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EFFECT OF CELL WALL DEGRADING ENZYMES AND CHEMICALS ON CORN STOVER PRESERVATION AND PRETREATMENT DURING ENSILAGE PROCESSING

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ABSTRACT

Bioconversion of corn stover on an industrial scale requires a safe and long-term storage method for large quantities of corn stover in order to supply biorefineries continuously year round. The ensilage process, which is a traditional crop storage method for ruminants in winter, is proposed as a preservation method for corn stover. Since cell wall saccharification and sugar fermentation occur naturally during the ensiling process, preserving corn stover as silage is expected to benefically pretreat the feedstock for downstream bioconversion. In this study, cell wall degrading enzymes and chemicals were added to stover silage in order to reduce pH and/or encourage lactic acid fermentation.

Through this study, the effect of size reduction was examined for coarse (as harvested), medium (<10mm), and fine (<5m) ground stover, with and without enzyme or chemical treatments. The pre-processing cost and the efficiency of enzyme attachment to corn stover will be influenced by stover size. To reduce the cost of enzyme additives, the lowest effective enzyme concentration was determined first. The purified enzyme mixture was applied at rates of 6.7, 13.4, 26.3, 53.4, and 106.9 international units (IU) hemicellulase activity per gram stover dry matter (DM). Relative to the lowest rate, the rate of 13.4 IUg^{-1} DM resulted in a significant decrease in pH and increase of lactic acid concentration, and shifted the dominant microbial regime from clostridia to *Lactobacillus* spp. The highest water soluble carbohydrate (WSC) content and hemicellulose degradation levels were obtained with coarse size stover, although the improved effect in coarse size was from the natural hydrolysis instead of the enzyme addition. The benefits of industrial enzyme mixtures were compared with those of purified enzymes, with similar results to the purified enzymes at a much lower cost. To reduce enzyme costs, the industrial enzyme prouducts were used for the following study.

There are numerous industrial enzyme mixtures available commercially, derived from different microbial sources and containing different enzyme components. The impacts of seven commercial enzyme mixtures were examined on corn stover preservation and pretreatment, with different combinations of microbial source and

enzyme components. These enzymes were produced by *Aspergillus niger*, *Trichoderma reesei*, and *Trichoderma longibrachiatum*. Treatments included three size grades of corn stover, two enzyme levels (1.67 IU g^{-1} DM and 5 IU g^{-1} DM based on hemicellulase), and various ratios of cellulase to hemicellulase (C:H) in products derived from each microbial source. Higher lactic acid content and lower pH were obtained with increasing C:H ratios, especially with *Trichoderma reesei* enzymes. The highest C:H ratio tested, 2.38, resulted in the most effective fermentation, with lactic acid the dominant product. Significant cellulose and hemicellulose degradation was observed in these high C:H ratio enzyme mixtures derived from *Trichoderma reesei*, indicating the additive rates could be reduced if preservation is the primary goal.

The positive effect of enzyme addition was demonstrated on a pilot scale and in an extended preservation period. Corn stover at the three different particle size ranges was ensiled with and without a commercial enzyme mixture in 20 L mini-silos. Triplicate silos were destructively sampled and analyzed on days 0, 1, 7, 21, 63, and 189. On days 0, 21, and 189, the triplicate samples were mixed evenly and assembled into particleboard using 10% ISU 2 resin, a soy-based adhesive. Enzymatic addition improved the ensiling process, as indicated by sustained lower pH, higher WSC concentration, and increased lactic acid production. The middle particle size range (<10 mm) demonstrated the most promising results during the ensiling process. Compared with fresh stover, the ensilage process increased the internal bond strength of stover particleboard by 32.6% and decreased water adsorption at 2 hr boiling and 24 hr soaking significantly. Particleboard panels produced from substrate ensiled with enzymes showed a significant reduction in water adsorption of 12.1% during 2 hr-boiling testing.

Compared with enzymes, chemical additives are currently more ecomonical. The effect of five chemicals, including sulfuric acid, formic acid, formaldehyde, ammonia, and urea, was evaluated for corn stover preservation and pretreatment. Treatments included 2, 4, and 8 g kg^{-1} DM of sulfuric acid, formic acid, and formaldehyde and 4, 8, and 16 g kg^{-1} DM of ammonia and urea, with each of these 15 chemical treatments applied to three different particle sizes of stover. Sulfuric acid, formic acid, and formaldehyde increased lactic acid concentration and decreased acetic acid concentration. Clostridia activities were inhibited at the 16 g kg^{-1} DM level of urea and ammonia.

Sulfuric acid, commonly considered as an economical and effective pretreatment reagent, was reexamined as a preservation and pretreatment additive over a 63 day period. The long term trial of sulfuric acid-treated silage showed that WSC increased over 63 days at the 16 g kg^{-1} DM level, although fermentation was almost inhibited at this level. Sulfuric acid, formic acid, formaldehyde, and ammonia increased sugar yield in enzymatic hydrolysis when compared to fresh stover and to control samples ensiled without chemical additives.

A mathematical model of corn stover silage was developed to predict the effect of enzyme addition on stover preservation. Cellulose degradation was first simulated by a hydrolysis kinetic model and integrated into a previous silage model developed by other researchers. A parameter to account for cellulose structural features, λ, was estimated as 0.56, implying 56% of cellulose was in the non-hydrolysable region and unavailable for enzymatic hydrolysis. With the integrated silage model, dynamic responses of several important chemical components of corn stover silage were predicted, including the concentration of cellulose, total water soluble carbohydrates, degraded sugars, and lactic acid. Acceptable predictions for these chemical constituents were obtained with the exception of lactic acid.

Ensiling with enzyme and chemical treatments has been demonstrated as a safe and effective preservation method with beneficial pretreatment effects for downstream bioconversion, such as particleboard manufacturing and sugar production. Integration of an improved ensiling process with a more efficient pretreatment method is required to increase the sugar yield of ensiled stover. If the crosslink in cell walls can be broken down by integrating pretreatment during the preservation period, bioconversion of corn stover can be carried out immediately without additional pretreatment.

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1. INTRODUCTION

The demand for transportation fuels, chemicals, and wood has increased dramatically in the last 50 years as the world population has exploded and consumption levels have increased (Rowell et al., 1997). Meanwhile, there is a growing concern about the predicted decline in production and eventual exhaustion of fossil fuels (Campbell and Laherrere, 1998), as well as environmental problems such as global warming caused by the use of fossil resources. The low growth rate of trees intensifies the pressure from a relatively low supply of wood. In order to meet the difference between the demand and supply of these resources and to solve related environmental problems, more and more researchers are looking at renewable resources as the feedstock for transportation fuels, chemicals, and natural fiber production (Collet et al., 2004; Ma and Hanna, 1999; and Leather, 2003). Agricultural residues have attracted a lot of attention because of their abundance, low cost, renewability, and biodegradability. The availability of agricultural residues in the U.S. was estimated at 192 million dry Mg/yr, with corn stover, soybean residue, and wheat straw as the major residues (Glassner et al., 1998).

Corn stover has been estimated as the most abundant agricultural residue in the U.S., with its approximately 139 million dry Mg/yr representing over 80% of total agricultural residues (Kadm and McMillan, 2003). However, current utilization of corn stover is focused on farm-based applications, such as field erosion control, animal feeding, barn bedding, and heating fuel. It has been reported that over 90% of corn stover is left in fields, although only a fraction of this residue is needed to minimize soil erosion (Sokhansanj et al., 2002). Less than 1% of stover is currently collected for industrial processing. When the harvested residue exceeds the farm management capacity, it will often be burned for disposal in the fields in developing countries as well as industrialized countries. Not only does this cause air pollution, but it also wastes the potential feedstock for industrial utilization. It is a challenge for researchers to utilize this annually abundant and undeveloped resource as the feedstock for value-added products manufacturing, such as transportation fuels, chemicals, and particle board.

Corn stover contains over 70% lignocellulosic fractions, which provide rich carbohydrates as the substrate for fermentable sugars, ethanol, and production of other

chemicals. Presently, a number of strategies are under development for bioconversion of corn stover into these value-added products. Kadam and McMillan (2003) examined the potential production of ethanol, pulp and particle board, and furfural from corn stover, with the conclusion that 11.37 billion liters of ethanol can be potentially produced from 40% of the harvestable corn stover with 50% of the theoretical ethanol yield. They also estimated that corn stover can replace the feedstock for 50% of both hardwood pulp and wood-based particleboard production.

Many aspects of large scale utilization of corn stover have been investigated, such as pretreatment, bioconversion, and board manufacturing (Karr and Holtzapple, 2000; Yang et al., 2001; and Wang and Sun, 2002). However, the issue of storage has not been adequately addressed. Industrial uses of corn stover will require long term storage up to one year, because the material harvested in one growing season has to supply the industrial processing refineries continuously with feedstock until the subsequent harvest. In temperate climates common to North America, corn and stover can only be harvested once per year. Current methods of corn stover storage use dry storage to minimize decomposition. However, the low moisture content, ranging from 14% to 33%, creates a high risk of fire with dry storage. Furthermore, up to 23% dry matter loss in weight has been reported in bale and stack storage for corn stover because of plant and microorganism respiration (Richey et al., 1982). Although storing at widely separated sites will reduce the loss in case of fire, the cost of facilities and transportation will make this method impractical. In order to solve these problems, an ensilage process has been proposed as a preservation method for large amounts of corn stover, since the high moisture content (up to 60%, dry basis, d.b.) can eliminate the risk of fire (Richard et al., 2001).

Ensilage is a traditional process used to preserve crops for ruminant feeding during the winter season. Through the ensiling process, the rate of decomposition and deterioration of crops is held to a very low level by limiting microbial activity through unfavorable environmental conditions. Low pH and anaerobic conditions are widely agreed upon as the principle requirements to obtain high quality silage. These conditions can be obtained through encouraging lactic acid fermentation and sealing the silage materials from the air.

Not only does ensilage provide a preservation method for crops, but saccharification of crop cell walls and mixed-acid fermentation also occur through the synergistic system of microorganisms that are present during the ensiling period. These reactions are expected to break down the physical and chemical structure of biomass and improve degradability for further bioprocessing. Therefore, ensilage is also proposed as a pretreatment process which can be beneficial for future conversion of corn stover into sugars or chemicals in industrial production (Richard et al., 2001).

However, the purpose and the desired characteristics of industrial corn stover storage are different from those of the most popular forage crops traditionally conserved as silage, such as ryegrass, timothy, alfalfa, and green harvested whole corn plants. Rather than as a means of food storage for ruminant feeding in winter, corn stover will be ensiled prior to industrial processing for chemicals or particleboard production. In addition, the very low content of fermentable sugars in corn stover, less than 1% dry matter (DM), may not always provide sufficient substrate lactic acid fermentation to obtain a low pH. Thus, further investigation and examination of corn stover ensilage is required because of these differences from traditional ensilage crops.

Enzyme, such as cellulase and hemicellulase, can degrade the lignocellulosic composition of the cell walls to fermentable sugars, which will support lactic acid bacteria growth to decrease the pH of silage. The effect of these fermentation stimulants have been successfully demonstrated in traditional silage of low-sugar-content crops (McDonald et al., 1991). Because biomass-destined corn stover silage will not be used to feed animals, some chemicals, such as sulfuric acid, formic acid, and urea can be added to the silage to weaken the linkages among the polysaccharides in the cell walls. Weakened linkages make these polysaccharides easier to degrade and thus should help produce fermentable sugars during downstream processing. Furthermore, these chemicals will inhibit microbial growth at an appropriate concentration, and this will decrease the decomposition by microbial metabolism.

To prepare for future utilization of corn stover for chemical and particleboard production on an industrial scale, this study examines ensilage as a storage method for biomass. Although ensilage is a well-known process for food preservation for ruminants, there are few studies on applying this preservation system to corn stover storage for

bioconversion to value-added products. Therefore, the aim of this study was to investigate the effectiveness of the ensilage process of corn stover on preservation and pretreatment for downstream processing. Enzymatic additives and chemicals have been applied to corn stover silage to encourage lactic acid fermentation, to weaken the linkages of polysaccharides, and to inhibit undesirable microbial growth. To evaluate these processes, the effects of these treatments on the chemical characteristics of corn stover silage and the growth of microorganisms in the silage have been examined. Finally, the physical properties of particle board produced from ensiled corn stover have also been analyzed. One outcome of this study is to provide a storage strategy of corn stover for industrial utilization. Furthermore, by investigating the production of sugars and particleboard from the treated corn stover silage, this study has examined the possibility of beneficial pretreatment during the ensiling process for downstream conversion.

2. LITERATURE REVIEW

2.1. General Background

As world population increases and living standards rise (Rowell et al., 1997; Campbell and Laherrere, 1998), the production of transportation fuels, chemicals, and particle boards from fossil fuels or wood are facing enormous pressures and challenges. These concerns, which include the limited resources, low rate of supply, increased demand, and environmental pollutions, drive researchers to look for new energy and fiber resources.

Since the 1980s biomass has been proposed as the primary replacement feedstock to substitute for fossil fuels in order to produce ethanol, hydrogen, and methane for transportation fuel. The high fiber content of biomass has similar physical properties and chemical composition to wood. The availability, renewability, and biodegradability of biomass are driving an extensive research effort focusing on the feasibility of substituting biomass for fossil fuels and wood. Glassner and Hettenhaus (1997) indicate that the most desirable biomass feedstocks should be low cost residues, by-products, or wastes from other processes. Agricultural residues are the most promising alternatives because they meet these requirements and also are the most abundant biomass resources available. The estimated quantities of agricultural residues annually in the U.S. were 139 million dry Mg whereas forest residues, mill residues, and urban wood wastes were 44, 90, and 36 Mg, respectively (Walsh et al., 1999).

Among available agricultural residues, corn stover was estimated to be the most abundant residue in the U.S., representing over 80% of the total amount (Kadam and McMillan, 2003; Walsh et al., 1999). Although other residues such as wheat and soybean straw contribute around 20% of total agricultural residues, corn stover is proposed as the most promising alternative feedstock to substitute for fossil fuels and wood. This is not only because of the broad availability, but also the concentrated production throughout the Midwestern U.S. (Walsh et al., 1999).

Technical issues involved in the processes of biomass pretreatment, bioconversion, extraction, and separation, as well as board manufacturing, have been investigated by many researchers. In the last 10 years, considerable research has been done on corn

stover as a substrate to produce ethanol, xylitol, furfural, lactic acid as well as particle boards (Riera et al., 1991; Kaar and Holtzapple, 2000; Wang and Sun, 2002; Kadam and McMillan, 2003). In recent years, several economic analyses and life cycle assessments of corn stover utilization strategies have also been initiated (Eggeman and Elander, 2005; Kim and Dale, 2005, and Wilke et al., 1981). However, an effective strategy for storage for large quantities of corn stover has not been adequately developed.

Because corn and stover are only harvested once a year in the temperate climates in North America, it is necessary to store large volumes of corn stover up to one year to supply the feedstock for year-round processing facilities. Current storage methods mainly rely on dry storage, in which a high risk of fire is inherent from either spontaneous combustion or cigarettes and lightning. Although widely dispersed storages will lessen the potential loss from fire, the associated expenditure on numerous storage facilities and inter-facility transportation makes this storage method unreasonable.

In the last few years, an ensilage method has been investigated to preserve corn stover as a feedstock for processing facilities (Richard et al., 2001; Shinners et al., 2003a). This method is favored because the high moisture content (higher than 60% wet basis, w.b.) can eliminate the risk of fire inherent with dry storage. The ensilage process is a traditional food preservation method for ruminants fed during the winter season, and has a history of over 2000 years. Although extensive research has examined the chemical and biological characteristics during ensilage of conventional forage crops, few results are available for the ensiling of corn stover. As corn stover has different characteristics from those traditional silage crops, more work will be required to study the feasibility of the ensilage process as an effective storage method for corn stover and to demonstrate the possible pretreatment benefits.

The following sections review corn stover yield in the U.S., the cost of corn stover as the feedstock, the potential for value-added products from corn stover, and the technical issues involved in the associated production processes. Several aspects of ensiling traditional forage crops are addressed, including the principles of ensilage, phases of the ensiling process, additives to improve silage quality, and previous mathematical models of the ensilage process. Finally, the current status of research on corn stover preservation is discussed.

2.2. Corn stover

There are about 32 million ha of corn planted annually in the U.S., covering more acreage than any other crop (USDA, 2002). The second and third most widely planted crops, soybeans and wheat, are planted on 29 and 24 million ha, respectively. The production of these crops for 2001 are as follows (in millions of Mg): corn 302 (bulk density, 70 pounds $bushel^{-1}$), soybeans 79 (bulk density, 60 pounds $bushel^{-1}$, and wheat 53 (bulk density, 60 pounds $bushel^{-1}$) (USDA, 2002). As these data show, corn is not only the most widely planted crop in the U.S., but also has the highest production among these three crops. Furthermore, a significant yield increase has been occurring for corn during the last 30 years, from 6.5 Mg ha^{-1} in 1971 to 10.8 Mg ha^{-1} in 2001 (USDA, 2001). Precision management, genetic development, and other biological and chemical techniques contributed to this yield increase.

Corn stover is the major byproduct of corn grain, and is presently utilized for low value applications. These are primarily on-farm applications, such as field erosion control, animal feed, and barn bedding. Currently, the most promising potential value of corn stover is as a renewable feedstock for fuels and bioethanol. To realize this utilization potential of corn stover and other biomass, in February 1, 2006, President Bush proposed a 22% increase in clean-energy research at the Department of Energy and 65% increase for research on biomass utilization.

2.2.1 Chemical characteristics of corn stover

Corn stover refers to the aboveground residue left in the field by harvest machinery, including stalks (50% dry basis, d.b.), leaves (20%), cobs (20%), and husks (10%), after corn grain is harvested (Myers and Underwood, 1992). Although the proportions of these residue fractions are variable, these parts of stover consist of similar cell wall structures and chemical components with small variations in composition. Cellulose, hemicellulose, lignin, a small amount of phenolic acids, and silica compose the main physical structure of cell wall, assisting the plant in standing up and protecting the plant's inner environment. Other chemical components include water soluble carbohydrates (WSC), starch, organic acids, protein, and non-protein nitrogen, but these are present in very small quantities, with the total less than 5-10% d.b. Of these

components, cellulose, hemicellulose, and lignin collectively contribute over 80-90% d.b. of corn stover. Generally, cellulose and hemicellulose serve as the substrates for further bioconversion for chemicals and transportation fuels. Lignin is a carbon-neutral combustible component with a high energy value. It can be used to generate electricity and/or steam without producing net CO_2, and can also be used in the production of construction material and asphalt binders.

2.2.1.1. Cellulose

Cellulose has been said to be the most abundant organic polymer on the earth, with annual production of 4×10^{10} Mg (Goyal et al., 1991). It is found mainly in the secondary cell wall of plants, and is the major structural component of higher plants (Robyt, 1997). Celluloses from all sources have the same linear polysaccharides of D-glucopyranose units linked without branches. The straight chains of cellulose rotate 180° every other β-1→4 glycosidic linkage, providing spatial sites to form intermolecular hydrogen bonds (Figure 2.1). Parallel cellulose chains are associated by these hydrogen bonds and van der Waals forces among molecules to produce three-dimensional microfibrils, in which a regular and repeating crystalline structure is interspersed by amorphous regions. The crystalline structure makes cellulose very water insoluble and impermeable to water, so that the highly associated microfibrils can act as an outside matrix to protect the inner environment of plant cells. This crystalline structure is one of the major limitations for cell wall hydrolysis.

The cellulose concentration of corn cobs has been reported in the range of 32.2-45.6% d.b. (Sun and Cheng, 2002; Foley, 1978) and 33.5-38.4% d.b. for corn stalks (Ladisch et al., 1983). When β-1→4 glycosidic linkages in this cellulose are broken down by enzymatic hydrolysis or moderate acids, glucan and glucose are released from the polysaccharide, which can be fermented into ethanol, lactic acid, or other chemicals. The details of cellulose hydrolysis will be discussed in Section 2.4.4.2.3.

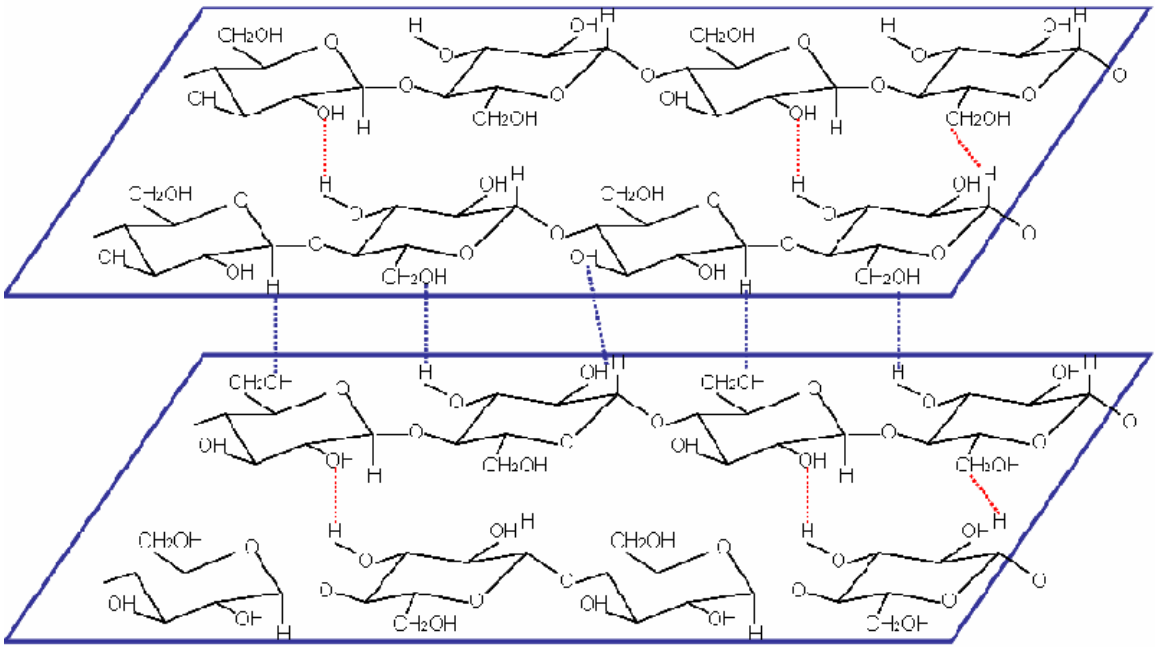


Figure 2.1 Structure of cellulose microfibrils with intermolecular hydrogen bonded cellulose chains (modified from Robyt, 1997).

2.2.1.2. Hemicelluloses

Hemicelluloses are a heterogeneous group of polysaccharides including four basic types: D-xyloglucans, D-xylans, D-mannans, and D-galactans. In each type, two to six various monomers are aggregated through β-1→4 and β-1→3 linkages in main chains and α-1→2, 3, and 6 linkages in branches. The monomer subunits can include D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucouronic acid. Hemicelluloses vary in subunits, compositions, polymer components, and concentrations from plant to plant and from one plant part to others.

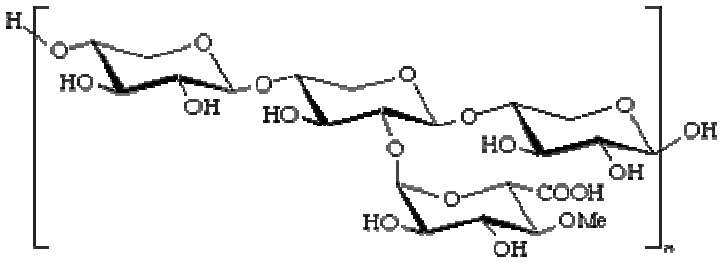


Figure 2.2 Structure of D-xylan fragment (Modified from Eriksson et al. 1990).

The hemicellulose concentration of corn stover has been reported as 35% d. b. (Sun and Cheng, 2002), with D-xylan (Figure 2.2) as the major type (Robyt, 1997). The main chain structure of D-xylan is similar to that of cellulose except that the monomer is xylose, which lacks a primary alcohol group at the C-5 site. Moreover, glucopyranosyl uronic acid units are linked to the main chain at every four or five xylose residue through a α-1→2 linkage. The absence of the primary alcohol group reduces the chances of formation of intermolecular hydrogen bonds and microfibrils. Lacking anintermolecular hydrogen bonds among the polysaccharide chains, xylan does not form a crystalline structure. Furthermore, uronic acids in the branches make xlyan an acidic polysaccharide. Thus, xylan is much more water soluble than cellulose and reactive to chemical treatment. However, the heterogeneous monomers and linkages of hemicellulose spatially hinder enzyme attachment, which reduces the effectiveness of hemicellulase during hydrolysis.

2.2.1.3. Lignin

Lignin and associated phenolic acids, although present in relatively small concentrations, play an important role in cell wall degradation. Unlignified or slightly lignified plant tissues can be degraded much more easily than intensively lignified tissues (Aisan et al., 1997). The complicated composition and structure of lignin greatly limits the complete understanding of lignin synthesis and degradation. In terms of chemical composition, lignin is historically divided into core lignin and non-core lignin. Core lignin includes the highly-condensed polymers formed by dehydrogenative polymerization of the hydroxycinnamyl alcohols, p-coumaryl alcohols, coniferyl alcohols, and sinapyl alcohols. Non-core lignin includes esterified or etherified phenolic acids bound to core lignin or to noncellulosic polysaccharides (Moore and Hatfield, 1994). The chemical structure of lignin is also very complicated (Fig. 2.3) (Sarkanen, 1970), as it is a three-dimensional cross-linked aromatic polymer made up from phenylpropane units. No single established structural scheme for lignin has been established thus far.

Lignin is mainly located in the middle lamella of the plant cell wall, cross-linked with hemicellulose directly or through phenolic acids. It provides vascular plants with strength and rigidity and helps the cell wall resist microbial attacks and enzymatic

hydrolysis. Lignin concentration differs considerably from plant to plant. Corn stover contains 10.00-14.67% d.b. lignin (Kaar and Holtzapple, 2000; Kim and Dale, 2004) while the lignin concentration of softwood stems range from 25-35% d.b. (Sun and Cheng, 2002). Different parts of corn stover have different levels of lignin with 10.9% in husks (Kurakake et al., 2001), 6.6% in cobs (Bhatti and Firkins, 2005), 11.8-20.8 % in node (Buxton et al., 1996). Furthermore, the lignin content also changes over time, increasing with the maturity of plants during the harvest season (Pordesimo et al., 2005).

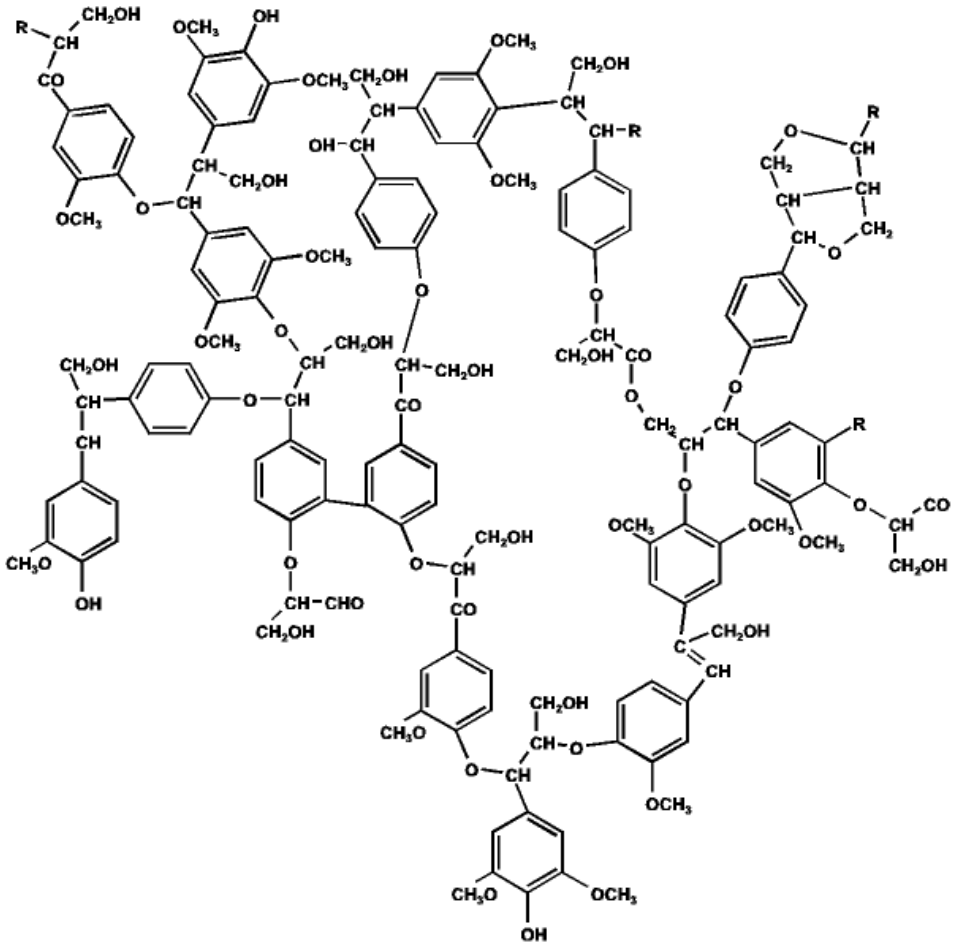


Figure 2.3 Partial lignin chemical bond structures (Modified from Sarkanen 1970).

2.2.2. Corn stover yield

Widely variable estimates of corn stover availability have been reported in the last few years. These estimates vary because of different yield estimation methods and

assumptions made about what fraction of corn stover can be sustainably harvested. Several recent estimates of the potential annual production of corn stover feedstock are presented in Table 2.1.

Approximately 1 kg of stover is produced per kg of grain (Lindstrom et al., 1981), and using corn grain production in 1997, Glassner et al. (1999) estimated 200 million dry Mg/year of corn stover were produced as above-ground residue. The available corn stover was estimated as 153 million dry Mg/year when 76% of 200 million dry Mg were assumed to be sustainably harvested (Glassner et al., 1998). These estimates are the highest reported so far, probably because all corn lands are assumed as no-till farming. No-till farming allows a larger fraction of stover to be harvested, as all the remaining residue stays on the surface to reduce water and wind erosion. Although up to 76 to 82% of stover can be recovered by commercial balers, soil conservation guidelines should be followed, leaving no less than 30% of surface of land covered by residues. Based on these guidelines, about 50% of the available corn stover could be harvested in southwest Iowa and more than 60% could be harvested for central Illinois (Hettenhaus et al., 2000a).

Table 2.1 Summary of corn stover availability estimates.

US (Mg)	Midwest (Mg)	Delivered cost ($/dry Mg)	Reference
153		35-40	Glassner et al. (1998, 1999)
120	108	<50	Walsh et al. (1999)
117-120			DiPardo (2000)
82			Kadam and McMillan (2003)
69			ORNL et al. (2005)

Kadam and McMillan (2003) estimated corn stover production as 204 million dry Mg/year using the 1:1 estimated ratio rule of stover to grain suggested by Lindstrom et al. (1981) and 217 million dry Mg/year using the 1.06:1 ratio reported by Nielsen (1995). Kadam and McMillan (2003) assumed about 35% of stover can be harvested from the field using traditional tilling techniques, whereas 68-75% will be available from no-till farming. They calculated an average harvestable fraction as 41% on a sustainable basis,

given that 17% of corn is currently grown using no-till techniques (CTIC, 1998). Based on these assumptions, they estimated the corn stover availability to be 82 million dry Mg/year in the U.S.

In a study of agricultural residue availability for 50 states in the U.S. (Walsh et al., 1999), corn stover was estimated to account for 71-99% (d.b.) of the residues in the Midwestern states, except for Kansas, where due to extensive wheat production, only 48% is corn stover. About 108 and 120 million dry Mg/year of corn stover would be available in the Midwest region and nationwide, respectively, when assuming 30-40% can be sustainably harvested. A similar availability was estimated by DiPardo (2000), with 117-120 million dry Mg/year of corn stover potentially harvested for ethanol production.

The Oak Ridge National Laboratory et al. (2005) studied sustainable biomass resources by analyzing with practical crop yields, tillage practices (20-40% no-till for major crops), residue collection technology (40% recovery), and expected grain to ethanol production. Around 16% of a total 1.1 billion dry Mg of biomass was agricultural residues that can be sustainably available for bioenergy and bioproducts. Corn stover is the single largest source of crop residues and has about 69 million dry Mg availability annually. This is about 66% of the 104 million dry Mg of crop residues.

The availability of corn stover depends not only on corn production and sustainably harvest practices, but also other factors such as topography, environmental constraints, weather conditions, and tradition. When considering corn stover as a feedstock for industrial utilization, the availability would further be limited by the accessible distance, transportation cost, labor requirements, and other competitive demands.

2.2.3 Impact of corn stover removal

Leaving corn stover in the field is believed to influence the performance of corn directly and indirectly with positive and negative effects. As it decomposes, stover is credited as a supplement of P and K nutrients, which are otherwise lost from soil due to plant growth and harvest. The credits of P and K nutrients from stover are not as valuable as expected, with stover concentrations only 0.1 and 1% (d.b.), respectively (Hettenhaus

et al., 2000b). Further, when animal manure is applied to fields on the basis of the N requirement, P is already in excess. Because the C/N ratio of stover (up to 30-70:1) is much higher than the optimal ratio for microbial degradation of 10:1, additional N may be required to prevent subsequent crops from suffering stress from N immobilization when fields are covered with stover.

Surface residue may also contribute to yield decreases by increasing weed pressure on the crop. Surface residue can slow down soil warming the next spring and keep soil wet, which will worsen weed control. Poor seed placement caused by residue interference can be another reason for decreased yield (Hettenhaus et al., 2000a). Residues may also impair water flow in irrigated fields. Although residue coverage is believed to supplement soil organic matter (SOM), it has been reported that over 80% (w/w) of stover coverage is lost as carbon dioxide, and the major contribution to SOM comes from the corn roots (Reicosky and Lindstrom, 1998).

In order to examine the effect of stover treatment on grain and stover yield, Linden et al. (2000) implemented two treatments of corn stover over 13 years. They included a no-residue treatment, in which all corn residues are removed using a silage chopper with only the stalk bottom and roots left, and the residue-returned treatment, in which all plant material except grain is left in the field. Three tillage systems, fall moldboard plow, fall chisel plow, and no-till, were randomly combined with the two residue treatments. The final results showed that there were no significant differences in corn yield and production for the two treatments over thirteen years, but there were decreases of corn yield from the no-residue treatments in several years when there was a smaller amount of rainfall during the growing season. The decreased yield reported by Linden et al. (2000) can be explained by the fact that surface residue assists soil in retaining moisture, which is helpful and useful for crop growth in a relatively dry year. Fall chisel plow tillage treatment increased the difference between the two residue treatments, which was attributed to the integral feature of the system and resulted in better use of water in marginally dry year. These results indicated a benefit from residue coverage under some conditions, and contradicted the claim of Hettenhaus et al. (2000a) that residue removal would not affect yield. Although the impacts of these treatments on corn yield were compared during the thirteen years, the soil quality, organic matter, and

nutrient content were not reported. A panel of USDA scientists studying residue availability cautioned that the long-term impact of residue removal on soil productivity should be determined first, before justifying the removal (Glassner et al., 1999).

2.2.4 Engineering of corn stover collection

After corn grain matures in the last month of the growing season, modern grain combines will strip grain and leave stover unshredded in the field, either as surface mulch or for further collection. Stover can also be shredded and windrowed in the field during grain harvest by using modified combines. In such a multi-pass collection system, stover will be collected in round or square bales and these bales will be transported to storage sites immediately or after a short-term delay. Since the physical characteristics of corn stover during the harvest period will determine the density of stover, and thus influence further collection steps, these characteristics (including mass distribution and moisture content) are discussed first.

2.2.4.1 Characteristics of corn stover in the field

During the last growing month of corn, the mass of above-ground parts of the whole plant increases from 7.0 to 12.3 dry Mg ha^{-1}, with an increase in grain mass from 0.5 to 6 dry Mg ha^{-1} (Sokhansanj et al., 2002). During this time, there is a slight decrease in the stover mass from 6.5 to 6 dry Mg ha^{-1}. Sokhansanj et al. (2002) reported that the ratio of grain mass to total mass changes from 0.45 to 0.55 during this last month of growth. Shinners et al. (2003a) reported a somewhat larger change in the last one and one-half months, from 0.41 to 0.62, averaging 0.57 when corn grain moisture ranged between 20-30% in the Upper Midwest. The average ratios obtained from a 10-year period of field data have been reported in the range of 0.35-0.75 (Linden et al., 2000). These differences can be caused by variations in grain yield, growth region, harvesting methods, stages of maturity, and harvest dates. Thus, although using the rule of thumb of one Mg of above-ground stover per Mg of grain harvested is reasonable, caution is still needed.

The moisture content of stover fractions decreases during the harvest season, although the extent of this decrease differs among the various fractions. The reduction of

moisture is greatest in the grain, followed by cob, husk, and leaves in that order. The moisture content of the stalk fraction remains at a high level through the harvest period. Edens et al. (2002) reported the average stover moisture content ranges from 40 to 60% (w.b.) when grain moisture is from 20% to 30% at harvest time. Shinners et al. (2003a) indicated a higher stover moisture content, ranging from 58-65% (w.b.) at the same grain moisture levels. A much higher moisture content range, 55-82% (w.b.), has been reported when grain moisture is around 34% (Johnson and Lamp, 1966). The study of Shinners et al. (2003a) also showed that the bottom half of the stalk remains at a high moisture level of 75% (w.b.) through out the harvest period. Even when the plant is totally dead and the grain is drying, moisture will still transfer from the root to the stalk because of evapotranspiration along the plant. As these data indicate, it will be difficult to directly harvest stover as a dry material, even at the end of the harvest season.

2.2.4.2 Corn stover collection and storage

Currently, corn stover is typically harvested and packaged into round or square bales as dry material following grain harvest and field drying. Storing stover as dry material can reduce decomposition and deterioration caused by active microorganisms at high moisture contents. But, according to Richey et al. (1982), there is a high dry matter loss of up to 23% (d.b.) for dry stover bale storage. When storing large amounts of stover for industrial utilization, fire is a potentially catastrophic risk. To address this concern, Sayler et al. (1993) recommended storing total annual feedstock in a dispersed fashion, with each storage site having less than 2% of total biomass so as to lower the risk of fire.

The major operations after grain harvesting typically include cutting and shredding, windrowing, raking, baling, and transporting bales to a storage site. Stover is shredded and spread around the field using combines to accelerate field drying. Then, the spread stover is raked into windrows for further baling. A flail shredder can be used to both shred and windrow stover to eliminate the raking step. Although the speed of the flail shredding operation is slower than that of shredding and then raking, the drying rate of stover is improved significantly. This is explained by the fact that plant stems are split open through mechanical forces during shredding, so that moisture trapped in pith can escape more easily. However, windrowing during shredding will decrease the drying rate,

because the compactness of windrows will prevent ventilation by wind and attenuate incoming solar radiation. Good weather during field drying is critical, and if it rains, shredding with windrowing will result in worse drying performance compared to standing stover without shredding and windrowing. Shinners et al. (2003a) stated that shredded stover lying down on the ground prevents water shedding, and windrowing intensifies water accumulation in stover. Conversely, standing stover allows water to flow down easily. In regions with rainy harvest seasons like the Upper Midwest, they suggested leaving stover upright before a shredding operation, which should then be followed by immediate baling. Delaying shredding until good weather, with low relative humidity, will result in the lowest possible moisture.

In order to reduce the number of the operations required to obtain biomass feedstock material, Shinners et al. (2003a) examined the efficacy of harvesting and storing corn while stover still wet. A flail shredder was used to shred and gather the stover right after the grain was harvested. The wet stover was chopped immediately with a forage harvester and ensiled in a silo or wrapped with plastic films as bales. The operations of raking and bale gathering operation were eliminated. Since there is no requirement for field drying, the three-pass system, including grain harvest, shredding/gathering, and chopping, can be modified into a two-pass and even a one-pass system. For example, a flail shredder can be incorporated into a crop harvest combine, thus only grain harvest and chopping are required. If the combine can chop and blow leaves and stalks into parallel container along with the grain harvester, both grain and stover can be harvested in just one pass.

The wet storage method has been demonstrated successfully and compared with harvesting stover through a 3-pass system (Shinners et al., 2003a). Less than 5% of dry matter loss was achieved by ensiling stover in bales wrapped by plastic films. Stover with moisture content up to 48% (w.b.) was also preserved well in a bag silo for 7 months, though over 10% dry mass was lost. Even at this higher range, these losses compare favorably with dry storage losses, which typically range from 17 to 23% (Richey et al., 1982). Losses are minimized by storage conditions that are inhospitable to microbial decomposition. In the wet storage systems, anaerobic conditions encourage an initial acid fermentation, which lowers pH, much like traditional ensiled storage of livestock fodder.

Improving this preservation strategy for corn stover destined for industrial utilization is the primary focus of this thesis.

2.2.5 Cost of corn stover as a feedstock

The cost of corn stover as a feedstock for future industrial utilization should include compensation to farmers for removed nutrients as well as the collecting, handling and hauling cost, transportation cost, and storage cost. Cost estimates will be significantly influenced not only by the calculation methods, but also by assumptions about refinery capacity, hauling distance, stover availability and competitive demand from other utilization options.

Sokhansanj et al. (2002) examined the stover cost including collection and transportation of corn stover within 5 miles, and estimated it as \$23.7 and \$25.9 dry Mg^{-1} for round baling and rectangular systems, respectively. Other reported costs of previous studies vary in the range of \$27.5-\$32.2 dry Mg^{-1}, depending on the stover yield, the assumed transportation distance, and the operation size. However, these prices didn't include the cost paid to the farmers who provide the stover and other factors.

Nutrient values of corn stover are credited as P, K, and C supplements, and their quantities change in proportion to corn biomass quantities. For example, the value of P and K increases from \$16 ha^{-1} to \$30 ha^{-1} when the corn yield increases from 10.2 $Mgha^{-1}$ to 15.7 Mg ha^{-1}. The nutrient value of corn stover has been reported by various researchers, and compensation for farmers has been estimated based on these values. Nielsen (1995) reported the nutrient value of corn stover at \$6.48 dry Mg^{-1}. Jose et al. (1996), and Schechinger and Hettenhaus (1999) estimated the value of \$9.60 and \$11.4 dry Mg^{-1}, respectively. Hettenhaus et al. (2000a) indicated that farmers will accept the compensation of \$25 ha^{-1} or less for stover harvested from fields with rich soils, where supplemental nutrients are not required. But when P and K are required, farmers will ask for more than \$50 ha^{-1} for compensation, assuming the real value of P and K is in the range of \$16-30 ha^{-1}. In a study by Perlack and Turhollow (2003), compensation for stover removal to the farmer was assumed at \$11 dry Mg^{-1}. This compensation is supposed to cover the value of removed nutrients, reasonable transportation costs, and

profit. The compensation is expected to fluctuate because of competition from other bio-based products and other uses.

Shinners et al. (2003b) compared the costs of harvest, storage, and transport of corn stover for dry and wet storage methods. A single-pass system with a modified combine crop unit for wet stover harvesting can eliminate field drying, increase the harvesting window, improve timeliness, reduce stover soil contamination, and save 20% harvesting and transportation costs compared with a conventional multi-pass dry stover harvester. Bag silos and wrapped silage bales were the most cost effective storage methods for wet stover with the lowest dry matter losses (5-7% d.b.). These storage costs were slightly less than storing dry bales indoors, although 50% greater than storing dry bales outdoors. The cost of transporting wet stover from the storage sites to processing sites was double that of transporting dry stover. Despite the transport disadvantages of the wet stover storage system, the final cost including harvest, stover, and transport of the single-pass wet stover system was estimated to be \$33.9 dry Mg^{-1}, a reduction of 26% compared to conventional dry storage system.

The total feedstock cost is also affected by the capacity of the facility. In the Perlack and Turhollow (2003) analysis, the cost gradually increased from \$47.4 dry Mg^{-1} for a 455 dry Mg day^{-1} facility to \$56.7 dry Mg^{-1} for a 2730 dry Mg/day facility because of the increased transportation and handling costs. The levels of stover availability in a region influence the total cost greatly, with a \$ 6-10 dry Mg^{-1} difference. Furthermore, the operation of transportation processes has an effect on the final cost. Perlack and Turhollow (2003) reported that delivering stover bales directly to the storage site is more economical than holding them at the field margin and moving them later for storage when the facility size was smaller than 2730 dry Mg day^{-1}.

2.3. Bioconversion of corn stover

Bioconversion of corn stover to value-added products has attracted a lot of attention in recent decades. The rich polysaccharide components of corn stover biomass, including cellulose and hemicellulose, provide potential sources for five and six-carbon sugars. Many products can be generated from these sugars through chemical modification or fermentation to generate value-added products from corn stover. For example, some

cellulose derivatives, such as cellulose acetates, cellulose nitrates, and rayon fiber can be produced from corn stover if purified cellulose can be separated from lignin and hemicellulose.

Although current industrial research interests are focusing on the application of corn stover as energy and chemical feedstock, traditional utilization of corn stover for animal feeding is still a major outlet. Ensiled with corn grain as silage, corn stover can supply the fiber required for ruminant digestion. The economic value of directly feeding fibrous feed to animals is increasing due to the development of new forage feeding strategies, enhanced understanding of animal nutrient requirements, and new methods to improve the nutritive value of fibrous feedstuffs (Berger et al., 1994).

2.3.1 Post-harvest treatment and animal feeding

Post-harvest treatment Corn stover contains over 80% fiber on a mass basis, including cellulose, hemicellulose, and lignin. Since there is not a large amount of starch and fructan in stover, the DM intake requirements by animals depend mainly on the digestibility of these polysaccharides and polymers in cell walls. To make corn stover a digestible and nutritional food for animals, some processing is required. The goals of this processing are to increase the acceptability and availability of the stover, to increase the daily feed intake, and to increase the digestibility of stover. This processing of corn stover can be mechanical, chemical, and biological.

Among mechanical treatments, grinding and pelleting are the most common methods. These treatments can decrease particle size and increase surface area and bulk density of leaf and stem fraction of stover. The increased surface area assists in the attachment of rumen microorganisms to the corn stover. The decreased particle size speeds the hydration of the feed and decreases the salivation of ruminants (Moore, 1964; Beardsley, 1964). The animal performance feed and feed intake have been reported to improve with ground forages (Beardsley, 1964). However, the nitrogen metabolism of ruminants is adversely influenced by grinding. The reduction in nitrogen availablity is explained by the fact that forage protein is degraded by heating during grinding (Thomson and Beever, 1980). Steam treatment is another effective process for disrupting the physical structure of cell wall, with typical processing at 5-40 kg cm^{-2} for less than 5

min (Berger et al., 1994). The acetyl esters of hemicellulose are cleaved under these conditions, releasing a small amount of acetic acid. This acid will lower the pH value of stover and may be beneficial for acid hydrolysis. The enhanced animal performance resulting from steam treated forage includes increased feed intake and daily gain.

Chemical treatment is generally considered the most beneficial strategy for mature and lignified material. The chemical additives used can be classified into two groups: hydrolytic and oxidative agents (Berger et al., 1994). NaOH, NH_3, and urea are classified as hydrolytic agents. They are believed to partially solubilize hemicellulose, lignin, and silica, as well as hydrolyze uronic and acetic acid esters. Access of microbial enzymes to substrates in the rumen is greatly enhanced by these treatments, while DM intake and digestibility of forage is also increased. The increased hydration rate results in a reduced lag time and increases the digestion rate. NH_3 and urea work as hydrolytic agents similar to NaOH. In addition, they are a supplement for nitrogen, which is often limiting in corn stover. Ozone, SO_2, chlorite, and hydrogen peroxide are oxidative agents. These chemicals can actively attack and degrade a major proportion of the cell wall lignin and cleave glycosidic links, which decreases lignin content and increases soluble carbohydrates. The application of mixtures of hydrolytic and oxidative agents has also been studied, with more promising results reported than for individual chemical agents.

Biological treatment strategies include inoculation of white-rot fungi and addition of cellulolytic enzymes. White-rot fungi are capable of degrading lignin more rapidly and extensively than any other microbial species. The digestibility of forage can be improved by white-rot fungi inoculation. Enzyme additions are also well known to degrade cell walls and increase water soluble carbohydrates for ruminants (Berger et al., 1994).

To a large extent, the post-harvest treatment of forage for animal feed is similar to pretreatment of corn stover for industrial bioconversion. Some similar treatment methods have been commonly used for these two applications. However, since the ultimate destinations of these two treatments are different, some differences are expected in these two treatment operations, including the expected extent of degradation, the effect on downstream steps, and environmental compatibility.

Animal feeding Ruminants, including cows, sheep, and goats, can digest cellulose and hemicellulose in one of their digestive stomachs, the rumen, by making use of

anaerobic microbes as digestive agents. The rumen operates with a relatively constant temperature of 37 °C and pH of 6.5, and maintains anaerobic conditions. The presence of fiber in the ruminant diet plays an important role in food digestibility, digestion rate control, forage intake, and the stability of cellulolytic microorganisms. Generally, fiber will stay in the rumen about 9-12 hrs. The cellulolytic bacteria and protozoa hydrolyze polysaccharides into disaccharides (such as cellobiose) and glucose units. These released sugars are fermented into volatile fatty acids and gases, dominated by carbon dioxide and methane, which are eructated into atmosphere. The fatty acids are assimilated into the bloodstream through the rumen wall and oxidized by the animal as its main source of energy.

2.3.2 Potential value-added products from corn stover

Bioethanol, chemicals, pulp and paper, and composite products are all potentially valuable products from stover. The prospects for corn stover utilization in these applications will be discussed in this section. Corn stover can also be used as a partial substrate for fungal cellulase and hemicellulase production after appropriate pretreatment. Other uses also have been reported, including as a composting amendment for C/N ratio adjustment, and as a mulch for preventing roadside erosion.

