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Ammonia Fiber Explosion (AFEX) Treatment of Corn Stover
&
Effects of AFEX Treatment on Plant-Produced Heterologous Cellulase

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AMMONIA FIBER EXPLOSION (AFEX) TREATMENT OF CORN STOVER
&
EFFECTS OF AFEX TREATMENT ON PLANT-PRODUCED HETEROLOGOUS CELLULASE

By

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ABSTRACT

AMMONIA FIBER EXPLOSION (AFEX) TREATMENT OF CORN STOVER
&
EFFECTS OF AFEX TREATMENT ON PLANT-PRODUCED HETEROLOGOUS CELLULASE

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The ammonia fiber explosion (AFEX) process treats lignocellulosic biomass with liquid ammonia under pressure followed by explosive pressure release to enhance conversion of structural carbohydrates (cellulose and hemicellulose) to fermentable sugars. The potential of ammonia fiber explosion (AFEX) treatment for enhancing the enzymatic conversion of lignocellulosic biomass is well recognized. However, optimizing the process conditions and parameters such as ammonia loading, moisture content of biomass, temperature and residence time is necessary for maximum effectiveness of this process. The effectiveness of the AFEX treatment was measured by the extent of enzymatic hydrolysis of the treated corn stover and also by the amount of ethanol produced during simultaneous saccharification and fermentation (SSF) process from these samples. Approximate optimal pretreatment conditions for corn stover were found to be temperature, 90°C; kg of ammonia: kg of dry corn stover, 1:1; moisture content of corn stover, 60% (dry weight basis [dwb]); and residence time (holding at target temperature), 5 minutes. Approximately 97% of the theoretical glucose yield was obtained during enzymatic hydrolysis of the optimal treated corn stover using 60 filter paper unit (FPU) of cellulase enzyme /g of glucan. The ethanol yield of optimally AFEX-treated corn stover has been increased up to 2.2 times over that of an untreated sample.

The cost of enzymes used for saccharification of cellulosic residues is dominant in the overall bioconversion process. One way to decrease this cost is to produce these enzymes in transgenic plants. In an effort to produce heterologous cellulases in transgenic corn plant, a research group from the Crop and Soil Sciences Department (at Michigan State University) and I constructed several different plasmid DNAs to target these enzymes to different compartments (cytosol or chloroplasts) of transgenic corn plants.

We have also investigated the effects of AFEX pretreatment, employing a range of treatment temperatures, moisture contents and ammonia loadings on the activity of plant-produced heterologous cellulase. The plant materials included transgenic tobacco plants expressing E1 (endoglucanase from *Acidothermus cellulolyticus*). The E1 activity was measured in untreated and AFEX-treated tobacco leaves to investigate the effects of the treatment on the activity of this enzyme. The maximum observed percent of retention of activity was about 36% for transgenic tobacco plant treated at 60°C, 0.5:1 ammonia loading ratio, and 60% moisture content (dwb).

DEDICATION

Dedicated to my parents, Rahimeh Arabi and Hossein Teymouri, who have continually encouraged and supported me throughout my entire life. To my husband, Seyed Nasrollah Alavi, whose love, support, encouragement and personal sacrifices help me to succeed in my endeavors. To my son Amir and my daughter Ayda, who are my inspirations.

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Chapter 1

General Introduction

As the human population continues to increase, the worldwide demand for energy and liquid fuels increases even faster. In the United States and much of the word the largest single portion of this energy demand is derived from petroleum. The earth's petroleum supply is finite, and it is becoming less accessible. As a result, many countries such as the United States, which are net importers of petroleum, seek to develop new sources of energy to reduce their dependency on foreign oil and to improve their economic strength. In addition to the economic issues, we are also faced with potentially great environmental consequences if we don't change our energy use patterns.

Utilization of solar energy and extracting energy from the carbon fixed by photosynthesis in plants offer an attractive solution to establish clean and sustainable resources for both energy demands and raw material needs. It has been estimated that 10^{11}-10^{12} tons of carbon are fixed annually around the world by photosynthesis of higher plants. Theoretically, this renewable resource can provide approximately 10 times our current energy demand and 100 times our current food needs (Grohman *et al.*, 1992).
The potential for using lignocellulosic biomass material to produce energy carriers such as electricity, gases, and transportation fuels is well recognized. Biomass energy produced in an efficient and sustainable manner can offer numerous economic, environmental and social benefits compared with fossil fuels.

