

The Pennsylvania State University

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**ENSILING CORN STOVER WITH ENZYMES AS A FEEDSTOCK
PRESERVATION METHOD FOR BIOCONVERSION**

A Dissertation in

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by

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ABSTRACT

Energy sustainability and environmental protection are two great challenges that face humanity. Biofuels from lignocellulosic biomass have been recognized as a potential solution for both of these interrelated issues. However, there is a serious bottleneck to economical and efficient ethanol production: the recalcitrance of lignocellulosic biomass due to lignin. This bottleneck has to be solved before cellulosic biofuels can play a significant role in a renewable energy society. To produce biofuels sustainably from lignocellulosic biomass, it will be necessary to store preserve large amounts of feedstock from seasonally harvested fields. Wet storage, and specifically ensilage, could serve as a promising platform for biological pretreatment, since saccharification of the cell wall occurs naturally by organic acids and amended enzymes during the ensilage process. In this study, the impact of the interaction between corn stover harvest seasons and cell wall degrading enzymes was investigated. These investigations included both experimental studies and model simulations of the impacts of feedstock, storage conditions and enzymes, including cellulase and hemicellulase or laccase, on the characteristics of stover silage. The objectives were to obtain a low pH, minimize dry matter losses, and create beneficial biochemical changes in the stover that would facilitate downstream pretreatment and bioconversion.

In temperate climates, the corn stover harvest can extend from early fall to early winter, during which time the chemical composition of stover varies significantly and influences the ensilage process. Identifying the optimum harvest

period can help maximize utilization of stover as a feedstock for bioethanol. The first investigations explored the effects of harvest date and enzyme addition along with possible interactions on the characteristics of corn stover silage. Corn stover was harvested five times in 2005 and eight times in 2006 throughout early fall and early winter. Samples 500g were subsequently ensiled at 37°C with and without the enzyme treatments at both field moisture and 60% moisture (w.b.). Dry matter loss, pH, water soluble carbohydrate and monosaccharides were analyzed on days 0, 1, 7, and 21. Samples were also subjected to reduced severity dilute acid pretreatment to quantify the conversion to simple sugars. Results demonstrated that harvest date had a significant impact on the quality of stover silage for bioconversion. The moisture content of corn stover, cob and corn were significantly influenced by harvest date, and at later harvest dates, moisture addition was critical for obtaining high quality silage. Results indicated that early fall was the best harvest time in terms of pH, dry matter, water soluble carbohydrate and monosaccharide as well as xylan conversion percentage. With respect to corn stover silage, the addition of enzymes significantly enhanced the positive effects.

The presence of lignin in ligninocellulosic biomass constrains and challenges the improvement of bioconversion techniques. A second set of investigations were performed to explore the influence of the lignin-degrading enzyme, laccase, on enzymatic ensiled corn stover. Tetramethylammonium hydroxide (TMAH) thermochemolysis and Gas Chromatography - Mass Spectroscopy (GC-MS) results documented molecular signals of lignin

decomposition in laccase-treated stover. Cellulose conversion through enzymatic hydrolysis improved with an increase in the laccase loading rate. This enhanced cellulose digestibility is believed to result from better exposure of cellulose to cellulase through structural changes of lignin, which makes more cellulase available for cellulose hydrolysis. The findings suggest that ensilage might provide a platform for biological pretreatment platform, partially hydrolyzing cellulose and hemicellulose into soluble sugars during the enzymatic ensilage process, and thus facilitating laccase penetration into complex biomass to enhance lignin degradation. These results serve as a first step to understanding the addition of multiple enzyme combinations during ensilage to maximize the utilization of corn stover as a biofuel and biochemical feedstock.

The final quality of enzymatically ensiled corn stover was significantly affected by its initial chemical composition, microbial population dynamics, enzyme activities, and thermal and physical conditions of silage fermentations. A comprehensive experimental study to better understand how the interactions of these factors govern silage quality requires a large number of trials and intensive analysis. A predictive enzymatic ensilage model, which was initially developed in a series of papers by Pitt and his colleagues in the late 1980s, was enhanced and then applied to simulate the dynamic behavior of pH, water soluble carbohydrates (WSC), cellulose, and the effects of enzyme additives on the major biochemical and microbial changes during the ensilage process. Estimated final pH, WSC and cellulose concentrations are in agreement with enzymatic silage experimental

results. The simulation results also demonstrated that the cellulose loading rate had a significant positive effect on the change of WSC. Results showed enzyme additives in the silage process enhanced the stability of long term storage. The optimal experimental conditions to obtain a high quality enzymatic corn stover silage can be achieved by adjusting the cellulase loading rate and operation temperature. The enhanced model could serve as a guide in designing silage systems (with or without enzyme additives) for large amounts of plant-based biomass.

In conclusion, this study demonstrated harvest seasons and cell wall degrading enzymes have strong effects on the characteristics of stover silage, and that ensilage technology was an effective preservation and pretreatment strategy for bioconversion of corn stover biomass. In order to further improve ensilage as a partial substitute for expensive and energy-intensive thermal and chemical pretreatment technologies, future work should focus on using biological strategies to deconstruct the recalcitrant structure of lignocellulosic biomass. If successful, such efforts could thereby improve bioconversion efficiency dramatically.

Key words: corn stover, enzyme, laccase, ensilage, harvest date, lignin depolymerization, pretreatment, bioconversion, modeling.

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Chapter 1

INTRODUCTION

Energy provides a focal point for several avenues of agricultural and biological engineering research. On the one hand, each year our agricultural system produces billions of tons of plant residue, and a large amount of these potential energy resources are wasted (Sheehan et al., 2004). On the other hand, society is faced with the tremendous problem of developing suitable long-term supplies of energy for sustainable development and existence. Bioconversion, the biological conversion of feedstocks into energy and materials, has the potential to provide an attractive solution to address these two critical problems simultaneously. Successful bioconversion strategies will increase the use of agricultural crops and forest resources as feedstocks for bioenergy and bioproducts, reduce foreign oil dependence, enhance income in America's rural region, provide more job opportunities, and help promote the environment (Perlack and Turhollow, 2005; DOE, 2007).

Corn stover, comprising over 80% of total domestic agricultural residues, is widely regarded as an ideal biomass feedstock because of its relatively low cost and the high volume of lignocellulose present in the biomass (Kadam and McMillan, 2003). The current availability of corn stover is estimated at 75 million dry tons per year (USDA/DOE, 2003), a majority of which could be available to ethanol plants in the near term. However, more than 90 percent of corn stover is left in farming fields; and less than 1% is utilized in industrial processing (Kim and Dale, 2004). The majority of corn stover

is not recovered, which effectively wastes renewable energy by ignoring potential biomass feedstock. Effective storage of corn stover, providing a high quality industrial feedstock continuously over the whole year, presents many challenges for researchers.

Stover with grain storage has been researched for decades for use as livestock bedding or fodder. Traditionally, dry storage was used to minimize decomposition. This method has been used for at least several hundred years for corn stover. But there are many disadvantages to dry storage, including the fact that procedures are labor intensive and there can be a high dry matter loss of up to 23% due to plant and microbial respiration (Richey et al., 1982). Furthermore, the low moisture content associated with dry storage creates a high risk of fire resulting from spontaneous combustion, stray sparks, lightning, or other fire triggering events. To solve these problems, Richard et al. (2001) proposed ensilage technology for biomass storage. This technology eliminates those storage risks, reduces dry matter loss, and offers a potential pretreatment opportunity as well (Richard et al., 2001).

The silage of corn stover is a very promising approach, because it provides an efficient and economical long-term storage method for large volume of plant-based material. It can minimize carbohydrate degradation and eliminate the risk of fire. Ensilage, essentially a lactic acid fermentation process, reduces the pH value and impedes secondary fermentations (Richard et al., 2001). In addition, ensilage is a natural pretreatment process because it partially saccharifies the biomass cell walls. However, if pH is not sufficiently low, some sugar fermentation occurs simultaneously through the

action of a synergistic system of microorganisms, producing organic acid which may or may not be valuable to downstream processes.

Although ensilage provides an attractive prospect for the preservation of corn stover, it still challenges researchers for two main reasons: first, desired characteristics of corn stover storage are distinct from those of forage crops, and second, owing to the very low content of fermentable sugars in corn stover (around 1 to 5% dry matter) it is very difficult to effectively initiate lactic acid fermentation to achieve a low pH (Ren et al., 2004).

To solve the above problems, enzyme additives, such as cellulase and hemicellulase, have been introduced in the process of ensiling corn stover to degrade the lignocellulosic cell wall components to fermentable sugars (Richard et al., 2002; Ren et al., 2004; 2005; 2006; 2007). This can bolster the growth of lactic acid bacteria and lower the pH value of the ensiling biomass. The positive effects of these fermentation stimulants for subsequent fiber utilization have been demonstrated by examining several physical properties, such as modulus of elasticity, water thickness swell, and internal bond strength of particleboard manufactured from ensiled corn stover with and without enzymes (Ren et al., 2004; 2005). However, whether the rate of sugar production has been enhanced or not has not been reported, and the impacts of silage with or without enzymes on bioconversion efficiency of corn stover are also unknown. Solutions to these two issues are crucial for the ethanol production and deserve further exploration.

Several related aspects of enzyme enhanced ensilage also warrant further investigation. In the prior studies the hemicellulose fraction of the corn stover has not

been degraded very thoroughly, nor has the cellulose fraction. Moreover, options for degrading lignin in corn stover have not been explored. The addition of lignin-degrading enzymes could possibly contribute to the downstream fermentation process, since such enzymes have proven effective in removing lignin from paper pulp (Kirk and Jeffries, 1996; and Tengerdy and Szakacs, 2003). The research on synergies among different enzymes will be required for a comprehensive project to achieve the improvement of the intermediate product (sugar) and the overall bioconversion efficiency to ethanol and other fuels and chemicals.

The interaction of storage with pretreatment may significantly help achieve these goals. Pretreatment has been demonstrated as a significant tool for biomass conversion, but it accounts for over 1/3 ~ 1/2 of the total energy required (Mosier et al., 2005). It may be possible to decrease the intensity of pretreatment to achieve cost-effective ethanol by combining enzymatic ensilage with appropriate pretreatment processes.

To enhance ensilage process for corn stover biomass conversion, this study will pursue a multi-pronged approach. An ideal harvest season will be identified to optimize silage quality for downstream bioconversion. Reduced-severity dilute acid pretreatment screening method will be applied to the stover samples to identify the optimal treatment. Other enzymes also will be selected and tested in an attempt to improve the sugar yield. To improve experimental efficiency and obtain effective and feasible solutions, empirical and numerical models of ensiled corn stover with related enzyme additives will be integrated by performing statistical data analysis and numerical simulations. The synergetic function of enzymes and ensilage technology in corn stover will be

comprehensively studied based on selected effect factors, such as pH, moisture content and water soluble carbohydrate concentration from the experiments and modeling analysis. The ultimate objective of this research is to enhance biomass preservation and bioconversion efficiency. The proposed research will give a better solution for ensiling corn stover with enzyme additives so as to lead to better biomass conversion to ethanol or alternative energy and chemical products.

Chapter 2

LITERATURE REVIEW

Both the energy crisis and environmental concerns provide strong motivation for scientists and engineers to develop alternative energy sources rather than continuing to rely on fossil fuels (DOE, 2008). Among potential energy alternatives, lignocellulosic biomass has been recognized as a potential feedstock for biofuels, and because of its large current volumes corn stover has been promoted as an important near term option (Kumar et al., 2008; Rubin, 2008; Kadam and McMillan, 2003). However, some crucial issues, such as bioconversion efficiency and large scale biomass storage, have to be addressed before society can shift its energy dependence away from petroleum to biofuel (Ragauskas et al., 2006). This literature review covers research concerning the characteristics of corn stover, solid state fermentation, ensiling corn stover with enzymes, ensilage modeling, and bioconversion of corn stover.

2.1 Corn stover

The chemical characteristics of corn stover play essential roles in determining how to implement biomass conversion to manufacture value-added products such as transportation fuels, chemicals, and particle board. Corn stover is composed of four components: stalk, leaf, cob, and husk (See Table 2-1).

Table 2-1: Dry matter distribution in corn residue (from Myers and Underwood, 1992).

Corn Residue	% Moisture	% of Residue D.M. Basis
Stalk	70-75	50
Leaf	20-25	20
Cob	50-55	20
Husk	45-50	10

By the time stover is harvested from the field, most of the mass remaining in these components is in the cell walls. Figure 2-1 shows the typical physical structure of plant cell wall, which mainly includes three structures: the middle lamella, primary cell wall and plasma membrane. To assess preservation and pretreatment effectiveness, the identification of these chemical components of corn stover is needed.

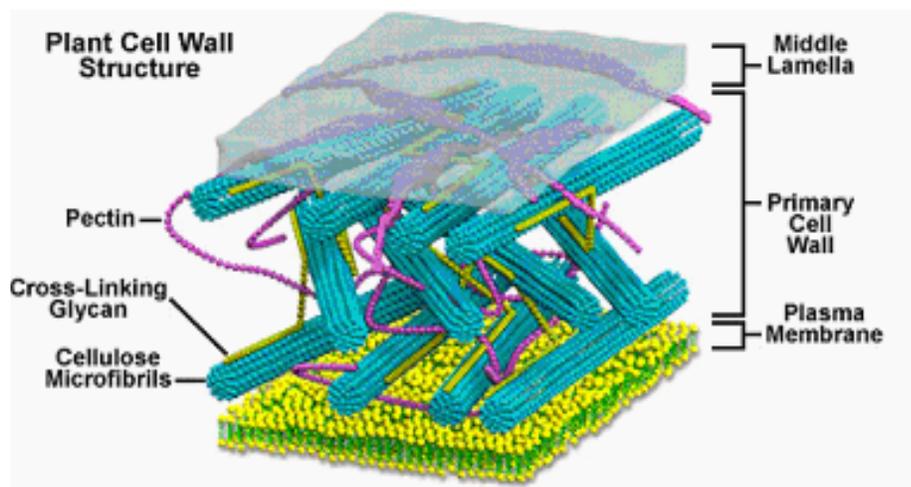


Figure 2-1: Physical structure of plant cell wall (Davidson, 1995).

2.1.1 Structural features of corn stover

Corn stover contains a variety of biochemical molecules including cellulose, hemicellulose, lignin, phenolic acids and silica, water soluble carbohydrates, starch, organic acids, protein, and non-protein nitrogen. Table 2-2 shows the percentage of dry weight composition of corn stover. The structural cell wall components, cellulose, hemicellulose and lignin, are more than 80-90% dry basis (d. b.) of corn stover.

Table 2-2: Percent dry weight compositions of herbaceous feedstocks (adapted from Wiselogel et al., 1996).

Feedstock	Glucan	Xylan	Galactan	Arabinan	Mannan	Lignin	Extractives	Ash
Corn stover ¹	36.4	18.0	1.0	3.0	0.6	16.6	7.3	9.7
Corn stover ²	40.9	21.5	1.0	1.8		16.7		6.3
Bagasse ³	40.2	21.1	0.5	1.9	0.3	25.2	4.4	4.0
Wheat straw	38.2	21.2	0.7	2.5	0.3	23.4	13.0	10.3

¹ Corn stover includes corn stalks and cobs; ² Corn stover does not include corn cobs; ³ Bagasse is the residue after the juice is extracted from sugar cane.

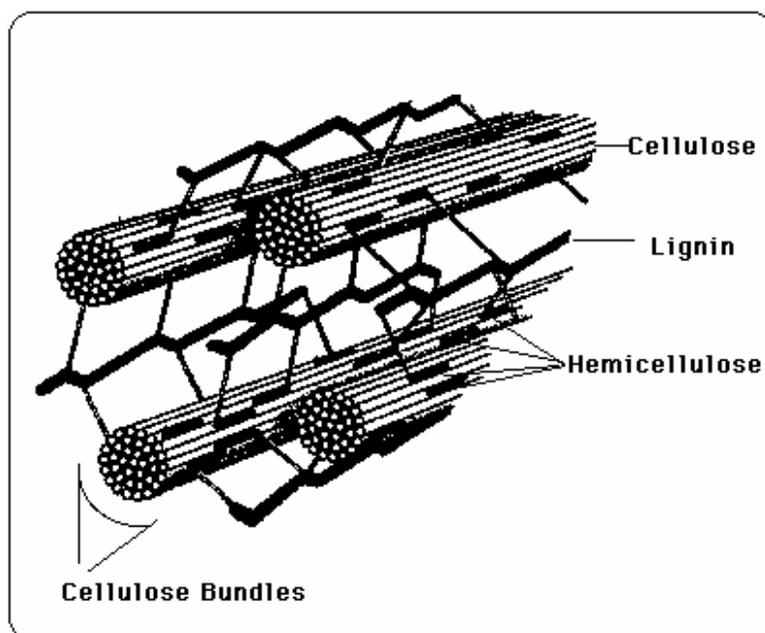


Figure 2-2: Plant cell wall composition (from Hamelinck et al., 2003).

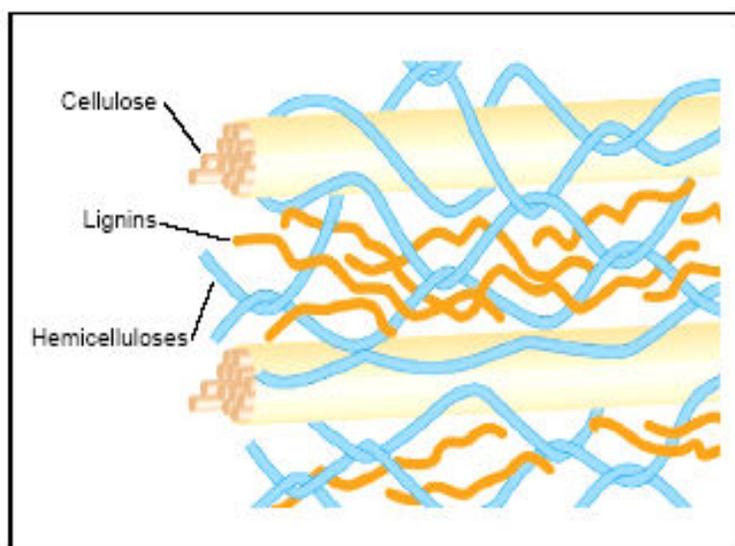


Figure 2-3: Schematic representation of the lignified secondary wall (from Boudet et al., 2003).

Figures 2-2 and 2-3 show the plant cell wall structure. A skeleton, formed by cellulose, functions as a matrix for hemicelluloses and encrusted lignin. All of these three main components are closely associated with each other (Palonen, 2004). The covalent cross-linkages between lignin and carbohydrates are known as “lignin-carbohydrate complexes (LCC)”, and are the major barriers to hydrolysis (Palonen, 2004; Kim and Lee, 2006).

2.1.1.1 Cellulose

Cellulose is the most abundant polymer on earth (Zaldivar et al., 2001). It comprises about 36.4 to 40.9 % of the dry weight of corn stover (Table 2-2).

Cellulose is characterized by identical linear polysaccharides of D-glucopyranose units linked by β -1, 4- glycosidic bonds (Figure 2-4), with every other linkage rotating 180° (Zaldivar et al., 2001). This structure is shown in. Crystalline microfibrils are formed by linkages, such as Van der Waals forces and hydrogen bonds, among cellulose chains (Palonen, 2004). Cellulose can be converted into glucose by dilute or concentrated acid or enzymes that can break down the linkages in this crystalline structure.

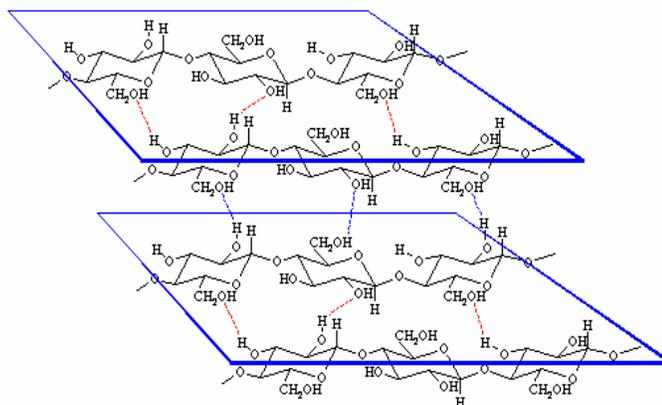


Figure 2-4: Structure of cellulose microfibrils with intermolecular hydrogen bonded cellulose chains (from Robyt, 1997).

2.1.1.2 Hemicelluloses

There are four basic types of hemicelluloses: xylans, mannans, galactans, xyloglucans. Xylans are the most common hemicelluloses (Figure 2-5). The monomers in hemicellulose are D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, 4-O-methyl-glucuronic, D-galacturonic and D-glucuronic acid. These sugars are linked by β -1, 4- (mainly) and β -1, 3-glycosidic bonds (Pérez et al., 2002). Unlike cellulose, hemicellulose has a branched chain, amorphous nature and various compositions, which depend on the part and species of the plant (Zaldivar et al., 2001). The hemicellulose components of corn stover are about 22.6 to 24.3% d.b., with xylan accounting for 21.5% d.b., as shown in Table 2-2. Compared with cellulose, hemicellulose does not aggregate and is easily hydrolyzed into simple sugars.

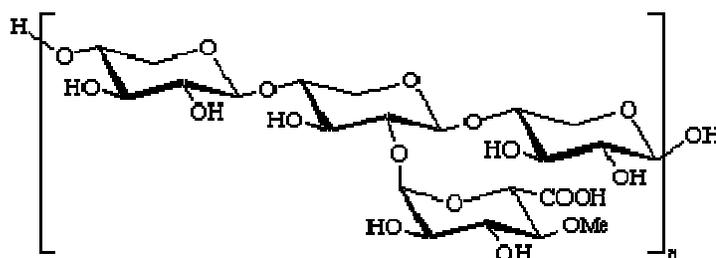


Figure 2-5: Structure of D-xylan fragment (from Eriksson et al. 1990)

2.1.1.3 Lignin

Lignin, the second most plentiful organic substance on earth, is a random three-dimensional network aromatic biopolymer, in which C-O-C and C-C linkages are main chemical bonds. There are three dominant precursor alcohols in lignin: p-hydroxycinnamyl (coumaryl) alcohol; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units. Among these units, β -O-4 type accounts for 50 % of total linkages, as seen between 1 and 2, 2 and 3, 4 and 5, 6 and 7 (Figure 2-6). Cross-linking between lignin and hemicellulose (Unit 5 in Figure 2-6) is formed by secondary reactions (Kirk and Farrell, 1987). While these major features of lignin are understood, the exact structure is very complex and amorphous and varies largely with species, age, and other conditions (Pérez, et.al, 2002; Palonen, 2004; Grabber, 2005; Kim and Lee, 2006).

In plants, lignin always interlaces with a carbohydrate polymer matrix of cellulose and hemicellulose, forming lignin-carbohydrate complexes (LCCs) with three kinds of linkages: ester, ether, and glycosidic bonds (Kim and Lee, 2006). The polysaccharide

matrix strongly influences both the chemical and 3-dimensional structure of lignin (Palonen, 2004). Although lignin is a hydrophobic polymer, the hydroxyl and methoxyl groups in lignin precursors may interact with cellulose microfibrils, according to experiments using the molecular dynamic simulations (Palonen, 2004). With such a unique impermeable and integral structure, lignin and LCC can supply plants with strength and structural support as well as imperviousness against water, light, oxidation and microbial invasion (Pérez et al., 2002; Hamelinck et al., 2003). Although much progress has been made recently, a comprehensive understanding of the biological decomposition of lignin is still incomplete (Van Soest, 1994; Grabber, 2005).

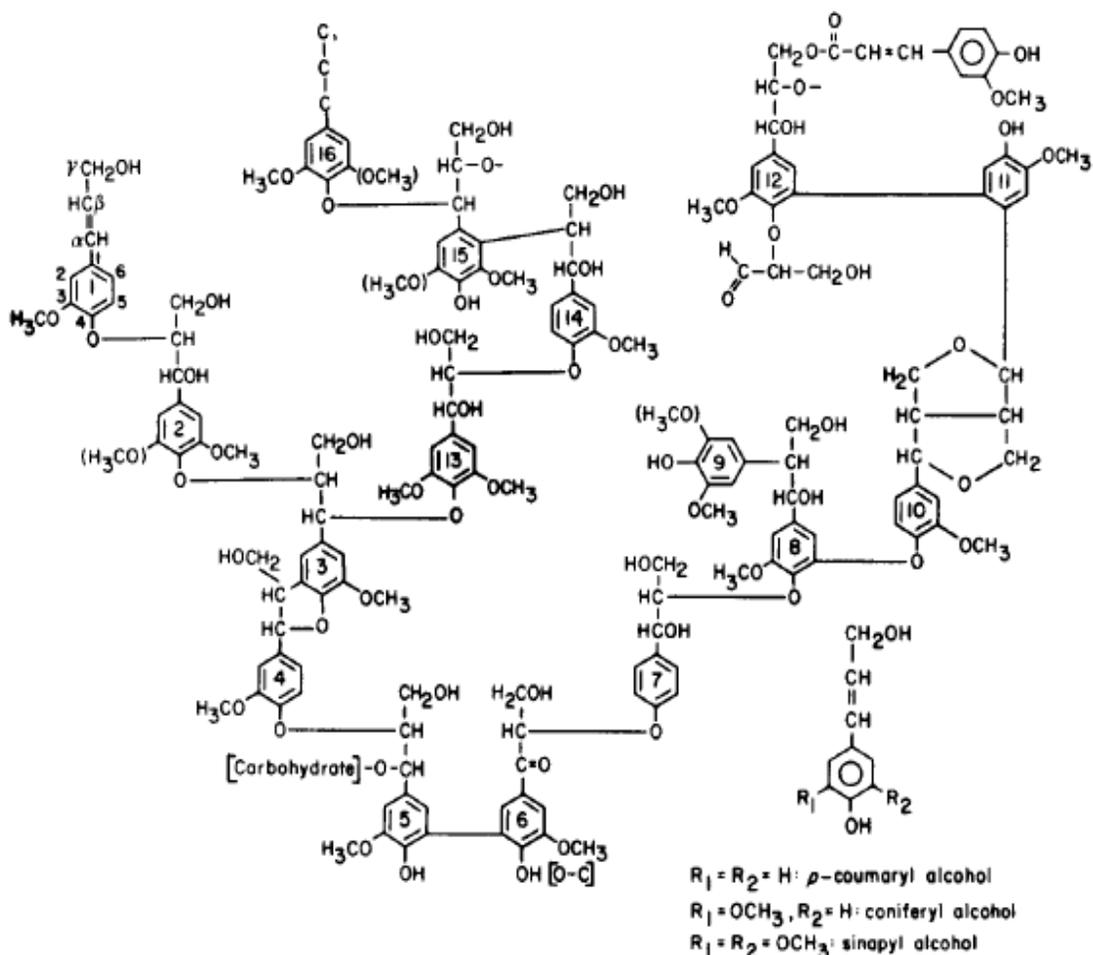


Figure 2-6: Schematic structural formula of lignin (from Kirk and Farrell, 1987)
 The three precursor alcohols are seen at the lower right.

Many researchers pointed out that prior lignin removal or alteration would significantly improve bioconversion efficiency, because lignin is a key barrier preventing enzymes from penetrating into cellulose (Orth and Tien, 1995; Hamelinck et al., 2003; Mosier et al., 2005; Kim and Lee, 2006). Current pretreatment delignification strategies include lignin removal and lignin structure alteration, both of which can decrease the intensity of the pretreatment and hydrolysis and thus save energy (Mosier et al., 2005;

Wyman et al., 2005). Some researchers indicate that the high temperature and acidic conditions in some pretreatment technologies change the chemical structure of lignin significantly (Palonen, 2004; Kim et al. 2008). For example, lignin was found to be agglomerated into smaller particles and separated from cellulose at temperatures higher than 200°C (Palonen, 2004).

The lignin separated by delignification processes can be used as a fuel with a high energy content, 26.3 MJ/OD kg, and is a candidate for use in engineering plastic and other polymeric materials (Kim and Lee, 2006; Grabber, 2005). The content of lignin in corn stover has been reported as 15.0- 25.0% w/w, and when separated it is relatively cleaner than that generated from a pulping process (Wiselogel et al., 1996; Kim and Dale, 2004; Kim and Lee, 2005, 2006).

2.2 Solid state fermentation

Ensilage processes are examples of Solid-State Fermentation (SSF) without inoculation. In general, SSF refers to the cultivation of microorganisms on moist solid supports. This structure can provide physical support and may also provide energy and a source of nutrients, such as carbon (Mitchell, 2002b; Couto and Sanroman, 2005). This fermentation environment, lacking free water, is close to the natural environment for many organisms. Besides these biological advantages, SSF also have some processing advantages, such as high-volume productivity of the end product, low energy demand for heating and easy aeration (Pandey et al., 2000; Mitchell, 2002b).

One prominent advantage of application of SSF techniques is that extremely cheap raw materials, for instance, the large volumes of agricultural and food wastes, could serve as the primary substrate for fermentation of a variety of products (Couto and Sanroman, 2005). SSF has been applied in a series of investigations for adding value to agricultural products and sub-products in Brazil since 1986. These projects converted agricultural wastes to value-added products such as bulk chemicals and value-added fine products, including ethanol, mushrooms, organic acid, amino-acid, enzymes, and single-cell protein (Raimbault, 1998). SSF can also play an important role in the bioconversion of lignocellulosic biomass, the very promising feedstock of biofuels, enzymes, and other biochemical products (Tengerdy et al., 2003).

In applications of the SSF process, microbial growth and product formation are significantly influenced by several important environmental factors, such as temperature, pH, moisture content, oxygen, and nutrients (Raimbault, 1998). For example, SSF processes have a low moisture content so that only some microorganisms, such as yeasts, fungi and some bacteria, can contribute to fermentation (Couto and Sanroman, 2005). And with limited water, the fermentation conditions, including temperature, pH, moisture, and substrate concentration, are difficult to control (Mitchell, 2002b). To overcome heat and mass transfer problems during SSF processes, Chinn and Nokes (2008) applied anaerobic thermophilic bacteria on lignocellulosic substrates. Their experiments showed that SSF maybe a more promising method for biochemical production than liquid substrate fermentation due to the more favorable environment and high product concentrations (Chinn and Nokes, 2008).

2.3 Silage

Typically, ensilage is an anaerobic solid-state fermentation that converts water-soluble sugars into organic acids (mostly lactic acid) by lactic acid bacteria (LAB). The organic acids decrease the pH and minimize secondary fermentation to achieve positive preservation effects, similar to the pickling process (Richard et al, 2001).

Ensilage technology has been used in preserving animal feed for ruminant livestock since ancient times (McDonald, 1981). Today this preservation technology not only has been used widely in forage storage but also has been applied to the long-term storage of feedstock for bioconversion, where several investigators have explored optimum processing strategies (Richard et al., 2001; 2002; Shinnars et al., 2007; Ren et al., 2004).

The ensiling process is composed of five basic procedures: gleaning biomass at the optimal harvest date; adjusting moisture content by wilting or adding water; chopping (if necessary); loading into a commercial or experimental-scale silo, compacting and sealing to block air and storing and then unloading for downstream bioconversion or animal feeding. Before loading, additives can be added if necessary (Weinberg and Ashbell, 2003).

During ensilage, the four main phases of biochemical or microbiological activities are identified as the initial aerobic process, anaerobic fermentation, anaerobic storage and aerobic uploading (Rooke and Hatfield, 2003; Weinberg and Ashbell, 2003).

- a. Aerobic process: During the period of filling and the short time after sealing, air is present in the silo, plants continue to respire, and the pH is 6.0-6.5.

Aerobic microorganisms, including fungi, yeasts, etc., are still active (Weinberg and Ashbell, 2003).

b. Anaerobic fermentation: Within this phase, LAB dominate the fermentation and convert water soluble carbohydrates into lactic acid and other organic acids. These acids accumulate during this process, so the pH decreases below 5.0. The exact pH value depends on the buffering capacity of the system and the initial plant constituents including sugar availability (Weinberg and Ashbell, 2003).

c. Anaerobic storage: If the pH decreases low enough to limit the growth of LAB and the silo is sealed well, the silage is relatively stable. Long periods of low pH anaerobic storage help destroy pathogens (Rooke and Hatfield, 2003; Weinberg and Ashbell, 2003).

d. Aerobic unloading: During this aerobic phase, aerobic microbial growth is reinitiated and eventually prevails. Microorganisms, mainly yeasts and acetic acid bacteria, proliferate and convert sugars, lactic acid, and other organic acids into CO₂, water and heat (Rooke and Hatfield, 2003; Weinberg and Ashbell, 2003).

2.3.1 Corn stover silage

Richard et al. (2001) proposed ensilage technology, a natural anaerobic fermentation process, for biomass storage, which eliminated dry storage risks and reduced dry matter loss, while offering a potential pretreatment effect. Although ensilage technology provides an

attractive prospect for corn stover long-term storage, it presents several challenges for research and development (Richard et al., 2001, 2002; Ren et al., 2004). First, the objectives and desired properties of corn stover storage for bioconversion are distinct from those for forage crops. The goal of forage crop silage is to increase digestibility for animal feeding, while that of bioconversion is to convert the feedstock to increase fermentable sugars for downstream bioconversion. In addition, moisture content and the fiber content of crop residues are different from those of green plants commonly used for animal feeding. Second, with very low content of fermentable sugars, around 1 to 5% in corn stover, it can be difficult to initiate the lactic acid fermentation. Third, the stability of long-term ensiled storage can not be guaranteed when there is such little available fermentable sugar.

To resolve these problems, enzyme additives, such as cellulase and hemicellulase, were introduced in the process of ensiling corn stover so as to degrade the lignocellulosic cell wall components to fermentable sugars (Richard et al., 2002). The positive effects of ensilage with enzymes, such as insuring a more stable storage process, leading to higher levels of water soluble carbohydrates, and quicker decrease in pH, demonstrated enzymatic ensilage could be a promising approach for biomass preservation and pretreatment for bioconversion (Richard et al., 2002).

Based on this potential of enzymes to enhance the ensilage process, comprehensive enzyme screening investigations were recently performed in Richard's research group (Ren et al., 2004, 2005, 2006, 2007). Specifically, comparison studies have been conducted to investigate the impacts of purified cellulase and industrial enzyme mixtures of cellulases with hemicellulases on the process and product

characteristics of ensiled corn stover. The experimental results showed that certain industrial enzyme products would work well in silage, and had benefits due to lower cost and the synergies between cellulase and hemicellulase. Seven commercial enzymes with various ratios of cellulase to hemicellulase (C:H), produced by *Aspergillus niger*, *Trichoderma reesei*, and *Trichoderma longibrachiatum*, were added to the corn stover ensilage process to determine the optimum enzyme mixture. *Trichoderma reesei* was the source the most effective enzyme in this screening study, with a ratio of C to H of 2.38, and produced a silage with high lactic acid content and low pH. Following these bench-scale short-term screening studies, larger-scale and longer-term trials also demonstrated positive impacts of enzyme addition, including lower pH and higher lactic acid and water soluble carbohydrate concentrations (Ren et al., 2007).

2.3.2 Ensilage models

To improve the quality of corn stover ensilage with or without enzymes, appropriate models should be designed to predict ensilage processes.

A qualitative model of the anaerobic phase of ensilage was presented by Neal and Thornley (1983) to simulate bacterial growth and changes in pH during ensilage biochemical and microbial processes. Using assumed parameters and five initial values, the numerical predictions generally agreed with the experimental results. However, the model predicted some unreasonable phenomena such as too low pH which does not often occurred in a real ensilage process. Some discrepancies were determined to be caused by the inaccurate or incorrectly chosen model parameters.

In order to understand better the basic biochemical and microbial processes during ensilage, a predictive quantitative model was developed by Pitt et al. (1985). Various dynamic mathematical relationships were established with respect to some primary processes in three different and interactive phases, aerobic, lag and fermentation, of the ensilage process. With the parameters determined from published silage experiments and pure culture bacterial research, the authors set up a framework to construct a quantitative empirical model of the ensilage process in lactate silages. However, the initial models had many limitations because of incompleteness of collected data. Leibensperger and Pitt (1987) extended the study to include growth of clostridia in wet forages and higher temperature. Existing experimental results were mapped to validate the models and show that the silages could be classified as clostridial, lactate or intermediate according to final concentrations of butyric acid and lactic acid. Three years later, Pitt extended these silage fermentation models by incorporating a kinetic model of enzymatic hydrolysis by incorporating cellulase and amylase into the previous silage model (Pitt, 1990). This model explored most of the important factors in enzymatic hydrolysis including pH, temperature, water activity (a_w), rate of cellulase activity, reaction rate, and concentration of substrates and end products. However, the estimated pH still deviated significantly from experimental results and more information is necessary to make the model widely applicable. Two modeling gaps of particular importance are the consumption of cellobiose and glucose by the lactic acid bacteria in silage, and the rate of WSC production by cellulase hydrolysis of cellulose.

In 2006, Ren developed a model to simulate the enzymatic ensilage process by combining the ensilage model of Pitt et al. (1985; Leibensperger and Pitt, 1987) with an enzymatic hydrolysis model (Gan et al., 2003). The resulting prediction of some chemical components of corn stover was acceptable, including the concentrations of cellulose, WSC, and degraded sugars. The assembled model was constructed based on several key assumptions: that liquid reaction systems are suitable representations of the ensilage process; that a single cellulase system can be used to simulate of the reactions of cellulases; and that all the kinetic rates for hydrolysis of hydrolysable cellulose microfibrils, inert cellulose microfibrils and different forms of sugars (released, inhibited and consumed) as well as pH are constant throughout the ensilage process. To the contrary, several of these assumptions seem to be contradicted by observations of real ensilage processes. For instance, pH experiences a dramatic reduction during the initial stage of ensilage, and can increase at later stages if secondary fermentations arise. In addition, it is well accepted that the cellulose hydrolysis rates for the enzymatic kinetic model are dynamic functions of pH, substrate and end-production concentration, water activity, temperature and other parameters (Pitt, 1990; Philippidis et al., 1992; 1993).

More recently, Rezaei (2008) applied the silage model (Pitt et al., 1985; Leibensperger and Pitt, 1987) to simulate organic acid production in a switchgrass ensilage process. Although the model predicted the concentration of lactic acid and acetic acid reasonably well, the relationship of pH with other parameters in the ensilage system was not considered. Instead, the pH of ensiled biomass was predetermined by three

segmental linear functions. Enzyme influences on the ensilage system also were not examined.

From this previous work it is apparent that Pitt, his colleagues, and successors have provided a solid modeling foundation that can be used to simulate the ensilage process with or without enzyme addition. However, certain aspects of the models could be improved by developing specific process-based submodels, and providing some supplemental information. Such modifications are required to integrate the whole enzymatic ensilage process so it can be simulated under specific thermal and biochemical conditions to predict the characteristics of ensiled corn stover.

One of the objectives of this study is to modify the enzymatic ensilage model by Pitt and his colleagues and then apply it to simulate the dynamic behavior of pH, WSC, cellulose, hemicellulose, lactic acid, and the effects of cellulases on the major biochemical and microbial changes during the ensilage process. A commercial engineering package, Stella, will be used to as a simulation tool to numerically solve all the differential equations involved in the hydrolysis model and ensilage model. The simulation results will be compared to experimental measurements to examine the feasibility and correctness of the enzymatic ensilage model. The optimal operating conditions required to obtain high quality of enzymatic silage of incoming corn stover will also then be suggested in terms of the adjustment of cellulase loading rate and operation temperature.

