr>
<tr>
<td>Glucan (%)</td>
<td>38.89 ± 6.72</td>
<td>34.80 ± 0.66</td>
<td>39.21 ± 2.55</td>
<td>34.38 ± 5.99</td>
</tr>
<tr>
<td>Xylan (%)</td>
<td>20.78 ± 0.30</td>
<td>23.59 ± 0.71</td>
<td>18.20 ±0.91</td>
<td>16.31 ± 0.60</td>
</tr>
<tr>
<td>Arabinan (%)</td>
<td>1.77 ± 0.04</td>
<td>2.39 ± 0.02</td>
<td>3.04 ± 0.26</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>AIL (%)</td>
<td>28.90±6.41</td>
<td>22.41±1.47</td>
<td>22.56±0.47</td>
<td>23.23±0.15</td>
</tr>
<tr>
<td>ASL (%)</td>
<td>3.12±0.26</td>
<td>2.61±0.14</td>
<td>2.60±0.21</td>
<td>3.16±0.97</td>
</tr>
</tbody>
</table>
3.3.2 Effect of alkali pretreatment on hydrolytic sugar production

Under the conditions of this study, hydrolysis of alkali pretreated biomass with 0.1 g/g Cellic® CTec2 generated more fermentable sugars than 0.1 g/g Alternafuel 200L (p<0.05) for all varieties except *M. × giganteus* (Table 3.5). Highest amount of sugar production (glucose and xylose) was observed from *S. arundinaceum* for both enzymes (Cellic® CTec2 – 639.42 mg/g, Alternafuel 200L – 334.33mg/g) while the lowest amount of fermentable sugars were observed from *M. × giganteus* at 212.8 mg/g with Cellic® CTec2 and 181.3 mg/g with Alternafuel 200L. These observations indicate that *M. × giganteus* is more recalcitrant to enzyme accessibility than the other varieties. Glucan conversions of over 100% with excessive enzyme loadings of 0.30 g/g were also observed in alkali pretreated samples. As mentioned earlier, these are believed to be due to the increased production and subsequent interference of hydrolysis by-products during HPLC analysis thus resulting in the overestimation of glucose (Cannella et al. 2012).

Comparison of glucan conversion efficiencies between alkali and ozone pretreated (AIL-W) samples suggests that hydrolysis of ozone pretreated *M. × giganteus* and *S. ravennae* had a higher glucan conversion (p<0.05) than alkali pretreated biomass. However in the case of *M. sinensis* ‘Gracillimus’ and *S. arundinaceum*, there were no significant differences (p>0.05) in the glucan conversion efficiencies of ozone (AIL-W) and alkali pretreated samples hydrolyzed with 0.1 g/g Cellic® CTec2.

Ozone pretreatment provides key advantages over other chemical pretreatments such that a) pretreatment occurs at room temperature and pressure, b) higher solid recoveries are obtained...
and c) waste stream consists of weak acids unlike the waste stream generated by alkali pretreatment which is highly toxic and requires a much complex treatment process prior to disposal or reuse (Quesada et al. 1998). Hence it can be inferred that ozone has the potential to be an efficient pretreatment method for energy grasses. Further investigation of ozonolysis at reduced ozone concentrations for shorter treatment times, identification of inhibitory products generated during ozonolysis and hydrolysis of the pretreated washed biomass at lower enzyme loadings (<10%) is recommended to make this pretreatment method functionally and economically feasible at process scale. An extensive economic analysis is also needed to enable investors of this technology to make informed decisions.
Table 3.6: Sugar yield and carbohydrate conversion after alkali pretreatment