One liquid fuel that has the potential to match the convenient attributes of petroleum fuels is ethanol produced from lignocellulosic biomass. A major problem in the commercialization of this potential is the inherent resistance of lignocellulosic

materials to conversion to fermentable sugars (Walter, 2000). There are two main techniques for producing fermentable sugars from lignocellulosic biomass acid hydrolysis and enzymatic hydrolysis. The required high temperature for acid hydrolysis results in significant sugar degradation and, hence, low yields. Enzymatic hydrolysis is attractive because of its specificity and absence of the competitive degradation, which result in higher yields (Wyman, 1996). With regard to enzymatic hydrolysis, although the yields are higher, the conversion rates are low and the enzyme is expensive. In order to improve the efficiency of both acid hydrolysis and enzymatic hydrolysis, a pretreatment step is necessary to make the cellulose fraction accessible to cellulases. In most pretreatments, delignification, removal of hemicellulose, and decreasing the crystallinity of cellulose produce more accessible surface area for cellulases to react with cellulose (Lamptey *et al.*,1986).

A major obstacle to the commercialization of enzymatic hydrolysis is the high cost of the enzyme. One way to reduce this cost is to use much less enzyme per unit of biomass hydrolyzed. It has been shown that this goal can be accomplished by optimizing the biomass pretreatment conditions. The Ammonia Fiber Explosion (AFEX) technique is a biomass pretreatment technique that has been shown to be an effective means of increasing the yields of fermentable sugars from biomass. Previous work has shown that the effective enzymatic hydrolysis of AFEX-treated biomass at enzyme loadings as low as 5 FPU/g of dry biomass can be achieved by adjusting the pretreatment parameters (Holtzapple *et al.*, 1991).

In the present research one of our foci was to optimize the AFEX treatment variables such as temperature, moisture content, ammonia loading, and residence time to

increase the efficiency of the enzymatic hydrolysis of AFEX-treated corn stover (includes all above ground portions of a corn plant except the grain and cob) at lower enzyme loading.

Another promising approach to reduce the enzyme cost is to genetically transform plants with the genes coding for these enzymes, in order to produce the desired enzymes. Transgenic plants may be an attractive and cost-effective alternative to microbial systems for production of biomolecules. Advances in biotechnology are enabling plants to be exploited as bioreactors for the production of proteins, carbohydrates, lipids, and industrial enzymes (Halliwell and Halliwell, 1995). Field-grown plants have the potential to produce biomolecules such as industrial enzymes in bulk quantities with minimal inputs of raw materials and energy (Verwoerd *et al.*, 1994). In collaboration with a research group from the Crop and Soil Sciences Department at Michigan State University, with the concept of producing cellulase in transgenic corn plant, three different DNA plasmids containing cellulase genes were constructed.

Plant proteins and enzymes produced in transgenic crops have very high value, therefore they may provide a significant byproduct credit in biomass refining. Extraction and recovery of these proteins in their active form or even releasing active cellulase from the plants during bioconversion could have a significant impact on overall lignocellulose conversion economics. As this technology continues to grow and improve the production levels of biomolecules in plants, the development of downstream processing technology to extract and to recover these biochemicals will determine progress. Therefore an integrated pretreatment that improves protein recovery from biomass and increases the digestibility of lignocellulosic biomass may significantly enhance the process economics.

Many pretreatments that increase the conversion of cellulose to fermentable sugars operate under harsh conditions (e.g., steam explosion and various acid processes) that tend to degrade the sugars, biomass proteins, and enzymes. However the AFEX process operates under relatively mild conditions and has shown a good potential for protein recovery from herbaceous crops with simultaneous conversion of cellulose and hemicellulose to simple sugars. (De La Rosa *et al.*, 1994)

The potential of the AFEX process in releasing active cellulase from transgenic plants during the bioconversion of the transgenic plants was examined in this research. Transgenic tobacco plants expressing cellulase E1 (endoglucanase from *Acidothermus cellulolyticus*) were used for this investigation.