2.4 Cell wall degrading enzymes (CWDE)

The two main reasons for using cell wall degrading enzymes as silage additives are as follows: first, to degrade the cell walls into fermentable water soluble carbohydrates (WSC), which promote lactic acid bacteria fermentation in cases where fermentable sugars are scarce; and second, to decrease the fiber content of silage or improve digestibility. Most previous interest in enzymes for silage focuses on cellulases and hemicellulases, generally from the fungi *Trichoderma longibrachiatum*, *Asperigillus niger*, *A. oryzae*, and *A. awamor*, and occasionally from bacterial sources (Kung et al., 2003).

2.4.1 Cellulases and hemicellulases

Cellulase is defined as a group of enzymes that catalyze the degradation of cellulose to soluble sugars and glucose (Bhat and Bhat, 1997). The cellulase complex includes endoglucanase (E.C. 3.2.1.4), exoglucanase or cellobiohydrolase (E.C. 3.2.1.91), and glucohydrolase or cellobiase (E.C. 3.2.1.21), all of which act synergistically to degrade cellulose (Kung et al., 2003). Research on the cellulase complex of *Trichoderma reesei* has shown that it effectively degrades crystalline and amorphous cellulose, chemically converting celluloses quantitatively to glucose (Bhat and Bhat, 1997).

Hemicellulase refers to an enzyme complex that catalyzes the degradation of hemicellulose to sugar. Hemicellulases include endo-1, 4-D-xylanases (E.C. 3.2.1.8) and exoxylosidases (E.C. 3.2.1.37) (Kung et al., 2003). The former, including nonarabinose and

arabinose releasing endoxylanases, randomly attack the xylan backbone and liberate shorter xylooligosaccharides; the latter can liberate xylose from the shorter xylooligomers and remove a single xylose molecule from the non-reducing end of a xylan (Kung et al., 2003). In *T. reesei*, several enzymes, such as xylanases and mannanase which degrade hemicellulose, have been identified (Palonen, 2004).

The synergies among cellulases to facilitate degradation of cellulose are well-known (Sun and Cheng, 2002). Murashima et al. (2003) reported the synergistic effects of cellulosomal xylanase and cellulases from *Clostridium cellulovorans* on plant cell wall degradation and explored the potential mechanism. The research result of Koukiekolo et al. (2005) showed that effective synergies among cellulases and hemicellulases from *Clostridium cellulovorans* increased the degradation of cellulose and hemicellulose of the plant cell wall.

2.4.2 Lignin-degrading enzymes

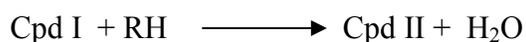
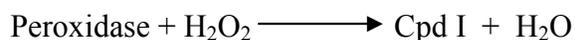
In nature, only filamentous fungi are able to degrade lignin (Kirk and Farrell, 1987). White-rot fungi are the best-known microorganisms that can efficiently degrade lignin by producing ligninolytic enzymes, peroxidase and laccases which use low-molecular-weight mediators to conduct lignin degradation.

The 4 different lignin-degrading enzymes are described below:

- a. Lignin peroxidases (LiPs, EC 1.11.1.14): LiP, discovered by Tien and Kirk in 1983, has been recognized as the most effective peroxidase, and can oxidize

phenolic and non-phenolic compounds, amines, aromatic ethers, etc. (Leonowicz et al., 2001; Pérez, et.al, 2002). LiP, isolated from white-rot fungi, is a glycoprotein with a heme group in its active center. The high molecular mass of LiP, ranging from 38 to 47 kDa, is too large to enter an intact plant cell, so it can only directly access exposed regions of the plant lumen or physically degraded biomass.

The catalytic cycle of ligninase was summarized by Tien (1987): Lignin peroxidase is initially oxidized by hydrogen peroxide to a two electron-oxidized intermediate, compound I. Compound (Cpd) I oxidizes the first substrate molecule by one electron, which then forms a free radical and an one electron-oxidized intermediate, compound II. This compound II then is reduced to ligninase via oxidizing another substrate by one electron.



The above reaction was stimulated by veratryl alcohol. The following have been proposed as possible reasons for this effect (Tien, et al., 1986; Kirk and Farrell, 1987):

- i. Veratryl alcohol may act as a substrate for ligninase;
- ii. Veratryl alcohol stimulates oxidation of other compounds;
- iii. Veratryl alcohol may also induce the synthesis of ligninolytic biocatalysts;

b. Manganese-dependent peroxidases (MnPs, EC 1.11.1.13): MnP, also isolated from *Phanerochaete chrysosporium*, resembles LiP. Both are extracellular and non-growth related proteins produced during the stationary stage, and are part of secondary metabolism caused by nutrient starvation. But MnP has somewhat higher molecular masses than LiP, ranging from 45 to 60 kDa. MnP can not oxidize non-phenolic units of lignin. The mechanism of MnP is to oxidize Mn(II) to Mn(III). The substrate Mn(II) must be chelated by organic acid chelators to stabilize the product Mn(III). Mn(III) is a strong oxidant, oxidizing phenolic compounds and leaving the active center (Pérez, et.al, 2002).

c. Versatile peroxidase (VP): VP has both manganese peroxidase and lignin peroxidase activities. With Mn(II) present, VP can oxidize hydroquinone in the absence of exogenous H₂O₂ (Takayoshi, 2004; Pérez, et.al, 2002).

d. Laccases (EC 1.10.3.2): They are four-copper enzymes, and the coppers belong to three different types (Figure 2-7). It is believed that laccase catalysis includes three main steps: (a) to reduce Type 1 Cu by substrate reduction; (b) to transfer internal electron from Type 1 Cu to Type 2 and Type 3; and (c) to reduce oxygen to water molecules. So Type 1 and Type 2 copper are responsible for electron capture and transfer, and Type 2 and Type 3 copper are involved in oxygen binding and then producing water. (Gianfreda et al., 1999; Call and Mücke, 1997).

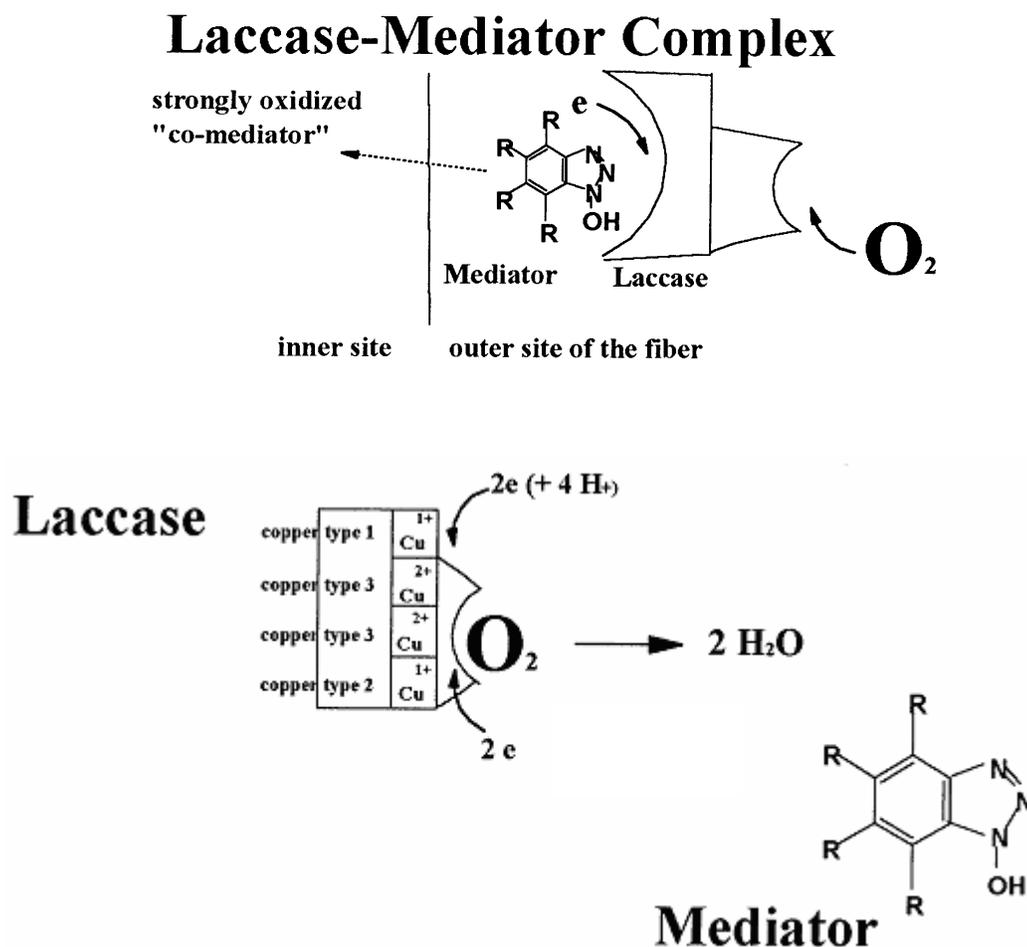


Figure 2-7: Reaction scheme of laccase (adapted from Call and Mücke, 1997).

In nature, laccase is produced by fungi, bacteria and plants. The main sources of laccases are fungi like *Agaricus*, *Tramees*, *Pholiota*, *Podospora*, *Rhizonia*, and *Neurospora* (Call and Mücke, 1997; Palonen, 2004). Laccase from different sources demonstrates a large variability in substrate specificity, molecular weight, optimum pH, etc. (Palonen, 2004). Laccases are rather unspecific enzymes, which react with some low-molecular mass compounds, called mediators, to cleave the ether bonds that link most aromatic subunits in lignin (Call and Mücke, 1997; Andrzej et al., 2001; Palonen, 2004).

The common characteristic of most effective mediators is they are N-heterocyclics bearing N-OH groups. Among these groups the most popular low-molecular mass mediators of laccases are ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] and 1-hydroxybenzotriazole (HBT) (Leonowicz et al., 2001).

The application of laccases to industrial lignin degradation has gained considerable attention in recent years, especially in the pulp and paper industry. Most studies have been laboratory research on the degradation of simplified lignin, whereby lignin has been separated by physical or chemical methods or synthesized (Kirk and Jeffries, 1996; Thompson, et al., 1998; Srebotnik and Hammel, 2000; Tengerdy and Szakacs, 2003; Camarero et al., 2004; Elegir et al., 2005). However, lignin-degrading enzymes still face challenges in the delignification of biomass that has not been modified by physical or chemical methods, because the large macromolecular size of lignin-degrading enzymes prevents them from contacting the lignin components in an intact plant cell wall (Kirk and Farrell, 1987; Pérez, et.al, 2002). To the best of our knowledge, very little research has examined the potential lignin-degrading enzymes to pretreat corn stover during storage. Similarly, synergies among hemicellulases, cellulases and ligninases during stover ensilage have not been reported as yet.

2.5 Pretreatment

Pretreatment refers to mechanical and physical operations to cut or grind the biomass, break the lignin seal, and destroy the crystalline structure of cellulose to make it more amenable to downstream treatment (Figure 2-8). Pretreatment can make biomass

more accessible to the hydrolytic enzymes which convert carbohydrates into fermentable sugars, as shown in Figure 2-8 (Hamelinck et al., 2003; Mosier et al., 2005; Zeng et al., 2007). Generally, without pretreatment hydrolysis yields are less than 20% of theoretical yields, while in the presence of pretreatment they are frequently more than 90% of theoretical (Lynd, 1996).

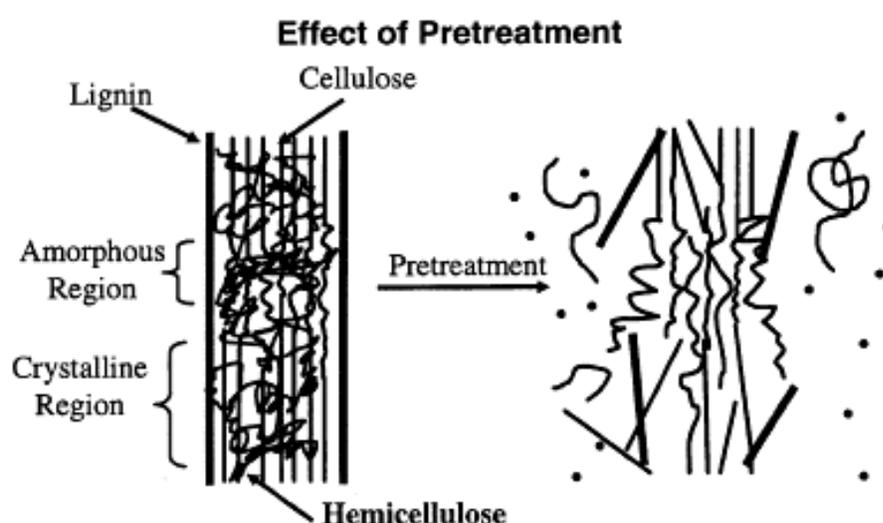


Figure 2-8: Schematic of pretreatment for lignocellulosic material (from Mosier et al., 2005).

Currently scientific understanding of pretreatment mechanisms is incomplete, so design of pretreatment technologies for lignocellulosic material is fundamentally dependent on empiricism. It has been believed that pretreatment efficacy is associated with the amount of hemicellulose and lignin removed by pretreatment processes (McMillan, 1994; Sun and Cheng, 2002). But this statement is contradicted by some data. For example, although over 50% of the corn husks lignin is dissolved in 5% NaOH pretreatment at 120 °C, this alkaline pretreatment was not particularly effective. In contrast, dilute acid pretreatment is associated with a small quantity of lignin removal,

but lead to high enzymatic hydrolysis yield (Lynd, 1996). In principle, an effective pretreatment strategy increases the enzymatic digestibility of biomass by disrupting cell wall physical barriers, reducing cellulose crystallinity, and detaching, desolving, or degrading lignin (Wyman et al., 2005; Mosier et al., 2005).

2.5.1 Dilute acid pretreatment

Among more than thirteen classified pretreatment processes, dilute acid has been considered one of the most cost-effective pretreatments to date (Eggeman and Dlander, 2005). The key features of this technology include 0.5-3.0% sulfuric acid, 10-40 (wt.%) solids concentrations, temperature of 130-200 °C, and pressure of 300-1500 kPa with reaction time spanning 2-30 minutes (Wyman et al., 2005).

Dilute acid pretreatment is regarded as a benchmark for other pretreatment methods (McMillan, 1994; Lynd, 1996). This method improves downstream glucose yields due to almost complete hydrolysis of hemicellulose (Wyman et al., 2005). High temperature dilute acid pretreatment (higher than 160 °C) effectively deals with low solids content (5-10%) in continuous-flow process; while low temperature (lower than 160 °C) is more appropriate for a high solids content (10-40%) batch process (Esteghlalian et al., 1997).

Dilute acid pretreatment has several advantages in achieving high reaction rates, improving cellulose hydrolysis, and achieving high xylan to xylose conversion yields (Sun and Cheng, 2002; Esteghlalian et al., 1997). Considering effects of this method on

the chemical composition and chemical or physical structure of lignocellulosic biomass, dilute acid pretreatment increases accessible surface, removes hemicellulose and alters lignin structure, so it is an effective pretreatment method (Mosier et al, 2005). It also has some limitations, such as energy consumption, a size reduction requirement (about 1-3 mm), high-cost corrosion resistant materials for construction, production of inhibitor compounds that affect downstream processes, and the acid itself must be neutralized before fermentation (US Department of Energy, 1993; Lynd, 1996; Mosier et al, 2005).

The usual industry goal of dilute acid pretreatment is to remove all the hemicellulose content by hydrolyzing it into xylose and other sugars and by dissolving or degrading lignin, thus enhancing the digestibility of cellulose in the residual solids. Recently, an alternative pretreatment method called “reduced severity dilute acid pretreatment” was designed by Corey W. Radtke (2006). The technique applies less intensive conditions that result in partial xylose conversion in order to compare the differences in pretreated biomass digestability. Comparisons of the conversion ratio of xylose/xytan provides a way to screen for more digestible feedstock, with higher ratios of xylose over original xytan due to less recalcitrant plant biomass or better harvest or storage processing (Hoskinson et al., 2006; Duguid et al., 2007;). This screening method includes two primary processes: washing and blending biomass, followed by dilute acid pretreatment under more moderate temperature and pressure conditions. Details of the method are described in Chapter 3 of this dissertation.

2.5.2 Biological pretreatment

Although dilute acid pretreatment is considered the current industry standard, it tends to be expensive, slow and relatively inefficient because it depends heavily on acid and heat treatment. Costs are high because this approach requires high pressure reactors made of corrosion-resistant materials. In addition, inhibitors are generated during this process and decrease the overall yield of the fermentation process (Rubin, 2008). Considering all the requirements of chemical pretreatment and the associated side-effects, there is renewed interest in evaluating biological pretreatment as an alternative for deconstruction of lignocellulose due to its environmental friendliness, potential high yield and cost-effectiveness in the long-run.

Biological pretreatment involves three major categories: microbial, enzyme, and other systems, such as termite gut and cow rumen (DOE, 2007).

a. Microbial strategy: A number of bioprospecting efforts are underway to evaluate and characterize microbial communities that can degrade lignocellulosic biomass. However, current research is limited to certain biomass degraders, such as white rot fungi and *Trichoderma reesei* for lignin and cellulose degradation, respectively (Rubin, 2008; Shi et al. 2008; 2009). For example, the white rot fungi *Phanerochaete Chrysosporium* was used for microbial pretreatment of cotton stalks by solid state cultivation (Shi et al. 2008; 2009). Unfortunately, such treatments are associated with long time periods, significant dry matter loss and complex metabolisms.

b. Enzyme strategy: Enzyme treatments, being added in a controlled manner, could potentially be managed to create optimal conditions for rapid lignocellulose degradation, which may not be achieved in free living systems (Geib et al., 2008). Compared to microbial strategies, enzyme treatment allows effective preservation of dry matter. There have been few reports on lignin decomposition by lignin-degrading enzymes, but the possibility of utilizing these enzymes to deconstruct lignin for improving bioconversion efficiency shows great potential.

c. Other systems, such as insects, ruminants, etc. Several plant consuming animals have gut flora that can degrade lignocellulose. For example, the termite gut possesses a highly efficient microbial community that can convert 95% of cellulose into simple sugars within 24 hours (DOE, 2007). Recently it was reported that the Asian longhorned beetle and the Pacific dampwood termite successfully degraded wood lignin within several hours (Geib et al., 2008). This discovery may contribute to identifying new, highly effective lignin-degrading enzymes from the gut of the insects instead of fungi.

2.5.3 Lignin analysis methods

Strategies for determining lignin structures involve three basic categories: gravimetric methods, spectrophotometric methods and noninvasive methods (Hatfield and Fukushima, 2005; Brinkmann et al., 2002)

a. Gravimetric methods depend on the separation of lignin, called acid insoluble lignin. Klason lignin and acid detergent fiber lignin (ADF) are two

techniques that are representative of these methods (Hatfield and Fukushima, 2005). Jung et al., (1999) compared the accuracy of lignin concentration estimates based on these Klason lignin and acid detergent lignin methods. The results indicate that Klason lignin, with substantially higher estimates than that of ADF, is the more accurate lignin analytical method.

b. Spectrophotometric methods, which are used to determine acid soluble lignin, rely on the decomposition of lignin into soluble degradation products and the determination of their absorbance in the UV. The acetyl bromide method (AB) and thoglycolic acid method (TGA) are two approaches (Brinkmann et al., 2002). Rather than standard commercial standards, extracted lignin standards, using an appropriate extraction method, are required for accurately quantifying lignin in unknown samples for both of these methods (Hatfield and Fukushima, 2005).

c. Noninvasive methods include infrared spectroscopy, near infrared spectroscopy (NIRS) and nuclear magnetic resonance spectroscopy (NMR) techniques. All of these spectral methods do not alter the sample chemically and only require milligram to gram quantities of sample material, but their accuracy depends on proper calibration (Hatfield and Fukushima, 2005).

Biomass from diverse sources may share a similar lignin content, but present different difficulties during the enzymatic hydrolysis process due to differences in lignin composition (Buranov and Mazza, 2008). For this reason, it is clear why new techniques have emerged to measure the amounts of lignin monomers and then identify the

characteristics of the biomass lignin. Among these new methods two of the most popular are the CuO oxidation method and a method using thermochemolysis with tetramethylammonium hydroxide (TMAH). Both methods show similar yield results in quantifying the lignin monomers. The TMAH method presents more sensitive results for the calculation of acid to aldehyde ratios, which signify the degree of lignin depolymerization or the oxidative degradation of lignin. The TMAH method also displays a larger dynamic range than the CuO oxidation procedure. Furthermore, the TMAH method is more suitable for analyzing a large number of samples (Hatcher et al., 1995). Due to the above advantages, TMAH methods have been actively used for detecting lignin depolymerization, including tracking lignin degradation by fungal and wood-feeding insects (Vane et al., 2001; Geib et al., 2008).

2.6 Hydrolysis of lignocellulosic biomass

After pretreatment, the cellulosic and hemicellulosic carbohydrates must be converted into simple sugars (Figure 2-9), which can be fermented to ethanol by microorganisms. Dilute acid and enzymes are two strategies to catalyze this conversion process.

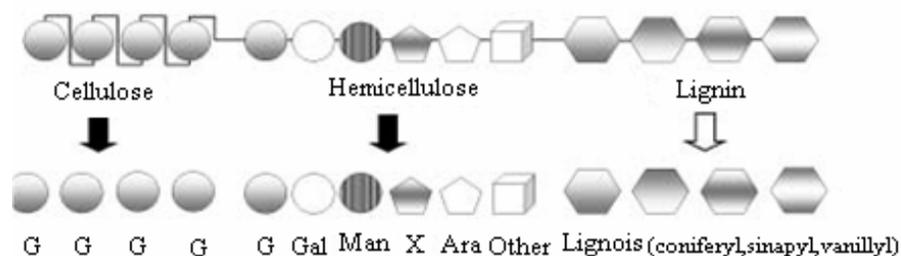
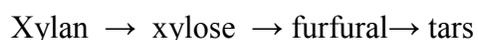


Figure 2-9: Source sugars for ethanol products. Dark arrows represent hydrolysis fermentable monomers, and light arrow represents hydrolysis process. G: Glucose, Gal: galactose, F: fructose, Man: manose, X: xylose; Ara: arainose, other: L-rhamnose, L-fucose, urnoic acids (from Zaldivar et al., 2001).

2.6.1 Acid hydrolysis

Under acidic conditions, the hydrolysis reactions proceed from cellulose and hemicellulose to simple sugars and eventually to tars (Schell and Duff, 1996):



The above reactions show that it is important to control the hydrolysis process. If hydrolysis conditions are very severe, a large amount of sugars will be converted into other chemicals, such as HMF, furfural and tars, which not only decrease the sugar yield but also inhibit downstream fermentation (Schell and Duff, 1996; Wyman, 1999; Hamelinck et al., 2003).

To avoid this sugar degradation, two stages of dilute acid hydrolysis have been developed: The first stage is designed to recover the 5-carbon sugars under mild condition, such as 0.7% sulfuric acid, 190 °C. The rest of the solids, including more

refractory cellulose, are then subjected to harsher conditions, such as 215 °C, but with a milder 0.4% acid, to obtain the 6-carbon sugars in the second stage. The residence time of each stage is three minutes. Typical sugar yields include 89% mannose, 82% galactose, 50% glucose (Hamelinck et al., 2003).

2.6.2 Enzymatic hydrolysis

Enzymatic hydrolysis is carried out by enzymes, such as cellulase and hemicellulase, which catalyze the reaction of cellulose and xylan to glucose (Schell and Duff, 1996).

There are many advantages of enzymatic hydrolysis: (a) Enzymes are 100% selective for the conversion (Schell and Duff, 1996); (b) Very mild condition, e.g. pH 4.8, temperature 45-50 °C (Sun and Cheng, 2002); (c) Utility cost is low; Low cost materials of construction (no corrosion problem) (Hamelinck et al., 2003); (d) Potential high product yield with no degradation of sugar (Schell and Duff, 1996); (e) Cost-effective in the long-run (Hamelinck et al., 2003); (f) No inhibition product for downstream fermentation (Lynd, 1996).

To optimize the enzymatic hydrolysis process, much research has been conducted on increasing the yield and rate of the reaction by investigation of influence of substrate concentration and improvement of enzyme activities. Sun and Cheng (2002) emphasized that enzymatic hydrolysis of cellulose is strongly influenced by substrate concentration. Low yields and rates are produced by low enzyme concentrations, while high enzyme dosage results in high cost and substrate inhibition. Although the cellulase enzyme

loadings relied on specific substrate characteristics, the typical loading dose ranged from 7 to 33 FPU/g substrate (Sun and Cheng, 2002; Hamelinck et al., 2003; Kim et al., 2008).

In addition, the susceptibility of substrates to cellulases is affected by structural features of the substrates, such as lignin content, surface area, and cellulose crystallinity (Hamelinck et al., 2003). According to recent research results, two promising methods to improve enzyme activities are by increasing enzyme loading and addition of surfactants. For example, Tween 80, used a surfactant, improved the rate of enzymatic hydrolysis of newspaper by 33% (Kaar and Holtzapple, 1998; Sun and Cheng, 2002; Castanon and Wilke, 2004).

2.7 Downstream process of biomass conversion to ethanol

The soluble sugars obtained from the hydrolysis process can be converted into ethanol by a fermentation process under oxygen-free conditions. A range of microorganisms, including certain bacteria, yeast, and fungi, can perform this function. The reactions are as follows (Hamelinck et al., 2003):



Hexoses can be easily fermented by some yeasts, such as *Saccharomyces cerevisiae*, while pentoses are quiet difficult for many organisms to ferment. Due to the high percentage of pentoses in most biomass hydrolyzates, efficient fermentation of pentoses is considered very important for the bioethanol industry (Olsson and Hahn-

Hägerdal, 1996). The ability of some wild yeast to ferment pentoses into ethanol was identified in the 1980s. Ho and her coworkers genetically engineered *Saccharomyces* sp. Strain 1400 and made the recombinant yeast able to efficiently coferment glucose and xylose to ethanol simultaneously (Ho, et al., 1998). Compared with yeasts, bacteria attracted people's attention owing to their high fermentation rate (Hamelinck et al., 2003).

Furthermore, purification of ethanol from fermentation is conducted by distillation, which typically includes counter-current contact between an ascending vapor stream and a descending liquid stream (Hamelinck et al., 2003; Lynd, 1996). A large amount of waste, including cellular biomass, lignin, gypsum, etc., is expected to be produced from lignocellulosic ethanol process (Zaldivar et al., 2001). Most of these solids will be used to produce heat and electricity.

2.8 State-of-the-art

Corn stover is actively being promoted as a feed stock for bioconversion, but few researchers have focused on long-term storage. Storage is a key step, as it determines the corn stover available for year-round bioconversion. Current dry storage has significant losses of water soluble carbohydrates as well as a high risk of catastrophic fire (Richard et al. 2001). In order to solve these problems, Richard (2001) proposed ensilage technology for biomass storage and pretreatment. Subsequent research demonstrated ensilage with enzymes is a promising approach for corn stover long-term storage. However, the effects of ensilage on the sugar conversion rate have not been explored, and

little is known about the influence of harvest season on ensiled stover quality. Very little research has examined the potential for lignin degradation of corn stover during its preservation process by ligninase or other lignolytic enzyme. Synergies among the hemicellulase, cellulase and ligninase on stover ensilage process have not been reported as yet. To demonstrate a complete system, this research will focus on increasing sugar yield as well as the enzymatic hydrolysis rate. Finally, few studies have modeled the ensilage process with enzymes. To fill this gap, an integrated enzymatic silage model will be modified and applied to predict key performance measures. The enhanced model could serve as a guide in the design of silage (with or without enzyme additives) for storing large amounts of plant-based biomass.

Chapter 3

THE EFFECT OF HARVEST DATE AND ENZYME ADDITION ON CORN STOVER BIOMASS ENSILAGE FOR BIOCONVERSION

3.1 ABSTRACT

Ensilage, a traditional forage preservation method for ruminants, has shown promise as a preservation strategy for storage of seasonally-harvested lignocellulosic biomass prior to bioconversion. In temperate climates, the corn stover harvest can extend from early fall to early winter, during which time the chemical composition of stover varies significantly and influences the ensilage process. Identifying the optimum harvest period can help maximize utilization of stover as a feedstock for bioethanol. The aim of this study was to explore the effects of harvest date and enzyme addition and their effect on mass losses and the quality characteristics of corn stover silage. Corn stover was harvested five times in 2005 and eight times in 2006 throughout early fall and early winter. Samples 500g were subsequently ensiled at 37°C with and without enzyme treatments at both field moisture and 60% moisture (w.b.). Dry matter loss, pH, water soluble carbohydrate and monosaccharides were analyzed on days 0, 1, 7 and 21. Ensiled samples were subjected to reduced severity dilute acid pretreatment to quantify conversion to sugars. Results demonstrated that harvest date has a significant impact on the quality of stover silage. The moisture content of corn stover, cob and corn were significantly influenced by harvest date, and at later harvest dates, moisture addition was critical for obtaining high quality silage. Results indicated that earlier fall was

the best harvest date in terms of pH, dry matter, water soluble carbohydrate and monosaccharide as well as xylan conversion. Furthermore, these parameters were significantly increased by cellulase enzyme addition.

Key words: harvest date, enzyme addition, corn stover, silage, bioconversion, ethanol

3.2 Introduction

Corn stover, accounting for 80% of total U.S. agricultural residues, is an attractive feedstock for bioconversion because of its low cost and high percentage of cellulose and hemicellulose, both of which can be converted into fermentable sugars for bioethanol production (Kadam and McMillan, 2003). Estimates indicate that over 4.8 billion gallons of ethanol per year could be produced from half of the available corn stover (Kadam and McMillan, 2003). However, only 1% of corn stover was utilized in industry (Kim et al., 2004). One challenge is storing a substantial amount of stover so that this annually harvested feedstock can be used in year-round manufacturing (Atchison and Hettenhaus, 2004). Ensiled storage is a practical alternative to traditional dry storage (Richard et al., 2001, 2002; Shinnars et al., 2007). Ensilage is an anaerobic solid-fermentation that converts water-soluble sugars into organic acids, thereby decreasing secondary fermentation. Its advantages include: application at or near field moisture content, elimination of field drying and soil contact, reduced fire risks and dry matter loss, and conversion of cellulose and hemicellulose into fermentable sugars, thereby potentially reducing pretreatment intensity in the downstream process (Richard et al., 2001; 2002).

There are two sets of criteria that should be considered in optimizing wet biomass storage strategies. Conservation of the mass (and energy) in the initial feedstock is clearly important to downstream uses, and during storage there is potential for shifts between (and especially from) solid to liquid and gas phases. Low dry matter loss is an indicator of mass conservation, and in ensilage is normally strongly correlated with low pH that will limit secondary fermentations (Richard et al. 2001; McDonald et al., 1991). When the downstream goal is bioconversion to ethanol, partial hydrolysis during storage could significantly decrease subsequent chemical pretreatment intensity requirements (Hoskinson et al., 2007). However, each of these criteria may also have negative aspects: the organic acids that lower pH can be inhibitory to fermentation organisms, while water soluble carbohydrates and monosaccharides are subject to decomposition and loss during post-storage handling if aerobic stability is not maintained (McDonald et al., 1991). Optimal strategies for wet storage will thus depend on the interactions and tensions between these two sets of criteria within the context of an integrated biorefinery system.

Moreover, initial biomass feedstock characteristics can significantly affect these criteria throughout the ensilage process. Feedstock differences can be obvious between species (e.g corn stover vs. wheat straw vs. switchgrass) and among different varieties and production locations for a single species (Coors et al., 2007; Schechinger and Thomas, 2005). For a single plant variety and location there can also be significant differences during the growing and harvest season as the plant matures and senesces (Clark et al., 2004). Although the effect of maturity on ensilage has been studied for conventional summer whole-plant corn silage, we are not aware of any prior research on the effects of harvest date

on fall harvested corn stover that does not include the grain. Similarly, little is known about the influence of enzyme addition and water addition on ensilage of mature crop residues at different harvest dates.

In this study we investigated the effect of harvest date and enzyme addition on ensilage of stover from corn grown in central Pennsylvania, in the northeast U.S. In temperate climates the harvest period of corn stover for bioconversion can stretch from early fall to early winter, during which the corn stover quality changes significantly. Understanding the effect of harvest dates on ensiled stover is thus critical for preserving the massive volumes required for industrial bioconversion facilities. Furthermore, little research has been conducted on water addition and enzyme addition during ensilage of stover for downstream bioconversion. The objective of this research was to investigate the effects of harvest dates and enzyme addition on stover quality and bioconversion rate, and to identify the optimum harvest period, thereby maximizing the potential utilization of stover as a feedstock for bioethanol.

3.3 Material and Methods

3.3.1 Corn stover harvest

The corn stover (Renk 232, Renk Seed Company, Sun Prairie, WI) was harvested at the Penn State Rock Springs Farm (Centre County, PA), from early fall to early winter in 2005 and again in 2006. In 2005, corn stover leaves and stalks, no cobs, were collected at five harvest dates and ensiled at 60% moisture (wet basis, w.b.). In 2006, stover from eight harvest dates was again ensiled at 60% moisture as well as field moisture (as harvested). In the 2006 year the cob was included with the stalks and leaves for a whole stover blend,

while in 2005 the cob was not part of the stover mix. In each year a forage plot harvest machine was used to harvest the corn stover after corn ears were hand-removed. The stover was cut approximately 15 cm above the soil, chopped and blown into a drum. In 2006 this stover was mixed with chopped corn cobs (by the ratio of cobs and stover), which were obtained from a corn thresher (R.L. Brownfield, Champaign, IL) and then were processed by a shredder (Model 246-645B000, MTD yard Machine, Cleveland, OH) into 1-3 cm pieces. Harvested corn stover was immediately used in silage experiments to minimize any chemical changes caused by temporary storage.

3.3.2 Moisture content measurement

Rapid and accurate measurement of moisture was required because corn stover was harvested and ensiled on the same day. The moisture content of stover, cob and corn was measured by the Vortex drying method (Buckmaster, 2005), which was compared with a traditional oven drying method (60°C or 105°C for 3 days until constant weight). The results showed that there was no significant difference in moisture content between these two methods.

3.3.3 Enzyme loading amount

To explore the effects of enzyme addition on stover silage, an industrial enzyme blend (Multifect A40, Genencor, Palo Alto, CA) that includes both cellulases and hemicellulases was applied to stover on the onset of ensilage.

Enzyme activities were measured according to Wood and Bhat (1988) and Bailey et al., (1992) by using carboxymethylcellulose (CMC) and birchwood 4-o-methyl glucuronoxylan (Roth 7500) as substrates of endo-1,4- β -glucanase and xylanase.

The lowest effective enzyme rate, 5 IU/g DM, was determined by Ren (2006). This was the minimum enzyme rate needed to produce a fermentation dominated by lactic acid and decrease the pH to below 4 (Ren, 2006). Lower concentrations sometimes had an acetic acid dominated fermentation with higher pH and risk of secondary fermentations to butyric and other organic acids that indicated spoilage in conventional ensilage (Ren, 2006).

3.3.4 Silage preparation

Corn stover was harvested and then ensiled on the same day. Five-hundred gram samples were subsampled from large, well mixed bins, and then packed tightly into 20 cm × 40 cm airtight bags made of polyethylene with treatments assigned randomly. Before packing, 60% moisture samples had water added at the required rates, and enzymes were added for those treatments. Triplicate samples were ensiled for all samples harvested in 2005 and the first three harvest times in 2006, while duplicates were ensiled for samples harvested the remaining five harvest dates in 2006. Samples were double bagged and each bag sealed with a vacuum sealer to guarantee anaerobic conditions for stover fermentation. Samples were subsequently ensiled for 21 days at $37\pm 1^\circ\text{C}$ at either field moisture or 60% moisture (w.b.), the later having been previously indicated as an optimal moisture content for stover ensilage (Richard et al., 2001). The moisture content of stover was adjusted by adding deionized water. Total mass loss, pH and dry matter were measured immediately on each sampling date. The remaining stover was frozen at -15°C and stored for later chemical analysis, which included water soluble carbohydrates (WSC) and monosaccharides. In all cases these sample analyses were completed in a randomized order of the treatments and replicates.

3.3.5 Weight loss and pH measurement

On 0, 1, 7 and 21 days, the stover was destructively sampled for weight and pH measurement. Corn stover weight loss refers to the weight difference between initial sample weight wet basis (w.b.) and the final stover weight (w.b.) after the ensilage process.

Before and after ensilage 10 gram of composite stover was subsampled from each bag after thorough mixing, and then mixed well with 100 mL deionized water for pH measurement by a pH meter (Seven Easy S20, Mettler Toledo, Leicester, United Kingdom) after 30 minutes of equilibration.

3.3.6 Dry matter

From each replicate sample, a composite sample consisting of 100 gram of stover was subsampled after thorough mixing and then dried at 60 °C in a forced air oven for two days. Dry matter loss was determined by calculation of the ratio of the dry matter mass difference relative to the initial dry matter mass, using Equation 3-1:

Dry matter loss (%) =

$$\frac{(Weight_{initial} (g) \times Moisture_{content_{initial}} (\%) - Weight_{final} (g) \times Moisture_{content_{final}} (\%))}{(Weight_{initial} (g) \times Moisture_{content_{initial}} (\%))} \times 100\% \quad (3-1)$$

3.3.7 Water soluble carbohydrate analysis

For this and all other chemical analysis composite samples were subsampled from the ensiled bags after thorough manual mixing, and then the analysis was completed in a randomized order. Water soluble carbohydrates were measured on a 25 gram composite subsample by the modified phenolsulfuric acid method (Guiragossian et al., 1977).

3.3.8 Monosaccharide analysis

For each replicate sample from days 0 and 21, 25 gram composite subsamples of stover were mixed with 250 mL of deionized water and shaken for 30 minutes at 200 rpm by an open air shaker (MaxQ 2000, Barnstead, Dubuque, IA). The liquid was then filtered through Whatman No.1 paper, centrifuged for 30 min at 4500 rpm, and then the supernatant was filtered through a 0.22 μm syringe filter. After being diluted 50 times, this liquid was measured for monosaccharides by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA). Monosaccharides analyzed included glucose, xylose, arabinose, galactose and ribose. The mobile phase was 4 mM NaOH with a flow rate of 0.50 mL/min and a running time of 73 min. A CarboPacTM PA20 (Dionex, Sunnyvale, CA) guard column and analytical column and a gold electrode chemical detector, (Dionex, Sunnyvale, CA) were used for identification and quantification of monosaccharides at 30 °C.

3.3.9 Compositional analysis

After through mixing a 30 gram composite stover was subsampled from each sample bag on days 0 and 21, dried at 45 °C in a forced air oven for 2 days, and then ground using a Wiley mill (laboratory mill model 4, Arthur H. Thomas Co., Philadelphia, PA) to pass through a 2 mm screen. Composition, including carbohydrates, acetyl, ethanol, ash, lignin, structural inorganics and uronic acid, was measured according to Chen et al. (2007).