<table>
<thead>
<tr>
<th>Variety</th>
<th>Enzyme</th>
<th>Glucose (mg/g)</th>
<th>Glucan conversion (%)</th>
<th>Xylose (mg/g)</th>
<th>Xylan conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. × giganteus</td>
<td>No enzyme</td>
<td>23.4±2.85</td>
<td>5.3±0.15</td>
<td>21.6±3.58</td>
<td>9.4±0.11</td>
</tr>
<tr>
<td></td>
<td>Enzyme A(^1)</td>
<td>154.5±16.41</td>
<td>32.8±3.49</td>
<td>58.2±7.84</td>
<td>23.1±3.12</td>
</tr>
<tr>
<td></td>
<td>Enzyme B(^2)</td>
<td>111.9±25.39</td>
<td>27.5±7.62</td>
<td>69.3±19.49</td>
<td>27.8±5.51</td>
</tr>
<tr>
<td>M. sinensis 'Gracillimus'</td>
<td>No enzyme</td>
<td>2.5±0.36</td>
<td>0.60±0.09</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
</tr>
<tr>
<td></td>
<td>Enzyme A</td>
<td>383.6±0.87</td>
<td>91.1±5.17</td>
<td>133.4±0.87</td>
<td>49.4±4.68</td>
</tr>
<tr>
<td></td>
<td>Enzyme B</td>
<td>198±19.35</td>
<td>42.2±8.45</td>
<td>42.2±21.24</td>
<td>16.8±8.45</td>
</tr>
<tr>
<td>S. arundinaceum</td>
<td>No enzyme</td>
<td>55.3±3.37</td>
<td>11.6±0.71</td>
<td>48.1±3.03</td>
<td>21.8±1.38</td>
</tr>
<tr>
<td></td>
<td>Enzyme A</td>
<td>467.9±34.52</td>
<td>85.3±11.54</td>
<td>171.4±4.89</td>
<td>77.8±2.22</td>
</tr>
<tr>
<td></td>
<td>Enzyme B</td>
<td>295.0±15.36</td>
<td>62.7±3.26</td>
<td>39.2±4.18</td>
<td>15.6±1.66</td>
</tr>
<tr>
<td>S. ravennae</td>
<td>No enzyme</td>
<td>22.1±1.08</td>
<td>3.8±2.31</td>
<td>17.6±2.29</td>
<td>8.4±1.04</td>
</tr>
<tr>
<td></td>
<td>Enzyme A</td>
<td>338.2±24.84</td>
<td>81.3±5.97</td>
<td>107.9±5.59</td>
<td>54.7±2.89</td>
</tr>
<tr>
<td></td>
<td>Enzyme B</td>
<td>213.0±27.08</td>
<td>51.2±6.51</td>
<td>29.2±2.03</td>
<td>14.8±1.03</td>
</tr>
</tbody>
</table>

\(^1\)Cellic® CTec2 at 0.1 g enzyme protein/g biomass (db)
\(^2\)Alternafuel 200L at 0.1 g enzyme protein/g biomass (db)
Acknowledgements:

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CHAPTER 4: 
OZONOLYSIS – PRELIMINARY KINETICS STUDY

1. INTRODUCTION:
Concerns about consequences of depleting oil and gas reserves coupled with their soaring demand and environmental impact have increased interest in alternative resources for production of fuels and chemicals. High oil prices in the past few years have further increased the significance of finding an alternate fuel that is cheaper, efficient and eco-friendly (Henstra et al. 2007). In this respect, plant biomass has been identified as the predominant sustainable source of organic carbon while bio-fuels derived from it are expected to be the key source of liquid fuels in the longer term (Huber et al. 2006b).
Lignocellulosic biomass constitutes the plant cell wall which is primarily composed of cellulose (30-50%), hemicellulose (15-35%) and lignin (10-30%) (da Costa Sousa et al. 2009). The structure and organization of these polymers makes the plant cell biologically resistant to various external factors- microbes and animals (Himmel et al. 2007). The complex network of cellulose, hemicellulose and lignin is a major barrier to the production of chemicals and fuels from lignocellulosic feedstocks such as miscanthus and switchgrass. The most crucial step in ethanol production from lignocellulosic biomass is the hydrolysis of cellulose and hemicellulose to monomeric sugars. Hydrolysis is best carried out by enzymes such as cellulase; however, efficient hydrolysis can be achieved only if cellulose and hemicellulose are easily accessible to the enzymes (Galbe and Zacchi 2007; Mosier et al. 2005). Although, pretreatment enhances the efficiency of hydrolysis by 10 folds
(Mosier et al. 2005) it accounts for 16-19% of the total capital investment and is the second largest expense for a lignocellulosic biorefinery after power plant generator costs (Aden et al. 2002; Wooley et al. 1999). In 2005, pretreatment was reported to cost 30 ¢/gallon ethanol produced through lignocellulosic conversion of biomass (Mosier et al. 2005). Pretreatment prior to hydrolysis, helps in breaking the lignin structure and disrupts cellulose crystallinity making cellulose more accessible to enzymes that convert the carbohydrates into fermentable sugars (Viikari et al. 2012). Without pretreatment, enzymatic hydrolysis of cellulose yields 20% less glucose than its maximum potential (Wright 1988).