The potential of the AFEX process as a biomass treatment technique that is able to preserve the biomass proteins in their bioactive form was explored in this study. We approached this goal by investigating the effects of the AFEX process on the activity of Rubisco, the most abundant plant protein in alfalfa plants.

The primary objectives of this research may be summarized as follows:

1) Investigate interplay between AFEX treatment conditions such as temperature, moisture content, ammonia loading, and residence time and their effects on the AFEX results.

2) Analyze AFEX-treated corn stover and evaluate the effects of the treatment on the enzymatic conversion of the samples to fermentable sugars as well as amount of ethanol produced during Simultaneous Saccharification and Fermentation (SSF) process.

3) Optimize AFEX treatment conditions for efficient conversion of corn stover to fermentable sugars.

4) Construct a series of DNA plasmids containing cellulase genes to be used by another research group to produce transgenic plants expressing these genes.

5) Investigate the effects of the AFEX treatment on the activity of plant-produced proteins such as Rubisco and cellulase.

Chapter 2

Literature Review

The increase in the price of petroleum feedstock has created opportunities for the development of combined biological and chemical processes that will produce liquid fuels and chemicals from alternate feedstock such as biomass. The term "biomass" generally refers to renewable organic material generated by plants through photosynthesis. Lignocellulosic biomass materials made up of three major organic fractions cellulose, hemicellulose and lignin. Cellulose and hemicellulose together compose about 65-75% of the overall biomass composition and significant portion of the remaining material is lignin (Wyman, 1996).

Cellulose is the world's most plentiful naturally occurring organic compound. Cellulose is an insoluble, unbranched, and linear polymer that is composed of many D-glucose subunits, linked end to end with β-1-4 glucosidic bonds (Figure 2.1). The general formula for cellulose is $(C_6H_{10}O_5)_n$ where (n) is the number of glucose units in the polymer molecule.

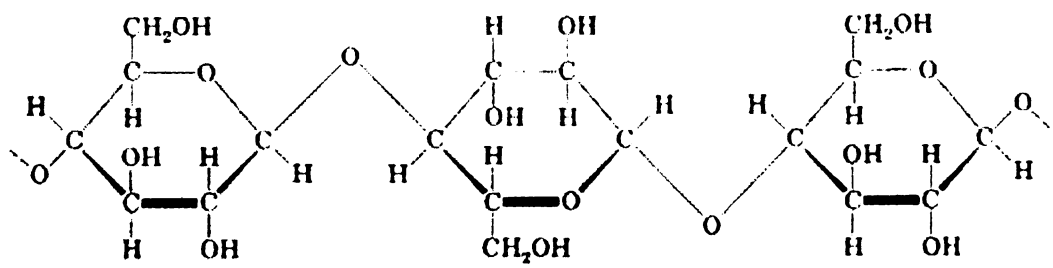


Figure 2.1: Cellulose structure

Cellulose chains are held together by hydrogen bonding and van der Waals forces, and they associate to form insoluble elementary fibrils that further aggregate to form microfibrils about 25nm wide (Campbell *et al.*,1999). These bonds and forces aggregate portions of the molecular chains into various degrees of lateral order ranging from perfect geometrical packing of the crystal lattice (crystalline region) to a random condition (amorphous region). The degree of crystallinity controls the chemical reactivity of cellulose; the crystalline regions are essentially not accessible to reactant molecules. However, crystallinity alone is not responsible for unreactive nature of cellulose. Cellulose microfibrils are surrounded by layers of lignin and hemicellulose, which also protect cellulose from degradation (Wyman, 1996).

Hemicellulose is another major class of cell wall matrix polysaccharides. Hemicelluloses can be subdivided into several groups on the basis of monosaccharide composition and ratio: 1) arabinans; 2) xylans (homo- and heteropolysaccharides including arabinoxylans, glucuronoxylans, arabinoglucuronoxylans); 3) glucans (homopolysaccharides includind β-(1-3)-D-glucan callose, and heteropolysaccharides such as arabinoglucans); 4) galactans (both homo- and heteropolysaccharides, e.g. arabinoglactans); 5) mannans (homo- heteropolysaccharides represented by glucomannans, galactomannas, and glucogalactomannanas); 6) fructosans and glucofructosans; 7) galacturonans, and 8) mannuronans (Tarchevsky and Marchenko, 1991). The main hemicellulosic component of monocotyledonous and dicotyledonous plants is represented by xylans with a back bone built of β-(1-4)-linked D-xylose residues bearing branches such as D-glucuronic acid, glucose, arabinose, and galactose (some of these sugars are presented in Figure 2.2). Unlike cellulose, which is a crystalline strong,

and resistant to hydrolysis, hemicellulose has an amorphous structure with little physical strength. It can be hydrolyzed easily by hemicellulases and mild acid to produce simple sugars (Tarchevsky and Marchenko, 1991).