3.3.10 Reduced severity dilute acid pretreatment

To evaluate the impact of enzyme enhanced ensilage on downstream processes, we applied a reduced severity dilute acid pretreatment that incorporates two steps: Washing and pretreatment (Hoskinson et al., 2007). These are implemented as follows:

3.3.10.1 Washing and blending biomass

Following Pierre Gy's modified sampling methodology, two types of solid samples were obtained (Pitard, 1989). Each silage bag was mixed thoroughly and a 60 g subsample of ensiled biomass was withdrawn for moisture content measurement, which was determined by oven drying at 60 °C as described earlier. In addition, 30 g of ensiled biomass was subsampled from each mixed silage bag for pretreatment. Then 300 mL of DI water was added and stirred for 50 s (30 at low speed and 20 s at high speed) by a Waring blender. The blended biomass was poured through a filter screen (hole size 0.8 x 0.8 mm) and the blender rinsed with 25 ml of DI water that was also filtered. The volume of the liquid passing the screen was measured and recorded, and two 50 ml vials of the liquids were stored in centrifuge tubes and frozen at -20 °C for later chemical analyses. The remaining solid biomass was washed twice with 750 of mL DI water to remove fermentation inhibitors formed during the silage process, such as lactic acid and acetic acid. The total biomass was then weighed and stored at 4 °C for downstream pretreatment.

3.3.10.2 Pretreatment

Reduced-severity pretreatment was performed on a 2.5 g dry weight composite subsample of the samples, from the following harvest dates in 2006: 10/25, 10/19, and 10/17, in Erlenmeyer flasks. The sample was digested with 0.8% sulfuric acid (w/w) and then autoclaved at 121 °C, 145 kPa for 30 minutes according to Hoskinson et al. (2007).

The liquids from both the washing and pretreatment steps, were hydrolyzed by H₂SO₄ to convert oligosaccharides to monosaccharides as described by Hoskinson et al. (2007). Samples were then diluted 1:150, filtered, and measured by ion chromatography for monosaccharides such as glucose and xylose as previous described. The concentrations of glucose and xylose were converted to glucan and xylan, respectively by using correction of factors 0.9 for glucose and 0.88 for xylose (Theander and Westerlund, 1986). The conversion rates were reported on a dry basis.

3.3.11 Statistical analysis

Data were analyzed using SAS 9.1 (SAS Institute Inc., Cary, NC) and Minitab (Minitab Inc., State College, PA). The ANOVA model included four factors: harvest date, enzyme treated or control, silage time, water addition or no water addition. The main and interactive effects of harvest date, enzyme treatment and water addition were tested using the F- test. Significance was determined at P< 0.05.

3.4 Results and Discussion

3.4.1 Initial moisture content of corn stover

In both 2005 and 2006 the corn stover field moisture content decreased with later harvest dates, although the progress of drying was somewhat different in each year. In 2005, the field moisture content of stover (without cob) was from 45.8% to 18% (w.b.) (Figure 3-1). In 2006, the moisture content of stover (before cob was added back in) ranged from 57.97% to 22.6% (w.b.), cob ranged from 37.7% to 15.44% (w.b), and the corn grain ranged from 25.6% to 16.20% (w.b.) (Figure 3-2). The moisture contents of stover, cob and corn were highly correlated ($r > 0.9$; $p\text{-value} < 0.05$). Although there was a general drying trend, in certain cases, the moisture content of stover did not vary greatly between two consecutive harvest dates, such as Sept. 29 and Oct. 11, 2005, likely because it rained before the second harvest date.

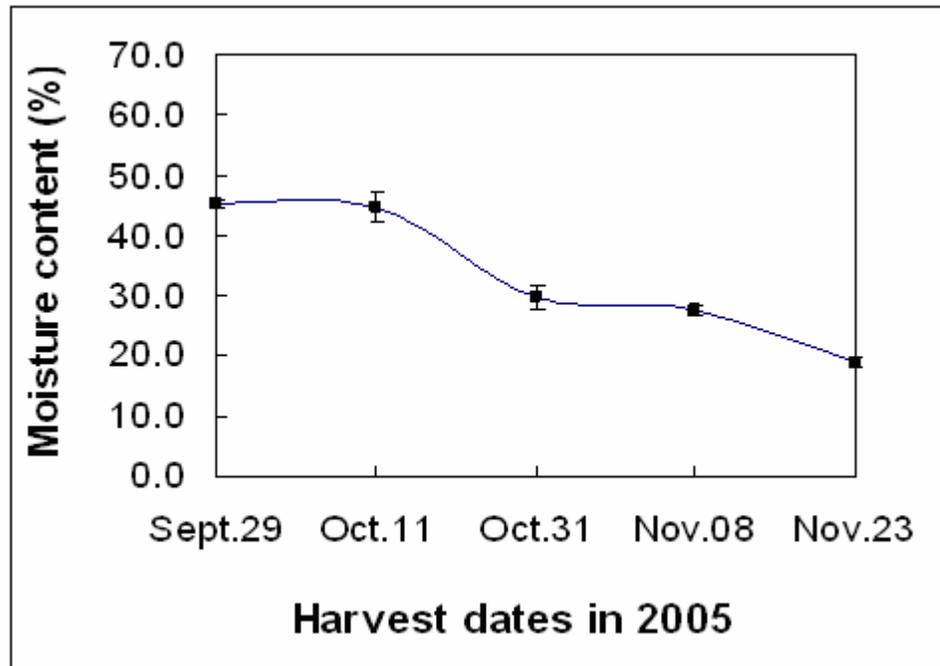


Figure 3-1: Field moisture content of stover (no cobs) from five harvest dates in 2005. These values were the initial moisture values for ensilage trials. (Note: Throughout this chapter, the error bar denotes the standard deviation of independent samples.)

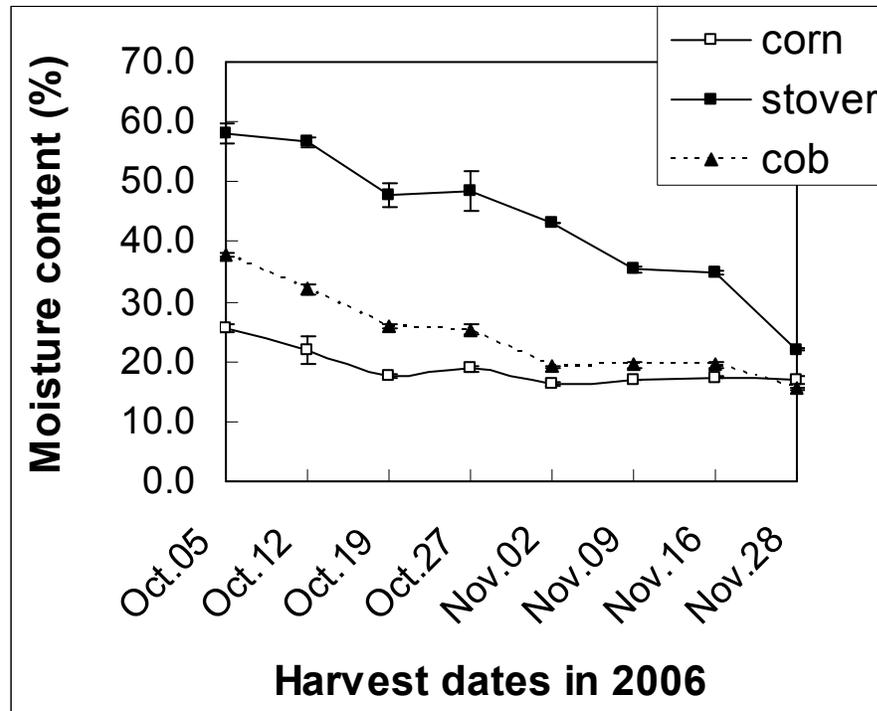


Figure 3-2: Initial moisture content of stover (with cobs) and corn from eight harvest dates in 2006 (Note: The error bar denotes the standard deviation of replicates)

3.4.2 pH change

Silage is a natural lactic acid fermentation process. A low pH normally indicates that lactic acid bacteria (LAB) dominate over the microbial ecosystem relative to various undesirable microorganisms, such as enterobacteria, clostridia and yeasts. LAB dominated fermentations quickly acidify the environment to limit these competing organisms and facilitates long term silage stability (McDonald et al, 1991). The critical pH for long-term preservation depends on the moisture content, buffering capacity of the biomass, and the temperature, and is at risk from even modest oxygen penetration and aerobic degradation. In general, if the dry matter content is above 200 g kg^{-1} , a pH of 4.0 can preserve the biomass

satisfactorily under anaerobic condition. This low pH increases the hydrogen ion concentration, which along with undissociated acids themselves inhibit undesirable bacteria (McDonald et al, 1991). In both 2005 and 2006, the pH of fresh corn stover decreased as the harvest season progressed from early fall to early winter. During ensilage, the pH value decreased dramatically on the first day from about 7 to around 4.5, and kept decreasing during the following 20 days. The rapid pH reduction in the first few days played a critical role on the subsequent silage process because it quickly acidified the environment, eliminating competing organisms and leading to a stable ensilage process. In 2005, the pH values of stover (without cob) on Sept. 29 and Oct. 11 after 21 days of ensilage were significantly lower than those of stover on other dates (Figure 3-3). The 2006 stover ensiled at 60% moisture had no significant difference in pH at 21 days among all the harvest dates (Figure 3-4). For 2006 stover ensiled at field moisture, the final pH of ensiled stover increased from Nov. 02 to Nov. 16 and by Nov. 29 was over pH 6 after 21 (Figure 3-5). This might be caused by reduced microbial activity due to low moisture content (Figure 3-2). Based on these results, it appears that while ensilage is generally robust and drives pH down to around 4 when initiated at 60% moisture (w.b.) (dry matter content of 400 g kg^{-1}), stable preservation cannot be assumed at the lower field moisture contents that occur late in the harvest season. In both years, the day 21 pH of the ensiled stover was not significantly changed by enzyme amendments. However, enzyme amendments did generally lower the pH of day 1 samples of ensiled stover, thus enhancing the stability of long term storage by quickly acidifying the stover environment and minimizing the activity of clostridia and other detrimental microorganisms.

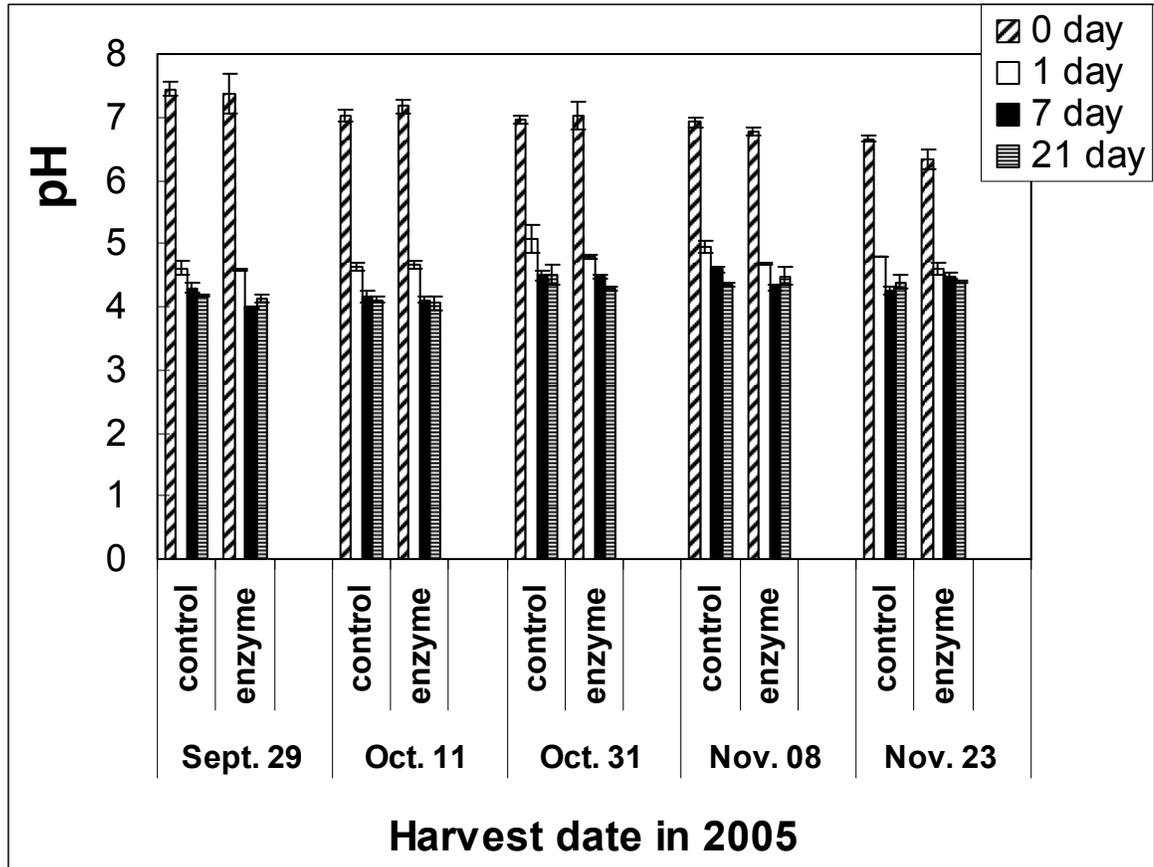


Figure 3-3: Stover pH change during 0, 1, 7, 21 days of ensilage at 60% moisture in 2005.

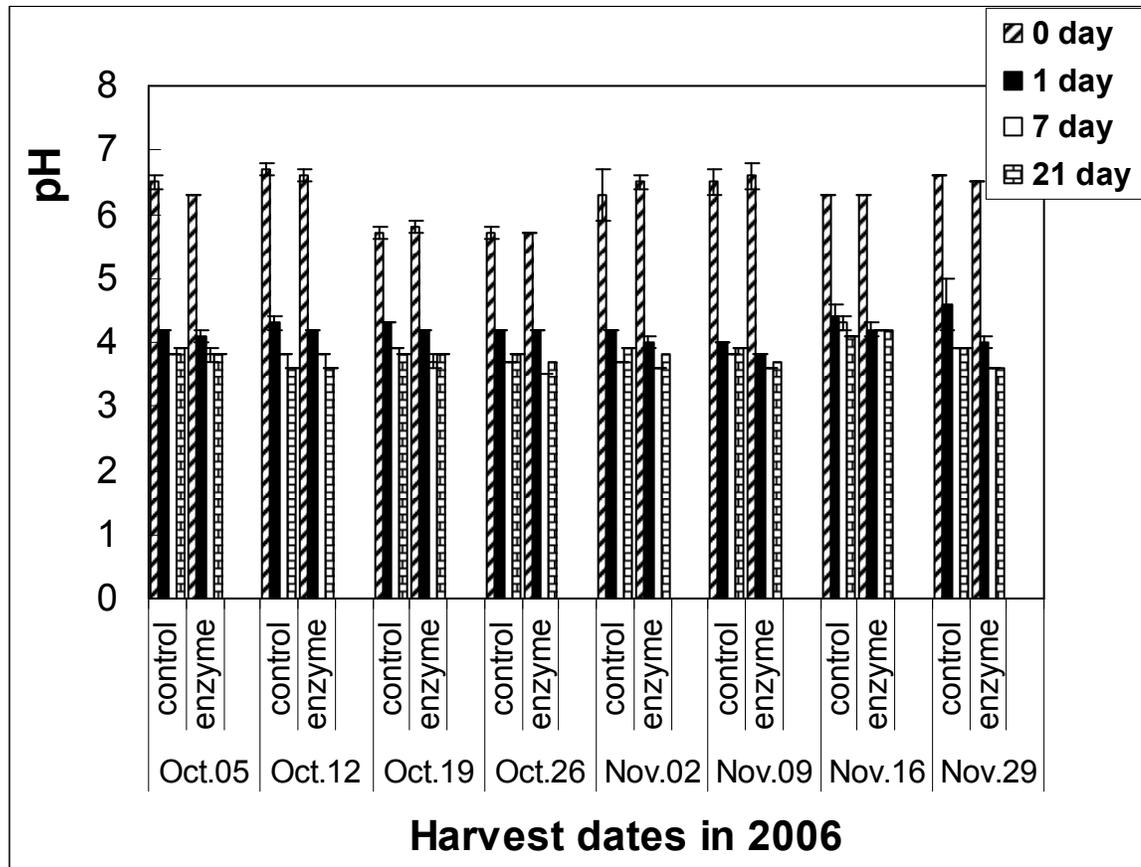


Figure 3-4: Stover pH change during 0, 1, 7, 21 days of ensilage at 60% moisture in 2006.

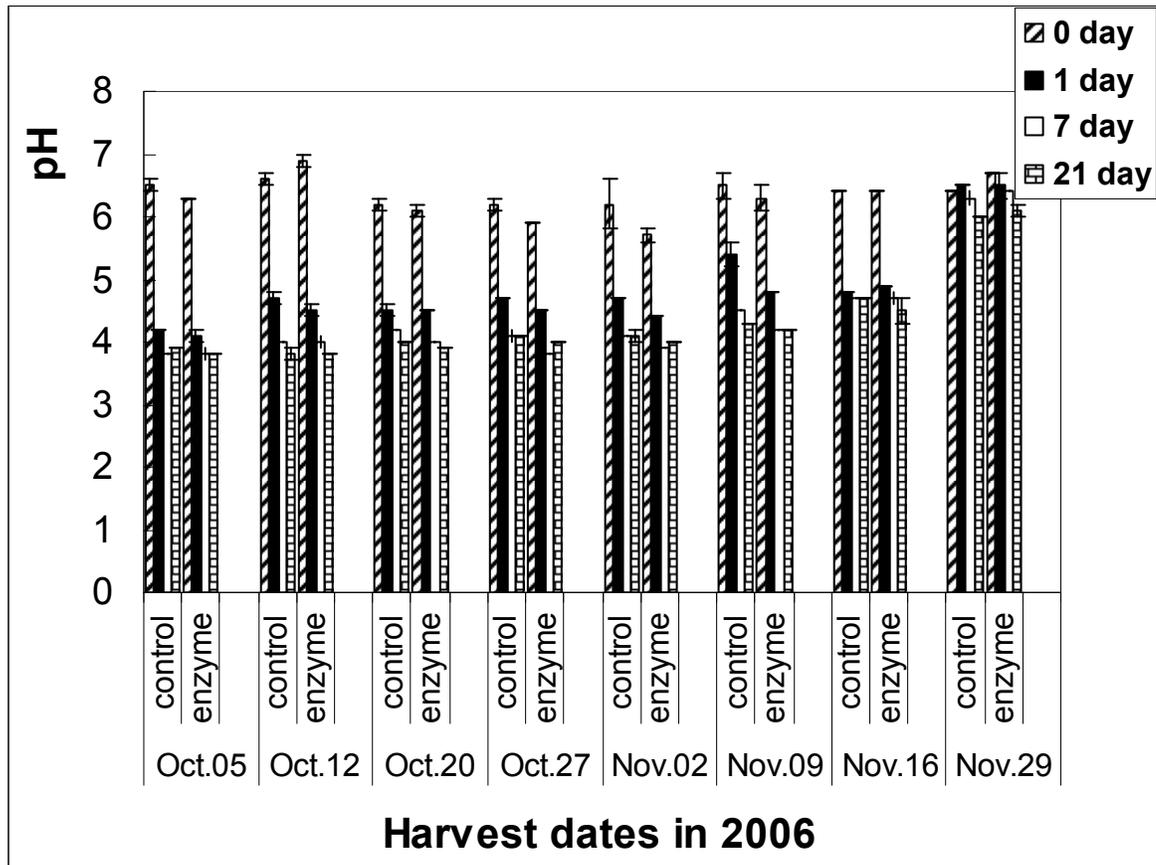


Figure 3-5: Stover pH change during 0, 1, 7, 21 days of ensilage with field moisture in 2006.

3.4.3 Dry matter loss

Corn stover is an attractive biomass resource because of the extensive corn acreage and large dry matter quantities per-acre. Given the quantities of biomass needed for energy, the magnitude of dry matter loss during storage is an important criterion to judge the effectiveness of different preservation methods. The dry matter loss of stover harvested in 2005 was quite different from 2006, as detailed below.

In 2005, these dry matter losses ranged from 3 to 12%, with the dry matter loss of stover harvested in the early fall significantly lower than that of stover harvested on other

dates. There was no significant difference of dry matter loss of the stover harvested on Sept. 29 between control and enzyme treatments. The dry matter loss of the stovers harvested on other dates and ensiled without enzyme addition was similar and there were no significant differences between them. The dry matter loss of enzyme-amended silage harvested on Oct. 11 and Oct. 31 was similar, but both were significantly higher than samples from Nov. 08 and Nov. 23 which in turn were higher than the Sept. 29 samples (Figure 3-6).

In 2006, for stover similarly ensiled at 60% moisture, the dry matter loss from all harvest dates was less than 2.5% and generally enzyme amendments minimized the dry matter loss (Figure 3-7). For stover at field moisture content, the dry matter loss from all the 2006 harvest dates was less than 4%, with the exception of 7% dry matter loss from the stover harvested on Nov. 16, 2006, and for these field moisture treatments, enzyme amendments had less consistent influence on dry matter loss (Figure 3-8). This high dry matter loss might have been caused by increased activity of clostridia relative to LAB, resulting from the low initial water soluble carbohydrate and moisture content. Clostridia consume lactic acid and produce higher level of acetic, butyric and isobutyric acid, resulting in a higher pH (4.72 in this case), and more dry matter loss than an LAB dominated fermentation. The lack of cobs in the 2005 stover is a likely cause of this dry matter loss difference between the two years, as cobs are known to have more available carbohydrate fractions than other stover fractions. The high water soluble carbohydrate content of cobs promotes lactic acid fermentation and minimizes dry matter loss (McDonald et al, 1991). But regardless of whether the cob was included, the amount of dry matter loss of ensiled corn stover was low relative to traditional dry storage, which can experience dry matter losses of up to 23% due to plant and microbial respiration (Richey et al., 1982).

In addition, low dry matter loss is associated with low losses of biomass energy content, and the latter is lower than the former. In the ensilage system the dry matter loss associated with organic acid production is almost completely recoverable, assuming lactic acid bacteria have dominated the fermentation. It has been reported that when homofermentative lactic acid bacteria are dominant, DM losses are lower than when heterofermentative organisms are dominant (McDonald et al., 1991). Comparing the dry matter loss from samples with and without enzyme addition, it was likely that enzyme addition encouraged a homofermentative lactic acid bacteria fermentation (McDonald et al., 1991). By significantly reducing dry matter losses, our results are consistent with previous research indicating ensilage with enzyme addition could be a promising strategy to preserve corn stover for later bioconversion (Richard, 2001).

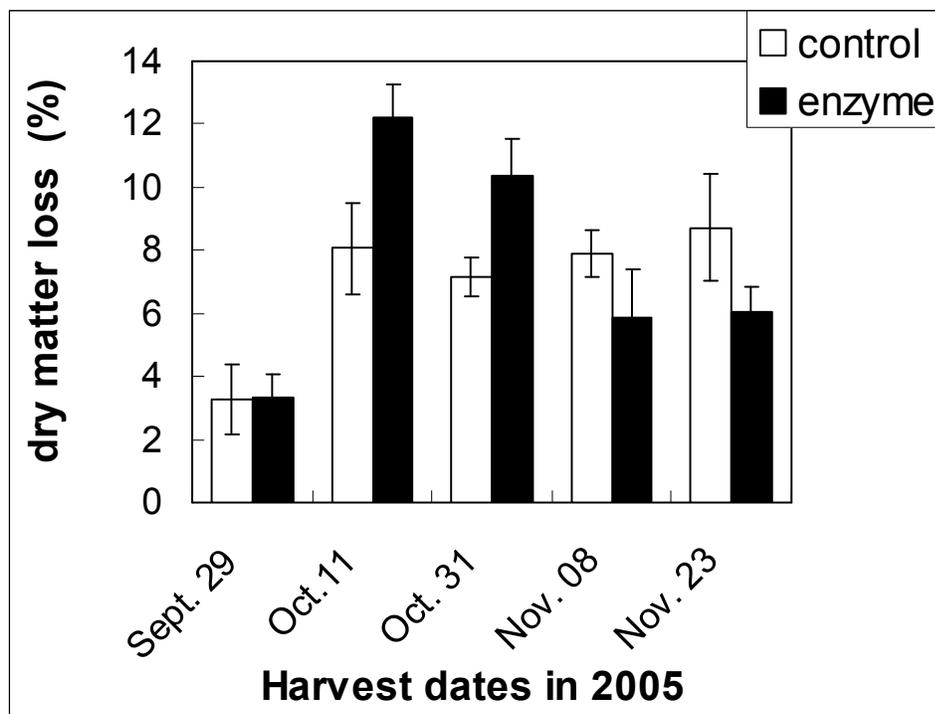


Figure 3-6: Dry matter loss percentage of 2005 stover (without cob) during 21 days of ensilage with 60% moisture.

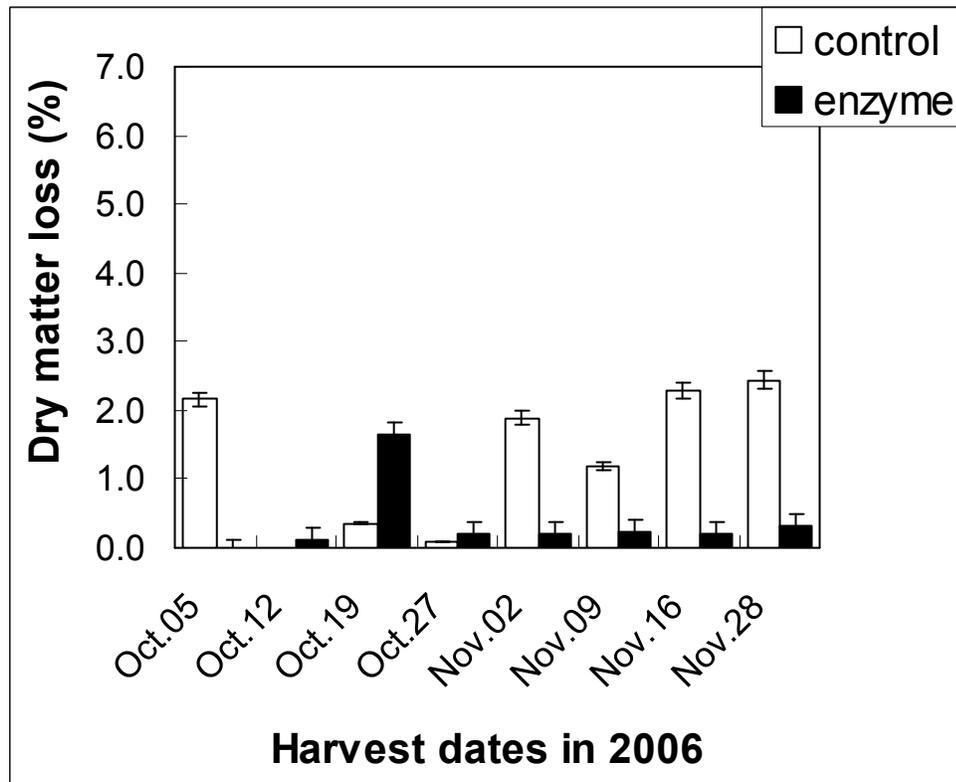


Figure 3-7: Dry matter loss percentage of 2006 stover (with cob) during 21 days of ensilage with 60% moisture.

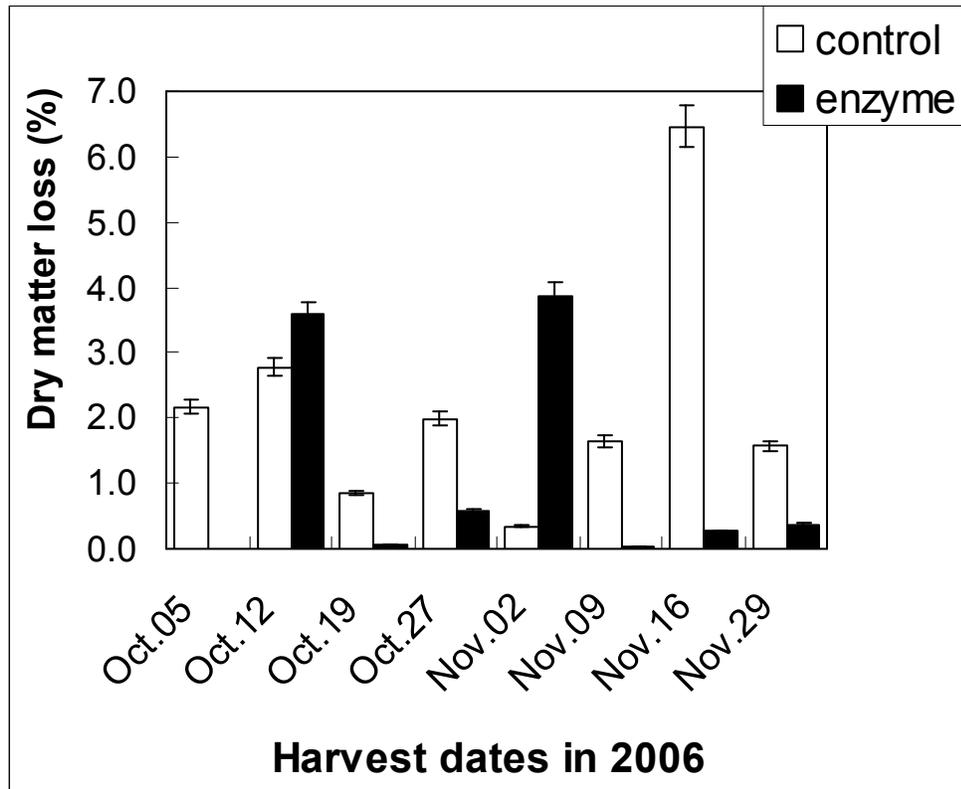


Figure 3-8: Dry matter loss percentage of 2006 stover (with cob) during 21 days of ensilage at field moisture.

3.4.4 Water soluble carbohydrate (WSC)

Water soluble carbohydrate, being a primary substrate of microorganisms, is an important parameter for evaluating the effects of harvest dates and enzyme addition on stover quality for bioconversion. The higher soluble carbohydrate levels found in wet biomass, the more the lactic acid bacteria become active. LAB are the most important group of species during ensiling, and determine whether it will be stable silage and a successful long term preservation process or not (McDonald et al., 1991).

A spectrophotometer (335907P-000, Thermo Scientific, Waltham, MA) was used to determine the WSC concentration. The calibration relationship between standard concentration and absorbance (ABS) can be described by the following linear equations:

1) For 2005 samples:

$$\text{ABS} = 0.0092X + 0.073 \quad (3-2)$$

where X = standard concentration (mg/ml), Degree of freedom (D.F.)=8 and $R^2=0.9913$. For 2006 samples:

$$\text{ABS} = 0.00868 \times X \quad (3-3)$$

where X = standard concentration (mg/ml), D.F.=10 and $R^2=.9960$.

In both years, the WSC was significantly increased by enzyme addition after 21 days of ensilage, with the exception of the Nov 26, 2006 field moisture treatment. The reason for this exception might be the low moisture content, 18.61%, which was apparently too low to fully initiate the lactic acid bacteria fermentation. Although the stover might be adequately stored under this anaerobic condition, because molds and yeasts are difficult to activate without oxygen, the lower acid levels and limited biological activity seem less likely to have a beneficial effect for biological pretreatment. In both years, the 21-day WSC of the enzyme-amended ensiled stover harvested in the early fall was significantly higher than that of ensiled stover harvested later in the season (Figures 3-9, 3-10, and 3-11). Without enzyme addition, less WSC was produced, putting the stability of long term storage at risk.

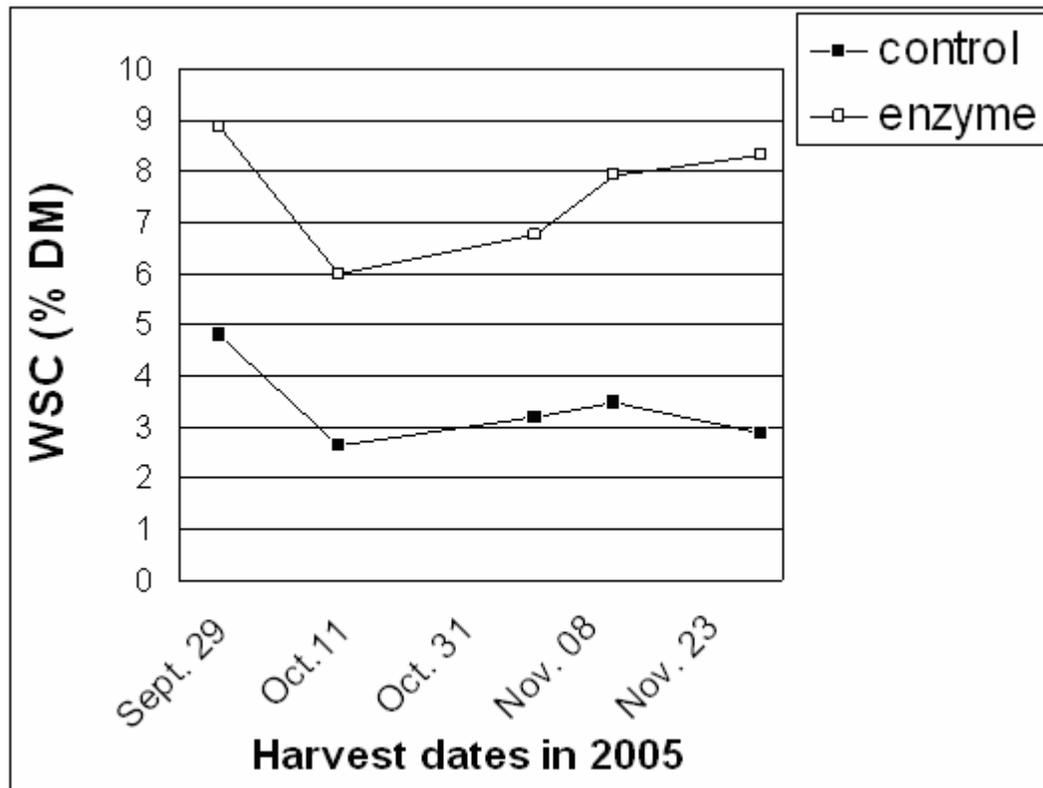


Figure 3-9: Water soluble carbohydrate (WSC) of 2005 stover (without cob) ensiled for 21 days at 60% moisture.

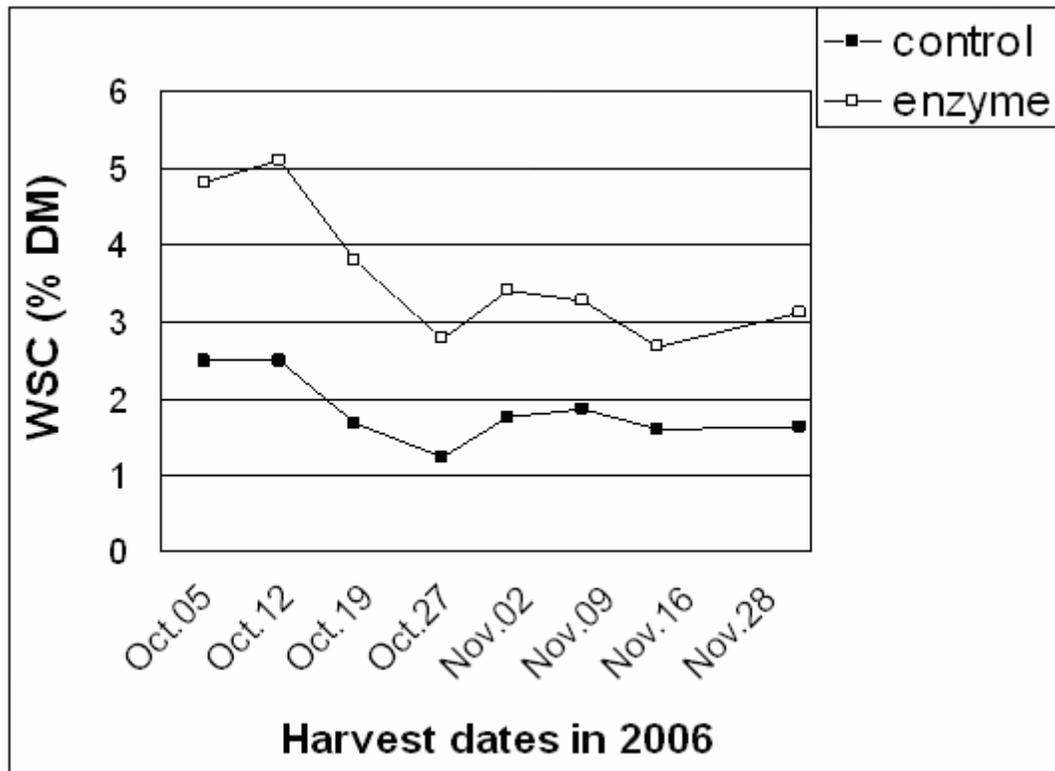


Figure 3-10: Water soluble carbohydrate (WSC) of 2006 stover (with cob) ensiled for 21 days at 60% moisture.

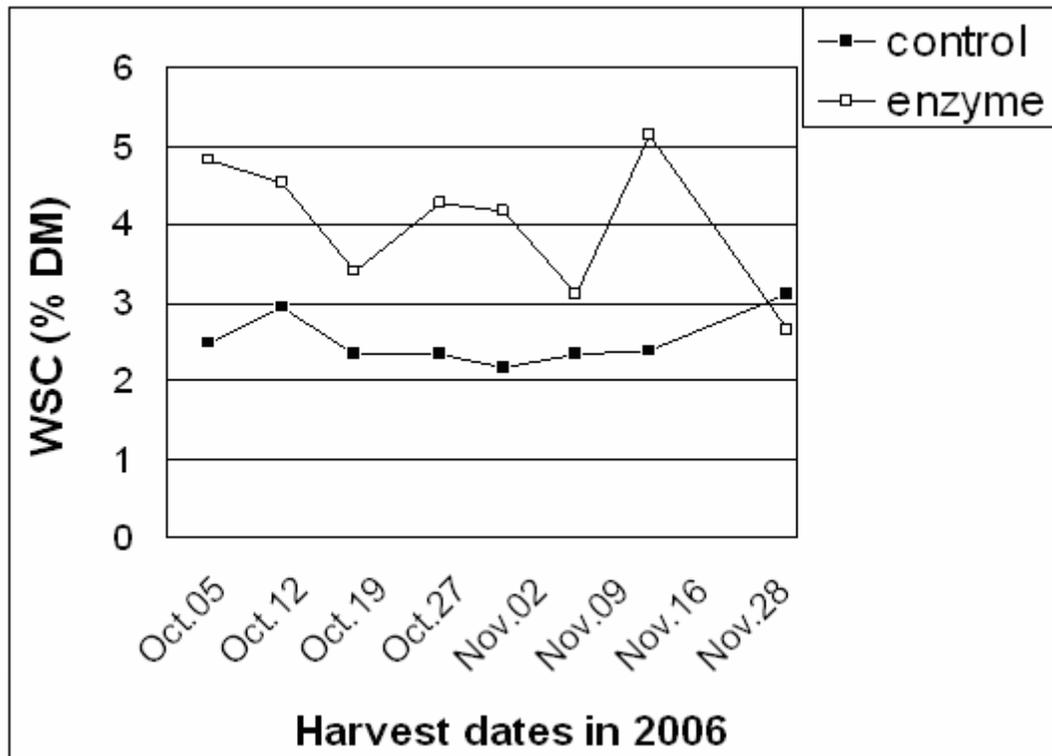


Figure 3-11: Water soluble carbohydrate (WSC) of 2006 stover (with cob) ensiled for 21th days at field moisture content.