In order to be effective, a pretreatment should a) avoid the need for size reduction of biomass, b) minimize sugar loss, c) preserve the hemicellulose fraction, d) maximize enzymatic convertibility/ hydrolysis, e) limit the formation of degradation products that might inhibit the enzymes or microorganisms used, f) minimize energy demand and g) be scalable to industrial size (Jorgensen et al. 2007; Mosier et al. 2005).

Ozonolysis is a pretreatment method in which lignocellulosic material is treated with ozone that is generated on-site. This method is known to effectively remove lignin and to some extent hemicellulose (Taherzadeh and Karimi 2008). Ozone is a very strong oxidizing agent that can be produced by passing oxygen through an electrical discharge where the oxygen molecules dissociate to form ozone (Eckert and Singh 1975). In a non–aqueous media, ozone reacts with organic substrates by cleaving the olefinic and activated aromatic bonds. However, in aqueous media, ozone abstracts an electron from easily oxidized organic substrates such as phenolates. This electron transfer produces either hydroxyl radicals or
superoxide as shown in equations 4.1 and 4.2, respectively (Ragnar et al. 1999). It is however hard to decide which of the radical species get formed initially as they can be easily transformed into each other.

Figure 4.1 shows the reaction mediated by superoxide radicals in the presence of oxygen and an organic substrate.

\[ RH + O_3 \rightarrow R + HO^- + O_2 \]  
(4.1)

\[ RH + O_3 \rightarrow RO + H^+ + O_2^- \]  
(4.2)
Figure 4.1: Chain reaction of ozone decomposition in the presence of oxygen and organic substrate such as phenol. Adapted from Ragnar et al. 1999.
It is believed that ozone has a high reactivity towards compounds having double bonds and functional groups with high electron densities. In lignocellulosic compounds the moiety that is most likely to be oxidized is lignin, owing to its high carbon-carbon double bonds (Garcia-Cubero et al. 2009).

Ozonolysis of lignocelluloses has several advantages such as a) absence of toxic residues in treated material, b) suitability for on-site generation, thus avoiding the problem of chemical supply and storage c) ozonation reaction takes place at ambient temperature and pressure, d) effective degradation/solubilization of lignin and hemicellulose without significant effect on cellulose, e) biodegradability of degradation fragments and potential for ozonolysis products like formic acid and acetic acid to be metabolized by animals (Neely 1984; Vidal and Molinier 1988).

Ozone has been used to pretreat a variety of lignocellulosic feedstocks and degrade lignin in bagasse, green hay, pine (Neely 1984), wheat straw, rye straw (Garcia-Cubero et al. 2009) and poplar sawdust (Vidal and Molinier 1988). Ozonolysis of wheat straw removed 60% of lignin and increased the rate of enzymatic hydrolysis by a factor of five (Binder and Scharf 1980). Treatment in poplar sawdust by ozone at 65mg/L decreased the lignin content from 29% to 8% which led to an increase in hydrolysis yield from 0% to 57% (Vidal and Molinier 1988). Garcia – Cubero et al. (2009) observed that ozonolysis increased enzymatic hydrolysis yield from 29 and 16% to 89 and 57% in wheat straw and rye straw, respectively, when treated with gaseous ozone at 2.7% (w/w). Of the parameters shown to impact ozonolysis, moisture content of the biomass (25-35%) and ozone concentration (2-6% w/w) are believed
to be significant for efficient ozonolysis (Neely 1984).