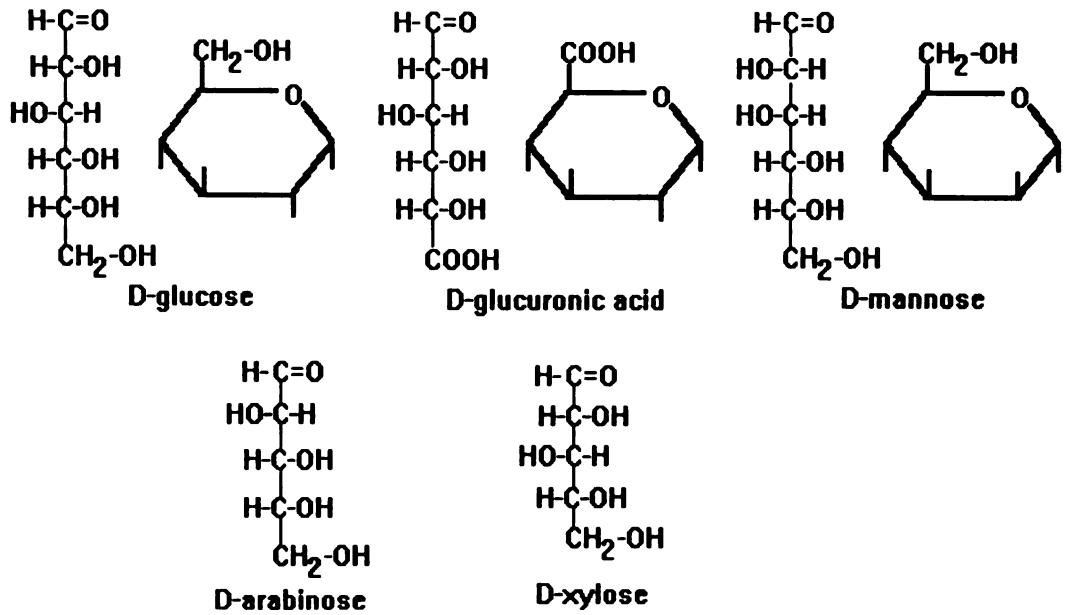


Figure 2.2. Some monomers of hemicellulose

Lignin is essentially a three-dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon –carbon bonds. It possesses a high molecular weight, and it is amorphous in nature. Lignin, hemicellulose and cellulose with extensive cross linking among them comprise the very rigid cell wall matrix of the plant (Tarchevsky and Marchenko, 1991).

The hydrolysis of polysaccharides in plant cell walls is far more challenging than hydrolysis of storage polysaccharides (starch). Due to intimate and strong associations with hemicellulose and lignin, cellulose cannot be liberated by simple mechanical treatments used successfully on starch granules.

Biomass Pretreatments

A key aspect of the overall conversion system for biological fuel production is the integration of plant substrates and pretreatments to provide easily hydrolysable cellulose. These treatments usually cause the destruction or severe modification of hemicelluloses and lignin. In most of these treatments the plant tissues are first converted into smaller particles to aid penetration of acidic and enzymatic catalysts. Chipping, shredding and milling steps are common to most treatments. Acid-catalyzed hydrolysis processes are more tolerant of larger particles because small acid molecules diffuse through cell walls and plant tissues much more rapidly than the relatively large enzyme molecules. However, even in acid hydrolysis, the use of large particles will decrease the yield of sugars (Torget *et al.*, 1988).