3.4.5 Monosaccharides

The total monosaccharide concentration, which includes arabinose, galactose, glucose, xylose, and ribose, was measured by IC. These monosaccharides may be consumed during ensilage fermentation or potentially benefit downstream processes that could convert them into chemicals or fuels. In both 2005 and 2006, there was no difference in total monosaccharides between control and enzyme treatment at time 0, but after 21 days of ensilage the monosaccharide levels were significantly increased in the enzyme treatments relative to those without enzymes. It is presumed that the structural sugars, cellulose and hemicellulose, were hydrolyzed into monosaccharides and oligosaccharides by enzyme during the silage process. For the 2005 season the monosaccharide content of stover

harvested in early fall was high after 21 days of ensilage. For most other harvest dates (Sept. 29, Oct. 31, Nov. 8 and Nov. 23) there was no significant difference among the monosaccharides of ensiled stover. However, the total monosaccharide content of the stover harvested on October 11 was significantly lower than those from other harvest dates (Figure 3-12). This reduction of total monosaccharides might indicate secondary fermentations and consumption by clostridia. Whatever the cause, this treatment also led to high 12.5% dry matter loss, 12%, as reported earlier. For the 2006 harvest, the total monosaccharide content of stover ensiled for 21 days was significantly higher than that of other harvest dates (Figure 3-13 and Figure 3-14). Therefore, in both years the best harvest date was in early fall, when the total monosaccharides was higher than that of other harvest dates. Harvest dates, water addition, enzyme addition and sampling dates and the interactions among them are significantly contributed to the total monosaccharides, with exception of water x enzyme, and water x enzyme x sampling dates interactions (See Appendix B).

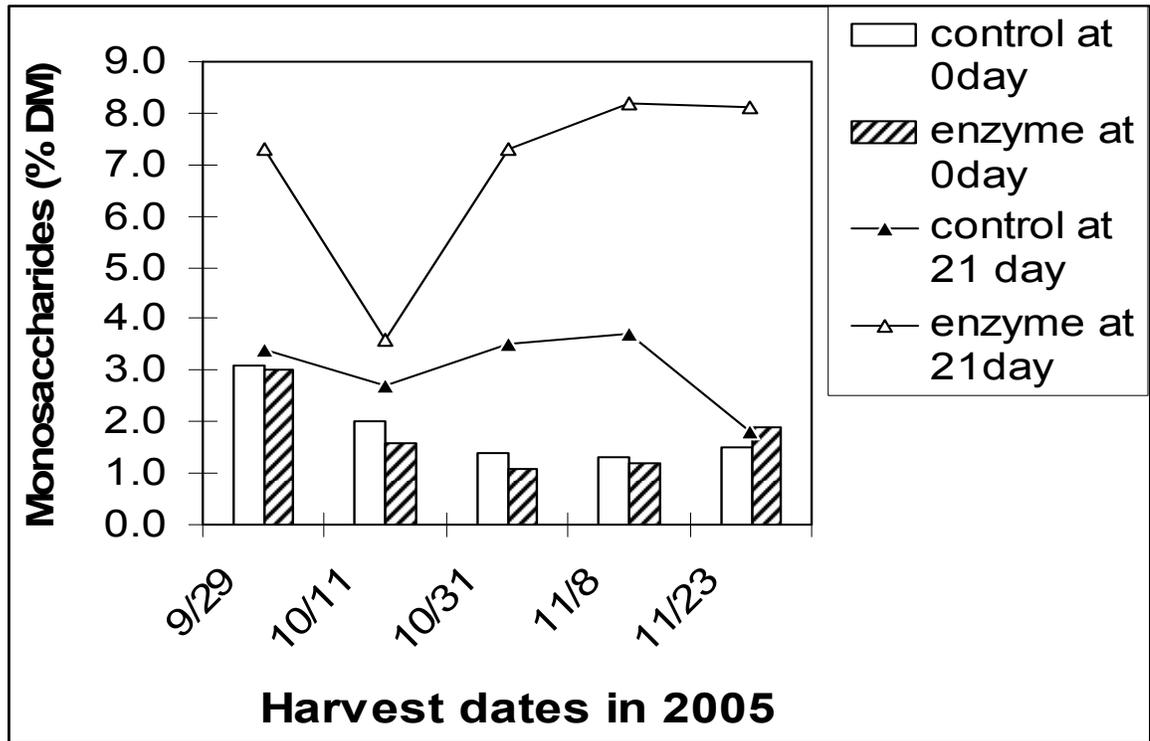


Figure 3-12: Monosaccharides of 2005 stover (without cob) ensiled at 60% moisture. Data are presented on days 0 and 21 in 2005.

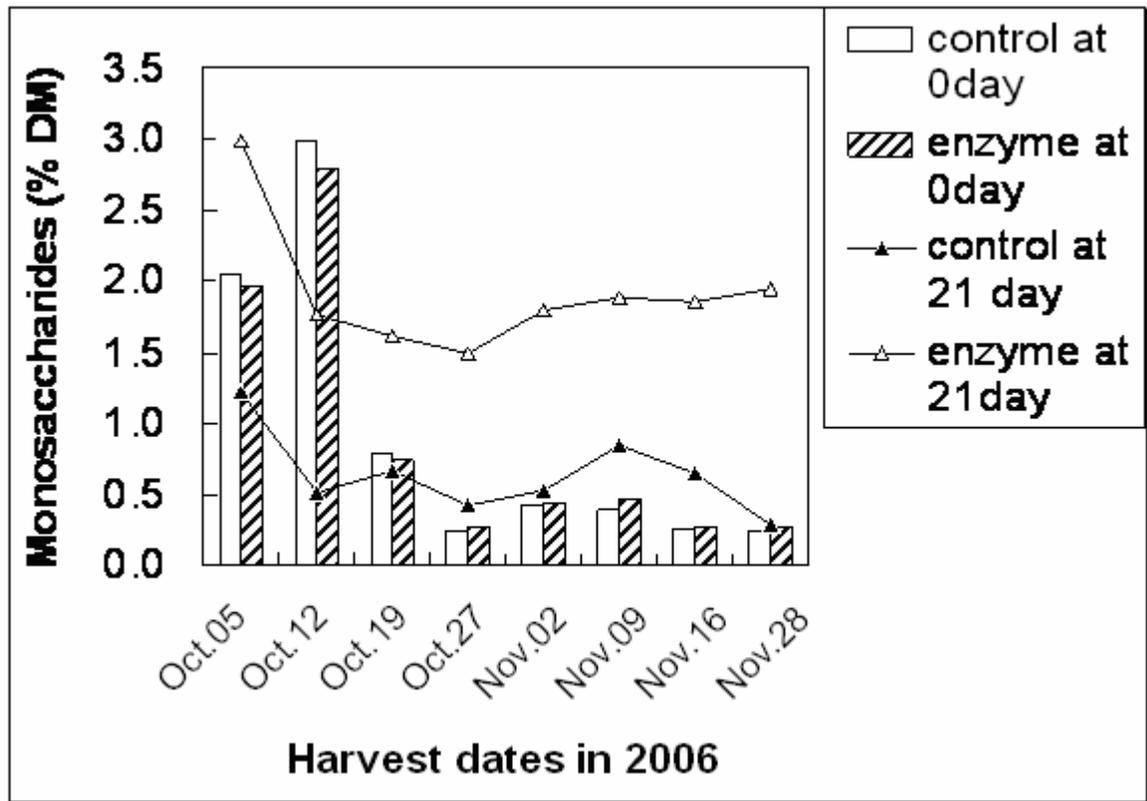


Figure 3-13: Monosaccharides of 2006 stover (with cob) ensiled at 60% moisture.

Data are presented on days 0 and 21 in 2006.

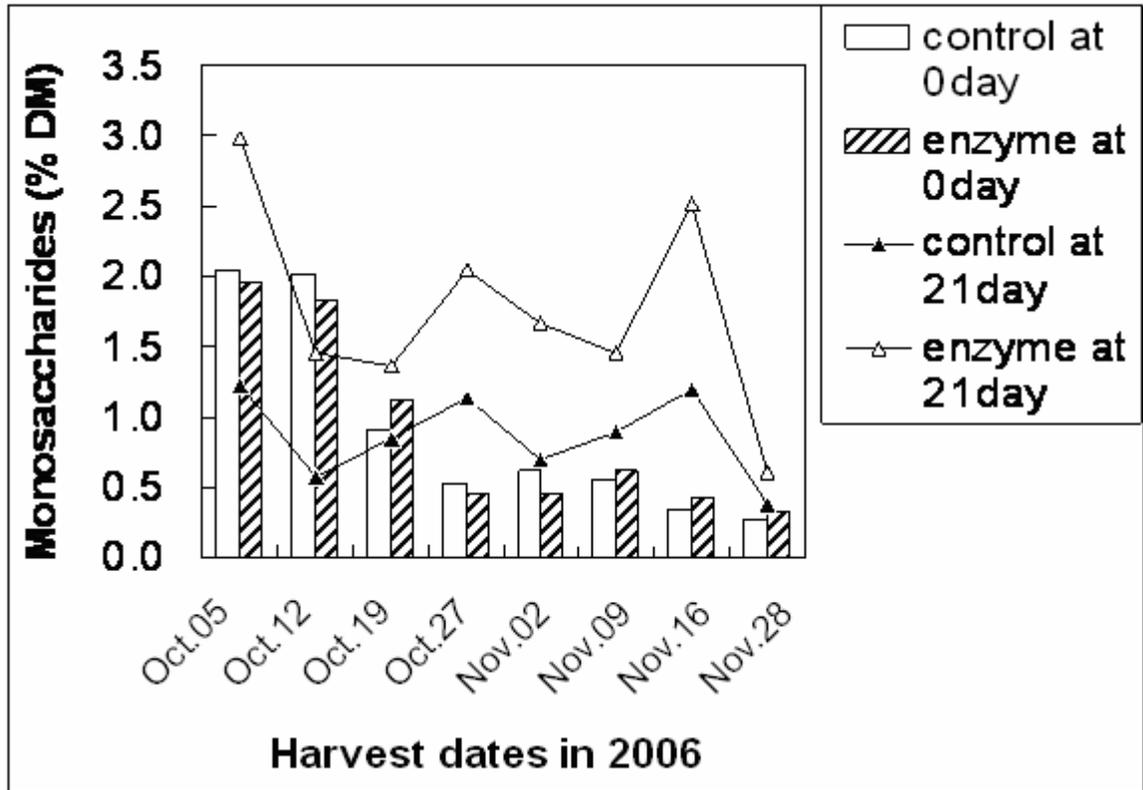


Figure 3-14: Monosaccharides of 2006 stover (with cob) ensiled at field moisture.

Data are presented on days 0 and 21 in 2006.

3.4.6 Compositional analysis and sugar conversion during pretreatment

For the 2006 trials, a more comprehensive compositional analysis of individual sugars and other constituents was performed in triplicate samples, and is summarized in Figure 3-15 and Figure 3-16. Glucans comprised the largest fraction, accounting for 33% of the stover dry weight. Xylose, lignin, protein, ethanol, arabinan, galactan accounted for 20%, 15%, 4%, 3%, 2%, and 1% , respectively. The “other” category accounted for 22% of the stover weight and was primarily composed of ash, uronic acid, acetyl, etc.

Polysaccharides, including glucan, xylan, arabinan, galactan, mannan, decreased after 21

days of ensilage, especially for those treatments with enzyme addition. This may have resulted from enzyme or organic acid-mediated polysaccharide hydrolysis into monosaccharides, disaccharides and oligosaccharides, such as glucose and sucrose. The resulting WSC fraction is also being converted to organic acids and alcohols, so these hydrolysis products do not necessarily accumulate.

Lignin content was not significantly changed by the ensilage process. But the protein and ethanol contents increased during ensilage for all treatments and harvest dates. This is likely due to both growth of microorganisms (indicated by protein) and ethanol production by yeast and other microbes.

Further conversion of xylan to xylose was investigated through reduced severity dilute acid pretreatment. Standard dilute acid pretreatment hydrolyzes essentially all the hemicellulose into xylose and other sugars while enhancing digestibility of cellulose in the residual solids. In contrast, reduced severity dilute acid pretreatment, designed by Corey W. Radtke, applies milder conditions to achieve moderate rates of xylose conversion in order to compare the differences in biomass recalcitrance: the higher conversion rate of xylose/xylan indicates more degradable biomass (Hoskinson et al., 2006; Duguid et al., 2007). As seen in Figure 3-17, 55 to 65% of the potential xylan was released as xylose from corn stover without ensilage. After 21 days of ensilage, around 80% of the xylan was recovered from the Oct. 5 harvest date, while recovery ranged from 65 to 75% for the later dates. For each date, there was trend for higher xylose release for the enzyme-treated ensilage samples, although this was not statistically significant. These results indicate that less pretreatment intensity will be required to achieve the same degree of saccharification on ensiled biomass.

This result has major importance for the cellulosic Biofuels industry, as downstream processing and saccharification required a considerable amount of energy. Pretreatment accounts for $1/3 - 1/2$ of the total energy required for bioconversion of cellulose biomass into ethanol, and a similar fraction of the capital costs of a biorefinery.

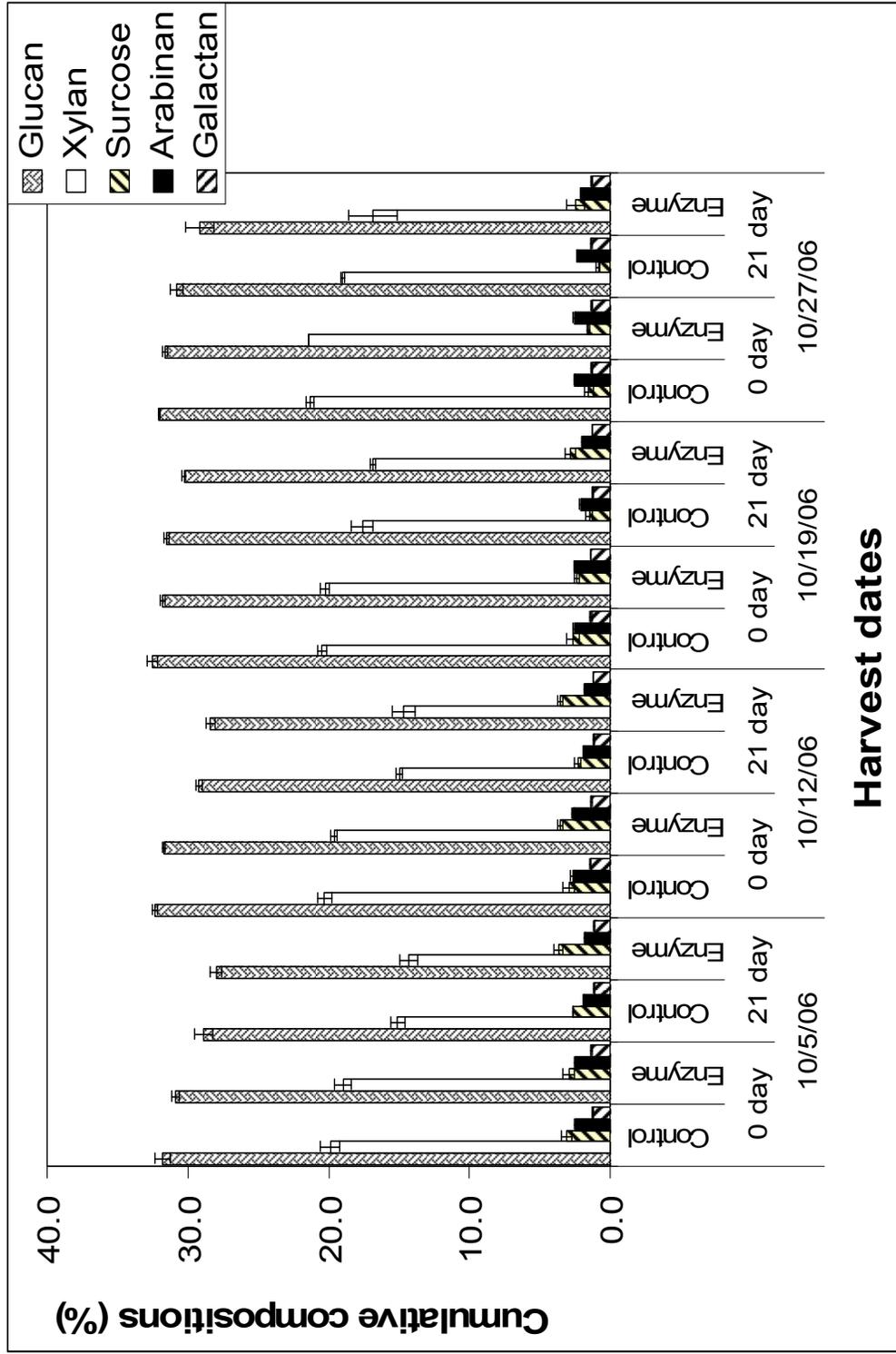


Figure 3-15: Compositional analysis of sugar content of 2006 stover (with cobs) on day 0 and after 21 days of ensilage.

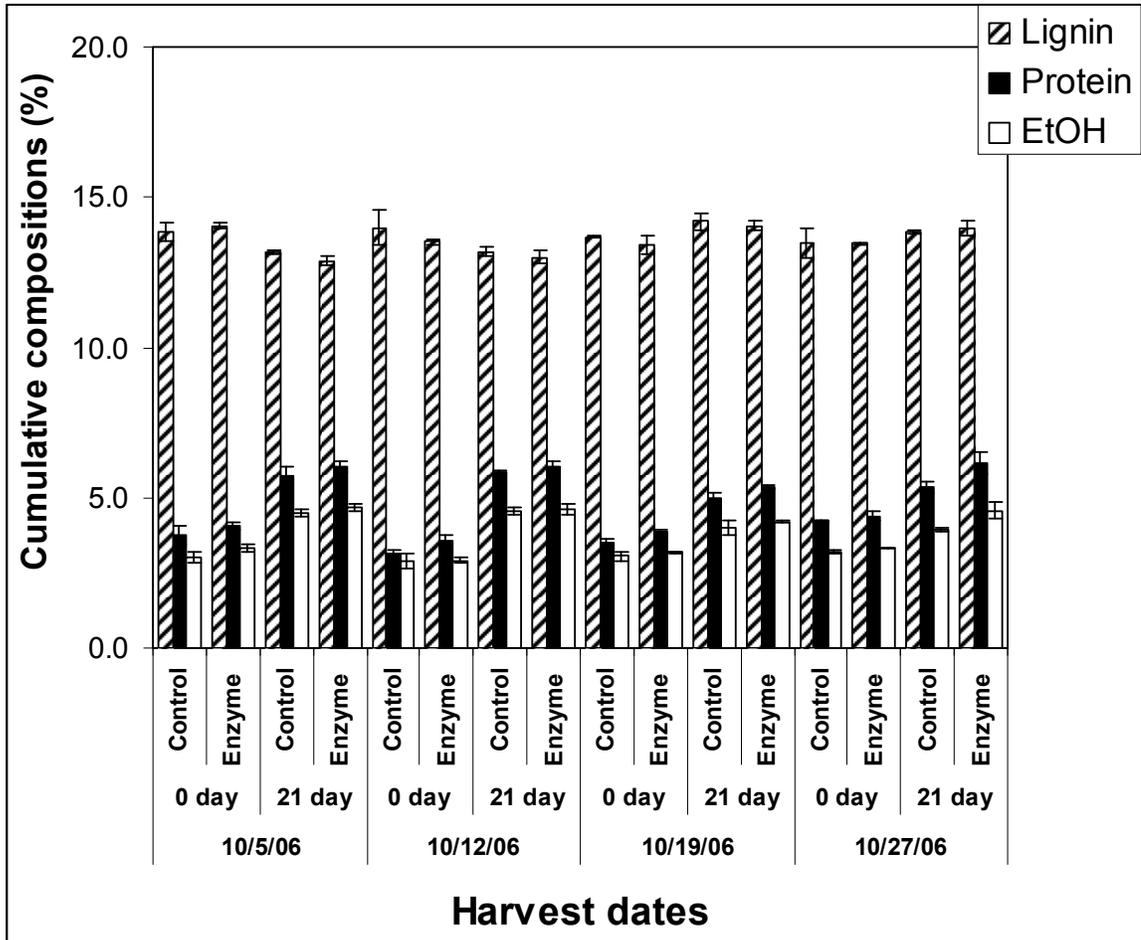


Figure 3-16: Compositional analysis of lignin, protein and ethanol content of 2006 stover (with cobs) on day 0 and after 21 days of ensilage.

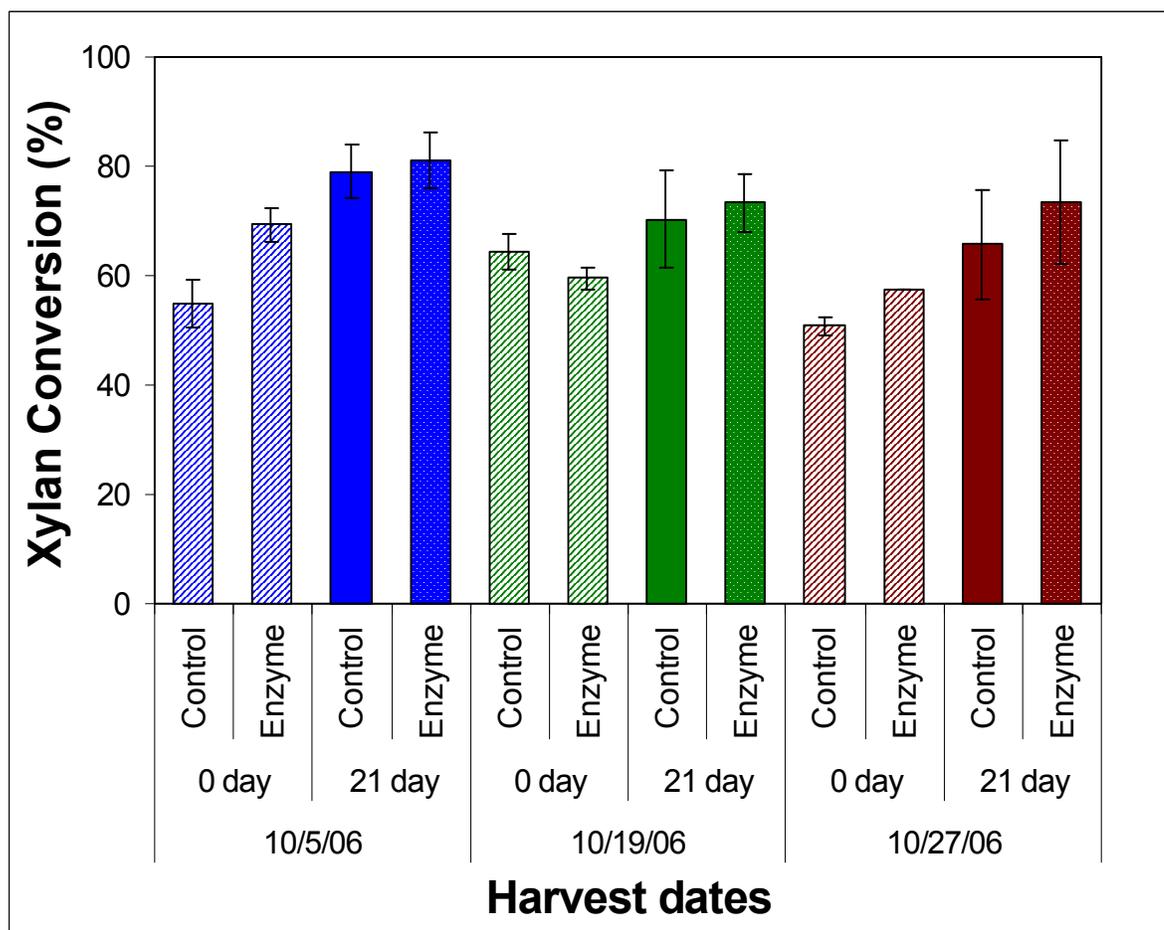


Figure 3-17: Xylose conversion percentage after reduced-severity dilute acid pretreatment of 2006 stover samples (with cobs).

3.5 Conclusions

The experimental results demonstrated that early fall was the best harvest time for bioconversion. The early fall harvested samples had lower pH and lower dry matter losses, and a higher level of water soluble carbohydrate and monosaccharides hydrolyzed from cellulose and hemicellulose. These benefits of ensilage were generally increased by amending the stover with cellulolytic enzymes prior to ensilage. Enzyme addition helped

to degrade cellulose into fermentable sugar, while water addition had a less consistent effect.

These findings were consistent with both compositional analysis and results from reduced severity pretreatment experiments. Composition analysis demonstrated that polysaccharides were decreased during 21 days of ensilage process, especially for samples with enzyme addition. Reduced severity pretreatment results indicated that xylan hydrolysis to xylose was significantly enhanced by ensilage, implying that less energy would be required for achieving similar pretreatment effects on ensiled corn stover than that for control. The highest xylan conversion rate after pretreatment, around 80%, was achieved from ensiled stover harvested in early fall.

This study demonstrated that biomass characteristics and downstream conversion rates are strongly influenced by both harvest dates and storage conditions. Early fall harvest of corn stover, combined with ensiled stover, provides distinct advantages for cellulosic bioconversion to fuels and chemicals relative to later harvest dates and immediate use. Further studies are needed to quantify the economic tradeoffs associated with harvest timing and storage techniques, including the use of enzymes to enhance the beneficial ensiled storage effects.

3.6 Acknowledgments

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Chapter 4

EFFECTS OF LACCASE ON LIGNIN DEPOLYMERIZATION AND ENZYMATIC HYDROLYSIS OF ENSILED CORN STOVER

4.1 Abstract

Enzymatic ensilage technology provides an effective long-term storage method for large amounts of plant-based biomass prior to bioconversion. However, the presence of lignin in lignocellulosic biomass constrains and challenges the improvement of current bioconversion techniques. The aim of this study was to explore the synergies of laccase, a lignin-degrading enzyme, with cellulase/hemicellulase amendments on ensiled corn stover. Molecular signals of lignin decomposition were observed by TetraMethylAmmonium Hydroxide thermochemolysis and Gas Chromatography - Mass Spectroscopy (TMAH-GC-MS) analysis. Cellulose conversion as measured by downstream enzymatic hydrolysis improved with increasing laccase loading rate. The enhanced cellulose digestibility, around 7 %, is believed to result from structural changes of lignin polymers, making cellulose more accessible for cellulase hydrolysis. The significant findings suggest that ensilage might provide a platform for biological pretreatment, and that by partially hydrolyzing cellulose and hemicellulose into soluble sugars, ensilage facilitates laccase penetration into the complex biomass structure to enhance lignin degradation. These results provide a promising indication of the potential

of enzyme amendments during ensilage to maximize utilization of corn stover for cellulosic biofuels and other downstream fermentations.

4.2 Introduction

Ensilage amended with cellulases and/or hemicellulases has been demonstrated as an effective and stable long-term storage strategy for lignocellulosic biomass, as well as a beneficial platform for downstream pretreatment (Richard et al., 2002; Ren, 2006; Richard et al., 2007; Oleskowicz-Popiel et al., 2008). However, saccharification of cellulose during and after ensilage is limited due to the presence of lignin in biomass. Lignin is a random three-dimensional network aromatic biopolymer, in which the primary chemical bonds are C-O-C and C-C linkages. Lignin is always intimately interlaced with hemicellulose in the plant cell wall, forming a matrix to envelop the crystalline cellulose microfibrils (Kirk and Farrell, 1987). Its complex structure and high molecular weight make lignin degradation very difficult (Orth and Tien, 1995; Hamelinck et al., 2004; Moiser et al., 2005; Kim and Lee, 2006).

Chemical and biological delignifications are two major methods of lignin depolymerization. The latter is considered superior to the former due to its friendly environmental characteristics and lower energy demand, but it is slow and often achieved at the expense of considerable dry matter loss (Lechnner and Papinutti, 2006; Shi et al., 2008). Within the category of lignin biodegradation, although enzymatic treatment challenges researches due to the high molecular mass of lignin-degrading enzymes besides high cost and the requirement of enzyme co-factors, it is more promising than

microbial treatment because enzymatic treatment requires simple apparatus and targets at specifically selected reaction, and removes the interfere of side reactions (Roberts et al., 1995). Enzymes have been proven effective in degrading the lignin of pulped fibers from the paper and pulping industry, as well as in laboratory studies of degradation of synthesized or purified lignin (Kirk and Jeffries, 1996; Thompson, et al., 1998; Srebotnik and Hammel, 2000; Tengerdy and Szakacs, 2003; Camarero et al., 2004; Elegir et al., 2005). In one such example, lignin was partially separated from paper fibers during normal heat treatment in the paper and pulping industry, which also facilitated access and surface contact of the lignin-degrading enzymes (Kirk and Jeffries, 1996; Tengerdy and Szakacs, 2003; Camarero et al., 2004). However, without such chemical or physical pretreatment, the efficacy of lignin-degrading enzymes has been constrained because the large macromolecular size of lignin-degrading enzymes limits access to the lignin components in an intact plant cell wall (Kirk and Farrell, 1987; Pérez, et.al, 2002). To our knowledge, there have been no prior studies on the use of ensilage or other biological pretreatment strategies to increase the accessibility of lignin-degrading enzymes to the plant cell wall. Similarly, synergies among the hemicellulase, cellulase and ligninase during the ensilage process have not previously been reported.

In this study, the potential effects of ensilage, a natural acid fermentation traditionally used to preserve livestock forages, as a platform for biological pretreatment was investigated. Specifically, the use of a lignin-degrading enzyme, laccase, to modify the lignin structure of enzymatically ensiled stover and enhance downstream hydrolysis of cellulose was examined. The impact of these treatments was indicated by amount of

water soluble carbohydrates (WSC) liberated by addition of H_2O_2 , while the modification of lignin was assayed offline by Tetramethylammonium hydroxide thermochemolysis followed by Gas Chromatography-Mass Spectrometry (TMAH-GC-MS). Downstream effects were assessed by enzymatic hydrolysis to quantify the digestibility of ensiled corn stover and explore the synergies of laccase treatment after ensilage with cellulase/hemicellulase. A series of experiments designed to investigate effects of the laccase system on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover are illustrated in Figure 4-1.

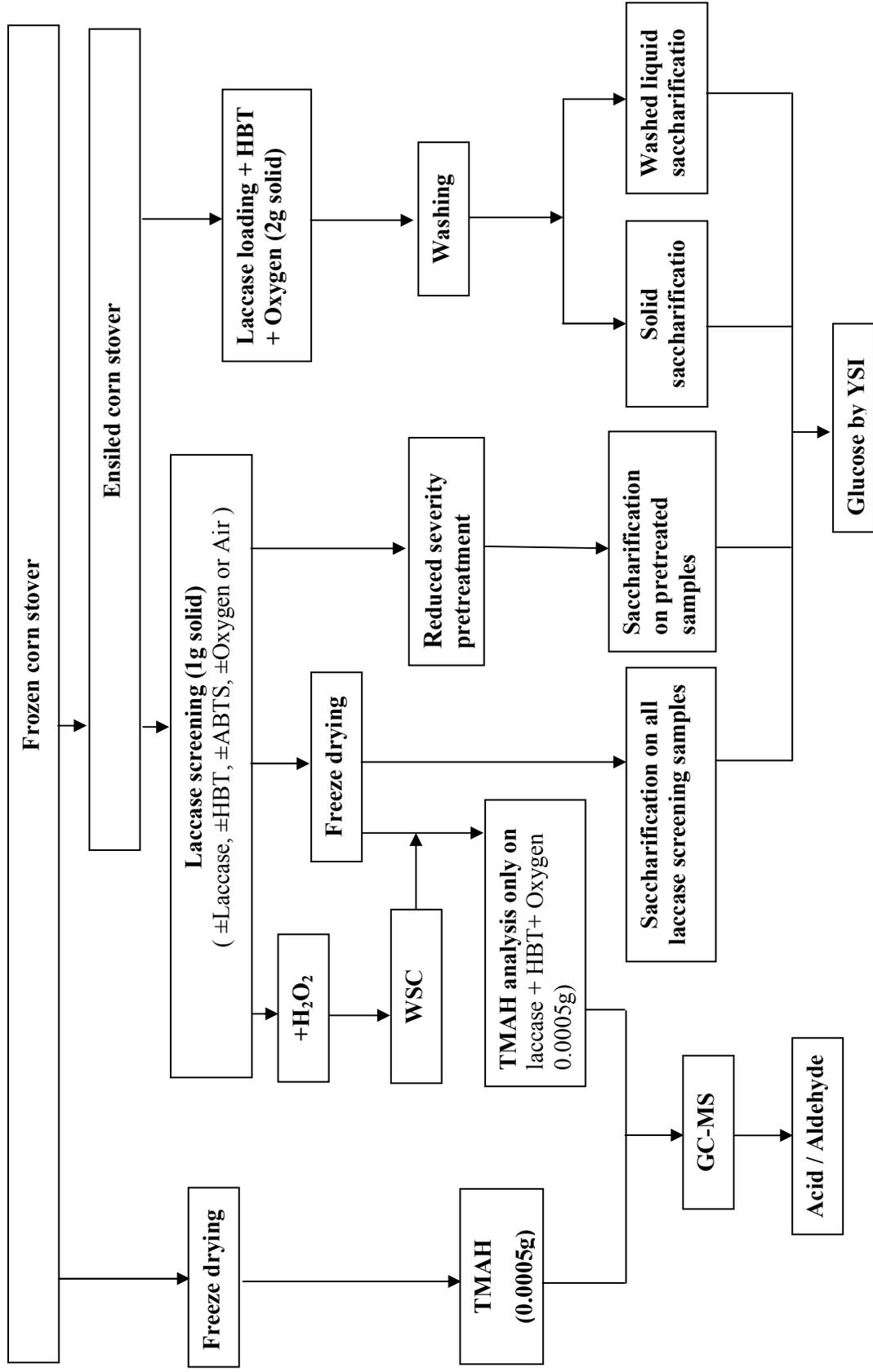


Figure 4-1: Flow chart of effects of laccase system on lignin depolymerization and enzymatic hydrolysis of ensiled corn stover

4.3 Material and methods

4.3.1. Material

4.3.1.1 Corn stover

The corn stover was harvested at the Penn State Rock Springs Farm (Centre County, PA), on October 5, 2006, which was identified as the optimal harvest time in the research described in the previous chapter. A forage plot harvest machine was used to harvest the corn stover after corn ears were hand-removed. The stover was cut approximately 15 cm above the soil, chopped and blown into a drum. Cobs were separated from the grain using a mechanical shelling machine and then chopped into 1-3 cm pieces. The chopped cob was mixed with the stalks and leaves at the field harvest ratio to recreate a whole stover blend. Fresh harvested corn stover was immediately frozen to minimize chemical changes caused by storage.

4.3.1.2 Enzymes

Multifect A40 (a cellulase and hemicellulase mixture) was provided by Genencor International (Palo Alto, CA) for use in the ensilage process. Spezyme was provided by Genencor International (Palo Alto, CA) and Novo 188 was purchase from Sigma, respectively for use in the saccharification assay. Laccase, from *Trameters versicolor* (*T. versicolor*), was purchased from Sigma-Aldrich for the lignin-degradation trials that were the focus of this set of experiments.

4.3.2. Enzymatic activity assay

Cellulase and hemicellulase activities of Multifect A40 were measured according to Wood and Bhat (1988) and Bailey et al. (1992). These methods use carboxymethylcellulose (CMC) and birchwood 4-o-methyl glucuronoxylan (Roth 7500) as substrates to indicate endo-1,4- β -glucanase and xylanase activities, respectively. One international unit of endoglucanase/xylanase refers to the amount of the enzyme that releases 1 μ mol of glucose/xylose from CMC/glucuronoxylan per minute (Wood and Bhat, 1988; Bailey et al., 1992). Cellulase and β -glucosidase activities of Spezyme CP and Novo 188 were determined according to Ghose (1987). Laccase activity was determined by monitoring 5 mM ABTS [2,2'-azinobis-(3-ethylbenzothiazoline -6-sulfonic acid)] oxidation at 40°C for 10 minutes at 420 nm (extinction coefficient, $\epsilon = 3.6 \times 10^4 \text{ cm}^{-1}\text{M}^{-1}$) in 50 mM sodium acetate, pH 4. One activity unit refers to the amount of enzyme that oxidizes 1 μ mol of ABTS per minute (Camarero et al., 2004; Elegir et al., 2005).

4.3.3. Cellulase enhanced ensilage experiments

To explore the effects of enzyme additives, corn stover was ensiled with and without Multifect A40, with laccase and mediators added after ensilage. The Multifect A40 was applied at an enzyme loading rate at 5 IU/ g DM, which summed the enzyme activities of the cellulase and xylanase components. This rate had previously been determined as the minimum effective rate of this enzyme for corn stover ensilage by Ren

(2006). Triplicate 500g samples were packed tightly into 20 cm×40 cm airtight bags made of polyethylene. Samples were double bagged and each bag was sealed with a vacuum sealer to guarantee anaerobic conditions for stover fermentation. Samples were subsequently ensiled in incubators for 21 days at 37±1°C (Richard et al., 2001). Total mass loss, pH and dry matter were measured immediately on each sampling date in random order. The remaining stover was frozen at -20 °C and stored for later chemical analysis and laccase system experiments.

4.3.4. Laccase system and experiments

4.3.4.1. Laccase and the role of mediators

Laccase (EC 1.10.3.2) is a class of copper containing enzymes produced by fungi, bacteria and plants. These rather nonspecific enzymes react with low molecular mass mediators with high redox potential. The mediators facilitate laccase penetration into the lignocellulosic complex for delignification, as shown in Figure 4-2 (Call and Mücke, 1997; Mester and Tien, 2000; Leonowicz et al., 2001; Palonen, 2004). A common characteristic of most effective mediators is the prevalence of N-heterocyclic bearing N-OH groups. The most popular low-molecular mass mediators of laccases are ABTS [2,2'-azinobis-(3-ethylbenzothiazoline -6-sulfonic acid)] and HBT (1-hydroxybenzotriazole) (Leonowicz et al., 2001).

Previously reported laccase loading rates have ranged widely, from 20 U to 80,000 U per gram of substrates, primarily for applications related to wood delignification in the

pulp and paper industry (Camarero et al., 2004; Call and Mücke, 1997; Sealey and Ragauskas, 1998). For example, Sealey and Ragauskas (1998) found a laccase loading rate of around 40,000 U per gram of kraft pulp was optimal in lignin degradation for 24 hour experiments. For our current studies of saccharification of ensiled corn stover with Multifect A40, the laccase loading rate might be lower due to ensilage, or higher since no previous heat or chemicals were applied. In the one previous bioenergy-related study found, two other lignin-degrading enzymes, 2,800 U/g, of manganese peroxidase (MnP) and 50 U/g lignin peroxidase (LiP), were applied to isolated poplar lignin in order to degrade the lignin substrate (Thompson et al., 1998).

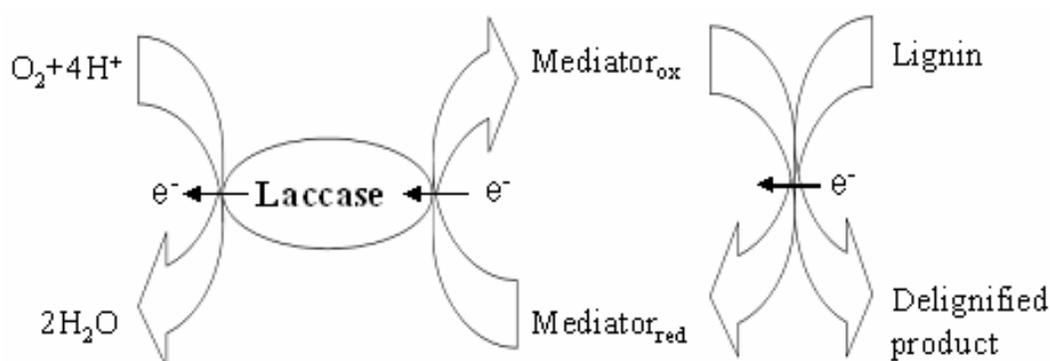


Figure 4-2: Schematic diagram of lignin degradation by laccase mediator-system (from Vicuna, 2000).