2.3.2.1 Ethanol

Interest in biomass energy has increased dramatically in the last few decades due to a foreseen decline in worldwide crude oil production in the next few decades. Ethanol has been widely proposed as a renewable energy to substitute for fossil oil. Ethanol can also be used in oxygenated fuel as an alternative to methyl tertiary butyl ether (MTBE). Annual production of ethanol in the U.S. in 2001 was about 6.82 billion liters, an increase of 20% compared to the production in 1999 (RFA, 2002). The production of ethanol from corn grain accounts for about 5.69 billion liters. Nearly all of this ethanol is sold as E10, a transportation fuel with 10% ethanol and 90% gasoline. Recently, U.S. auto-manufacturers announced plans to greatly expand the number of vehicles which can use ethanol blend E85 (85% ethanol and 15% gasoline) and gasoline flexibly (Sun and Cheng, 2002). However, the high cost of the current feedstock, corn grain, accounts for two

thirds of total ethanol cost, making ethanol less competitive than fossil fuels without economic incentives and government subsidies.

In order to reduce the feedstock cost for ethanol production, extensive research has examined the possibility of producing ethanol from corn stover, given stover's low cost and enormous availability. The bioconversion of corn stover to ethanol has been demonstrated to be feasible and promising. However, due to technical issues involved in this bioconversion process, it is not yet economical on an industrial scale, with considerable costs associated with pretreatment, enzymatic hydrolysis, and substrate purification. Intensive research funding from industrial sources and government agencies is being invested into this promising field, and significant reduction of processing cost is expected in the next 5-10 years.

The potential ethanol production from corn stover has been estimated by several researchers working on the optimization of conversion processes. According to a report by McAloon et al. (2000), the yield of ethanol from corn stover is estimated in the range of 316-491 l dry Mg^{-1} by using a "theoretical ethanol yield calculator" (U.S. DOE,1999). This tool was developed to estimate the ethanol yield from lignocellulosic material. Based on an estimated 82 million dry Mg/year availability of corn stover (Kadam and McMillan, 2003), the potential ethanol production from 40% of the available corn stover is calculated as 11.4 billion liters, using 50% of the theoretical stover ethanol yield from corn stover (Kadam and McMillan, 2003). Kim and Dale (2004) reported that over 1 billion liters of ethanol can be produced from wasted corn grain and corn stover in North America if these feedstocks were utilized fully. Estimates of ethanol production from corn stover vary widely because the stover availability, the fractions of stover used for ethanol production, and assumed ethanol yield are determined differently by various researchers.

2.3.2.2 Chemicals

Although many chemicals can potentially be produced from corn stover as long as they can be synthesized or fermented from reducing sugars; such as furfural and xylitol. Both of these chemicals can be economically produced from corn stover with current technology. Biomass derived furfural and xylitol can be commercialized in the current

market, because other comparable synthesis routes from cheaper sources, such as fossil fuels, have not been developed. This list of economically viable products will expand enormously as oil prices increase and anticipated biomass conversion breakthroughs are achieved.

2.3.2.2.1. Furfural

Furfural, 2-furaldehyde, has been used as a selective extractive solvent in the lubricating oil sector of the petroleum industry for many years. It can also be converted to furfuryl alcohol and tetrahydrofuran. The former is used to generate "furan resins" for the metal casting industry and the latter is used as a solvent in the resins and plastics industry.

Furfural is produced by acid hydrolyzing pentosans contained in agricultural residues into xylose, followed by dehydrating xylose to furfural. The technology to use corncobs as substrate to produce furfural has been successfully developed, and large factories have been built around the world. Although part of the residue after furfural production can be burned to heat the steam needed during furfural production, excess residues cause disposal problems. Riera et al. (1991) suggested the residue can be applied as a humic fertilizer after an oxiammoniation process to neutralize the acid left from acid hydrolysis and increase the nitrogen content.

2.3.2.2.2. Xylitol

Xylitol is a compound with sweetness comparable to sucrose, but without the dietary calories. Since it is not metabolized and absorbed by the human body, it has been proposed as a sugar substitute for diabetic and trauma patients. Furthermore, its unique pharmacological properties can help prevent tooth decay and ear infections in children. These potentially wide applications of xylitol in the food industry have attracted a lot of interest from researchers trying to develop large scale production pathways.

Xylitol, a five-carbon xylose alcohol, can be generated from catalytic reduction of xylose or xylose-rich hemicellulose hydrolysates through fermentation by filamentous fungi and bacteria. Corn stover has a high concentration of xylan, which can be hydrolyzed to provide xylose for fermentation. Buhner and Agblevor (2001) used dilute sulfuric acid at 121°C to release 73% of the potential xylose from xylan chains. After

neutralization of the hydrolysates with $Ca(OH)_2$ and treatment with activated carbon, the hydrolysates were fermented by *Candida tropicalis* into xylitol. Although the xylitol yield of 0.23 g g^{-1} obtained from the hydrolysates was lower than the yield from mixed model sugars, 0.34 g g^{-1}, the feasibility of production xylitol from corn stover has been demonstrated successfully.

2.3.2.3 Pulp and paper

The papermaking potential of corn stover has a long history, having been demonstrated back in the 18th century. Pulp from corn stover can be blended with wood pulps and various grades of stover/wood paper were produced for newspapers and magazines in the 1920s. However, problems with storage, pollution, and economic factors eventually closed the one mill operating. In order to eliminate pollution problems associated with pulping, the "organosolv process" has been studied for the last 10 years. This process applies ethanol/water to digest raw material and produced carbohydrates and lignin as the main valuable byproducts.

Presently, pulp production from agricultural biomass is less than 250,000 Mg per year, representing less than 1% of total pulp production in the U.S. (Kadam and McMillan, 2003; Wong, 1997). Rowell et al. (1997) estimated that more than 95% pure pulp can be produced from corn stover through alkaline sulfide and sulfite pulping (Rowell et al., 1997).

2.3.2.4 Composite products

Bio-based composites are another potentially important product from corn stover. Composites are any combination of two or more materials held together by some type of mastic or matrix. Composites produced from baggasse and wheat straw have been commercialized all over the world, and are widely used as furniture core stock. A low-density insulation board from corn stover was produced in Dubuque, Iowa, for several decades in the middle of the last century. Kadam and McMillan (2003) estimated the demand of corn stover for medium-density fiberboard (MDF) and particleboard production as 2.2 and 6.8 million dry Mg yr^{-1} respectively, if 100% of the composites in the market were produced from corn stover.

The big challenge in using corn stalks to produce particleboard is the expensive resin binder. Economic factors associated with the resins have led to failures of several firms. Many researchers have attempted to develop more economical resins for corn stover utilization. Chow et al. (1999) examined several different resins, comapring the water resistance and dimensional stability of thermoplastic composites using recycled high-density polyethylene (HDPE) and polypropylene as binder. The results showed that most of the polypropylene composites of corn stalk had a lower average water adsorption, thickness swell, and linear expansion than those of the HDPE composites, based on ASTM standards D 955 and 1037. The thickness, width, and length shrinkages of polypropylene composites were 5, 1.5, and 0.5%, respectively, whereas the 10% virgin HDPE composite specimens cannot survive the 2-hour water-boil test. Wang and Sun (2002) also reported the qualities of low density particleboard produced from wheat straw and corn pith using methylene diphenyl diisocyanate (MDI) and NaOH-modified soy protein isolate (SPI) as resins. NaOH-modified SPI increased the tensile strength and compressive strength of the particleboard 54% and 43% respectively. They concluded that the particleboard developed in this study had the potential to be used as ceiling panels, core materials, and bulletin boards.

Although all of these corn stover derived products have promising prospects in industrial application, bioethanol is the most focused topic due to the increased concern of energy crisis and national security. The technical phases involved in ethanol production include pretreatment, hydrolysis, and fermentation. Recently, integrated strategies of combining two separate phases have been initiated. The following sections will introduce the development of these phases in recent years.

2.3.3 Hydrolysis of corn stover to sugars

Any bioconversion strategy used to produce ethanol or chemicals from corn stover will release some fraction of fermentable and/or reducing sugars from the cell wall. Chemical and enzymatic hydrolysis are the two major methods used to degrade cell wall polysaccharides in corn stover into their constituent sugars.

Chemical hydrolysis is an effective and economical operation. A solution of 75% sulfuric acid can break down the crosslink between cellulose, hemicellulose, and lignin completely. However, separation units are required to remove the extra reactants and by-products generated by chemical hydrolysis before the hydrolysates can be sent for sugar fermentation. Otherwise, many of these chemicals will inhibit the fermentation process. Furthermore, this process is expensive, requiring a high reaction temperature, anti-corrosion treatment on equipment, and waste management.

Enzymatic hydrolysis is biocompatible, environmentally friendly and also efficient for specific glycosidic linkages. However, several factors limit the wide application of enzymes for corn stover hydrolysis. First, the expense of current enzymes can be prohibitive on an industrial scale, at least to produce low value-added sugars from low-cost corn stover. These costs have dropped rapidly over the last few years, but further reductions are needed. Second, crosslinking among lignin and hemicellulose restrains the access of enzymes to cellulose and hemicelluloses in the cell wall. In addition, hemicelluloses are complicated hetero-polysaccharides with various monomers and hetero-linkages on branch and main chains. Auxiliary enzymes may be required to break down branch linkages to expose the bonding site on the backbone structure for primary function enzymes. For example, purified xylanase can be used to hydrolyze the resistant substrate xylan, but in one study only 18% of the potential xylose was released from corn fiber xylan after treating with xylanase for 72 h (Hespell et al, 1997). In contrast, complete enzyme systems derived from yeast-like fungus *Aureboasidium* have been reported to release 70% of xylose and all arabinose and glucose in 48 hr (McMillan, 1994).

To effectively and economically bioconvert corn stover through enzymatic hydrolysis, additional processing is required to address these limitations. Various pretreatment strategies have been developed to break the crosslink between polysacchrides and polymers and/or remove lignin and hemicellulose before enzymatic hydrolysis.

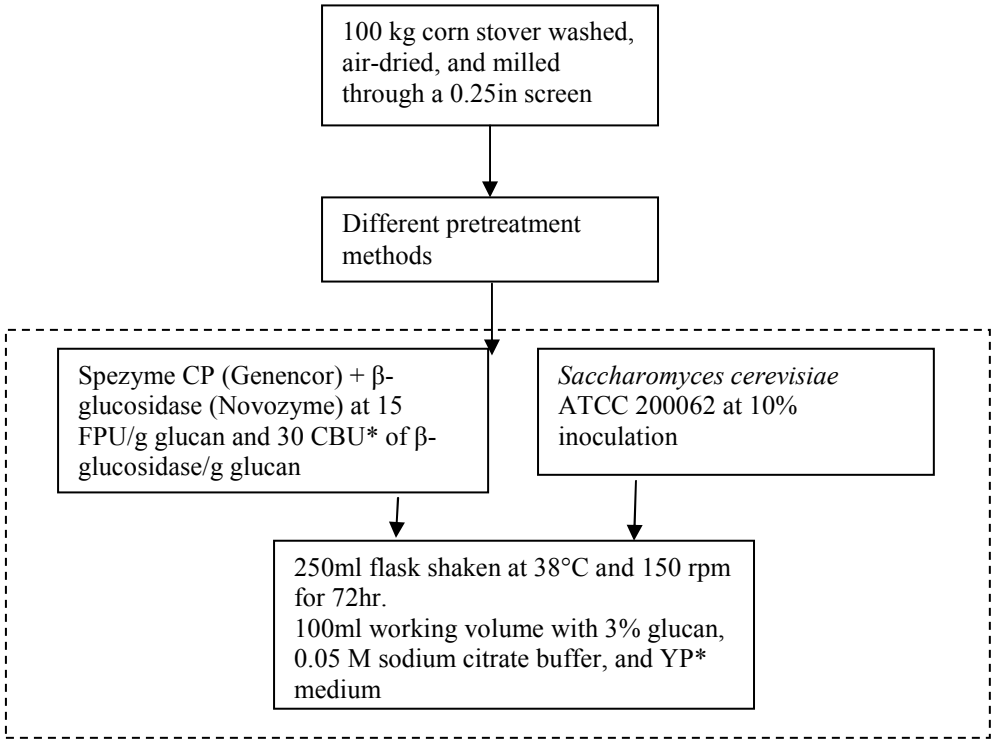
2.3.3.1 Pretreatment

It has been widely accepted that pretreatment of corn stover is required to obtain theoretical sugar yield by enzymatic hydrolysis. The purposes of pretreatment are to break the links among lignin and hemicellulose; loosen and disrupt the crystal region of cellulose; and increase the porosity of the raw materials. Pretreatment methods can be physical, chemical, physical-chemical, or biological. A successful pretreatment method should have these characteristics: 1) increases the efficacy of subsequent hydrolysis, 2) avoids degradation or loss of carbohydrates, 3) avoids the formation of inhibitory byproducts, and 4) cost-effective (Sun and Cheng, 2002).

More than ten different pretreatment methods have been developed and optimized for various lignocellulosic biomass (Sun and Cheng, 2002; Lloyd and Wyman, 2005; Liu and Wyman, 2005; Mosier et al., 2005a; Teymouri et al., 2005; Kim and Lee, 2005; Kim and Holtzapple, 2005). The features of nine promising pretreatment technologies for lignocellulosic material were summarized by Mosier et al. (2005b), including uncatalyzed steam explosion, liquid hot water, pH controlled hot water, flow-through liquid hot water, dilute acid, flow-through acid, ammonia fiber/freeze explosion (AFEX/FIBEX), ammonia recycle percolation (ARP), and lime pretreatment. Although the effects of these various pretreatment methods on chemical composition and chemical/physical structure of corn stover have been qualitatively compared, quantitative comparison of their effects on downstream enzymatic hydrolysis is difficult because of the different substrate characteristics, enzyme loading, and hydrolysis conditions investigated (Wyman et al, 2005). For example, Kaar and Holtzapple (2000) chose calcium hydroxide in the form of pebble quicklime for corn stover pretreatment. Conversion yields approaching 100% of total polysaccharides were obtained at an enzyme loading rate of 25 FPU (filter paper unit)/g dry biomass with optimized pretreatment parameters. This is much higher than the 77.5% of total polysaccharides previously reported by MacDonald et al. (1983), who used a dilute sodium hydroxide pretreatment followed by an enzyme rate of 2400 FPU/g dry biomass. In addition to this difference in enzyme loading levels, the hydrolyzing enzymes themselves were also different. Kaar and Holtzapple (2000) employed β-glucosidase and cellulase while cellulase and hemicellulase were used in the study of MacDonald et al. (1983). Therefore, although the sodium hydroxide pretreatment had

lower sugar yield with a much higher enzyme rate than those of calcium hydroxide, it is hard to claim that latter method had a better pretreatment effect. The lower sugar yield may also be explained by the various efficiencies of the enzymes used and the synergies of the enzyme mixtures.

To compare these pretreatment technologies on the same basis and understand the relationship between these methods and other subsequent operations, a multi-institutional research team studied the effect of seven leading biomass pretreatments on a single uniform batch of corn stover feedstock. Dependent variables included the sugar recovery rate, monosaccharide fate, and subsequent ethanol yield (Wyman et al., 2005a; Lloyd and Wyman, 2005; Liu and Wyan, 2005; Mosier et al., 2005a; Teymouri et al., 2005; Lim and Lee, 2005; Kim and Holtzapple, 2005; Wyman et al., 2005b). Identical analytical methods and a consistent approach for data interpretation were employed by the members of the group. The common process for stover preparation, pretreatment, enzymatic hydrolysis and simultaneous saccharification and fermentation (SSF) is provided in Figure 2.4. The reaction conditions of the pretreatment technologies, sugar yields, and the pros and cons of each technology are summarized in the Table 2.2



* CBU: cellobioase unit; YP medium: 10% yeast extract and 20% peptone

Figure 2.4 A flow chart illustrating pretreatment, saccharification and fermentation of corn stover.

Table 2.2 Technologies, representative reaction conditions, sugar yields, and pros and cons for corn stover pretreatment.

Pretreatment technology	Chemicals used	Temperature, °C	Pressure, atm	Reaction times, min	Concentration of solids, wt.%	Results	Pros and Cons	References
Dilute sulfuric acid-cocurrent	0.22-0.98% sulfuric acid	140-200	3-15	2-30	10-40	over 92.5% of sugars recovered at 15FPU/g glucan 15% sugar released in pretreatment as glucose	High sugar recovery Costly materials of construction Neutralization of hydrolyzate required Slow cellulose digestion by enzymes Binding of enzymes to lignin	Lloyd and Wyman, 2005
Flowthrough pretreatment	water	200	20-24	24	2-4	96.6% of sugar recovered at 15FPU/g glucan	No extraneous reagents are needed Large amount of water consumed High energy requirement	Liu and Wyman, 2005
pH controlled water pretreatment	water	170-200	6-14	5-20	16	87.2% of sugar recovered at 15FPU/g glucan 88% of theoretical ethanol yield	No extraneous reagents are needed Minimized degradation of sugars to aldehydes Large amount of water consumed High energy requirement	Mosier et al., 2005
AFEX/FIBEX*	100% (1:1) anhydrous ammonia	60-110	15-20	<5	62.5	94.4% of sugar recovered at 15FPU/g glucan 96% of theoretical ethanol yield	Moderate temperature Minimized sugar degradation with high sugar yield High cost of ammonia and recovery operation	Teymouri et al., 2005
ARP*	10-15 wt. % ammonia	110-170	9-17	10-20	15-30	89.4% of sugar recovered at 15FPU/g glucan 84% of theoretical ethanol yield	Ammonia is separated and recycled Large extent of delignification Less efficient for softwood based pulp mill sludge	Kim and Lee, 2005
Lime	0.5g $Ca(OH)_2$/g biomass	25-55	1-6	4 weeks	5-20	86.8% of sugar recovered at 15 FPU/g glucan	Lower temperature and pressure than other pretreatments Longer pretreatment time Chemicals are expensive and difficult to recycle	Kim and Holtzapple, 2005

* AFEX/FIBEX : ammonia fiber/freeze explosion; ARP: ammonia recycle percolation

2.3.3.2 Enzymatic hydrolysis

Many researchers have hydrolyzed corn stover using cell wall degrading enzymes after pretreatment. Various conversion yields have been reported because of the differing effects of applied pretreatments, the enzyme types and activities, and hydrolysis conditions. For example, a 60% conversion yield of total polysaccharides with 13 FPU cellulase g^{-1} dry mass is obtained after a dilute sulfuric acid pretreatment (Wilke et al., 1981). Elshafei et al. (1991) reported 100% cellulose conversion with 3000 FPU cellulase g^{-1} dry mass. Montross and Crofcheck (2003) obtained over 80% cellulose conversion by applying cellulase at the rate of 13.25 FPU cellulase g^{-1} dry mass. However, in these studies, the activities of the specific cellulase and/or hemicellulase enzymes were not elucidated. Generally, commercially available enzymes contain both cellulase and hemicellulase in various ratios. Some of these studies only monitored glucose concentration during hydrolysis, and the reducing sugars degraded from hemicellulose, such as xylose and arabinose, were not investigated. Given these discrepancies, it is difficult to identify an optimum strategy for enzymatic hydrolysis from the existing literature.

The cost of cell wall degrading enzymes is the major barrier to industrial bioconversion of corn stover into sugars. As recently as 2000 the enzyme cost was 13.2¢ for each liter of ethanol production (Hettenhaus et al., 2000a). Hettenhaus et al. (200a) predicted the cost could be reduced to 1.32¢ per liter if 25-50 full time equivalent researchers could focus on this aspect conversion, but the R&D investment was viewed as a significant investment risk. If 30% of total available corn stover could be converted into ethanol with a factor of 10 improvements in enzyme performance, they estimated an enzyme market of $400 million could be created. However, only $17 million profit could be generated from the ethanol produced by fermenting the sugars derived from corn stover. The large discrepancy between the required investment and the potential for profit is a major constraint to implementation on an industrial scale. Nevertheless, continuing advances in technology and processing will reduce the costs of bioconversion processes, and they are expected to become competitive soon.

2.3.4 Ethanol production from corn stover

Researchers have been developing strategies for bioconversion of corn stover to ethanol since the 1970s. A tentative processing scheme for ethanol production from corn stover was presented by Wilke et al. (1981), consisting of pretreatment, enzyme production, enzymatic hydrolysis, and ethanol fermentation, successively. This initial strategy provided the basis for further development in the bioconversion of corn stover biomass.

Wilke et al. (1981) analyzed the cost of ethanol production through the processes in their initial shceme (Table 2.3 and Table 2.4). The processing cost was estimated as \$0.47 l^{-1} ethanol, with a yield of 100 l ethanol/Mg corn stover. The by-product credits from yeast cake and mycelium (\$22 kg^{-1} respectively) were not considered into this cost. Their analysis indicated that the cost of ethanol was primarily dependent on four factors: the cost of biomass, the extent of enzymatic hydrolysis, the recovery yield of enzymes, and potential utilization of xylose.

Table 2.3 The cost of glucose production from corn stover (1252 Mg corn stover day^{-1}, Wilke et al., 1981).

Glucose production from corn stover						
	Milling	Acid pretreatment	Hydrolysis	Enzyme recovery	Enzyme make-up	Total
Fixed capital cost (× \$ 1000)	3375	5150	8684	1937	10261	29,407
Annual capital-related costs (× \$ 1000)	810	1236	1798	465	2463	6771
Annual labor-related costs (× \$ 1000)	96	191	191	96	191	768
Annual utilities costs (×\$ 1000)	109	451	657	62	62	2088
Annual material costs (× \$ 1000)	-	1238	5	-	-	4452
Annual manufacturing cost (× \$ 1000)	1105	3116	2697	623	623	14100
Glucose cost(\$/kg)	0.016	0.049	0.042	0.01	0.10	0.22

Table 2.4 The cost of ethanol production from corn stover (Wilke et al., 1981).

Ethanol production from glucose	Sugar concentration	Fermen-tation	Dis-tillation	Medium	Glucose	Methane	Total
Capital investment (× $ 1000)	800	2500	500			3800	7000
95% Ethanol cost($/liter)	0.014	0.02	0.0079	0.055	0.36	0.017	0.47

More recently, ethanol production through simultaneous saccharification and fermentation (SSF) by *Clostridium thermocellum* has attracted intensive interest because of its evident advantages over the multiple-step process of fungal enzymatic hydrolysis and yeasts fermentation (Lynd, 1989). Although the low ethanol tolerance (1.5% v/v) and yield (0.08-0.29 g/g) of wildtype *C. thermocellum* limit its commercialization, screening of strains and optimization of techniques are expected to overcome these limitations (Sudha Rani et al., 1996). Thermophilic and anaerobic *C. thermocellum* strains, SS21 and SS22 have been reported to produce 0.37 and 0.33g ethanol per g cellulose. These two strains can be tolerant of 4.0 and 5.0% (v/v) ethanol and up to 7.0 and 8.0% (v/v) with an increase of culture age (Sudha Rani et al., 1998).

To reduce the processing and production costs, maximizing the potential of ethanol production from the hydrolysed monosaccharides should also be considered. The previous strategies relied on metabolic pathways that only convert the 6-carbon sugar glucose to ethanol, which is the case with commercial production of ethanol from grain today. The hydrolyzed sugars from corn stover include a considerable amount of xylose, arabinose, and galactose, which are 5-carbon sugars. Fermentation of these hemicellulose derived sugars into ethanol will increase the conversion yield of corn stover significantly. Over the last decade, one of the most significant advances in the biomass conversion field is the development of microorganisms capable of converting xylose and other 5-carbon sugars into ethanol at high yields.

Xylose utilization strategies have been examined in *Saccharomyces cerevisiae* (Lynd et al., 2002). Other researchers examined the xylose fermentation to ethanol using a recombinant strain of *Pachysolen tannophilus* (Kuyper et al., 2004). Several metabolism pathways of this strain have been explored, and one of which is illustrated below (Figure 2.5). Xylose is first converted to xylulose, which is dissimilated via the pentose–phosphate pathway after phosphorylation. Overall ethanol yield of 0.39 kg/kg

and a specific ethanol-production rate of 0.06 kg/kg h were observed, which are comparable to those of glucose fermentation. Xylitol is produced as a by-product with an overall yield of 0.14 kg/kg. The development of several engineered microorganisms is advancing rapidly, and these are being considered for use in commercial processes.

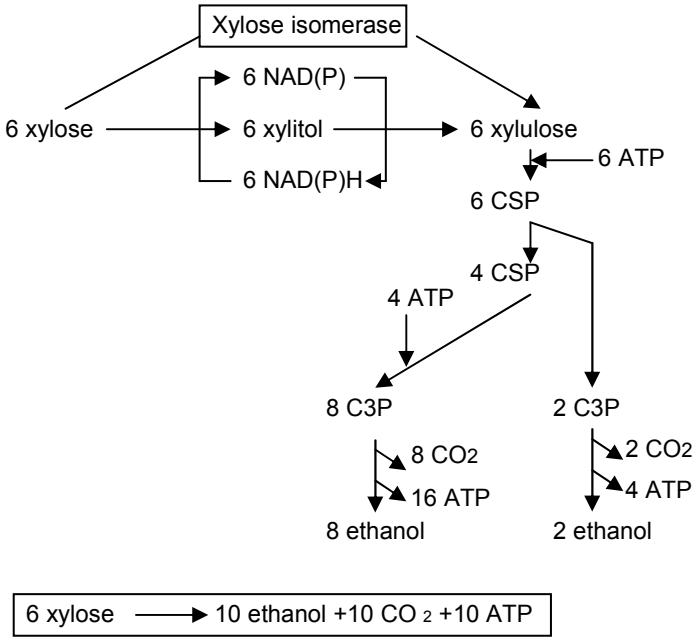


Figure 2.5 Metabolic pathway for alcoholic fermentation of xylose with a calculated redox balance (Modified from Kuyper et al., 2004).

2.4. Silage

Although several aspects of large scale utilization of corn stover, such as pretreatment, bioconversion, and board manufacturing, have previously been investigated (Karr and Holtzapple, 2000; Yang et al., 2001; and Wang and Sun, 2002), long-term storage is one essential step that has not been adequately addressed. Industrial uses of corn stover will require long term storage of up to one year, because the material harvested in one growing season has to supply the industrial processing refineries continuously with feedstock over the whole year. Current methods use dry storage to minimize decomposition of corn stover. However, the low moisture content of dry stover, ranging from 14% to 33%, creates a high risk of fire with dry storage. Furthermore, up to 23% dry matter loss has been reported in bale and stack storage for corn stover due to

plant and microbial respiration (Richey et al., 1982). Although storing at widely separated sites will reduce the loss in case of fire, the cost of facilities and transportation may make this method unpractical. In order to solve these problems, an ensilage process has been proposed as a preservation method for the large amounts of corn stover. The high moisture content, up to 60% (d.b.), can eliminate the risk of fire entirely (Richard et al., 2001). Further, saccharification of cellulose and hemicellulose, and fermentation of water soluble carbohydrates to organic acids, will naturally occur during the ensiling process. These features suggest that ensilage of corn stover can also be considered as a pretreatment strategy for biomass feedstock destined for downstream conversion to value-added products. This dissertation explores both of these options, evaluating strategies for ensilage that provide both storage and pretreatment benefits.

2.4.1 Principles of ensiling

Ensiling is a traditional preservation process for crops that are not readily available for ruminant feeding during the winter season. During ensiling, the rate of decomposition and deterioration of crops is reduced to a very low level by inhibiting microbial activity through low pH and anaerobic conditions. These conditions can be obtained by encouraging lactic acid fermentation and sealing the silage materials from air.

Under anaerobic condition, fermentation will occur if sufficient sugars are available. Water soluble carbohydrates (WSC) in crops are fermented by naturally occurring lactic acid bacteria (LAB) into organic acids: predominantly lactic and acetic acids. A quick pH decrease to 4.0-4.5 can be obtained, with these organic acids, especially lactic acid with a pKa of 3.86. Undesirable microbial growth, including clostridia and enterobacteria, can be significantly inhibited at pH values below 4. Clostridia and enterobacteria are undesirable, because they can consume lactic acid, cause pH to increase, and activate other microorganisms to result in further biomass loss. Some of the enterobacteria are pathogens which are detrimental for people, animals, and plants.

Sealing a silage pile to insure anaerobic conditions is critical to inhibit yeast and mold germination at such high moisture level. These microorganisms can deteriorate biomass with the presence of oxygen at high moisture contents. Exclusion of oxygen can

also prevent plant autorespiration and reduce the degradation associated with plant enzymes. Good quality silage can be stored at low pH and anaerobic conditions for one year or even longer.

2.4.2 Four phases of ensiling

The ensiling process can be segregated into four sequential phases: the field phase, an aerobic loading phase, stable storage phase, and an unloading phase for utilization (Weinberg and Ashbell, 2003). Several significant biochemical and microbiological changes occur during these phases.

Field phase After harvesting, the dry matter (DM) concentration of crops should be in the range of 300-400 g kg^{-1} biomass (w.b.) through field drying. High DM can inhibit clostridia growth and prevent the production of effluent from silos, especially tower silos. The major components of silage effluent are WSC, true protein, lactic acid and acetic acid. Some mineral elements, such as potassium, calcium, magnesium and copper, are also contained in the effluent. This effluent represents a nutrition loss in conventional silage, and is a potential water pollution source because of the high BOD values. According to the regression relation between DM content and effluent production reported by Bastiman (1976), no effluent will be produced with a DM concentration over 290 g kg^{-1} herbage (w.b.). The time required for field drying is mainly dependent on weather conditions and the type of mechanical treatment. Two or three days drying period is reported to be sufficient to obtain a DM content of 350 to 450 g kg^{-1} for grass under good drying condition (McDonald et al., 1991). Chemical additives, such as desiccants and volatile fatty acids, and thermal treatment using hot gases and steam are also used to increase the drying rate of crops.

During field wilting, some of the water soluble carbohydrates will be consumed by microbial respiration. Lactic acid bacteria (LAB) multiply during this period, as harvest and cutting equipment liberates sap and provides more uniform inoculation (Henderson et al., 1972; Fenton, 1987; McDonald et al., 1991; Pitt et al., 1985). Other microorganisms, such as yeasts and enterobacteria, will also develop under humid and warm conditions during the drying period.

Aerobic loading phase After field drying, crops are loaded and stored in sealed silos until unloading for feeding or other uses. Silos come in a variety of types, including tower silos, clamp or bunker silos, and big wrapped bales. Of these various types, tower silos are probably the most effective, with minimal air exposure of the silage. In addition, pressure from the biomass weight aids the consolidation of silage and reduces air penetration. However, too high pressure will cause serious effluent problems and structural damage to silos, especially on the bottom wall of the tower. Other types of silos are also used according to farmer's preferences and tradition. Silos should be filled quickly and compactly to minimize trapping air in crops, and to insure a high degree of consolidation to prevent future air infiltration. In a filled and well sealed silo, 90% of the initial atmospheric oxygen will be used up in 15 min through plant and microbial respiration, with only 0.27% of glucose calculated to be lost through respiration of this entrapped oxygen (McDonald and Whittenbury, 1973). Sealing should be done immediately after loading to minimize air penetration and the resulting WSC loss. Delaying sealing for 24 hr has been reported to lead to more WSC consumption by aerobic microbial metabolism, leaving less available WSC for the ensiling process. As a result, less lactic acid will be produced, and clostridia are more likely to dominate the fermentation during storage. However, a contradictory finding has been reported by Adogla-Bessa et al. (1995, 1999), who observed that delaying silo sealing by 24 h improved lactic acid fermentation and enhanced the aerobic stability of wheat silages later when the silos were opened for unloading. They explained that the high temperatures caused by plant autorespiration prior to sealing encouraged the proliferation of lactic acid bacteria and thus their higher prevalence during subsequent.

Stable storage Significant changes of biochemistry and microbiology occur in the first several days of ensiling under anaerobic conditions. The type of fermentation initiated in this period will dominate the whole ensilage process and determine the final quality of silage. When WSC is sufficient, homolactic fermentation will be initiated, and maximum counts of lactic acid bacteria can be reached at 10^9 CFU/g dry biomass within two to seven days (McDonald et al., 1991). Due to the formation of the major product, lactic acid, a quick drop of pH from 6-8 to below 5 will be obtained.

If the WSC in crops is insufficient to initiate homolactic fermentation, volatile organic acids, lactic acid, and even amino acids will be metabolized through heterolactic fermentation. The resulting final metabolic products can include lactic acid, ethanol, acetate, 2, 3-butanediol, succinate, ornithine, and acetoin. These products will increase the buffering capacity of the silage considerably, so that the pH can not drop quickly and effectively. Clostridia and enterobacteria will be encouraged in such conditions. Much greater losses of DM and energy have been reported when ensilage is dominated by clostridia and enterobacteria. Carbon dioxide and hydrogen production from these secondary fermentations can partially contribute to these high losses.

Air infiltration during the ensiling process will activate yeasts and molds, potentially resulting in DM losses of nearly 40% and producing CO_2, heat and ethanol that change the composition of the final metabolic products (McDonald et al., 1965). Heat generated from oxidation of DM by these microorganisms will raise silage temperatures and accelerate some detrimental reactions. For example, amino acids or proteins can bind with WSC in Millard reactions, while new linkages among carboxyl groups and hydroxyl groups in amino acids will also be formed. These linkages will decrease the solubility of protein and resist proteolysis during subsequent animal feeding or bioconversion processes.

Unloading phase for utilization When a silo is opened and silage is unloaded for utilization, any aerobic microorganisms which remain dormant during ensiling are activated by the invasion of air. Losses of DM and nutritional value result from the oxidation of lactate, acetate, and WSC by these microorganisms. The indicators of aerobic deterioration include a temperature rise, CO_2 production, and the appearance of yeast and mold. This deterioration can be minimized by renewing the unloaded face often and compacting the remainder. Consumption of the silage in the same day as it is removed from the silo will shorten the development time of the detrimental microorganisms. Short field drying, quick filling, and immediate sealing before ensiling will also limit the deterioration in the unloading phase. Volatile fatty acids, such as isovaleric acid and caproic acid, have been used as silage additives to successfully enhance aerobic stability of silage.

Ensiling of corn stover for biomass will follow the four ensiling phases of traditional forage crops. However, the biological and chemical characteristics of stover silage during these phases can be expected to be significantly different from those of conventional crops due to the initial low content of WSC in stover and the different intended use. These distinct aspects of stover silage will be considered in the proposed research.

2.4.3 Microorganisms important to ensiling

The chemical and biochemical changes occurring during these four phases are caused by the activities of diverse and adaptive microbial ecosystem. Understanding the development and change of microorganisms involved in the ensilage process can improve the process and final quality of the silage. Although the total number of bacteria on fresh grass has been shown to vary between 10^6 and 10^9 cells g^{-1} DM, the great majority of these bacteria are strict aerobes and contribute little or nothing to silage preservation. After anaerobic conditions are obtained, the anaerobic microorganisms (lactic acid bacteria, enterobacteria, clostridia, yeasts) begin to proliferate and compete for available nutrients. The changes in the first few days are vital in determining the dominate fermentation regime throughout the whole preservation period (McDonald et al., 1991). If the initial substrate level is appropriate, the silage will be quickly acidified by lactic acid fermentation to such a degree that the other organisms can not compete, and the biomass is consequently preserved.

2.4.3.1 Lactic acid bacteria

Lactic acid bacteria are gram-positive rods and cocci. They anaerobically ferment sugar into lactic acid and/or acetic acid as their main products. They are aerotolerant anaerobes since they grow anaerobically, but are not sensitive to oxygen and can grow in its presence as well as in its absence (McDonald et al., 1991). An important feature of the lactic acid bacteria in silage is their ability to lower the silage pH. Some species, such as *Pediococcus* and *Lactobacilli*, can reduce the medium pH to 3.5.

It is surprising that lactic acid bacteria can dominate fermentation within just 2-4 days of ensiling, since the number of lactic acid bacteria on fresh growing crops is

extremely low. A rapid increase in numbers of lactic acid bacteria has been reported to occur from harvesting to ensiling. Two possible explanations are inoculation from farm equipment, followed by multiplication in the sap liberated during harvest. However, Pahlow and Ruser (1988) demonstrated that these inoculation and substrate sources can not result in such large increase in LAB numbers. In addition, the short time span during this period is not enough for such rapid bacterial multiplication. They suggested that the LAB counts reported previously for the fresh crops may be underestimating the true count, because of loss in viability before crop samples are analyzed in the lab.

The substrates for lactic acid fermentation under anaerobic conditions include a wide range of sugars: glucose, xylose, fructose, arabinose, as well as lactose. Lactic acid bacteria are divided intro three physiologically distinct groups according to their metabolism pathways: 1) obligate homofermenters, 2) facultative heterofermenters, and 3) obligate heterofermenters. Obligate homofermenters convert hexoses exclusively to lactic acid without fermenting pentoses and gluconate. Only fructose bisphosphate aldolase is present in their enzyme systems. Facultative heterofermenters ferment hexoses exclusively into lactic acid, but also convert pentoses into lactic acid and acetic acid with an inducible phosphoketolase. Obligate heterofermenters ferment hexoses to lactic acid, acetic acid, and/or ethanol and carbon dioxide. The proliferation of these three LAB bacterial groups will be dependent on the initial sugar type, composition, and concentration (McDonald et al., 1991).

2.4.3.2 Clostridia

Clostridia are gram-positive, sproulating, strict anaerobic bacteria. They can ferment sugars, lactic acid or proteins into fatty acids (McDonald et al., 1991). The inoculation of clostridia is thought to be from soil contamination since clostridia numbers on green plant material are generally quite low. Manure slurry is probably another possible source of clostridia contamination because manure can contain significant levels of spores.

Clostridia can be classified into two groups: saccharolytic clostridia and proteolytic clostridia, according to the substrates it assimilates. The former mainly ferments sugars and organic acids and the latter ferments amino acids, while some

clostridia have both types of activity. Most lab methods for clostridia enumeration only detect spores, but there is a poor correlation between spore numbers and the products of clostridia fermentation. Measurement of vegetative clostridial activity requires biochemical or molecular techniques.

During the ensiling process, clostridia activity is encouraged by low DM content, high silage temperature, low WSC content, high pH value and high buffering capacity of the silage crop. Clostridia can compete for the substrate with lactic acid bacteria (LAB), consume the degraded sugars by enzymatic hydrolysis, and ferment lactic acid. Clostridia growth disrupts the preservation process by converting lactic acid into butyric acid, initiating a chain that reduces the effectiveness of crop preservation. Since butyric acid is a weaker acid than lactic acid, clostridia growth will result in an increase of pH. This increased pH will activate undesirable bacterial growth, including more clostridia and enterobacteria, and the resulting microbial decomposition will cause biomass loss. Clostridia also reduce the nutritional value of the silage by proteolysis of protein and amino acids, which is especially a problem in ruminant feed. Furthermore, for conventional animal feeding, clostridia spores contaminate milk and cause “late blow” in hard cheeses. The existence of clostridia is also a health hazard for animals fed with silage, and has been reported to be responsible for botulism in horses and cattle. Because of all these problems, domination of clostridia in corn stover silage can be viewed as a failure of the preservation system.

The optimum pH for clostridia growth is 7-7.4, and they can not tolerate strongly acidic conditions. If a sufficient amount of lactic acid is produced to lower the pH to a critical level, clostridia growth is inhibited. A pH of 4.2 is usually considered sufficiently low in conventional ensilage processes.

2.4.3.3 Yeasts and molds

In a conventional forage ensilage system, mold and yeast may persist and increase during aerobic phases, including the field phase, the loading phase, and the unloading phase. However, some fermentative yeasts can exist in the silage storage phase and convert glucose into ethanol anaerobically. The growth of these yeasts is not inhibited by typical silage pH because their optimum pH is in the range of 3.5-6.5. However, short-

chain organic acids, such as lactic acid and acetic acid, can depress the activities of these yeasts.

Low pH and anaerobic conditions are not favorable for mold growth either. The existence of molds may be observed at the corner of a silo or on the surfaces of silage, in areas that are possibly exposed to air. Molds will not only consume water soluble carbohydrates and lactic acid, but some cellulosic strains can also degrade the cell wall to cause biomass loss. Some molds produce mycotoxins that can be harmful to people and animals.

2.4.4 Silage additives

Because of the low content of WSC in stover, difficulties in initiating lactic acid fermentation in stover silage can be expected. For traditional forage crops with low content WSC, cell wall degrading enzymes and acidic additives are used to improve preservation of the crops. In the current research, these additives were applied to stover silage to examine their effects on preservation and pretreatment of corn stover for downstream conversion. Before investigating the feasibility of using these additives in stover silage, typical applications in common forage crops will be discussed.

Additives are sometimes applied during forage harvesting or silo filling to ensure good silage quality. Over forty kinds of additives, including lactic acid bacteria, enzymes, carbohydrate sources, acids, urea, and polymers have been used, acting by various mechanisms (McDonald et al., 1991). These additives help ensure good quality silage by encouraging lactic acid fermentation, inhibiting undesirable microbial growth, enhancing aerobic stability, enriching nutritional value, or absorbing effluent. These additives can be classified into five categories according to their functions: fermentation stimulants, fermentation inhibitors, aerobic deterioration inhibitors, nutrients, and absorbents (McDonald et al., 1991). Some additives actually have multiple functions. For example, LAB inoculation and some chemicals can encourage lactic acid fermentation and also enhance aerobic stability.

Acidic additives belong to the category of fermentation inhibitors because they reduce the pH value of silages to a very low level to restrain microbial growth and totally inhibit the activity of proteolytic enzymes. Before investigating the use of these additives for stover preservation, previous research results will be reviewed. However, it is

important to remember that in this previous research, the recommended levels of the acidic additives were based on the goal of feeding the silage to ruminants. Industrial corn stover preservation has a different goal, with different opportunities and constraints.

2.4.4.1. Acidic additives

The use of mineral acids as additives has a long history, being first advocated in 1885 (Watson and Nash, 1960). A few decades later Virtanen (1933) described the AIV process (named after A. I. Virtanen), which estimates the amount of acid needed to lower the pH of silage to 3.5. Playne and McDonald (1966) designed another method in which the pH value of crop is first reduced to 3 by 0.1 M hydrochloric acid and then titrated to 6 with 0.1 M sodium hydroxide. Sulfuric acid, hydrochloric acid and phosphoric acid or their acid salts are the major components of commercial acidic additives. Although the initial purpose of these additives was to suppress all fermentation by inhibiting microbial activity, they are currently used at lower levels to encourage the natural lactic acid fermentation process. Because LAB have a higher degree of acid tolerance than clostridia and enterobacteria, appropriately lowering pH by acids can inhibit these undesirable bacteria, retaining any the available substrate for the LAB. The effect of these additives on LAB growth depends on the initial WSC content in silage, the composition of the additives, active components, and the concentration of applied additives. Sulfuric acid has been reported to inhibit the activity of undesirable bacteria such as enterobacteria and clostridia (McDonald et al., 1991).

Organic acids, especially formic acid, are known to have antibacterial action owing to both the hydrogen concentration effect and selective bactericidal action of the undissociated acid. The effect of formic acid on microbial growth and chemical composition of silages varies widely depending on the rate applied. McDonald et al. (1991) concluded that while low levels assisted lactic acid fermentation, a high level severely restricted the fermentation. The intermediate level inhibited lactic acid bacteria more than enterobactria, since the later can generate formic acid and thus more easily tolerate it. Increased levels of formic acid resulted in decreasing levels of lactic and acetic acids and increasing WSC. When high levels were applied, WSC can even exceed the original concentration in silage, probably due to acid hydrolysis of polysaccharides.

Because of variations in the buffering capacity and sugar content of substrate, identical levels of formic acid can result in positive or negative effect on fermentation and chemical composition on various species of crops.

Other organic acids, such as lactic acid, benzoate, and acrylic acid have also been examined with respect to their inhibition ability to inhibit bacteria of various species (McDonald et al., 1991). Higher molecular weight fatty acids, including decanoic, dodecanoic, tetradecanoic and so on, have also been investigated as silage additives, although disappointing results were obtained.

Corrosion, handling problems and human health risks are associated with acidic additives. Acid salts can be used as a safer, less corrosive substitute for mineral acids or formic acid. Because of the lower acid content, the salts of acidic additives must be applied at a higher rate to obtain a similar effect on silage quality. Ammonium tetraformate, a complex acid salt, has been commercialized as a silage additive, although studies could not demonstrate the same efficiency as formic acid on various species of crops, even at a higher rate (Drysdale and Berry, 1980).

2.4.4.2. Cell wall degrading enzymes

In recent years, extensive research has focused on two groups of additives: the application of cell wall degrading enzymes, and inoculation of lactic acid bacteria, because of their ability to improve the digestibility of crops, milk yield of cows, and body weight gain by ruminants. Although both LAB inoculation and enzyme addition share these benefits, only enzyme amendments will be reviewed here and were investigated in the present study.

The purpose of adding enzymes in silage is to encourage lactic acid fermentation by degrading cell wall into WSC. This method of supplementing WSC is essential to initiate lactic acid fermentation for crops with low sugar content. Addition of the enzymes can also improve the rate and extent of silage digestion for ruminants.

2.4.4.2.1. Effect of cell wall degrading enzymes

The commercial enzymes used as silage additives generally contain cellulase, hemicellulase, α-amylase, pectinase, etc. The cellulase and hemicelluase in the enzyme

mixtures play a major role on cell wall degradation and result in hexose and pentose sugar production. As discussed previously, plant have various structural arrangements of cellulose and hemicellulose, and these will response differently to cellulolytic and hemicellulolytic enzymes. Van Vuuren et al. (1989) demonstrated that the degradation of the cellulose fraction increased by 14% in alfalfa silage with an enzyme treatment. In the same experiment hemicellulose degradation only increased by 2%, although the activities of cellulase and hemicellulase were equivalent in the treatment. It seems that cellulose degradation is more sensitive to cellulase addition than is hemicellulose to hemicellulase. Dewar et al. (1963) suggested the complex structure and various monomers of branch chains of hemicellulose increased the difficulty of enzymatic hydrolysis by impeding the spatial access of enzymes. The addition of hemicellulase mostly increases the hydrolysis rate of the side chains instead of enhancing the hydrolysis of the β-1→4 linkages in the main chains. The fact that cellulase can improve degradation of cellulose to a greater extent can be attributed to the simple linear structure of cellulose, with uniform β-1, 4 linkages throughout.

Before applying cell wall degrading enzymes as silage additives, it can be helpful to determine the optimal pH and temperature as well as the thermal stability of these enzymes to make sure they will still be active at the acidic pH values of the silage process. Colombatto et al. (2004a) examined these parameters of two industrial enzyme products derived from *Trichoderma* before they applied the enzymes to silage. The optimum pH and temperature of the xylanase and endoglucanase in these two enzyme products were determined as 5.6 and 45°C. Three enzyme product concentrations plus a control treatment were applied to the silage. Increasing the concentration of the two enzyme products increased fiber degradation. All silages were well preserved, as evidenced by final pH values below 4.0. The thermal stability of xylanase was greater at lower pH values, while higher temperatures reduced stability. However, this negative impact took time to manifest, and was not noticeable in the initial 48h incubation time. Furthermore, the inhibiting effect of high temperature significantly decreased as pH rapidly dropped.

Colombatto et al. (2004b) compared the effect of three enzymes derived from psychrophilic (*Flavobacterium xylanivorum*), mesophilic (*Trichoderma reesei*), and thermophilic (*Thermoascus aurantiacus*) sources on maize silage. Although enzymes

derived from the thermophilic organism exhibited higher stability than their mesophilic counterparts, they stated that the addition of enzymes from the mesophilic source tends to result in a lower pH than that of the thermophilic sources.

The effect of enzymes on the silage composition and stability of whole-crop wheat harvested at the four different maturities was investigated by Adogla-Bessa et al. (1999). They found that the effect of enzymes was more pronounced in less mature crops, while silage stability increased as crops matured. In the same experiment, increasing levels of enzymes, from 366 to 1099 ml Mg^{-1} DM, did not influence silage composition. This was in contrast to the earlier work of Weinberg et al. (1995), who found a significant linear regression with the log of enzyme concentration for pH, WSC, lactate, neutral detergent fiber (NDF) and acid detergent fiber (ADF).

Since enzymes was firstly proposed as additives to encourage lactic acid fermentation in 1961 (Olsen and Voelker, 1961), numerous studies have focused on the effects of enzyme application for various forage crops. Some of these research results are summarized in Table 2.5, which presents the enzyme parameters including types, rates, and activities, as well as the subsequent effects on silage quality. Although it is evident that enzyme addition can improve lactic acid fermentation in various crop silages and lower pH value, it is difficult to compare the effect of specific enzymes of an enzyme sources because only some of these studies elucidate the enzyme types and activities. Furthermore, the enzyme levels used in these investigations varied.

Table 2.5 Effect of cell wall degrading enzyme additives on crop silages.