Although ozonolysis has been reported to be an effective pretreatment method, and the reactivity of ozone has been explored in general, a clear understanding of its mechanism and reaction kinetics during interaction with lignocellulosic materials is little understood. Hence this study was undertaken to investigate the reaction kinetics of ozone and its effect on lignin over time in a column packed with a layer of lignocellulosic biomass. The feedstocks chosen were energy grasses including *M. ×giganteus*, *M. sinensis* ‘Gracillimus’, *S. arundinaceum* and *S. ravennae* owing to their higher sugar content relative to other lignocellulosic materials. The conditions for ozonolysis were based on previous studies on the energy grasses which indicated that ozone had potential to be used for pretreatment of these feedstocks to enhance subsequent hydrolysis.

2. Materials and Methods:

2.1 Biomass Preparation:

Energy grasses from the Mountain Horticultural Crops Research and Extension Center (Mills River, NC) harvested in December 2009 were dried at 45°C for 72 h and ground to pass 2mm sieve using a Wiley mill (Model: 4, Thomas, Philadelphia, PA, USA) and stored in ziplock bags at room temperature until further use.

2.2 Pretreatment:

2.2.1 Ozone Pretreatment:

Thirty percent moisture (dry basis) was added to 1 g each of ground, moisture free energy
grass samples in triplicate and allowed to equilibrate for an hour before treatment with ozone. Ozonolysis was performed in a glass column reactor tube (Ace glassware, Product no: 5813 – 26) of diameter 2.5 cm and length 30 cm. One end of the tube was plugged with glasswool and the sample was placed over it. Ozone was produced by an ozone generator (Model: OL80 A, Ozone lab instrument, Canada) fed with industrial grade oxygen (Airgas National Welders, Raleigh, NC). The flow rate of oxygen from the cylinder to the ozone generator was maintained at 0.25 L/min using a mass flow controller (Model no: FMA5516, Omega.) and samples were pretreated for 10, 20, 30, 60, 90 and 120 min at 40mg/L. Ozone at the outlet of the reactor was logged on a per min basis using a hand held ozone sensor (Aeroqual, Series 500, New Zealand).

Ozone consumption data for pretreating 5 gram of biomass samples were measured for a pretreatment time of 120 minutes to generate correlations with prior experiments on optimization of ozonolysis conditions. Pretreated samples were analyzed for changes in lignin and sugar concentration.

2.3 Analytical Methods:

Total solids, acid insoluble lignin (AIL), acid soluble lignin (ASL) and ash were determined using the procedure described by National Renewable Energy Laboratory (NREL) (Sluiter et al. 2006; Sluiter et al. 2005a; Sluiter et al. 2005b). Monomeric sugars including glucose, xylose and arabinose in the untreated and pretreated solids were determined using HPLC (Dionex Corporation, Sunnyvale, CA, Model: UltiMate 3000) with a refractive index (RI)

96
detector. An Aminex HPX-87H column was used at 65 °C with 0.005 M sulfuric acid at 0.6ml/min as eluant.

2.4 Statistical Analysis:

All experiments in this study were performed in triplicate. Statistical analysis was done using SAS® 9.2 (SAS Institute, Cary, NC). Significance of change in lignin concentration over time was performed by the Tukey-Kramer test at 95% confidence level using PROC MIXED.

3. RESULTS AND DISCUSSION:

Energy grass samples used in the study contained on average 20.8-23.3% acid insoluble lignin and 53.5-59.1% carbohydrates. Table 4.1 shows the change in acid insoluble concentration for the different varieties over time as a result of ozonolysis. Ozonolysis of 1 gm for 2 hours ranged between 8.13 - 23.66%, which was lower than lignin reductions of 29.2-42.2% observed during ozonolysis of 5g biomass (Chapter 3). Limited loss of acid insoluble lignin (1.7-26.0%) accompanied by complete ozone consumption (10mg/min) was observed for all the varieties at all time periods. Nevertheless, change in lignin concentration over time was significantly different (p<0.05) from untreated biomass after 10, 20, 30 and 60min of pretreatment for M. sinensis ‘Gracillimus’, S. ravennae, M. × giganteus, and S. arundinaceum, respectively.