4.3.4.2. Laccase system screening trial: mediator and oxygen interactions

Stover, ensiled for 21 days with Multifect A40 as previously described, was ground by Geno-grinder 2000 (BT&C/OPS Diagnostics, Bridgewater, NJ) at a speed of 200 rotations per minute for 9 minutes. Triplicate 1 g samples of the ground stover were

transferred to 60 mL serum bottles and then mixed with 20 mL sodium acetate buffer (pH 4, 50 mM) with 0.05% Tween-80 added as a surfactant. For the oxygen treatments, the sodium acetate buffer (pH 4, 50 mM) was saturated with oxygen by bubbling O₂ for 20 minutes. After adding all the components to the serum bottles, O₂ bubbling continued on the slurry for an additional 2 minutes, and then the bottles were capped using rubber and aluminum seals. For the air treatment, the slurry was stirred for 20 minutes to saturate it with air and then the bottles were capped.

All chemicals were ACS grade. ABTS and HBT, purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), respectively, were used as enzyme mediators. Tween 80 was purchased from Sigma-Aldrich. Laccase and a mediator, either ABTS or HBT, were applied in triplicate to six treatments, as shown in the detailed design in Table 4-1. The resulting slurry was incubated in a rotary water bath at 90 rpm and 40 ± 1°C for 48 hours.

Table 4-1: Laccase with and without mediator treatments.

	Trt1	Trt2	Trt3	Trt4	Trt5	Trt6
Laccase (U/g)	0	4400	4400	2200	2200	4400
HBT/ABTS (g)	0	0	0.05	0	0.05	0.05
Gas supply	Oxygen	Oxygen	Oxygen	Oxygen	Oxygen	Air

Note: Trt denotes treatment.

During incubation, a 1 mL aliquot was sampled at 48 hours by 1.0 mL pipet, transferred to a 1.5 mL microcentrifuge tube, cooled quickly on ice, and then centrifuged for 5 minutes. The supernatant was filtered through a 0.22 µm syringe filter for water

soluble carbohydrates (WSC), which were measured by the modified phenol sulfuric acid method (Gruiragossian et al., 1977).

Halliwell reported that in acidic condition, pH around 4, hydrogen peroxide with metal salt could solubilize cellulose-containing material, such as grass and straw (1965). Gould's research demonstrated that alkaline solutions of hydrogen peroxide could solubilize lignin and hemicellulose of lignocellulosic crop residues, but not cellulose (1985). In this experiment, we applied hydrogen peroxide in acidic condition to hydrolyze cellulose in order to compare the degree of delignification of samples in terms of WSC released by hydrogen peroxide after laccase system treatments. A 4 mL aliquot from every serum bottle was sampled at 48 hours. Half of these samples were treated with 1.2% H₂O₂, then all samples were incubated for 2 hours at 40°C. A 1 mL aliquot from each sample was analyzed for soluble sugar content and monosaccharides as described in the previous chapter. The preferred treatment was selected based on the highest soluble sugar content following H₂O₂ treatment. The remainders of the non-H₂O₂ treated samples were freeze dried for subsequent analyses including the TMAH and saccharification assays described in the following sections.

4.3.5. Tetramethylammonium hydroxide (TMAH) thermochemolysis and GC-MS assay

4.3.5.1. TMAH thermochemolysis

As shown in Figure 4-3, tetramethylammonium hydroxide (TMAH) thermochemolysis depolymerizes lignin into its aromatic subunits by breaking down β–

O-4 linkages, and also methylates all ring hydroxyls (Geib et al., 2008). GC-MS analysis of the TMAH thermochemolysis products indicates the acid to aldehyde ratios of the various types of lignin subunits. By cleaving some of the C_α-C_β bonds, lignin depolymerization increases the ratio of acids to aldehydes, providing a biochemical measure of the impact of the laccase system.

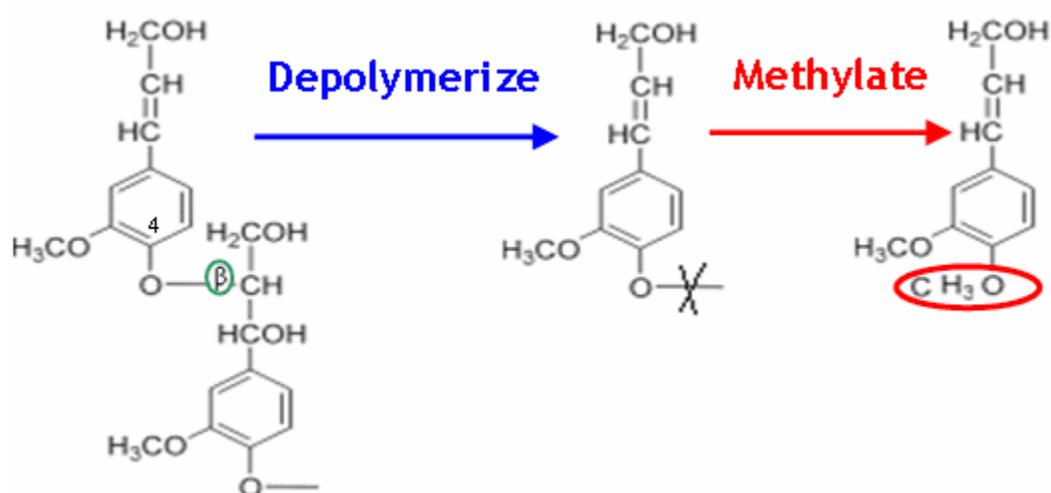


Figure 4-3: Illustration of mechanisms of TMAH thermochemolysis.

Both the non-ensiled corn stover, and laccase-treated ensiled corn stover (amended with Multifect A40) were freeze-dried to remove water for TMAH analysis, which was conducted according to Geib et al. (2008). This assay was done on freeze dried samples that had not been exposed to H₂O₂. Briefly, approximately 0.5 mg sample was placed in a borosilicate glass tube along with 150 μL of TMAH (25% TMAH in methanol, Fisher Scientific). After being purged with N₂ and covered with foil, the

mixture sat overnight to allow full contact between the reagent and sample before removing methanol under vacuum. The glass tube was flame-sealed under vacuum on a manifold and then baked at 250°C for 30 min. After cooling, the samples were extracted by ethyl acetate, filtered through sterilized glass wool, and then analyzed by Gas Chromatography-Mass Spectrometry (GC -MS).

4.3.5.2. TMAH-GC-MS measurements and analysis

GC-MS analysis was performed on a Waters GCT GC-MS system (Waters Corporation, Bellerica, MA) using a fused silica capillary column (with length and inner diameter of 20 m and 0.15 mm, respectively) coated with a 5% phenylpolysiloxane and 95% methylpolysiloxane stationary phase (Varian VF5-mas coated, 0.15 μ m). The column was run at a constant volume flow rate (0.5 mL/min), using helium as the carrier gas. The 1 μ L samples were injected at 260°C with a split ratio of 5. The oven temperature was programmed from an initial temperature of 40°C to a temperature of 290°C, ramping up at 15°C/min, and then held at 290°C for 7 min. giving a total run time of 25 min. The ionization source was electron ionization, with electron energy of 70 eV, mass range m/z between 35 and 650, and a cycle time of 1 second after the 4 min solvent delay. Data acquisition and analysis were performed using Waters MassLynx software (version 4.0). Chromatograms from the GC/ -MS were used to quantify the six acids and aldehydes of interest: 4 -methoxybenzaldehyde (P4) , 4-methoxybenzoic acid (P6), 4-dimethoxybenzaldehyde (G4), 3,4-dimethoxybenzoic acid (G6), 3,4,5-trimethoxybenzaldehyde (S4) and 3,4,5-trimethoxybenzoic acid (S6). The peaks were

integrated using masses of 95, 165, 136, 166, 195, 196, 211 and 266. Acid/aldehyde (Ad/Al) parameters, defined as the ratios of P6 and P4, G6 and G4, and S6 and S4, provide a measure of the state of lignin degradation (Geib et al., 2008; Vane et al., 2001; Hatcher et al., 1995).

4.3.6. Saccharification Assays

After biological treatment of ensiled stover by laccase system, the digestibility of ensiled stover was assayed by enzymatic hydrolysis to determine whether the laccase treatments improved cellulose saccharification downstream using Spezyme CP and Novo 188 cellulases.

4.3.6.1. Saccharification of laccase system screening samples

Two sets of saccharification assays were performed on samples that had been exposed to the laccase system screening trial described in section 4.3.4. These included saccharification of samples that were freeze dried directly after incubation with the laccase system, and fresh samples from those same trials that were instead immediately exposed to reduced-severity dilute acid pretreatment and saccharification.

For the freeze dried samples, the NREL LAP 009 method was used on samples from each laccase system treatment (Brown and Torget, 1996). This procedure, detailed in section 4.3.6.2 below, was modified slightly by setting the total volume in each vial to

5.0 mL to accommodate the smaller amount of solid sample (0.04 g) obtained from the upstream process.

After the laccase plus HBT treatment described above, fresh samples were subjected to a reduced-severity dilute acid pretreatment intended to magnify the potential impacts of laccase treatment on downstream saccharification. Fresh samples were used for this assay to minimize any negative impacts from freeze drying on the saccharification process. Sample processing and analytical constraints precluded running all the assays on fresh samples, and no direct comparison of freeze-dried versus fresh samples (without reduced-severity pretreatment) was made.

The procedure used for reduced-severity pretreatment was described by Hoskinson et al. (2007), and was modified by adjusting 0.8% H₂SO₄ to 0.15% H₂SO₄. After pretreatment with this acid for 30 minutes at 121 °C and 0.14 MPa, the samples, each with 0.1 g solid in 4 mL slurry, were cooled and adjusted to pH 4.8 by addition of NaOH, and then diluted to double volume by sodium citrate buffer (0.1 M, pH 4.8). Assuming minimal changes of cellulose during laccase and pretreatment, the appropriate amounts of Spezyme CP, 15 FPU/ g cellulose, and Novo188, 60 IU/g cellulose, were added to each bottle along with tetracycline and cycloheximide as antibiotics as recommended by the NREL LAP 009 method (Brown and Torget, 1996). The samples were incubated in a shaker water bath at 50 °C for 72 hours. At 0 and 72 h, 0.5 mL aliquots were sampled and centrifuged for glucose analysis by YSI glucose analyzer (YSI Incorporated, Yellow Springs, Ohio).

4.3.6.2 Laccase - HBT system optimization

A second set of experiments was performed on the most promising laccase system from the screening study (laccase + HBT mediator + oxygen) to investigate the influence of laccase loading rates. This trial differed from the screening study in two important ways. First, a larger sample size was used to minimize sampling error associated with the very small (0.04 g solid, approximately 0.015 g cellulose) samples tested in the previous enzymatic hydrolysis assays. The National Renewable Energy Laboratory (NREL) recommends that biomass samples for enzymatic hydrolysis assays should be 0.3 g solid (based on 30% cellulose concentration). Second, washing steps were introduced after laccase and HBT treatment to reduce any potential influence of residues from the laccase system on the downstream enzymatic hydrolysis process.

4.3.6.2.1 Laccase loading rate experiment

Stover was ensiled for 21 days with Multifect A40 as previously described, then ground using the Geno-grinder 2000 (BT&C/OPS Diagnostics, Bridgewater, NJ) at the speed of 200 rotations per minute for 4.5 minutes. 2 g samples of the ground stover were added at a 10% (w/w) concentration to 60 mL serum bottles containing oxygen saturated sodium acetate buffer (pH 4, 50 mM) and 0.05% Tween-80 as a surfactant. The designated loading rates of laccase as well as 5% 1-hydroxybenzotriazole (HBT) mediator were added to each bottle. Triplicate bottles were prepared for each treatment. The solid/liquid slurry was bubbled with oxygen for 2 minutes and then incubated in a rotary water bath at 90 rpm and $40 \pm 1^\circ\text{C}$ for 48 hours. Aliquots of 0.5 mL were taken at

0 and 48 hours and immediately chilled on ice, then centrifuged at 14,000 rpm for 5 minutes. Glucose concentration was determined on the supernatant using the YSI glucose analyzer.

4.3.6.2.2 Washing

After the 48 hour incubation with laccase and mediators, the substrate was centrifuged at 47,000 rpm for 30 min at 4 °C, and then the solids were washed twice by re-suspending in 17.5 mL nano-pure water (18.2 megohm-cm, Barnstead, Dubuque, Iowa) and centrifuged again in order to reduce any influence of laccase and mediator on the downstream process. The supernatant of the laccase treated samples and also the washed liquid were both subjected to glucose analysis for a direct measurement of the sugar released by laccase treatment. Saccharification results are reported on both a solid basis and a gross basis (solids plus washed liquids) based on cellulose content as determined through fiber analysis.

4.3.6.2.3 Saccharification assays for laccase loading rate optimization trials

Enzymatic hydrolysis was carried out according to NREL Laboratory Analytical procedure LAP 009 (Brown and Torget, 1996). Solid samples after washing were added to 25 mL of 0.05M citrate buffer (pH~4.8) containing 40 µg/mL tetracycline and 30 µg/mL cycloheximide in 50 mL centrifuge tubes. The supernatant and washing liquid (approximately 50 mL) was also added to citrate buffer with Spezyme CP and Novo 188

and antibiotics as previously described. After sealing with a cap and Para-film, the tubes were suspended in a shaking water bath at 90 rpm and $50 \pm 1^\circ\text{C}$ for 3 days. Glucose assays were conducted on the supernatant of samples drawn at 0 and 72 hours.

4.4 Results and discussion

This study examined how laccase and mediators influenced lignin decomposition and enzyme digestibility of ensiled corn stover. Detailed changes in the chemical structure of stover lignin were measured by TMAH-GC-MS analysis, while saccharification assays indicated how digestibility of ensiled stover changed in different laccase mediator systems and at different laccase loading rates.

4.4.1 Enzymatic (cellulase and hemicellulase) silage treatment

The observations of enzymatic silage experiments were consistent with previous research (Chapter 3). The dry matter was well preserved and dry matter loss was minimized by enzyme enhanced silage; moreover, as shown in Figure 4-4, monosaccharides and water soluble carbohydrate (WSC) were doubled by hydrolyzing cellulose and hemicellulose by cellulase, hemicellulase and organic acid during the silage process. Therefore, the corn stover was not only well-preserved, but also was partially biologically pretreated. Accordingly, ensilage could serve as a platform for further biological pretreatment, such as the lignin-degrading enzyme treatments evaluated in this chapter. By creating channels and opening the lumen, the ensilage process may provide

opportunities for these enzymes, whose molecular weight is too large to enter an intact plant cell wall, to penetrate the cell wall structure and access and degrade corn stover lignin.

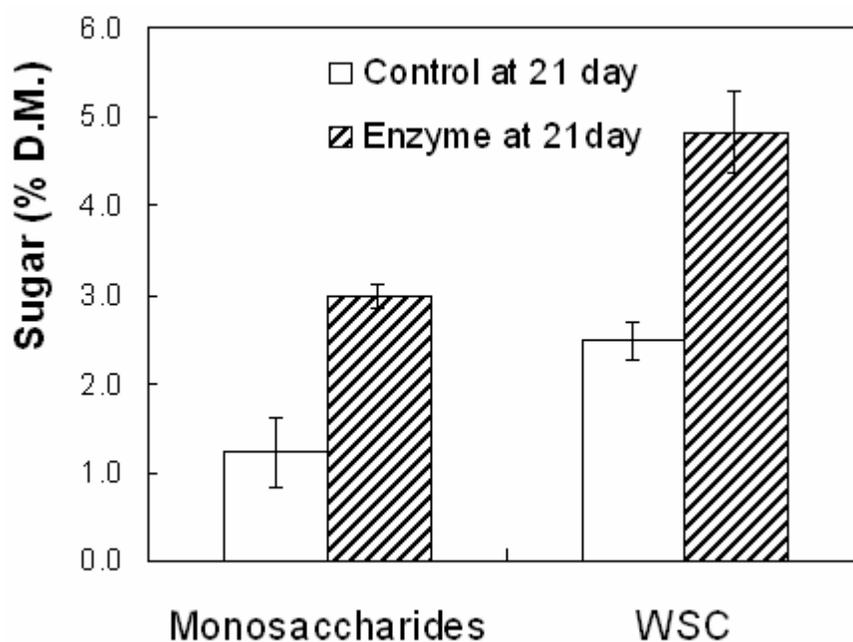


Figure 4-4: Monosaccharides and WSC of ensiled stover with and without cellulase/hemicellulase on day 21. (Note: Throughout this chapter, the error bar denotes the standard deviation of independent samples.)

4.4.2 Laccase experiments

4.4.2.1 Laccase system screening trial results

For the six laccase + mediator + oxygen/air treatments, to isolate the effects of mediators, WSC induced by laccase with or without mediator was subtracted from the

corresponding samples to determine the net release of WSC from additions of mediator to the laccase-mediator system (Table 4-2). Ensiled corn stover was used as control for the following two sets of experiments: laccase with ABTS and laccase with HBT.

Table 4-2: WSC of ensiled stover with laccase and HBT/ABTS treatments.

	Treatments	WSC at 0 hours (mg/0.4 g dry stover) with S.D.	WSC at 48 hours (mg/0.4 g dry stover) With S.D.	Net increased in WSC (mg/0.4g dry stover) with S.D.
Non-H2O2	O ₂	19.8 ± 0.4	41.7 ± 0.9	21.9 ± 0.5
	4,400U/g+O ₂	121.0 ± 2.3	149.5 ± 4.8	28.5 ± 2.5
	4,400U/g+HBT+O ₂	120.0 ± 6.0	147.4 ± 12.6	27.4 ± 6.6
	2,200U/g+O ₂	68.2 ± 2.2	90.9 ± 4.5	22.7 ± 2.4
	2,200U/g+HBT+O ₂	71.5 ± 0.4	97.9 ± 0.8	26.4 ± 0.4
	4,400U/g+HBT+Air	120.8 ± 2.1	169.0 ± 4.3	28.2 ± 2.3
H2O2	O ₂	22.5 ± 2.7	47.3 ± 5.6	24.9 ± 2.9
	4,400U/g+O ₂	117.7 ± 2.9	142.5 ± 6.1	24.8 ± 3.2
	4,400U/g+HBT+O ₂	191.3 ± 4.5	297.4 ± 9.5	106.1 ± 5.0
	2,200U/g+O ₂	65.2 ± 3.5	84.6 ± 7.4	19.4 ± 3.9
	2,200U/g+HBT+O ₂	67.7 ± 3.9	89.8 ± 8.2	22.2 ± 4.3
	4,400U/g+HBT+Air	129.3 ± 14.9	166.8 ± 31.4	37.6 ± 16.5
Non-H2O2	O ₂	20.0 ± 0.7	42.0 ± 1.6	22.1 ± 0.8
	4,400U/g+O ₂	107.4 ± 7.3	120.9 ± 15.3	13.5 ± 8.1
	4,400U/g+ABTS+O ₂	111.4 ± 3.0	129.2 ± 6.4	17.9 ± 3.4
	2,200U/g+O ₂	65.6 ± 0.2	85.6 ± 0.5	19.9 ± 0.3
	2,200U/g+ABTS+O ₂	63.7 ± 1.3	81.6 ± 2.7	17.8 ± 1.4
	4,400U/g+ABTS+Air	107.9 ± 3.0	121.9 ± 6.4	14.0 ± 3.3
H2O2	O ₂	18.8 ± 2.7	39.7 ± 5.8	20.8 ± 3.0
	4,400U/g+O ₂	110.2 ± 6.9	126.7 ± 14.6	16.5 ± 7.7
	4,400U/g+ABTS+O ₂	102.3 ± 7.7	114.9 ± 8.0	12.6 ± 6.5
	2,200U/g+O ₂	63.9 ± 2.8	81.8 ± 5.9	18.0 ± 3.1
	2,200U/g+ABTS+O ₂	63.2 ± 4.5	80.3 ± 9.5	17.2 ± 5.0
	4,400U/g+ABTS+Air	105.2 ± 3.3	116.3 ± 6.9	11.0 ± 3.6

Note: S.D. denotes standard deviation.

Experimental results for samples with laccase and ABTS treatments are shown in Figure 4-5. There was no significant difference in WSC between the samples with and

without hydrogen peroxide. The net amount of WSC was similar for all the treatments for ensiled corn stover and low relative to 600 mg per dry gram that was theoretically possible based on fiber analysis (Chapter 3). It is possible that ABTS is not a proper mediator for laccase delignification of ensiled corn stover. This finding is consistent with the application of laccase system in the pulp and paper industry, where it has been reported that ABTS is less able to promote lignin decomposition than HBT (Camarero et al., 2004; Morozova et al., 2007).

The WSC results for laccase systems with the HBT mediator are provided in Figure 4-6. In treatments without hydrogen peroxide, the laccase - HBT system did not significantly change the WSC level. However, among the samples that were exposed to hydrogen peroxide, the 4,400 IU/g laccase loading rate in combination with HBT experienced dramatic increases in WSC. The underlying mechanism might be that the lignin structure was partially decomposed by the laccase and HBT system, which made the stover cell wall structure more accessible and then partial cellulose and hemicellulose were hydrolyzed into sugar by addition of hydrogen peroxide. This result suggests that the cell wall structure was degraded by a combination of cellulases (added during ensilage) and the post-ensilage laccase treatment. Such a strategy might reduce the downstream energy requirements for pretreatment and enzyme costs for saccharification. Among the treatments tested, the 4,400U/g laccase loading with HBT as a mediator is the most promising approach for lignin decomposition.

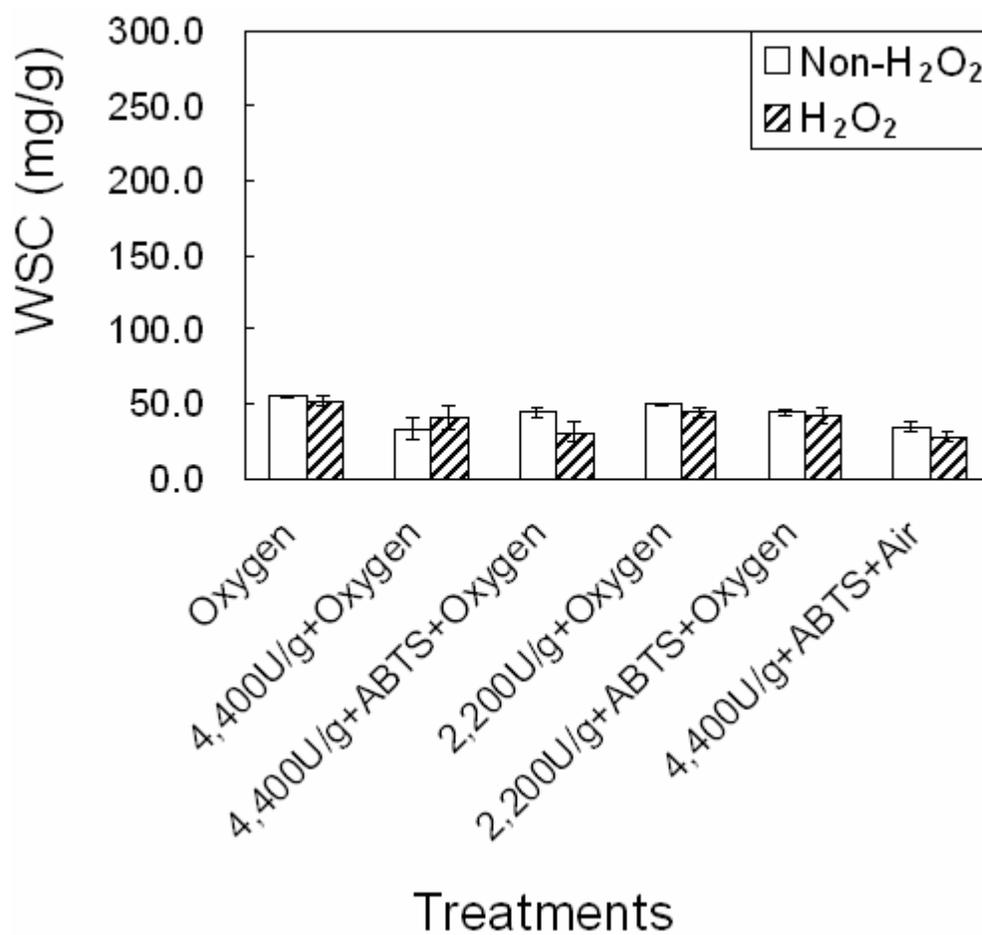


Figure 4-5: Water Soluble Carbohydrates (WSC) released from samples with laccase (LA) and 2,2'azino-bis-(3-ethylbenzothiazoline -6-sulfonic acid (ABTS) treatment. All samples were ensiled first, including the oxygen-only control.

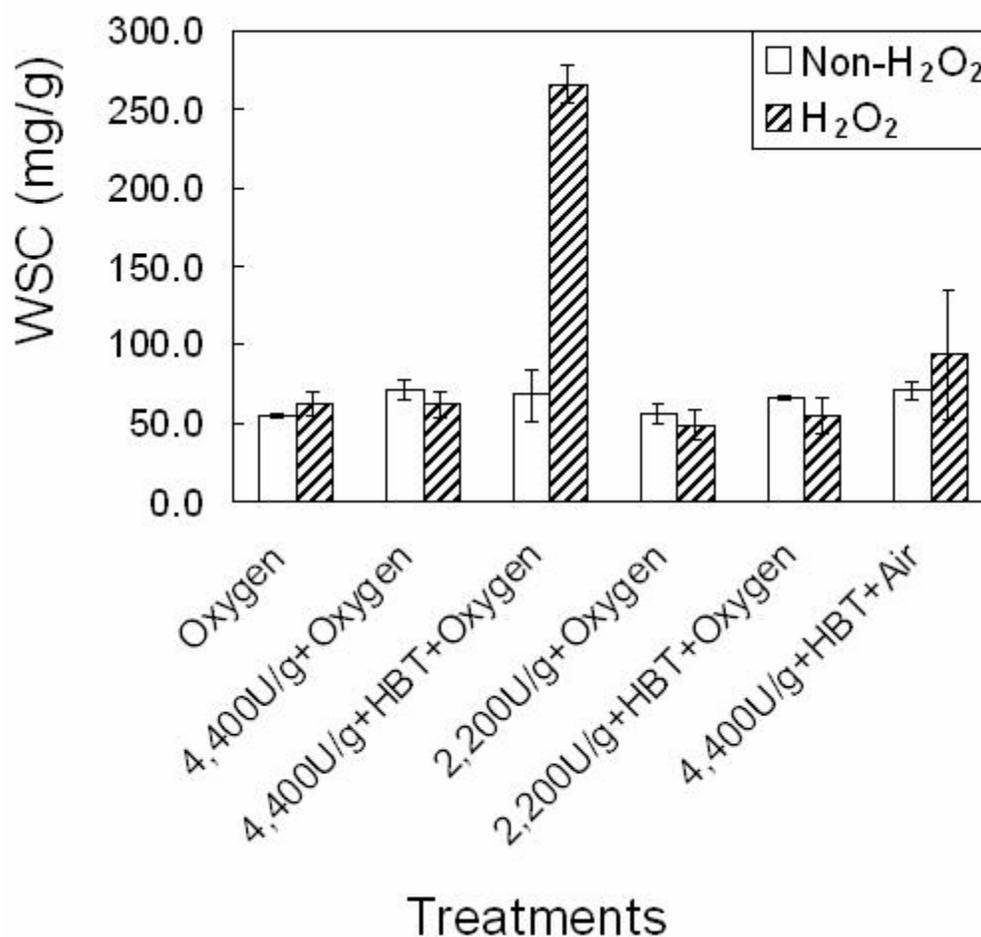


Figure 4-6: Water Soluble Carbohydrates released from samples with laccase (LA) and 1-hydroxybenzotriazole (HBT) treatment. All samples were ensiled first, including the oxygen-only control.

4.4.2.2 TMAH GC-MS results

In order to determine whether the lignin structure of corn stover was altered by the combination of ensilage and treatment 3 (4,400 U/g laccase + 0.05 g HBT + oxygen), TMAH thermochemolysis was used to examine the characteristic compositions of native (not ensiled) and treated corn stover.

4.4.2.2.1 TMAH-GC-MS on native corn stover

TMAH reaction breaks the β -O-4 linkages of lignin's aromatic subunits and also methylates all ring hydroxyls, through which S4, G4, and P4 are the main products. The GC-MS results, shown in Figure 4-7, indicate that corn stover lignin can be classified as a *p*-hydroxyphenyl-guaiacyl-syringyl (P-G-S) lignin because the major TMAH thermochemolysis products were composed of methylated *p*-hydroxyphenyl, guaiacyl and syringyl derivatives. This observation is consistent with the result from Lapierre, 1993, in which the CuO oxidation method, an alternative to TMAH thermochemolysis, showed all three of these monolignol units of corn lignin present in significant amounts (Buranov and Mazza, 2008). For this research, the focus is on the ratio change of acid to aldehyde, including P6/P4, G6/G4 and S6/S4, the total derivatives of P, G and S were not explored. But Lapierre's research included all the derivatives from P, G and S. The resulting ratio of P, G and S units was 4:35:61 (Lapierre, 1993), but this was on mature maize stalks that did not include the leaves or ground cob included in our stover mix. Although this ratio of P, G, and S units is expected to vary due to differences in the maturity and type of the corn stover, the two methods gave very similar results on the total yield of lignin monomers. From a previous comparative study on the ratios of acid to aldehyde monomers, TMAH thermochemolysis was found to be more sensitive than the thioacidolysis method because the former displayed a larger dynamic range (Hatcher et al., 1995).

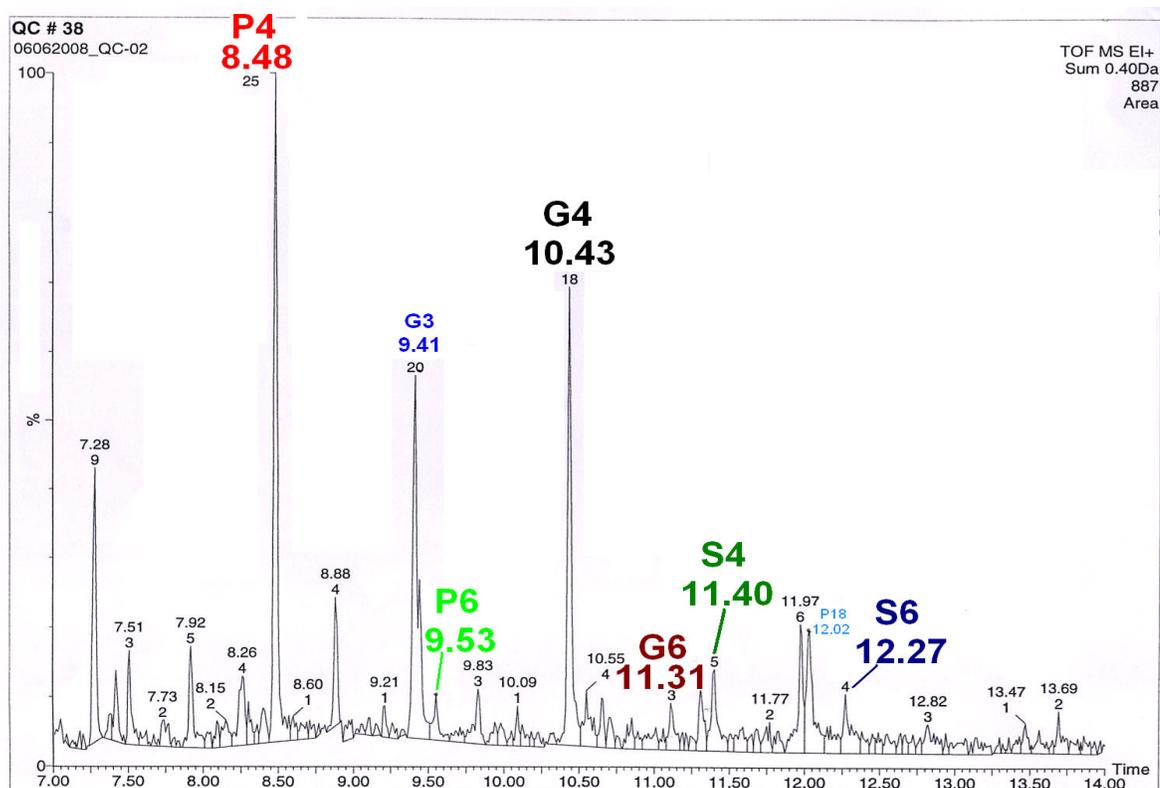


Figure 4-7: Partial chromatogram of the selected ion current for the TMAH thermochemolysis products from native corn stover (Note: The GC/TOF-MS chromatogram was integrated using masses 95, 165, 136, 166, 195, 196, 211 and 266).

4.4.2.2.2 TMAH-GC-MS on laccase treated ensiled corn stover

When fungi use enzymes to degrade lignin there are three dominant reactions: propyl side chain oxidation, ring hydroxylation and demethylation (Geib et al., 2008; Filley et al., 2000). White rot fungi (wrf) mainly rely on side chain oxidation, leading to C_{α} - C_{β} cleavage/depolymerization of lignin. This side chain oxidation increases the ratio of acid to aldehyde by producing S6 from S4; G6 from G4; and P6 from P4 (Geib et al.,

2008; Filley et al., 2000). Thus we expect that the ratio of acid to aldehyde will be higher if laccase, from the white rot fungi *T. versicolor*, contributes to lignin depolymerization by cleaving the C_α-C_β bonds of lignin.

In the native corn stover (not ensiled) samples, the aldehydes P4, G4, and S4 dominated the TMAH reaction products from lignin (see Figure 4-7). Figure 4-8 illustrates these reactions, with the TMAH breaking β-O-4 linkages and methylating all ring hydroxyls for both native and degraded lignin. During TMAH thermochemolysis the degraded lignin products are converted to S6, G6 and P6 acids, with a corresponding decrease in the S4, G4 and P4 aldehydes.

After ensilage and laccase treatment, all three aldehydes decreased, while the acid forms increased (see Figure 4-9 and Table 4-3). We hypothesize two underlying mechanisms may contribute to these results: 1) the added enzymes plus organic acids produced during ensilage degraded cellulose and especially hemicellulose, eroding channels that allowed more laccase to enter the complex lignocellulosic substrate; and 2) laccase from white rot fungi, resulted in C_α-C_β cleavage/depolymerization of lignin in the stover cell walls. The increasing ratios of acids to aldehydes reported in Table 4-3 demonstrated that the combined treatment (ensilage + laccase + mediator) contributed to lignin degradation.

Comparing the results of TMAH-GC-MS analyses on the thermochemolysis products from both native and laccase-treated ensiled corn stover (Table 4-3), there was a relative increase in S6 and significant increase in both G6 and P6 in samples from laccase degraded ensiled corn stover. The acid/aldehyde ratios (Ad/Al)_G, (Ad/Al)_S, and (Ad/Al)_P

for degraded corn stover were 0.56, 1.56 and 1.14, respectively, while they were 0.22, 0.93 and 0.16 for native corn stover (Figure 10). The increases of $(Ad/Al)_G$, $(Ad/Al)_S$ and $(Ad/Al)_P$ in laccase-treated ensiled corn stover provide evidence that the laccase system induced significant lignin side chain oxidation, cleaving the $C_\alpha-C_\beta$ bonds located on the aliphatic side chain. Assuming that these lignin structural changes primarily occurred during the 2 days of laccase exposure (but even allowing for some degradation during the 21 days of ensilage), degradation was more rapid than the previously reported white-rot fungal induced degradation of wheat straw, the ratio of acid to aldehyde for G and S changed from 1.0 to 6.4 and 1.7 to 3.1, respectively, after 272 days (Vane et al., 2002).

Table 4-3: Peak area of the TMAH thermochemolysis products from stover.

Subunits	P4	P6	G4	G6	S4	S6
Non-ensiled1	24.83	2.42	18.19	2.99	5.31	4.27
Non-ensiled2	14.78	2.94	10.86	2.35	3.32	1.73
Non-ensiled3	7.55	1.30	9.22	2.62	2.03	2.98
Ensiled+LA1	7.65	6.48	6.06	3.92	2.06	1.91
Ensiled+LA2	13.10	11.03	11.75	5.00	3.34	2.95
Ensiled+LA3	5.02	9.46	5.32	2.25	1.69	3.65
Ensiled+LA4	69.93	38.14	136.68	108.77	45.24	129.42
Ensiled+LA5	7.25	10.22	5.3	3.17	3.81	5.54
Ensiled+LA6	10.66	13.97	9.69	4.65	4.76	5.04

Note: GC-MS measurements were performed on ensiled corn stover treated with laccase (4,400U/g) +HBT+Oxygen as previously described. The original triplicate samples were subsampled into duplicates, with pairs Ensiled+LA1 and Ensiled+LA2, Ensiled+LA3 and Ensiled+LA4, and Ensiled+LA5 and Ensiled+LA6 at tabulated above from the same group. The peak area of each component varied due to the different masses used for GC-MS analysis, but the ratio of Acid to Aldehyde was relative consistent within each duplicate measurement. The average ratios of Acid to Aldehyde of not ensiled, and laccased-treated ensiled corn stover were demonstrated in Figure 4-10.

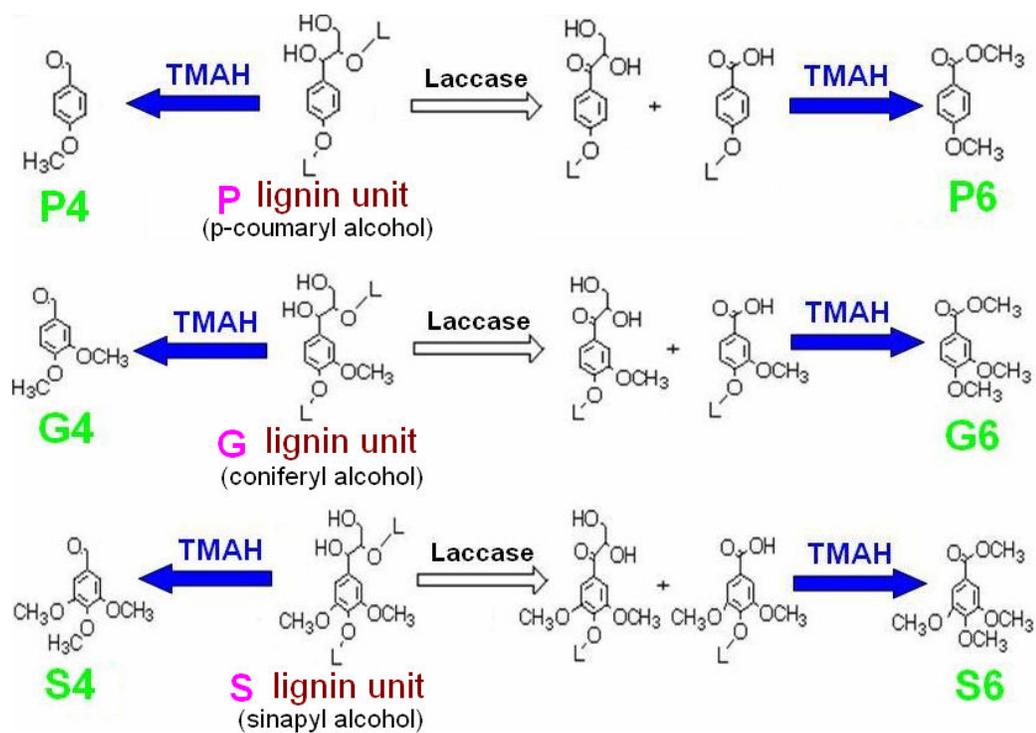


Figure 4-8: Mechanism of TMAH-GC-MS on lignin analysis (adapted from Geib et al., 2008). Teramethylammonium hydroxide (TMAH) creates a molecular signal of lignin degradation, with the shift from aldehyde to acid pyrolysis derivatives measured by GC/TOF-MS.