Crop	Enzyme types	Enzyme rates	Enzyme activities	Effect on silage quality	Silage aerobic stability	Other treatment	References
Whole-crop wheat	Mixture of cellulase, carboxymethyl cellulase, cellobiase, and xylanase	366, 733, and 1099 ml Mg^{-1} DM	Cellulase 3 IU/ml, carboxymethyl cellulase 58 IU/ml, cellobiase, 7 IU/ml, and xylanase 217.6 IU/ml.	The concentration of lactic acid and acetic acid increased; increased enzyme rates didn't affect silage composition.	N	Delayed silo sealing 24 hr	Adogla-Bessa and Owen, 1995
Orchardgrass and alfalfa	Cellulase	Cellulase 2, 10, and 20 ml kg^{-1}, DM	Carboxymethylcellulase 2500 IU/ml	More WSC and lactic acid production was obtained with the lower pH value; The effect was more significant on orchardgrass than alfalfa.	N	Pectinase, bacterial inoculation contained *L. plantarum* and *P. Cerevisiae*.	Nadeau et al., 2000
Timothy and alfalfa	*Acremonium* cellulase	0.01%	N	The cellulase enhanced the degradation of parenchyma tissue while sclerenchyma remained intact.	N	*L. casei* bacteria inoculation	Aisan et al., 1997
Corn	Maize-all® enzyme	11g Mg^{-1}	N	The lactic acid concentration is increased significantly	N		Dönmez et al., 2003
Peas and wheat	Cellulast® (cellulase) and Viscozyme® (hemicellulase plus pectinase)	0.2, 1, and 2 ml/kg forage	Cellulast 74 IU/ml and Viscozyme 120 FBG/ml	Pea silages generated significant correlation between enzymes levels and pH and WSC after 4 days while wheat needed 45 days. A correlation for lactic acid was obtained for both crops after 45 days.	Increased Aerobic deterioration	Pectinase	Weinberg et al., 1995
Whole-crop wheat	Mixture of cellulase, carboxymethyl cellulase, cellobiase, and xylanase,	1750, 3500, and 15503 ml/t	Cellulase 2 IU/ml, carboxymethyl cellulase 54 IU/ml, cellobiase, 3 IU/ml, and xylanase 497 IU/ml.	WSC and lactic acid concentration increased and the pH value reduced with the increase of enzyme levels.	High enzyme level enhanced aerobic stability	Urea	Adogla-Bessa et al., 1999
Bermudagrass	Mixture of cellulase, α-amylase, and hemicellulase	2g/t fresh forage	N	Enzyme had no effect on fiber degradation, pH, and organic acids except for the increased butyrate.	N	Homofermentative LAB	Mandebvu et al., 1999

NUC: novo cellulose units; FBG: fungal β-glucanase units; N: not specified.

2.4.4.2.2. Combinations of enzymes and other additives

Combinations of enzymes and other additives, such as lactic acid bacteria, urea, or formic acid, have been demonstrated to improve the fermentation quality and digestibility of silages through several types of synergistic mechanisms. Inoculating homofermentative lactic acid bacteria (LAB) with enzymes can ferment the exclusively hydrolyzed WSC to lactic acid. Positive results on silage preservation have been obtained from ryegrass, Rhodes grass and barley straw silage which were treated with cellulase and inoculated with LAB (Asian et al., 1997). Increased silage intake and milk production by Jersey cows has been reported by Haigh (1998) after combining LAB inoculation with enzymes in oat silage. However, the impact of this combination on aerobic stability is inconsistent, which probably can be attributed to the different crop types and the DM content.

Combining cell wall degrading enzymes with urea has been reported to improve degradation of the cell wall and ruminant digestibility, decrease DM losses, and enhance aerobic stability more than enzymes or urea alone. The complimentary effect of enzymes and urea can be explained by the sequential action of the individual components. The initial enzymatic cell wall degradation occurring in the early stage of ensilage is followed by additional degradation of lignocellulose linkages by ammonia, which is gradually produced from urea during the next two weeks of ensiling. Manure has been used instead of urea and combined with enzymes as a silage additive mixture and similar results were obtained.

Nadeau et al. (2000) treated orchardgrass and alfalfa with cellulase and formic acid and investigated the digestion kinetics of the cell wall in ruminants. The application of formic acid restricted silage fermentation by direct acidification. The nutrients and hydrolyzed WSC were effectively preserved for susequent ruminant utilization. The combination of cellulase and formic acid resulted in greater total cell-wall degradation and digestion in ruminants than application of either additive individually. Formic acid has also been suggested as a way to adjust the pH value of silage to the optimal level for more effective enzymatic hydrolysis (Henderson, 1993).

2.4.4.2.3. Characteristics of cell wall degrading enzymes

Despite intensive studies that have demonstrated the effectiveness of enzymes for stimulating lactic acid fermentation and preserving silage, less than 20% of the theoretical enzyme activities are typically exploited during the hydrolysis process (Lynd et al., 2002). The β glycosidic linkages in cellulose and hemicellulose are partially inaccessible for enzymes because of the small spatial dimensions of the heterogeneous matrix. Furthermore, cellulose and hemicellulose are associated with each other and surrounded by lignin. These chemical and physical barriers delay and weaken the efficiencies of the enzymes. Effective pretreatment, as stated before, can convert lignocellulosic biomass from its natural recalcitrant form to a form in which enzymatic hydrolysis is much more effective. The resulting increased conversion is attributed to increased surface area accessible to enzymes, enhanced solubilization, and redistribution of lignin achieved through pretreatment (Walker and Wilson, 1991).

Although these studies have demonstrated that the efficiencies of enzymatic hydrolysis can be significantly improved by changing the structural features of the biomass in those physical and chemical pretreatments, extensive research is still needed to focus on understanding the mechanisms of enzymatic hydrolysis in order to enhance the enzyme activity by protein engineering and enhancing the synergy between cellulase systems.

Characteristics of fungal cellulases

Fungi are the major cellulase-producing microorganisms used in commercial production. Representative cellulolytic fungi include: *Acremonium cellulolyticus, Aspergillus acculeatus, Aspergillus fumigatus, Aspergillus niger, Fusarium solani, Irpex lacteus, Penicillium funmiculosum, Phanerochaete, Cchrysosporium, Schizophyllum commune, Sclerotium rolfsii, Sporotrichum cellulophilum, Talaromyces emersonii, Thielavia terrestris, Trichoderma koningii, Trichoderma reesei,* and *Trichoderma viride* (Miyamoto, 1997). The cellulase system produced by fungal microorganisms can be divided into three major categories: endoglucanase, cellobiohydrolase, and β-glucosidase (Walker and Wilson, 1991). The generalized cellulolysis scheme by these cellulase systems has been partially elucidated. Endoglycanase, also known as

carboxymethylcellulase (CM-celluase), initially and randomly attack the amorphous regions along the cellulose fiber. The length of cellulose chain is reduced rapidly and multiple non-reducing sites for cellobiohydrolase attack are generated at the broken points. Cellobiohydrolase, also named exoglucanase, not only can generate cellobiose from the non-reducing sites produced by CM-cellulase, but also can degrade the crystalline region of cellulose into cellobiose and oligomers. Finally, β-glucosidase will produce glucose from produced cellobiose and other cello-oligosaccharides (Figure 2.6).

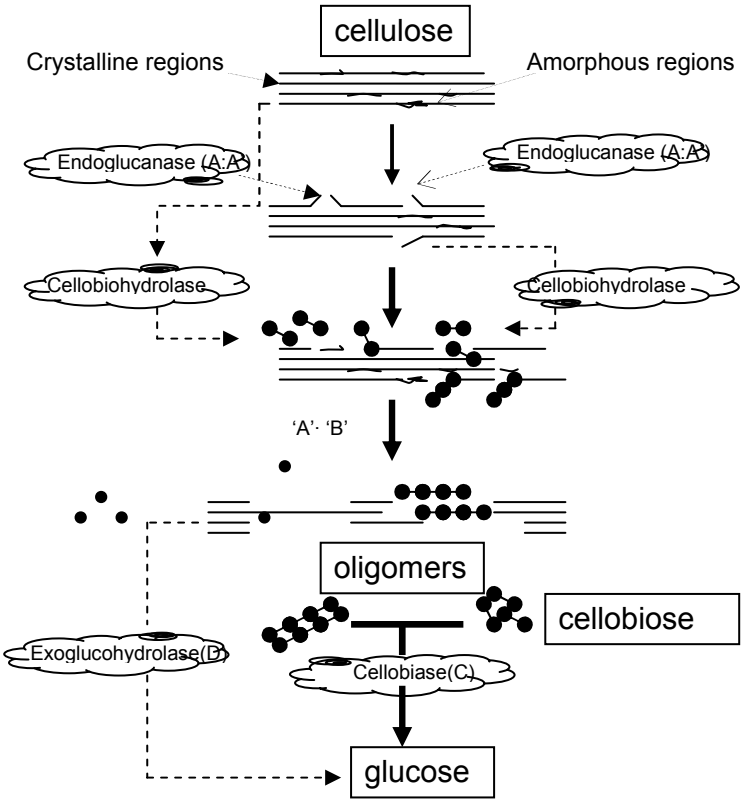


Figure 2.6 Generalized scheme for cellulolysis (A: A′, two forms of endoglucanases; B: B′, two forms of cellobiohydrolase; C, cellobiase; and D, exoglucohydrolase) (Modified from Goyal et al., 1991).

Different fungal sources of enzymes have an effect on the efficacy of cell wall degradation. It was predicted by Goyal et al. (1991) that anaerobic fungi would have higher specific activities than aerobic cellulases because anaerobes have less energy

available for protein synthesis, consistant with the evolutionary principle of retention of energy efficiency. Currently, the genus of *Trichoderma* and *Aspergillus* are mostly used to produce purified and crude enzymes for food industry, animal feed, and pulp and paper industry (Bhat, 2000). McDonald et al. (1991) concluded that *Trichoderma reesei* had a more complete enzyme system than *Aspergillus niger* for degrading cellulose to glucose. Actually, *Trichoderma* produce relatively large quantities of endoglucanase and cellobiohydrolase, but only low levels of β-glucosidase. *Aspergillus* produce relatively large quantities of endoglucanase and β-glucosidase with low levels of cellobiohydrolase production (Miyamoto, 1997). Thus, *Aspergillus* cellulases will be less effective in hydrolyzing the crystalline zones in cellulose due to their lower content of cellobiohydrolase.

Characteristics of bacterial cellulases

Compared to fungi cellulases, the studies of bacterial cellulases are rare because far fewer genus of bacteria can produce cellulases. Although bacterial cellulases are comprised of a number of distinct enzymes from those in fungi cellulases, such as cellodextrinase, these enzymes can still have analogous degradation of cellulose. They can be classed into two categories according to their hydrolysis functions: exo- and endoglucannases, which will hydrolyze cellulose in similar ways to fungi cellulases. Three representative genus of cellulolytic bacteria have been identified, including *Clostridium thermocellum*, *Ruminococcus albus,* and *Streptomyces*. *Clostridium thermocellum*, an anaerobic and thermophilic bacteria, has been investigated intensively by numerous researchers because of the ability of *C. thermocellum* to produce cellulase, hydrolyze cellulose into glucose, and also to ferment glucose into ethanol through the process called simultaneous saccharification and fermentation (SSF) (Lynd et al., 1989).

Synergism

The phenomenon of synergism of cellulases refers to the extent that the enzymatic hydrolysis resulting from a combination of several cellulases is greater than the sum of the hydrolysis by individual cellulases (Woodward, 1991). As described previously (Figure 2.6), the cooperation between cellobiohydrolase and endoglucanase, combining

with the final hydrolysis by β-glucosidase, the primary basis for this synergy. However, the actual synergism is much more complicated than this scheme. Two of the three major categories of cellulase systems each include several distinct enzymes. For example, six distinct endoglucannases (Endo I, II, III, IV, V, and VI), three cellobiohydrolases (Exo I, II, and III), and a β-glucosidase have been identified electrophoretically from *T.viride* cellulases by Beldman et al. (1985). Not only does synergism exist between cellulase components from a homogenous microbial source, cross synergism between the components from various species can also occur. Endoglucannases and β-glucosidase generated by some pseudo-cellulolytic microorganism exhibit synergetic cooperation with cellobiohydrolase produced by *T. koningii* and *F. solani* (Wood, 1969).

The degree of synergism is dependent on the enzyme's source, the features of the substrate, and the synergetic components. The degree is also influenced by the concentration of cellulases. According to a review by Woodward (1991), there is an optimum concentration of cellulases that maximizes of synergism before the substrate is saturated by enzymes. Thus far, four distinct forms of synergism have been observed among endoglucanase, exoglucanase, β-glucosidase, and carbohydrate binding modules, all of which accelerate and help complete the process of enzymatic hydrolysis (Bayer et al., 1994). But more research is needed to understand which mechanisms in synergism are more important, and how synergism is related to carbohydrate binding modules and catalytic actions. This study will investigate the effect of the synergism between cellulase and hemicellulase, and synergism between these enzyme components on stover preservation.

2.4.4.3 Impact of chemical structure of corn stover on enzymatic hydrolysis

The physical and chemical structure of cell walls, as well as the enzyme activities, individual composition and kinetics, all determine the rate and extent of the hydrolysis reaction. Key factors that limit the rate and extent of enzymatic hydrolysis are presented in Table 2.6.

The spatial orientation of cellulose and hemicellulose in the cell wall, the physical association of lignin with noncellulosic polysaccharides, and the covalent links between lignin and the polysaccharides are the major constraints on enzymatic hydrolysis (Figure 2.7). Hydrolysis of hemicellulose can increase the effective surface area of cellulose

fibrils and therefore enhance cellulose hydrolysis. Therefore, although hemicellulose hydrolysis mostly produces five carbon sugar monomers, which are not used by current commercial ethanol fermentation strains, hydrolysis of hemicellulose is still valuable for bioconversion of cellulolysic biomass to sugars. The heterogeneous characteristics of hemicellulose backbone and branch chains, substituted with acetyl esters and different monomers such as arabinose, fucose, glucuronic acid, and mannose, are also a limitation to enzymatic hydrolysis. The hemicellulose backbones associate with cellulose by hydrogen bonds, resulting in a highly complex structure with multiple orders of spatial structure. Side-chain substitution is both a steric hindrance for hydrolysis and an enzymatic hindrance.

Table 2.6 Substrate and enzyme related factors that influence the enzymatic hydrolysis of cellulose in lignocellulosic feedstocks (Esteghlalian et al., 2000).

Enzyme related factors	Substrate related factors
1. Reaction heterogeneity (soluble enzyme vs. insoluble substrate) 2. Irreversible binding of enzymes onto lignin 3. Gradual loss of synergism in cellulase mixture 4. Substrate dependence of synergism and binding (specificity) of enzyme components 5. End-product inhibition 6. Thermal inactivation of enzyme	1. Cellulose crystallinity (CrI) 2. Cellulose degree of polymerization (DP) 3. Feedstock particle size 4. Lignin barrier (content and distribution) 5. Substrate's available surface area (pore volume) 6. Cell wall thickness (coarseness)

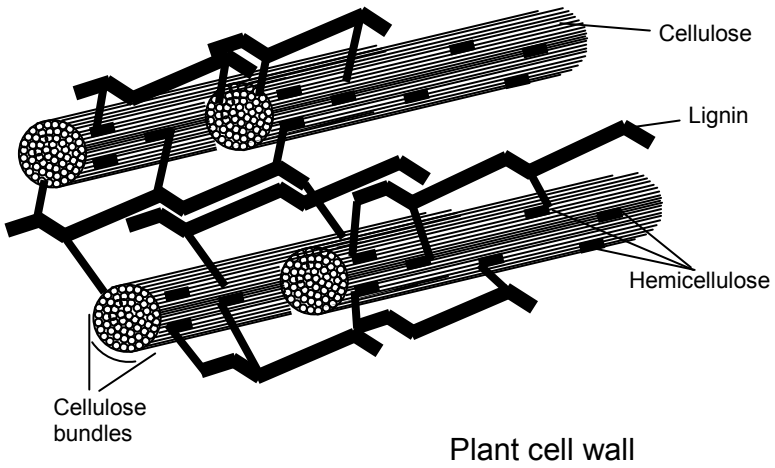


Figure 2.7 The three dimensional structure of cell wall (Modified from Hawaii, 1994).

The close physical crosslinking of lignin and polysaccharides hinders the accessibility of the polysaccharides to enzymes (Moore and Hatfield, 1994). This steric interference of lignin with enzymes is considered to be the primary constraint by lignin to enzymatic hydrolysis. The irreversible binding of enzymes onto lignin is another factor leading to inefficient hydrolysis. Furthermore, polysaccharides linked to lignin by covalent bonds, such as glycosidic, ether, and ester cross-linkages, or cinnamic acid bridges, can not be broken down by enzymes because of spatial and chemical obstacles, and non-specific hydrolysis activity.

2.4.5 Models of the silage process

Two models of ensiling as dominated either lactic acid bacteria or clostridia were developed by Pitt and Muck (1985) and Leibensperger and Pitt (1987), respectively. These models simulated the change rate of the key output variables, such as pH, water soluble carbohydrates, lactic acid bacteria and so on. The ensilage process was divided into three phases: aerobic respriation, lag plant cell lysis and slow bacteria growth, and stable fermentation. The chemical and biological processes modeled during these three phases include oxygen consumption, growth and death of lactic acid bacteria and clostridia, change of pH, and hemicellulose hydrolysis. Inputs and outputs of each process and related environmental control factors are presented in Table 2.7.

Table 2.7 Phases, processes, inputs and outputs and environmental control factors (Pitt and Muck, 1985).

Phase	Process	Input to process	Out of process	Environmental factors
Aerobic	respiration	WSC, O_2	CO_2, H_2O, heat	T, pH, dry matter, CO_2, O_2
	hemicellulose hydrolysis	HC	WSC	T, pH, dry matter, CO_2, O_2
	proteolysis	PN	NPC	T, pH, dry matter, PN
Lag	plant cell lysis			T, aw
	lag in bacterial growth			aw
	hemicellulose hydrolysis	HC	WSC	T, pH
	proteolysis	PN	NPN	T, pH, dry matter, PN
Fermentation	growth of LAB	WSC	LAB, L, A	T, pH, aw, WSC
	death of LAB	LAB	WSC	T, pH, aw
	change in pH	L, A	H+	pH, $ß_h$, Cl,Ca
	hemicellulose hydrolysis	HC	WSC	T,pH
	proteolysis	PN	NPN	T,pH,dry matter, PN

WSC: water soluble carbohydrates, d: dry matter, PN: protein nitrogen, HC: hemicellulose, NPN: non-protein nitrogen, LAB: lactic acid bacteria, L: lactic acid, A: Acetic acid, $ß_h$: buffer index of herbage.

To briefly introduce the approach of these two models, we take the rate changes of lactic acid bacteria as an example. The mass change rate of lactic acid bacteria per g silage is defined as:

$$\frac{dC_{LB}}{dt} = (\mu_g - \mu_b)C_{LB} \quad (1)$$

Where C_{LB}: the concentration of lactic acid bacteria, g bacteria g^{-1} silage; μ_g: specific growth rate, g new bacteria g^{-1} total bacteria h^{-1}; μ_b: specific death rate, g dead bacteria g^{-1} total bacteria h^{-1}.

μ_g and μ_b are each functions of the maximum growth/death rate as influenced by temperature, pH, water activity, and the substrate of water soluble carbohydrates. They are calculated as:

$$\mu_g = \mu_g^{\max} f_{g,pH} f_{g,aw} f_{g,wsc} \tag{2}$$

$$\mu_b = \mu_b^{\max} f_{b,pH} \tag{3}$$

$\mu_g^{\max}$ is the maximum growth rate, which is described by Arrhenius equation:

$$\mu_g^{\max} = \begin{cases} e^{27.3-8344.4/T}, & 288 \le T \le 303K \\ e^{17.2-5280.7/T}, & 303 \le T \le 308K \end{cases} \tag{4}$$

$f_{g,pH}$, $f_{g,aw}$, and $f_{g,wsc}$ are the fractions of the maximum rate affected by pH, water activity, and WSC content. The pH and water activity correction factors are defined by polynomial regression models while the WSC content factor is a Mechaelis-Menton expression.

Since there is no quantitative information for the death rate of any silage lactic acid bacteria, $\mu_b^{\max}$ is estimated from the growth rate at the critical pH value, where the highest LAB is obtained. At this critical pH value the death rate equals to the growth rate, and bacterial population in the silage is at a maximum.

The critical pH at which growth rate and death rate are equal, pH*, is dependent on the DM concentration of silage. An empirical expression for pH* is

$$pH^* = \begin{cases} 4.21 & d \le 0.2 \\ 3.23 + 4.93 \times d & 0.2 < dry\ \ matter < 0.45 \end{cases} \tag{5}$$

d is the dry matter content on the wet basis, g/g silage;

The death rate at pH* is termed as μ_b^*, which equals to μ_g^*

$$\mu_b^* = \mu_g^* = \mu_g^{\max} f_{g,pH*} f_{g,aw} f_{g,wsc} \tag{6}$$

Since $\mu_b^* = \mu_b^{\max} f_{b,,pH*}$ at the specific pH* value, the maximum death rate $\mu_b^{\max}$ can be expressed as:

$$\mu_b^{\max} = \mu_b^* / f_{b,pH*} \tag{7}$$

The death rate of lactic acid bacteria can be determined as $\mu_b = \mu_b^{\max} \times f_{b,pH}$

For any given initial lactic acid bacteria mass concentration, it is then easy to determine the growth rate, death rate, and the rate of change of the LAB mass concentration during the ensilage process.

2.5. Ensiling of corn stover

During the ensiling process, some saccharification of crop cell wall and sugar fermentation naturally occurs, mediated by the synergistic system of microorganisms that are present. Thus, conservation of corn stover through ensilage for future processing will not only preserve the corn stover feedstock, but will also provide some extent of pretreatment. This pretreatment may be beneficial for future conversion of corn stover into sugars or chemicals in industrial production (Richard et al., 2001).

However, the purpose of industrial corn stover storage and the initial and desired stover characteristics are different from those of the most popular forage crops, such as ryegrass, timothy, alfalfa, and green harvested whole corn plants traditionally conserved as silage. As an industrial biomass feedstock, corn stover will be ensiled prior to fuel, chemical, or particleboard production, rather than for ruminant feeding in winter. In addition, the very low content of fermentable sugars in corn stover, less than 1% dry matter (DM), may not always effectively initiate a robust lactic acid fermentation to obtain low pH. Although there is a large body of research on ensiled storage of forages, further investigations of corn stover ensilage are required because of these differences from traditional ensilage crops and products.

One concerned feed ensilage study of particular relevance is that of Yang et al. (2001), who combined solid state fermentation with ensiling in order to increase the nutritional value and palatability of corn stover for animal feed. Corn stover provided the substrate for cellulosic bacteria to produce cell wall degrading enzymes in solid state fermentation. The produced enzymes and fermented corn stover were ensiled without separation. Through this combined 2-step fermentation, the cost for purchasing commercialized enzymes was greatly reduced. A rapid drop of pH, high levels of lactic acid, partial degradation of cell wall, and production of sugars were obtained through the process.

Focusing on industrial biomass storage, Richard et al. (2001) examined the effect of five various moisture contents of corn stover, ranging from 53% to 85%, on silage

quality. High pH values (larger than 5) and low levels of overall acids (less than 3% d.b.) were caused by the low concentration of fermentable sugars in the fiberized corn stover tested. The chemical characteristics of the final silage samples suggested higher moisture levels caused secondary clostridia fermentation. Based on this initial study of corn stover ensiling, Richard et al. (2002) tried to apply cell wall degrading enzymes to encourage lactic acid fermentation in stover with a moisture content of 60% w.b. Purified cellulase, hemicellulase, and industrial enzymes were applied on corn stover, which was sealed for 21 days. The enzyme addition partially hydrolyzed the cell wall into fermentable sugars. The lowest pH (around 4) was obtained with the treatment of hemicellulase and a mixture of cellulase and hemicellulase. A higher level of lactic acid (up to 6.5% d.b.) demonstrated the efficacy of enzymes addition. More than 6% d.b. WSC was produced at the end of 21 days.

Because industrial corn stover silage will not be used to feed animals, some chemicals, such as sulfuric acid, formic acid, and urea can be added at high levels to the silage without concern of detrimental impact on animals. Two effects can be expected from these acidic additives. The first is to lower the pH value of stover silage to a level which is not suitable for microbial growth, so as to inhibit fermentation and conserve the biomass. The second, and more valuable effect, is to weaken the linkages among the polysaccharides in the cell walls, and thus to encourage lactic acid fermentation. The weakened bonds will provide easier degradation of the cell wall to produce fermentable sugars both during ensilage and subsequent downstream conversion processing.

2.6. Summary

Corn stover has attracted a lot of interest in recent years because of its availability and increasing feasibility for use as a feedstock for ethanol, chemical, pulp and paper, and biocomposites production. The low cost, rich lignocellulosic components, enormous availability, and concentrated regional production in the U.S. provide substantial potential for corn stover on an industrial scale. Economic and social benefits can be obtained from the value-added products generated from corn stover, compared to the present relatively low value uses.

Several researchers have examined the environmental impact of corn stover removal and engineering aspects of stover collection. The costs of corn stover, including the nutrient value, harvesting and handling costs, and transportation costs, have been estimated based on various assumptions and calculation methods. Several potential value-added products, including ethanol, furfural, xylitol, pulp and paper, and composites, can be and have been generated commercially from corn stover. Technical issues about pretreatment of the raw material, chemical and enzymatic hydrolysis, and final fermentation or modification, have been widely investigated. However, the essential step about how to store the seasonal raw material to supply a year-round biorefinery facility has not been appropriately addressed.

In order to preserve corn stover for ethanol, chemical and particleboard production in future conversion processes on an industrial scale, ensilage has been proposed as a storage method. Ensilage will reduce the risk of fire and dry matter loss, which are inherent in dry storage. As WSCs are naturally released from the raw material and subsequently fermented during the ensiling process, ensilage of corn stover may also provide some beneficial pretreatment of corn stover. Five categories of additives have been used to improve the quality of crop silage. Acidic additives and cell wall degrading enzymes are discussed in this review because they have the potential to either better preserve stover silage by encouraging lactic acid fermentation, and/or improve enzymatic hydrolysis in downstream processing by providing pretreatment during the ensiling process. Cell wall degrading enzymes can be produced from fungi and bacteria with various combinations of specific enzyme produced by different organisms. The efficacy of these enzymes can vary significantly due to the microbial sources of the enzymes, active components in enzyme systems, and synergism between these components.

Although ensilage is a well-known process of forage preservation for ruminants, there are few studies on applying this preservation system to corn stover storage for future bioconversion to value-added products. The initial studies investigating the ensilage system of corn stover have successfully demonstrated the efficacy of this system. However, the insufficient WSC content in corn stover may not consistently initiate a robust lactic acid fermentation. Poor fermentation can result in excessive biomass DM

loss and complicated fermentation products, which are not favorable for further industrial utilization.

The proposed research will investigate the effect of cell wall degrading enzymes and chemicals, such as acidic additives, on corn stover preservation and pretreatment in an ensilage system. These additives are not only expected to improve the preservation of corn stover by initiating lactic acid fermentation and/or by lowering the pH value, but also by enhancing the sugar yield and the structural properties of composites made from ensiled stover.

3. OPTIMIZING ENZYME RATES FOR PRESERVATION AND PRETREATMENT OF ENSILED CORN STOVER

3.1 Abstract

Ensilage has been proposed as a preservation and pretreatment method for corn stover prior to further bioconversion into sugars, fuels, and fiber products. Ensilage is a truncated solid-state fermentation process in which anaerobically produced organic acids accumulate to reduce pH and thus limit microbial activity. Corn stover has a relatively low concentration of the water soluble carbohydrates (WSC) needed to initiate and sustain this fermentation. Adding supplemental cell wall degrading enzymes can increase the availability of 5- and 6-carbon sugars, both to facilitate ensilage and for further pretreatment and hydrolysis. This study investigated the use of purified and industrial enzyme mixtures with cellulase:hemicellulase activity ratios of 0.7:1 and 0.8:1 respectively. The purified mixture was applied at rates of 6.7, 13.4, 26.3, 53.4, and 106.9 international units (IU) hemicellulase activity per gram stover dry matter (DM). Several chemical characteristics and fiber fractions were measured to evaluate the quality of the resulting stover silage, including pH, WSC, lactic acid, volatile organic acids, cellulose, and hemicellulose. Relative to the lowest rate, the rate of 13.4 IUg^{-1} DM resulted in a significant decrease in pH and increase of lactic acid concentration, and shifted the dominant microbial regime from clostridia to *Lactobacillus* spp. These effects continued at higher enzyme rates, but with diminishing returns, while WSC continued to increase linearly with increasing enzyme rate with the highest concentration of WSC in the range of 4.69-5.25 %DM. The ensiling process influenced the degradation of the different fiber fractions in various ways. Hemicellulose degradation was much higher than that of cellulose under natural ensilage conditions. Cellulose was more sensitive to the applied enzyme mixture than hemicellulose, resulting in significantly greater fiber degradation at low enzyme rates.

The effect of size reduction was examined for coarse (as harvested), medium (<0.01 m), and fine (<0.005m) ground stover, with and without the enzyme treatment.

The highest WSC content and hemicellulose degradation levels were obtained with coarse size stover, although the lactic acid concentration at this size was intermediate.

This study also compares the effect of industrial and purified enzymes on the composition of stover silage. The industrial enzyme mixture was applied at rates of 13.4 and 53.4 IU hemicellulase activity g^{-1} DM. Industrial enzyme mixtures is necessary for economical large-scale implementation, and in this study achieved similar results to the purified enzyme at a much lower cost.

3.2 Introduction

Corn stover, including the stalks, leaves, and cob, is a byproduct of maize production that is normally left in the field when the grain is harvested. This organic residue contributes to soil organic matter and reduces soil erosion, but in many cropping situations significant amounts of stover can be harvested at sustainable rates (ORNL et al., 2005). Approximately 69 million dry Mg/year of corn stover are available for such harvest in the US, representing approximately 65% of the total agricultural residue (ORNL et al., 2005). Currently, only about 10% of this potential resource is collected, and is used primarily as animal feed, barn bedding, and heating fuel. Less than 1% of stover currently collected is used for industrial processing. When the harvested residue exceeds the farm's management capacity, it is sometimes disposed of by open burning beside the fields, generating thick smoke and harmful air pollution. This smoke may contain carbon dioxide, carbon monoxide, particles, hydrocarbons, and nitrogen oxides. In order to make full use of this low cost, abundant, and biorenewable resource, several recent studies have focused on developing corn stover as an alternative feedstock for ethanol, chemicals, and particle board manufacturing (Wilke et al., 1981; Buhner and Agblevor, 2001; Riera et al., 1991; Wang and Sun, 2002; Chow et al., 1999). The potential productions of ethanol, pulp and particle board, and furfural from corn stover have been examined. The results of these studies indicate (Kadam and McMillan, 2003) that 11.4 billion liters of ethanol could be produced from 40% of the harvestable corn stover, assuming only 50% of the theoretical ethanol yield is achieved. Meanwhile, 50% of the feedstock for both hardwood pulp and wood-based particleboard could be replaced by corn stover.

Corn stover contains over 80% (w/w) lignocellulosic fractions, and these consist of 32.2-45.6% cellulose and around 35% hemicellulose along with 5-10 % lignin (Sun and Cheng, 2002; Ladisch et al., 1983). The abundant polysaccharides serve as a substrate for sugar production and further bioconversion into ethanol and other chemicals. Regardless of whether these production pathways are through biological fermentation or chemical modification, five- or six-carbon monosaccharides are required as the intermediaries.

Currently, many studies are focused on the technical challenge of degrading polysaccharides into monosaccharides by breaking down the glycosidic linkages (Mosier et al, 2005; MacDonald et al., 1983; Kaar and Holtzapple, 2000). However, the future utilization of corn stover on an industrial scale also requires the safe and effective long-term storage of the feedstock, and suitable preparation of the stover for the following bioconversion steps. Because stover can only be harvested once per year in temperate climates in North America, the harvested biomass must to be stored to provide a continuous supply for industrial refineries. Currently, corn stover is stored in bales and stacks, where the low moisture content (14-33%, Sokhansanj et al., 2002) presents a high risk of fire. Furthermore, typical dry matter loss during dry storage is 17-23 % (Richey et al., 1982). In order to overcome these limitations, an ensilage process has been proposed as a preservation method for large quantities of corn stover, since the high moisture content of silage (up to 60% (dry basis, d.b.)) completely removes the risk of fire (Richard et al., 2001).

Ensilage is a traditional preservation process for crops that are fed to ruminants during the winter season. Low pH and anaerobic conditions are required to inhibit microbial activity and minimize biomass decomposition (McDonald et al., 1991). Lactic acid production is encouraged over other potential acids as with a pKa of 3.87, it is the most effective at lowering the pH value of the stover. Saccharification of the crop cell wall and immediate fermentation have been reported during the ensiling process, because of synergies between microbial activity, plant enzymes, and hydrolysis by the acids produced (Dewar et al., 1963). Preservation of corn stover through the ensiling process is therefore also proposed as a pretreatment process, which can be beneficial for future

conversion of corn stover into sugars or chemicals in industrial production (Richard et al., 2001).

Minimizing dry matter loss during storage is important for efficient use of biomass feedstocks. Shinners et al. (Shinners et al., 2003) reported around 3.6% DM loss in wrapped bales sealed by plastic film, although the moisture content, 38.9% (w.b.) was not high enough to completely remove the fire risk. Our previous study examined the ensilage of corn stover over a wide range of moisture contents (53~85%)(Richard et al., 2001). Under these conditions, a secondary fermentation by clostridia was observed, and the acetic acid production overwhelmed lactic acid production, resulting in a final pH value greater than 5. Cell wall degrading enzymes, such as cellulases and hemicellulases, have been shown to encourage lactic acid fermentation, and hence improve the quality of conventional crop silages (Dewar et al., 1963; Zahiroddini et al, 2004; Colombatto et al., 2004; Jaster and Moore, 1990; Morrison, 1979; Henderson and McDonald, 1977). Richard et al. (Richard et al., 2002) reported a significant effect of various enzyme mixtures on corn stover silage; they observed considerable increases of fermentable sugars and lactic acid. Yang et al. (Yang et al., 2001) combined solid state fermentation for enzyme production with an ensiling process to produce enzymes for corn straw bioconversion. In that study, the cellulose and hemicellulose were degraded 38% and 21% respectively.

Only one or two enzyme levels were used in these studies; the relation between enzyme levels and quality of corn stover silage has not been reported. Economics will require use of the lowest effective enzyme rate for a corn stover silage system, as high price of commercial enzymes could be prohibitive. This study also compares the effectiveness of industrial enzymes to that of more expensive purified enzymes. Finally, the effect of particle size reduction of corn stover was investigated, because the corn stover particle size will influence the cost of pre-processing, as well as the contact efficiency of enzymes with the substrate.

3.3 Material and Methods

3.3.1 Corn stover and silage preparation

The corn stover, harvested in fall 2002, was ground through a Art's-Way hammer mill (Art's-Way, Armstrong, IA) 0, 1 and 2 times to obtain three size grades. Coarse corn stover, once moderately milled (<10 mm) and twice finely milled (<5 mm) are indicated as sizes C, M, and F size, respectively. The size distrubtion of the three size grades is provided in Table 3.1. Initial pH, moisture content, and fiber composition of the C, M, and F sizes are shown in Table 3.2. The material contained 16-20% moisture (wet basis, w.b.) and was adjusted to 60% (w.b.) by adding water, which was previously determined to be the optimum condition (Richard et al., 2001).

Table 3.1 The particle size distribution of coarse, medium, and fine sizes of ground stover

		Coarse	Medium	Fine
Arithmetic mean particle size (mm)		19.3	2.8	1.1
U.S. Mesh	Screen size (mm)	cumulative % retained		
-	25.40	71.1	0.0	0.0
-	12.70	83.2	6.2	0.0
3	6.73	91.2	15.8	0.5
6	3.35	95.3	51.4	6.8
12	1.68	96.3	75.7	35.8
20	0.84	100.0	96.1	75.9
40	0.42		97.8	86.5
70	0.21		99.1	95.4
Pan	Pan		100.0	100.0

Each enzyme treated corn stover or control sample was packed tightly into a 20cm × 35cm polyethylene bag (200 g dry matter, DM, mixed with 300 g water), vacuumed under 25 inch mercury vacuum, and immediately heat-sealed. Each treatment was run in triplicate and was incubated at 37 ±1ºC for 21 days. Dry matter and pH measurements were taken immediately at the end of the fermentation period. The remaining stover was frozen and stored for later analysis of lactic acid, volatile fatty acids, and fiber fractions, as described in the section 3.2.3.

Table 3.2 Chemical characteristics and fiber composition of three sizes of corn stover.

Stover size	Coarse	Medium	Fine
pH	7.2	6.8	7.1
WSC* (% d.b.)	1.6	1.8	3.3
NDF** (% d.b.)	83.7	74.8	72.0
ADF† (% d.b.)	48.6	42.1	41.3
Lignin (% d.b.)	4.3	3.2	3.6
Ash (% d.b.)	0.91	3	3.4
Cellulose (% d.b.) (= ADF – Lignin-Ash)	43.5	35.9	34.3
Hemicellulose (% d.b.) (= NDF – ADF)	35.1	32.7	30.7

* Water soluble carbohydrates (WSC)

** Neutral detergent fiber (NDF); † Acid detergent fiber (ADF);

The analysis method for chemical and fiber composition was described in the following section 3.3.3.

3.3.2 Enzyme treatment

The purified enzyme (Sigma H-2125) from *Aspergillus niger* was purchased from Sigma (St. Louis, MO). The industrial enzyme Safizym fl300, a mixture of hemicellulase and cellulase was provided by Saf Agri (Milwaukee, WI). The endoglucanase and hemicellulase in enzymes were measured as described by Sharrock (Sharrock, 1988) and Bailey et al. (1992), using medium viscosity carboxymethylcellulose and 1% (w/v) birchwood 4-*o*-methyl glucuronoxylan (Roth 7500) as substrate, respectively. The enzyme activity was shown in Table 3.3. Appropriately diluted enzymes were incubated with 0.5 ml of substrate at 50°C and pH 4.8 (50mM citrate buffer) for 30 min. The reducing sugars, xylose and glucose, were determined by the DNS method (Miller, 1959). One international unit of endoglucanase/hemicellulase is defined as the amount of the enzyme which releases 1μmol glucose/xylose from carboxymethylcellulose /glucuronoxylan in one minute.

Table 3.3 Characterization of enzyme products added to corn stover.

	Sigma H-2125 (IU/g enzyme)	Safizym fl300 (IU/ml enzyme)
Microbial source	*Aspergillus niger*	*Trichoderma longibrachiatum*
Endoglucanase	1200	646.8
Hemicellulase	1670	803.3
Ratio of endoglucanase to hemicellulase	0.7	0.8

Five rates of Sigma H-2125 were applied to the C, M, and F sized stover. The levels, based on the xylanase activity, were 6.7, 13.4, 26.3, 53.4, and 106.9 IU hemicellulase g^{-1} DM. Two rates of Safizym fl300, 13.4 and 53.4 IU g^{-1} DM, were applied only to the M size stover to examine the improvement effect of induatrial enzymes.

3.3.3 Chemical analysis

Dry matter was determined by drying 100 g of fresh samples at 60ºC in a forced air oven for 48 h, while pH was determined using a pH electrode on samples at a 10:1 (H_2O:sample) mass dilution. Lactic acid and volatile fatty acids were determined using gas-liquid chromatography with SP-1000/1200-H_3PO_4 columns (Supelco, Inc., Bellefonte, PA) and a flame ionization detector. The operating temperatures for the oven, injector, and flame detector were 120, 170, and 180ºC respectively. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined by the method of Vogel et al. (1999). Hemicellulose content was calculated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL. Water soluble carbohydrates (WSC) were determined by the modified phenol-sulfuric acid method described by Guiragossian et al. (Guiragossian et al., 1977).

3.3.4 Statistical analysis

Data were analyzed using generalized linear model (GLM) procedure of Statistical Analysis System (SAS) edition 9.1 (SAS, 1999). Difference between treatments were determined by Tukey's test. The acid production was regressed on log (enzyme concentration +1) using the regression (REG) procedure of SAS. Significance of

all analyses was declared at a 5% probability level. The following chapters use the same methods of statistical analysis method except as otherwise indicated.

3.4 Results and Discussion

3.4.1 Chemical characteristics of corn stover silage

The pH values at the five rates of enzyme were significantly lower ($P < 0.05$) than the control samples, which were in the range from 5.02 to 5.23 (Table 3.4). In general, an increase in the level of enzyme resulted in a decline of pH values ($P < 0.0001$), though some exceptions occurred with the C size stover. The size of the stover appeared to influence ($P = 0.0278$) the pH value, with the M size stover having the lowest pH value. Unsurprisingly, the lowest pH was obtained at the highest enzyme rate. The reduction of pH with increasing enzyme rate was greatly reduced above the 13.4 IU g^{-1} DM level. No lactic acid was produced in the control samples, which can be attributed to the low WSC content of the corn stover. Homolactic fermentation cannot be sustained with such low levels of available sugars (McDonald et al., 1991). Even if some lactic acid is produced, other bacteria, such as clostridia and enterobacteria, are unrestrained at moderate pH values and will consume it immediately. Although the F size stover had a WSC content of 3.3% DM, this was not high enough to enable a lactic acid dominated fermentation. In general, the concentration of lactic acid increased with increasing enzyme rate, with some exceptions for the rate of 26.3 IU/g DM that were consistent with the exceptions in pH change.

Similar increases in lactic acid concentration, associated with decreases in pH, have previously been observed in conventional crop silages, such as orchardgrass and alfalfa (Nadeau et al., 2000), peas and wheat (Weinberg et al., 1995), and whole-crop wheat (Adogla-Bessa et al., 1999). However, the 16-fold increase in enzyme rate (106.9 IU/g DM compared with 6.7 IU g^{-1} DM) was not reflected by a corresponding increase in lactic acid. Lactic acid concentrations did increase rapidly with increasing enzyme rates up to a rate of 13.4 IU g^{-1} DM, but above that experienced diminishing returns. The C size stover had the lowest lactic acid concentration ($P < 0.0001$), while there was no significant difference between the M and F sizes.

Table 3.4 Chemical composition of corn stover silage for the stover sizes and enzyme treatments tested.

Enzyme	Stover size	Enzyme rate (IU/g DM)	pH	LA* (% d.b.)	AcA (% d.b.)	BA (% d.b.)	iBA (% d.b.)	PA (% d.b.)
Sigma H-2125	Coarse	Control	5.08a**	0a	1.87a	0.91a	0.19a	0.11a
		6.7	4.57ab	0.72ab	1.72a	0.92ab	0.11ab	0.06ab
		13.4	4.26b	1.80bc	0.85b	0.09c	0.01c	0.05b
		26.3	4.30b	1.70bc	0.77b	0.07c	0.01c	0.05b
		53.4	4.08b	2.37c	0.73b	0.06c	0c	0.02b
		106.9	4.19b	1.86bc	0.60b	0.02d	0c	0c
Sigma H-2125	Medium	Control	5.02a	0a	1.28a	2.0a	0.26a	0.21a
		6.7	4.41b	1.97b	1.63a	0.08b	0b	0.02b
		13.4	4.29bc	2.46bc	1.41a	0.03b	0b	0.01b
		26.3	4.20cbd	2.52c	1.45a	0b	0b	0b
		53.4	4.12cd	3.2d	1.17a	0b	0b	0b
		106.9	4.05d	3.18d	1.18a	0b	0b	0b
Sigma H-2125	Fine	Control	5.23a	0a	1.44a	2.7a	0.21a	0.42a
		6.7	4.42b	1.97b	1.80a	0.64b	0b	0.01b
		13.4	4.37b	2.88b	1.49a	0.22bc	0b	0b
		26.3	4.35b	2.38b	1.63a	0c	0b	0b
		53.4	4.36b	2.89b	1.30a	0c	0b	0b
		106.9	4.12b	3.64b	1.32a	0c	0b	0b
Safizym fl300	Medium	13.4	4.34a	2.76a	1.33a	0.04a	0a	0a
		53.4	4.25a	3.29b	1.15a	0.04a	0a	0.02a

*Organic acids are abbreviated as follows: lactic acid (LA), acetic acid (AcA), butyric acid (BA). isobutyric acid (iBA), and propionic acid (PA)

** Means in the same column within a size treatment with different letters are significantly different ($P<0.05$).

The acetic acid contentration was reduced by enzyme addition to the C size stover, and continued to decrease with increasing enzyme rates. The M and F sizes, however, had higher acetic acid concentrations than the control at the 6.7, 13.4, and 26.3 IU g^{-1} DM rates. Such mixed results are not unusual. Adogla-Bessa and Owen (1995) reported that low rate enzyme treatments increased acetic acid and lactic acid concentrations as compared to the control samples, and noted that increased enzyme levels did not change the composition of whole-crop wheat silage. However, when higher

enzyme levels were applied by the same group in another study of wheat silage (Adogla-Bessa et al., 1999), increased enzyme levels enhanced lactic acid content, but had no effect on acetic acid. Their acetic acid content was higher than the lactic acid content in all treatments.

Three possible metabolic pathways of acetic acid production could be causing these effects: 1) heterolactic fermentation of sugars; 2) fermentation of organic acids, including lactic acid, by lactic acid bacteria; and 3) fermentation of nitrogenous compounds by proteolytic clostridia. In conventional ensilage of forage crops, homolactic fermentation is enabled by sufficient WSC and becomes dominant among the competitive fermentation pathways under anaerobic conditions. However, the limited available sugar in corn stover silage can lead to a mixed fermentation process, as evidenced by the higher concentration of acetic acid than lactic acid produced in the control. Although moderate levels of enzyme were shown to lower the acetic acid concentration and increase the lactic acid concentration significantly, the ratio of these two concentrations did not change greatly with the further increases in the applied enzyme rate. This indicates that increased enzyme addition did not cause a complete shift from mixed fermentation to homolactic fermentation.

Significantly lower levels of butyrate and isobutyrate ($P < 0.0001$) were obtained beyond the rate of 13.4 IU g^{-1} DM on C size stover and 6.7 IU g^{-1} DM on the M and F size stover. Butyrate and isobutyrate are products of secondary fermentation by clostridia, which converts fermentable sugars and lactic acid into these acids. Mead (1971) concluded that saccharolytic clostridia were exclusively responsible for the production of butyrate. Enzyme addition increases the lactic to butyric acid ratio as compared to the control. This indicates a conversion from clostridia fermentation to lactic acid bacterial fermentation.

Significant linear regressions were obtained on the logarithm of enzyme rate plus 1 for pH, lactic acid and acetic acid with exceptions of acetic acid on M and F size ($P<0.05$). Similar regressions were observed in pea and wheat silage on an enzyme mixture additive, containing cellulase, hemicellulase, plus pectinase (Weinberg et al., 1995). Although the cellulases in this previous study were measured in NCU (novo cellulose unit) and FBG (fungal β-glucanase unit), and thus cannot be directly compared

with CMCase activity as defined in this study, Weinberg et al. (1995) did observe a linear regression between chemical characteristics and the log of enzyme levels.

3.4.2 Water soluble carbohydrates

The WSC content increased gradually with increasing enzyme rate (Figure 3.1). Linear regressions demonstrated significant relationships between enzyme rate and WSC at the C, M, and F sizes ($P < 0.0001$, $P < 0.0001$, and $P = 0.0005$, respectively). The WSC content in silage comes from several sources, including the original WSC in the stover, any added fermentable sugars, and sugars hydrolyzed from lignocellulose by plant enzymes, organic acids, and saccharolytic bacteria. The final WSC content of the silage depends not only on inputs from these sources, but also on outputs, e.g. the WSC consumption during sugar fermentations. Therefore, exact measurements of WSC inputs from degradation of the cell wall by enzymes are difficult to achieve, as some of the sugars released through hydrolysis of the cell wall will be partially fermented by bacteria (McDonald et al., 1991). The final WSC content is indicative of the relative balance between production and utilization. The data from the present study suggest that WSC generation from lignocellulose fractions dominate the final enzyme-treated silage, and can be modeled with a linear regression.

Although the initial WSC in the control samples differed between the three sizes, the final contents after 21 days of ensiling were similar at around 1% DM. Following the enzyme treatments, the C size stover, which had the lowest initial WSC, had the highest WSC content ($P < 0.0001$) followed by M and, finally, F. However, this higher sugar content in the C size did not correspond to an enhancement of lactic acid production; rather, the lactic acid concentration was the lowest among the three sizes. The extra WSC in the C size can be attributed to pentoses hydrolyzed from hemicellulose, as demonstrated in the following section on fiber degradation. Some of these sugars, such as xylose and arabinose, are not fermented by lactic acid bacteria to a significant degree (Kandler, 1983; Barre, 1978) because the process requires the induction of additional metabolic enzymes. Furthermore, less WSC is consumed in the C size, as can be inferred from the lower lactic acid and acetic acid contents relative to the other sizes.

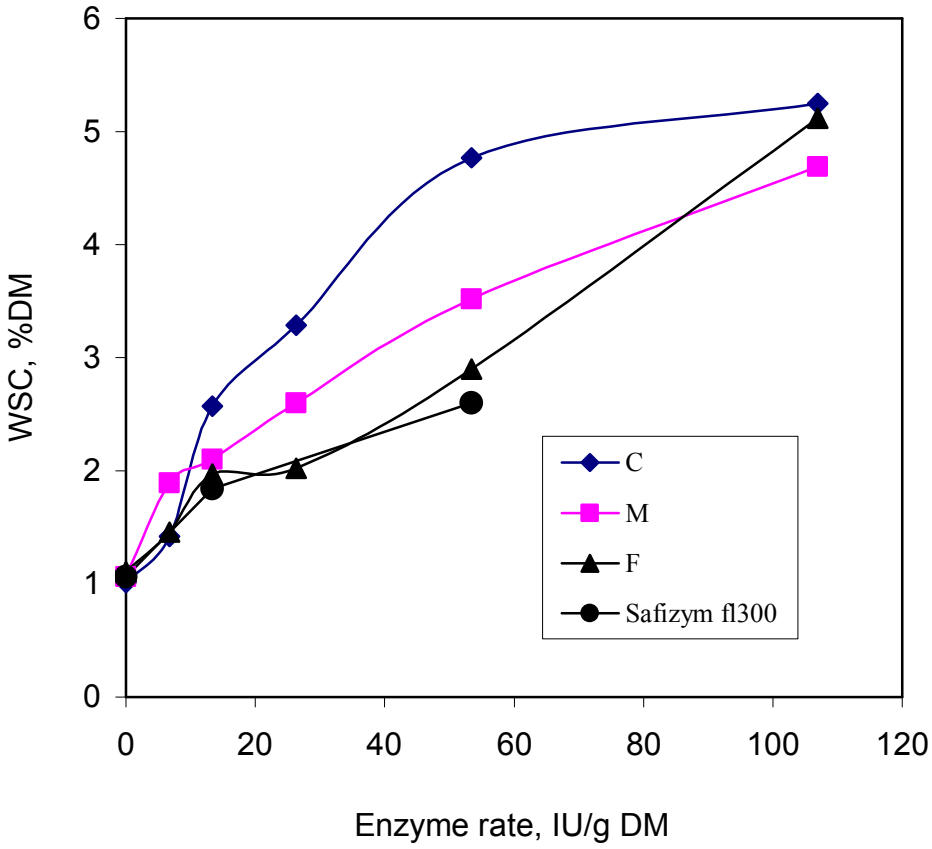


Figure 3.1 Water soluble carbohydrates produced during corn stover ensiling for varied purified enzyme and industrial enzyme application rates (C for coarse size, M for medium size, and F for fine size).