The minimal delignification associated with complete utilization of supplied ozone gas, indicates that ozone gas was being used by other reactions which gained precedence over delignification. It is hypothesized that ozone reacted with the initial lignin degradation
products instantaneously, which potentially got further fragmented over time (Holen et al. 1998; Mbachu and Manley 1981). In a previous study on identifying optimal conditions for ozonolysis and subsequent enzymatic hydrolysis of energy grasses, syringaldehyde and vanillin were observed in the sample wash water after pretreatment (Chapter 2 and 3). A study on ozonation of corn stalks has shown that ozone oxidized the initially generated oxyaromatics from lignin and further transformed them into shorter aliphatic carboxylic acids (Quesada et al. 1999). Degradation products identified by GC/MS from the ozonolysis of corn stalk were identified as glycolic, oxalic, malonic, glyoxalic, glycric, p-hydroxybenzoic and malic acid. Vanillin and p-hydroxybenzaldehyde were also detected. It was also observed that a higher rate of delignification occurred at the beginning than at the end of the reaction. Ozonation of poplar sawdust at 45% moisture content yielded oxalic, formic, glycolic, glyoxalic, succinic, glycric, malonic, p-hydroxybenzoic, fumaric and propanoic acids (Euphrosine-Moy et al. 1991; Lasry et al. 1990) while ozonation of coastal Bermuda grass and Kentucky 31 tall fescue at 50% moisture content produced caproic, levulinic, p-hydroxybenzoic, vanillic, azelaic and malonic acids along with p-hydroxybenzaldehyde, vanillin and hydroquinone (Morrison and Akin 1990). Thus the ranges of ozonation products are influenced by both substrate and moisture content. Moreover, the reaction rate of these aliphatic acids with ozone are influenced by functional groups other than carboxylic groups present in the molecule (Holen et al. 1998).
Table 4.1: Percent acid insoluble lignin content of energy cane sample before and after ozonolysis

<table>
<thead>
<tr>
<th>Time</th>
<th>$M. \times$giganteus</th>
<th>$M. sinensis$</th>
<th>$S. arundinaceum$</th>
<th>$S. ravennae$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>‘Gracillimus’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>23.3± 0.57</td>
<td>21.8± 0.20</td>
<td>20.8±0.06</td>
<td>22.6 ± 0.26</td>
</tr>
<tr>
<td>10 min</td>
<td>21.6± 0.83</td>
<td>18.9±0.56</td>
<td>20.4±0.22</td>
<td>19.9± 0.59</td>
</tr>
<tr>
<td>20 min</td>
<td>19.7±2.17</td>
<td>17.6±0.12</td>
<td>19.7±0.21</td>
<td>17.6±1.78</td>
</tr>
<tr>
<td>30 min</td>
<td>18.2±0.96</td>
<td>17.2±0.58</td>
<td>19.5±0.77</td>
<td>17.7±0.90</td>
</tr>
<tr>
<td>60 min</td>
<td>20.1±1.00</td>
<td>17.3±0.29</td>
<td>19.0±0.38</td>
<td>16.7 0.91</td>
</tr>
<tr>
<td>90 min</td>
<td>17.5±0.18</td>
<td>17.5±0.01</td>
<td>19.2±0.71</td>
<td>17.3 ± 0.68</td>
</tr>
<tr>
<td>120 min</td>
<td>19.2± 0.01</td>
<td>18.1±0.60</td>
<td>19.1±0.53</td>
<td>17.2 ± 0.66</td>
</tr>
</tbody>
</table>
Figure 4.2 and Table 4.2 show the glucan and xylan contents of pretreated and untreated solids. Glucan content of the pretreated solids recovered from the different treatment times was found to be either equal to or more (p>0.05) than the glucan concentration in the untreated samples. Minimal change (p>0.05) in concentration of xylose from pretreated solids, was observed over time for all the varieties.

Increase in glucan concentration in the pretreated solids may be attributed to depolymerization of cellulose and to a lesser extent hemicellulose, resulting in products such as gluconic acid, glucornic acid and lactones which can interfere with analytical methods. Gluconic acid is known to co-elute with glucose under the conditions used in this study for HPLC analysis (Cannella et al. 2012) and can thus result in over-estimation of glucan content. It has been noted that cellulose degradation by hydroxyl radicals produced during ozone-lignin reactions are more pronounced than by self decomposition of ozone (Kang et al. 1995; Lind et al. 1997).
Figure 4.2: Glucan concentration (%) in untreated and pretreated energy grasses.
Table 4.2: Percent xylan content after ozonolysis of energy grasses.