For enzymatic hydrolysis, the minimal pretreatment requirement is the production of fine particles with a high surface area accessible to cellulase enzymes. Small particles can be produced by explosive decompression of steam or gases, but to penetrate cell walls and to make cellulose surfaces more accessible, we also need to modify other components in plant cell walls such as lignin and hemicellulose, which are recognized as major barriers to enzymatic hydrolysis (Grohmann *et al.*, 1992).

There are three major classes of biomass treatments, which are described below.

Physical Treatment

The most common physical treatment is ball milling. This technique provides a dramatic increase in the rates of hydrolysis and in the yields of fermentable sugars. Shearing and compressive forces involved in milling reduce crystallinity, decrease the mean degree of polymerization, and increase surface area (Abraham and Kurup, 1997).

But this physical treatment demands expensive electrical energy, which is a major drawback for this technique (Holtzapple *et al.*, 1991).

Chemical Treatment

Chemical treatment with strong acids or bases also effectively increases the hydrolysis of cellulose. These chemicals are generally quite corrosive and expensive. In addition, they are often toxic or inhibitory to microorganisms, so that they have to be removed. These problems increase the expense and difficulty of such chemical treatments (Schell and Duff, 1996).

Physicochemical Treatment

In physicochemical treatment both physical and chemical aspects are involved. This technique has the advantage of physical treatment without the expense of high energy use in ball milling. The two common examples of physicochemical treatment are steam explosion and ammonia fiber explosion (AFEX).

In steam explosion, lignocellulosic materials are saturated with water under pressure (300-500 psi) at elevated temperature (215-260°C). When the pressure is released, the water evaporates rapidly and makes the cellulose fibers separate, which in turn increases the surface area for subsequent hydrolysis. Even though the technique is effective, it demands considerable thermal energy. In addition, due to the high temperatures, some of the sugars are degraded (Carrasco *et al.*, 1994).

The ammonia fiber explosion process (AFEX) treats lignocellulosic biomass materials with liquid anhydrous ammonia under pressure and moderate temperatures (60-90°C) for a few minutes and then the pressure is rapidly released. The rapid

decompression expands the fiber structure of the biomass and significantly increases the surface area available for enzymatic attack (Dale and Moreira, 1983).

This pretreatment has been shown to greatly enhance the susceptibility of biomass to enzymatic hydrolysis. The mechanism by which the enhancement occurs has not been elucidated yet, but presumably involves changes in physical and chemical structure. It is known that anhydrous ammonia in either liquid or gaseous form is a strong cellulose swelling agent which can change the cellulose fiber structure from crystalline cellulose I (native crystalline cellulose) to crystalline cellulose III (ammonia-treated cellulose). Anhydrous ammonia penetrates cellulose and reacts with the hydroxyl groups after breaking the hydrogen bonds (Barry *et al.*, 1936). Through its penetration in to the crystallites the distances between the cellulose chains increases. The cellulose III crystallites obtained are smaller than those of cellulose I, 16-23Å as against 50-80Å (Lewin and Roldan, 1971). Ammonia can react with the lignocellulosic biomass by ammonolysis of the ester crosslinks of uronic acid with the xylan units, cleaving the bonds linking hemicellulose and lignin, and cleaving the C-O and C-C bonds of lignin macromolecules to produce smaller soluble fragments (Chou, 1986).

Cellulose Hydrolysis

Bioethanol can be produced by converting the complex cellulosic and hemicellulosic polysaccharides into simple sugars, which can be fermented to ethanol by various microorganisms. Complex polysaccharides can be hydrolyzed by acid or enzymes.

Dilute acid hydrolysis is inexpensive and relatively simple, but the high temperatures, ranging from 140°C to 260°C, may result in the formation of sugar

degradation products. Concentrated acids also can be used to hydrolyze the lignocellulosic material and, since the temperature is lower (100- 120°C), high sugars yields are obtained with little degradation of products. However concentrated acid hydrolysis requires later neutralization of the acid solution and recovery of the acid, both of which are expensive processes (Schell and Duff, 1996). On the other hand, enzymatic hydrolysis of cellulose is attractive because of its specificity and absence of competitive degradation, which results in high yield of sugars.