3.4.3 Fiber degradation

The fiber analysis of treated and non-treated stover silage is presented in Table 3.5. The NDF and ADF fractions decreased as the applied enzyme rate increased, with no significant differences between the three sizes. The ADL fraction did not change with enzyme treatment, as the lignin and ash fraction cannot be hydrolysed.

Hemicellulose and cellulose are the sources of five and six carbon sugars for downstream bioconversion. The ensilage process has been reported to have a degradation effect on cell wall polysaccharides, especially on hemicellulose (McDonald et al., 1991). In this study, an average of 17.2, 7.1, and 10.4% of the hemicellulose was degraded in C, M, and F sizes in control samples, respectively, while the corresponding cellulose

degradation was 5.5, 5.1, and 2.3%. This agrees with observations by Yahaya et al. (2001), who investigated the ensilage of orchardgrass and lucerne, reporting 17.2~19.8%

Table 3.5 Fiber composition of ensiled corn stover.

	Stover size	Rate of enzyme (IU/g DM)	NDF** (% DM)	ADF** (% DM)	ADL** (% DM)
Sigma H-2125	Coarse	control	82.3a*	50.8a	4.3a
		6.7	76.7b	45.4b	4.3a
		13.4	74.5bc	44.0bc	4.0a
		26.3	72.8cd	43.2bc	4.3a
		53.4	70.8d	43.0bc	4.2a
		106.9	70.0d	41.9c	3.8a
	Medium	control	76.5a	43.1a	3.3a
		6.7	67.7b	38.4b	3.0a
		13.4	66.4bc	37.5bc	2.9a
		26.3	66.6bcd	37.8bc	3.0a
		53.4	65.3bcde	37.2bc	3.0a
		106.9	62.2ce	35.2c	2.9a
	Fine	control	74.9a	44.9a	3.5a
		6.7	70.6b	41.3b	3.3a
		13.4	69.0b	40.3bc	3.3a
		26.3	68.6bc	40.2bc	3.3a
		53.4	65.7c	38.5c	3.2a
		106.9	67.7d	36.1d	3.1a
Safizym fl300	Medium	13.4	69.0a	39.0a	3.5a
		53.4	64.8b	36.7b	3.2b

* Means in the same column within a size treatment with different letters are significantly different ($P<0.05$).

** NDF: netural detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin

hemicellulose degradation and only 0.5~3.3% cellulose degradation. Kawamura et al. (2001) found a 21.8% loss of hemicellulose and a 7.9% loss of cellulose during the fermentation of Italian ryegrass. Although the extent of degradation varies widely for different crop silages (11-54% for hemicellulose and 0.5-5% for cellulose) (McDonald et al., 1960; McDonald et al., 1962; Morrison, 1979; McDonald et al., 1991; Yahaya et al., 2001), these results demonstrate a significant preferential degradation of hemicellulose over cellulose during the ensiling processes.

The enzyme treatments were expected to increase the polysaccharide degradation significantly ($P < 0.0001$), and the extent of hydrolysis was indeed observed to improve with increases in the applied enzyme rates. The addition of enzymes had different impacts on cellulose and hemicellulose degradation (Figure 3.2). The degradation of these individual polysaccharide fractions is defined as the decrease of polysaccharide concentration over the ensiling divided by the initial concentration. Cellulose hydrolysis increased about 185~468% at the rate of 6.7 IU g^{-1} DM, while hemicellulose hydrolysis increased only 3.5~63.7%. Beyond this initial threshold, further incremental effects of higher enzyme rates were similar for these two fiber fractions. Although the enzyme amendments had higher hemicellulase activity than cellulase, the increase in hemicellulose degradation did not surpass that of cellulose. The same results were reported by Van Vuuren (1989). This phenomenon can be explained by the different structures of cellulose and hemicellulose. The linear structure of cellulose, which has uniform β-1, 4 glycosidic linkages, makes cellulase enzymes specific and effective biocatalysts. Hemicellulose is complex, with various monosaccharide units, hetero-linkages, and different branch lengths. These differences account for the increased sensitivity of cellulose to enzyme treatment, especially at low enzyme rates.

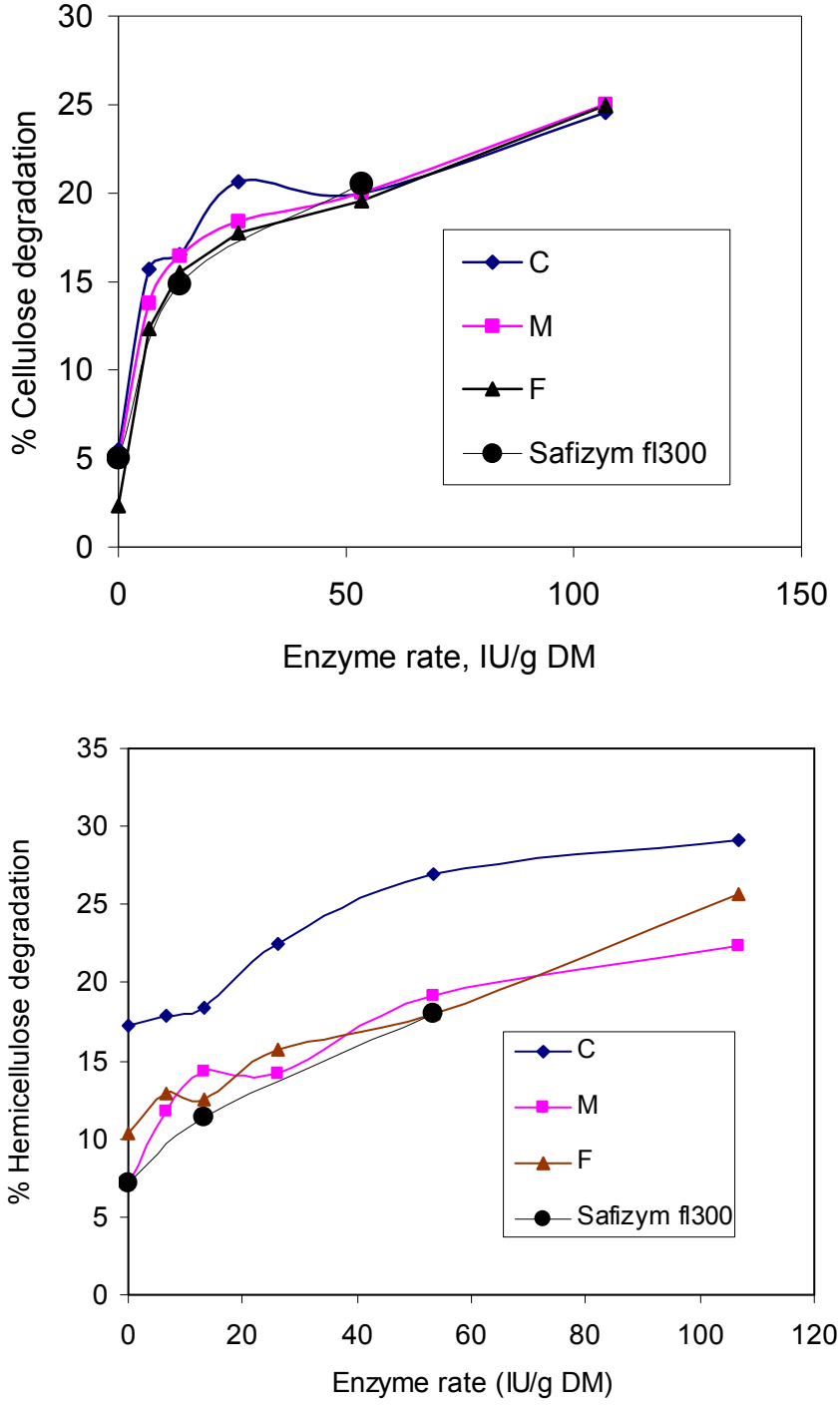


Figure 3.2 Degradation of cellulose and hemicellulose with increasing enzyme levels of purified and industrial enzymes (C for coarse size, M for medium size, and F for fine size).

Cellulose degradation was similar across the three corn stover sizes. For hemicellulose, the C size stover experienced significantly higher degradation ($P < 0.0001$) in both the control and enzyme treated samples. However, the difference in percent hemicellulose hydrolyzed between the control and any of the five enzyme rates was the lowest in the C size stover, followed by F and M. Therefore, the observed increase in total hydrolysis in the C size stover was not caused by the enzyme treatments, but rather was a result of naturally occurring saccharification during the ensilage process that the control samples experienced as well. The reason that the C size had the highest natural hemicelluose degradation is not clear. One possibility is that the higher moisture content per surface area unit in the C size enhanced saccharification by some facultative anaerobic bacteria and increased the diffusion of plant enzymes.

The products of cell wall hydrolysis were mainly five and six carbon sugars and oligosaccharides. Glucose is the sole monomer produced from cellulose, while a mixture of monomers, including glucose, xylose, arabinose, and mannose, are produced from hemicellulose. The higher losses of the hemicellulose fraction from the C size stover corresponded to increases in WSC, which probably was largely composed of pentoses.

3.4.5 Dry matter loss of silage stover

Corn stover size had a significant effect on dry matter loss in control samples, with the lowest DM loss of 5.5 % in the medium size samples and higest DM loss of 10.1% in the coarse size samples (Figure 3.3). This result agreed with the lowest and highest hemicellulose degradation in the medium and coarse sized control samples, respectively. Increasing enzyme concentration resulted in variable results on DM loss. The lowest DM loss of 1.5 % was obtained at an enzyme concentration of 6.7 IU g^{-1} DM in the medium size, 6.2 % in the coarse size and 6.6 % in the fine size both at 13.4 IU g^{-1} DM.

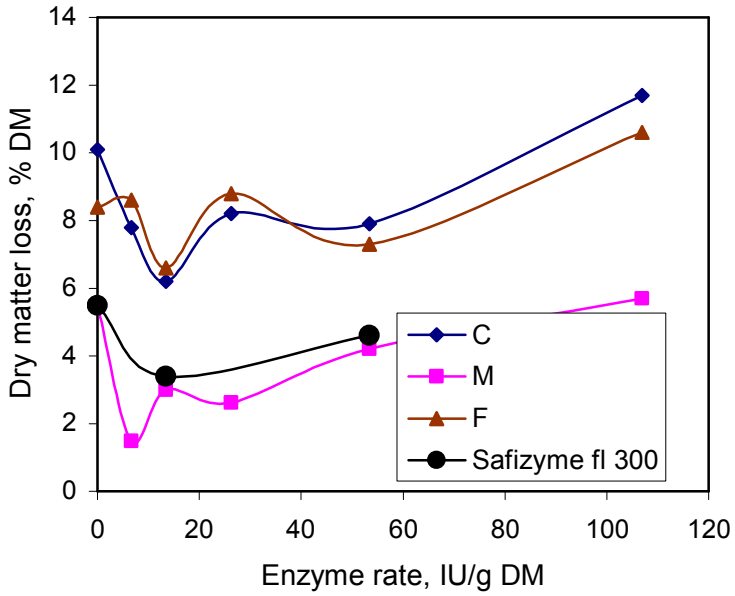


Figure 3.3 Dry matter loss with increasing enzyme levels of purified and industrial enzymes (C for coarse size, M for medium size, and F for fine size).

3.4.4 Industrial enzyme

The industrial enzyme Safizym fl300, represented by the black circles in plots, had a similar effect on chemical and fiber composition as with the purified enzyme when applied to the M size stover. Increased application levels of the industrial enzyme followed the same trends as the purified enzyme. The much lower price of the industrial enzyme makes it more attractive for large scale biomass storage.

3.5 Conclusions

Enzyme treatment showed positive effects for both the preservation and pretreatment aspects of corn stover ensilage. Enzyme additives significantly lowered the pH of silage after 21 days of ensiling. Cell wall degradation, particularly enzymatic cellulose degradation, increased considerably, with 22 – 29% of each fiber fraction hydrolyzed at the higher enzyme rates. The effect of enzyme additives on the characteristics of stover silage became more significant with increasing enzyme rates. The enzyme rate of 13.4 IU g^{-1} DM lowered the pH to around 4.3 – 4.4, and resulted in a

comparable lactic acid concentration to the 106.9 IUg^{-1} DM rate. The dominance of lactic acid production over butyric acid production was significant at this rate, and indicated a shift in primary fermentation from clostridia to lactic acid bacteria.

The stover size significantly affected pH, lactic acid concentration, WSC content, and hemicellulose degradation. Although the C size stover yielded the lowest lactic acid concentration, it had the highest WSC and hemicellulose hydrolysis levels. Considering the preprocessing phase and downstream bioconversion, C size stover offers the most promising benefits from the view of economics and efficiency. Coarse stover does not require any grind or chopping steps, which saves time, labor, and mill equipment. Enzyme addition and mixing might even be conducted by modified machinery during the wet stover harvest process. High levels of hemicellulose degradation and WSC should make further pretreatment of biomass, saccharificaion and sugar fermentation more effective and economical.

The industrial enzyme tested here was shown to be as effective as the purified enzyme at similar rates of hemicellulase and cellulase activity. At an enzyme amendment rate of 13.4 IU g^{-1}, the costs associated with the industrial enzyme tested are on the order of $63 per Mg of stover ensiled. With overall biomass storage costs estimated at $21.6 per Mg (Sokhansanj et al., 2002), this would be a significant addition. Minimizing the total costs of biomass storage and conversion will require optimizing the amended enzyme systems, lowering costs of enzyme production, and/or reducing costs of subsequent pretreatment to capitalize on the pretreatment effects that occurred.

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4. INVESTIGATION OF THE IMPACT OF ENZYME CHARACTERISTICS ON CORN STOVER PRESERVATION AND PRETREATMENT

4.1 Abstract

An anaerobic fermentation process similar to ensilage has been proposed as a preservation and pretreatment method for lignicellulosic biomass prior to industrial bioprocessing. To initiate this process, cell wall degrading enzymes may be required to supplement the low sugar content in biomass materials harvested during senescence. This study investigated the impacts on corn stover preservation and pretreatment of 7 commercial enzyme mixtures. The enzyme products were derived from three microbial sources: *Aspergillus niger*, *Trichoderma reesei*, and *Trichoderma longibrachiatum*. Treatments included three size grades of corn stover (< 10 cm, < 1 cm, < 0.5 cm), two enzyme levels (1.67 IU/g DM and 5 IU/g DM based on hemicellulase), and various ratios of cellulase to hemicellulase (C:H) in products derived from each microbial source. Higher lactic acid content and lower pH were obtained with increasing C:H ratios, especially with *Trichoderma reesei* enzymes. The highest C:H ratio tested, 2.38, resulted in the most effective fermentation, with lactic acid the dominant product. Significant cellulose and hemicellulose degradation was observed in these high C:H ratio enzyme mixtures derived from *Trichoderma reesei* indicating the additive rates could be reduced if preservation is the primary goal. More intensive enzymatic pretreatment during storage may complement industrial pretreatment strategies, creating synergies that could reduce total bioconversion costs.

4.2 Introduction

In recent years, corn stover, the above-ground residue of maize plants grown for grain, has attracted intensive interest as lignocellulosic feedstock for bioethanol production because of its considerable availability and biorenewability (Kadam and McMillan, 2003). Around 69 million dry Mg/year can be sustainably harvested in the U.S. (ORNL et al., 2005). Over 85% of the available corn stover is concentrated in Midwestern states (Walsh et al., 1999), which would reduce industrial-scale harvesting

and transportation costs as biomass based industries develop and mature. The rich content of polysaccharides in corn stover can be hydrolyzed to five and six carbon sugars for fermentation and chemical modification to produce value-added products (Sudha et al., 1998; Riera et al., 1991, Buhner and Agblevor, 1999). Corn stover can also serve as fiber feedstock for particle board manufacturing (Wang and Sun, 2002; Chow et al., 1999). Considerable efforts have been carried out to develop bioconversion processes and utilization strategies for corn stover (Green and Feng, 2005; Hu and Yu, 2005; Teymouri, 2005).

Bioconversion of corn stover from its raw form as a plant in the field to final commercial products requires four vital processing phases: harvesting, storage, pretreatment, and bioconversion. Addition steps provide links between these phases, such as transportation of the stover, neutralization of pretreatment chemicals and removal of toxic by-products before sugar fermentation. Although technical issues involved in the processes of pretreatment and bioconversion have been extensively investigated, the storage phase has received little attention to date. Since corn stover in the U.S. can only be harvested once a year, storage is needed to preserve large quantities of stover to provide a continuous supply to future biorefineries. Minimum criteria for storage include: minimizing dry matter loss, and reducing risk of fire. In addition, it would be preferable if beneficial pretreatment for downstream bioconversion could occur during the preservation period.

Ensilage, a traditional crop preservation method for ruminant feed, has been examined as a preservation method for corn stover (Ren et al., 2006; Richard et al. 2002). The high moisture content of silage (up to 60% wet basis) eliminates the risk of accidental fire. A robust lactic acid fermentation initially results in rapidly declining pH, and this low pH then inhibits most microbial activity and dry matter loss as long as anaerobic conditions are maintained. Ensiled storage can provide stable storage with minimal dry matter deterioration for as long as one year (McDonald et al., 1991). Acid hydrolysis of the cell wall by lactic and other produced acids can occur during the entire storage period, which may be beneficial for downstream cell wall degradation. However, the low sugar content of corn stover makes it difficult to obtain a robust lactic acid fermentation, resulting in more moderate pH that permits undesirable microbial growth,

such as clostridia, whose secondary fermentations reduce stability and degrade the biomass. Amending the corn stover ensilage process with cell-wall degrading enzymes has been shown to generate lower pH values, increase fiber hydrolysis to sugars, and conserve water soluble carbohydrates (WSC), thus providing partial pretreatment for downstream bioconversion into sugar platform chemicals and fuels (Ren et al., 2004). Enzyme treatment has also been shown to improve downstream manufacturing of stover based biocomposite materials. Particle board made from ensiled stover had enhanced physical strength and dimensional stability (Ren et al., 2006).

Enzymes prepared from different aerobic fungi have been observed to have various impacts on improving silage quality, although mechanistic investigations in an ensilage context are lacking. When *Trichoderma reesei* and *Aspergillus niger* were compared as amendments for ryegrass-clover silage, the most active degradation of cellulose and lower pH were found with *T. reesei* (Henderson and McDonald, 1977). However, the activity of individual enzyme components was not discussed or quantified in their study. Different microorganisms produce different types and proportions of individual enzymes, and their overall activity is a function of the characteristics and composition of these enzyme components. *T. reesei* excretes a complete set of cellulases with appreciable levels of endoglucanase and cellobiohydrolase (Allen and Roche, 1989). However, the level of β-glucosidase is not sufficient to thoroughly hydrolyze cellobiose, thus limiting the complete saccharification of cellulose to glucose (Sternberg et al., 1977). *A. niger* is another widely studied and commercially-used enzyme producer. The β-glucosidase productivity was found to be 4.8 times higher that of *T. reesei* (Flachner et al., 1999). But the activities of endoglucanase and cellobiohydrolase are weaker compared to *T. reesei*, even with genetically improved mutants (Kang et al., 2004; Kim et al., 1997). A recent study of enzymes was completed on maize silage to examine the effects of enzymes derived from *Flavobacterium xylanivorum*, *T. reesei*, and *Thermoascus acrantiacus*, which are psychrophilic, mesophilic or thermophilic organisms, respectively (Colombatto et al., 2004). This study found that *T. reesei* and *T. acrantiacus* reduced pH values and cellulose content significantly more than *F. xylanivorum*. These differences may also be caused by the various ratios and activities of individual enzyme components, including xylanase, endoglucanase, exoglucanase, and β-glucosidase, acting in

synergistic mixtures.

Similarly, there have been few detailed investigations of the effect of combinations of cellulase and hemicellulase enzymes on silage. The cross-linked spatial orientation of cellulose and hemicellulose in the cell wall is such that complete enzymatic hydrolysis of biomass requires synergistic interactions of cellulases and hemicellulases (Mielenz, 2001). Since most commercial enzyme additives are already mixtures of cellulase and hemicellulase, the effect of these additives on silage chemical composition is actually an integrated effect of these synergistic interactions. As one example of such synergy, hydrolysis of hemicellulose has been reported to increase the effective surface area of cellulose fibrils and, therefore, enhance cellulose hydrolysis (White et al., 1993). But we are not aware of any previous studies that have attempted to optimize the ratios of these enzymes to maximize the synergistic effects.

Effective and economical application of commercial enzymes for corn stover preservation requires a full characterization of the enzyme additives, including the microbial organisms they are derived from and the ratios of individual enzymes in each mixture. The present study 1) investigated and characterized cell wall degrading enzymes from three microbial sources: *T. reesei, A. niger,* and *T. longibrachiatum*; and 2) examined the effect of various ratios of cellulase to hemicellulase from these sources on the biochemical transformations of corn stover during ensilage. Since stover particle size might influence the contact efficiency and hydrolysis efficacy of the enzymes (Mooney et al., 1999; Allan et al., 1991), particle size was considered as an additional treatment variable in this study.

4.3 Material and Methods

4.3.1 Corn Stover and silage preparation

Corn stover was harvested by chopping, windrowing, and baling in the fall of 2002. Stover was then fiberized through a Art's-Way hammer mill (Art's-Way, Armstrong, IA) 0, 1 and 2 times to obtain three size grades of samples. The original coarse corn stover, once milled (0.5-1.0 cm) and twice milled (0.1-0.5 cm) stover sizes are called course, medium, and fine, respectively. The samples contained 16-20% moisture (w.b.) and were adjusted to 60% (w.b.) by adding water. A moisture level of

60% (w.b.)was previously determined to be the optimum moisture to minimize clostridia and secondary fermentations in a previous study (Richard et al., 2001).

Six replicates of each treatment (a complete factorial of size fraction x enzyme treatment x enzyme rate) were prepared, with three replicates of each treatment destructively sampled on day 0 and the other three destructively sampled on day 21. For each replicate, 500 g of treated sample was packed tightly into a 20cm×35cm polyethylene bag (200 g dry mass mixed with 300 g water) which was placed under 25 inch mercury vacuum and heat sealed immediately. Samples were incubated at 37 ±1ºC for 21 days. At the end of this preservation period samples were taken for dry matter (DM) and pH measurement. The remainder of each sample was stored frozen for later analysis of lactic acid, volatile fatty acids, water soluble carbohydrates, and fiber fractions.

4.3.2 Industrial enzyme additives

Seven enzymes derived from three different microbial organisms were added to the three size grades of corn stover at two different levels. These seven enzymes were chosen from an initial pool of 15 commercial enzymes to represent a diversity of microbial sources and a wide ratio of cellulase to hemicellulase. A description of the seven enzyme characteristics is summarized in Table 4.1.

Endo-1,4-β-glucanase, cellobiohydrolase, and cellobiase were measured according to the methods described by Wood and Bhat (1988) by using carboxymethylcellulose, avicel, and cellobiose as substrate, respectively. Hemicellulase measurement used 1% birchwood 4-*o*-methyl glucoronoxylan (Roth 7500) as substrates (Bailey et al., 1992). The enzymes were applied in liquid solution with water to adjust moisture content and were mixed evenly with the stover. The amount of enzymes applied was based on two constant hemicellulase activities: 1.67 IU g^{-1} dry mass and 5.0 IU g^{-1} dry mass. The data in each column is the measured activity for each enzyme, with the ratio of the component to hemicellulase in parenthesis. Every enzyme has the same units as presented for hemicellulase. The last column is the code representing each enzyme, with a letter for microbial source and number for the ratio of endo-1,4-β-glucanase to hemicellulase (C:H). Endo-1,4-β-glucanase was chosen to quantify cellulase based on the

facts that: 1) the order of the ratios of filter paper unit to hemicellulase of these seven enzymes is the same as that of the ratio of Endo-1,4-glucanase:H; 2) endo-1,4-β-glucanase initiates the degradation of cellulose by randomly and rapidly shortening the cellulose chain.

Table 4.1 Characteristics of industrial enzymes used to pretreat corn stover.

Microbial source	Hemicellulase	Endo-1,4-β-glucanase	Cellobiohydrolase	Cellobioase	Code C:H
Aspergillus niger	24805 U g^{-1}	1904 (0.08*)	42.9 (0.002)	87.4 (0.003)	AN0.08
	1075 Ug^{-1}	384 (0.36)	34.6 (0.03)	84.3 (0.080)	AN0.36
Trichoderma reesei	5712 U ml^{-1}	109 (0.02)	5.31 (0.001)	3.1 (0.0005)	TR0.02
	1219 U g^{-1}	2607 (2.14)	247.1 (0.20)	36.6 (0.03)	TR2.14
	116 U ml^{-1}	278 (2.38)	45.8 (0.39)	5.4 (0.047)	TR2.38
Trichoderma longibrachiatum	18624 U g^{-1}	5144 (0.28)	50.9 (0.003)	79.5 (0.004)	TL0.28
	390 U ml^{-1}	543 (1.39)	18.2 (0.05)	7.0 (0.018)	TL1.39

* the ratio of individual cellulase activity to hemicellulase activity.

4.3.3 Chemical analysis of silages

Physical characteristics and chemical composition of stover silage were analyzed using the same methods described in section 3.3.3.

4.4 Results and Discussion

4.4.1 Characterization of initial feedstocks

The initial characteristics of each stover size are shown in Table 4.2. There were some variations in fiber composition among the three sizes. These may result from different extents of natural degradation occurring on the three sizes before they were used for experiments. The fine size had the lowest concentration of cellulose and hemicellulose. The corollary to this reduced fiber content in the fine size was a higher ash concentration.

Table 4.2 Initial pH, fiber fraction, and water soluble carbohydrates (WSC) of each stover size fraction.

	Coarse	Medium	Fine
pH	7.32±0.067*	7.77±0.078	7.49±0.017
WSC** (% d.b.)	1.32±0.05	1.84±0.09	1.88±0.17
NDF[#] (% d.b.)	80.72±0.08	75.61±0.32	72.72±0.75
ADF[†] (% d.b.)	48.32±0.48	43.07±0.45	43.12±1.16
ADL[‡] (% d.b.)	5.08±0.13	5.03±0.21	7.56±0.70
Ash (% d.b.)	1.01±0.10	1.62±0.10	4.10±0.94
Cellulose (% d.b.)	43.24±0.57	38.04±0.23	35.56±1.85
Hemicellulose (% d.b.)	32.40±0.41	32.54±0.19	29.59±0.49

* average ± standard error

** Water soluble carbohydrates (WSC)

[#] Neutral detergent fiber (NDF); [†] Acid detergent fiber (ADF); [‡] Acid detergent lignin (ADL)

4.4.2 pH value of stover silage

The pH value of stover silage dropped to 3.79-4.76 after a 21-day ensilage process. Increasing C:H ratio resulted in lower final pH values within all three microbial sources of enzymes on medium size stover (Figure 4.1). However, the enzyme treatments with a low C:H ratio, especially at low enzyme concentrations, did not reduce pH significantly compared to the control sample (P=0.814 for TR0.02; P=0.114 for AN0.08) (pH value around 4.73-4.8). The TR treatment with the highest C:H ratio of 2.38 had the lowest pH value, 3.79, which can guarantee low levels of microbial activity and high preservation quality. For ensiled storage of biomass, low pH is desired not only for preservation purposes, but also to create an acidic condition to enhance hydrolysis. These

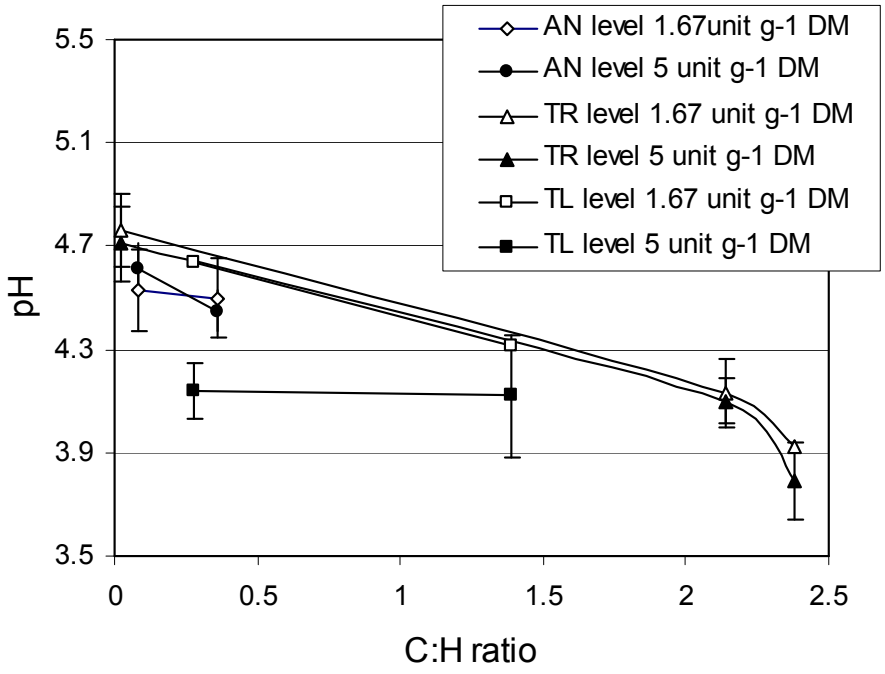

Figure 4.1 The pH change of ensiled corn stover versus the C:H ratio of enzyme additives for the medium size stover. (AN represents enzymes from the *A. niger* microbial source; TR represents enzymes from *T. reesei*; and TL represents enzymes from *T. longibrachiatum*. Each enzyme product with a certain C:H ratio is represented by data points, with the white color for low enzyme concentrations and black for the high concentration.).

acids partially break down the glycosidic linkages of microfibrils of cell walls, especially hemicelluose, during long-term storage. Dewar et al. (1963), who investigated hemicellulose degradation at various pH levels for 90 days, suggested that considerable hydrolysis of hemicelluloses can be obtained at pH 4. This can be presumed to be a beneficial pretreatment for downstream degradation.

Differences among microbial sources are reflected in the observation that higher C:H ratios did not guarantee a lower pH value. For example, at 1.67IU g^{-1} DM, TL0.28 did not resulted in a significantly lower pH value than that of AN0.08 (P=0.407).

The enzyme complexes produced by different microbial sources generated different responses in terms of pH. Increasing the level of the TR enzymes did not significantly reduce pH (P=0.713), suggesting that this enzyme system's hemicellulase component was already sufficient at the lower 1.67 IU/g DM rate. Increasing C:H ratio did increase the response for the TR enzymes, demonstrating the importance of synergistic effects. For TL enzymes, the increase of enzyme levels had a more significant effect in reduced pH value at the C:H ratio of 0.28 than at a C:H ratio of 1.39, suggesting that hemicellulase sufficiency for this enzyme complex occurred between these two rates, and that cellulase was sufficient for both C:H ratios at the higher 1.67 IU g^{-1} DM hemicellulase rate. Only two relatively low C:H ratios were tested for the AN enzymes. For this enzyme source there was not a significant effect of hemicellulase level suggesting that enzyme concentration was sufficient at the lower rate. Although not significant, there was a slight trend with increasing C:H, which might have been significant if enzyme mixtures had been available over a larger C:H range.

4.4.3 Chemical composition of corn stover silage

The nature of the mixed fermentation process and the quality of ensiled stover are reflected in the final chemical composition. Combinations of high concentrations of lactic acid and low concentrations of butyric acid in silage indicate a more active lactic acid fermentation and more dormant secondary fermentations (Leibensperger and Pitt, 1987, Ren et al. 2006). The chemical composition of stover samples treated and stored for 21 days are presented in Figures 4.2-4.4. Each figure is for one stover size, and includes results for all 7 enzyme types at the 1.67 IU/g DM hemicellulase rate as well as the

control. For each microbial source, increases of C:H ratio generally resulted in higher concentrations of lactic acid and lower concentrations of butyric acid. This trend was most significant for all three sizes of stover treated with enzymes from the TR microbial source ($P<0.001$ for lactic acid and $P=0.013$ for butyric acid). The fraction of lactic acid in the total acid products was highest for the TR 2.38 treatment in the medium size, where it was 80%. This represented the most efficient treatment for silage preservation,

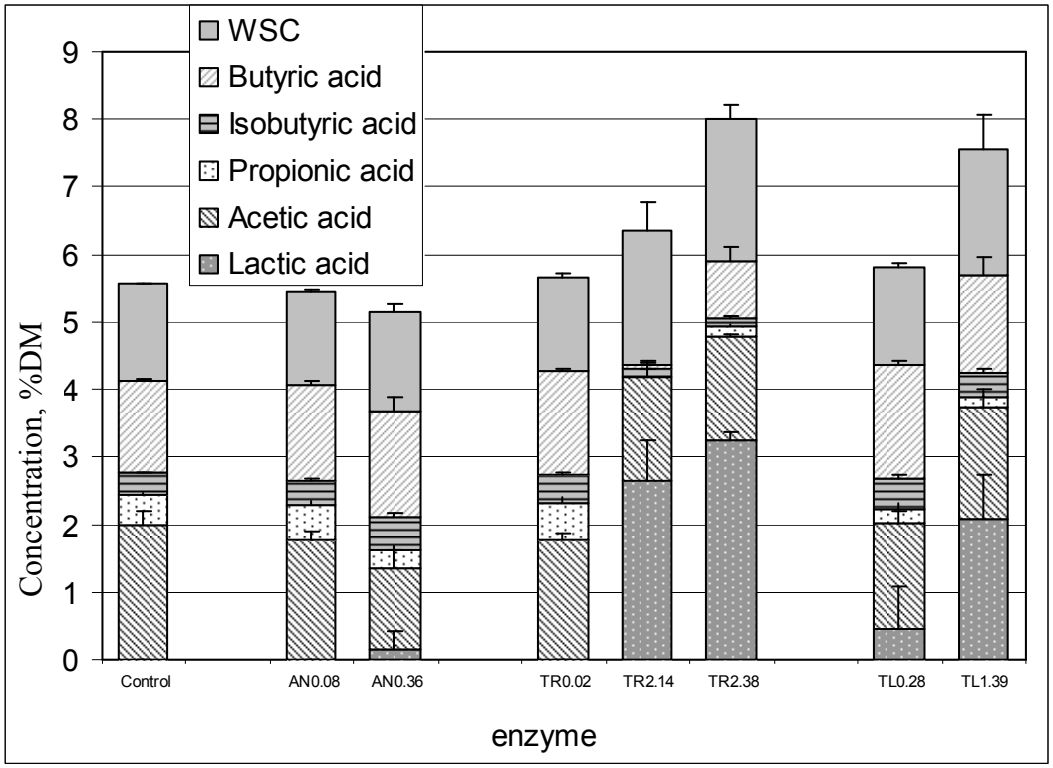

Figure 4.2 Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the fine size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase (C:H) of the enzyme products).

as fermented WSCs were primarily metabolized into lactic acid to lower the pH. For the enzymes derived from AN and TL microbial sources, increasing the C:H ratio only increased the lactic acid concentration, without consistently decreasing the amount of

acetic acid and butyric acid. The fraction of lactic acid was not improved by the increased C:H ratio because of similar or greater increases in total acid products.

For silage preservation purposes, the enzyme treatments would ideally exclusively encourage lactic acid fermentation with little or no contribution to secondary fermentation. If the enzyme treatment is not sufficient to encourage lactic acid production and reduce the pH low enough to inhibit clostridia, enzymatically hydrolyzed sugars will be mostly converted to an undesirable acid mixture (Rauramaa et al., 1987). Mandebvu et al. (1999) found that the treatment of bermudagrass silage with enzymes increased the butyric acid concentration without improving lactic acid or WSC production. Jakhmola et al. (1990) reported that the addition of cellulase had no significant effect on the forage of perennial ryegrass, and a mixture of perennial ryegrass and white clover mixed with shredded barley straw. Inadequate enzyme levels may also encourage yeast counts and ethanol production (Rauramaa et al., 1987). Enzyme treatments will not be beneficial for preservation if degraded sugars are not sufficient to initiate a dominate lactic acid fermentation. If hydrolyzed sugars are instead allowed to be assimilated by undesirable microorganisms, these treatments could lead to substantial DM loss. Environmental and chemical conditions in the first hours and days of a silage process are crucial for establishing and maintaining a lactic acid dominated system and an appropriate microbial ecosystem.

For all three stover sizes, some enzyme treatments with a low ratio of C:H (AN0.08 AN0.36, TR0.02, and TL0.28) did not produce much lactic acid. It is interesting that for the medium size stover, the lactic acid produced by AN0.08, TR0.02, and TL0.28 treatments was even lower than that of the control samples, though some of these differences were not statistically significant ($P=0.074$ for AN0.08; $P=0.007$ for TR0.02; and $P=0.21$ for TL0.28). Only TR2.14, TR2.38, and TL1.39, which had higher C:H ratios, enhanced lactic acid concentration significantly ($P<0.001$ for TR2.14; $P<0.001$ for TR2.38; and $P=0.033$ for TL1.39). Cellulases play a more important role in silage preservation than hemicellulase, since most of the hydrolyzed sugars come from the cellulose fraction (McDonald et al., 1991). Ren et al. (2006) observed that cellulose degradation is more sensitive to enzyme addition than hemicellulose. This difference was attributed to the heterogeneous structure of the hemicellulose and hemicellulase

specificity. Thus for the purpose of encouraging lactic acid fermentation, a certain amount of cellulase is required in the enzyme mixture. A higher ratio of C:H can result in an elevated lactic acid content in the final silage composition.

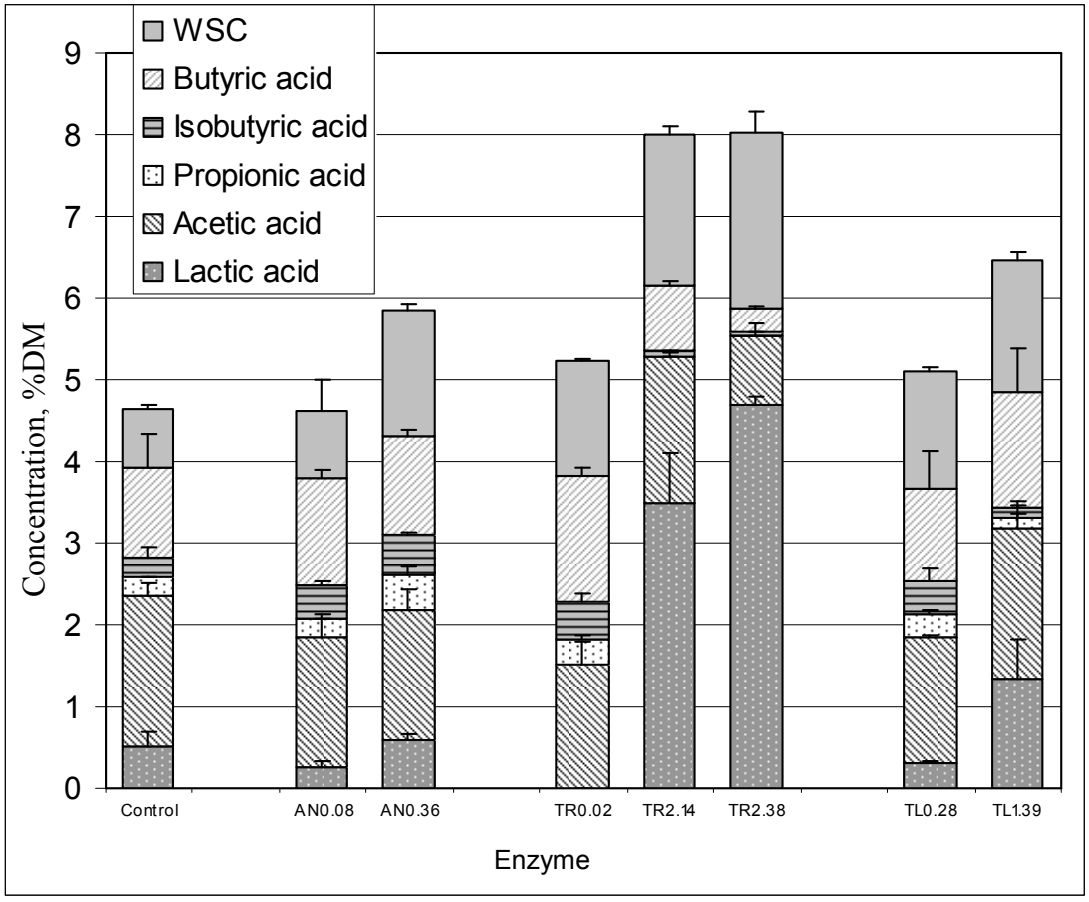

Figure 4.3 Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase (C:H) of the enzyme products).

It is difficult to examine the effects of microbial enzyme sources on stover silage using the present enzyme selection. These enzymes were produced and separated by different processes, and therefore cannot be assumed to have the same composition for individual enzyme components even when produced by the same microbial source.

However, there do appear to be some consistent results that may provide useful insights. Notably, when choosing enzymes available in the market for enzyme screening work, enzymes from AN always have a lower ratio of C:H compared to those of TR. Our results indicated that cellulases produced by AN always have lower activity of endo-1,4-β-glucanase g^{-1} enzyme than those of TR when the comparison is based on the same hemicellulase activity , which is consistent with the work of Kang et al. (2004) and Kim et al. (1997).

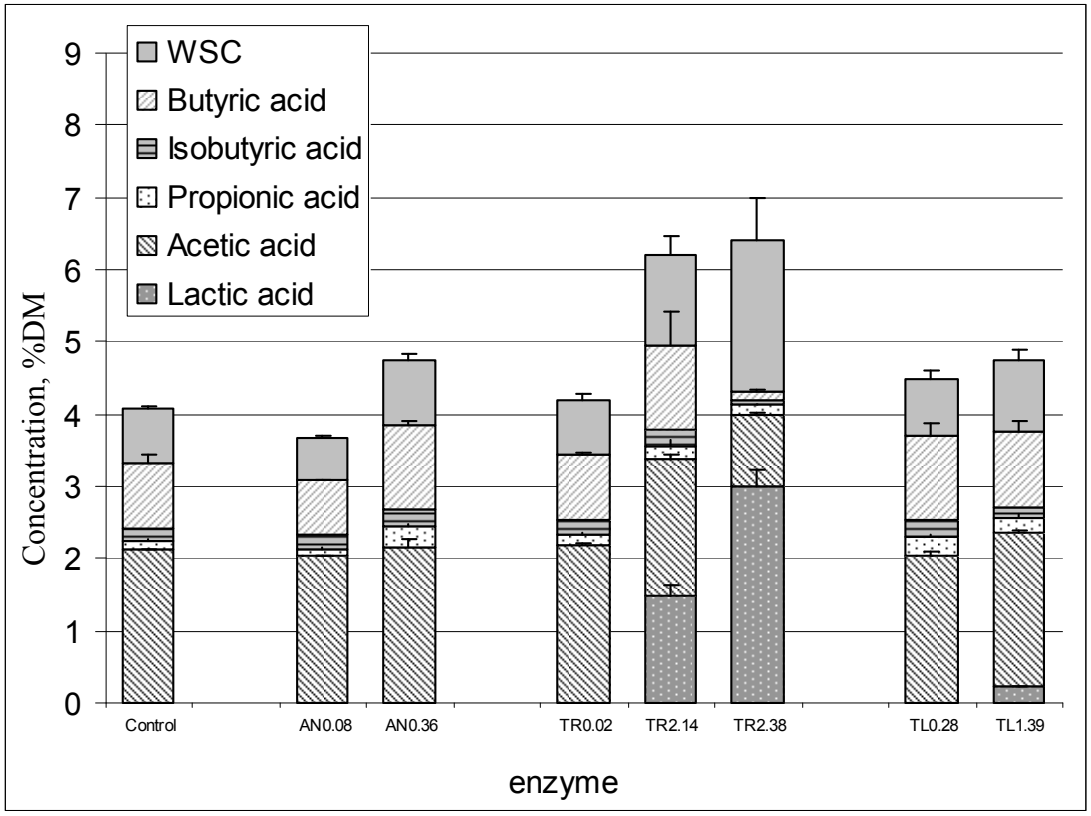

Figure 4.4 Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the coarse size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase (C:H) of the enzyme products).

An expected effect of enzyme addition is increased hydrolysis of the cell wall, releasing sugars that increase WSC content (Nadeau et al., 2000; Adogla-Bessa et al., 1999). However, in this study AN and TL enzymes did not enhance WSC content significantly in fine and coarse when compared to control samples (P=0.66 for AN in fine size; P=0.93 for AN in coarse size; P=0.227 for TL in fine size; P=0.402 for TL in coarse size). Only in medium size, AN0.36, TL0.28, and TL1.39 treatments resulted in significant increase of WSC compared to control samples (P<0.001). TR enzymes did significantly increase WSC content in each of the three sizes with the increase of C:H ratio (P=0.006 for fine size; P=0.002 for medium size; and P=0.03 for coarse size). The different effects of enzyme addition on WSC content depend on the fate of degraded WSC during the ensilage process (McDonald et al., 1991). The WSC content is the difference between the amount of initial WSC and hydrolyzed WSC, and the amount of the WSC metabolized by microorganisms (Morrison, 1988; Henderson and McDonald, 1971). The amount of both hydrolyzed WSC and metabolized WSC is influenced by the activity of microbial communities, especially lactic acid bacteria (LAB), throughout the ensilage process. This microbial consumption of hydrolyzed glucose can reduce or eliminate the glucose inhibition effects on cellobioase, resulting in an accelerated and more complete hydrolysis of the cell wall (Lynd et al., 2002).

The net result of these inputs and outputs to the WSC pool is determined by the the initial biomass characteristics including its WSC content, the amounts of cellulose and hemicellulose hydrolyzed to WSC by native and introduced enzymes, the population dynamics of vegetative microorganisms, and the metabolic pathways used by the microorganisms. For enzymes with a low ratio of C:H, such as AN0.08, TR0.02, and TR0.28, the relatively constant WSC can be attributed to a low level of cellulase activity that is not sufficient to generate large amounts of WSCs. Low levels of WSCs early in the ensilage process limited lactic acid production and the resulting pH decline, allowing secondary fermentations by clostridia to dominate in these treatments. For TR2.14 and TR2.38, a significant amount of lactic acid was fermented from the available WSCs in silage, and although this lactic acid consumed WSCs, low pH and reduced secondary fermentation resulted in a final WSC that was still significantly higher than the control samples (P<0.001). Previous long term trials have demonstrated that cell wall hydrolysis

continues throughout the ensilage process (Ren et al., 2006). The conversion of some of this sugar to lactic acid encourages more sugar production, resulting in higher final WSC concentrations in these treatments.

4.4.4 Fiber degradation of corn stover silage

Degradation of cellulose and hemicellulose at the two levels of enzyme addition are presented in Figure 4.5 and Figure 4.6. Degradation is reported as the difference between initial and final concentrations divided by the initial concentration (all on a DM basis), and thus assumes negligible dry matter loss. The average dry matter loss in previous expriemnts was 2.7% d.b. with the highest DM loss of 6.1% d.b. Fine and coarse size obtained the similar results of cellulose degradation with medium size while resulting in no significant increase of hemicellulose degradation. In the control sample, only hemicellulose degradation was observed. This is consistent with ensilage results for orchardgrass and lucerne (Yahaya et al., 2001), Italian ryegrass (Kawamura et al., 2001), and perennial ryegrass (Morrison 1979). All these studies reported an overwhelming preferential degradation of hemicellulose relative to cellulose. The degradation of hemicellulose during the ensilage process is catalyzed by indigenous plant hemicellulases, bacterial enzymes produced during ensilage, and acid hydrolysis by produced acids (McDonald et al., 1960). Although plant enzymes have been credited as contributers to sugar production (Heron et al., 1986; Ohyama and Masaki, 1977; Pitt et al., 1985), experimental results have been inconsistent. Bousset et al. (1977) did not observe hemicellulase activity in sterilized silage, and argued that if such activity did exist, the activity should be low because of compartmentalization, plasmolysis, and the short life of these enzymes. Dewar et al. (1963) found that plant enzymes lost most of their activities after three days of ensiling. Direct acid hydrolysis has been suggested by Dewar et al. (1963) and Morrison (1979) to be mainly responsible for the degradation of hemicellulose. High levels of hemicellulose degradation have been reported to occur at a pH level of 4 (Dewar et al., 1963). Cellulose degradation at this pH is much lower, since cellulose has more resistant microfibrils with extensive crystalline regions, and relatively moderate acid hydrolysis can not break down the beta 1-4 glycosidic links buried in this three-dimensional structure.