<table>
<thead>
<tr>
<th>Time</th>
<th>M. × giganteus</th>
<th>M. sinensis 'Gracillimus'</th>
<th>S. arundinaceum</th>
<th>S. ravennae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>16.9 ± 1.72</td>
<td>17.2 ± 2.34</td>
<td>18.5 ± 1.75</td>
<td>18.2 ± 1.68</td>
</tr>
<tr>
<td>10 min</td>
<td>13.5 ± 0.86</td>
<td>16.3 ± 1.78</td>
<td>16.1 ± 1.64</td>
<td>18.7 ± 1.63</td>
</tr>
<tr>
<td>20 min</td>
<td>14.7 ± 0.52</td>
<td>19.7 ± 0.82</td>
<td>18.0 ± 1.30</td>
<td>21.6 ± 2.31</td>
</tr>
<tr>
<td>30 min</td>
<td>14.9 ± 1.34</td>
<td>18.5 ± 2.76</td>
<td>18.6 ± 1.30</td>
<td>20.5 ± 2.89</td>
</tr>
<tr>
<td>60 min</td>
<td>13.6 ± 0.66</td>
<td>18.2 ± 0.31</td>
<td>17.2 ± 1.91</td>
<td>21.2 ± 1.36</td>
</tr>
<tr>
<td>90 min</td>
<td>14.1 ± 0.17</td>
<td>20.8 ± 0.51</td>
<td>18.4 ± 0.21</td>
<td>19.5 ± 2.56</td>
</tr>
<tr>
<td>120 min</td>
<td>12.8 ± 1.99</td>
<td>19.4 ± 1.51</td>
<td>16.4 ± 2.47</td>
<td>18.2 ± 1.68</td>
</tr>
</tbody>
</table>
Various mechanisms have been proposed for the formation of hydroxyl radicals as shown in figures 4.3, 4.4 and 4.5 (Kang et al. 1995).

a) Formation of hydroxyl radicals via hydrogen abstraction from a phenolic compound.

\[
R\text{OCH}_3\text{OH} + O_3 \rightarrow HO_3^- + ROCH_3\text{O}^-
\]

\[
HO_3^- \rightarrow HO^- + O_2
\]

Figure 4.3: Formation of hydroxyl radicals by hydrogen abstraction.

b) Formation of hydroxyl radicals via phenolate formation and electron transfer.

\[
ROCH_3O^- + O_3 \rightarrow O_3^- + ROCH_3O^-
\]

\[
O_3^- + H^+ \rightarrow OH^+ + O_2
\]

Figure 4.4: Hydroxyl radicals formation by electron transfer.

c) Formation of hydroxyl radicals via organic peroxides, which are produced during ozonolysis of phenolic and non-phenolic structures.
Reaction of ozone with cellulose can occur via a tree radical chain mechanism or an ionic mechanism (Katai and Schuerch 1966). The ionic mechanism of ozone on cellulose has two pathways- (1) oxidative cleavage of the glycosidic linkage yielding gluconolactone or methyl esters and (2) hydrolytic cleavage yielding glucose (Katai and Schuerch 1966; Pan et al. 1981; Sakai and Uprichard 1991). Studies on ozonation of glucose and cellobiose (cellulose model compounds) in aqueous solution, proved that ozonation of glucose produced gluconic acid and its lactones as main products while NMR analysis of oxidation of cellobiose indicated attack at the free reducing end with minor glycosidic linkage scission (Holen et al. 1997). Gluconic acid produced from glucose reacted further with ozone at a rate equal or higher than the glucose-ozone reaction and produced products such as D-arabino-2-hexulosonic acids, D-xylo-4-hexulosonic acid, L-guluronic acid and tartaric acid.
Figure 4.6 shows the formation of gluconic acid from glucose.