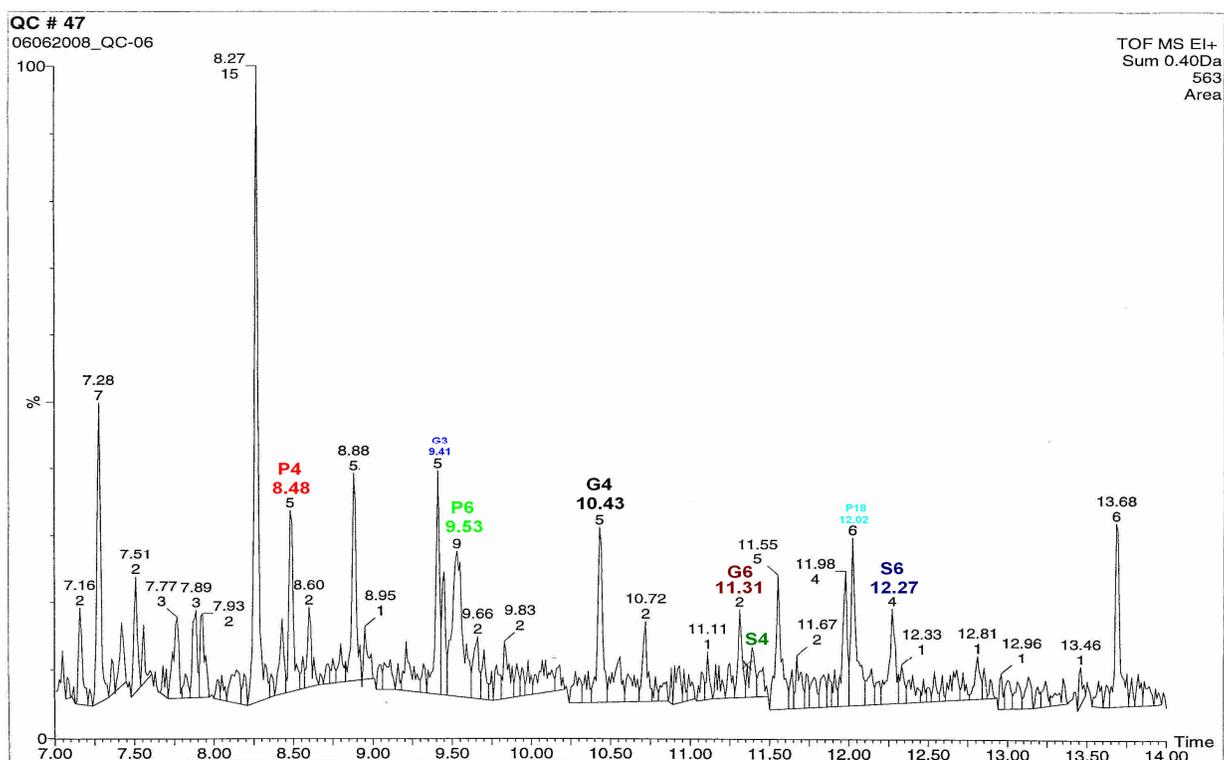


Figure 4-9: Partial chromatogram of the selected ion current for the TMAH thermochemolysis products from degraded corn stover. (Note: The GC/TOF-MS chromatograms were integrated using masses 95, 165, 136, 166, 195, 196, 211 and 266.).

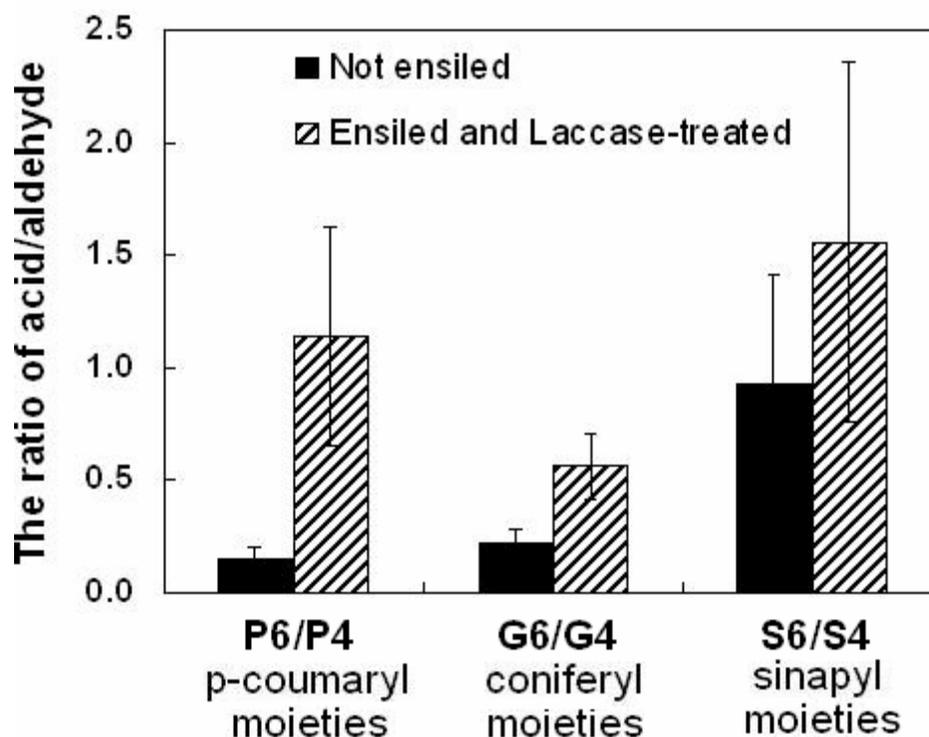


Figure 4-10: The ratio of Acid/Aldehyde of non-ensiled stover and ensiled and laccase-treated stover.

4.4.3 Saccharification Results

Lignin degradation by the laccase system and ensilage was demonstrated by TMAH-GC-MS analysis. While native lignin content is strongly correlated with biomass recalcitrance to saccharification, it is important to determine the extent to which enzymatic degradation to monoglignol units would also have beneficial downstream effects. The following results of standard saccharification assays using enzymatic hydrolysis illustrate the impacts of these laccase treatments on ensiled stover.

4.4.3.1 Saccharification results for laccase screening study

4.4.3.1.1 Freeze-dried samples

On these freeze dried samples the cellulose conversion rates from all the treatments had similar values (Figure 4-11) and there was no significant difference among them based on an ANOVA one way Tukey test, with $P < 0.05$. This similarity among the cellulose conversion rates of all the treatments might be caused by the following two reasons. First, all the samples may have been affected by freeze drying before enzymatic hydrolysis. Procedure LAP 009 from NREL recommends that after pretreatment the biomass should not experience any drying whatsoever prior to enzyme digestibility assays. The concern is that irreversible pore collapse could happen in the micro-structure of the biomass, and that would influence enzyme access to and glucose release from cellulose (Brown and Torget, 1996). Second, although lignin degradation was observed by TMAH-GC-MS analysis, such structural changes might be not be sufficient to directly increase enzymatic hydrolysis in the absence of any further chemical pretreatment.

4.4.3.1.2 Fresh samples exposed to reduced-severity dilute acid pretreatment

To avoid any negative effects associated with freeze drying and explore synergies with chemical pretreatment, liquid slurry samples were immediately exposed to reduced-severity dilute acid pretreatment without freeze drying, and then subjected to enzymatic hydrolysis, which increased the overall cellulose hydrolysis by almost 300%. This cellulose conversion rate represents the sum of the effect of the pretreatment and

saccharification and is based on the initial cellulose content of the ensilage. Despite the changes, the cellulose conversion rates were still not significantly different among the various treatments based on ANOVA one way Tukey test, with $P < 0.05$ (Figure 4-12). The relatively low saccharification rates and similarity among treatments might be attributed to inhibition associated with the organic acids and other compounds from ensilage, as well as residues of laccase and mediators in those treatments (Palmqvist et al., 1996). If the residue of laccase and mediator were to inhibit the downstream enzymatic hydrolysis, this could counter any benefits the laccase system might otherwise bestow. Washing the solids prior to enzymatic hydrolysis might be needed to reduce this residue effect.

The relatively small sample size used for these experiments may also have been a factor, increasing the variability among replicates and obscuring any small differences that did occur. These saccharification experiments started with the equivalent of 0.015 g of cellulose, while the original NREL LAP 009 (Brown and Torget, 1996) recommended starting with the equivalent of 0.1 g of cellulose for enzymatic hydrolysis. Increasing sample size might help minimize sampling and measurement errors. This was done for the loading rate optimization trials reported on in the next section.

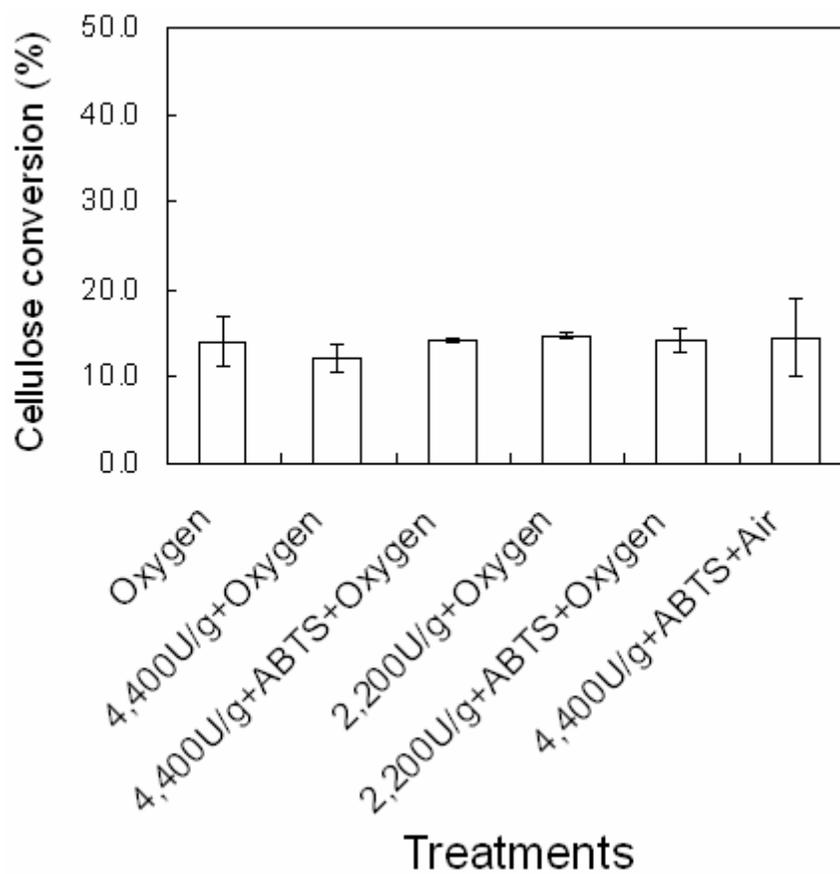


Figure 4-11: Cellulose conversion rate of the ensiled and laccase-treated samples after freeze-drying. U/g is the unit of laccase applied on the samples.

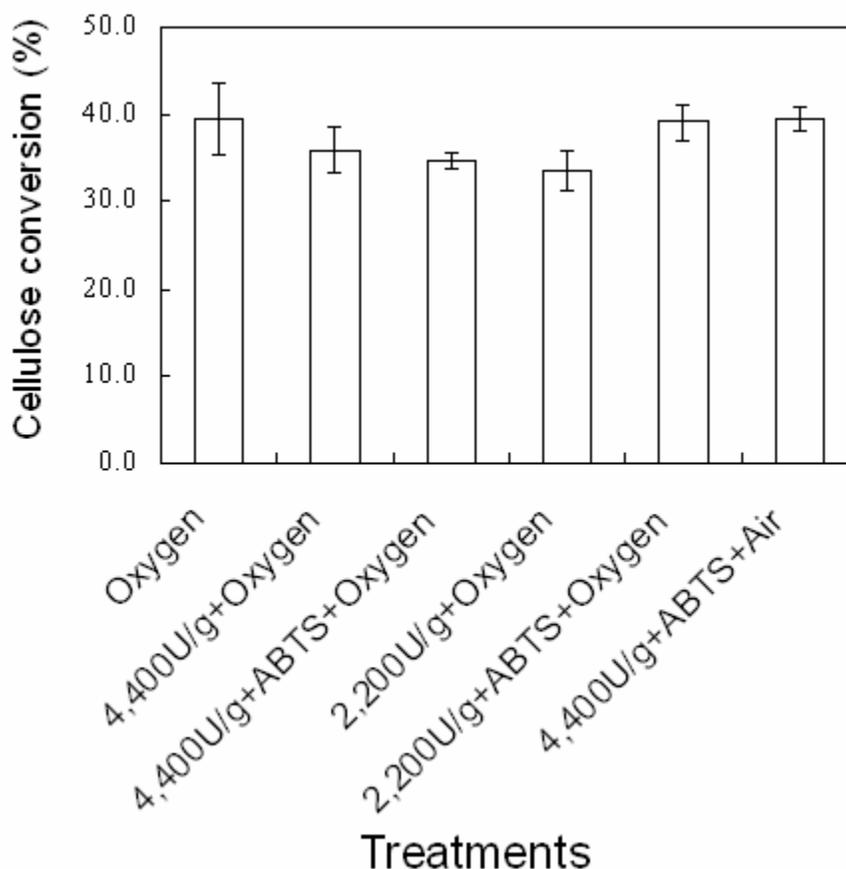


Figure 4-12: Cellulose conversion rate of the ensiled and laccase-treated samples after reduce-severity acid pretreatment. U/g indicates the units of laccase applied per gram of samples as defined in Appendix C.

4.4.3.2 Saccharification results for laccase loading rate optimization trial

The laccase loading rate optimization experiments were performed with larger sample sizes (2 g), with the entire samples saccharified to reduce sampling errors. The solids were also washed after laccase treatment to reduce potential inhibitions associated with the enzymes, mediators, or ensilage byproducts. Several different models were

tested to fit the cellulose conversion (y) on either a washed solids or gross basis (including both washed solids and the washing liquid) as a function of laccase loading rate (U/g). Models tested included polynomial, exponential, logarithm, and the Michaelis-Menten equation. These fitting function parameters and associated coefficients of determination were obtained as,

(a) quadratic polynomial:

$$y = -4 \times 10^{-7} U^2 + 2.9 \times 10^{-3} U + 22.059; R^2 = 0.949 \quad (\text{washed solids})$$

$$y = -4 \times 10^{-7} U^2 + 3.3 \times 10^{-3} U + 24.625; R^2 = 0.992 \quad (\text{gross basis})$$

(b) Exponential function:

$$y = 22.51 \times e^{6 \times 10^{-5} U}; R^2 = 0.817 \quad (\text{washed solids})$$

$$y = 25.119 \times e^{6 \times 10^{-5} U}; R^2 = 0.897 \quad (\text{gross basis})$$

(c) Logarithmic function:

$$y = 13.970 + 3.732 \times \log_{10}[113.208 + U]; R^2 = 0.971 \quad (\text{washed solids})$$

$$y = 4.413 + 7.394 \times \log_{10}[516.678 + U]; R^2 = 0.998 \quad (\text{gross basis})$$

(d) Modified Michaelis-Menten equation (Nelson and Cox, 2005)

$$y = 21.06 + \frac{6.904U}{496.5 + U}; R^2 = 0.923 \quad (\text{washed solids})$$

$$y = 24.34 + \frac{10.587U}{1975.63 + U}; R^2 = 0.996 \quad (\text{gross basis})$$

Based on the criteria of maximize R^2 , it seems any one of these functions, the quadratic polynomial, exponential function and modified Michaelis-Menten equation, would be appropriate. However, the quadratic polynomial equation predicts negative cellulose conversion and the exponential function predicts more than 100% cellulose conversion as the laccase loading goes to extremely large values. These physically impossible predictions make these two models not attractive for a system that has not been experimentally tested over the entire potential range. The modified Michaelis-Menten equation, which was proposed to approximately describe the kinetics of many enzymes (Michaelis and Menten, 1913), provides more generally realistic results. Taking

$$y = 24.519 + \frac{10.725U}{2195.99 + U}$$

as an example, the total cellulose conversion can be

considered to be the sum of cellulose conversion without laccase loading plus additional cellulose conversion due to the laccase-system. In addition, the modified Michaelis-Menten equations are monotonic and steady-state functions. As laccase loading rate goes to infinity, the cellulose conversions approach asymptotic values of 27.96, and 34.93 for washed solids and gross basis, respectively. All of these properties make the modified Michaelis-Menten equation the best choice to fit cellulose conversion as a function of laccase loading rate.

As shown in Figure 4-13, the enzymatic digestibility of washed solids was correlated with the laccase loading rate. As loading rate increased from 0 to 4,400 U/g, the enzymatic digestibility increased from 21.06% to 27.85% for washed solids from laccase-treated ensiled stover (Figure 4-13). The modified Michaelis-Menten equation fit the loading curve well ($R^2=0.923$) within the experimental range between 0 and 4,400

U/g, and showed that the digestibility of washed solids almost reached its estimated maximum cellulose conversion. This model also suggested the digestibility of washed solids would not increase much further at laccase loading rates beyond 4,400 U/g, which implied our experimental loading rate almost achieved the maximum value of 27.96%.

On a gross basis (considering both washed solids and the washing liquid), measured cellulose conversion of the ensiled corn stover increased from 24.34% to 31.73% as the laccase loading rate increased from 0 to 4,400 U/g, as shown in Figure 4-14. The modified Michaelis-Menten equation ($R^2=0.996$) fit the loading curve well, and predicts that digestibility of ensiled stover almost reached the estimated maximum value of 34.93 when the loading rate reached 4,400 U/g.

To choose a preferable laccase loading rate, it is critical to examine the minimum statistically significant loading rate relative to relevant bioconversion indicators. This rate was assessed by the enzymatic saccharification of washed solids and the ensiled stover. For washed solids in Figure 4-13, the cellulose conversion percentage was increased by 5.5% when the laccase loading rate was changed from 0 to 2,200 U/g. However, the cellulose conversion percentage increased less than 2% when the laccase loading rate was doubled from 2,200 U/g to 4,400U/g. This is similar to the change of the cellulose conversion percentage of the ensiled stover based on analysis of Figure 4-14. Therefore, the minimum statistically significant loading rate may indeed be around 2200 U/g.

The enhanced digestibility of laccase-treated ensiled corn stover is believed to result from better exposure of cellulose to cellulase through the structural changes of lignin, providing better access of cellulase to the substrate so it can further react with

cellulose. In addition, structural changes may make lignin less able to competitively absorb cellulase, which in turn would allow more cellulase to be available for cellulose hydrolysis (Gusakov and Sinitsyn, 1992).

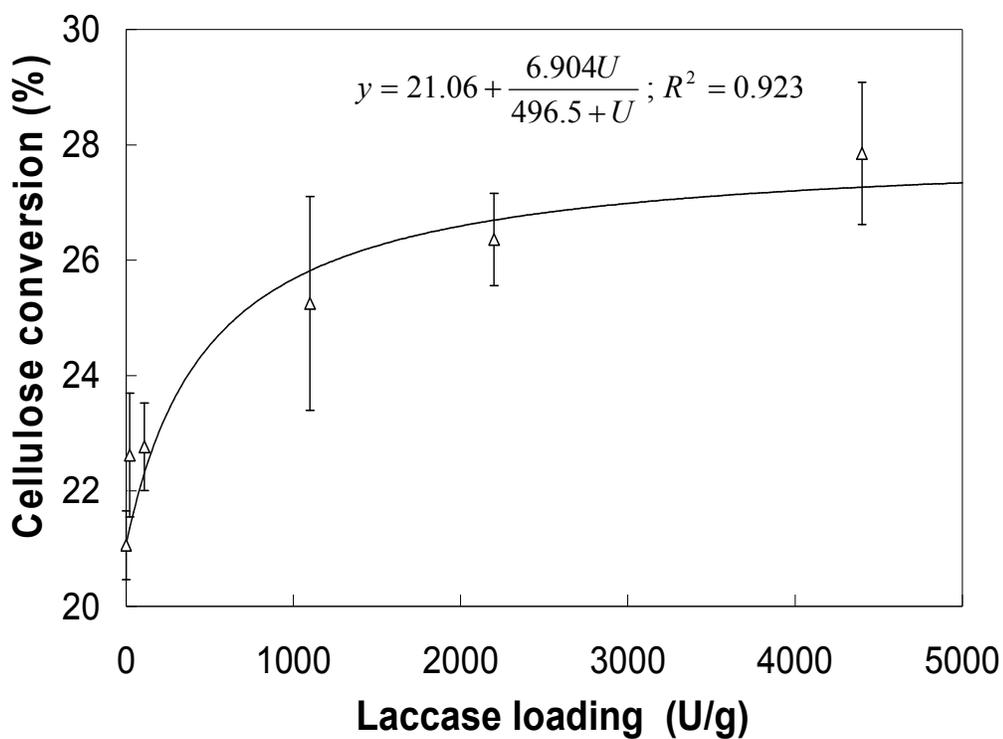


Figure 4-13: Cellulose conversion rate of washed solids as a function of laccase loading rate. All samples were previously ensiled.

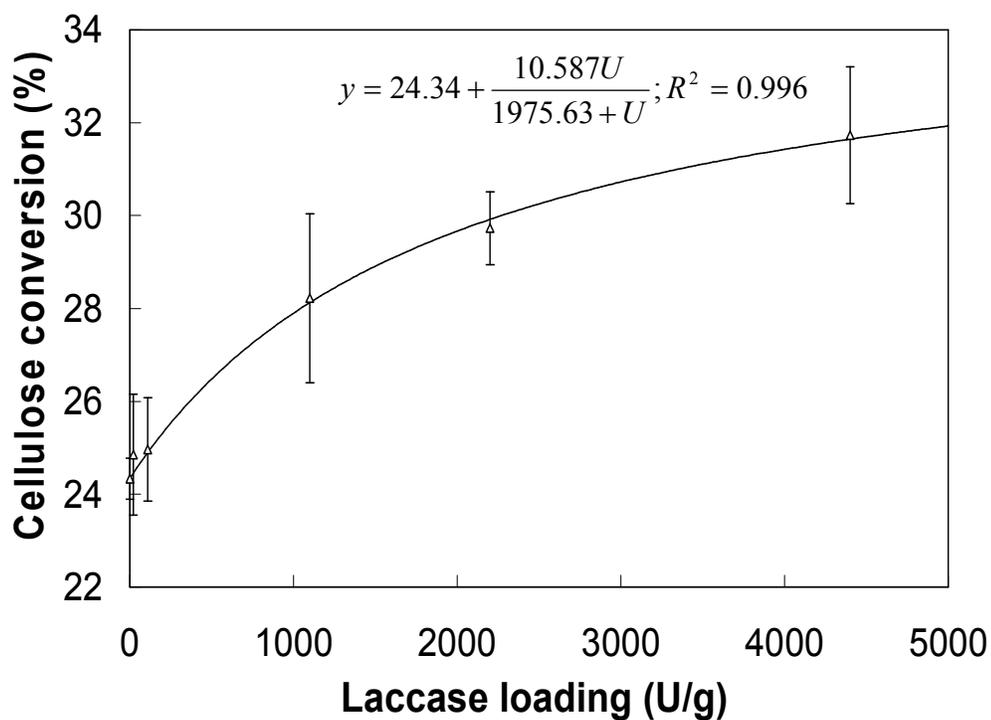


Figure 4-14: Cellulose conversion rate of the ensiled corn stover on a gross basis (washed solids plus washing liquid) as a function of laccase loading rate.

4.5 Conclusions

This study is the first demonstration that laccase, a lignin-degrading enzyme, directly contributes to lignin modification in a natural biomass substrate. By demonstrating this effect on ensiled corn stover, the results extend previous laccase studies on lignin that had been isolated from plant cell walls by physical or chemical processes.

TMAH-GC-MS was shown to be a useful analytical technique for monitoring the molecular changes in lignin structure. TMAH-GC-MS analysis indicated that the combination of ensilage, laccase and HBT mediator, was able to oxidatively degrade ensiled corn stover lignin, resulting in an increased ratio of lignin derived phenolic carboxylic acids to aldehydes.

Water soluble carbohydrate levels in ensiled stover were enhanced by laccase in the presence of HBT mediator. The ABTS mediator did not have a significant effect, even at high laccase loading rates. Saccharification studies on washed samples, where any inhibitory effects would be reduced, indicated that this synergistic effect of laccase plus the HBT mediator was strongest with lower rates of laccase addition, with benefits leveling off between 2000 and 4000 U/g.

Given the short time that the ensiled stover was treated by the laccase-mediator system, it was surprising to observe such a significant change in the lignin structure and digestibility. It is possible that ensilage enhanced the effects of biological pretreatment by providing channels for laccase to enter the complex biomass, and because cellulose and hemicellulose were partially hydrolyzed into soluble sugars by cellulase, hemicellulase and organic acid during the ensilage process. We expect this synergistic effect could be further enhanced by optimizing the reaction conditions during laccase treatment, such as by adjusting oxygen partial pressure, mediators and additives.

4.6 Acknowledgments

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Chapter 5

SIMULATION OF CORN STOVER ENSILAGE WITH ENZYME AMENDMENTS

5.1 Abstract

Ensilage technology can provide an effective long-term storage strategy for corn stover and other biomass feedstocks prior to bioconversion. The final quality of ensiled corn stover depends on its initial chemical composition, microbial populations, thermal and physical conditions, and whether supplemental enzymes are added to enhance the silage fermentation. A comprehensive experimental study to better understand how these variables interact to affect silage quality would require a large number of trials and intensive analysis. The objective of this investigation is to modify a predictive dairy silage model initially developed by Pitt and his colleagues in the late of 1980s, and then to simulate the dynamic behavior of pH, water soluble carbohydrates (WSC), cellulose, and other major biochemical and microbial processes as effected by initial substrate, enzyme amendments, and environmental conditions. Estimates of final pH, WSC and cellulose concentrations are in agreement with experimental measurements of enzyme-amended ensilage. The simulation results also demonstrate that cellulase loading rate had a significant positive effect on the change of WSC, and that these enzyme additives can enhance biomass stability during long term storage. The optimal experimental conditions required to obtain high quality corn stover silage can be achieved by adjustment of

cellulase loading rate and storage temperature. This study can help guide the design of ensilage systems for large-scale storage of plant-based biomass.

5.2 Introduction

The energy crisis has been gaining world-wide attention since the 1970s. Currently the United States consumes around 140 billion gallons of ground transportation fuel per year. It has been projected that energy demand would grow by more than 50% by 2025 (Somerville, 2006; Kumar et al., 2008). Among all the potential energy alternatives to satisfy this growing demand of energy and reduce the environmental problems associated with petroleum, bio-fuels from lignocellulosic plants have been identified as among the most promising near-term solutions. Excluding existing agricultural and forestry production for food and fiber, it has been estimated that the United States could produce about 130 billion gallons of ethanol per year sustainably from corn stover and perennial crops, while the world as a whole could potentially produce 1000-50000 billion gallons (Kumar et al., 2008; Mariam, 2006; Somerville, 2006). Besides being a promising cost-competitive fuel feedstock, producing domestic bio-fuel on such large scale would have considerable political and economic advantages, increasing the self-sufficiency of nations around the globe (Somerville, 2006).

The lignocellulosic biomass in corn stover has been recognized as an important near-term option for beginning to develop a bio-based economy. However, some crucial issues, including biomass feedstock availability, biomass harvest and storage technologies, bioconversion efficiency, and cost need to be determined before energy

resources can shift away from petroleum to renewable biomass resources (Mariam, 2006). Among these issues, effective storage of large volumes of corn stover and other seasonally harvested lignocellulosic crops presents one of the major and essential challenges for bio-fuel development. Ensilage, a traditional forage preservation method for ruminant feed, is considered a promising biomass preservation and pretreatment strategy because it reduces storage risks and dry matter loss (Richard et al., 2000; 2001; Shinnars et al., 2007). Amending ensilage with cellulase enzymes was further demonstrated as an effective strategy of corn stover preservation and pretreatment for bioconversion in several previous studies (Ren, 2006; Richard et al., 2007; Chen et al., 2008). These experimental studies demonstrated that enzyme-amended silage fermentation is a complex process and results in a high degree of variability of ensiled corn stover quality. Silage quality was shown to depend on many factors, including the harvest date, initial chemical composition of the incoming corn stover, moisture content, microbial populations, silage temperature, enzyme loading rate and other operational conditions (McDonald et al., 1991; Charmley, 2001; Ren, 2006; Shinner et al., 2007). A comprehensive experimental study of the effects all these interacting factors on silage quality would require a large number of trials and intensive analysis. To greatly reduce uncertainty about conditions required to achieve quality targets for ensiled corn stover, while simultaneously reducing the intensity of the associated experiments, a predictive simulation model is needed. Such a model can also lead to a better understanding of biochemical and microbial processes occurring during corn stover ensilage, and serve as an efficient first step in optimizing the ensilage conditions for downstream bioconversion to ethanol and other value-added products.

There is a small body of literature dealing with modeling silage processes, initially developed for the livestock feeding industry. For example, Neal and Thornley (1983) developed a qualitative model of the anaerobic phase of ensilage; Ren (2006) implemented a predictive model of enzyme-amended ensilage based on a model by Pitt and coworkers (Pitt et al. 1985; Leibensperger and Pitt 1987); and Rezaei (2008) expanded this to provide a quantitative model of organic acid production from switchgrass ensilage. However these models suffered a lot of constraints and application limitations. First of all, pH, one of the most important parameters during the ensilage process, was not adequately treated or reported. Neal and Thornley's (1983) model of the anaerobic phase of ensilage estimated too low a pH, which did not often occur in practical ensilage processes. In Rezaei's (2008) work, pH was predetermined by use of experimental data and implemented as time-dependent segmental linear functions in aerobic, lag and fermentation three phases. And there was no report of the change of pH during enzyme-amended ensilage in Ren's (2006) modeling simulations. An inaccurate estimated or presumed pH could lead to erroneous or inappropriate estimates of the concentrations of water soluble carbohydrates, all of which might reduce the credibility of these models in real-world applications.

In addition, little research has been conducted to study the influence of enzyme additives on the major biochemical and microbial changes during the corn stover ensilage process. Ensilage processes considered by Neal and Thornley (1983) and Rezaei (2008) did not take account of the enzyme effects at all, because their models were used to simulate the anaerobic phase of silage and organic acid production from switchgrass

ensilage, respectively, without any enzyme amendments. Ren's model did indeed simulate enzyme-amended silage of corn stover by combining an ensilage model (Pitt et al. 1985; Leibensperger and Pitt 1987) with a model of enzyme hydrolysis kinetics (Gan et al., 2003). In this previous work the hydrolysis kinetic rates for hydrolysable cellulose microfibrils, inert cellulose microfibrils, and different categories of sugar (released, inhibited and consumed) as well as pH were taken as constants during the ensilage processes (Gan et al., 2003; Ren, 2006). However, the experimental data previously reported in this thesis demonstrates that the cell wall structure of corn stover changes during ensilage. Since the cellulose hydrolysis rates for enzymatic kinetics models are dynamic functions of pH, substrate and end-product concentrations, water activity (a_w), temperature and other parameters (Pitt, 1990; Philippidis et al., 1992, 1993), changes in substrate characteristics would change that dynamic. Cellulose hydrolysis rates are known to be dependent on the structure of cell wall of corn stover, and enzyme accessibility to the crystalline region of the cellulose, where hemicellulose and lignin are highly intertwined (Lee and Fan, 1982; Pitt, 1990; Bayer et al., 1998; Esteghlalian et al. 2000).

Since previous models of the silage process have mostly been extensions of the framework developed by Pitt and his colleagues in the late of 1980s, a review of their approach provides an important foundation for further work. Their first predictive quantitative model was developed to describe the basic biochemical and microbial processes during ensilage (Pitt et al., 1985). Various dynamic mathematical relationships were established with respect to three different and interactive phases: aerobic, lag, and

fermentation during the ensilage process. Using parameters determined from published silage experiments and pure culture bacterial research, the authors set up a framework to construct a quantitative empirical model of the typical lactate-dominated ensilage process. The initial model was extended by Leibensperger and Pitt (1987) to include growth of clostridia in wet forages and higher temperature ranges. Prior experimental results were mapped to validate the models and showed that the ensilage process could be classified as clostridial, lactate or intermediate according to final concentrations of butyric acid and lactic acid. Three years later, Pitt expanded these silage fermentation models by incorporating a dynamic enzymatic hydrolysis model with cellulase and amylase. In this more biochemically explicit silage model, the most important factors affecting enzymatic hydrolysis of biomass were examined, including temperature, pH, a_w , time, concentration of substrates and end products. The cellulase reactions were modeled in two steps, simulating the multiple enzymes required. The models developed by Pitt and his colleagues paved solid ground in simulating the enzymatic components of the ensilage process for plant-based biomass. Parts of their model were later used by Ren (2006) and Rezaei (2008). However, additional information, such as the rates of use of cellobiose and glucose by lactic acid bacteria (LAB), and the appropriate formulation of the WSC released by cellulase hydrolysis, will also need to be included in order to make the model applicable.

The intent of the present study is to modify the enzymatic ensilage model by Pitt and his colleagues and then use to simulate the dynamic behavior of pH, water soluble carbohydrate (WSC), cellulose content and the effects of enzyme additives on the major

biochemical and microbial changes during the ensilage process. A commercial engineering package, Stella (Version 8.0, High Performance System Inc., Lebanon, NH), is used to as a simulation tool to numerically solve all the differential equations involved in the ensilage model, including submodels for hydrolysis and other processes. The simulation results will be compared to the experimental measurements to examine the feasibility and correctness of the enzymatic ensilage model. The model will then be used to explore optimal strategies to obtain high quality silage from corn stover by adjusting cellulase loading rate and operation temperature.

5.3 Modeling the enzymatic ensilage process

Previous experiments in Chapter 3 and Chapter 4 demonstrated that the addition of cellulase enzymes at low concentrations can enhance the cellulose degradation. In natural fungal degradation systems there are three functional enzyme types needed to hydrolyze cellulose to glucose: endoglucanases to break the long chains and crystalline structure of cellulose; exoglucanase to hydrolyze from the ends of the resulting shorter glucose chains the two-glucose units called cellobiose; and β -glucosidase to decouple that cellobiose into individual glucose molecules. A simplified two-stage mechanism lumping endo- and exo-glucanase as a single cellulose hydrolyses reaction, was modeled by Philippidis et al. (1992) as:

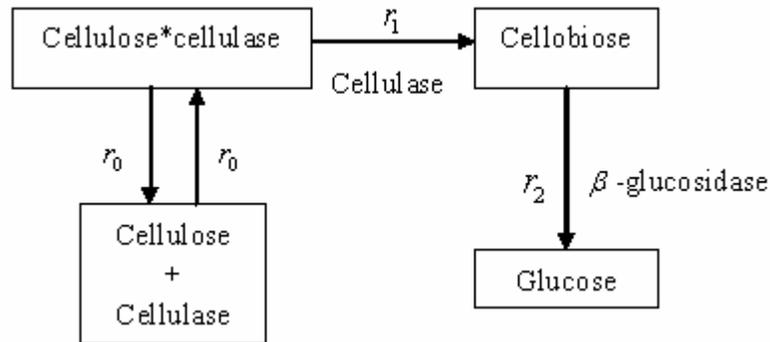


Figure 5-1: Schematic mechanism of cellulase hydrolysis of cellulose in absence of ensilage process (adapted from Philippidis et al., 1992).

This simplified model shows the cellulose is hydrolyzed to cellobiose by cellulase at the hydrolysis rate r_1 . Cellobiose is furthermore hydrolyzed to glucose by β -glucosidase at the rate r_2 (Philippidis et al., 1992). To take account of the consumption of cellobiose and glucose by the lactic acid bacteria during the ensilage process (Pitt, 1990), the rate of change of cellulose ($C_{C,e}$), cellullobiose concentration ($C_{CB,e}$) and glucose concentration ($C_{G,e}$) could be respectively derived to be

$$\frac{dC_{C,e}}{dt} = -r_1 \quad (5-1)$$

$$\frac{dC_{CB,e}}{dt} = r_1 - r_2 - \frac{dC_{CB,g}}{dt} \quad (5-2)$$

$$\frac{dC_{G,e}}{dt} = r_2 - \frac{dC_{G,g}}{dt} \quad (5-3)$$

Here $\frac{dC_{CB,g}}{dt}$ and $\frac{dC_{G,g}}{dt}$ are respectively the rates of use of cellobiose and glucose

by lactic acid bacteria in silage. Hydrolysis rates r_1 and r_2 are determined by enzyme

activities (μ_{C1}, μ_{C2}) and their concentrations (C_{EC1}, C_{EC2}) at each step, where enzyme activities are a time dependent function of pH, temperature T , water activity a_w , and substrate and end-product concentrations. Here the mathematic formulations of enzyme activities are taken the same forms given by Pitt (1990) but with different parameters, which are calculated from our experiments as follows,

$$r_1 = \mu_{C1} C_{EC1}, r_2 = \mu_{C2} C_{EC2} \quad (5-4)$$

We use the enzyme activities given in Pitt (1990), but modify the relative rate of cellulase activity as dependent on substrate and end-product concentrations by considering our specific plant-based biomass: corn stover. These and associated initial parameter values of are listed in Table 5-1.

In addition to the kinetic model of enzymatic hydrolysis of cellulose described above, other modules are needed to model major microbial and biochemical processes during ensilage. The model constructed here mainly focuses on the time course of pH, change in water soluble carbohydrate and cellulose, the growth and death of lactic acid bacteria, and their production of lactic and acetic acids. Other processes, such as aerobic respiration, hydrolysis of hemicellulose, proteolysis, and production of clostridia are implicitly considered by adjusting parameters in the model.

The change in the concentration of water soluble carbohydrate (C_{WSC}) in an ensilage process without added enzymes was included in the original model of Pitt et al. (1985). By combining enzyme effects on cellulose hydrolysis (Equations 5-1-3), the rate

of change of water soluble carbohydrate in enzymatic (cellulase amended) ensilage process can be formulated as Equation 5-5 (Pitt, 1990):

$$\frac{dC_{WSC}}{dt} = -\frac{dC_{WSC,g}}{dt} + \frac{dC_{WSC,H}}{dt} + \frac{dC_{WSC,a}}{dt} + \frac{dC_{WSC,b}}{dt} + \frac{dC_{WSC,c}}{dt} \quad (5-5)$$

where $\frac{dC_{WSC,g}}{dt}$, $\frac{dC_{WSC,H}}{dt}$, $\frac{dC_{WSC,a}}{dt}$ and $\frac{dC_{WSC,b}}{dt}$, are respectively the rates of

WSC consumption associated with the growth of the lactic acid bacteria, and increases of WSC by hemicellulase hydrolysis of hemicellulose, chemical hydrolysis and bacterial death. These were described by Equations (46), (18), (16) and (47) respectively in Pitt's model (1985).