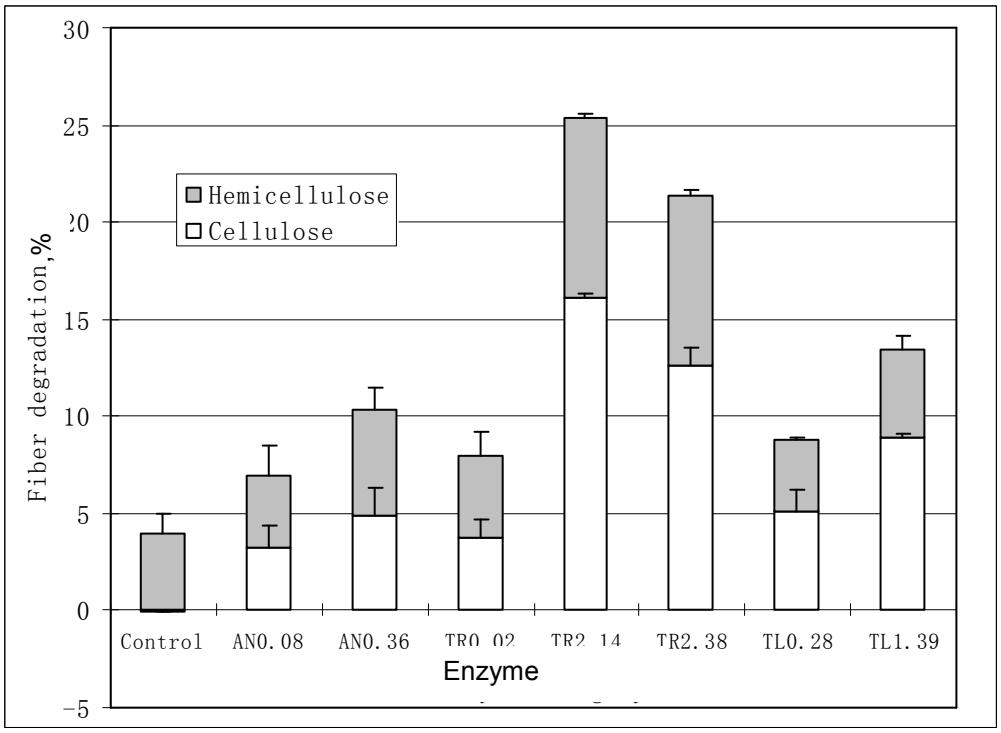

Figure 4.5 Degradation of cellulose and hemicellulose with different enzyme treatments at the level of 1.67 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase (C:H) of the enzyme products).

It is not surprising that enzyme additions increased cellulose degradation significantly. For treatments with enzymes from the same microbial source, cellulose degradation increased with the increase of C:H ratio, except for TR2.38. This anomalous result may be an artifact of higher DM loss in the TR 2.38 treatment. Increased cell wall degradation would have reduced the DM basis for the measured concentrations, resulting in a higher percentage of cellulose in the silage, and a corresponding smaller apparent cellulose loss. Comparing across microbial sources, the cellulose degradation in the TL0.28 treatment was higher than that with AN0.36 at the 5 IU/g DM hemicellulase level, even though the latter had a higher C:H ratio. A similar but smaller difference between these treatments was observed at the 1.67 IU/g DM level. This was also the case

when comparing TR0.02 and AN0.08 treatments, with the lower C:H ratio from the *T. reesei* source resulting in higher cellulose degradation. However, we need to be cautious before definitively concluding that enzymes from *T. longibrachiatum* and *T. reesei* are more effective at cellulose degradation than those of *A. niger* at the same C:H ratio. Cellulose degradation is a complicated synergistic process that includes contributions by at least 6 individual enzyme components (Lynd et al., 2002). Complete elucidation of the effects of the microbial source of enzymes on cell wall degradation requires enzyme characterization on the molecular level.

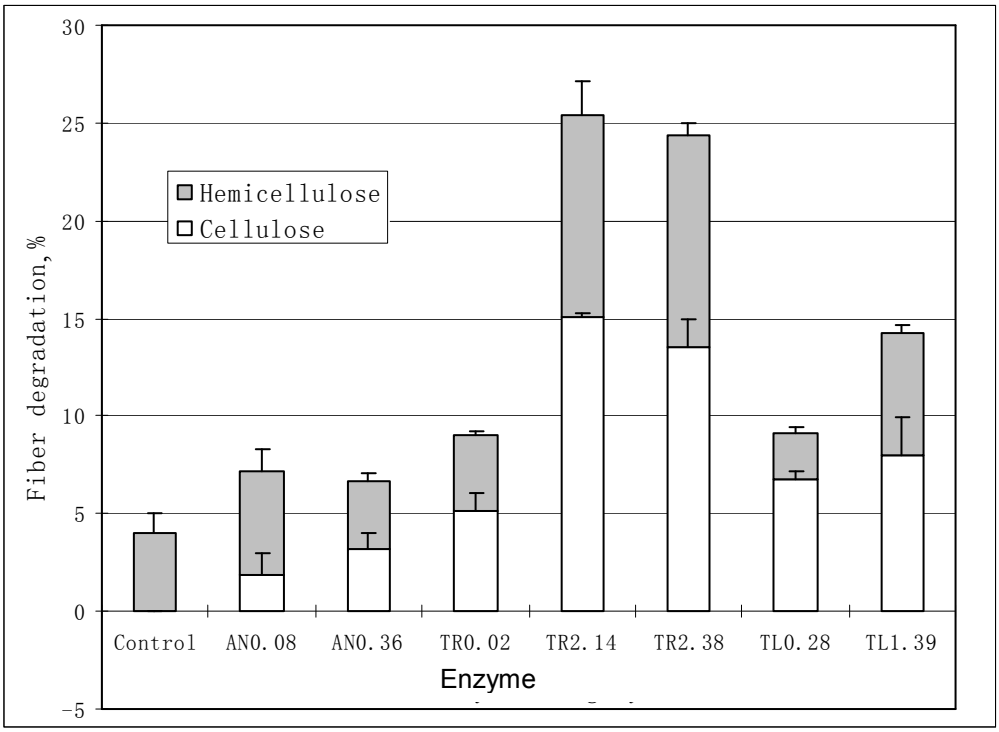

Figure 4.6 Degradation of cellulose and hemicellulose with different enzyme treatments at the level of 5 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase (C:H) of the enzyme products).

Significant increases in the degradation of hemicellulose were only observed for the treatments with TR2.14 and TR2.38 in medium size. Other enzyme treatments did not

significantly enhance hemicellulose degradation. Similar results have been reported by Ren et al. (2006) and Van Vurren et al. (1989). The increased hemicellulose degradation observed with TR2.14 and TR2.38 in medium size is probably because the higher lactic acid concentration and lower pH value (below 4.0), enhancing acid hydrolysis as previously discussed. The higher degradation of cellulose observed with these treatments may also have contributed, by partially removing the structural hindrance around hemicellulose and increasing access of hemicellulase to the substrate (White et al., 1993).

Increasing the hemicellulase enzyme level did not change fiber degradation significantly. The chemical composition of stover silage for enzyme treatments at the higher hemicellulase level were also similar to previously reported data at the lower (1.67 IU g^{-1}) level (Ren et al., 2006). To decrease the cost of enzyme additives in full-scale industrial applications, the lower level of enzyme amendment is preferable.

4.5 Conclusion

Mixtures of different ratios of cellulase and hemicellulase enzymes from different microbial sources had varying effects on fiber hydrolysis during ensiled storage of corn stover biomass feedstock. To facilitate comparisons among treatments, mixtures were normalized on the basis of hemicellulase activity. For each enzyme mixture and C:H ratio, two hemicellulase levels were tested, 1.67 IU g^{-1} and 5 IU g^{-1}, and results indicated the lower level of hemicellulase was sufficient to achieve the most beneficial effects. At each of these hemicellulase levels, the ratio of cellulase to hemicellulase was important for improving the quality of stover silage. Increasing ratios of C:H reduced pH, increased lactic acid concentration, and decreased butyric acid concentration.

Successful development of ensilage as a biomass storage strategy will require minimizing dry matter loss. Dry matter loss often results from secondary fermentations, which can be suppressed by high concentrations of lactic acid and the resulting reduced pH. In order to increase the concentration of lactic acid and suppress these secondary fermentations, a critical C:H ratio is required. Mixtures at or above this critical C:H ratio will have sufficient cellulase to hydrolyze glucose for fermentation into lactic acid. For the enzyme mixtures tested, this critical C:H ratio was somewhere in the vicinity of 2.14,

but is likely to vary somewhat depending on the specific synergistic interactions of enzymes from a particular microbial source.

One of the potential benefits of an ensiled storage process would be *in-situ* pretreatment and hydrolysis of polymers during storage to produce water soluble carbohydrates for downstream bioconversion. This benefit was observed in many of our ensilage treatments. The WSC content of stover silage depended on the ratio of C:H in the applied enzymes as well as the size of stover material. For each of the stover sizes tested, enzymes from *T. reesei* increased WSC content at increasing rates with increasing C:H ratios. Results for other stover sizes and microbial sources were less consistent, but similar trends were observed. The effect of the microbial source of enzyme mixtures can not be completely elucidated based on our current results. But enzymes mixtures derived from *T. reesei* and *T. longibrachiatum* appear to hydrolyze more cellulose than those derived from *A. niger*, even when the ratio of C:H in the former mixtures is less than that of the latter. These differences suggest that optimized enzyme mixtures can provide significant pretreatment benefits during ensiled biomass storage.

Hemicellulose is more easily hydrolyzed than cellulose by the acid conditions that prevail during the normal ensilage process. Thus it was not surprising that the addition of enzymes improved cellulose degradation more significantly than that of hemicellulose. However, this improved cellulose degradation also contributed to lower pH and presumably increased access of hemicellulase to substrate, resulting in considerably improved hydrolysis of hemicellulose for the high C:H treatments. Development of microbial strains that can convert both 5- and 6-carbon sugars to ethanol and other value-added chemicals makes increased hemicellulose hydrolysis important for maximizing product yields.

Because the hemicellulase enzyme concentration did not have a significant influence on pH, chemical composition, or fiber degradation of the final stover silage, minimizing the enzyme treatment level should maximize economic returns. For the high C:H ratios of 2.l4 and 2.38 in mixtures derived from *T. reesei*, the low 1.67 IU g^{-1} hemicellulase level appears more than sufficient to achieve positive results. Further research to optimize these levels and the increase in synergies among enzyme mixture

components appears likely to result in attractive ensilage strategies for industrial storage of large volumes of biomass feedstocks.

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5. ENSILING CORN STOVER: EFFECT OF FEEDSTOCK PRESERVATION ON PARTICLEBOARD PERFORMANCE[1]

5.1 Abstract

Ensilage is a truncated solid-state fermentation in which anaerobically produced organic acids accumulate to reduce pH and limit microbial activity. Ensilage can be used to both preserve and pretreat biomass feedstock for further downstream conversion into chemicals, fuels, and/or fiber products. This study examined the ensilage of enzyme treated corn stover as a feedstock for particleboard manufacturing. Corn stover at three different particle size ranges (<100 mm, <10 mm, and <5 mm) was ensiled with and without a commercial enzyme mixture having a cellulase:hemicellulase ratio of 2.54:1, applied at a hemicellulase rate of 1670 IU/kg dry mass. Triplicate 20 L mini-silos were destructively sampled and analyzed on days 0, 1, 7, 21, 63, and 189. Analysis included produced organic acids and water soluble carbohydrates, fiber fractions, pH, and microorganisms including *Lactobacillus spp.* and clostridia were monitored. On days 0, 21, and 189, the triplicate samples were mixed evenly and assembled into particleboard using 10% ISU 2 resin, a soy-based adhesive. Particleboard panels were subjected to industry standard tests for modulus of rupture (MOR), modulus of elasticity (MOE), internal bonding strength (IB), thickness swell (TS), and water absorption at 2 hr boiling and 24 hr soaking. Enzymatic addition did improve the ensilage process, as indicated by sustained lower pH ($P < 0.0001$), higher water soluble carbohydrates ($P < 0.05$), and increased lactic acid production ($P < 0.0001$). The middle particle size range (<10 mm) demonstrated the most promising results during the ensiling process. Compared with fresh stover, the ensilage process did increase IB of stover particleboard by 32.62% ($P < 0.05$) and decrease water adsorption at 2 hr boiling and 24 hr soaking significantly ($P < 0.05$). Particleboard panels produced from substrate ensiled with enzymes, showed a

[1] A version of this chapter was published as Ren, H., T.L. Richard, Z. Chen, M. Kuo, Y. Bian, K.J. Moore, and P. Patrick. 2006. Ensiling Corn Stover: Effect of Feedstock Preservation on Particleboard Performance. *Biotechnology Progress* 22(1):78-85.

significant reduction in water adsorption of 12.10% at 2 hr-boiling testing. Based on these results, ensilage can be used as a long-term feedstock preservation method for particleboard production from corn stover. Enzyme amended ensilage not only improved stover preservation, but also enhanced the properties of particleboard products.

5.2 Introduction

The demand for fiber products has increased dramatically in the last 50 years as the world population has exploded and consumption levels have increased, especially in developing countries (Rowell et al., 1997). Manufacturing composites from agricultural residue is attractive as an alternative production strategy to releave some of the pressure from increased demand and the low growth rate of trees. Among the available agricultural residues, corn stover is the most abundant, with an estimated 90 million dry Mg $year^{-1}$ in the United States alone (NRC, 2000; Glassner et al., 1998).

Corn stover contains over 80% fiber mass, which combined with its widespread availability, has motivated several investigations of composites using stover (the stalk, leaves, husk and cob) and its various component fractions. High density particleboard has been made from maize husk and cob (Sampathrajan et al., 1992), low density particleboard has been produced from corn pith (30% combined with wheat straw (70%) (Wang and Sun, 2002), and thermoplastic composites have been made from cornstalk fibers cemented by high-density polyethylene (HDPE) (Chow et al., 1999). Particleboard made from corn stover has been tested in a variety of applications, including floorboards, desk core, and bulletin boards. Enough stover is available to supply all these applications and more. Estimates indicate that complete substitution of stover for wood fiber in the medium density fiberboard (MDF) and particleboard markets would require 9 million dry Mg of corn stover per year, or less than 10% of the quantity available (Kadam and McMillan, 2003).

Although there appears to be considerable potential for the use of corn stover in composite materials, most of the research to date has been carried out at a laboratory scale. Implementation at an industrial scale faces several challenges, not least of which is the need for safe and effective long-term storage of the biomass feedstock. Corn stover is harvested only once a year in North America, and industrial utilization requires storage of

large volumes of stover to allow year-round manufacturing. Currently, corn stover is stored in dry bales and stacks, with moisture contents ranging from 14-33% (Sokhansanj et al., 2002). Relatively high moisture, whether caused by insufficient field drying or precipitation and high humidity, encourages microbial degradation and can result in dry matter losses up to 23% (Richey e al., 1982). This aerobic degradation not only destroys the lignicellulosic feedstock, but also generates heat that increases the risk of catastrophic fire. Storing at widely separated sites will reduce the loss in case of fire, though this increases the facility and transportation costs. In order to solve these problems, ensilage has been proposed to preserve large quantities of corn stover. Under ensiled conditions the moisture content is high (up to 60% dry basis, d.b.) thus eliminating the risk of fire (Richard et al., 2001), while dry matter losses are typically less than 10%.

Ensilage is traditionally used to preserve crops for feeding ruminants during winter. Traditional crop silage depends on low pH and anaerobic conditions to inhibit undesirable microbial growth and prevent deterioration (McDonald et al., 1991). Under anaerobic conditions, a synergistic system of naturally occurring microorganisms and plant enzymes hydrolyze crop cell wall polymers and ferment the resulting sugars (Henderson, 1993). Organic acids are produced from this sugar fermentation, usually dominated by lactic acid, and result in the necessary pH drop. Low pH inhibits microbial growth and reduces undesirable organisms, such as clostridia. Degradation of cell wall structural carbohydrates and fermentation of degraded sugars are the major processes required to convert biomass to chemicals and fuels, so synergies between ensiled storage and downstream pretreatment and conversion appear promising (Richard et al., 2002). However, the impact of this storage strategy on fiber composite properties is currently unknown.

Ensilage could change either the quantities or the qualities of various fiber constituents (cellulose, hemicellulose, and lignin) of stover. With respect to quantities, ensilage is expected to hydrolyze hemicellulose selectively over cellulose. In a conventional ensilage process without additives, a much as half of the initial hemicellulose content is degraded (McDonald et al., 1960) while cellulose losses are less than 5% (Morrison, 1979). Hemicellulose has a higher water solubility and swelling ability than cellulose, so reducing hemicellulose content may result in less water

adsorption; this should improve the dimensional stability of the final composite products. However, this benefit may be negated by a decrease in the quality (length, strength, etc.) of the fiber constituents that remain.

Shinners et al. (2003) reported successful ensilage of fresh corn stover in horizontal bagged silos and wrapped bales sealed with plastic, but there is some concern that these results may not be replicable in low-cost bunkers silos or other configurations that are not as effectively sealed. An initial investigation in our lab with unamended stover resulted in undesirable secondary fermentations, with higher concentrations of acetate than lactic acid, increasing concentrations of butyric and isobutryic acid with time, and pH exceeding 5 by the end of the eight week trial (Richard et al., 2001). To insure a robust lactic acid fermentation on corn stover silage, we then investigated using cell wall degrading enzymes as a fermentation stimulant (Richard et al., 2002). In that initial enzyme amended study, the supplemental enzymes improved the production of fermentable sugars by a factor of six and increased fermentation of the sugars into lactic acid by a factor of 12 in 0.5L mini-silos during a 21-day ensilage process. Although these additional results were promising, additional experimentation is needed to verify the efficacy of enzyme addition through longer term corn stover ensilage on a larger scale.

Developing effective strategies to preserve and pretreat corn stover as biomass feedstock requires integration with both the upstream harvest and collection process, as well as downstream processing to produce composites and other co-products. The present study 1) investigated the feasibility of enzyme-enhanced ensilage to preserve corn stover on a pilot scale; 2) enumerated microorganisms in stover silage to identify the influence of enzyme addition on the fermentation type; and 3) manufactured and evaluated composites produced from fresh stover as well as ensiled stover with and without enzyme additives. Because stover particle size might influence the contact efficiency of the enzymes with corn stover and thus the cost and efficacy of pre-processing, we included the effect of particle size reduction as an additional treatment variable.

5.3 Material and Methods

5.3.1 Preparation of corn stover silage

This study examined six ensiled treatments, including three stover particle sizes, each with and without an enzyme supplement. Composite boards were also manufactured from an unensiled control. The corn stover, harvested in fall 2002 in Ames, IA, was ground through a Art's-Way hammer mill (Art's-Way, Armstrong, IA) to obtain three size grades. Coarse (as baled, <10 cm); medium milled (< 10 mm) and finely milled (< 5 mm) are indicated as sizes C, M, and F, respectively. The stover had been stored dry and contained 16-20% moisture (w.b.), which was adjusted to 60% (w.b.) by adding water. The enzyme treatments used Genencor Multifect A40 (Genencor International, Palo Alto, CA), a mixture of cellulase and hemicellulase with respective activities in the ratio 2.54:1 respectively. In this study the enzyme supplement was applied at a hemicellulase rate of 1670 IU kg^{-1} dry mass, which reduced the enzyme cost to approximately 3 cents per kg dry mass. For each replicate, 5 kg (w.b.) of treated sample was packed tightly into a plastic bag within a 20 L mini-silo, partially evacuated to no greater than 150 Torr, and immediately heat sealed. Silos were incubated up to 189 days at 37ºC, which is the traditional standard temperature for ensilage investigations (Hunter and Bushnell, 1916). For each of the six treatments, triplicate mini-silos were destructively sampled and analyzed on 0, 1, 7, 21, 63, and 189 days.

5.3.2 Physical and chemical analysis of stover silage

Physical characteristics and chemical composition of stover silage were analyzed using the same methods described in section 3.3.3.

5.3.3 Microbial populations

Microbial analysis was carried out on a sample mixed from the three replicated mini-silos of each treatment. A 20-g composite silage sample was diluted with 200 g deionized (DI) water and macerated for 1 min at 18,000 rpm rate in a sterile homogenizer (Waring, New Hartford, CT). The extract was further diluted into a dilution series in 1 g $liter^{-1}$ peptone. Enumeration of *Lactobacilli* used a pour plate technique on Rogosa SL agar (Rogosa et al., 1951; Difco, Sparks, MD). Agar plates were incubated at 37ºC for

72h. Clostridia spores were enumerated on reinforced clostridial agar (CM 151, Oxoid, Basingstoke, Hampshire, England) supplemented with 0.2 g L^{-1} of cycloserine to inhibit *Bacillus* growth after incubation in an anaerobic tank at 37ºC for 72 h.

5.3.4 Particleboard manufacturing

On days 21 and 189 triplicate mini-silos of each treatment were combined and mixed evenly, dried, and ground to 20 mesh (0.84 mm) after sampling for the standard chemical analyses described previously. Fresh stover that had not been ensiled was also dried and ground through the same procedure as that of ensiled stover. A soy-based resin, ISU2 (Kuo and Stokke, 2001), was used as the adhesive and added at 10% dry matter weight. Resin and stover were mixed for 15 minutes in a custom mixing chamber. An 885 g mixture was hand-fed into a 30.5 × 30.5 cm board forming box to obtain a final density about 1 g cm^{-3}. Each particleboard was pressed for 7 minutes at 200°C using a Wabash MPI V150H-18-PX press (Wabash, Indiana). Two replicate boards were produced from each treatment on the three sampling dates, and three test panels were cut from each replicate board. Particleboard panels were subjected to standard ASTM D-1037 (ASTM, 1999) tests for modulus of rupture (MOR), modulus of elasticity (MOE), and internal bond (IB). The thickness swell (TS) and water absorption after 2-hr-boiling and 24-hr-soaks were measured using two additional 10.2 × 12.7 cm specimens from the same duplicate boards.

5.4 Results and Discussion

5.4.1 Silage of corn stover

In a previous study of a 21-day ensilage by Richard et al. (2002), it was concluded that the most rapid pH decline happened in first 7 days and remained at that level from days 7 to 21. This was confirmed and extended by the six-month ensilage results (Table 5.1). The pH on day 0 averaged 7.77 ± 0.28. The pH value dropped quickly during the first 7 days, with the most rapid decrease during the first day. The enzyme treatment decreased pH value significantly throughout the experiment relative to the control samples ($P < 0.0001$). The samples without enzymes stabilized at pH around 4.6-4.9 after day 21 while the enzyme treated samples stabilized at about pH 4.0-4.5. Rapid and deep

drops in pH, especially in the first day, tended to persist throughout the whole preservation period. The rapid and deep drop in pH for the enzyme treated sample implies these enzymes enhanced hydrolysis and facilitated acid production from the beginning of the ensilage process. The addition of enzymes caused a greater pH drop for the medium size stover relative to the course and fine sizes. For the various sized treatments without enzymes, pH values were not significantly different. Although the effect of grinding was not consistent across treatments, moderate grinding does appear to have positive impact on enzymatic hydrolysis of polymers, which would be expected as a result of increased surface area.

Table 5.1 The pH change of ensiled corn stover with and without enzyme treatment.

	Enzyme			Control		
Time (day)	Coarse	Medium	Fine	Coarse	Medium	Fine
0	7.50c*	7.77b	8.05a	7.50c	7.77b	8.05a
1	5.25c	4.81d	5.34c	6.36a	6.12b	6.51a
7	4.66d	3.93e	4.61d	5.03c	4.95b	5.18a
21	4.54c	3.97e	4.45d	4.77a	4.63b	4.84a
63	4.54a	4.00c	4.25b	4.54a	4.64a	4.73a
189	4.50cd	4.05e	4.42d	4.87a	4.65bc	4.78ab

*Means in the same row with different letters are significantly different ($P<0.05$)

5.4.2 Organic Acids and Water Soluble Carbohydrates

The effect of the enzyme treatment on WSC and the produced acids on three particle size treatments is shown in Figures 5.1 through 5.3. For all three sizes, enzyme addition increased WSC and lactic acid concentration significantly throughout the preservation period relative to the untreated silage ($P < 0.0001$). Differences in WSC, lactic acid, and other organic acid concentrations were observed for the different stover size grades, and within each stover size these concentrations also varied over time.

With the exception of the medium size enzyme amended treatment, WSC experienced the highest concentrations on days 0 or 1, then decreased slowly over time.

An increase in WSC was often observed on the initial day, presumably caused by microbial saccharification and/or enzymatic degradation. Both mechanisms are expected to peak during the first day, microbial saccharification because the system is initially aerobic until pore oxygen is consumed, and enzymatic hydrolysis because the enzymes have not had much opportunity to degrade. Although the enzyme additives have been reported to remain active during the ensiling process, the efficacy and thermal stability of enzymes decrease with time (Colombatto et al., 2004), which partially explains the decrease of WSC of enzyme treated samples. Over time the WSC produced during the initial period is consumed by *Lactobacillus* spp. and clostridia, fermenting the WSC into organic acids. These results were consistent with those reported by Meeske and Basson (1999), who supplemented silage with amylase, a starch degrading enzyme. They found WSC content decreased continually over 40 days of ensiling, following a similar progression both with and without amylase, but with a slower reduction in WSC content between days 2 and 5 in the enzyme treated samples.

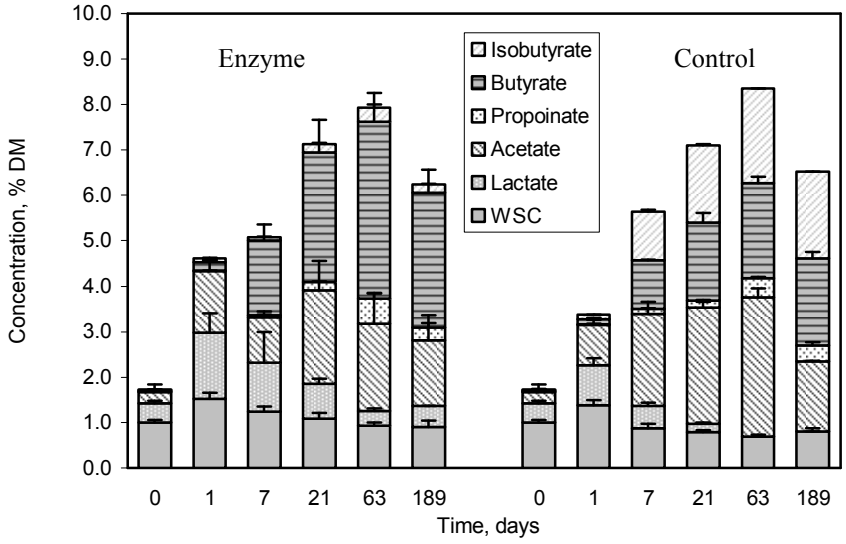

Figure 5.1 Time course of water soluble carbohydrate and organic acid concentrations with and without enzyme treatment for the coarse (as baled) size stover

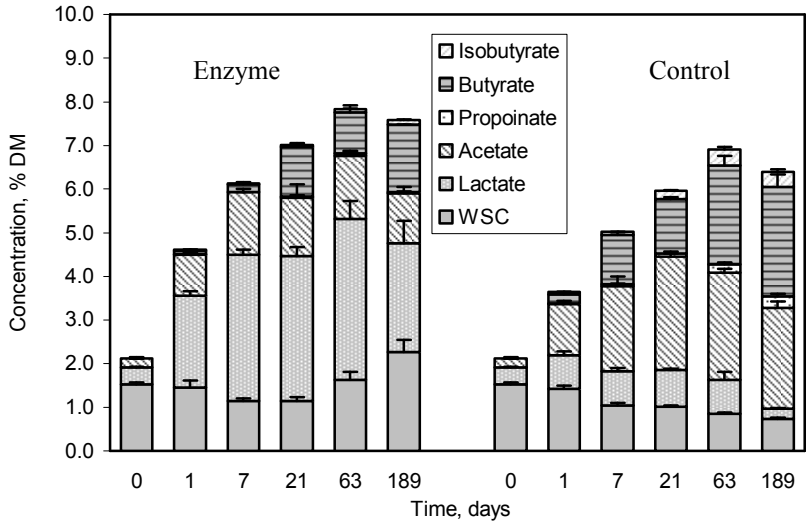

Figure 5.2 Time course of water soluble carbohydrate and organic acid concentrations with and without enzyme treatment for the medium size (<10 mm) stover.

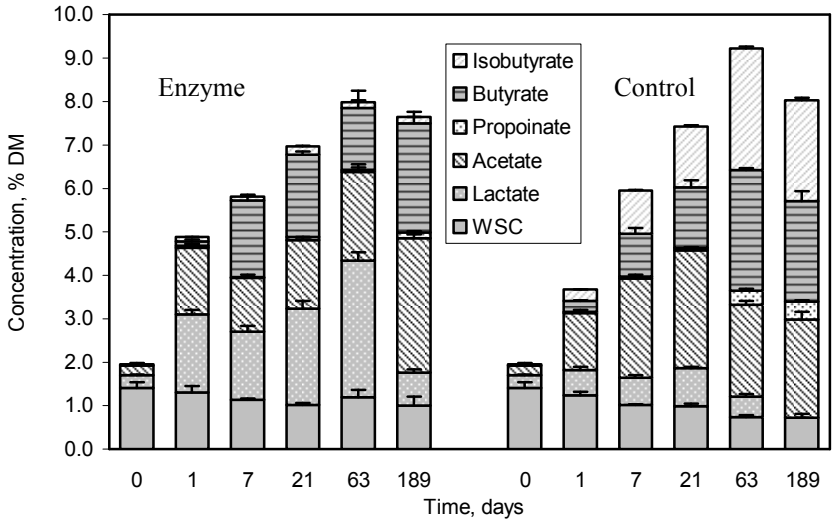

Figure 5.3 Time course of water soluble carbohydrate and organic acid concentrations with and without enzyme treatment for the fine size (< 5 mm) stover.

There were two exceptions to the generally consistent decline in WSC changes during the course of the 189 day trial. In the medium and finely ground enzyme amended treatments, WSC decreased in the first 21 days to 1.14 and 1.01%, respectively, and then increased slowly to 2.27 and 1.19% (d.b.) at the end of the process. Net decreases in

WSC during the initial 21 days indicates active fermentation by *Lactobacillus* spp. and clostridia at rates greater than the enzymatic hydrolysis of cellulose and hemicellulose. Increases in WSC after 21 days imply either an increase in hydrolysis (unlikely) or a decline in the fermentation rate. A decline in fermentation rate is likely due to microbial aging, as evidenced by decreasing bacterial counts (see below). Although the coarse and fine sizes had similar concentrations of WSC, the higher lactic acid production in the fine size replicates indicates that there was greater enzymatic hydrolysis in this treatment, since hydrolysis is the source of both WSC and the organic acids.

In conventional ensilage, lactic acid is normally the dominant acid produced. In this study lactic acid concentrations were greater than those of all other acids combined in the enzyme amended medium size treatment, and lactate was the largest single acid in the enzyme amended fine size treatment through day 63. However, the other treatments were dominated by acetate, butyrate, and isobutyrate. These are likely evidence of secondary fermentations by clostridia, which convert fermentable sugars and lactic acid into these other organic acids. Although it was historically thought that clostridia were exclusively responsible for the production of butyrate, modern molecular tools indicate other organisms can also create that metabolite (Louis e al., 2004).

Isobutyrate was generally much lower with enzymes than without, particularly for the coarse and fine sizes. Isobutyrate is generally fermented from amino acids by proteolytic clostridia. The additional glucose and lactic acid made available by enzyme addition would encourage saccharolytic clostridia, thus inhibiting proteolytic clostridia due to competition for proton donors in metabolism. The low pH value of enzyme treated samples also inhibits proteolytic clostridia relative to saccharilytic clostridia (Anderson and Jackson, 1970). Evidence for pH mediated competition was evident in the samples without added enzymes, where the coarse and fine sizes had significantly higher pH as well as isobutyrate concentrations than the medium size. Although the addition of enzymes did not increase the total fermented products much compared with the control samples, enzyme treatment did shift the dominant fermentation pathways from proteolytic metabolism by clostridia to lactic acid fermentation.

For all treatments, the combined WSC and organic acid concentrations peaked with the day 63 samples, and was lower on day 189. Subsequent declines may result

from the small but measurable permeability of the mini-silo bags and containers to oxygen, which over those intervening four months could have allowed some aerobic metabolism of these components to CO_2 and H_2O. Although most samples did not show overt evidence of oxidation (referred to as spoilage in conventional ensilage), there was sometimes some evidence of aerobic conversion, particularly near the heat-sealed seams. From a practical perspective, this evidence reinforces the need to effectively exclude oxygen in long term ensiled storage systems.

5.4.3 Fiber Degradation

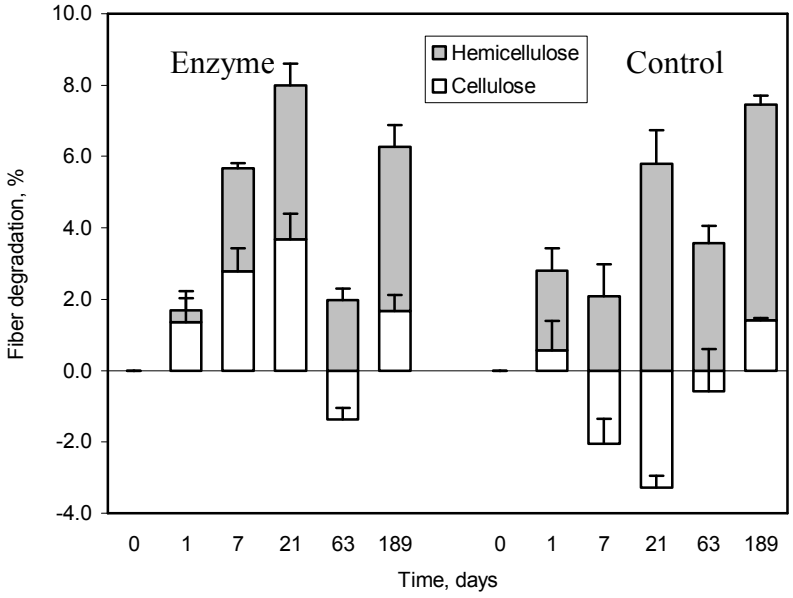

Figure 5.4 Time course of concentration reductions of cellulose and hemicellulose with and without enzyme treatment for the coarse (as baled) stover size.

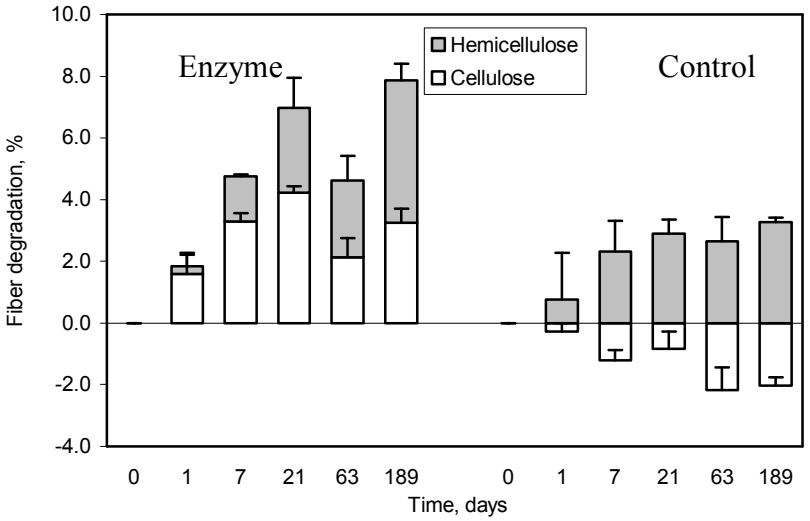

Figure 5.5 Time course of concentration reductions of cellulose and hemicellulose with and without enzyme treatment for the medium (< 10 mm) stover size.

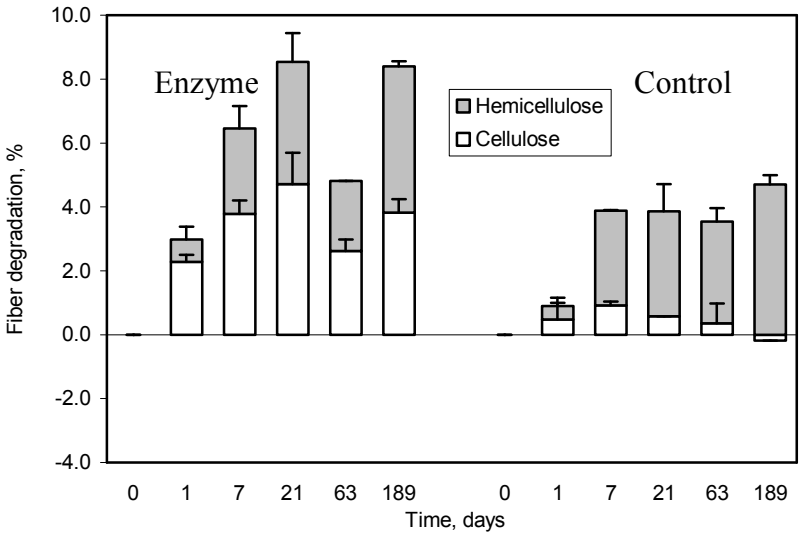

Figure 5.6 Time course of concentration reductions of cellulose and hemicellulose with and without enzyme treatment for the fine (< 5 mm) stover size.

Degradation of cellulose and hemicellulose with and without addition of enzymes for the coarse, medium and fine sized stover are illustrated in Figures 5.4, 5.5, and 5.6

respectively. Not surprisingly, the enzyme treatments significantly increased polysaccharide degradation ($P < 0.0001$), with the highest total change in concentration in the range of 6.28 to 8.49 %, compared to the range of 1.25 to 7.46 % in the non-enzyme treatments. Interestingly, there was an increase in cellulose concentration of the control during ensiling. Richard et al. (2001) found a similar result and suggested the decrease of biodegradable constituents and thus of total mass may have caused the increase in cellulose percentage.

A higher level of hemicellulose hydrolysis than cellulose hydrolysis was observed in control samples. This agrees with the observation of Yahaya et al. (2001) who investigated ensilage of orchardgrass and lucerne, reporting 17.2~19.8% hemicellulose degradation while only 0.5~3.3% degradation for cellulose. Kawamura et al. (2001) also found 21.8% loss of hemicellulose and 7.9% loss of cellulose during the fermentation of Italian ryegrass. MacDonald et al. (1991) concluded that hemicellulose in cell walls is hydrolyzed more easily than cellulose during anaerobic fermentation.

The addition of enzymes had its greatest impact on increased cellulose degradation, with a smaller or even little effect on hemicellulose hydrolysis. The same results had been reported by Van Vuuren (1989). The ratio of cellulase to hemicellulase in the enzyme treatment, 2.54:1, may partially explain this effect. The uniform β-1, 4 glycosidic linkage in cellulose chain may also have played an important role. This simple linear structure makes cellulase biocatalysis specific and efficient, relative to the complex structure of hemicellulose, with various monosaccharide units, hetero-linkages, and different branch lengths.

When comparing WSC and organic acid production with polysaccharide degradation, we found that sum of degraded polysaccharides was essentially equivalent to the sum of these fermented products in the enzyme treated samples. However, a similar mass balance for the treatments without enzymes indicated more polysaccharide degradation than accounted for in the products we measured. Other fermentation products, like ethanol, succinate, CO_2, and water, probably explain this result. It was interesting to note that the production of organic acid was greater than expected from stoichiometric degradation of the lignocellulosic fraction, especially on control samples. Henderson (1993) stated that proteins, amino acids and organic acids all probably

contributed to the production of fermented acids. In our case this difference may be related to the production of acids from catabolism of amino acids and amides by proteolytic clostridia.

5.4.4 Lactic Acid Bacteria and Clostridia

The initial counts of *Lactobacillus* spp. on day 0 were 7.1 ± 0.8 $\log_{10}$ CFU g^{-1}. This value was a little higher than the range of 5-6 $\log_{10}$ CFU/g previously reported for ensilage by Pahlow (1989). There has been a trend of higher numbers of *Lactobacillus* spp. on harvested crops in recent years (McDonald et al., 1991), which may be caused by finer chopping in harvest machinery, encouraging natural growth of *Lactobacillus* spp. during harvest and transport.

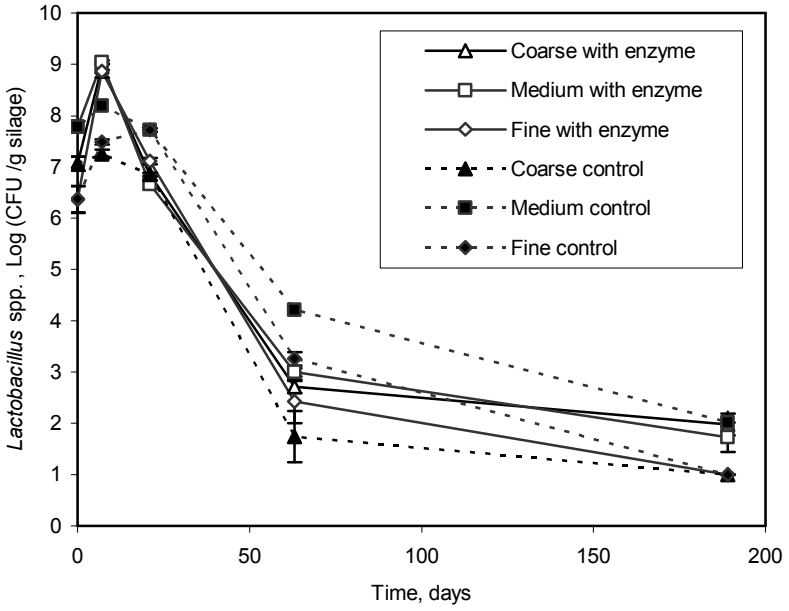

Figure 5.7 Population of Lactobacillus spp. during ensiling with and without enzymes.

The addition of enzymes had an effect on *Lactobacillus* spp. growth during the six-month experiment. The counts of *Lactobacillus* spp. increased to peak values by day 7 (Figure 5.7), which explains the rapid accumulation of lactic acid and corresponding

pH decrease during these first 7 days (Figures 5.1, 5.2, and 5.3 and Table 5.1). The peak counts of *Lactobacillus* spp. were 10 times higher for the enzyme treated silage than for the non-enzyme control.

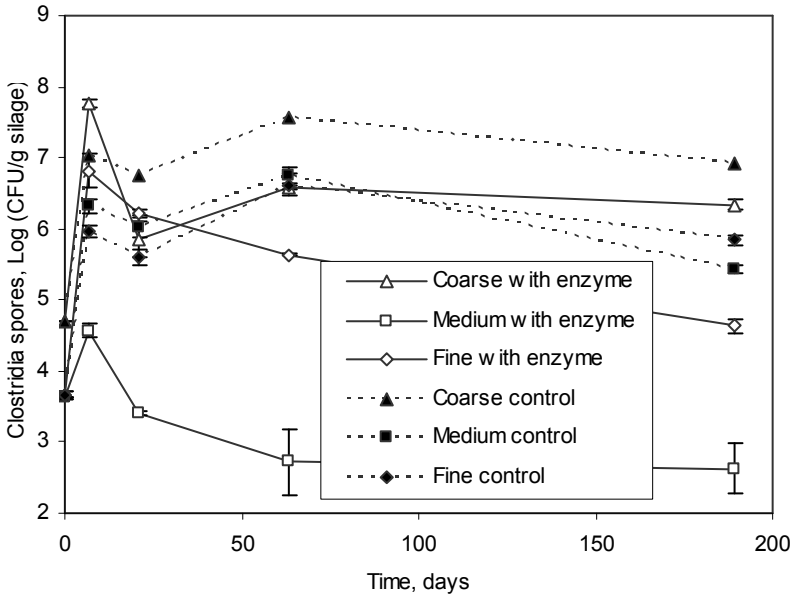

Figure 5.8 Population of clostridia spores during ensiling with and without enzymes.

Other than those peak values, the *Lactobacillus* spp. counts for the non-enzyme treatments were similar to those of the enzyme treatments, although more variable with respect to stover particle size. Among these non-enzyme treatments, the medium size stover treatments obtained the highest counts during the entire period, including having the highest initial counts. The coarse size stover, which presumably had the lowest surface area to be colonized by microorganisms, generally resulted in the lowest counts of *Lactobacillus* spp., although its initial count was intermediate.

A pH of 4.2 is typically required to inhibit growth of clostridia, with their optimum pH in the range of 7.0-7.4 (Pelczar and Reid, 1972). Clostridia can be killed in a few hours in the presence of oxygen, and only spores survive under aerobic conditions. The growth of clostridia may therefore only partially correlate with the number of clostridia spores. In this study, the products of clostridia fermentation were weakly

associated with the spore numbers. The highest levels of acetate and butyrate were observed on day 21 and day 189, respectively. The peak value of clostridia spores, however, occurred on day 63.

The order of clostridia spore counts of three sizes with the enzyme treatment (Figure 5.8) was consistent with that of pH (Table 5.1). The medium size, with a pH 3.9, had the most significant reduction in clostridia spore numbers. This combination of low pH and reduced clostridia spores for the medium particle size also resulted in the highest lactic acid level (*data not shown*). The apparent advantage of the medium particle size may result from increased surface area (relative to the coarse size) and greater water content per unit surface area (relative to the fine size). Greater surface area is important for enzyme contact, and available water is important for enzyme transport.

5.4.5 Physical properties of corn stover particleboard

The basic physical strength and dimensional stability of the particleboard was characterized by modulus of rupture (MOR), modulus of elasticity (MOE), internal bond (IB), thickness swell (TS), and water adsorption measurements. Figure 5.9 shows the MOR and MOE of ensiled particleboards with and without the enzyme treatment, as well as the fresh unensiled stover control. The t-test results indicated ensiled stover had similar MOR and MOE as fresh stover.

Although enzyme addition during the ensiling process increased MOE by 10.79 % compared to unamended ensiled stover, this difference was not significant at the 5% level ($P = 0.062$). For particleboard from ensiled stover samples, the length of ensilage decreased MOR significantly both in enzyme and non-enzyme treatments ($P = 0.04$), reducing MOR 16.46% between 21 and 189 days. The extended ensiling process allowed more time for interaction with water, cellulytic bacteria, and other fermentation microorganisms, which all of which would be expected to soften and degrade of the fiber microstructure, reduce particle size, and increase surface area. Ensiled medium size stover (< 10mm) achieved the highest MOE among these ensiled treatments. The MOR of corn stover particleboard was comparable to the 16.5 MPa American National Standards Institute high density fiberboard standard for MOR (ANSI, 1993), and MOE was higher than the 2400 MPa ANSI MOE standard for all ensilage treatments.

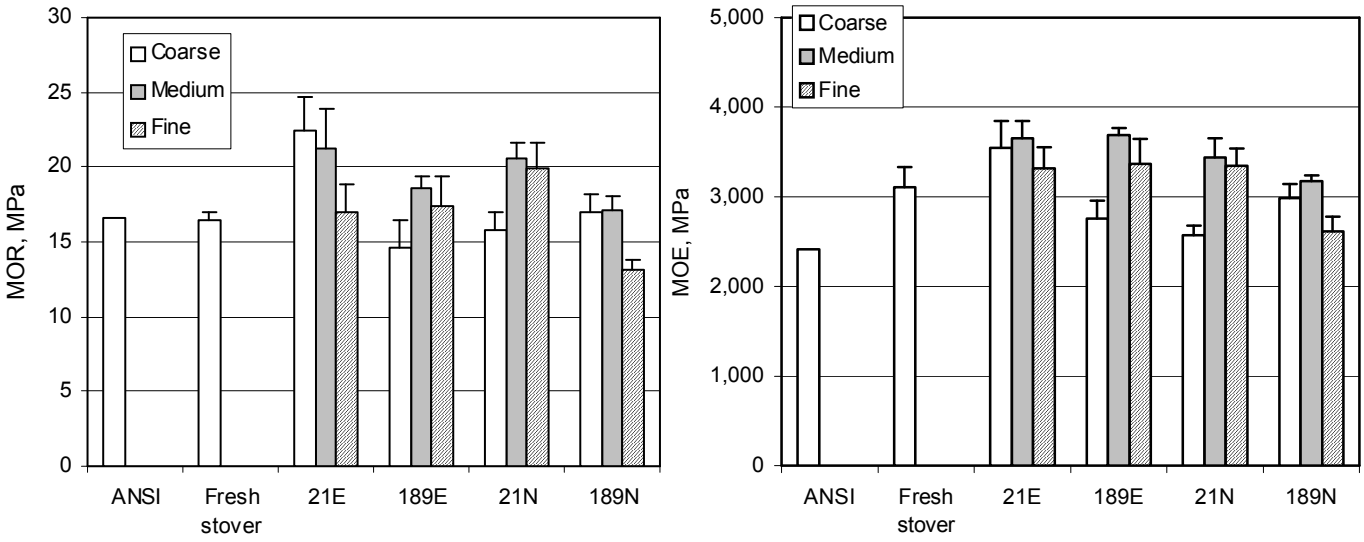

Figure 5.9 Modulus of rupture (MOR) and modulus of elasticity (MOE) of particleboards made from fresh stover and ensiled corn stover with and without enzyme treatment. (21 and 189 represent days of the ensiling period; E and N represent the enzyme and non-enzyme treatments respectively).

Internal bonding strength was significantly affected by ensiling process ($P = 0.03$) (Figure 5.10). Boards made from fresh stover had an IB value of 793 kPa, while ensiled stover boards had IB values ranging from 958 to 1593 kPa, for an average increase of 33%. The ensiled treatment also improved the IB of corn stover particleboard to values above the ANSI IB standard of 896 kPa. Several biologically mediated mechanisms may have operated in synergy with the soy-based adhesive, such as increased surface area from the hydrolysis of structural carbohydrates and the production of bio-adhesives in microbial biofilms.