\[
\begin{align*}
\text{H-} & \quad \text{C=O} \\
\text{(CHOH)}_4 & \quad \text{OH}^- \rightarrow \quad \text{C=O} \\
\text{(CHOH)}_4 & \quad \text{OH}^- \rightarrow \quad \text{COOH} \\
\text{CH}_2\text{OH} & \quad \text{D-glucose} \\
\text{CH}_2\text{OH} & \quad \text{D-gluconic acid}
\end{align*}
\]

Figure 4.6: D-gluconic acid formation from D-glucose

3.1 Kinetics of delignification:

Since lignin is the key component in lignocellulosic materials that is impacted by ozone, the impact of ozonolysis on lignin concentration in energy grasses over time was analyzed. Information on reaction rates was obtained to possibly help in the development of more efficient pretreatment processes. Equation 4.3 represents the relation between change in lignin concentration and time as observed in this study (Fogler 1992).

\[
\ln \left( \frac{C_0}{C_t} \right) = kt \quad \text{(4.3)}
\]

Where,

\( C_0 \) = initial lignin concentration (%)  \\
\( C_t \) = lignin concentration (%) at time \( t \) (min)  \\
\( k \) = overall reaction rate constant (% min\(^{-1}\))

Figure 4.7 shows the plot of \( \ln \left( \frac{C_0}{C_t} \right) \) versus time for pretreated samples. It was observed that the reaction followed first order kinetics for the first 30 minutes as change in lignin
concentration over time was linear. The slopes of the curves represent the rate constant \( k \).

From the figure it can be observed that *S. ravennae* had a higher reaction rate \((k = 0.012)\) while *S. arundinaceum* had the lowest reaction rate \((k = 0.0021)\). The coefficients of determination \((R^2)\) of more than 0.95 for all varieties except *M. sinensis* ‘Gracillimus’ indicated that first order kinetics can be used to represent lignin degradation on account of ozonolysis well.

4. CONCLUSION:

Limited delignification of lignin along with complete consumption of ozone and increase in glucan content was observed with ozonation of energy grasses for all the different varieties. It is hypothesized that the hydroxyl radicals produced during the ozone-lignin interactions depolymerized cellulose and produced by-products such as gluconic acid, glucose and other aliphatic organic acids. Furthermore, it is also believed that the initial compounds formed during ozone-lignin interactions, resulted in continual consumption of ozone and produced additional by-products. Future kinetic studies with varying quantities of biomass in a packed bed are required for a better understanding of the lignin degradation kinetics.
Figure 4.7: Delignification efficiency by ozonolysis of energy grasses

- M. × giganteus: Slope = 0.0084, $R^2 = 0.99$
- M. gracillimus: Slope = 0.0091, $R^2 = 0.87$
- S. arundinaceum: Slope = 0.0021, $R^2 = 0.96$
- S. ravennae: Slope = 0.012, $R^2 = 0.99$
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Holen A, Christensen P, Moe S. Investigation of reaction products from the ozonation of cellulose model compounds by NMR-spectroscopy; 1997. TAPPI Proceedings.


CHAPTER 5: CONCLUSION AND FUTURE WORK

In this study, four energy grasses varieties, *Miscanthus ×giganteus*, *Miscanthus sinensis* ‘Gracillimus’, *Saccharum arundinaceum* and *Saccharum ravennae* were used as lignocellulosic feedstock for fermentable sugar production by ozone pretreatment and enzymatic hydrolysis. The major conclusions are as listed below:

1. Ozone pretreatment was conducted at three different ozone concentrations (40, 50 and 58 mg/L) using two reactor configurations – Uniflow and Flip. Pretreatment conditions were optimized based on lignin content and glucan recovery in the pretreated solids. Ozonolysis proved to be an effective pretreatment method as it removed up to 59.9% lignin without cellulose degradation. However, enhanced sugar yields after hydrolysis of pretreated solids were not observed and this was speculated to be the inhibitory effect of lignin degradation products on the enzyme.