The final term in Equation 5-5, $\frac{dC_{WSC,c}}{dt}$ is the rate of release of WSC by cellulase hydrolysis of cellulose, and can be obtained from Equation 5-1 as,

$$\frac{dC_{WSC,c}}{dt} = -\frac{dC_{C,e}}{dt} = r_1 \quad (5-6)$$

The expression used in Equation 5-6 implies that reductions in cellulose reduction are reflected in the release of WSC due to cellulase hydrolysis.

There is a lack of quantitative information on the death rates for any lactic acid bacteria in silage, as pointed out by Pitt et al. (1985) and Ren (2006). In the absence of data, models use an empirical formulation based on the specific death rate μ_b to describe the rate of WSC created by bacterial death $\frac{dC_{WSC,b}}{dt}$. In the previous models μ_b was simply considered as a fraction of the maximum death rate, using a linear function

dependent on pH. However, this is an incomplete formulation because the specific death rate is definitely a time-dependent function, with the death rate changing during the ensilage process as substrate concentrations fluctuate. By using this simplified formulation of μ_b , the corn stover enzymatic ensilage model of Ren (2006) resulted in large discrepancies between estimated and experimental WSC concentrations. Given these discrepancies, a dynamic specific death rate μ_b is formulated for our model according to our experimental observations as,

$$\mu_b = \begin{cases} 0.33\mu_b^{[\max]} f_{b,pH} & t \leq 48.0 \text{ hour} \\ 0.55\mu_b^{[\max]} f_{b,pH} & 48.0 < t \leq 72.0 \text{ hour} \\ \mu_b^{[\max]} f_{b,pH} & \text{other} \end{cases} \quad (5-7)$$

, where $f_{b,pH}$, and $\mu_b^{[\max]}$ were given by Equations (41) and (44) respectively in Pitt (1985).

The ensilage literature also lacks any prior mathematic descriptions of $\frac{dC_{CB,g}}{dt}$ and $\frac{dC_{G,g}}{dt}$. Based on their common functions of facilitating bacterial growth, the equations used to describe $\frac{dC_{CB,g}}{dt}$ and $\frac{dC_{G,g}}{dt}$ were assumed to take the same form as $\frac{dC_{WSC,g}}{dt}$, with the factor $f_{g,WSC}$ (due to effect of WSC concentration, in Equation (35) Pitt et al. (1985)) replaced with the new factors $f_{g,CB}$ and $f_{g,G}$ of cellobiose concentration and glucose effect, respectively. Thus $\frac{dC_{CB,g}}{dt}$ and $\frac{dC_{G,g}}{dt}$ are formulated as follows,

$$\frac{dC_{CB,g}}{dt} = \frac{dC_{WSC,g}}{dt} \frac{f_{g,CB}}{f_{g,WSC}} \quad (5-8)$$

$$\frac{dC_{G,g}}{dt} = \frac{dC_{WSC,g}}{dt} \frac{f_{g,G}}{f_{g,WSC}} \quad (5-9)$$

$$\text{where } f_{g,CB} = \frac{C_{CB}}{K_g + C_{CB}}, \text{ and } f_{g,G} = \frac{C_G}{K_g + C_G}.$$

As for the rate of change of pH, which is dependent on the changing concentrations of lactic acid and acetic acid, its mathematic formulation was also expressed in the form of Equation (54) in Pitt's model. This rate of pH change can be derived as,

$$\frac{dpH}{dt} = - \frac{10^{pH} \left(\frac{1}{(10^{pK_A} + 10^{pH}) \omega_A} \frac{dC_A}{dt} + \frac{1}{(10^{pK_L} + 10^{pH}) \omega_L} \frac{dC_L}{dt} \right)}{\beta + \log 10 \frac{10^{(pK_A + pH)} C_A}{(10^{pK_A} + 10^{pH})^2 \omega_A} + \log 10 \frac{10^{(pK_L + pH)} C_L}{(10^{pK_L} + 10^{pH})^2 \omega_L}} \quad (5-10)$$

,with the rate of change of lactic and acetic acids given by Equations 48-50 and other parameters in Pitt's model (1985).

To sum up, a quantitative model for corn stover enzymatic ensilage has been constructed using the mathematical representations described by Equations 5-1~5-10 above. The model simulates ensilage as an integrated process with simultaneous consideration of the influences of enzyme additives on the ensilage process. By taking account of the dynamic changes in pH described by Equation 5-10 as well as enzyme interactions in the ensilage process, the model incorporates important processes not included in the models presented by Ren (2006) and Rezaei (2008).

5.4 Simulation and discussion

Once the entire mathematical formulation of the enzymatic ensilage process is derived and forms a closed system of equations, an appropriate numerical approach or commercial computational package is needed to solve these equations and simulate the process. Careful examination of the constructed model indicates the system is composed of a series of unsteady (time-dependent) nonlinear, first order differential equations. For models of this type, Euler's explicit method and the Runge-Kutta algorithm are good candidates for numerical implementation. These methods are also completely integrated in Stella[®] 8, an engineering application package developed by High Performance Systems, Inc. Stella[®] 8 provides an integrated interface, where codes, graphics and flow charts are organically integrated into a user friendly environment. It presents important structural relationships, feedback loops, and model elements in a storytelling manner that highlights selected building blocks in a timed sequence. Stella modeling is widely used in many fields, such as Pharmaceutical Sciences (Chen et al. 1999), Ecosystem Science (Canham et al. 2003) and Environmental Engineering and Science (Morrison et al. 2006). Recently, Rezaei (2008) used it in modeling organic acid production in ensilage, where the pH is predetermined by fitting experimental data and the effects of enzyme additions on the ensilage system were not included. In this chapter, Rezaei's Stella model is extended to model enzymatic ensilage of corn stover. The time-course of pH, WSC, cellulose, lactic and acetic acid, and the effects of enzymes on these important parameters are simulated and validated in comparison with our experimental measurements.

5.4.1 Conventions and modeling structures

Stella modeling is mainly made up by four elements, namely stocks, flows, converters and connectors (Figure 5-2).

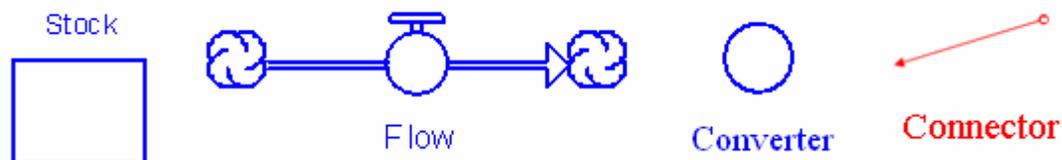


Figure 5-2: Primary elements used in Stella modeling.

The stocks are accumulators that reflect the amounts that they are gaining from and losing to other processes over time. The flows are used to describe activities or changes causing modification of the stocks. The converters modify the activities within the system and also act as transmitters. The connectors are used to as communicators to connect the stocks to the converters, the stocks to flows, flows to each other, converters to flows and converters to other converters. By presenting models in these graphic elements, Stella exhibits structural relationships, building blocks and simulated parameters in a highly integrated interface, which then is beneficial to implement code debugging and result analysis.

The model of enzymatic ensilage of corn stover mainly includes five interactive subordinate modules: the change of lactic acid concentration, dynamic pH, the change of water soluble carbohydrates, the change of enzymatic activities, and one auxiliary model. These five mutually coupled submodels can be found in Figures 5-3 to Figures 5-7.

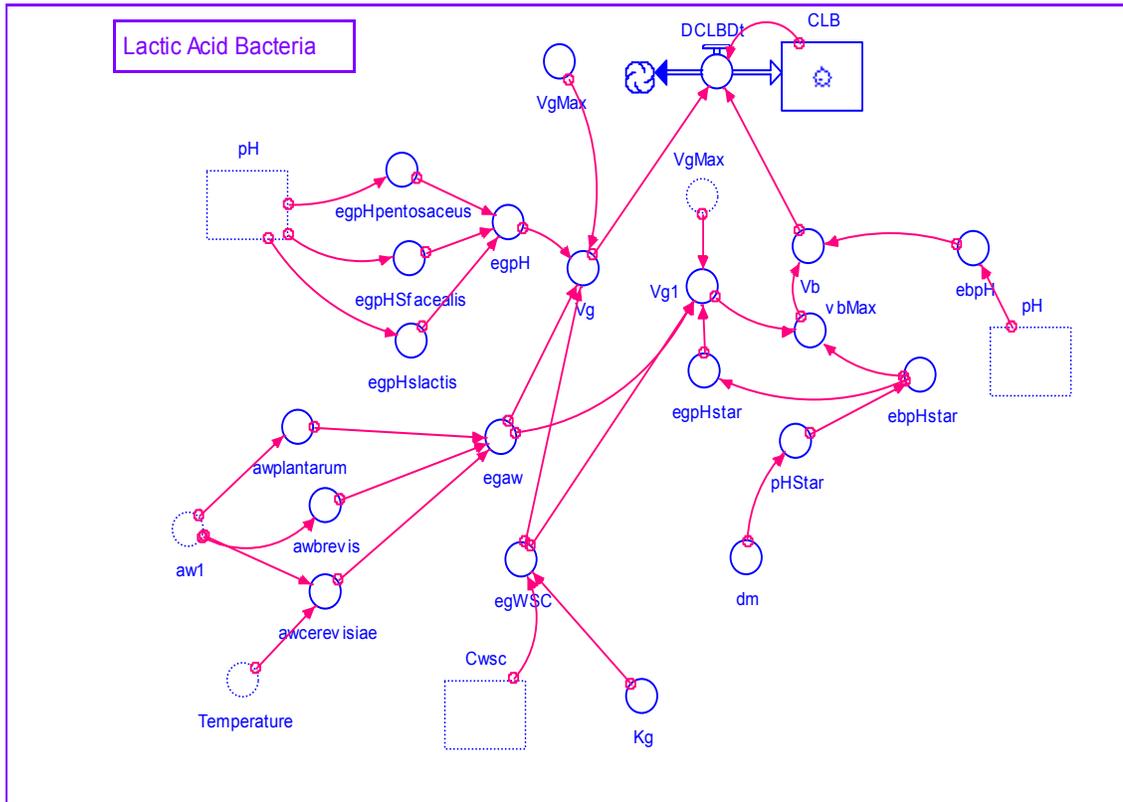


Figure 5-3: Submodel of concentration of lactic acid.

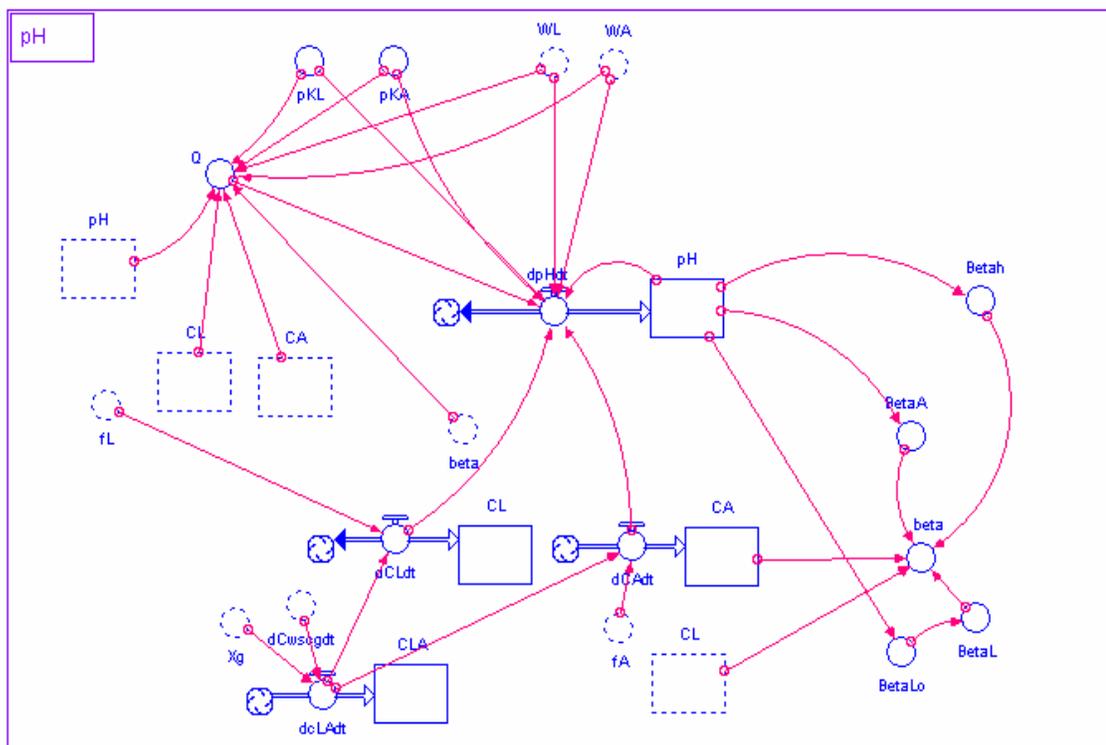


Figure 5-4: Submodel for pH dynamics.

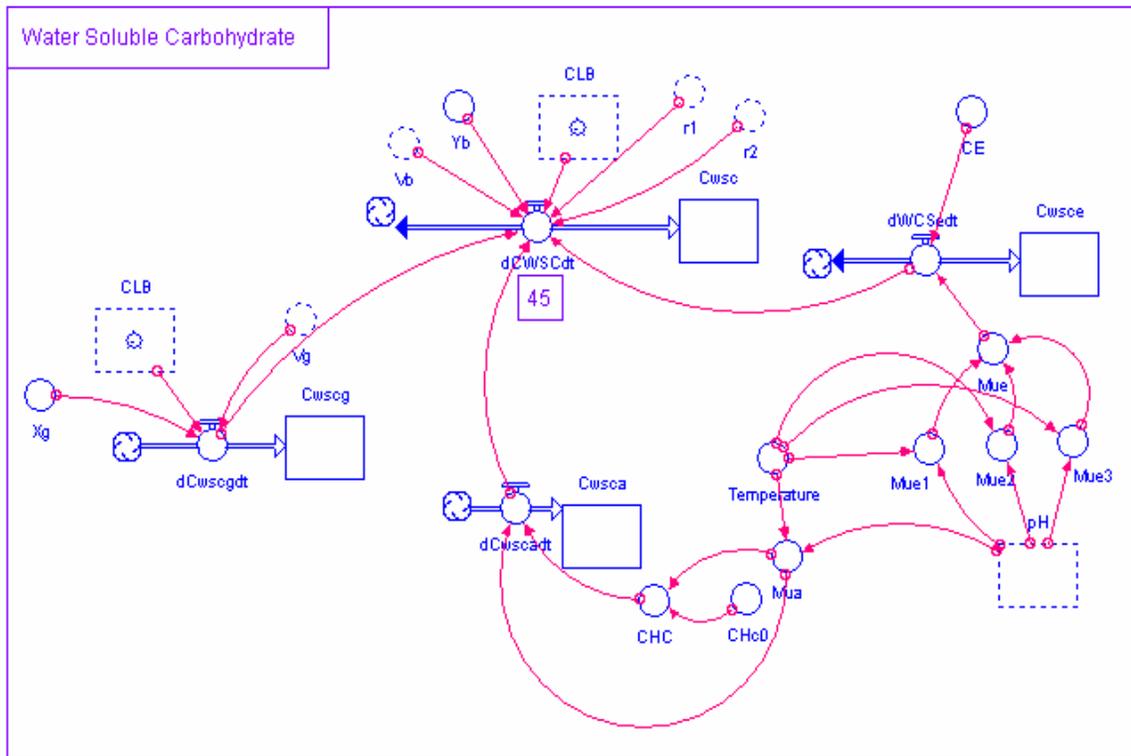


Figure 5-5: Water soluble carbohydrate submodel.

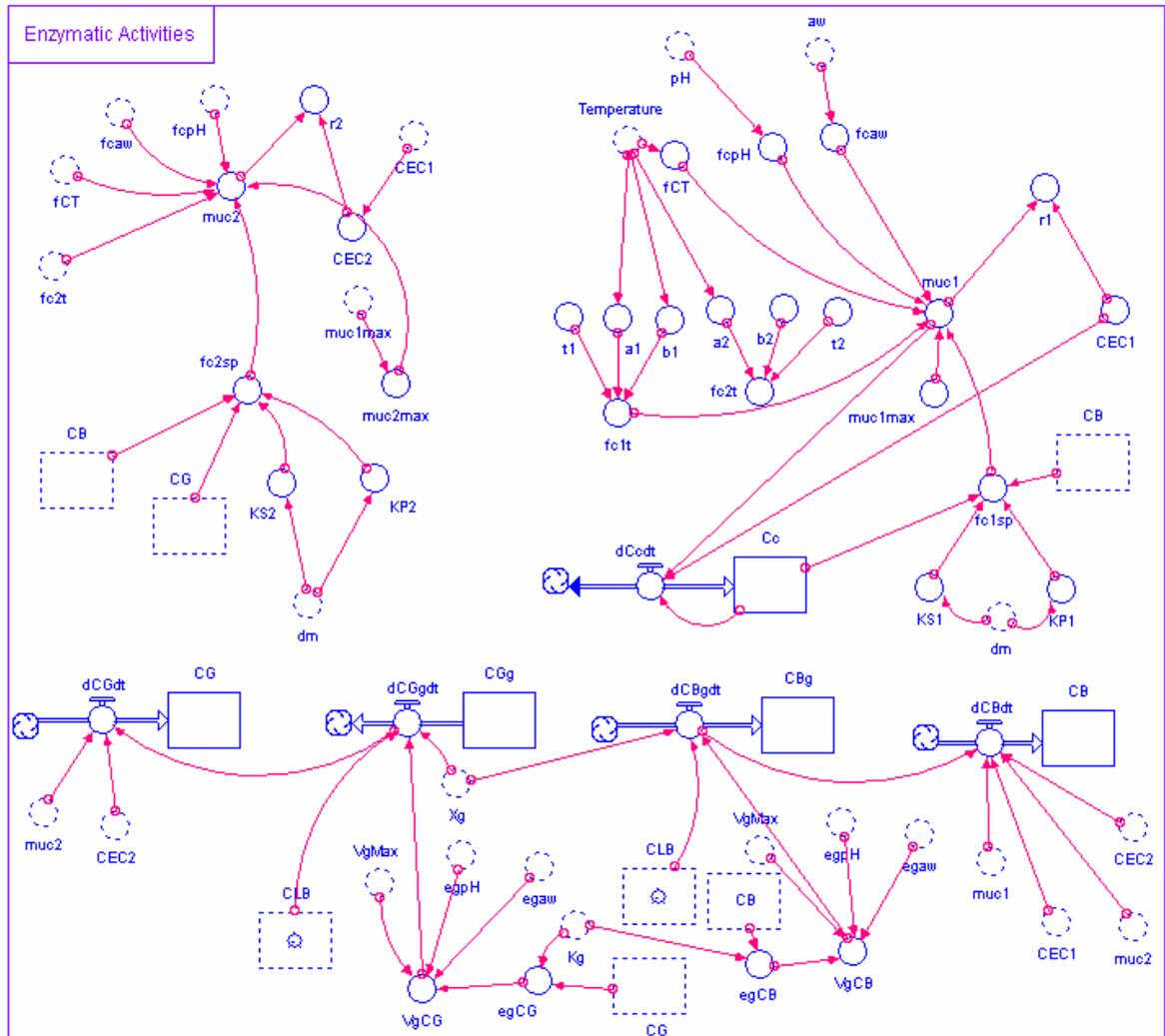


Figure 5-6: Enzymatic activity submodel.

Table 5-1: Input parameters for the enzyme-enhanced ensilage model.

Input Parameter	Value	Parameter Source
Initial cellulase concentration at step 1, C_{EC1}	7.0×10^{-3} g/g silage	From 2006 , 2007 experiments
Cellulase concentration at step 2 , C_{EC2}	$0.169 \times C_{EC1}$ g/g silage	(Pitt, 1990)
Temperature, T	310 °K	From 2006 , 2007 experiments
Initial LAB concentration, C_{LAB}	8×10^{-5} g/g silage	(Ren, 2006)
Cellulase hydrolysis rate, r_1	$r_1 = \mu_{c1} C_{EC1}$	(Pitt, 1990)
Cellulase hydrolysis rate, r_2	$r_2 = \mu_{c2} C_{EC1}$	(Pitt, 1990)
Initial cellulase activity at step 1 , μ_{c1}^{\max}	0.02 (g product/g enzyme per hour)	From 2006, 2007 experiments
Initial cellulase activity at step 2 , μ_{c2}^{\max}	$\mu_{c1}^{\max} = \mu_{c2}^{\max}$	(Pitt, 1990)
Initial dry matter, $D.M.$	0.40	From 2006, 2007 experiments
Initial glucose, C_G	$0.52 \times C_{WSC}$	From 2007 experiments
Initial cellobiose, C_{CB}	$0.05 \times C_{WSC}$	Assumption*

Note*: Sensitivity analysis indicates that initial cellobiose concentrations (ranging from 0.01 to $0.1 \times C_{WSC}$) have little effect on simulation results.

5.4.2 Results and Discussion

To test the model, simulations were run under the initial concentrations of WSC, cellulose, hemicellulose, glucose, cellubiose, and pH value from experiments as given in Table 5-2 and other shared initial conditions as indicated in Table 5-1. The estimated final (21 day) results were then compared with the corresponding experimental data (Table 5-3). Estimated 21 day results of representative parameters, including cellulose, hemicellulose, WSC, and pH are in good agreement with their experimental counterparts. Although simulated concentrations of lactic acid were not directly compared due to lack of corresponding experimental measurements, the accurate prediction of other important parameters and mass balance considerations give us some confidence in those results. In addition to comparing these final products, the submodels describing the change of pH and enzymatic hydrolysis submodels are investigated in the following sections.

Table 5-2: Initial conditions of experiments and model simulation.

Experiment No.	1	2	3	4	5
Harvest dates	9/29/05	10/11/05	10/31/05	11/08/05	11/23/05
Cwsc	0.0658	0.0558	0.0337	0.0403	0.0438
Cc	0.3972	0.4255	0.4272	0.4144	0.4262
CcH	0.2376	0.2381	0.2482	0.2325	0.2521
pH	7.3	7.2	7.0	6.8	6.3
CG	0.0342	0.0290	0.0175	0.0210	0.0228
CCB	0.0066	0.0056	0.0034	0.0040	0.0044

Note: Cwsc: the concentration of WSC, Cc: the concentration of cellulose, CcH: the concentration of hemicellulose, CG: the concentration of glucose, CCB: the concentration of cellubiose. The concentration unit is g/ g d.b. silage.

Throughout this chapter, experiments the trials labeled 1, 2, 3, 4 and 5 correspond to the five different harvest dates in 2005: Sept. 29, Oct. 11, Oct. 31, Nov. 08 and Nov. 23, respectively. The initial values of the parameters in Table 5-2 were acquired from stover harvested on these dates (Chapter 3).

Table 5-3: Observed and estimated results for WSC, cellulose, hemicellulose, lactic acid and pH of corn stover after 21 days of enzyme-enhanced ensilage.

	Experiment No.	1	2	3	4	5
Cwsc	Observed	0.0888	0.0599	0.0674	0.0790	0.0833
	Estimated	0.0867	0.0856	0.0860	0.0858	0.0864
Cc	Observed	0.363	0.400	0.391	0.379	0.377
	Estimated	0.367	0.395	0.397	0.384	0.396
CcH	Observed	0.217	0.229	0.220	0.220	0.225
	Estimated	0.230	0.230	0.240	0.230	0.250
pH	Observed	4.13	4.10	4.20	4.38	4.29
	Estimated	4.13	4.13	4.14	4.14	4.14
C _L	Estimated	0.14	0.14	0.14	0.14	0.13

Note: C_L: the concentration of lactic acid (g/g silage).

5.4.2.1 The validation of pH

The change in pH with respect to time during ensilage is perhaps the most critical parameter to model accurately, both because it reflects the changes the concentration of WSC, lactic acid and acetic acid as well as the growth of bacteria over time (Pitt et al., 1990).

Figure 5-8 is a plot of experimental and simulated pH during the enzymatic ensilage process, with four experimental pH values measured at days 0, 1, 7 and 21 in experiment No. 1, with the solid line of pH change estimated under the corresponding initial conditions of that experiment (Table 5-2). The results demonstrate that the model reflected the pH change of ensiled stover well, with a small standard error (0.03) between estimated and experimental results. The measured data from all five groups of experiments were also used to validate the pH change of the integrated enzymatic ensilage model, with the results shown in Figure 5-9. Linear regression analysis on estimated results and experimental data throughout the ensilage process yields an average ratio of estimated to observed slope $k = 0.97$, with a coefficient of determination $R^2 = 0.94$ and standard error (s.e.) $s.e. = 0.06$ between estimated and experimental results. All three of these important indices indicate that the model adequately represents the pH change during enzymatic ensilage process. The predicted pH values are a little smaller than experimental values for trials No. 4 and No.5 (Table 5-3). This discrepancy might result from the model overestimating the buffering capacity of the biomass.

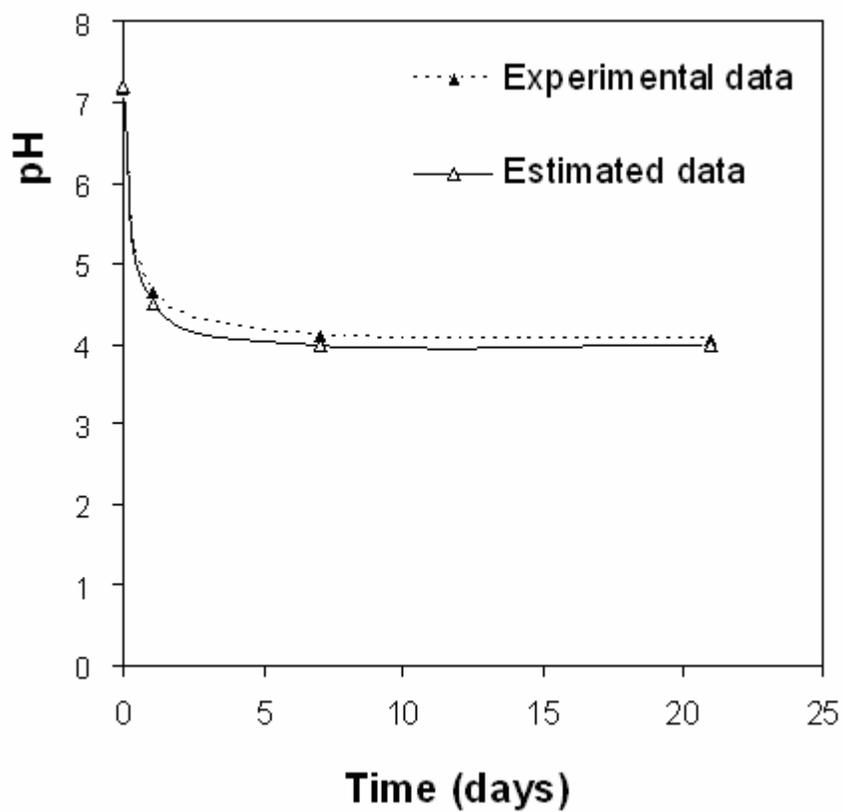


Figure 5-8: Experimental and estimated pH time course during ensilage process.

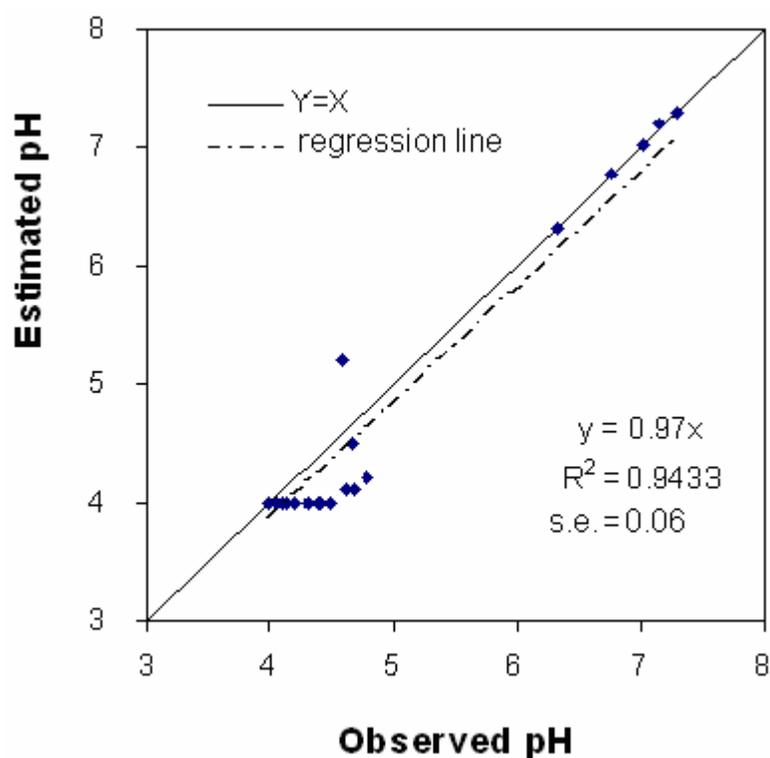


Figure 5-9: Estimated versus observed pH.

Evolution of pH during the ensilage process (Figure 5-8) demonstrated a rapid change of pH during the initial stage, which was caused by the production of lactic acid through anaerobic fermentation. Specifically, after oxygen was consumed by aerobic microorganisms, including fungi, yeasts, etc., lactic acid bacteria dominated the fermentation and converted WSC into lactic acid and other organic acids, which resulted in pH of ensiled stover quickly dropping from around pH 7 to around pH 4.5 during the first day of ensilage. This rapid pH reduction in the first few days is important to the subsequent silage process because the high hydrogen ion concentration, along with the undissociated acids themselves, inhibit undesirable microorganisms such as enterobacteria, clostridia and yeasts (McDonald, et al., 1991). The pH continued to decreasing to around pH 4 during the 21 days of ensilage, which was sufficient to limit

the growth of lactic acid bacteria and thus led to a long-term stable storage process. These long periods of low pH anaerobic conditions can also contribute to destroying pathogens that might be in the biomass (Weinberg and Ashbell, 2003).

5.4.2.2 Validation of the hydrolysis model

The enzymatic ensilage model was also validated by comparing experimental and estimated cellulose concentrations from 0 days to 21 of the ensilage process, for all five trials indicated in Table 5-2 (Figure 5-10). Linear regression analysis (with $R^2 = 0.81$, $s.e. = 0.19$, and the slope $k = 1.01$) indicated that the model was reasonable. The model overestimated the cellulose concentration by about 1%, probably because the effects of organic acids and plant enzymes on cellulose degradation were not considered. Generally, estimated cellulose reductions were reasonably accurate for the ensiled stover.

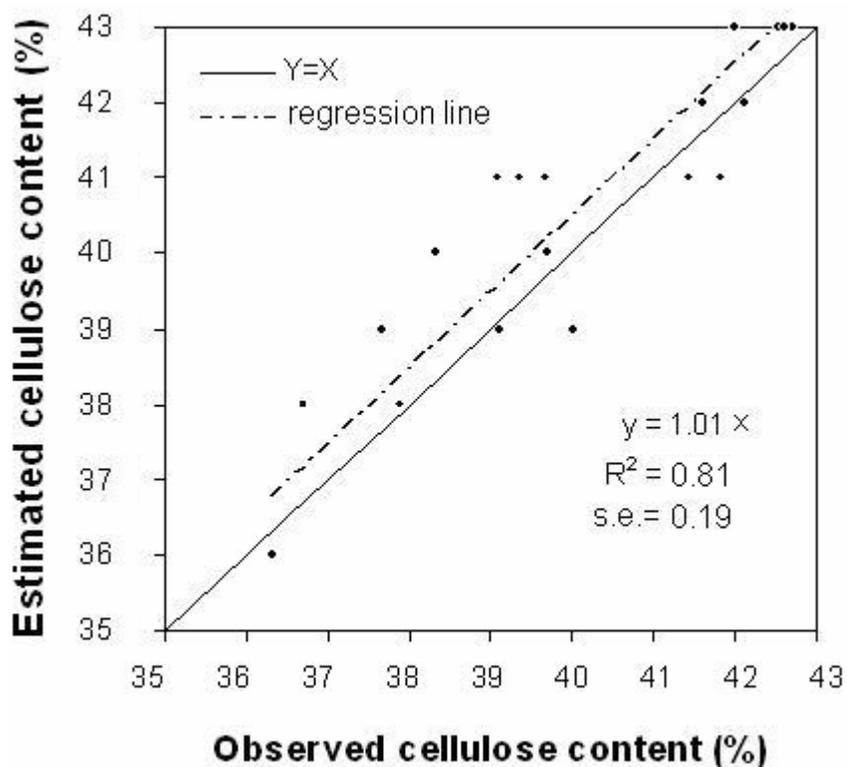


Figure 5-10: Estimated versus observed cellulose content.

In addition, experiments of the ensilage process without enzyme additives showed there was no significant cellulose reduction during 21 days ensilage as shown in Table 5-4. This was consistent with the model of ensilage with no enzyme. As for enzymatic silage model, there was around 4% cellulose reduction with cellulase addition as listed in Table 5-2 and Table 5-3. This cellulase-enhanced cellulose degradation partially contributed to the increase of WSC. Meanwhile, simulations clearly demonstrated that enzymatic ensilage contributed to increasing WSC, whereas WSC was decreasing during the ensilage process (without enzyme additives) as shown in Table 5-4. In addition, estimated C_{wsc} values were similar to each other but observed C_{wsc} were different for enzymatic ensilage as shown in Table 5-4. These similarities among estimated C_{wsc}

values are caused by similar totals of the input data for C_c and C_{cH} in the different runs. The discrepancy between observed and estimated C_{wsc} might result from other factors, such as lignin content and the death rate of lactic acid bacteria. For example, lignin content was not considered in the model. Therefore the influence of lignin and other factors should be included to improve the model in the future work.

Table 5-4: Estimated results of the concentration of WSC, hemicellulose, and pH of corn stover after 21 days of ensilage

No.	1	2	3	4	5
Cc	0.3972	0.4255	0.4272	0.4144	0.4262
CcH	0.2376	0.2381	0.2482	0.2325	0.2521
Cc+CcH	0.6348	0.6636	0.6754	0.6469	0.6783
Estimated	0.0867	0.0856	0.086	0.0858	0.0864

Note: Cc: the concentration of cellulose, CcH: the concentration of hemicellulose. The concentration unit is g/ g d.b. silage.

5.4.3 Sensitivity analysis on the model of specific death rate μ_b

In this ensilage model, we proposed a modified model of specific death rate μ_b , described as Equation (5-7). To evaluate its robustness, we performed numerical perturbation analysis on the ensilage model by considering the parameterized $\mu_b^{(\varepsilon_1, \varepsilon_2)}$,

$$\mu_b^{(\varepsilon_1, \varepsilon_2)} = \begin{cases} (0.33 + \varepsilon_1) \mu_b^{[\max]} f_{b,pH} & t \leq 48.0 \text{ hour} \\ (0.55 + \varepsilon_2) \mu_b^{[\max]} f_{b,pH} & 48.0 < t \leq 72.0 \text{ hour} \\ \mu_b^{[\max]} f_{b,pH} & \text{other} \end{cases} \quad (5-11)$$

where two perturbed parameters ε_1 and ε_2 are within the range of $-0.33 \leq \varepsilon_1 \leq 0.67$ and $-0.55 \leq \varepsilon_2 \leq 0.45$, respectively. The boundary values of these two intervals correspond to two extreme cases. The left boundary corresponds to a negligible specific death rate, while the right boundary corresponding to maximum specific bacteria death rate.

Taking pH as a target, and using Harvest Data 9/29/05 as a typical example, we evaluate the impacts of these two parameters. We ran models 100 times for a 10 x 10 combination of ε_1 and ε_2 as shown in Table 5-5.

Table 5-5: Estimated pH of corn stover after 21 days of ensilage at 100 computational ($\varepsilon_1 \times \varepsilon_2$) grids.

$\varepsilon_1 \backslash \varepsilon_2$	-0.55	-0.44	-0.33	-0.22	-0.11	0	0.15	0.25	0.35	0.45
-0.33	3.82	3.83	3.83	3.84	3.84	3.85	3.86	3.86	3.86	3.87
-0.22	3.87	3.88	3.89	3.90	3.91	3.92	3.93	3.93	3.94	3.94
-0.11	3.94	3.96	3.98	3.99	4.00	4.01	4.02	4.03	4.03	4.03
0	4.03	4.06	4.09	4.11	4.12	4.13	4.14	4.15	4.15	4.16
0.11	4.15	4.19	4.22	4.25	4.26	4.27	4.28	4.28	4.29	4.29
0.22	4.27	4.33	4.37	4.39	4.41	4.42	4.43	4.43	4.44	4.44
0.33	4.38	4.44	4.49	4.52	4.55	4.57	4.58	4.58	4.58	4.58
0.44	4.48	4.63	4.7	4.72	4.72	4.72	4.72	4.72	4.72	4.72
0.55	4.69	4.81	4.84	4.84	4.84	4.84	4.84	4.84	4.84	4.84
0.67	4.92	4.94	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95

Note: When ε_1 and ε_2 belong to the highlighted area, the error of estimated pH to observed pH is not larger than 4%.

The table shows the model is more sensitive to ε_1 (for a fixed value of ε_2 , pH varies largely due to the variations of ε_1), and much less sensitive to variations in ε_2 . This sensitivity is consistent with our experimental observations: pH dramatically changes during the first one or two days ensilage, and quickly reaches a constant plateau, which implies the model is more sensitive to ε_1 and less sensitive to ε_2 . Sensitivity analysis of the individual factors ε_1 and ε_2 is further demonstrated in Figure 5-11. This shows that at $\varepsilon_2 = 0$, pH changed from 3.85 to 4.95 when ε_1 increased from -0.33 to 0.67; and for $\varepsilon_1 = 0$, pH changed from 4.03 to 4.16 while ε_2 varied from -0.55 to 0.45.

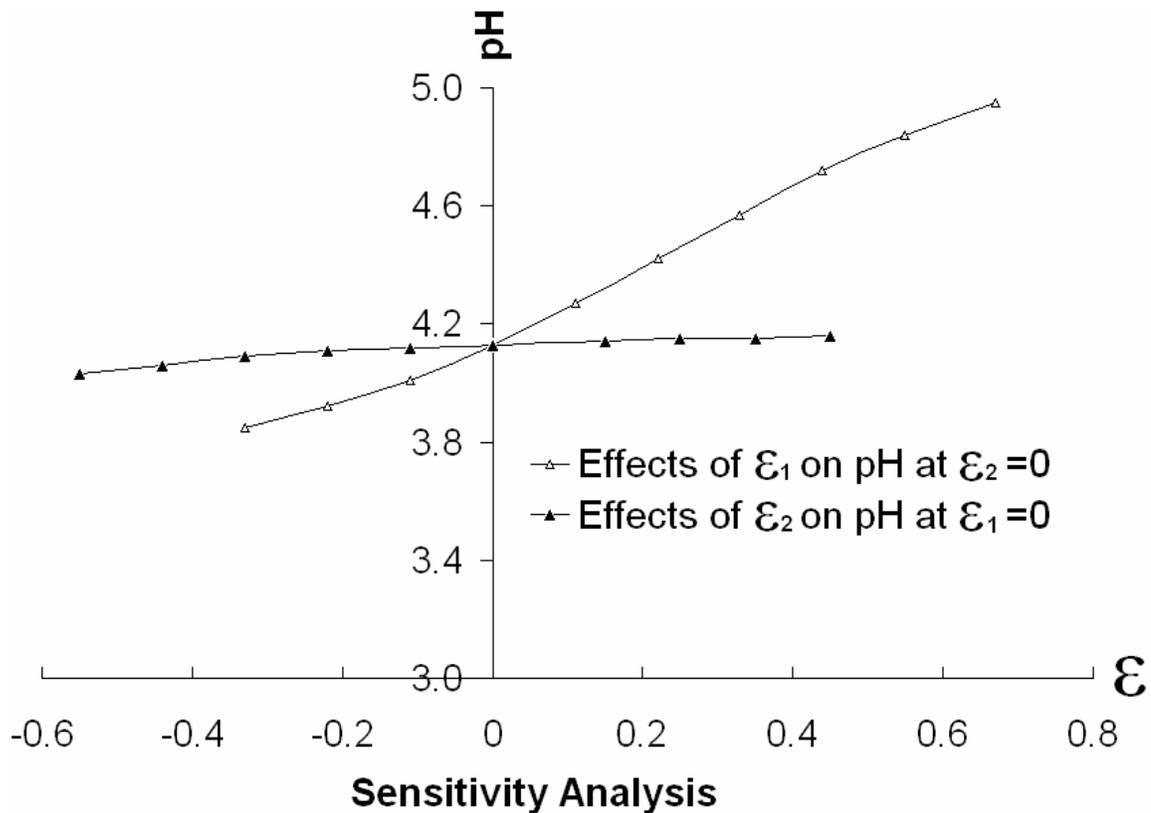


Figure 5-11: Estimated pH of corn stover after 21 days of ensilage at different ε_1 or ε_2 values.