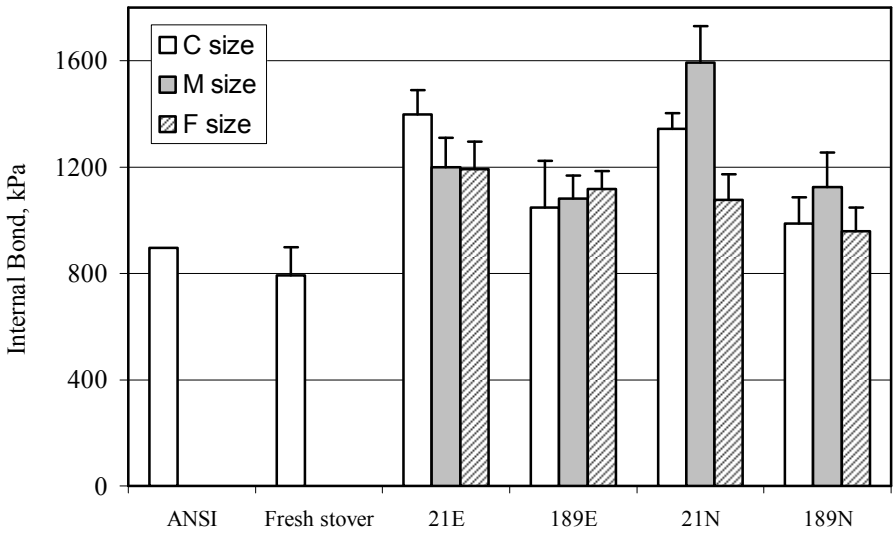

Figure 5.10 Internal bond strength (IB) of particleboards made from fresh stover and ensiled corn stover with and without enzyme treatment (21 and 189 represent days of the ensiling period; E and N represent the enzyme and non-enzyme treatments respectively; C, M, and F represent coarse, medium, and fine size of stover).

The addition of enzymes did not show much impact on IB values. IB values of 21-day-ensilage stover particleboards were higher than those of 189-day-ensilage stover by 29% ($P = 0.001$). This may have been associated with reduced microbial activity, as microbial populations decreased between 21 and 189 days. Microbial protein concentration may be higher on day 21 than on day 189, which has been reported to provide adhesive benefits for particleboard material (Bian et al., 2002).

The suitability of particleboard for interior and exterior use is based on thickness swelling (TS) and water adsorption. Even used inside in controlled environments, high humidity and accidental spills and leaks can lead to water stress and damage to composites. Thickness swelling and water adsorption are expressed as the percentage increase over the specimens' initial thickness and weight respectively, after submersing specimens for 2 hrs in boiling water or 24 hrs in cold water. Figures 5.11 and 5.12 illustrate TS and water adsorption for the control and ensiled treatments. The ensiling process had a significant effect on thickness swelling in the 24-hr-soak test ($P = 0.0005$), achieving an

average TS of 15.3%, which was much lower than that of the particleboards made from fresh stover, 34.0%. For 2-hr-boiling testing, ensiling resulted in consistently lower TS values, but the ensilage effect was not significant at the 5% criteria (P =0.08). Ensiled pretreatment resulted in less adsorbed water than fresh stover, both in the 2 hours boiling test (P = 0.02) and in cold water for 24 hrs (P = 0.01). This is another indication that the ensiling process can contribute to the effectiveness of the adhesive system. Particleboards made after 21 days of ensiling gave significantly less TS and water adsorption than those ensiled for 189 days in the 2-hr-boiling testing (P = 0.03 and P = 0.001 respectively). Enzyme addition during the ensiling process resulted in 12.1% less water adsorption after boiling for 2 hrs (P = 0.05).

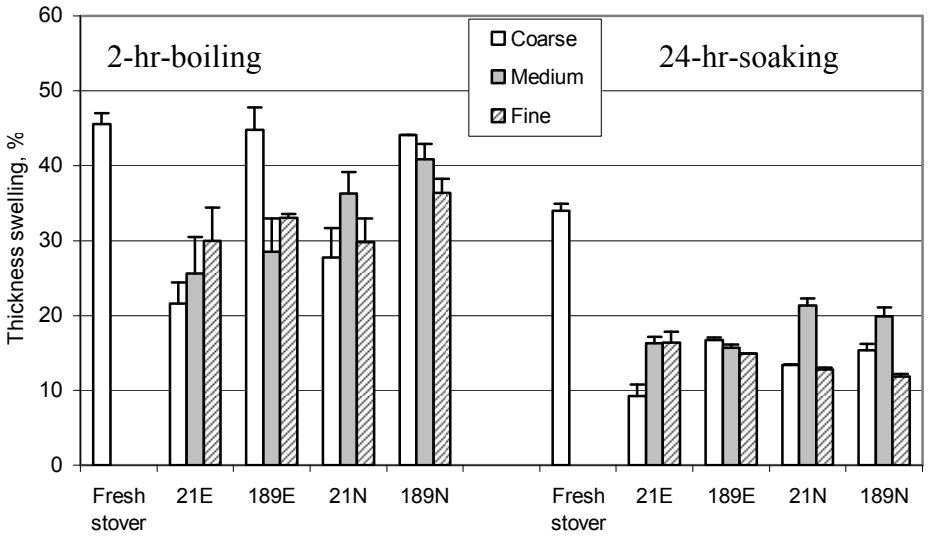

Figure 5.11 Thickness swelling (TS) after 2-hr-boiling and 24-hr-soaking testing of particleboards from ensiled corn stover with and without enzyme treatment (21 and 189 representing days of the ensiling period; E and N representing the enzyme and non-enzyme treatment respectively).

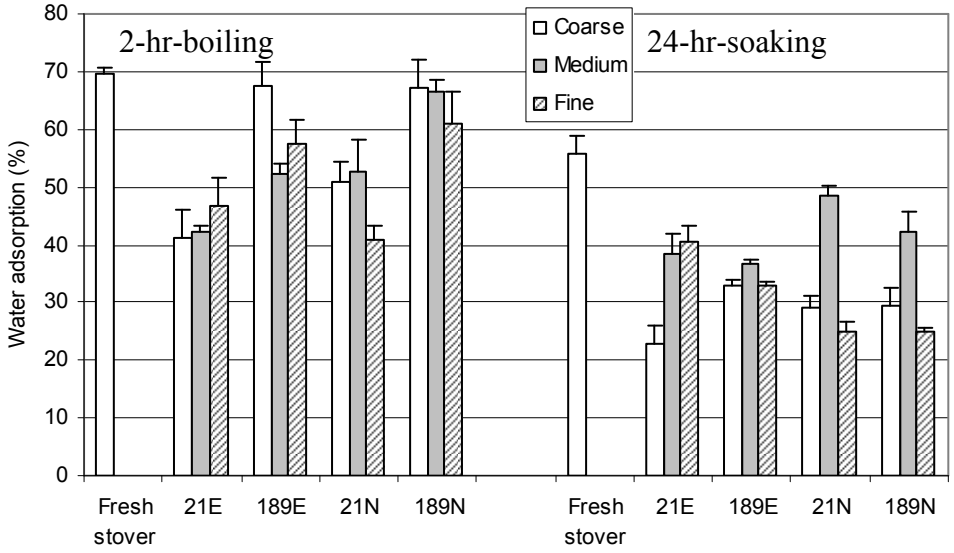

Figure 5.12 Water adsorption after 2-hr-boiling and 24-hr-soaking testing of particleboards from ensiled corn stover with and without enzyme treatment (21 and 189 representing ensiling period; E and N representing the enzyme and non-enzyme treatment respectively).

5.5 Conclusions

This investigation of the long term impact of enzyme treatment on corn stover bioconversion provided useful information about cell wall degradation and chemicals production during the fermentation. Addition of hemicellulase and cellulase enzymes encouraged lactic acid fermentation significantly, reducing pH to 3.9-4.5, and guaranteed stable six-month biomass preservation as long as anaerobic conditions prevailed. The enzymatic amendment resulted in lactic acid being the dominant organic acid among the fermented products, with sufficient WSC hydrolyzed from the lignocellulosic component of corn stover to initiate a robust *Lactobacillus* spp. fermentation. This enzyme treatment also resulted in a higher production of total organic acids converted from cell wall constituents.

The degradation of hemicellulose and cellulose was enhanced significantly by the addition of enzymes. Although more hemicellulose than cellulose was hydrolyzed in all treatments over the course of the 189 day trial, cellulose hydrolysis was more responsive to the enzyme treatment than hemicellulose

Enzymatic treatment enhanced the peak counts of *Lactobacillus* spp. by a full order of magnitude. The counts of clostridia spores were effectively inhibited by enzyme addition, especially on the medium particle size. This enzyme treated medium particle size showed the most promising results for both bioconversion and microorganism selectivity. These apparent advantages for the medium particle size suggest a tradeoff between substrate surface area and enzymatic transport in solid-state fermentation, a promising avenue for future research.

The ensiled treatments had several positive impacts on the mechanical properties and dimensional stability of the manufactured particleboards relative to boards made from unensiled stover. The ensilage process resulted in a significant increase in internal bond strength, while significantly decreasing thickness swelling and water adsorption in 24-hr-soaking testing. The enzyme treatments had the lowest water adsorption, and may increase the modulus of elasticity, although the later effect was not statistically strong. The extended ensilage period of 189 days resulted in lower modulus of rupture and higher thickness swelling and water adsorption in both 2-hr boiling and 24-hr soaking tests. Thus while short-term ensilage improves board properties in several respects, these benefits tend to weaken over time.

Particleboard made from corn stover had MOR values comparable to the ANSI standard. The MOE and IB of boards made with ensiled stover tested significantly higher than ANSI standard for high density fiberboard. These results indicate that corn stover has promising potential as a feedstock for particleboard manufacturing.

Although the ensiling process did degrade hemicellulose selectively over cellulose when no enzymes were added, the enzyme treatment with higher cellulase activity compensated for this selectivity and resulted in roughly equivalent degradation rates. The weakened selectivity may explain the lack of improvement in particleboard properties for the enzyme treated boards in 2-hr-boiling and 24-hr-soak tests.

Although ensilage appears a promising approach for long-term storage of corn stover and presumably other biomass crops, storage conditions must be aligned with the ultimate product use. For board manufacturing and other fiber applications the hydrolysis needed to insure adequate initial fermentation should target the hemicellulose fraction to minimize reductions in mechanical strength. The increased microbial activity resulting from enzyme enhanced ensilage can complement bio-based adhesives to provide biocomposite materials for a more sustainable world.

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6. EFFECTS OF CHEMICAL ADDITIVES ON CORN STOVER BIOMASS PRESERVATION AND PRETREATMENT

6.1 Abstract

Ensiling is attracting increasing interest as a feedstock preservation method for corn stover prior to further bioconversion into value-added chemicals and energy products. The addition of chemicals during the ensiling process is proposed not only to improve preservation, but also to provide partial pretreatment of the cell wall and improve sugar yield in downstream enzymatic hydrolysis. This study examined the effect of five chemicals, including sulfuric acid, formic acid, formaldehyde, ammonia, and urea, on corn stover preservation and pretreatment. Treatments included 2, 4, and 8 g kg^{-1} dry matter (DM) of sulfuric acid, formic acid, and formaldehyde and 4, 8, and 16 g kg^{-1} DM of ammonia and urea, with each of these 15 chemical treatments applied to stover of three particle size distribution. Sulfuric acid, formic acid, and formaldehyde increased lactic acid concentration and decreased acetic acid concentration. Although urea and ammonia increased the final pH significantly compared to control samples, clostridia activities were inhibited at the 16 g kg^{-1} DM level. A long term trial of sulfuric acid-treated silage showed that water soluble carbohydrates (WSC) increased over the 63 day period at the 16 g kg^{-1} DM level, although fermentation was almost inhibited at this level. Sulfuric acid, formic acid, formaldehyde, and ammonia increased sugar yield from enzymatic hydrolysis when compared to fresh stover and to control samples ensiled without chemical additives. The highest sugar yield of 19% was obtained in ammonia treatment.

6.2 Introduction

Corn stover is regarded as one of the most promising biomass feedstocks for ethanol, chemical, and fiberboard production, due to its high availability in the U.S. (Kadam and McMillan, 2003; Sokhansanj et al., 2002; Wang and Sun, 2002). Among these value-added products, ethanol production from corn stover is of particular importance because of the increasing demand for renewable transportation fuels and the low cost of this feedstock (Moiser et al., 2005; Perlack and Turhollow, 2003). According

to the Department of Energy (2005), over 69 million dry Mg of stover can be sustainably harvested in the U.S., assuming 20-60% no-till practice and 40% removal of residue. The annual potential ethanol production from corn stover is estimated as 11 billion liters (Kadam and McMillan 2003), which is equivalent to the current grain ethanol production capacity of 11 billion liters in the U.S. (Mosier et al., 2005).

The production of industrially acceptable yields of ethanol from corn stover requires several steps, including feedstock harvesting and preparation, pretreatment, enzymatic hydrolysis, and ethanol fermentation (Esteghlalian et al., 2000). Of these steps, pretreatment has been the most intensively studied topic over the past five years for two main reasons: first, it significantly affects the cost and efficiency of feedstock preparation and enzymatic hydrolysis (Wyman et al., 2005). Many pretreatment methods require preliminary size reduction, and any acids or chemicals added in pretreatment have to be neutralized or removed before enzymes are added for saccarification; second, the pretreatment itself is one of the most costly steps, due to capital costs, energy-intensive operating conditions, and instrumention requirements. Improved pretreatment and the integration of pretreatment with other processing steps is expected to reduce the overall conversion costs significantly.

Although many of the major steps of corn stover bioconversion have been investigated extensively (Wyman, 1999; Wyman et al., 2005; Yang et al., 2001; and Wang and Sun, 2002), the storage of feedstock prior to pretreatment has received little attention thus far. Because stover can only be harvested once per year in most of the U.S., future industrial ethanol production requires the safe storage of large quantities of stover to supply year-round manufacturing facilities.

To minimize the dry matter (DM) loss and eliminate the fire risk associated with corn stover preservation, ensiling was proposed as a biomass feedstock preservation method by Richard et al. (2001). Subsequent studies have shown it to be a safe and practical long-term storage method (Shinners et al. 2003). During ensiling, corn stover is not only preserved, but also pretreated by microorganisms and associated biochemicals in ways that can enhance downstream conversion processes. The stover cell wall can be degraded by microorganisms, plant enzymes, and organic acids produced during the ensiling process; this is beneficial for future saccharification and bioconversion. Enzyme

additives, such as cellulase and hemicellulase, have been shown to enhance the degradation of the lignocellulosic cell wall, improving the conversion of biomass to fermentable sugars and organic acids during short and long-term ensilage (Richard et al., 2002; Chapter 5). Ensiling can also improve fiber qualities for subsequent biocomposite manufacturing. Particleboard made from ensiled corn stover had an improved modulus of elasticity and internal bond strength, as well as less thickness swell and water adsorption in soaking tests compared to particleboard manufactured from fresh stover (Chapter 5).

Ensiling is a widely used process for animal feeds and forages; and several chemical additives have been investigated in that context to reduce dry matter loss and improve nutritional quality. Some of these additives, including sulfuric acid, formic acid, and formaldehyde, can act as fermentation inhibitors at moderate to high levels. Sulfuric acid and formic acid quickly reduce the pH of silage to a low level and consequently suppress microbial growth, inhibit proteolytic enzyme activity, and preserve the biomass. Formaldehyde, applied in an aqueous solution as formalin, acts directly as a sterilizing agent because of its bacteriostatic properties. Although the initial purpose of these additives was to suppress all fermentation by inhibiting microbial activity, they were found to encourage the natural lactic acid fermentation process if added at the appropriate level. Because lactic acid bacteria (LAB) tolerate higher levels of acidity than clostridia and enterobacteria, lowering the pH with the acids can inhibit competitors, thus making more of the substrate available to LAB. In particular, sulfuric acid has been reported to inhibit the activity of undesirable bacteria such as enterobacteria and clostridia (McDonald et al., 1991). However, the effect of this and other additives on LAB growth and forage quality depends on the initial composition of ensiled crops and the active components and concentrations of the applied additives.

Urea and ammonia have been used as nutrient additives to supplement the crude protein in forage crops destined for ruminant feed. Although the final pH value of silage is higher when these additives are used, higher levels of fermentation acids are produced, which lower the otherwise increased buffering capacity caused by urea and ammonia. Ammonia has been shown to increase the stability of silage (Clewen and Young, 1982). Furthermore, the alkalinity of these additives is expected to improve digestability by degrading the cell wall.

Based on these previous experiences with ensiling crops for animal feed, adding acid or alkaline chemicals during ensiling of biomass feedstocks is expected to provide pretreatment benefits and improve subsequent enzymatic hydrolysis. The purpose of this study is to evaluate chemical additives as part of an integrated system for corn stover preservation, pretreatment, and downstream bioconversion to sugars. To examine the effect of chemical additives on pretreatment during the corn stover preservation period, the research presented in this study addresses three main objectives. First, the effect of five chemicals on the preservation of corn stover was evaluated during the fermentation phase of ensiling. Next, the kinetics of chemical transformations and fiber degradation were studied in sulfuric acid treated silage over an extended 63day period. Third, downstream yield of sugar was investigated through enzymatic hydrolysis of ensiled corn stover treated with each of the five chemical additives. In addition to overall sugar yield, the monosaccharide composition of these degraded sugars was characterized.

6.3 Materials and Methods

6.3.1 Corn stover silage preparation

Corn stover, harvested in the fall of 2002, was ground through a hammer mill (Art's-Way, Armstrong, IA) to obtain three size grades of samples. The coarse harvested corn stover, once milled (0.5-1.0 cm), and twice milled (0.1-0.5 cm) stover are referred to as coarse, medium, and and fine sizes respectively. The stover was stored and milled dry at 16-20% moisture (w.b.) and was adjusted to 60% (w.b.) by adding water. Each 500 g (200 g dry matter, DM, mixed with 300 g water) sample of chemically treated or control stover was packed tightly into a 20cm×35cm polyethylene bag and placed under 25 inch mercury vacuum for 3 minutes, and immediately heat-sealed. The chemical and fiber composition of corn stover before ensiling was reported in Table 6.1.

Table 6.1 Chemical characteristics and fiber composition of three sizes of corn stover.

Stover size	Coarse	Medium	Fine
pH	7.1	8.2	8.4
WSC* (% d.b.)	1.52	1.81	1.57
NDF** (% d.b.)	73.3	73.0	74.0
ADF† (% d.b.)	40.3	40.9	42.5
Lignin (% d.b.)	2.3	2.7	3.1
Ash (% d.b.)	1.3	1.2	1.75
Cellulose (% d.b.) (= ADF – Lignin-Ash)	36.8	37.0	37.7
Hemicellulose (% d.b.) (= NDF – ADF)	32.9	32.6	31.5

* Water soluble carbohydrates (WSC)

** Neutral detergent fiber (NDF); † Acid detergent fiber (ADF);

The analysis method for chemical and fiber composition was described in the section 3.3.3.

6.3.2 Experimental design

This study included three experiments. The first experiment surveyed the effect of the five chemicals on corn stover and the pretreatment process. Treatments with sulfuric acid, formic acid, and formaldehyde were done at the 0, 2, 4, and 8 g kg^{-1} DM levels, while those with ammonia and urea were done at the 0, 4, 8, and 16 g kg^{-1} DM levels. Each treatment was performed on three replicate samples from each of the three different stover sizes. The sealed mini-silage bags were incubated at 37 ±1ºC for 21 days.

The second experiment investigated long term impacts of the ensiling process on stover treated with sulfuric acid. Four levels of sulfuric acid – 0, 4, 8, 16 g kg^{-1} DM – were applied to the medium size stover, and three replicates at each treatment level were destructively sampled and analyzed at 0, 1, 7, 21, and 63 days.

Samples from the first two experiments were analyzed for dry matter and pH immediately after the bag was opened. Samples were then frozen and stored for later analysis of lactic acid, volatile fatty acids, water soluble carbohydrates (WSC), and fiber fractions as described in section 3.2.3.

The third experiment investigated the effect of silage amendments on subsequent enzymatic hydrolysis of corn stover. Treatments included fresh stover, ensiled stover without additives, stover with each of the five chemical treatments (treated with 8 g chemical kg^{-1} DM), and stover with cell wall degrading enzymes (Chapter 5). These eight treatments were enzymatically hydrolyzed to examine the effect of each treatment on sugar yield in downstream bioconversion subsequent to biomass storage. The hydrolysis enzyme was Multifect A40 (Genencor, Cedar Rapids, IA), a mixture of cellulase and hemicellulase, with a ratio of cellulase to hemicellulase of 2.54. A diluted enzyme solution (100 ml in 0.05M sodium acetate) was added to 10 g dry samples in 250 ml of plastic bottles. The enzyme was applied at a level of 75 filter paper units (FPU)/g dry matter (DM). One mL of sodium azide (5mM) was added to inhibit microbial growth during enzymatic hydrolysis. The plastic bottles were shaken at a rate of 200 rpm in an incubator at 50 °C for 90 hrs. After enzymatic hydrolysis, the solution was centrifuged and filtered for monosaccharide analysis on high performance liquid chromatography (HPLC). The sugar yields of ensiled corn stover treated with each chemical or the enzymes were compared to those of fresh stover and of ensiled stover without any additives.

6.3.3 Analysis of hydrolyzed sugars

Monosaccharides, including glucose, xylose, mannose, arabinose, and galactose, were separated and quantified by HPLC with a CarboPac PA 20 column (Dionex, Inc., Sunnyvale, CA) and an electrochemical detector, Dionex ED40. The mobile phase was 2 mmol NaOH and the flow rate was 0.5 ml/min.

6.4 Results and Discussion

The pH value of the control sample was approximately 5 after 21 days (Figure 6.1), which is not usually considered low enough to inhibit clostridia and enterobacteria and minimize dry matter loss. Sulfuric acid decreased the pH at each of the examined levels ($P=0.01$). A similar effect on pH was not obtained with formic acid ($P=0.4$), probably because formic acid is a much weaker acid than sulfuric acid. Formaldehyde showed an inconsistent effect on pH, having the lowest pH value of 4.53 at 8 g kg^{-1} DM.

It was not surprising that the pH value was higher with the increasing levels of urea and ammonia, because of the alkalinity of these compounds. However, ammonia had a more dramatic effect on pH at 16 g kg^{-1} DM than urea because urea must release ammonia in order to generate a hydroxide group and this reduces the pH.

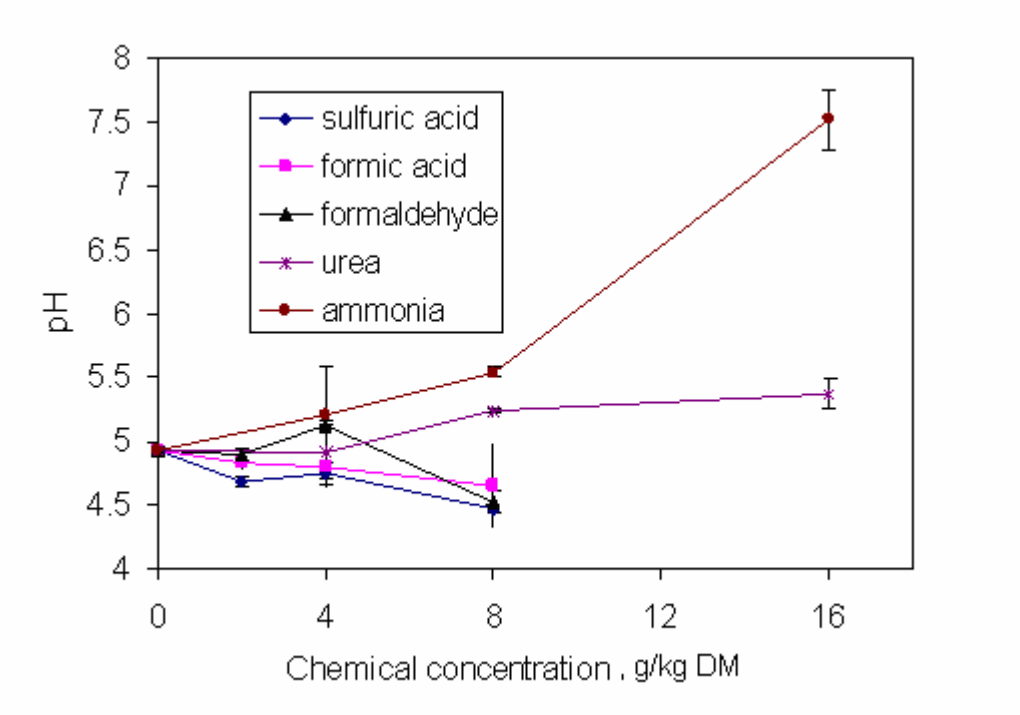

Figure 6.1 The day 21 pH value of medium sized corn stover silage treated with increasing levels of five chemicals.

6.4.1 Chemical composition of corn stover silage

The effectiveness of ensilage on biomass preservation is generally characterized by the concentration of lactic acid and volatile fatty acids, as these acids are indicators of the activity of ensilage microorganisms during the preservation period. The chemicals applied in this study are expected to either encourage lactic acid fermentation or impede all kinds of fermentation to minimize dry matter loss. Simultaneously, beneficial pretreatment of the cell wall by these chemicals is anticipated. The acid concentrations in chemically treated coarse, medium, and fine sized stover are presented in Figures 6.2 – 6.4.

The addition of sulfuric acid increased lactic acid and decreased acetic acid compared to the control samples. Butyric acid was reduced significantly with increasing sulfuric acid concentration in the coarse and medium sizes (P=0.02 and P=0.006, respectively). Since butyric acid is a sign of clostridia fermentation, this result supported Henderson's conclusion (1993) that an appropriate level of sulfuric acid can inhibit the activity of clostridia. However, in the fine size sample, the concentration of butyric acid did not statistically change with the increase of sulfuric acid (P=0.135). The total content of fermented organic acids, including lactic acid, acetic acid, propionic acid, butyric acid, and isobutyric acid, decreased with increasing levels of sulfuric acid, and the total acid content in the coarse and fine sizes was lower than those of control samples. Increased levels of sulfuric acid inhibited fermentation and generated less products; demonstrating that corn stover can be preserved while reducing fermentation products. Water soluble carbohydrates (WSC) in all three sizes remained constant with increasing sulfuric acid concentration. However, this does not mean that no cell wall was pretreated and degraded by sulfuric acid, because degraded sugars may have been consumed immediately, or, if the degradation products were oligosaccharides, they would not have been measured as WSC (Ren et al., 2005).

Formic acid showed different effects on lactic acid fermentation in the three sizes of stover. Lactic acid increased 30-46 times more than the control sample in the medium and fine sizes while the extent of increase of 1.3-6.2 times was more limited in the coarse size. Barry et al. (1978) reported a similarly positive effect of formic acid on lactic acid production in a study of lucerne silage, though effective preservation was only obtained at high level acid levels (6 ml kg^{-1} DM). Other researchers (Wilson and Wilkins, 1973; Henderson and McDonald, 1971) also observed similar fermentation patterns in several formic acid-treated crops. However, these results differed from those of Carpintero et al. (1979) for ryegrass-clover crop. They found that the lactic acid fermentation was restricted by formic acid application and WSC were higher than the initial WSC content in crops. It is now known that the fermentation pattern associated with adding formic acid is not only influenced by the acid levels, but also determined by the original WSC level in crops. McDonald et al. (1991) summarized that a crop low in WSC will follow Barry's

fermentation type when treated with formic acid, while lactic acid and acetic acid will be reduced and WSC will be preserved in a crop rich in WSC.

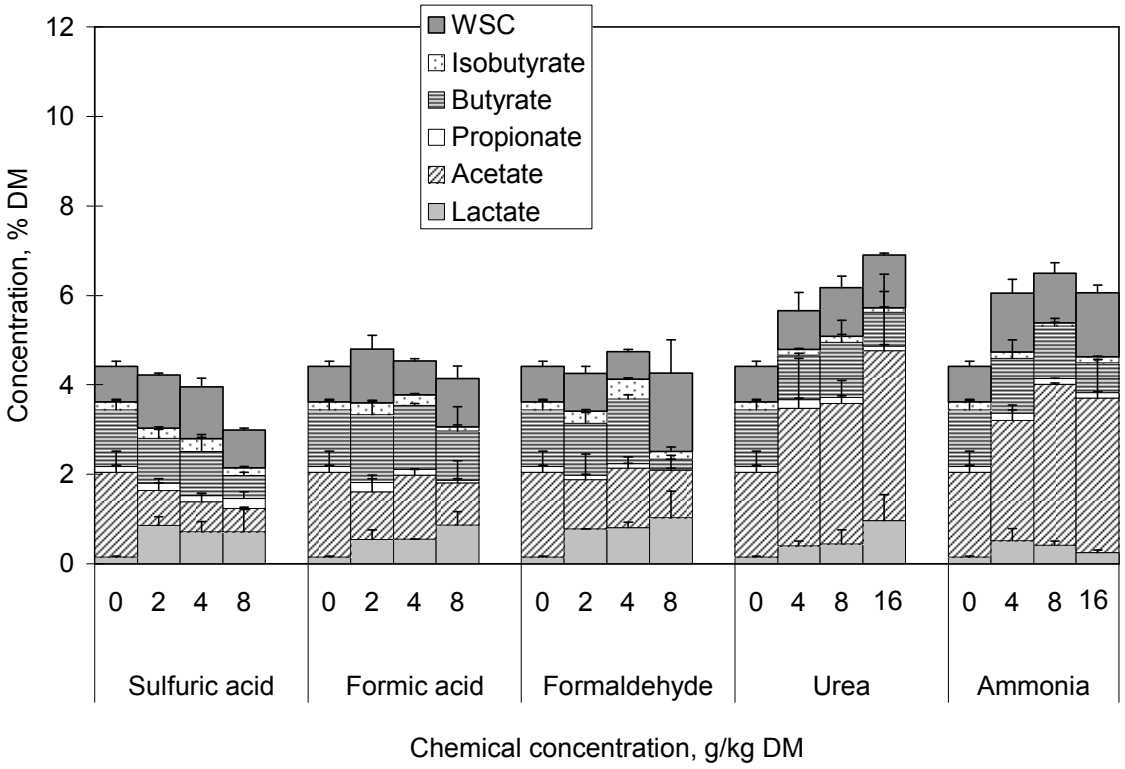

Figure 6.2 Post fermentation concentration of organic acids and water soluble carbohydrates for increasing levels of five chemicals in coarse size stover.

The stover size had a significant effect on lactic acid fermentation ($P<0.001$), with the lowest lactic acid content in the coarse size. The low surface area in the coarse size prevents the formic acid from contacting all of the polysaccharides, resulting in less degraded sugar for lactic acid fermentation. Although the medium and fine sizes had higher concentrations of fermented acids than the coarse size, there was no corresponding effect on dry matter loss (data not shown). Higher levels of lactic acid can prevent a resurgence of clostridia during the end of the storage period, thereby preventing significant dry matter loss during the final, potentially aerobic, transport and size reduction processes. Other fermented products that were not measured in this analysis, such as ethanol, formic acid, succinic acid, and CO_2, may be correspondingly higher in the coarse size than in the medium and fine sizes.

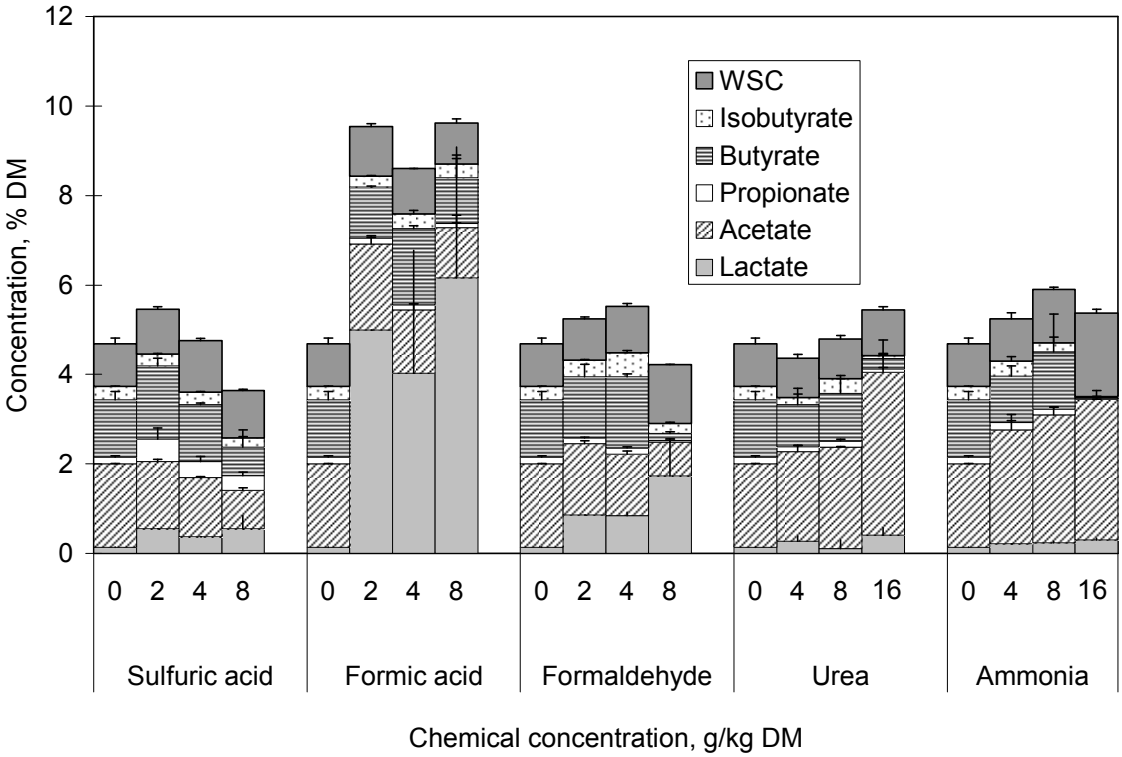

Figure 6.3 Post fermentation concentration of organic acids and water soluble carbohydrates with increasing levels of five chemicals in medium size stover.

Although formic acid is weaker than sulfuric acid in acidity, the higher lactic acid content in the formic-acid treated silage led to comparable pH values for the formic and sulfuric acid treated medium size stover (Figure 6.1). However, sulfuric acid and formic acid had different impacts on corn stover pretreatment benefits during the preservation period, as will be further discussed below.

Formaldehyde gradually increased lactic acid and decreased acetic acid with increasing treatment levels in the coarse and medium sizes. Butyric acid at 2 and 4 g kg^{-1} DM of formaldehyde was comparable to the control sample, but almost disappeared at 8 g kg-1 DM of formaldehyde. This result agrees with the studies by Kaiser et al. (1981) and McDonald et al. (1983), who found that low levels of formaldehyde preferentially stimulated clostridia growth over that of LAB because the former is more resistant to low concentrations of formaldehyde. At 8 g kg^{-1} DM of formaldehyde, water soluble carbohydrates were significantly higher than at other levels in all three sizes of stover,

and these other levels were comparable to the initial content before storage. Furthermore, the level of 8 g kg^{-1} DM gave the lowest total concentration of the produced acids, but with the highest fraction of lactic acid. This result differs slightly from that in the summary of Wilkins's study (1974), in which fermentation in ryegrass silage was totally restricted at a rate of 9 g kg^{-1} DM (originally reported as 8 liter Mg^{-1} DM) of formaldehyde. In that summary, the production of these organic acids was negligible and WSC were preserved. This difference in lactic acid fermentation can be attributed to the ensilage conditions, including the initial WSC content, the dry matter content, the buffering capacity, and the chemical concentration, any of which can alter LAB tolerance of formaldehyde. The study of formaldehyde-treated lucerne silage by Barry et al. (1978) supports this conclusion.

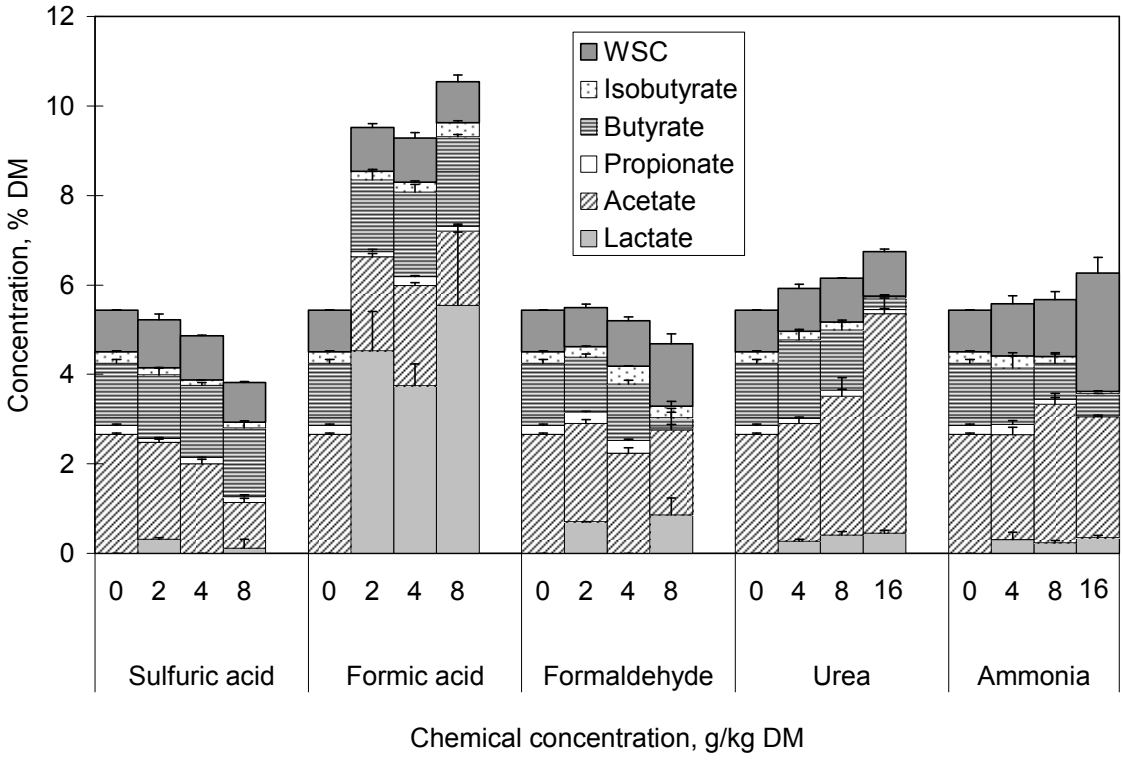

Figure 6.4 Post fermentation concentration of organic acids and water soluble carbohydrates with increasing levels of five chemicals in fine size stover.

Urea and ammonia had a similar effect on the production of organic acids during stover ensiling. Acetic acid increased with increasing chemical additive concentrations

(P<0.01 and P=0.005 for urea and ammonia respectively), while there was no significant change for lactic acid (P=0.628 and P=0.643). Butyric acid was relatively constant at the 0, 4, and 8 g kg^{-1} DM levels, but significantly reduced at 16 g kg^{-1} DM. It is particularly noteworthy that ammonia treatment at 16 g kg^{-1} DM enhanced WSC significantly over other levels. The theory that this increase is due to degradation of the cell wall is supported by our fiber data presented in the following section. In conventional corn silage the application of urea and ammonia has primarily been promoted as a nutritional supplement, and the reported effects on the chemical composition of silage vary (Kung et al., 1984; Thomas et al., 1975; Lessard et al., 1978). McDonald et al. (1991) suggested that differences in initial WSC content and variations in applied chemical levels, dry matter content, and buffering capacity may explain these differences.

6.4.2 Fiber degradation of corn stover silage

The degradation of corn stover hemicellulose during ensilage was significantly higher than that of cellulose in control samples (Figure 6.5). Similar results have been observed in previous studies of corn stover preservation (Ren et al., 2004; Ren et al., 2005; Richard et al., 2002). The addition of sulfuric acid increased cellulose degradation significantly compared to the control samples (P=0.001), but above 2 g kg^{-1} there was no significant increase of the degradation with increasing chemical concentration (P=0.386). For the chemical treatment concentrations in this study, the degradation of cellulose was always less than 10%. Higher concentrations of sulfuric acid would be expected to significantly increase cellulose degradation. In traditional dilute sulfuric acid pretreatment methods, the final sulfuric acid concentration is in the 100-600 g kg^{-1} DM range (Lloyd and Wyman, 2005; Bhandari et al., 1984), and this method results in over 90% of cellulose degrading to sugar monomers in the subsequent enzymatic hydrolysis. Hemicellulose degradation increased with addition of sulfuric acid at the 2 g kg^{-1} DM level when compared to the control samples, but decreased as the sulfuric acid concentration was further increased. Degradation of hemicellulose of corn stover in silage is mainly attributed to plant enzyme activity, acid hydrolysis, and cellulolysic microorganisms (McDonald et al., 1991). When sulfuric acid is added at very low levels, acid hydrolysis of hemicellulose can be encouraged without evidence of inhibition of

plant enzymes or microbial activity. But as the sulfuric acid concentration is further increased, hemicellulose degradation will be reduced, because the negative effect of the inhibition of plant enzymes and cellulolysic microorganisms exceeds the positive effect of increased acid hydrolysis. This may explain why 2 g kg^{-1} sulfuric acid had the highest fiber degradation in these treatments.

Cellulose degradation was also enhanced by the addition of formic acid, presumably through a mechanism similar to that of sulfuric acid. However, because formic acid is a weaker acid than sulfuric acid, less cellulose degradation should occur at any given concentration. As anticipated, hemicellulose degradation with formic acid followed a similar pattern to that of sulfuric acid, but to a lesser extent.

Higher levels of formaldehyde improved cellulose degradation, but did not change the hemicellulose degradation dramatically (P=0.43). Similar cellulose degradation for formalin treated ryegrass has been reported for formaldehyde levels above 8 L Mg^{-1} DM (McDonald et al., 1991).

Urea and ammonia act as weak bases during corn stover preservation and pretreatment. The addition of these chemicals had little effect on cellulose degradation, which implies that the crystalline structure is not broken down under these conditions. However, the hemicellulose degradation did improve significantly with increasing ammonia concentrations (P=0.028). Among all these chemical treatments, the highest rate of hemicellulose degradation, 18.7%, was observed at 16 g kg^{-1} DM of ammonia.

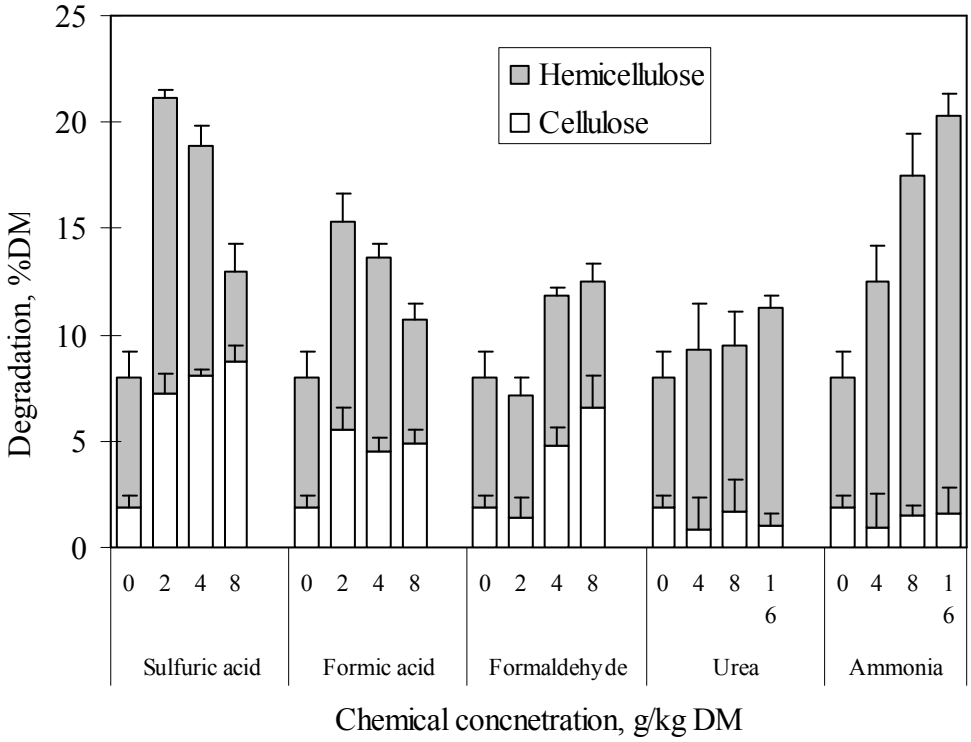

Figure 6.5 Degradation of cellulose and hemicellulose over 21 days with increasing levels of five chemicals in medium size stover.

Summarizing the fiber degradation results of all these chemical treatments, it is apparent that adding sulfuric acid or ammonia during corn stover ensilage can provide beneficial pretreatment for cell wall degradation while maintaining a low dry matter loss of 3-5%. However, to completely eliminate the need for pretreatment steps following corn stover storage, the sugar yields during saccarification need to be extremely high. This issue will be addressed later in this article, after first examining the long term kinetics of the sulfuric acid treatment.

6.4.3 Long term changes in the chemical composition of corn stover silage treated with sulfuric acid

Prior to the downstream saccharification and fermentation of stover, pretreatment is generally carried out to break the links between cellulose, hemicellulose, and lignin, to loosen the cellulose crystal structure, and to increase the surface area of the raw material.

Dilute sulfuric acid was chosen for the kinetics studies, in part because it is viewed as cost-effective reagent for pretreatment. In this study we used rates far lower than the 100 to 600 g kg^{-1} typically used for rapid chemical pretreatment, hypothesizing that the combination of time, microbial, and enzymatic processes during ensiled storage would have synergistic effects. Sulfuric acid addition during preservation is expected to degrade the cell wall, conserve WSC, and inhibit microbial growth. The pH of the stover during the period is illustrated in Figure 6.6.

The pH value dropped quickly in the first day for the control samples and the 4 g sulfuric acid kg^{-1} DM treated stover. The application of high levels of sulfuric acid, 8 and 16 g kg^{-1} DM, resulted in very low initial pH values of 3.79 and 2.44 respectively. For the control and 4 g kg^{-1} DM treatments, pH remained low for the first three weeks, then slowly increased until the end of the 63 day trial, probably due to consumption of lactic acid and production of butyric acid (a weaker acid) by clostridia. At 8 g kg^{-1} DM the pH increased in the first day to 4.24 and continued increasing until 4.91 at the end of the period, while the 16 g sulfuric acid kg^{-1} DM level resulted in a relatively constant pH of 2.68 throughout the 63 day period. At these higher treatment levels, the increase in pH at the beginning of the ensilage process can be explained by the production of organic acids, which are weaker acids than sulfuric acid. The pH increase after 21 days can be attributed to the proliferation of clostridia in the later stages of the ensiling process (McDonald et al., 1991).

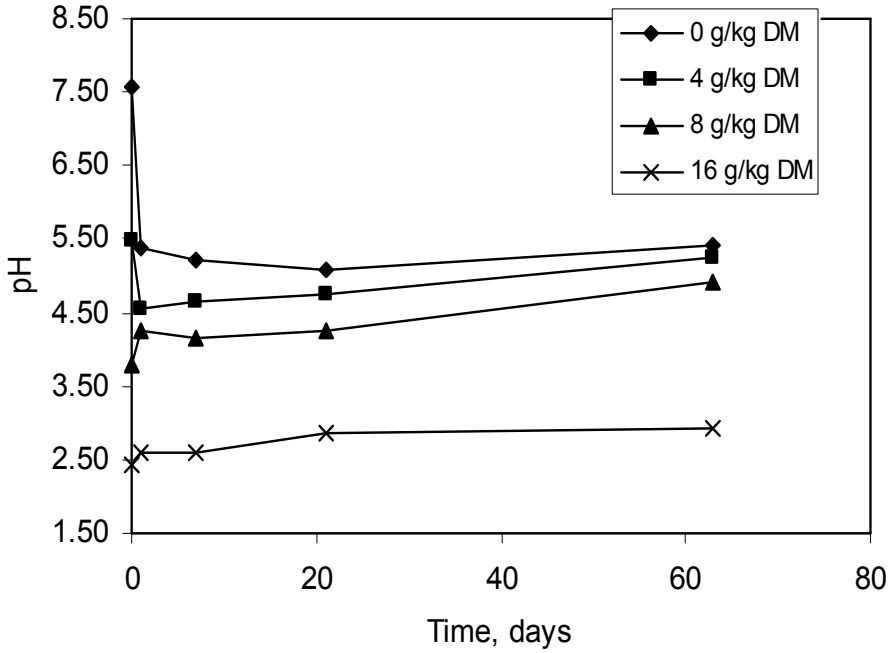

Figure 6.6 Corn stover pH over time for varying sulfuric acid concentration.

The chemical composition of corn stover during the ensiling period is shown in Figure 6.7. Lactic acid increased within one day for the 4 g sulfuric acid/kg DM treatment and in the control samples. This lactic acid was consumed more quickly during the rest of the period in the control samples relative to the 4 g sulfuric acid/kg DM samples. This decrease in lactic acid concentration was accompanied by an increase of butyric and isobutyric acid. The lactic acid was totally consumed within 21 days for the control samples and 63 days for the 4 and 8 g kg^{-1} DM samples. Acetic acid, butyric acid, and isobutyric acid concentrations decreased as sulfuric acid levels were increased, reaching undetectable concentrations at the 16 g kg^{-1} DM sulfuric acid treatment. Fermentation was almost completely inhibited at the highest sulfuric acid concentration of 16 g kg^{-1} DM, as indicated by the very low levels of organic acids. Water soluble carbohydrates were maintained at relatively constant levels in all treatments ($P=0.146$, $P=0.077$, and $P=0.021$), except in the 16 g kg^{-1} DM samples, where WSC content increased linearly with time after one day ($P<0.001$). Because the pH value at this level was about 2.5, plant enzyme and microbial activities were completely inhibited. Thus the

additional WSC appears to directly result from saccharification of the cell wall by sulfuric acid.