2. Energy grasses were pretreated at optimized conditions (AIL and GLU) and hydrolysis was conducted at three different enzyme loadings. Effect of washing the solids after pretreatment to overcome the inhibitory effect of lignin degraded products was also studied. For all the varieties, pretreatment conditions optimized for minimum lignin content followed by washing the solids with water (AIL-W) and hydrolysis with 0.1g/g enzyme loading of Cellic® CTec2 proved to be statistically significant (p<0.05) for maximum glucan conversion. Increasing the enzyme loading from 0.1g/g to 0.2g/g did not have a statistically significant effect on glucan conversion. *M. ×giganteus* proved to be the most recalcitrant variety.
3. Hydrolysis of energy grasses at higher enzyme loading (0.3g/g) had >100% glucan conversion and this was due to overestimation of glucose by the HPLC, due to co-elution of gluconic acid.

4. Energy grasses pretreated with 1% NaOH for 60 min at 121°C followed by hydrolysis with 0.1g/g enzyme, served as a baseline comparison against ozonolysis. Comparison of glucan conversion efficiencies between alkali and ozone pretreated (AIL-W) samples suggested that hydrolysis of ozone pretreated *M. ×giganteus* and *S. ravennae* had a higher glucan conversion (p<0.05) than alkali pretreated biomass.

5. Ozone kinetics study was conducted to understand the change in lignin and glucan over time. Study was conducted using 1g of samples and very minimal delignification was observed over time. Glucan content increased for some of the varieties, which indicated plausible depolymerization of cellulose.

Ozone pretreatment of energy grasses proved to be effective in removing lignin and in increasing glucan yield after hydrolysis. However, further studies are required to gain a better understanding of the overall process and to make it economically attractive for capital investors. Some of the suggestions for future work are:

1. Results reported in this study were based on ozonolysis for 120 minutes. However, further study needs to be done on reducing the pretreatment time, to make this process economically less energy intensive.

2. Pretreatment at 40mg/L of ozone proved to be statistically significant in maximizing glucan conversion and delignifying the energy grasses. Future investigation could be
done on lower ozone concentrations.

3. Identification of lignin degradation products that inhibited the enzyme during hydrolysis is an area that needs to be investigated. Such an investigation would enable better understanding of reaction kinetics and mechanism of ozone with the samples and thus help in increasing the sugar yields.

4. Further research on reducing the enzyme loading for enzymatic hydrolysis, will make the process economically attractive and feasible.

5. Additionally, an extensive economic analysis of a completely optimized process would enable better decisions by investors.
APPENDIX A

Model SAS code for pretreatment optimization

```sas
ODS PDF FILE='insoluble concentration.pdf';
proc MIXED data=a;
  class trt variety Sample concentration;
  model Acid_insoluble_lignin = trt|concentration|variety/
    outp=outmx residual ddfm=satterth htype=1;
  repeated subject= trt*concentration group=trt;
  lsmeans trt|concentration|variety/diff adjust=Tukey;
run;
ODS PDF CLOSE;

ODS PDF FILE='glucose concentration.pdf';
proc MIXED data=a;
  class trt variety Sample concentration;
  model glucose = trt|concentration|variety/
    outp=outmx residual ddfm=satterth htype=1;
  repeated subject= trt*concentration group=trt;
  lsmeans trt|concentration|variety/diff adjust=Tukey;
run;
ODS PDF CLOSE;
```
APPENDIX B

HPLC chromatogram of lignin degradation products in wash liquid

Peak 1 – Syringaldehyde, Peak 2 – Vanillin, Peak 3 – unknown compound and Peak 4 – unknown compound.
### Appendix C

Glucose yield in untreated and ozone pretreated samples at higher enzyme loadings.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Enzyme loading (%)</th>
<th>Glucose yield (mg/g untreated biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>AIL-U</td>
</tr>
<tr>
<td>M. × giganteus</td>
<td>20</td>
<td>336.8±10.15</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>481.3±30.30</td>
</tr>
<tr>
<td>M. sinensis ‘Gracillimus’</td>
<td>20</td>
<td>333.2±7.35</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>441.5±26.28</td>
</tr>
<tr>
<td>S. arundinaceum</td>
<td>20</td>
<td>333.3±12.60</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>382.6±65.95</td>
</tr>
<tr>
<td>S. ravennae</td>
<td>20</td>
<td>334.6±7.56</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>499.9±27.63</td>
</tr>
</tbody>
</table>

*Hydrolysis done at 25% enzyme loading

n/a – hydrolysis not performed.