Using the experimental value $pH=4.13$ as an indicator, the model suggests reasonable ranges of ε_1 and ε_2 would be $[-0.11, 0.11]$ and $[-0.33, 0.35]$ (highlighted by yellow colors in Table 5-5), respectively. When ε_1 and ε_2 are within this region, the error of estimated pH to observed pH is not bigger than 4%, as calculated by

$$\left(\frac{pH_{estimated} - pH_{observed}}{pH_{observed}} \times 100\% \right).$$

According to this sensitivity analysis and our simulation results, the proposed model of specific death rate μ_b is sufficient to reproduce the main ensilage processes. To make the model more robust, there needs more information of the specific death rate μ_b on the first ensilage day, on which dramatically varied pH happened.

5.4.4 Model prediction beyond experiments

Having successfully completed the validation exercises, the model can be used to examine the influence of the key factors on the enzymatic silage process. The model should help reveal where important knowledge is lacking, evaluate existing management facilities, better understand basic mechanisms and enhance process design, thereby improving the impact of ensiled storage on the downstream processing into ethanol or other fuels and chemicals. In this subsection, the model is used to investigate the effects of different cellulase loading rates and ensilage temperatures on the characteristics of ensiled corn stover in terms of the two key responses, the concentration of WSC and

cellulose. All the simulations here were run under the initial conditions listed in Table 5-1 and trial No. 1, whose stover was harvested on Sept. 29, 2005 in Table 5-2.

5.4.4.1 The effect of cellulase loading rate

As shown in Figure 5-12 and Figure 5-13, the cellulose concentration decreased and the WSC concentration increased as the enzyme loading rate increased from 1.4×10^{-3} to 7.0×10^{-2} g/g silage. This predicted result demonstrates the conversion of cellulose in corn stover to WSC by cellulase during the ensilage process. The model also indicates that the cellulose degradation rate would not change significantly when enzyme loading increased above 1.4×10^{-2} g/g silage. It can be seen from the fitted $y = 0.3951x^{0.2902}$ that at $x = 1.4 \times 10^{-2}$ the rate of decrease is five times more than that at $x = 1.4 \times 10^{-3}$. The lower enzyme loading used in the experimental trials, 7.0×10^{-3} g/g silage, might be a better choice, because this lower enzyme loading was enough to hydrolyze sufficient cellulose into WSC to initiate lactic acid fermentation and maintain a long-term stable storage. With the price of cellulase continuously going down, it may make economic sense to increase enzyme loading to maximize the effects of biological pretreatment during ensilage.

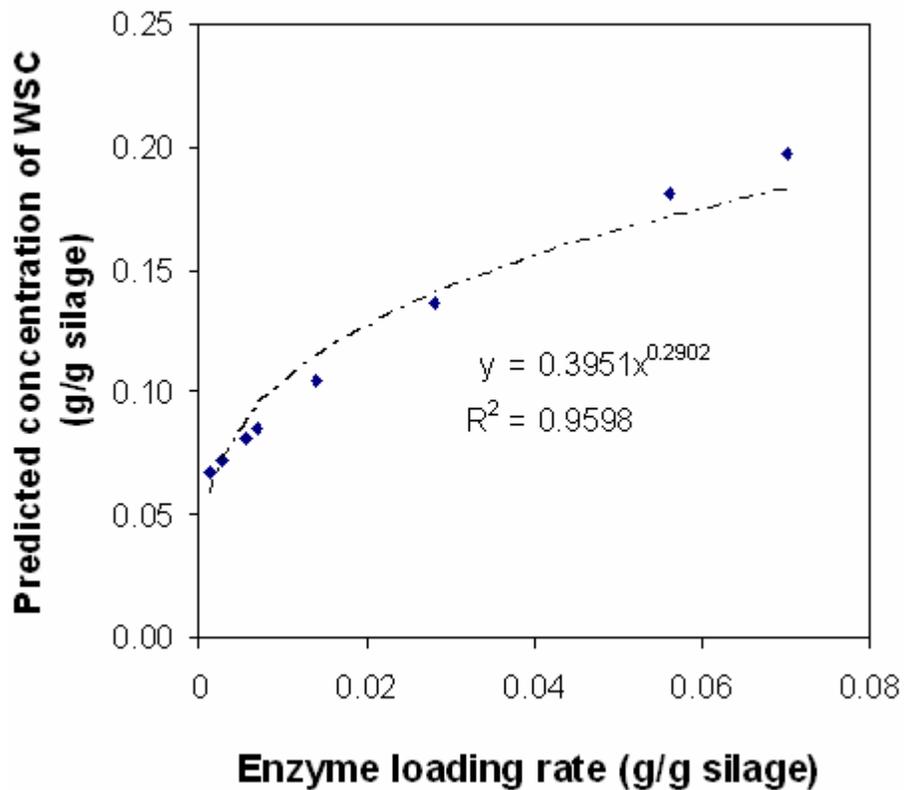


Figure 5-12: Predicted concentration of WSC with respect to cellulase loading rate (The discrete solutions were denoted by model simulation, and the analytic regression is fitted from the discrete solutions).

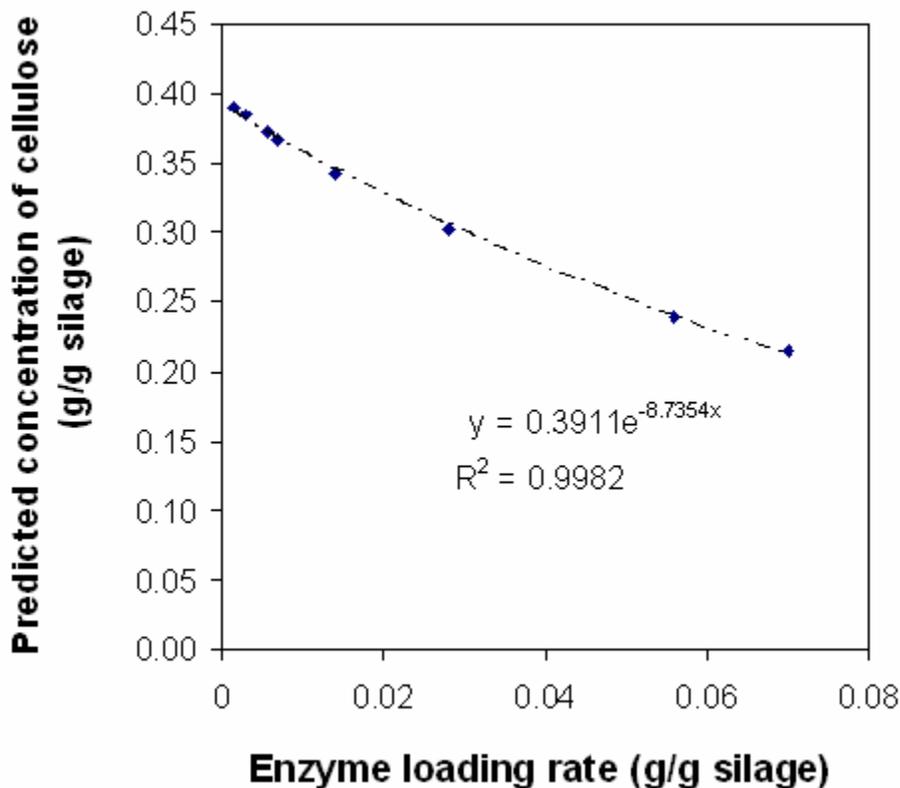


Figure 5-13: Predicted concentration of cellulose with respect to cellulase loading rate. The discrete solutions were denoted by model simulation, and the regression analysis is fitted from the discrete solutions.

5.4.4.2 Effect of temperature

The optimum temperature range for the various lactic acid bacteria is different, but most species grow from 15 to 50°C. The fermentation temperature not only influences the behavior of lactic acid bacteria but also has important effects on cellulase performance. Thus it is important to investigate the effects of temperature and to look for an optimum as indicated by cellulose conversion and WSC accumulation.

Model simulations were performed at the following temperatures: 15, 20, 25, 30, 37, 45, 50°C. The concentration of WSC increased as fermentation temperature changed from 15 to 37°C, where it reached the highest concentration, and then decreased when temperature continued to increase. Similarly, the concentration of cellulose decreased until the temperature reached 37°C and then started to increase under higher temperature conditions. This demonstrated that 37°C is the optimum temperature for enzymatic corn stover silage. Because the temperature of large biomass storages are likely to vary with both ambient temperatures and biological activity, this results suggests that control of silage temperature in corn stover silage will be critical to achieve high biomass quality for downstream use.

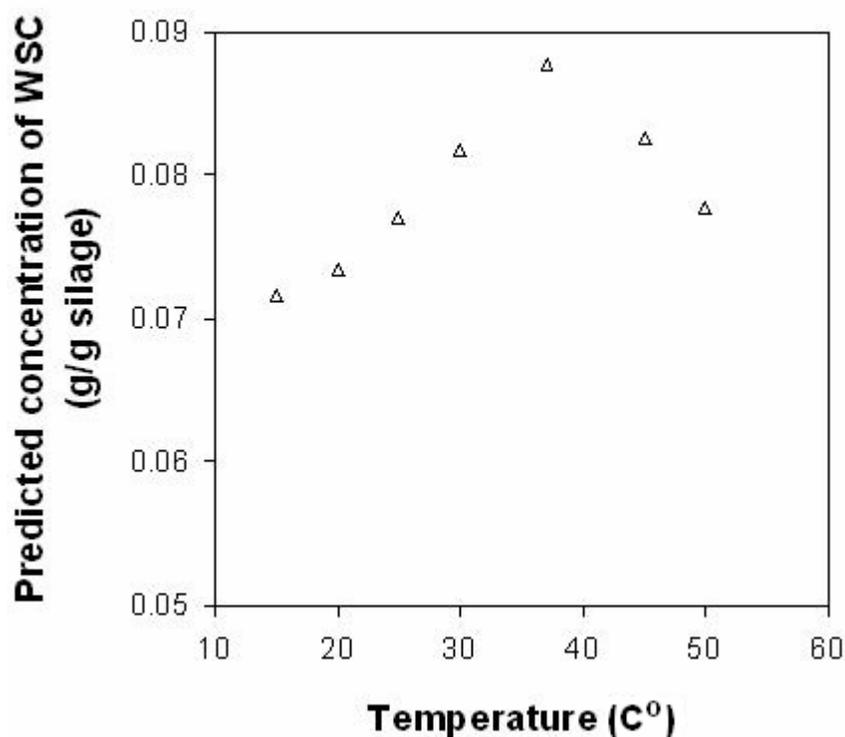


Figure 5-14: Predicted concentration of WSC with respect to temperature (The discrete

points are achieved by running the model under different temperatures.

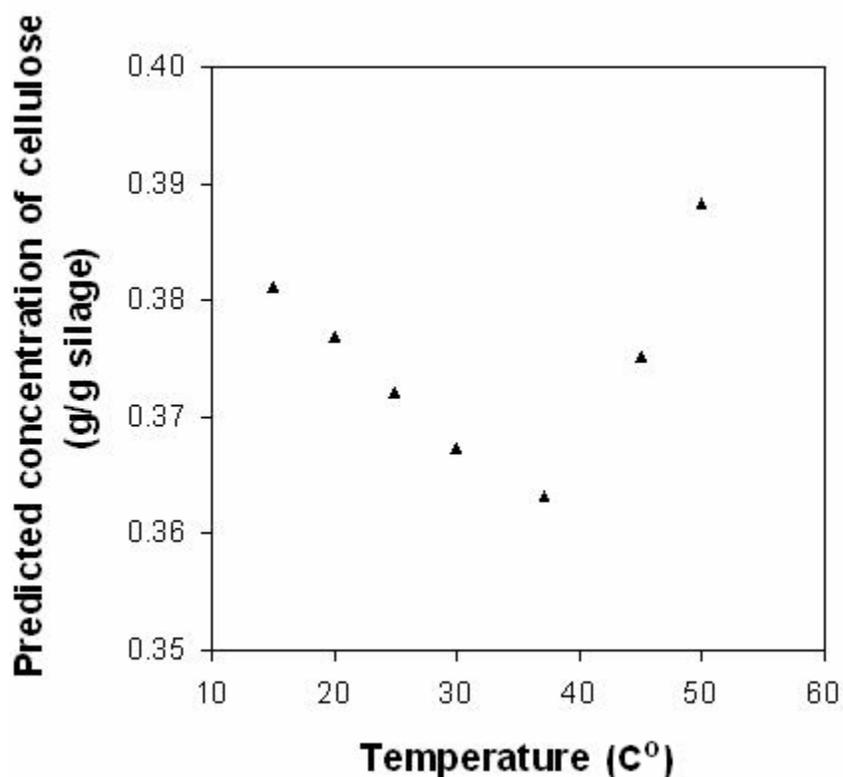


Figure 5-15: Predicted concentration of cellulose with respect to temperature. The discrete points are achieved by running the model under different temperatures.

5.5 Conclusion

The study modified and integrated the enzymatic ensilage model, which was originally developed by Pitt and coworkers (1985) and was recently extended by Ren (2006) and Rezaei (2008). The two main modifications were to construct an empirical expression for the death rate of lactic acid bacteria and to formulate the rates of conversion of cellulose and cellobiose to complete the enzymatic hydrolysis model. The

improved model was then used to simulate the enzymatic ensilage process. The model was first validated under different initial experimental conditions, comparing the estimated and observed dynamic responses of several important chemical components including total water soluble carbohydrates, concentrations of cellulose and hemicellulose, and pH. Comparison with experimental enzymatic studies demonstrated the accuracy and feasibility of the model. Simulations also demonstrated moderate cellulase loading rates provided substantial WSC for the fermentation process, which then stabilized the ensilage process, and that excess cellulase would have little benefit. By examining the effects of the temperature and enzymatic loading rate on the corn stover ensilage, it was found the optimal conditions for these are 37°C and 0.007~0.014(g/g silage), respectively. The correspondance of these optimal conditions with our experimental study conditions in Chapter 2 and Chapter 3 indicates that the experimental results should be very helpful in further optimization of the process. Thus this integrated model appears likely to lead to a better understanding of the enzymatic ensilage process, and to provide useful guidelines for the design of ensilage processes (with or without enzyme additives) for large amounts of plant-based biomass.

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Chapter 6

CONCLUSIONS AND RECOMMENDATIONS

The global challenges of energy sustainability and environmental protection currently spur scientists and engineers to develop alternative energy sources rather than continuing to rely on fossil fuels. Among potential energy alternatives, lignocellulosic biomass has been recognized as a potential source for biofuels, which can provide an effective solution to transportation energy needs. Among the many potential lignocellulosic biomass feedstocks, corn stover provides an important near term option. In order to produce sustainable biofuels from lignocellulosic biomass, preservation of large amounts of feedstock is crucial. The bioethanol industry requires continuous year-long supplies of a high quality feedstock, while many crops will be harvested during only a short season each year. Enzymatic (with cellulase and hemicellulase) ensilage technology has been demonstrated as an effective strategy of corn stover preservation storage. However, extending the benefits of enzymatic ensilage technology beyond preservation to actually improve downstream bioconversion efficiency may depend on the degree of lignin degradation, as lignin is a primary constraint to the hydrolysis of cellulose to fermentable sugars. Successfully degrading lignin in lignocellulosic biomass presents well-known challenges that in the past have proven difficult to solve, but the long times and unique environment of ensiled storage may provide a platform to achieve that goal. Ultimately, the final quality of enzymatic ensiled corn stover will depend on

both the initial chemical composition of incoming corn stover and optimizing additives and process conditions.

In this study, the impact of interactions between corn stover harvest season and cell wall degrading enzymes, were investigated in order to understand the effects on stover silage characteristics and facilitate downstream pretreatment and bioconversion processes. Several cell wall degrading enzymes were evaluated, including cellulase, hemicellulase, and laccase, the later including a mediator and oxygen in a laccase system, A predictive enzymatic ensilage model was developed and evaluated to better understanding the multiple, interactive chemical and biological subprocesses occurring during corn stover ensilage. This model could serve as a guide in designing effective biomass preservation methods.

The first study examined the effect of harvest date and enzyme addition on the characteristics of ensiled corn stover. The sugar conversion rate was also quantified by reduced severity dilute acid pretreatment. Early fall was identified as the best harvest time to ensile stover in terms of dynamic responses of pH, dry matter, water soluble carbohydrate, cumulative composition of stover, and monosaccharide as well as xylan conversion percentages. With respect to corn stover silage, the addition of enzymes significantly enhanced the positive effects. Further research to measure organic acids may help support the early harvest season preference and expand our understanding of unexpected data obtained from other chemical analyses.

This first study of harvest date and enzyme addition demonstrated no significant differences in xylan removal with enzymatic ensilage relative to fresh corn stover. This similar bioconversion efficiency could be due to recalcitrance associated with the lignin

in corn stover. In order to improve the enzymatic ensilage technology, a second study explored the impacts of the lignin-degrading enzyme laccase on lignin depolymerization and cellulose digestibility of ensiled corn stover. The molecular changes associated with lignin resulting from treatment with lignin-degrading enzyme were successfully monitored by TMAH-GC-MS. The laccase system increased sugar yield of ensiled stover during saccharification and potentially decreased the required pretreatment intensity. These findings suggest that the laccase system degraded the lignin fraction in enzymatic ensiled corn stover. If this lignin degradation has downstream benefits, then ensilage could serve as a platform for biological pretreatment. Considering the short duration time the stover was exposed to the laccase with mediator treatments (48 hours), it was surprising to observe such significant depolymerization in the lignin structure. Such deconstruction of ensiled stover was further verified by a downstream saccharification experiment, which showed that higher sugar conversion rate was associated with higher laccase loading rate. However, from a practical standpoint the required laccase loading rate was too high to be applied to the bioethanol industry, at least without dramatic reductions the laccase price. To enhance lignin depolymerization and bioconversion efficiency, the laccase system will need to be optimized, controlling the reaction conditions by adjusting oxygen partial pressure, mediators and additives. In addition, strategies which exploit potential synergies among laccase and other lignin-degrading enzymes, such as lignin peroxidase and manganese peroxidase, might achieve better results with a much lower loading rate.

These experimental investigations of the effect of harvest date and enzyme addition on the characteristics of ensiled stover not only demonstrated that the enzymatic

ensilage technology is capable of providing an attractive long-term storage method for large amounts of corn stover, but only showed that the final quality of enzymatically ensiled corn stover depends on its initial chemical compositions and microbial populations. To better understand how these factors govern silage quality and reduce potential experimental complexity, an enzymatic ensilage model was developed. The modeling framework was originally developed by Pitt and his colleagues for the dairy industry in the late of 1980s and the early of 1990s, was modified and used to simulate the enzymatic ensilage process. Dynamic responses of pH, water soluble carbohydrates and cellulose concentration, as well as several important chemical components of corn stover, were predicted reasonably well when compared with experimental observations. The validation study demonstrated that adding enzymes to the silage process can enhance the stability of long-term storage. Beyond the validation, the integrated model has been applied to study the effects of cellulase loading rate and temperature on the characteristics of ensiled corn stover. Results suggested the optimal experimental conditions for temperature and enzymatic loading rate were 37 °C and 0.007~0.014 (g/g silage) in order to obtain high quality silage from incoming corn stover. The model could serve to increase our understanding of the enzymatic ensilage process, and provide an attractive tool for design of silage storage systems for large amounts of plant-based biomass.

As it was pointed out in Chapter 3, some possible processes occurring during ensilage are not explicitly considered in the model. These include aerobic respiration, hydrolysis of hemicellulose, proteolysis, and production of clostridia. Therefore one obvious goal of future modeling research will be to develop the associated subordinate

models for these processes and incorporate them into the current framework to allow a more complete model of the ensilage process. Another potentially important gap results from the lack of quantitative information on the death rates for lactic acid bacteria in silage. The current model assumed an empirical formulation of the specific death rate to account for the water soluble carbohydrates released by bacterial death. Although the empirical formulation works fine here, a better understanding of the population dynamics and metabolic response of lactic acid bacterial is needed. A more mechanistic microbial population submodel would require more experiments to study the rates of death or growth of lactic acid bacterial as a function of pH, temperature and water activity as well as the concentration of water soluble carbohydrates. Last and most important given the results of this dissertation, it will be important to improve and extend the current model to consider the functional effects of lignin-degrading enzymes and mediators on ensiled corn stover. A successfully integrated model that incorporated the synergies of hemicellulase, cellulase and lignin-grading enzymes would provide insight and design advantages for both ensilage and post-ensilage downstream processes. Such a model could be a great help in improving and controlling the enzymatic ensilage technology. The deeper understanding of ensiled storage generated by this research and the follow-on research proposed will provide a great step in the pursuit of effective bioconversion strategies to transform agricultural feedstocks into energy and materials.

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Appendix A

STELLA MODEL CODE

$$aw(t) = aw(t - dt) + (dawdt) * dt$$

$$\text{INIT } aw = 1 - .03 * dm / (1 - dm)$$

INFLOWS:

$$dawdt = (-aw) * (1 - 2 * Xg) / (10 * (1 - dm)) * dCwscgdt$$

$$CA(t) = CA(t - dt) + (dCAdt) * dt$$

$$\text{INIT } CA = 0.015$$

INFLOWS:

$$dCAdt = fA * dcLAdt$$

$$CB(t) = CB(t - dt) + (dCBdt) * dt$$

$$\text{INIT } CB = 0.0044$$

INFLOWS:

$$dCBdt = muc1 * CEC1 - muc2 * CEC2 - dCBgdt$$

$$CBg(t) = CBg(t - dt) + (dCBgdt) * dt$$

$$\text{INIT } CBg = 0.000$$

INFLOWS:

$$dCBgdt = CLB * VgCB / Xg$$

$$Cc(t) = Cc(t - dt) + (dCc dt) * dt$$

$$\text{INIT } Cc = 0.4262$$

INFLOWS:

$$dCcdt = \text{if } Cc < 0.35 * 0.54$$

then 0

$$\text{else } -\text{muc1} * \text{CEC1}$$

$$CG(t) = CG(t - dt) + (dCGdt) * dt$$

$$\text{INIT } CG = 0.0228$$

INFLOWS:

$$dCGdt = \text{muc2} * \text{CEC2} - dCGgdt$$

$$CGg(t) = CGg(t - dt) + (-dCGgdt) * dt$$

$$\text{INIT } CGg = 0.000$$

OUTFLOWS:

$$dCGgdt = CLB * VgCG / Xg$$

$$CL(t) = CL(t - dt) + (dCLdt) * dt$$

$$\text{INIT } CL = 0.015$$

INFLOWS:

$$dCLdt = fL * dcLAdt$$

$$CLA(t) = CLA(t - dt) + (dcLAdt) * dt$$

$$\text{INIT } CLA = 0.03$$

INFLOWS:

$$dcLAdt = (1 - Xg) * dCwscgdt$$

$$CLB(t) = CLB(t - dt) + (DCLBDt) * dt$$

$$INIT CLB = 8 * 10^{(-5)}$$

INFLOWS:

$$DCLBDt = (Vg - Vb) * CLB$$

$$Cwsc(t) = Cwsc(t - dt) + (dCWSCdt) * dt$$

$$INIT Cwsc = 0.0438$$

INFLOWS:

$$dCWSCdt = -dCwscgdt + Yb * Vb * CLB + dCwscadt + dWCSedt + r1$$

$$Cwsca(t) = Cwsca(t - dt) + (dCwscadt) * dt$$

$$INIT Cwsca = 0.000$$

INFLOWS:

$$dCwscadt = Mua * CHC$$

$$Cwsce(t) = Cwsce(t - dt) + (dWCSedt) * dt$$

$$INIT Cwsce = 0$$

INFLOWS:

$$dWCSedt = Mue * CE$$

$$Cwscg(t) = Cwscg(t - dt) + (dCwscgdt) * dt$$

$$INIT Cwscg = 0.000$$

INFLOWS:

$$dCwscgdt = CLB * Vg / Xg$$

$$\text{pH}(t) = \text{pH}(t - dt) + (dpHdt) * dt$$

$$\text{INIT pH} = 6.3$$

INFLOWS:

$$\begin{aligned} dpHdt = & -(10^{\text{pH}}) * (\\ & 1 / (WL * (10^{\text{pH}} + 10^{\text{pKL}})) * dCLdt \\ & + 1 / (WA * (10^{\text{pH}} + 10^{\text{pKA}})) * dCA dt \\ &) / Q \end{aligned}$$

$$a1 = \text{EXP}(-41.33 + (14896 / \text{Temperature}))$$

$$a2 = \text{EXP}(-115.66 + (38210 / \text{Temperature}))$$

$$aw1 = aw$$

$$awbrevis = -18.33 + 19.59 * aw1 - 246.2 * (aw1 - .97)^2$$

$$awcerevisiae = -10.42 + 11.47 * aw1 - 0.1398 * (\text{Temperature} - 273) + 0.1394 * aw1 * (\text{Temperature} - 273)$$

$$awplantarum = -5.55 + 6.64 * aw1 - 268.7 * (aw1 - 0.98)^2$$

$$b1 = \text{if } \text{Temperature} < 316.77 \text{ then } 0 \text{ else } 21.17 - .0668 * \text{Temperature}$$

$$b2 = 0$$

$$\text{beta} = \text{BetaL} * \text{CL} + \text{CA} * \text{BetaA} + (1 - \text{CL} - \text{CA}) * \text{Betah}$$

$$\text{BetaA} = 2.303 * (10^{(\text{pH} - 4.76)}) / (1 + 10^{(\text{pH} - 4.76)})^2 / 60.05$$

$$\text{Betah} = (0.7715 * 10^{(\text{pH} - 4.5)}) / (1 + 10^{(\text{pH} - 4.5)})^2 / 1000$$

$$\text{BetaL} = \text{BetaLo} / 90.08$$

$$\text{BetaLo} = 2.303 * 10^{(\text{pH} - 3.86)} / ((1 + 10^{(\text{pH} - 3.86)})^2)$$

$$\text{CE} = 0.02$$

$$\text{CEC1} = 7 * 10^{(-3)}$$

$$\text{CEC2} = 0.169 * \text{CEC1}$$

$$\text{CHC} = \text{CHc0} * \text{EXP}(-\text{Mua} * \text{time})$$

```

CHc0 = 0.2521

dm = 0.4*EXP(-time/10000)

ebpH = if pH<5.04 then 1
else if (5.04<=pH) and (pH<=5.98)
then 12.98-6.44*pH+1.289*(pH)^2-0.0959*pH^3
else if (5.98<pH) and (pH<=7.6)
then 15.09-4.96*pH+0.409*(pH)^2
else 1

ebpHstar = if (5.98<pHStar) & ( pHStar<=7.6 )
then 15.09-4.96*pHStar+0.409*pHStar^2
else if (5.04<=pHStar) and (pHStar<=5.98 )
then 12.98-6.44*pHStar+1.289*pHStar^2-0.0959*pHStar^3
else if pHStar<5.04
then 1
else 1

egaw = MEAN(awbrevis,awcerevisiae,awplantarum)

egCB = CB/(Kg+CB)
egCG = CG/(Kg+CG)

egpH = .4*egpHpentosaceus+.4*egpHSfacealis+.2*egpHslactis

egpHpentosaceus = if ( pH>=3.7 ) and (pH<=6.30)
then -3.45+1.27*pH-0.0899*(pH)^2
else if ( pH>6.3 )
then -23.2+7.62*pH-0.6*(pH)^2
else 0

egpHSfacealis = if (pH>= 3.72) and (pH<=10)

```

```

then -3.48+1.27*pH-0.09*(pH)^2
else 0

egpHslactis = if (pH>=4.0) and (pH<=4.5)
then 0.160*(pH-4)+0.64*(pH-4)^2
else if (pH>4.5) and (pH<=8.2)
then -7.19+2.54*pH-0.196*(pH)^2
else 0

egpHstar = ebpHstar

egWSC = Cwsc/(Cwsc+Kg)

fA = 1-fL

fc1sp = 0.44*Cc/(0.44*Cc+KS1+(KS1/KP1)*CB)

fc1t = if 0<=time<t1 then EXP((b1-(1/a1))*t1)
else EXP(b1-(time/a1))

fc2sp = CB/(CB+KS2+(KS2/KP2)*CG)

fc2t = if 0<=time<t2 then EXP((b2-(1/a2))*t2)
else EXP(b2-(time/a2))

fcaw = if aw>=.9999
then 0.04
else if .9835<=aw and aw<0.9999
then -9.132+11.235*aw+.5149*LOG10(1-aw)
else if .9777<=aw and aw<0.9835
then 1
else -9.022+10.25*aw

fcpH = if 2.86<=pH and pH<6.74
then 1-0.2667*(pH-4.8)^2

```

```

else 0

fCT = if Temperature>=313.6

then 1

else EXP(-4448.14*((1/Temperature)-(1/313.16)))

fL = if (0<=Cwsc/dm) and (Cwsc/dm<0.01) and (pH<=5)

then 2.454-.391*pH+51*Cwsc

else if (0<Cwsc/dm) and (Cwsc/dm<=0.01) and pH>5

then 0.499+51*Cwsc

else if (Cwsc/dm>0.01) and (pH<=5.75)

then 1.58-.153*pH

else if (Cwsc/dm>0.01) and (pH>=5.75)

then 0.7

else 1

Kg = 3*10^(-5)

KP1 = 1.66*10^(-3)*(1-dm)

KP2 = 8.8*10^(-5)*(1-dm)

KS1 = 3.3*10^(-3)*(1-dm)

KS2 = 5.3*10^(-4)*(1-dm)

Mua = 9.4*10^(-6)+0.00113*EXP(-pH)+2.27*10^(-7)*(Temperature-273)

muc1 = muc1max*fCT*fcpH*fcaw*fc1sp*fc1t

muc1max = 0.02

muc2 = muc2max*fCT*fcpH*fcaw*fc2sp*fc2t

muc2max = muc1max

Mue = (Mue1+Mue2+Mue3)/3

Mue1 = if (time>=0) & (time<=168)

```

```

then -0.183+0.0665*pH-0.00597*pH^2+0.00128*(Temperature-273)-2.03*10^(-
    5)*(Temperature-273)^2
else if ( time<=500 )
then 0.0363-0.0108*pH+0.00104*(pH)^2-3.86*10^(-4)*(Temperature-273)+8.43*10^(-
    6)*(Temperature-273)^2
else 0
Mue2 = if( time>=0) & (time<=168)
then-0.111+0.0387*pH-0.00326*pH^2+9.05*10^(-4)*(Temperature-273)-1.17*10^(-
    5)*(Temperature-273)^2
else if time<=500
then 0.0104+0.00188*pH-6.96*10^(-5)*pH^2-7.28*10^(-4)*(Temperature-273)+1.12*10^(-
    5)*(Temperature-273)^2
else 0
Mue3 = if( time>=0) & (time<=168 )
then -0.043+0.0137*pH-0.00104*pH^2+7.21*10^(-4)*(Temperature-273)-1.0*10^(-
    5)*(Temperature-273)^2
else if time<=500
then -0.0392+0.0208*pH-0.00203*pH^2-3.26*10^(-4)*(Temperature-273)+4.58*10^(-
    6)*(Temperature-273)^2
else 0
pHStar = 3.23+1.2*dm
pKA = 4.76
pKL = 3.86
Q = beta+ 10^(pH+pKA)*CA*2.303/(WA *(10^pKA+10^pH)^2)
+ 10^(pH+pKL)*CL*2.303/(WL *(10^pKL+10^pH)^2)

```

```

r1 = if time<=500 then muc1*CEC1
else muc1*CEC1*EXP(-(time-500)/100)
r2 = if time<=500 then muc2*CEC2
else muc2*CEC2*EXP(-(time-500)/10)
t1 = 1
t2 = 0
Temperature = 310
Vb = if time <=24.0 then 0.33* ebpH*vbMax
else if time>24.0 and time<=48.0 then 0.34*ebpH*vbMax
else if time> 48 and time <=72.0 then 0.55*ebpH*vbMax
else 1*ebpH*vbMax
vbMax = Vg1/ebpHstar
Vg = if time<=2.0 then 0
else VgMax*egpH*egaw*egWSC
Vg1 = egpHstar*VgMax*egaw*egWSC
VgCB = VgMax*egpH*egaw*egCB
VgCG = VgMax*egpH*egaw*egCG
VgMax = 1.0
WA = 60.05
WB = 88.12
WE = 46.06
WH2O = 18
WL = 90.08
WM = 182.17

```

$$\text{WNH3} = 17.01$$

$$\text{Wwsc} = 180.15$$

$$\text{Xg} = 0.185$$

$$\text{Yb} = .166$$

Appendix B

STATISTIC MODEL AND ANALYSIS OF VARIANCE TABLE

Statistic model for the split plot design in Chapter 3 is:

$$y_{ijkl} = \mu + D_i + W_j + En_k + S_l + R_m + (DW)_{ij} + (DEn)_{ik} + (DS)_{il} + (DWEEn)_{ijk} \\ + (DWS)_{ijl} + (WEnS)_{jkl} + (DWEEnS)_{ijkl} + \varepsilon_{ijklm}$$

$$\left. \begin{array}{l} i = 1,2,3,4,5 \\ j = 1,2 \\ k = 1,2 \\ l = 1,2 \\ m = 1,2,3 \end{array} \right\}$$

Note: D-Harvest dates, W-water addition, En-enzyme addition, S-sample dates, R- replicates.

SAS analysis of variance table:

Source	DF	Type I SS	Mean Square	F Value	Pr > F
D	7	19677.81	2811.12	90.44	<.0001
W	1	4755.52	4755.52	152.99	<.0001
D*W	6	3151.49	525.25	16.9	<.0001
En	1	9438.88	9438.88	303.66	<.0001
D*En	7	664.27	94.9	3.05	0.0068
W*En	1	0.42	0.42	0.01	0.9076
D*W*En	6	430.28	71.71	2.31	0.0421
S	1	4579.81	4579.81	147.34	<.0001
D*S	7	21494.48	3070.64	98.79	<.0001
W*S	1	460.81	460.81	14.82	0.0002
H*W*S	6	1819.45	303.24	9.76	<.0001
En*S	1	10230.63	10230.63	329.13	<.0001
H*En*S	7	925.83	132.26	4.25	0.0005
W*En*S	1	41.4	41.4	1.33	0.252
H*W*En*S	6	569.49	94.92	3.05	0.0098

Appendix C**LIST OF SYMBOLS**

a_w : water activity

C_A : acetic acid concentration

C_C : cellulose concentration

$C_{C,e}$: cellulose concentration associated with cellulose hydrolysis

C_{CB} : cellobiose concentration

$C_{CB,e}$: cellobiose concentration associated with cellobiose hydrolysis

$C_{CB,g}$: cellobiose concentration associated with growth of lactic acid bacteria

C_{CH} : hemicellulose concentration

C_{ECi} ($i = 1,2$) : Cellulase concentrations at two steps

C_G : glucose concentration

$C_{G,e}$: glucose concentration associated with glucose cellobiose hydrolysis

$C_{G,g}$: glucose concentration associated with growth of lactic acid bacteria

C_L : lactic acid concentration

C_{WSC} : concentration of water soluble carbohydrate

$C_{WSC,a}$: WSC concentration associated with chemical hydrolysis

$C_{WSC,b}$: WSC concentration associated with death of lactic acid bacteria

$C_{WSC,c}$: WSC concentration associated with cellulose hydrolysis

$C_{WSC,g}$: WSC concentration associated with growth of lactic acid bacteria

$C_{WSC,H}$: WSC concentration associated with hemicellulose hydrolysis

d.b : dry basis

$D.M.$: dry matter

f_{g,a_w} : fraction of the maximum growth lactic acid bacteria as dependent on a_w

$f_{b,pH}$: fraction of the death rate of lactic acid bacteria as dependent on pH

$f_{g,CB}$: fraction of cellobiose used for the growth lactic acid bacteria

$f_{g,G}$: fraction of glucose used for the growth lactic acid bacteria

$f_{g,WSC}$: fraction of water soluble carbohydrate used for the growth lactic acid bacteria

K_g : Michaelis-Menten constant

k : slope

pK_A : constant

pK_L : constant

R^2 : coefficient of determination

r_1 : hydrolysis rate of cellulose to cellobiose by cellulase

r_2 : hydrolysis rate of cellobiose to glucose by β -glucosidase

$s.e.$: standard error

T : temperature

t : time

U/g : unit of enzymatic activity

w.b. : wet basis

β : buffer index of silage

μ_b : specific death rate of lactic acid bacteria

$\mu_b^{[\max]}$: maximum specific death rate of lactic acid bacteria

$\mu_{C_i} (i = 1,2)$: Cellulase activities at two steps

$\mu_{C_i}^{\max} (i = 1,2)$: initial cellulase activities at two steps

μ_g : specific growth death rate of lactic acid bacteria

$\mu_g^{[\max]}$: maximum specific growth death rate of lactic acid bacteria

ω_A : molecular weight of acetic acid

ω_L : molecular weight of lactic acid

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RESEARCH AND WORK EXPERIENCES

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SELECTED PUBLICATIONS

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Chen, Q., T.L. Richard, M. Marshall, I.D. Darku, L.M. Wendt, C.W. Radtke. 2009. The effect of harvest date and enzyme addition on corn stover biomass ensilage for bioconversion. *Biofuels*. (To be submitted)
Chen, Q., F. Rezaei, T.L. Richard. 2009. Simulation of corn stover ensilage with amendments. 2009. *Biological Engineering*. (To be submitted)
H. You, **Qin Chen**, and Y.Gao. Studies on optimal processing parameters of the extraction of propolis flavonoids, 2002. *Food Science*, 23(8):172-174. (In Chinese)
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Chen, Q., M. Marshall, M. Tien, T. L. Richard. 2008. Enzymatic silage for corn stover storage and pretreatment into bio-ethanol. Presented at 2008 NE Renewable Energy Conference. Aug. 26- Aug. 28, State College, PA.
7 Total presentations and poster (Complete List Available Upon Request)

PATENTS

- Y. Gao, **Q. Chen**, H. You, R. Ruan, etc. Chinese Patent Application No. 200410015807.9, The method of extracting active ingredients from the leaves, stem and vine of sweet potato.
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