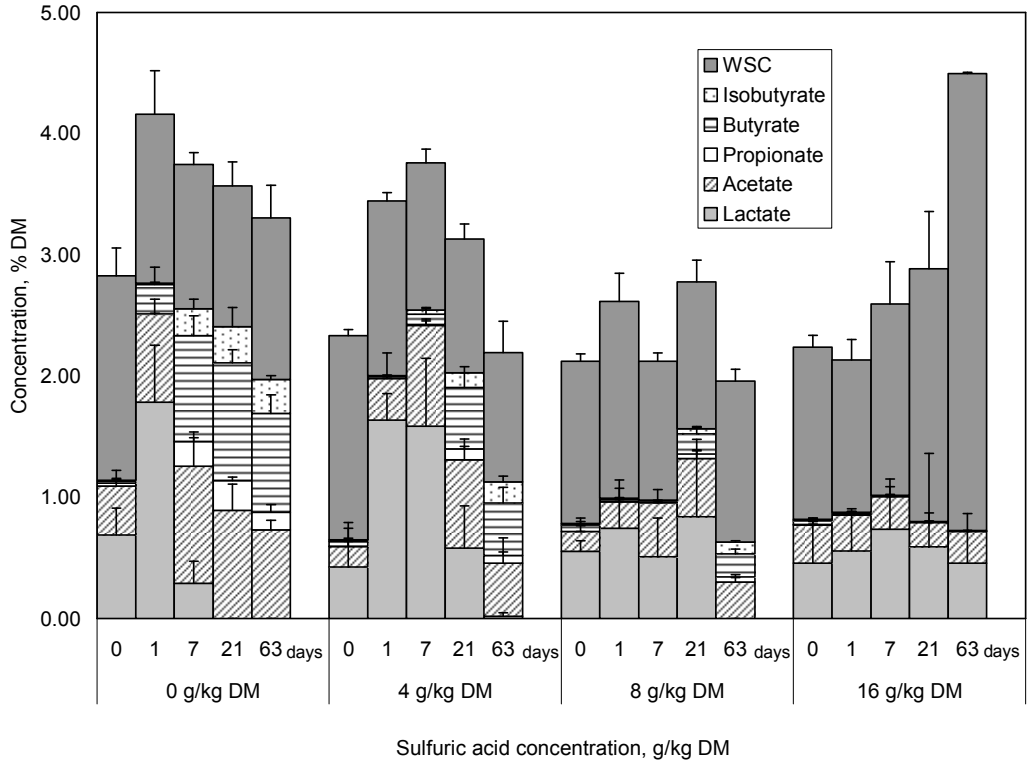

Figure 6.7 Concentrations of water soluble carbohydrate (WSC) and organic acids over time for four different sulfuric acid concentrations.

The effect of sulfuric acid levels on cell wall degradation can be illustrated by the reduction of the fiber fractions in the cell wall as shown in Figure 6.8. The degradation of hemicellulose increased at 63 days for all treatment levels, and the highest degradation percentage (21.4%) was obtained at the 16 g kg^{-1} DM level. However, an increase in WSC was only observed at 63 days for the 16 g kg^{-1} DM level. Hemicellulose degradation in other samples did not result in similar increases in WSC. Two possible reasons have been proposed to explain this phenomenon (Chapter 5). First, the amount of WSC produced from degraded hemicellulose may have been similar to the simultaneous consumption of WSC by microorganisms, resulting in little net change. Second, oligosaccharides may be produced from acid hydrolysis of corn stover at low levels of sulfuric acid, while monomer sugars and disaccharides are released at higher levels.

Hemicellulose degradation at the 4 and 8 g kg^{-1} DM levels was lower than that of the control samples, again reflecting the acid's role in inhibiting plant enzyme and microorganism activity. Only when the sulfuric acid level was increased to 16g kg^{-1} DM did the improved acid hydrolysis exceed the acid inhibition effect, resulting in higher degradation of hemicellulose than in the control samples.

Cellulose degradation was not increased substantially with increased levels of sulfuric acid (P=0.456). The crystalline structure of cellulose makes this fiber fraction very resistant to chemical hydrolysis. Similar reactions of these fiber fractions to ensilage processing and additives have previously been reported (Yahaya et al., 2001; Kawamura et al., 2001). Generally, a higher level of hemicellulose than cellulose hydrolysis was obtained in both control and acid-treated samples.

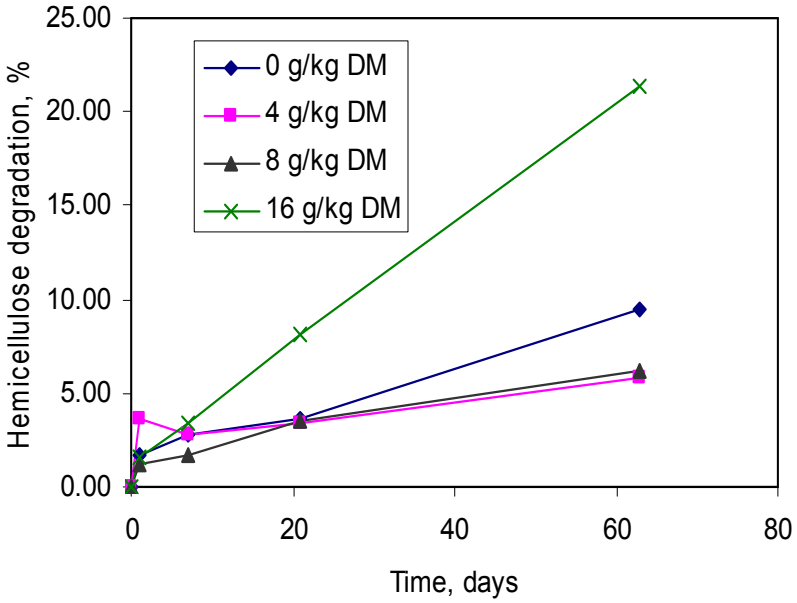

Figure 6.8 Hemicellulose degradation over time for four levels of sulfuric acid.

6.4.4 Sugar yield from ensiled corn stover by enzymatic hydrolysis

In order to examine the hypothesis that the ensiling process and chemical treatment can improve sugar yield in downstream bioconversion, ensiled stover samples treated with sulfuric acid, formic acid, formaldehyde, urea, ammonia, and cell wall degrading enzyme were enzymatically hydrolyzed.

Sugar yield was calculated as the amount of sugar monomers hydrolyzed from ensiled stover divided by the maximum theoretical amount of monomer sugars that could have been recovered from that quantity of treated stover. Ensiling did not significantly improve sugar production when compared to the control sample with the fresh stover (Figure 6.9). Stover ensiled with urea and that with enzyme additives actually produced less sugar than fresh stover. This is probably because the fraction which can be easily hydrolyzed by the enzyme was already degraded and consumed by microorganisms during ensiling. We did not know the reason for lower sugar yield of urea treatment and it should be investigated in further study. The other four chemical additives – sulfuric acid, formic acid, formaldehyde, and ammonia – enhanced sugar yields significantly ($P<0.001$). However, these increases mainly came in the forms of xylose and mannose. A significant increase in glucose was only observed with the sulfuric acid treatment ($P=0.001$).

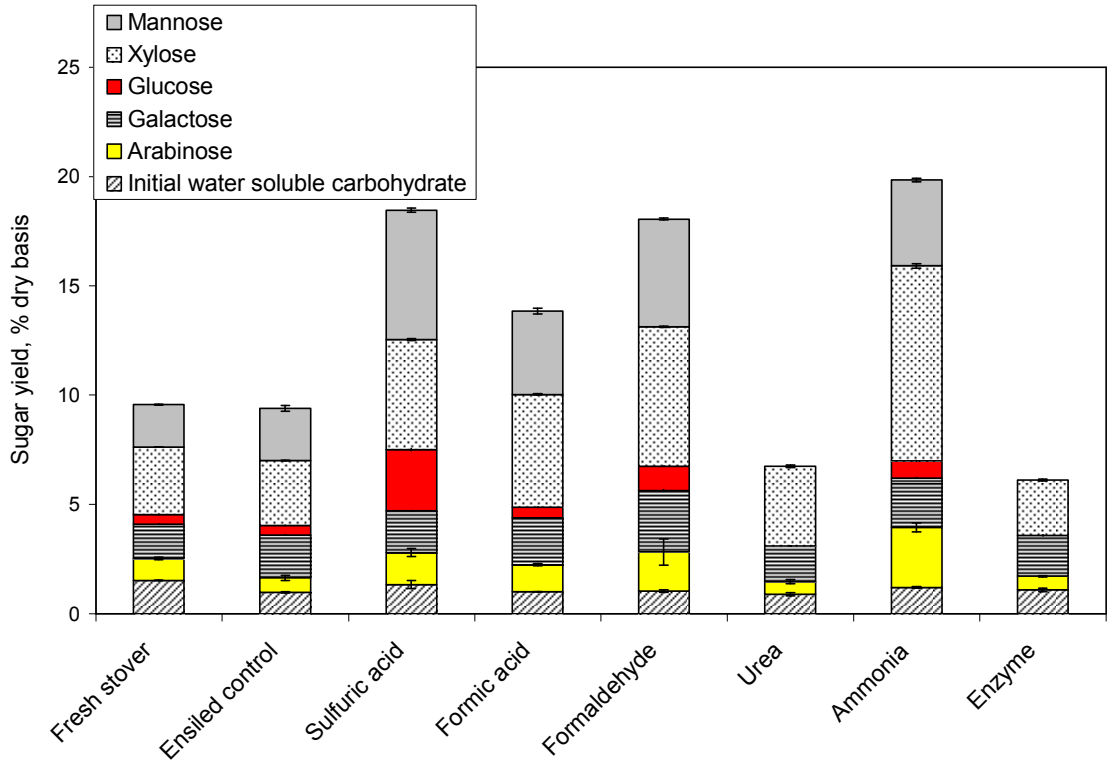

Figure 6.9 Sugar yield of enzymatic hydrolysis of ensiled corn stover treated with various additives.

Although ensiling with chemical additives can improve sugar yield, even the highest yield of 18.6% for the ammonia treatment is not sufficient for industrial

bioconversion. This low yield of glucose suggests that the microstructure of the cell wall was not effectively destroyed during the mild ensiling process, and remained semi-intact during the subsequent enzymatic attack. Based on these results, future efforts should be aimed at integrating ensiled preservation of corn stover with appropriate pretreatment strategies to cost effectively remove the lignin fraction, destroy the crystalline structure of cellulose, and/or increase the stover surface area.

6.5 Conclusions

This study investigated the effects of chemical additives on corn stover preservation and pretreatment. The sugar yield from enzymatic hydrolysis of treated stover was also examined. The addition of formic acid and formaldehyde encouraged lactic acid fermentation and inhibited clostridia growth. Sulfuric acid limited mixed fermentation, as indicated by reduced butyric acid and acetic acid concentrations. Corn stover was preserved at the tested levels of these chemical additives. High levels of urea and ammonia (16 g kg^{-1} DM) can inhibit undesirable microorganisms, as indicated by low butyric acid concentrations in these samples. The analysis of fiber degradation showed that sulfuric acid was effective at enhancing cellulose degradation, though hemicellulose degradation was limited as plant enzymes and microorganisms were suppressed at low pH values. At the level of 16 g kg^{-1} DM, ammonia obtained the highest (21%) hemicellulose degradation with little effect on cellulose.

During a longer, 63 day trial, the sulfuric acid-treated stover silage had a similar chemical composition at 21 days to the corresponding end date of the first experiment. The addition of sulfuric acid slowed the consumption of lactic acid and the production of acetic and butyric acids. The fermentation of silage was totally inhibited at the rate of 16 g kg^{-1} DM due to a very low pH of 2.5. WSC content increased throughout the 63 days in the 16 g sulfuric acid kg^{-1} DM samples, and this WSC increase was associated with significant hemicellulose degradation.

The potential for beneficial pretreatment during the ensiling process was examined by enzymatically hydrolyzing ensiled stover. Although the ensiling process did not significantly improve sugar yields, the treatments with sulfuric acid, formic acid, formaldehyde, and ammonia did increase the total sugar production. However, the utility

of the ensiling process as a pretreatment for sugar conversion was not fully realized. Because the sugar yields were not high enough to meet industrial needs, a new strategy of integrating effective ensiling preservation and more efficient pretreatment must be developed to obtain sugar yields closer to the theoretical maximum.

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7. MODELING THE EFFECT OF ENZYME ADDITION ON CELLULOSE DEGRADATION AND THE ENSILAGE PROCESS

7.1 Abstract

An integrated silage model was developed to simulate the impact of enzyme addition on the ensiling process, combining previously developed silage models with an enzymatic hydrolysis model. Cellulose structural features were characterized by the parameter, λ, the ratio of the nonhydrolyzable region to total cellulose in corn stover. The content of cellulose degradation and water soluble carbohydrate production during ensiling were predicted with an acceptable accuracy. To develop a more complete and predictive model of enzyme treated ensilage, further development was recommended to focus on the simulation of cellulose degradation by cellulolytic bacteria, better understanding of death rate of lactic acid bacteria, and other poorly documented mechanisms..

7.2 Introduction

Chapter 3, 4 and 5 demonstrated that enzyme additions could have a positive effect on corn stover preservation and pretreatment. However, even with supplemental enzymes, the final quality of ensiled corn stover is still largely dependent on the initial conditions, including the chemical composition of corn stover, its moisture status, and the initial microbial populations. A comprehensive experimental evaluation of the effects of enzyme treatments under different initial conditions would require a large number of trials and intensive analysis. A predictive simulation model could provide an efficient alternative for exploring the influence of enzyme additives during corn stover ensilage and gaining insight into process parameters and system dynamics.

Previous silage models (Pitt et al., 1985; Leibensperger and Pitt 1987) simulated the major microbial and biochemical changes during ensiling, as previously reviewed in section 2.4.5. Microbial populations and activities simulated for ensiling were restricted to Lactic Acid Bacteria (LAB) and clostridia. These two models predicted the final pH value and chemical composition of silage with different initial conditions, including crop

type, dry matter (DM) content, water soluble carbohydrate (WSC) content, and fiber content. Validation of these models demonstrated acceptable accuracy, with average ratios of modeled to observed WSCs and lactic acid concentrations in the range of 1.01-1.07. Although additives and microbial inocula were not directly included in these models, the authors suggested adjusting the initial conditions to incorporate their effects. For example, inoculation of LAB could be modeled by increasing the initial bacterial concentration; addition of sugars could be modeled by enhancing the initial content of water soluble carbohydrates.

While these strategies might work for bacterial, chemical, or nutrient additives, the effects of enzyme addition cannot be accurately predicted by changing only the initial conditions of the models. Enzymatic hydrolysis of cell walls occurs throughout the ensiling process, as long as the enzymes retain their biocatalytic activity. The net rate of sugar accumulation from the hydrolysis reaction is continuously changing, both by changes in the metabolism of released sugars by microorganisms, and by sugar product inhibition of enzyme activities. For an accurate representation of these interconnected processes, enzymatic hydrolysis of cell walls must be incorporated into the silage models in a dynamic fashion.

Corn stover is the biomass feedstock considered in this investigation of enzymatic hydrolysis during the ensilage process. Stover has a physical structure which constrains hydrolysis by hindering the access of enzymes to cellulose and hemicellulose, and thus decreasing enzyme activity. Crosslinking between cellulose, hemicellulose, and lignin inhibits the spatial orientation of enzymes to the cell wall polymers, resulting in inefficient enzymatic hydrolysis. Although hemicellulose and cellulose both can be degraded by the added enzymes, our experimental investigation demonstrated that degradation of cellulose is more sensitive to enzyme addition than that of hemicellulose (Chapter 5). When the hemicellulase concentration in added enzyme was lower than 6.7 IU g^{-1} DM, there was no significant increase in hemicellulose degradation relative to the control. This result was attributed to the complexity of the chemical structure and glycosidic linkages in hemicellulose, as well as crosslinking with other polymers.

Since the effects of enzyme addition at low concentrations will mainly come from enhanced cellulose degradation, the model developed in this chapter focuses on

simulating enzymatic hydrolysis of cellulose. Effects on hemicellulose hydrolysis are only indirectly accounted for in this version of the model, which would need to be further expanded to be applicable for enzyme additions at higher hemicellulase rates. Nonetheless, economically efficient enzyme additions are likely to be at the low levels where the model is applicable (Chapter 3), so that the model should prove useful in developing and assessing enzyme amendment strategies to minimize biomass feedstock costs.

7.3 A mathematical model of enzymatic hydrolysis of cellulose

The cellulose that constitutes much of the cell wall of corn stover is insoluble, structured, and closely associated with hemicellulose and lignin. The crystalline region in cellulose and the crosslinkages among various polysaccharides and polymers have been demonstrated as the most important structural features influencing the accessibility of enzymes and determining the cellulose hydrolysis rate (Esteghlalian et al., 2000). Cellulases absorb onto the active enzyme binding sites through the cellulase binding domain (CBD) and form a cellulase-cellulose complex (Bayer et al., 1994). Catalytic reactions occur at the binding sites as the enzyme moves along the cellulose chains. Although several distinct protein components are present in cellulases, there is no preferential binding of individual components during the hydrolysis process (Converse and Optekar, 1993). The attachment of cellulases to phenolic acid and lignin will result in irreversible adsorption, and enzyme activity was even reported to drop because of non-productive adsorption on lignin (Lee and Fan 1982). These structural and biochemical limitations allow only a proportion of cellulose to be accessed and hydrolyzed by enzymes.

Glucose and cellulobiose are known to inhibit cellulolytic enzymes (Kastel'yanos et al., 1995). However, there is no agreement on the inhibition mechanism. Three patterns of inhibition have been observed, suggesting competitive, non-competitive and combined inhibition may occur under different circumstances (Ghose and Das 1971; Gregg and Saddler, 1996; Holtzapple et al., 1984; and Gusakov and Sinitsyn, 1992). There are also some disputes on the extent of inhibition relative to glucose and cellobiose. Kastel'yanos et al. (1995) reported that glucose decreased the extent of hydrolysis and greatly

decreased the initial rate. However, Ghose and Das (1971) found that cellulose hydrolysis was only slightly inhibited by glucose, while significantly inhibited by cellobiose even at very low concentrations. In the model developed in this chapter, product inhibition will not differentiate between glucose and cellobiose. All the released sugars, including glucose, cellobiose, and oligosaccharides, are considered as a single inhibition product.

All the aforementioned factors influence the kinetics of cellulose hydrolysis in corn stover. Quantification of these factors is difficult since they are not only related to each other through hydrolysis kinetics, but are also associated with microbial activity during the ensilage process. To simulate the kinetics of hydrolysis mathematically, these factors and relationships are modeled in a mechanistic framework, with individual parameters drawn from the literature. Before coupling the resulting hydrolysis model with the previously developed ensilage model (Pitt et al., 1985; Leibensperger and Pitt 1987), a parameter that accounts for the structural features of corn stover was estimated. This parameter is the ratio of non-hydrolysable region to the total cellulose of corn stover (λ). This structural parameter combines the effects of available active cellulose surfaces, crosslinking of polysaccharides and polymers, as well as stover particle size. The hydrolysis model is then combined with the ensilage model to predict the effect of enzyme addition on the chemical composition of corn stover silage.

The main assumptions of this model are:

1) Moisture content is high enough for a thin water film covering the surface of corn stover to provide a local aqueous phase for enzyme adsorption and desorption. Kinetic parameters (k_1, k_{-1}, k_2, k_3, k_{-3}, k_4, k_{-4}) previously obtained in liquid reaction systems are thus still suitable for hydrolysis of corn stover during the ensilage process.

2) The individual protein components of cellulase enzymes, endoglucanase, exoglucanase, and β-glucosidase, express a combined cellulolytic activity, and can thus be aggregated and modeled as a single complete cellulase system (E).

3) The cellulose of corn stover (S) can be divided into a hydrolysable region containing exposed cellulose microfibrils (Sca) and a nonhydrolysable region containing inert cellulose microfibrils (Scn).

4) The released sugar product (P_1) can be regarded as part of the water soluble carbohydrate pool, and is available for lactic acid bacteria and clostridia fermentation in the coupled ensilage model.

5) The sugar present in corn stover silage (P_2) is the sum of the initial sugar content (P_{20}) and released sugar (P_1) minus the sugar consumed by microorganisms (P_3).

6) The sugar (P_2) inhibition is reversible and competitive. A complex (EP_2) of product and enzyme forms during inhibition.

Under these assumptions, the resulting reaction scheme for hydrolysis kinetics of a heterogeneous cellulose substrate can be represented by Figure 7.1:

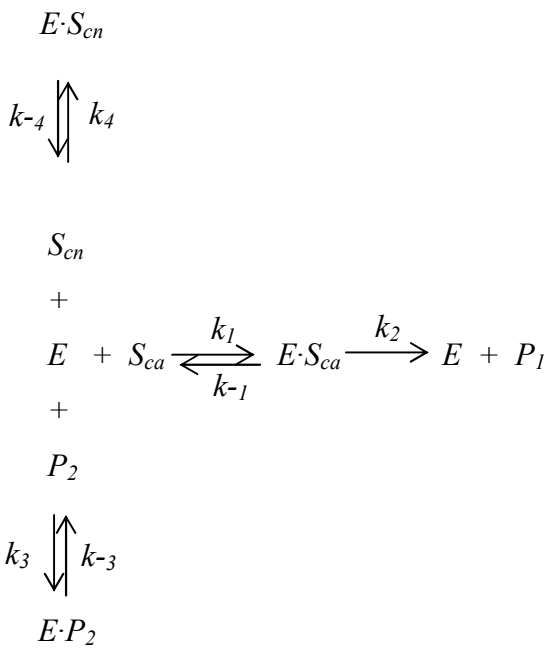

Figure 7.1 The model of hydrolysis kinetics of a heterogeneous cellulose substrate.

In this scheme, k_1 and k_{-1} are the rate constants for the reversible formation of ES_{ca}; k_2 is the rate constant of product formation; k_3 and k_{-3} are the rate constants for the competitive product inhibition with the formation of enzyme-product complex EP_2; and k_4 and k_{-4} are the rate constants for the formation of the non-productive complex, ES_{cn}. All the values of these parameters are adopted from the previously reported results of Gan et al. (2003) (Table 7.1):

Table 7.1 Adopted values of kinetic rate constants (Gan et al., 2003).

Rate constant	value
k_1	0.20 l $g^{-1}hr^{-1}$
k_{-1}	0.05 hr^{-1}
k_2	9.05 hr^{-1}
k_3	0.1 l g^{-1} hr^{-1}
k_{-3}	0.03 hr^{-1}
k_4	0.02 l g^{-1} hr^{-1}
k_{-4}	0.002 hr^{-1}

In a closed ensilage system, the transient state concentrations of the involved substances can be expressed by six first-order differential equations:

$$\frac{d(E \cdot S_{ca})}{dt} = k_1(E)(S_{ca}) - k_{-1}(E \cdot S_{ca}) - k_2(E \cdot S_{ca})$$
$$\frac{d(E \cdot S_{cn})}{dt} = k_4(E)(S_{cn}) - k_{-4}(E \cdot S_{cn})$$
$$\frac{d(S_{ca})}{dt} = k_{-1}(E \cdot S_{ca}) - k_1(E)(S_{ca})$$
$$\frac{d(S_{cn})}{dt} = k_{-4}(E \cdot S_{cn}) - k_4(E)(S_{cn})$$
$$\frac{d(P_1)}{dt} = k_2(E \cdot S_{ca})$$
$$\frac{d(E \cdot P_2)}{dt} = k_3(E)(P_2) - k_{-3}(E \cdot P_2)$$
$$\frac{d(P_2)}{dt} = \frac{d(P_1)}{dt} - \frac{d(P_3)}{dt}$$

The cellulose of corn stover (S) is assumed to consist of hydrolysable (S_{ca}) and non-hydrolysable (S_{cn}) regions. The ratio of initial S_{cn0} to the total of initial S_0, λ, is the key parameter estimated from this model. This ratio represents the structural features of corn stover associated with enzyme hydrolysis.

$$\lambda = \frac{(S_{cn0})}{(S_0)}$$

and also

$$(S_{ca0}) = (S_0)(1-\lambda)$$

Furthermore, at any time

$$(S) = (S_{ca}) + (S_{cn}) + (E \cdot S_{cn}) + (E \cdot S_{ca})$$

The change of sugar consumed by microorganisms (P_3) is termed as y(3) in the coupled models as shown in Appendix 3.

These equations were numerically analyzed using MATLAB's ODE45 method (MATLAB version 7.3) with the enzyme conservation of mass equation:

$$(E_0) = (E) + (E \cdot S_{ca}) + (E \cdot S_{cn}) + (E \cdot P_2)$$

The initial conditions used in the analysis of the differential equations are:

At t = 0, ES_{ca} = 0, ES_{cn} = 0, S_{ca} = S×(1-λ), S_{cn} = S×λ, P_1 = 0, P_2= initial WSC concentration, EP_2=0, E = E_0, S = S_0,

Among these variables, only the S (cellulose concentration) and P_2 (WSC) can be measured with current analytical methods, while the latter changes with the activities of LAB and clostridia. Since λ is a feature of the cellulose, the value of the parameter was estimated using a nonlinear least-square routine by minimizing the sum of square errors (SSE):

$$SSE = \sum_{i}^{2} \sum_{j}^{n} (y_j - \hat{y}_j)^2$$

Where y_j is a datapoint for cellulose concentration from experimental data; $\hat{y}_j$ is the predicted value of that cellulose concentration; i is the experiment number; and j is the date the sample was taken during the ensilage period.

7.4 Materials and Methods

Two different sets of experiments were used for developing and analyzing the mathematical model. The corn stover for these experiments was harvested in fall 2002 and fall 2004 and stored in bales. The 2002 stover was used for estimation of the

structural parameter λ, while the 2004 stover was used for model validation. The coarse stover contained 5-16 % moisture (w.b.) and were adjusted to 60% (w.b.) by adding water. The corn stover substrate from 2002 and 2004 had different cellulose concentrations: 258 g l^{-1} and 285 g l^{-1} respectively (Table 7.2). The cellulose concentration is calculated as the mass of cellulose divided by the water content in corn stover. Enzyme solutions were diluted to an appropriate concentration and added into corn stover when adjusting the moisture content to 60% wet basis, which was the optimal ensilage level determined in a previous study (Richard et al., 2001).

An industrial enzyme, Deerland Cellulase TR (Deerland Inc., Kennesaw, GA), was used as the enzyme additive. This enzyme has an appropriate combination of high enzyme activity in filter paper units (101 FPU g^{-1} enzyme) and a high ratio of cellulase to hemicellulase (2.14), as determined in Chapter 4. The activity of filter paper units (FPUs) represents the combined performance of the individual enzyme components. This measure can better represent the complete cellulase system (E) as described in the model assumptions, compared to endoglucanase, exoglucanase, and β-glucosidase. The FPUs of the enzyme were measured according to the methods described by Wood and Bhat (1988), using No 1. filter paper as substrate.

In the experimental dataset 1 (2002 stover), the enzyme concentrations (enzyme mass/water content in stover silage) were 0.82g l^{-1} and 1.29g l^{-1} respectively. In the experimental set 2 (2004 stover), the same enzymes were added at 1.29g l^{-1} and 1.87g l^{-1}. Three replicates were ensiled for destructive sampling on each sampling date. For each replicate, 500 g of treated sample was packed tightly into a 20cm×35cm polyethylene bag (200 g dry matter mixed with 300 g water), and the bags were vacuumed at 25 inch mercury vacuum for 3 minutes before immediate heat sealing. Samples were incubated at 37 ±1ºC for 21 days. On each sampling date subsamples were immediately processed for dry matter (DM) and pH measurements, while the remainder of the replicate was frozen for later analysis as described elsewhere (section 3.3.3).

All the differential equations involved in the hydrolysis model and ensilage model were numerically analyzed in MATLAB. The software code is presented in Appendix A.

7.5 Results and Discussion

7.5.1 Estimation of nonhydrolyzable region fraction, λ

The effect of the nonhydrolyzable region fraction, λ, on the sum of square errors is illustrated in Figure 7.2. The best λ was obtained at value of 0.56. A structural parameter of 0.56 indicates that at the beginning of hydrolysis, the hydrolysable region is 44% of the total cellulose mass. This is a reasonable value when considering typical sugar yields from enzymatic hydrolysis of corn stover without pretreatment. Montross and Crofcheck (2004) reported conversion efficiencies of cellulose in corn cobs, leaves, and stalks were in the range of 29.6-55.9%. Table 7.2 presents the experimental and simulation results for the parameter estimation dataset.

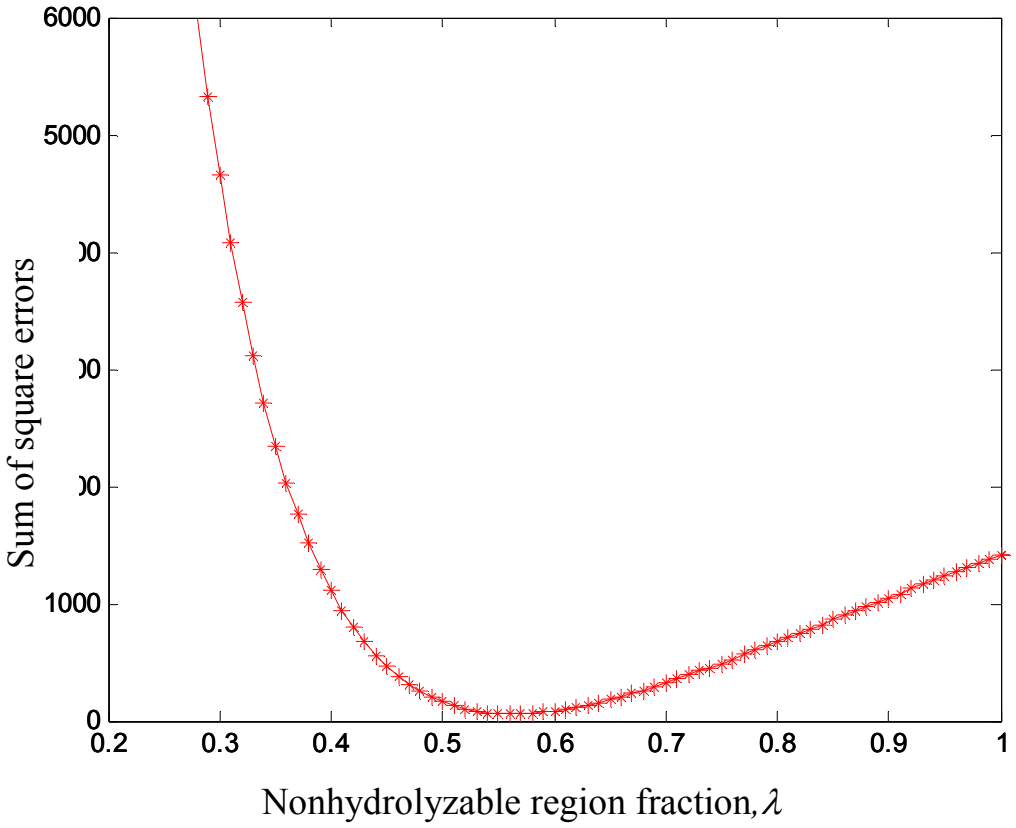

Figure 7.2 Effect of nonhydrolyzable region fraction, λ, on model errors.

Table 7.2 Cellulose concentrations used to estimate the structural parameter λ.

	E=0.82g/l, S=258g/l*		E=1.29g/l, S=258g/l	
Time, Days	Observed	Estimated	Observed	Estimated
0	258.0		258.0	
1	253.8	251.5	250.6	247.9
3	254.6	250.9	250.4	247.0
7	250.5	249.8	244.0	245.3
14	246.0	247.8	238.9	242.3
21	244.7	245.9	239.7	239.4

*E is the enzyme concentration; S is the cellulose concentration.

7.5.2 The validation of the hydrolysis model

The experimental dataset from stover harvested in 2004 was used to validate the coupled hydrolysis and ensilage model, with the results of cellulose degradation presented in Table 7.3. Simulation results and experimental data throughout ensiling were evaluated (Figures 7.3). The r^2 values were 0.88 and 0.97 respectively, and the slopes of the plots were 1.52 and 1.33 respectively. The good correlation and plot slopes near 1.0 indicated the model adequately represented the cellulose degradation process.

Table 7.3 Cellulose concentrations (g l^{-1}) used for model validation.

	E=1.29/l, S=285g/l*		E=1.87g/l, S=285g/l	
Time, Days	Observed	Estimated	Observed	Estimated
0	285.5		285.5	
1	274.5	275.4	271.2	271.1
3	275.0	274.5	269.1	269.8
7	267.1	272.7	264.6	267.4
14	265.1	269.7	261.9	263.2
21	262.2	266.8	254.4	259.2

*E is the enzyme concentration; S is the cellulose concentration.

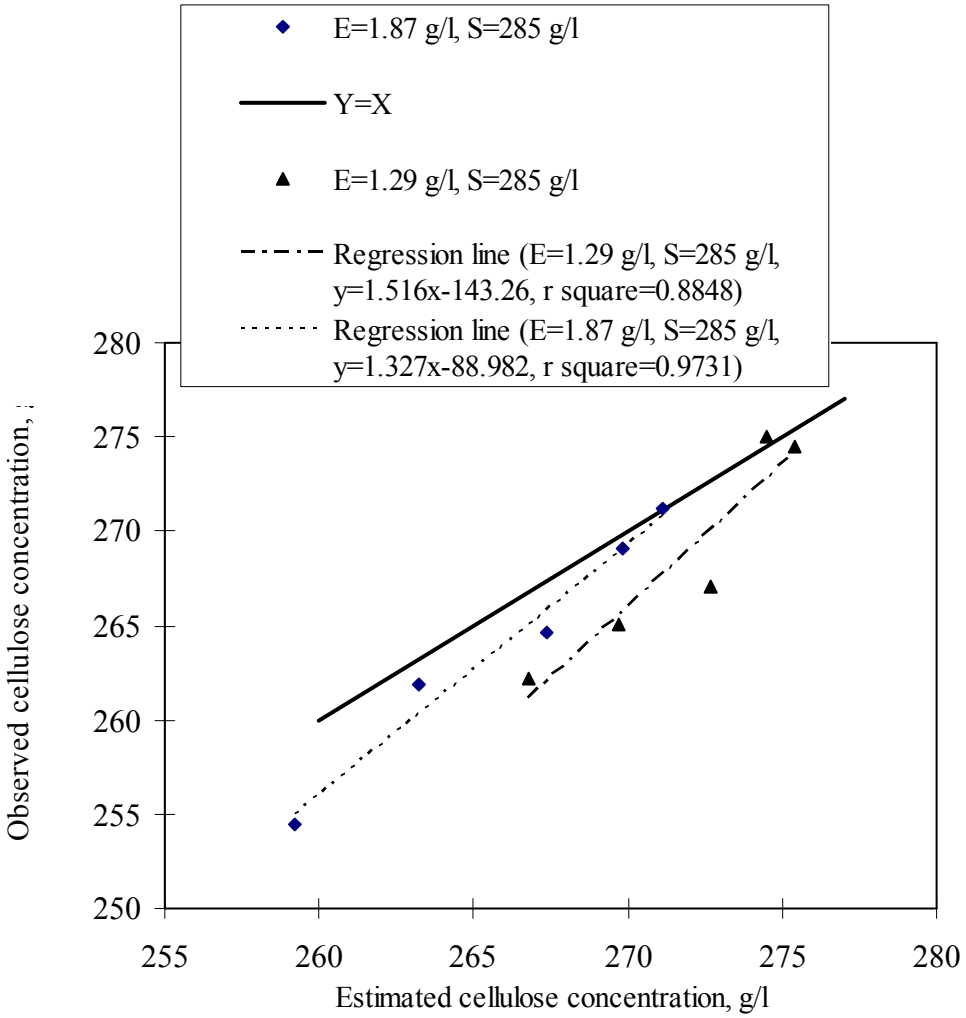

Figure 7.3 Comparison of estimated values to observed data with an added enzyme concentration of 1.29 and 1.87 g l^{-1} (E represents enzyme concentration, S represents cellulose concentration).

7.5.3 The effect of cellulose concentration

The simulation results obtained with the two different initial cellulose (S_0) concentration are compared in Figure 7.4. The increase of S_0 did not change the extent of hydrolysis during the ensilage process. This was expected because the cellulose was in excess even at the low cellulose concentration (S=258 g l^{-1}) when the enzyme concentration is 1.29 g l^{-1}. Thus the reaction balances do not change with increased substrate under these conditions.

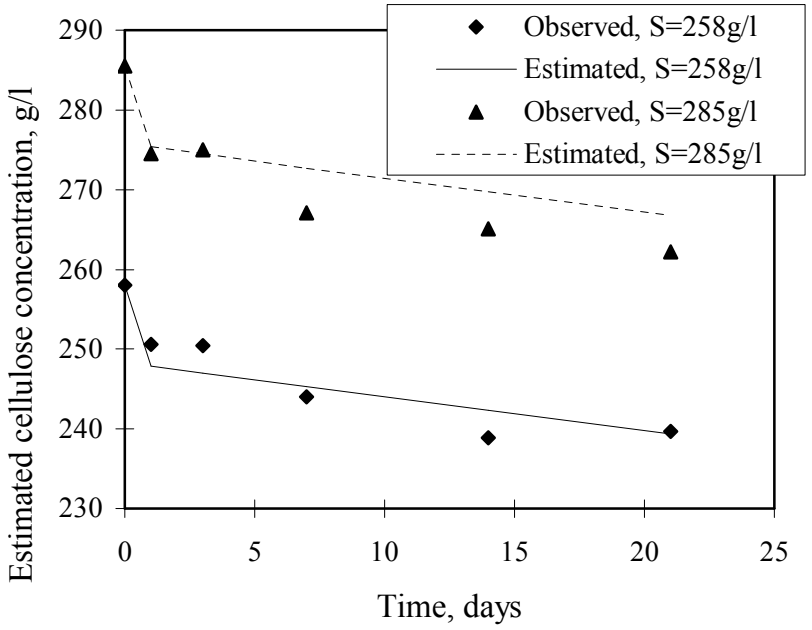

Figure 7.4 Cellulose concentration over time for different initial cellulose concentrations (S represents cellulose concentration).

7.5.4 The effect of enzyme concentration

Although the experimental data points fluctuated from the simulation results on several sampling dates, the increase of enzyme concentration did consistently increase cellulose degradation as shown in Figure 7.5. However, the measured cellulose concentration was consistently lower than the simulated value after day 3, especially with the enzyme concentration of 1.29 g l^{-1}. This was probably caused by some unmeasured factors, such as the activity of cellulolytic bacteria and acid hydrolysis. With the added enzymes about a half of the cellulose degradation was achieved in the first day, according to both experimental and simulation results. These results agreed with separate experimental cellulose degradation data previously shown in Figures 5.4, 5.5, and 5.6. At the beginning of the silage process, sugar product inhibition on enzyme activity was low, and the hydrolysis occurred at a relatively rapid rate ($k_2 = 9.05$). As the sugars produced from the cellulose degradation accumulate, the product inhibition begins to depress enzyme activity and the rate of hydrolysis is reduced.

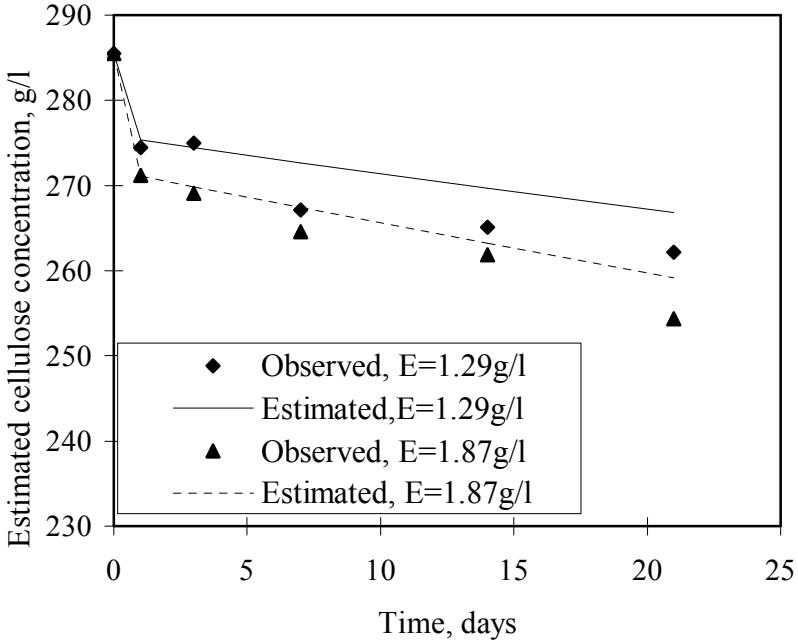

Figure 7.5 Cellulose concentration over time with different enzyme concentrations (E represents enzyme concentration).

7.5.5 The change of sugars during ensilage process

The estimated and experimental results for total sugars (P_2) are shown for two rates of enzyme addition to stover in Figures 7.6 and 7.7. The simulation lines fit the experimental datapoints reasonably well, except for the last two dates at the lower enzyme concentration of 1.29g/l. In should be noted that the simulation plots of sugar concentrations showed different patterns with the two different enzyme concentrations. Several factors contributed to this difference. First, with these two enzyme concentrations, the quanitities of degraded sugars differed, which resulted in different growth rates of lactic acid bacteria (LAB), and led to different rates of sugar consumption. Second, the initial pH of the silage was different for the two enzyme concentrations. The enzyme concentration of 1.29g/l had an initial pH value of 6.12, while the enzyme concentration of 1.87g/l experienced an initial pH value of 5.77. The growth rate and death rate of LAB are strongly influenced by pH during the ensilage process, and consequently, sugar consumption by LAB differed significantly under these two conditions. The combination

of these factors resulted in differing impacts on sugar production and consumption dynamics, with the net result reflected in the water soluble carbohydrate pool.

The degraded sugars (P_1) from cellulose were also simulated and illustrated in Figures 7.7 and 7.8. Although this sugar content cannot be directly measured, it is apparent from net increases in the WSC pool that the sugar production increased during the ensilage period, and that increasing the enzyme concentration enhanced sugar production through increased cellulose hydrolysis.

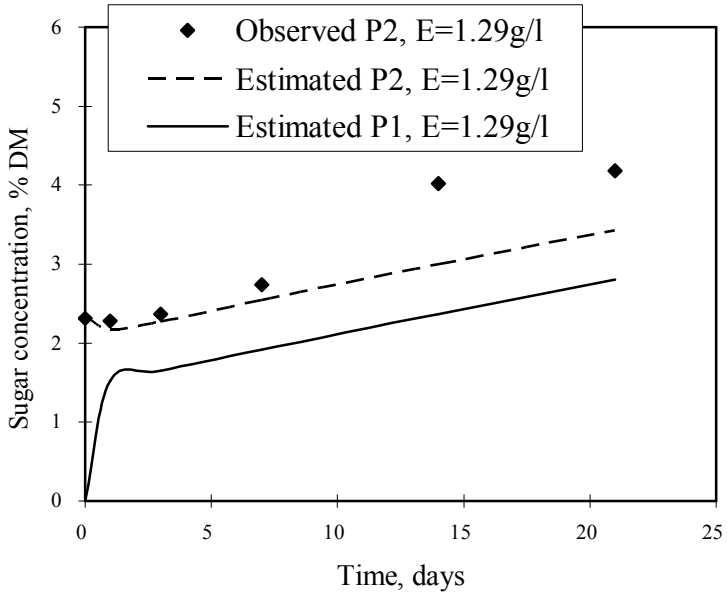

Figure 7.6 The change of sugars during ensiling with an enzyme concentration of 1.29 g/l (P1, degraded sugars from cellulose; P2, total sugars in silage; E, enzyme concentration).

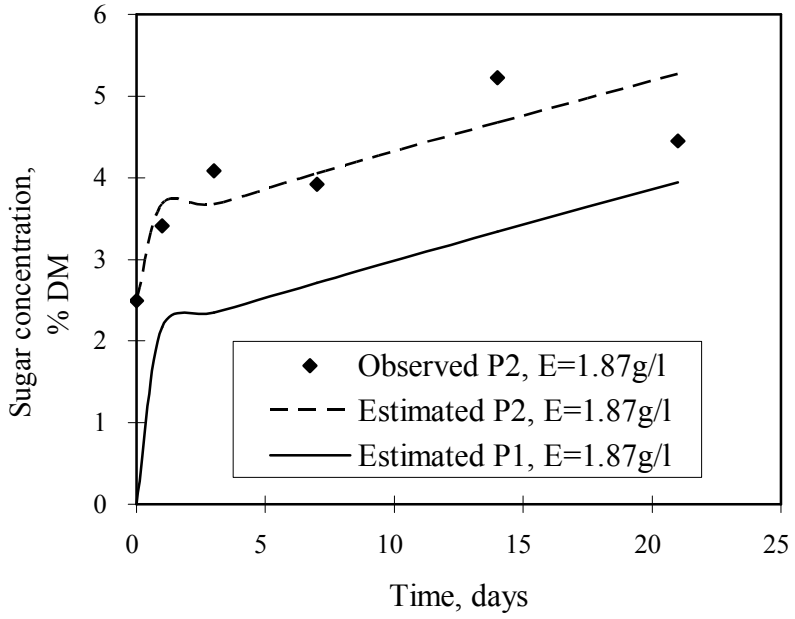

Figure 7.7 The change of sugars during the ensilage process with an enzyme concentration of 1.879g/l (P1, degraded sugars from cellulose; P2, total sugars in silage; E, enzyme concentration).

7.5.6 Simulation of lactic acid production

In the original articles describing the silage model developed by Pitt et al (1985) and Leibensperger and Pitt (1987), validation of the predicted individual acid concentration was not reported. Instead, lactic acid and acetic acid were each predicted as a ratio of the acid of interest over the sum of lactic plus acetic acid content. The simulated ratios for lactic acid at the end of 21 days with the two enzyme amendment levels of 1.29 and 1.87 g l^{-1} were 3.27 and 3.22, while the measured ratios were 5.06 and 7.0, respectively. The experimental results for lactic acid concentration and simulated values are compared in Figure 7.8. Although the simulated ratios were within a factor of 2 of the measured ratios, the simulated concentration values are about 10 times lower than the true values. Furthermore, continuing dynamic changes in lactic acid concentration during the ensilage process were not expressed in the simulation plots. This discrepancy may be due to the simulation model's approach to LAB population dynamics. In the model the death rate of LAB is estimated as a function of the maximum death rate. For estimating

the maximum death rate, they assumed the death rate equals the growth rate at the moment that the population of bacteria is at a maximum. This assumption resulted in the counts of lactic acid bacteria dropping very quickly after they achieved the peak count, so that lactic acid remained constant after that moment. However, this assumption underestimates the persistance of lactic acid bacteria in inhospitable environments. Lactic acid bacteria have been reported to lower their maintenance energy to survive when the environment is undesirable (Renault et al., 1988). Thus in reality the counts of the bacteria will decrease more slowly after the maximum counts are obtained, while lactic acid continues to be produced.

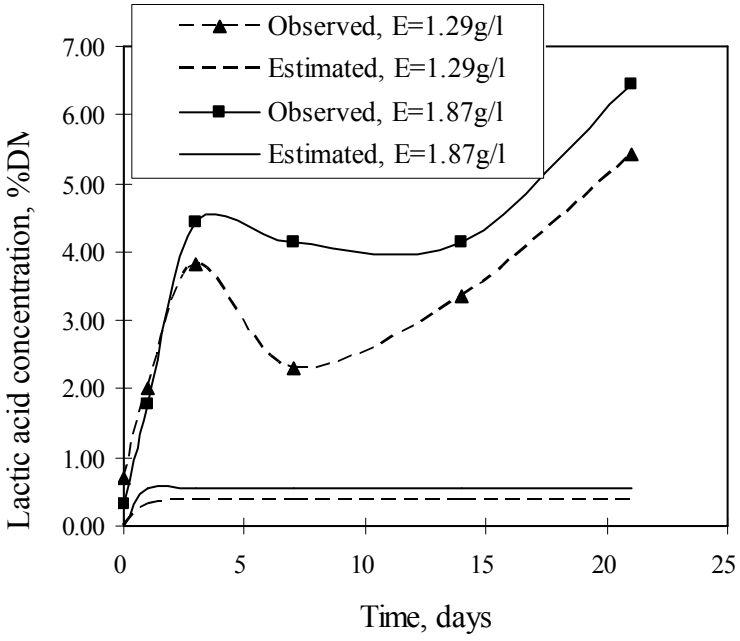

Figure 7.8 Observed and estimated results for lactic acid concentration over time for two enzyme concentrations.

7.6 Conclusion

This study developed an integrated model to simulate the ensilage process as affected by enzyme additions. Cellulose degradation was simulated by developing a hydrolysis kinetic model based on known mechanisms, including product inhibition and the existence of a non-hydrolysable region in lignocellulosic biomass. A parameter to account for cellulose structural features, λ, was estimated as 0.56, implying 56% of cellulose was in the non-hydrolysable region and unavailable for enzymatic hydrolysis.

With enzymatic hydrolysis integrated into a fermentation model, dynamic responses of several important chemical components of corn stover silage are predicted, including the concentration of cellulose, total water soluble carbohydrates, degraded sugars, and lactic acid. Acceptable prediction for these chemical constitutents were obtained with the exception of lactic acid. The model's assumption about the death rate of lactic acid bacteria resulted in an underestimation of ongoing lactic acid production and the resulting lactic acid concentration. Better understanding of the population dynamics and metabolic response of lactic acid bacteria to extreme environments is needed to improve the prediction of lactic acid and presumably other organic acid components as well.

7.7 Recommendations for improvement

This simulation study provides a foundation for further investigations of the effect of enzyme additions on the ensilage process. In this initial effort, several simplifications were made to reduce the complexity of the model. For example, cellulose degradation in the absence of enzyme addition was assumed to be zero, which was not always the case in previous experiment al results. Hemicellulose degradation by enzyme addition was also not considered in this model. To develop a more complete and predictive model of enzyme amended ensilage processes, several aspects should be considered for further model development:

1. Simulation of hemicellulose degradation at high enzyme concentrations;
2. Simulation of cellulose degradation by cellulolytic bacteria.
3. Investigation of the death rate of lactic acid bacteria death;
4. Inclusion of lignin content as an input for the model, since lignin content has been reported to influence cellulose degradation by enzymatic hydrolysis;
5. Simulation of sugar consumption by clostridia, since in the current model lactic acid was considered as the only substrate for clostridia;
6. Simulation of cellulose and hemicellulose degradation by chemical additives and produced organic acids.

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8. CONCLUSIONS AND RECOMMENDATIONS

A safe and efficient storage method is needed to preserve corn stover and supply biorefineries continuously throughout the year. Ensilage has been proposed as an alternative preservation method to dry storage. Due to the low content of water soluble carbohydrates (WSC) in corn stover, additives can be used to improve the quality of preserved stover. This study investigated the effect of cell wall degrading enzymes and chemicals on bioconversion of corn stover during ensilage process. Recommendations based on the analysis of results of the various treatments can help in directing further studies.

The first study examined the effect of enzyme concentration on stover silage. Enzyme treatment showed positive effects for both the preservation and pretreatment aspects of corn stover silage. Enzyme additives resulted in significantly lower silage pH after 21 days of ensiling. Cell wall degradation, particularly enzymatic cellulose degradation, increased considerably, with 22 – 29% of each fiber fraction hydrolyzed at the higher enzyme rates. The effect of enzyme additives on the characteristics of stover silage became more significant with increasing enzyme rates. The enzyme rate of 13.4 IU g^{-1} DM lowered the pH to approximately 4.3 – 4.4, and resulted in a comparable lactic acid concentration to the 106.9 IUg^{-1} DM rate. The dominance of lactic acid production over butyric acid production was significant at this rate, and indicated a shift in primary fermentation from clostridia to lactic acid bacteria.

Stover particle size significantly affected pH, lactic acid concentration, WSC content, and hemicellulose degradation. Although the coarse size yielded the lowest lactic acid concentration, it resulted in the highest WSC concentration and hemicellulose hydrolysis levels. Considering the preprocessing phase and downstream bioconversion, coarse size stover offers the most promising benefits from the view of economics and efficiency of manufacturing. Coarse stover does not require any grind or chopping steps, which saves time, labor, and mill equipment. Enzyme addition and mixing might even be conducted by modified machinery during the wet stover harvest period. High levels of hemicellulose degradation and WSC should make further pretreatment of biomass, saccharificaion and sugar fermentation more effective and economical. The industrial

enzyme tested here was shown to be as effective as the purified enzyme at similar rates of hemicellulase and cellulase activity. At an enzyme amendment rate of 13.4 IU g^{-1}, the costs associated with the industrial enzyme tested are on the order of $63 per Mg of stover ensiled. With overall biomass storage costs estimated at $21.6 per Mg, this would be a significant addition. To minimize the total costs of biomass storage and conversion will require optimizing the amended enzyme systems, lowering costs of enzyme production, and/or reducing costs of subsequent pretreatment to capitalize on the pretreatment effects that occurred.

In the second study, mixtures of different ratios of cellulase and hemicellulase enzymes from different microbial sources were used as treatments. They had varying effects on fiber hydrolysis during ensiled storage of corn stover. To facilitate comparisons among treatments, mixtures were normalized on the basis of hemicellulase activity. For each enzyme mixture and C:H ratio, two hemicellulase levels were tested, 1.67 IU g^{-1} and 5 IU g^{-1}. Results indicated the lower level of hemicellulase was sufficient to achieve most beneficial effects. At each of these hemicellulase levels, the ratio of cellulase to hemicellulase was important for improving the quality of stover silage. Increasing ratios of C:H reduced pH, increased lactic acid concentration, and decreased butyric acid concentration.

Successful development of ensilage as a biomass storage strategy will require minimizing dry matter loss. Dry matter loss often results from secondary fermentations, which can be suppressed by high concentrations of lactic acid and the resulting reduced pH. In order to increase the concentration of lactic acid and suppress these secondary fermentations, a critical C:H ratio is required. Mixtures at or above this critical C:H ratio will have sufficient cellulase to hydrolyze glucose for fermentation into lactic acid. For the enzyme mixtures tested, this critical C:H ratio was approximately 2.14, but likely to vary somewhat depending on the specific synergistic interactions of enzymes from a particular microbial source.

One of the potential benefits of an ensiled storage process would be *in-situ* pretreatment and hydrolysis of polymers during storage to produce WSC for downstream bioconversion. This benefit was observed in many of our ensilage treatments. The WSC content of stover silage depended on the ratio of C:H in the applied enzymes as well as

the size of stover material. For each of the stover sizes tested, enzymes from *T. reesei* increased WSC content at increasing rates with increasing C:H ratios. Results for other stover sizes and microbial sources were less consistent, but similar trends were observed. The effect of the microbial source of enzyme mixtures can not be completely elucidated based on our current results. However, enzyme mixtures derived from *T. reesei* and *T. longibrachiatum* appear to hydrolyze more cellulose than those derived from *A. niger*, even when the ratio of C:H in the former mixtures is less than that of the latter. These differences suggest that optimized enzyme mixtures can provide significant pretreatment benefits during ensiled biomass storage.

Hemicellulose is more easily hydrolyzed than cellulose by the acid conditions that prevail during the normal ensiling process. Thus it was not surprising that the addition of enzymes improved cellulose degradation more significantly than that of hemicellulose. However, this improved cellulose degradation also contributed to lower pH and presumably increased access of hemicellulase to substrate, resulting in considerably improved hydrolysis of hemicellulose for the high C:H treatments. Development of microbial strains that can convert both 5- and 6-carbon sugars to ethanol and other value-added chemicals makes increased hemicellulose hydrolysis important for maximizing product yields. Because the hemicellulase enzyme concentration did not have a significant influence on pH, chemical composition, or fiber degradation of the final stover silage, minimizing the enzyme treatment level should maximize economic returns. For the high C:H ratios of 2.l4 and 2.38 in mixtures derived from *T. reesei*, the low 1.67 IUg^{-1} hemicellulase level appears more than sufficient to achieve positive results. Further research to optimize these levels and increase synergies among enzyme mixture components appears likely to result in attractive ensilage strategies for industrial storage of large volumes of biomass feedstocks.

In order to validate the effect of enzyme addition during long storage periods, the third study with enzymes extended the preservation period to 189 days. Addition of hemicellulase and cellulase enzymes encouraged lactic acid fermentation significantly, reducing pH to 3.9-4.5, guaranteeing stable six-month biomass preservation as long as anaerobic conditions prevailed. The enzymatic amendment resulted in lactic acid being the dominant organic acid among the fermented products, with sufficient WSC

hydrolyzed from the lignocellulosic component of corn stover to initiate a robust *Lactobacillus* spp. fermentation. This enzyme treatment also resulted in a higher production of total organic acids converted from cell wall constituents.

Enzymatic treatment enhanced the peak counts of *Lactobacillus* spp. by a full order of magnitude. The counts of clostridia spores were effectively inhibited by enzyme addition, especially on the medium particle size. This medium size stover treated with enzyme showed the most promising results for both bioconversion and microorganism selectivity. These apparent advantages for the medium particle size suggest a trade-off between substrate surface area and enzymatic transport in solid-state fermentation, a promising avenue for future research.

The ensiled treatments had several positive impacts on the mechanical properties and dimensional stability of the manufactured particleboards relative to boards made from unensiled stover. Ensiled stover led to a significant increase in internal bond strength, while significantly decreasing thickness swelling and water adsorption in 24-hr-soaking testing. The enzyme treatments had the lowest water adsorption, and may increase the modulus of elasticity, although the later effect was not statistically significant. The extended ensilage period of 189 days resulted in a lower modulus of rupture and higher thickness swelling and water adsorption in both 2-hr boiling and 24-hr soaking tests. Thus while short-term ensilage improves board properties in several respects, these benefits tend to weaken over time.

Although ensiling did degrade hemicellulose selectively over cellulose when no enzymes were added, the enzyme treatment with higher cellulase activity compensated for this selectivity and resulted in roughly equivalent degradation rates. The weakened selectivity may explain the lack of improvement in particleboard properties for the enzyme treated boards in 2-hr-boiling and 24-hr-soak tests.

Although ensiling appears to be a promising approach for long-term storage of corn stover and presumably other biomass crops, storage conditions must be aligned with the ultimate product use. For board manufacturing and other fiber applications, the hydrolysis needs to insure adequate initial fermentation of the hemicellulose fraction to minimize reductions in mechanical strength. The increased microbial activity resulting

from enzyme enhanced ensilage can complement bio-based adhesives to provide biocomposite materials.

The fourth study examined the effects of chemical additives on corn stover preservation and pretreatment. The sugar yield from enzymatic hydrolysis of treated stover was also investigated. The addition of formic acid and formaldehyde encouraged lactic acid fermentation and inhibited clostridia growth. Sulfuric acid limited mixed fermentation, as indicated by reduced butyric acid and acetic acid concentrations. Corn stover was preserved at the tested levels of these chemical additives. High levels ($16 g kg^{-1}$ DM) of urea and ammonia can inhibit undesirable microorganisms, as indicated by low butyric acid concentrations in these samples. The analysis of fiber degradation showed that sulfuric acid was effective at enhancing cellulose degradation, though hemicellulose degradation was limited as plant enzymes and microorganisms were suppressed at low pH values. At the level of 16 g kg^{-1} DM ammonia, the highest (21%) hemicellulose degradation was obtained with little effect on cellulose degradation.

During a longer 63 day trial, the sulfuric acid-treated stover silage had a similar chemical composition at 21 days to the corresponding end date of the first experiment. The addition of sulfuric acid slowed the consumption of lactic acid and the production of acetic and butyric acids. Fermentation was totally inhibited at the rate of 16 g kg^{-1} DM due to a very low pH of 2.5. WSC content increased throughout the 63 days in the 16 g sulfuric acid kg^{-1} DM samples, and this WSC increase was associated with significant hemicellulose degradation.

The potential for beneficial pretreatment during ensiling was examined by enzymatically hydrolyzing ensiled stover. Ensiling did not significantly improve sugar yields, however, the treatments with sulfuric acid, formic acid, formaldehyde, and ammonia did increase the total sugar production. The anticipated utility of the ensiling process as a pretreatment for sugar conversion was not fully realized.

Finally, an integrated model was developed to simulate the ensilage process as affected by enzyme additions. Cellulose degradation was simulated by developing a hydrolysis kinetic model based on known mechanisms, including product inhibition and the existence of a non-hydrolysable region in lignocellulosic biomass. A parameter to account for cellulose structural features, λ, was estimated as 0.56, implying 56% of

cellulose was in the non-hydrolysable region and unavailable for enzymatic hydrolysis. With the integrated ensilage model, dynamic responses of several important chemical components of corn stover silage are predicted, including the concentration of cellulose, total WSC, degraded sugars, and lactic acid. Acceptable prediction for these chemical constitutents were obtained with the exception of lactic acid.

One of the assumptions of the integrated silage model about the death rate of lactic acid bacteria resulted in an underestimation of ongoing lactic acid production and the resulting lactic acid concentration. Better understanding of the population dynamics and metabolic response of lactic acid bacteria to extreme environments is needed to improve the prediction of lactic acid and presumably other organic acid components as well.

Because the sugar yields of ensiled stover in downstream bioconversion were not high enough to meet industrial needs, a new strategy of integrating effective ensiled preservation and more efficient pretreatment must be developed to obtain sugar yields closer to the theoretical maximum. Since crosslinks between cellulose, hemicellulose, and lignin are the major limitation for enzymatic hydrolysis, reducing lignin content is expected to improve the sugar yields. Chemicals, such as SO_2 and ozone, or lignase can be used to remove this limitation. Furthermore, the synergism between cell wall degrading enzymes and lignase deserves to be examined. The positive interaction between polysaccharide hydrolysis and lignin reduction is anticipated to improve the cell wall degradation of corn stover during preservation periods.

The rich carbohydrate content of lignocellulosic materials make corn stover not only an appropriate feedstock for ethanol and particleboard production, but also a digestible and nutritional food for animal feeding after appropriate pretreatments. The proportions of these final products of corn stover can be adjusted according to the relative market demands. The solid residues after ethanol production can be used as fertilizer to supplement soil nutrients. Extensive investigation is required to develop integrated strategies for combining these potential uses of ensiled stover to obtain cost-efficient and profit-maximizing bioconversion systems.

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APPENDIX 1, MATLAB FILE FOR λ ESTIMATION

ren.m

```
global r S;
Q=zeros(100,1);
M=zeros(100,1);
W=zeros(100,1);
R=zeros(100,1);

fprintf('Welcome to the model of crop ensilage process\n')
global O2 CO2 ph T c cr d r t1 s hc0 lc lgl lgc aw s1 O21 CO21 T1 aw1 ph1 sa1 se1
ph=6.62%input('please input the initial pH value')
T=310%input('please input the initial Temperature value, K')
c=2%input('please input the crop category: 1 for grass; 2 for corn; and 3 for legume')
d=0.40%input('please input the DM content')
r=0.01%input('please input r')
cr=8%input('please identify the crop category, 1 for alfalfa; 2 for ryegrass; 3 for white clover; 4 for red clover; 5 for timothy; 6 for cocksfoot; 7 for fescue; and 8 for corn')
s=0.0081%input('please input the initial water soluble carbohydrates,g/g silage')
hc0=0.16%input('please input the initial hemicellulose content, g/g silage')

ph1=ph;
t1=0;

y0=zeros(7,1);
%dydt = [-miur*ko*roh*d/1000/roo/r          %y1, oxygen concentration;
%       miur*roh*d/1000/roc/r     %y2, carbon dioxide concentration;
%       -miur*ks*d/1000                                    %y3, wsc;
%       miur*kj*d/ch ];                                    %y4, T, temperature;
%       miua*hc                                                %y5, sugar produced by chemical hydrolysis;
%       miue*ce                                                %y6, sugar produced by enzymatic hydrolysis;
%       0                                                      %y7;
%       (vg-vb)*y(8)                                %y8, LAB concentration;
%       (miug-miub)*y(9)                            %y9, clostridia concentration;
%       0                                  %y10, lactic acid concentration;
%       y(9)*miug/0.231                    %y11, lactic acid used by clostirdia;
%       0                          %y12, butyric acid production;
%       0                          %y13, NH3 production by clostridia;
%       0                          %y14, sugar except mannitol used for LAB growth;
%       0                          %y15, mannitol used for LAB growth;
%       0                          %y16, end production;
%       0                          %y17, acetic acid produced;
%       0                          %y18, lactic acid produced;
%       0                          %y19, mannitol produced;
%       0                          %y20, ethanol;
```

```
%       0                       %y21, CO2 produced by LAB;
%       0                       %y22, mannitol;
%       0                       %y23, fraction of ammonia released by non-clostridia sources as a function of time;
%       0                       %y24, NH3 released by LAB;
%       0                       %y25, NH3;
%       0                       %y26, NH3 associated with hydrogen;
%       0                       %y27, dissociated lactic acid;
%       0                       %y28, dissociated acetic acid;
%       0                       %y29, dissociated butyric acid;
%       0                       %y30, pH;
%       0                       %y31, non-protein nitrogen;
%       0                       %y32, protein nitrogen;
%       0               %y33, sugar from cellulose degradation
%       with enzyme];

y0(1)=0.21;
y0(2)=0;
y0(3)=s;
y0(4)=T;
%initial water activity;
if (c==1) | (c==2)
  y0(5)=1-0.03*d/(1-d);
elseif c==3
  y0(5)=1-0.04*d/(1-d);
end
y0(6)=0;
y0(7)=0;

tspan=[0:1:240]; %specify the time range and units you are using

[t,y]=ode45(@f,tspan,y0);

nn=length(y(:,7));
T1=0;
for i=1:nn-1
  if t(i)<=t1 & t(i+1)>t1
    O21=y(i,1)
    CO21=y(i,2)
    s1=y(i,3)
    T1=y(i,4)
    aw1=y(i,5)
    sa1=y(i,6)
    se1=y(i,7)
  end
end

y0=zeros(56,1);
```

```
y0(1)=O21;
y0(2)=CO21;
y0(3)=s;
y0(4)=T1;
y0(5)=sa1;
y0(6)=se1;
y0(7)=aw1;
y0(8)=0.0000001%input('please input the initial lactic acid bacteria concentration, g/g silage');
y0(9)=0.0000005%input('please input the initial clostridia concentration, g/g silage');
y0(10)=0%input('please input the initial lactic acid concentration, g/g silage');
y0(11)=0;
y0(12)=0%input('please input the initial butyric acid concentration, g/g silage');
y0(17)=0%input('please input the initial acetic acid concentration,g/g silage');
y0(22)=0%input('please input the initial mannitol concentration, g/g silage');
y0(27)=y0(10)*10^(ph-3.86)/(1+10^(ph-3.86));
y0(28)=y0(17)*10^(ph-4.76)/(1+10^(ph-4.76));
y0(29)=y0(12)*10^(ph-4.81)/(1+10^(ph-4.81));
y0(30)=ph1;
y0(31)=0.05%input('please input the initial non-protein nitrogen concentration, g/g silage');
y0(32)=0.05%input('please input the initial protein nitrogen concentration, g/g silage');
y0(39)=1.868;
y0(40)=285;
y0(55)=258;
y0(56)=258;
S=258;
S12=253.82;
S13=254.62;
S14=250.47;
S15=245.95;
S16=244.71;

S22=250.56;
S23=250.39;
S24=243.95;
S25=238.90;
S26=239.69;
i=1;
rm=0.01;
while i<=100
   rs=rm*i;
   y0(3)=0.0087;
   y0(30)=7.16;
   y0(43)=(1-rs)*S;
   y0(44)=rs*S;
   y0(47)=0.8179;
   [t,y]=RKmain(@haiyu1,t1,504+t1,0.05,y0,0);
   M(i)=(y(55,481)-S12)^2+(y(55,1441)-S13)^2+(y(55,3361)-S14)^2+(y(55,6721)-S15)^2+(y(55,10081)-S16)^2;
```

```
    y0(3)=0.0081
    y0(30)=7.15;
    y0(50)=(1-rs)*S;
    y0(51)=rs*S;
    y0(54)=1.289;
    [t,y]=RKmain(@haiyu2,t1,504+t1,0.05,y0,0);
    W(i)=(y(56,481)-S22)^2+(y(56,1441)-S23)^2+(y(56,3361)-S24)^2+(y(56,6721)-S25)^2+(y(56,10081)-S26)^2;
    Q(i)=M(i)+W(i)
    R(i)=rs;
    i=i+1;
end
figure;
plot(R,Q,'r*-');
title('the change of sum of square with r','FontSize',12,'FontWeight','bold');
xlabel('r','FontSize',12,'FontWeight','bold');
ylabel('sum of square','FontSize',12,'FontWeight','bold');
k=find(Q==min(Q));
R(k);
rs=R(k)
    y0(3)=0.0087;
    y0(30)=7.16;
    y0(43)=(1-rs)*S;
    y0(44)=rs*S;
    y0(47)=0.8179;
    [t,y]=RKmain(@haiyu1,t1,504+t1,0.05,y0,0);
    y0(3)=0.0081
    y0(30)=7.15;
    y0(50)=(1-rs)*S;
    y0(51)=rs*S;
    y0(54)=1.289;
    [t,y]=RKmain(@haiyu2,t1,504+t1,0.05,y0,0);
S=285.51;
    y0(3)=0.0093;
    y0(30)=6.12;
    y0(56)=285.51;
    y0(50)=(1-rs)*S;
    y0(51)=rs*S;
    y0(54)=1.289;
    tspan=[t1:0.05:504+t1]; %specify the time range the unit you are using
[t,y]=RKmain(@haiyu2,t1,504+t1,0.05,y0,0);
    y0(3)=0.01;
    y0(30)=5.77;
    y0(40)=285.51
    y0(35)=(1-rs)*S;
    y0(36)=rs*S;
    y0(39)=1.868;
tspan=[t1:0.05:504+t1];
```

```
[t,y]=RKmain(@haiyu3,t1,504+t1,0.05,y0,0);
```

APPENDIX 2, MATLAB FILE FOR AEROBIC RESPIRATION

f.m

```
function dydt=f(t,y)
global O2 CO2 ph T c cr d r s t1 aw1 ph1 hc0

dydt=zeros(7,1);
O2=y(1);
CO2=y(2);
s=y(3);
T=y(4);


%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%;

%Aerobic respiration;
%miurm, miaximum respiration rate
if c==1 | c==2
  miurm=5.6;   %mg CO2/g DM/hr;
else
  miurm=7.0;   %mg CO2/g DM/hr;
end
%frph, pH
frph=(ph-3)/3.5;
%frt, temperature
if T>=278 & T<=298
   frt=0.178*exp(0.069*(T-273));
else
   frt=1.0;
end
%frd, DM
if c==1 | c==2
  if d<0.167
     frd=1.0;
  elseif d>0.167 & d<0.8
     frd=1.42-2.73*d+1.21*d^2;
  else
     frd=0.01;
  end
else
   if d<0.217
      frd=1.0;
   elseif d>=0.217 & d<=0.7
      frd=1.58-2.89*d+1.05*d^2;
   else
```

```
      frd=0.072;
   end
end
%fro, oxygen;
kr=0.055;        %constant, m3 O2/m3 gas;
fro=y(1)/(kr+y(1));
%frc, carbon dioxide
if y(2)>=0.1 & y(2)<=0.21
   frc=1.222-2.22*y(2);
else
   frc=1.0;
end
roh=3000000/(3-d);                    %density of herbage, g/m3;
ch=d*1.89+(1-d)*4.19;                  %herbage specific heat, kJ/kg/K;
ko=0.727;                             %g O2 used/g CO2 produced;
roo=101.325*1000*32/8.314/T;          %density of O2, g/m3;
roc=101.325*1000*44/8.314/T;          %densigy of CO2, g/m3;
ks=0.682;                             %g WSC used/g CO2 produced;
kj=10.9;                              %kJ released/g CO2 produced;
% environmental factors;
miur=miurm*frph*frt*frd*fro*frc;

%hydrolysis of hemicellulose;
% chemical hydrolysis;
miua=0.0000094+0.00113*exp(-ph1)+0.000000227*(T-273);     %g WSC/g hemic./hr;
%dydt(6) = miua*hc0*exp(-miua*t)   % y5, sugar produced by chemical hydrolysis, g WSC/g silage;
%enzymatic hydrolysis;
%italian ryegrass;
if t>=0 & t<=72
    miue1=-0.183+0.0665*ph1-0.00597*(ph1)^2+0.00128*(T-273)-0.0000203*(T-273)^2;
elseif t>72 & t<=168
   miue1=0.0363-0.0108*ph1+0.00104*(ph1)^2-0.000386*(T-273)+0.00000843*(T-273)^2;
else
    miue1=0;    %g WSC/g extract/hr;
end
%perenial ryegrass;
if t>=0 & t<=72
    miue2=-0.111+0.0387*(ph1)-0.00326*(ph1)^2+0.000905*(T-273)-0.0000117*(T-273)^2;
elseif t>72 & t<=168
    miue2=0.0104+0.00188*(ph1)-0.0000696*(ph1)^2-0.000728*(T-273)+0.0000112*(T-273)^2;
else
    miue2=0;
end
%cocksfoot;
if t>=0 & t<=72
    miue3=-0.043+0.0137*ph1-0.00104*(ph1)^2+0.000721*(T-273)-0.00001*(T-273)^2;
elseif t>72 & t<=168
```

```
    miue3=-0.0392+0.0208*ph1-0.00203*(ph1)^2-0.000326*(T-273)+0.00000458*(T-273)^2;
else
    miue3=0;
end
miue=1/3*(miue1+miue2+miue3);
%ce, concentration of extract, g extract/g silage;
if c==1 | c==2
  ce=0.01875*d;          %concentration of extract, g extract/g silage;
else
  ce=0.002*d;
end
%dydt(7) = miue*ce   %y6, sugar produced by enzymatic hydrolysis;
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%;
% aerobic respiration period;
if y(1)>0.01
  dydt(1) = -miur*ko*roh*d/1000/roo/r; %y1, oxygen concentration, m3 O2/m3 gas;
  dydt(2) = miur*roh*d/1000/roc/r;  %y2, carbon dioxide concentration, m3 CO2/m3 gas;
  dydt(3)=-miur*ks*d/1000; %y3, WSC concentration, g wsc/g silage;
  dydt(4) = miur*kj*d/ch;   %y4, T, temperature, K;
  dydt(5)=-y(5)*18/(1-d)*dydt(3)/180.15   % aw change during aerobic period
  dydt(6)=miua*hc0*exp(-miua*t);       %WSC chemically hydrolized
  dydt(7)=miue*ce;        %y7, sugar produced by enzymatic hydrolysis;
  t1=t;
elseif y(1)<=0.01
   dydt(1)=0;
   dydt(2)=0;
   dydt(3)=0;
   dydt(4)=0;
   dydt(5)=0;
   dydt(6)=0;
   dydt(7)=0;
end
```

APPENDIX 3, MATLAB FILE FOR INTEGRATED MODEL

haiyu1.m

```
function dydt=haiyu1(t,y)
global ph T1 c cr d r s hc0 t1 lc lgl lgc aw O21 s1 CO21 T1 aw1 sa1 se1 ph1
global r S
            dydt=zeros(56,1);
  %At=2.718282^(0.00000000008*t^4-0.00000009*t^3+0.00004*t^2-0.0071*t+4.55555)/100;
  dydt(41)=0.2*y(47)*y(43)-0.05*y(41)-9.05*y(41);
  dydt(42)=0.02*y(47)*y(44)-0.002*y(42);
  dydt(43)=0.05*y(41)-0.2*y(47)*y(43);
  dydt(44)=0.002*y(42)-0.02*y(47)*y(44);
  dydt(45)=9.05*y(41);
  dydt(46)=0.1*y(47)*y(3)-0.03*y(46);
  dydt(47)=0.05*y(41)+0.002*y(42)+0.03*y(46)+9.05*y(41)-0.2*y(47)*y(43)-0.1*y(47)*y(3)-0.02*y(47)*y(44);
  dydt(55)=dydt(43)+dydt(44)+dydt(41)+dydt(42);

  dydt(48)=0.2*y(54)*y(50)-0.05*y(48)-9.05*y(48);
  dydt(49)=0.02*y(54)*y(51)-0.002*y(49);
  dydt(50)=0.05*y(48)-0.2*y(54)*y(50);
  dydt(51)=0.002*y(49)-0.02*y(54)*y(51);
  dydt(52)=9.05*y(48);
  dydt(53)=0.1*y(54)*y(3)-0.03*y(53);
  dydt(54)=0.05*y(48)+0.002*y(49)+0.03*y(53)+9.05*y(48)-0.2*y(54)*y(50)-0.1*y(54)*y(3)-0.02*y(54)*y(51);
  dydt(56)=dydt(50)+dydt(51)+dydt(48)+dydt(49);

  dydt(33)=0.2*y(39)*y(35)-0.05*y(33)-9.05*y(33);
  dydt(34)=0.02*y(39)*y(36)-0.002*y(34);
  dydt(35)=0.05*y(33)-0.2*y(39)*y(35);
  dydt(36)=0.002*y(34)-0.02*y(39)*y(36);
  dydt(37)=9.05*y(33);
  dydt(38)=0.1*y(39)*y(3)-0.03*y(38);
  dydt(39)=0.05*y(33)+0.002*y(34)+0.03*y(38)+9.05*y(33)-0.2*y(39)*y(35)-0.1*y(39)*y(3)-0.02*y(39)*y(36);
  dydt(40)=dydt(35)+dydt(36)+dydt(33)+dydt(34);

aw=y(7);
ph=y(30);

%Lag in plant cell lysis and bacterial growth;
%plant cell lysis;
if c==1 | c==2
  lc=-2.76+4490000000000000*exp(-35.4*aw1)+199000000*exp(-0.0586*T1); %hr;
else
  if T1<=308 & aw1>=0.986
    lc=64.7-0.205*T1+0.015*(T1-303)^2
  elseif T1<=308 & aw1<0.986
    lc=672.1-0.205*T1+0.015*(T1-303)^2-616*aw1;
```

```
   elseif T1>308 & aw1<0.986
      lc=609.3-616*aw1;
   elseif T1>308 & aw1>=0.986
      lc=1.92;
   end
end
%awm, minimum aw for growth;
if  ph>=4.0 & ph<=6.4
    awm=1.145-0.05452*ph+0.004259*(ph)^2;
elseif ph1>6.4 %& ph<6.9
    awm=0.9706;
elseif ph<4.0
    awm=0.9951;
end
%lag in LAB growth;
if  aw1<0.990
    lgl=-101.4+104.6*aw1+6310*(aw1-0.99)^2;
else
    lgl=2.0;
end
%lag in clostridia growth;
if T1>=288.8 & T1<=310.2 & ph1>=6 & ph1<=7.2 & aw1>awm
    lgc=exp(102.8-0.1348*T1-59.8*aw1);
elseif T1>310.2 & T1<=319.2 & ph1>=6 & ph1<=7.2 & aw1>awm
    lgc=exp(60.99-59.80*aw1);
elseif T1>=288.8 & T1<=310.2 & ph1>=5.5 & ph1<=6 & aw1>awm
    lgc=-53+318/ph+exp(102.8-0.1348*T1-59.8*aw1);
elseif T1>310.2 & T1<=319.2 & ph1>=5.5 & ph1<6.0 & aw1>awm
    lgc=-53+318/ph+exp(60.99-59.80*aw1);
else
    dydt(9)=0;
end

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%;

%hydrolysis of hemicellulose;
% chemical hydrolysis;
miua=0.0000094+0.00113*exp(-ph)+0.000000227*(T1-273);     %g WSC/g hemic./hr;
dydt(5) = miua*hc0*exp(-miua*t);   % y5, sugar produced by chemical hydrolysis, g WSC/g silage;
%enzymatic hydrolysis;
%italian ryegrass;
if t>=t1 & t<=72
     miue1=-0.183+0.0665*ph-0.00597*(ph)^2+0.00128*(T1-273)-0.0000203*(T1-273)^2;
elseif t>72 & t<=168
    miue1=0.0363-0.0108*ph+0.00104*(ph)^2-0.000386*(T1-273)+0.00000843*(T1-273)^2;
else
    miue1=0;    %g WSC/g extract/hr;
```

```
end
%perennial ryegrass;
if t>=t1 & t<=72
    miue2=-0.111+0.0387*(ph)-0.00326*(ph)^2+0.000905*(T1-273)-0.0000117*(T1-273)^2;
elseif t>72 & t<=168
    miue2=0.0104+0.00188*(ph)-0.0000696*(ph)^2-0.000728*(T1-273)+0.0000112*(T1-273)^2;
else
    miue2=0;
end
%cocksfoot;
if t>=t1 & t<=72
    miue3=-0.043+0.0137*ph-0.00104*(ph)^2+0.000721*(T1-273)-0.00001*(T1-273)^2;
elseif t>72 & t<=168
    miue3=-0.0392+0.0208*ph-0.00203*(ph)^2-0.000326*(T1-273)+0.00000458*(T1-273)^2;
else
    miue3=0;
end
miue=1/3*(miue1+miue2+miue3);
%ce, concentration of extract, g extract/g silage;
if c==1 | c==2
  ce=0.01875*d;        %concentration of extract, g extract/g silage;
else
  ce=0.002*d;
end
dydt(6) = miue*ce;   %y6, sugar produced by enzymatic hydrolysis;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%;

%bacterial growth rate;
% growth rate of lactic acid bacteria;
%vgm, the maximum growth rate;  hr-1;
if T1>=288 & T1<=303
   vgm=exp(27.287-8344.4/T1)*0.975;
elseif T1>=303 & T1<=320
   vgm=exp(17.168-5280.7/T1)*0.975;
end

%egt, temperature factor;
if T1>=288 & T1<=303
   egt=exp(27.287-8344.4/T1)/1.023;
elseif T1>303 & T1<=308
   egt=exp(17.168-5280.7/T1)/1.023;
elseif T1>308 & T1<=313
   egt=11.965-0.0356*T1;
end
```

```
%egph, pH factor;
%ph factor of P. pentosaceus;
egph1=0;
if ph<3.7
    egph1=0;
elseif ph>=3.7 & ph<6.3
   egph1=-3.45+1.27*ph-0.0899*(ph)^2;
elseif ph>=6.3 & ph<=7.3
   egph1=-23.2+7.62*ph-0.6*(ph)^2;
elseif ph>7.3
   egph1=0;
end
%ph factor of S. faecalis;
egph2=0;
if ph<3.72
   egph2=0;
elseif ph>=3.72 & ph<=10
    egph2=-3.48+1.27*ph-0.09*(ph)^2;
elseif ph>10
    egph2=0;
end

%ph factor of S.lactis;
egph3=0;
if ph<4
   egph3=0;
elseif ph>=4 & ph<=4.5
   egph3=0.16*(ph-4)+0.64*(ph-4.0)^2;
elseif ph>4.5 & ph<=8.2
   egph3=-7.19+2.54*ph-0.196*(ph)^2;
elseif ph>8.2
   egph3=0;
end
egph=0.4*egph1+0.4*egph2+0.2*egph3;
%egaw, water activity factor;
%water activity factor of L.plantarum;
egaw1=0;
if aw>=0.9326 & aw<=0.99
   egaw1=-5.55+6.64*aw-268.7*(aw-0.98)^2;
elseif aw>0.99
   egaw1=1;
elseif aw<0.9326
   egaw1=0;
end
%water activity factor of L. brevis;
egaw2=0;
if aw>=0.9445 & aw<=0.995
```

```
   egaw2=-18.33+19.59*aw-246.2*(aw-0.97)^2;
elseif aw>0.995
    egaw2=1;
elseif aw<0.9445
    egaw2=0;
end
%water activity factor of P. cerevisiae;
egaw3=-10.42+11.47*aw-0.1398*(T1-273)+0.1394*aw*(T1-273);
egaw=1/3*(egaw1+egaw2+egaw3);
%egs, sugar factor
kg=0.000022; %g wsc/g silage;
if y(3)>0
   egs=1;
else
   egs=0;
end

% growth rate of lactic acid bacteria;
if t<t1+lc+lgl
   vg=0;   %hr-1;
else
   vg=vgm*egph*egaw*egs;
end

%Death rate of lactic acid bacteria
%phs, pH*;
if d<=0.2
   phs=4.21;
elseif d >0.2 & d<0.45
   phs=3.23+4.93*d;
end
%egphs, pH* factor;
%phs factor of P. pentosaceus;
if phs>=3.7 & phs<6.3
   egphs1=-3.45+1.27*phs-0.0899*(phs)^2;
elseif phs>=6.3 & phs<=7.3
   egphs1=-23.2+7.62*phs-0.6*(phs)^2;
elseif phs<3.7
   egphs1=0;
elseif phs>7.3
   egphs1=0;
end
%ph factor of S. faecalis;
if phs>=3.72 & phs<=10
    egphs2=-3.48+1.27*phs-0.09*(phs)^2;
elseif phs<3.72
    egphs2=0;
```

```
elseif phs>10
   egphs2=0;
end
%phs factor of S.lactis;
if phs<4
  egphs3=0;
elseif phs>8.2
  egphs3=0;
elseif phs>=4 & phs<=4.5
  egphs3=0.16*(phs-4)+0.64*(phs-4.0)^2;
elseif phs>4.5 & phs<=8.2
  egphs3=-7.19+2.54*phs-0.196*(phs)^2;
end
egphs=0.4*egphs1+0.4*egphs2+0.2*egphs3;
%vbm, the maximum death rate of LAB, hr-1;
vg1=vgm*egphs;
%ebphs, pH* factor for vbm;
if phs>5.98 & phs<=7.6
  ebphs=15.09-4.96*phs+0.409*(phs)^2;
elseif phs>=5.04 & phs<=5.98
  ebphs=12.98-6.44*phs+1.289*(phs)^2-0.0959*(phs)^3;
elseif phs<5.04
  ebphs=1.0;
elseif phs>7.6
  ebphs=1.0;
end
vbm=vg1/ebphs;
%ebph, pH factor
ebph=0;
if ph<5.04
  ebph=1.0;
elseif ph>=5.04 & ph<=5.98
  ebph=12.98-6.44*ph+1.289*(ph)^2-0.0959*(ph)^3;
elseif ph>5.98 & ph<=7.6
  ebph=15.09-4.96*ph+0.409*(ph)^2;
elseif ph>7.6
  ebph=1.0;
end
%Death rate of lactic acid bacteria, hr-1;
if t<t1+lc+lgl
  vb=0;
else
  vb=vbm*ebph;
end

dydt(8)=(vg-vb)*y(8);          %y8, LAB concentration, g bacteria/g silage;
```

```
%Growth rage of clostridia;
miugm=2.39; %h-1
%fgt, temperature factor;
if T1>=285.7 & T1<=293.2
  fgt=exp(122.25-36178/T1)/2.0604;
elseif T1>293.2 & T1<=310.2
  fgt=exp(32.384-9820/T1)/2.0604;
elseif T1>310.2 & T1<=318.2
  fgt=1;
end
%phm, minimum ph value;
phm=0;
if aw>0.9706 & aw<0.9951
  phm=(0.05452-(0.01704*aw-0.01654)^0.5)/0.008518;
elseif aw>=0.9951 %& aw<=1
  phm=4.0;
elseif aw<=0.9706
  phm=6.4;
end
%fgph, pH factor;
fgph=0;
if ph>=phm & ph<=6.5
  fgph=(ph-phm)/(6.5-phm);
elseif ph>6.5 %& ph<=7.5
  fgph=1.0;
elseif ph<phm
  fgph=0;
end
%fgaw, water activity factor;
A=0.04278*((aw-awm)/(0.995-awm))+0.9522;
fgaw=0;
if aw>awm & A<0.9932
  fgaw=290.5-459.7*A+170.53*A^3;
elseif aw>awm & A>=0.9932
  fgaw=1.0;
elseif aw<=awm
  fgaw=0;
end

%fgl, lactic acid factor;
if y(10)>0
  fgl=1.0;
else
  fgl=0;
end
%Growth rage of clostridia, hr-1;
miug=miugm*fgt*fgph*fgaw*fgl;
```

```
%Death rate of clostridia;
%miubm, maximum death rate, hr-1;
miubm=0.521; %h-1;
%fbt, Temperature factor;
if T1>=285.7 & T1<=293.2
   fbt=exp(122.25-36178/T1)/2.0604;
elseif T1>293.2 & T1<=310.2
   fbt=exp(32.384-9820/T1)/2.0604;
elseif T1>310.2 & T1<=318.2
   fbt=1;
end
%fbph, ph factor;
fbph=0;
if ph>=5.56 & ph<6.5
   fbph=12.98-6.44*(ph-0.52)+1.289*(ph-0.52)^2-0.0959*(ph-0.52)^3;
elseif ph<5.56
   fbph=1.0;
elseif ph>=6.5 %& ph<7.5
   fbph=0.056;
end
%Death rate of clostridia, hr-1;
if y(9)>0
miub=miubm*fbt*fbph;
else
miub=0;
end

if t<t1+lc+lgc
   dydt(9)=0;
elseif t>=t1+lc+lgc
   dydt(9)=(miug-miub)*y(9); %y9, clostridia concentration, g bacteria/g silage;
end
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%

%substrate use and end product formation by bacteria;
%substrate use by clostridia, y11;
if t<t1+lc+lgc
   dydt(11)=0;
else
   dydt(11)=y(9)*miug/0.231;      %y11, lactic acid used by clostirdia, g/g silage;
end
%production by clostridia;
%butyric acid;
if t<=t1+lc+lgc
   dydt(12)=0;
else
```

```
  dydt(12) = 0.5*(1-0.231)*88.12/90.08*dydt(11);   %y12, g/g silage;
end
%NH3;
if t<t1+lc+lgc
  dydt(13)=0;
elseif y(12)>=0.000345 & y(12)<=0.0124
  dydt(13)=(49.3*y(12)-0.017)*dydt(12);       %y13, g/g silage;
elseif y(12)<0.000345
  dydt(13)=0;                          %y13;
end

%substrate used by LAB;
if t<=t1+lc+lgl
  dydt(14)=0;
  dydt(15)=0;
else
%y14, sugar used for LAB growth, g/g silage;
%y15, mannitol used for LAB growth, g/g silage;
  if y(3)>0
  dydt(14)=y(8)*vg/0.185;
  dydt(15)=0;
  elseif y(3)==0
  dydt(14)=0;
  dydt(15)=y(8)*vg/0.185;
  end
end

%Production and distribution of end products, g/g silage;
if t<t1+lc+lgl
  dydt(16)=0;
else
  dydt(16)= (1-0.185)*(dydt(14)+dydt(15));
end
% product fraction;
% fl, g LA produced per g lactic acid plus acetic acid;
fl=0;
if y(3)/d<=0.01 & ph<=5
  fl=2.454-0.391*ph+50*y(3);
elseif y(3)/d<=0.01 & ph>5
  fl=0.499+51*y(3);
elseif y(3)/d>0.01 & ph<=5.75
  fl=1.58-0.153*ph;
elseif y(3)/d>0.01 & ph>5.75
  fl=0.7;
end
%fa,
fa=1-fl;
```

```
%fet, ethanol fraction;
fet=0.0514;
%fma, mannitol fraction;
fma=2.87;
%fce, CO2 produced from pathway 3 per g acetic acid;
fce=fet*44/46.07;
%fcm, CO2 produced from pathway 4 per g acetic acid;
fcm=fma*44/2/182.18;
%fat, the fraction of acetic acid;
if fa>0
   fat=(1-fce-fet)/(fl/fa+fcm+fma+1);
else
   fat=0;
end

%change in products;
%Acetic acid, g/g silage;
if t<t1+lc+lgl
   dydt(17)=0;
else
   dydt(17)=fat*dydt(16);
end
%lactic acid produced, g/g silage;
if fa>0
   dydt(18)=fat*fl/fa*dydt(16);
elseif fa==0
   dydt(18)=(1-fet-fce)*dydt(16);
end
%mannitol produced, g/g silage;
dydt(19)=fma*fat*dydt(16);
%ethanol produced, g/g silage;
dydt(20)=fet*dydt(16);
%CO2 produced by LAB, g/g silage;
dydt(21)=(fce+(fat*fcm))*dydt(16);
%lactic acid, g/g silage;
if t<t1+lc+lgl
  dydt(10)=0;
else
  dydt(10)=dydt(18)-dydt(11);
end
%WSC;
if  t<=t1+lgl+lc   %y3, wsc in aerobic process, g/g silage;
   dydt(3)=dydt(45)*0.3/500;
elseif t>=t1+lgl+lc & t<t1+lgc+lc
   dydt(3)= -dydt(14)+0.166*vb*y(8)+dydt(45)*0.3/500;%+dydt(5)+dydt(6);
elseif t>=t1+lgc+lc
    dydt(3)= -dydt(14)+0.166*(miub*y(9)+vb*y(8))+dydt(45)*0.3/500;%+dydt(5)+dydt(6);
```

```
end
%mannitol, g/g silage;
dydt(22)=dydt(19)-dydt(15);
%change in NH3;
%fraction of NH3 produced from LAB;
if t>t1+lc+lgl
  dydt(23)=0;
else
  dydt(23)=0.00974*exp(-0.00929*t);
end
%NH3 produced from LAB;
if c==1 | c==2
  cfnl=(0.00405-0.00564*d)*d;
elseif c==3
  cfnl=(0.00493-0.00563*d)*d;
end
cin=0.000285*d;
dydt(24)=(cfnl-cin)*dydt(23);
%NH3, g/g silage;
dydt(25)=dydt(24)+dydt(13);

%change in aw;
dydt(7)=-y(7)*18/(1-
d)*(dydt(3)/180.15+dydt(10)/90.08+dydt(17)/60.05+dydt(12)/88.12+dydt(22)/182.18+dydt(20)/46.07+dydt(25)/17.01);

%change in pH;
%dissociated acids;
dydt(26)=0.325*dydt(24)+1*dydt(13);
dydt(27)=dydt(10)*10^(ph-3.86)/(1+10^(ph-3.86))+y(10)*10^(ph-3.86)*log(10)*dydt(30)/(1+10^(ph-3.86))^2;
dydt(28)=dydt(17)*10^(ph-4.76)/(1+10^(ph-4.76))+y(17)*10^(ph-4.76)*log(10)*dydt(30)/(1+10^(ph-4.76))^2;
dydt(29)=dydt(12)*10^(ph-4.81)/(1+10^(ph-4.81))+y(10)*10^(ph-4.81)*log(10)*dydt(30)/(1+10^(ph-4.81))^2;
%y(27)=y(10)*10^(ph-3.86)/(1+10^(ph-3.86));
%y(28)=y(17)*10^(ph-4.76)/(1+10^(ph-4.76));
%y(29)=y(12)*10^(ph-4.81)/(1+10^(ph-4.81));
%bt, bufer index;
btl=1/90.08*2.303*10^(ph-3.86)/(1+10^(ph-3.86))^2;
bta=1/60.05*2.303*10^(ph-4.76)/(1+10^(ph-4.76))^2;
btb=1/88.12*2.303*10^(ph-4.81)/(1+10^(ph-4.81))^2;
%bth, buffer index of herbage;
if cr==1
  bth=(2.928-0.8612*ph+0.06619*(ph)^2)/1000*d;
elseif cr==2
  bth=(1.504-0.4452*ph+0.03524*(ph)^2)/1000*d;
elseif cr==3
  bth=(2.893-0.8781*ph+0.06952*(ph)^2)/1000*d;
elseif cr==4
  bth=(46.9*exp(-ph)-(0.197-46.9*exp(-ph))*btl*90.08)/1000*d;
```

```
elseif cr==5
  bth=(361.86*exp(-1.63*ph)-(0.0154-222*exp(-1.63*ph))*btl*90.08)/1000*d;
elseif cr==6
  bth=(26.4*exp(-ph)-(0.101-26.4*exp(-ph))*btl*90.8)/1000*d;
elseif cr==7
  bth=(36.2*exp(-ph)-(0.0816-36.2*exp(-ph))*btl*90.8)/1000*d;
elseif cr==8
  bth=(0.7715*10^(ph-4.5)/(1+10^(ph-4.5))^2)/1000*d;
end
bt=y(10)*btl+y(17)*bta+y(12)*btb+(1-y(10)-y(17)-y(12))*bth;
if t<t1+lc+lgl
  dydt(30)=0;
else
  dydt(30)=-1/bt*(dydt(27)/90.08+dydt(28)/60.05+dydt(29)/88.12+dydt(26)/17.01);
end

%proteolysis;
% proteolysis rate, g NPN/g DM/hr;
%miupm, the maximum proteolysis rate;
if c==1 | c==2
  miupm=1.37;
elseif c==3;
  miupm=3.74;
end
%environmental factors;
%fpph, ph;
fpph=0;
if ph<=6  %& ph>=4
  fpph=0.184-0.327*ph+0.0774*(ph)^2-0.0276*(ph-5.22)^3;
elseif ph>6
  fpph=-0.229+0.624*ph-0.069*(ph)^2+0.0887*(ph-6.71)^3;
end
%fpt, temperature;
fpt=exp(19.613-6259.9/T1);  % T<=319;
%fpd, dry matter;
if d>=0.14 & d<=0.55
  fpd=0.066+0.0074*d^(-2.47);
elseif d<0.14
  fpd=1;
end
%fppn, protein nitrogen;
fppn=0;
if y(32)>0.0018
  fppn=(y(32)-0.0018)/((0.0026-0.0018)+(y(32)-0.0018));
elseif y(32)>=0 & y(32)<=0.0018
  fppn=0;
end
```

```
% proteolysis rate;
miup=miupm*fpph*fpt*fpd*fppn;
dydt(31)=miup*d/1000;
dydt(32)=-dydt(31);

%y(2), CO2 change during ensilage process;
if  t<=t1+lgl+lc
   dydt(2)=0;
   elseif t>=t1+lc+lgl & t<t1+lc+lgc
   dydt(2)=dydt(21);
   elseif t>=t1+lc+lgc
   dydt(2)=dydt(21)+(1-0.231)*44/90.08*dydt(18);
end
```

APPENDIX 4, MATLAB FILE FOR STEPLENGH ADJUSTMENT

RKmain.m

```
function [x,y] = RKmain(RKfun,xa,xb,hh,yy,ff)
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Runge-Kutta ODE solution with specific step size                  %
%           RKfun     : First order divergence
%           xa,xb     : Integration range
%           hh                : Step size
%           yy                : Initial value on 'xa'
%           ff                : 0: fixed step size 1: adjustable               %
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
i=1;
h=hh;
dlta=0.01;
xx=xa;
y(:,1)=yy;
flag=ff;
while xb>xx
  if flag==1
    ya=RKstep(RKfun,xx,yy,h,args);
    yb=RKstep(RKfun,xx,yy,h/2,args);
    yb=RKstep(RKfun,xx+h/2,yb,h/2,args);
    dltb=max(abs(yb-ya));
    %If the step is too small, enlarge it till dlt>creteria
    %Don't worry! It will come back.
    while dltb<dlta
      h=h*(dlta/dltb)^0.2;
      ya=RKstep(RKfun,xx,yy,h);
      yb=RKstep(RKfun,xx,yy,h/2);
      yb=RKstep(RKfun,xx+h/2,yb,h/2);
      dltb=max(abs(yb-ya));
    end
    %If too large, correct it
    while dltb>dlta
      h=h*(dlta/dltb)^0.2;
      %Prevent the h from exceeding the range
      %     abs(tm-t) for the last point
      h=min(h,abs(xb-xx));
      ya=RKstep(RKfun,xx,yy,h);
      yb=RKstep(RKfun,xx,yy,h/2);
      yb=RKstep(RKfun,xx+h/2,yb,h/2);
      dltb=max(abs(yb-ya));
    end
    yy=yb;
```

```
    else
      yy=RKstep(RKfun,xx,yy,h);
    end

    i=i+1;
    y(:,i)=yy;
    xx=xx+h;
    x(i)=xx;
end
```

APPENDIX 5, MATLAB FILE FOR STEPLENGH ADJUSTMENT

RKstep

```
function f=RKstep(RKfun,x,y,h)
ka=h*feval(RKfun,x,y);
kb=h*feval(RKfun,x+h/2,y+ka/2);
kc=h*feval(RKfun,x+h/2,y+kb/2);
kd=h*feval(RKfun,x+h,y+kc);
f=y+ka/6+kb/3+kc/3+kd/6;
```

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