Cellulase refers to a family of enzymes that act synergistically to hydrolyze cellulose to glucose. Cellulases are widely distributed throughout the biosphere and are most manifest in fungal and microbial organisms. Enzymatic degradation of cellulose to glucose is generally accomplished by the synergistic action of three distinct classes of cellulase enzymes: Endo-1,4-β-glucanases (EC 3.2.1.4), Exo-1,4-β-D-glucanases or 1,4-β-D-glucan cellobiohydrolases (EC3.2.1.91), and β-D-glucosidases (EC 3.2.1.21) (Wilke *et al.*, 1983).

The rate and the extent of enzymatic conversion of biomass are limited, by end-product inhibition of cellulase by glucose and cellobiose formed during the breakdown of cellulose. Several methods have been proposed to overcome this inhibitory problem. These include the use of high concentration of enzymes (Ishihara *et al.*, 1991), the elimination of sugars from the hydrolysate by ultrafiltration (Tan *et al.*, 1986), or the simultaneous saccharification and fermentation (SSF) of the substrate (Saddler *et al.*, 1982), and supplementation of cellulases with β-glucosidase that converts cellobiose into glucose (Wyman *et al.*, 1986, Spindler *et al.*, 1988, Spinder *et al.*, 1992; Breuil *et al.*, 1990). It has been shown that both the endoglucanases and cellobiohydrolases are

inhibited by increased concentrations of cellobiose, whereas β-glucosidase is more sensitive to glucose accumulation. Although the glucanases are also inhibited by glucose, the drop in the overall enzyme activity of the system due to glucose accumulation is still relatively small compared with that associated with cellobiose accumulation (Breuil *et al.*, 1990; Holtzapple *et al.*, 1990).

Furthermore, the accumulation of glucose was minimized by using the simultaneous saccharification and fermentation (SSF) process, developed by the Gulf Oil Corporation (Emert and Ratzen 1980). In this process the glucose becomes simply an intermediate, which lowers the glucose concentration than in any one-stage saccharification process, provides less inhibition to the rate of conversion of the cellulose (Wyman, 1999).

Fermentation of sugars to ethanol

Simple sugars such as glucose can be fermented to ethanol using yeasts and other microbes. The most common yeast used in the fermentation of glucose is *Saccharomyces cerevisiae*. The stoichiometric equation of glucose fermentation to ethanol is:

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$$

Usually the fermentation proceed until the ethanol concentration reaches ~10%-12%, when the activity of the yeast becomes inhibited by the ethanol. In practice, the ethanol yield from sugar is about 46% by weight; as opposed to the 51.1% theoretical yield (Wyman, 1999).

The development of simultaneous saccharification and fermentation (SSF) was a major breakthrough in cellulose utilization. In this process hydrolysis and fermentation are combined in one vessel (Walter, 2000). The presence of yeast along with the enzyme minimizes the inhibitory effects of sugars. The sugars produced during the process slow down the action of cellulase enzyme, which in turn lowers the ethanol production. Additional benefits are that the number of fermentation vessels is reduced, which lowers the capital cost of the process. Furthermore the presence of ethanol makes the mixture less vulnerable to contamination by unwanted microorganisms.

Biomass pretreatment in livestock producing industry

High protein crops such as alfalfa and other forages (which contain 10%-20% protein) are particularly important in the livestock industry (De La Rosa *et al.*, 1994). Feed-grade protein is worth four times more per unit weight than carbohydrates; therefore, any effort to increase the digestibility of these feeds and at the same time

preserve their protein in their useable form might significantly improve the economics of livestock production. Many biomass pretreatments have been used to improve feed quality by increasing the digestibility of the feed. Unfortunately, most of these pretreatments use relatively high temperatures, which are detrimental to feed grade protein in biomass.

Ammonia fiber explosion treatment has shown great potential for improving the digestibility of biomass by ruminants (Weaver, 1998, Gollapalli, 2001). In addition to enhancing substrate digestibility, the ammonia bound to the biomass during the reaction can serve as a source of nitrogen for the animal. Only ruminants can use non-protein nitrogen as a source of nitrogen for synthesis of protein, therefore less protein needs to be added to the animal's diet. AFEX treatment has also shown potential to be used as an integrated process that allows more protein extraction from biomass as well as increasing the digestibility of biomass compared to the untreated biomass. The quality of biomass feed will be significantly improved if the biomass proteins are preserved in useable form after the treatment.

Chapter 3

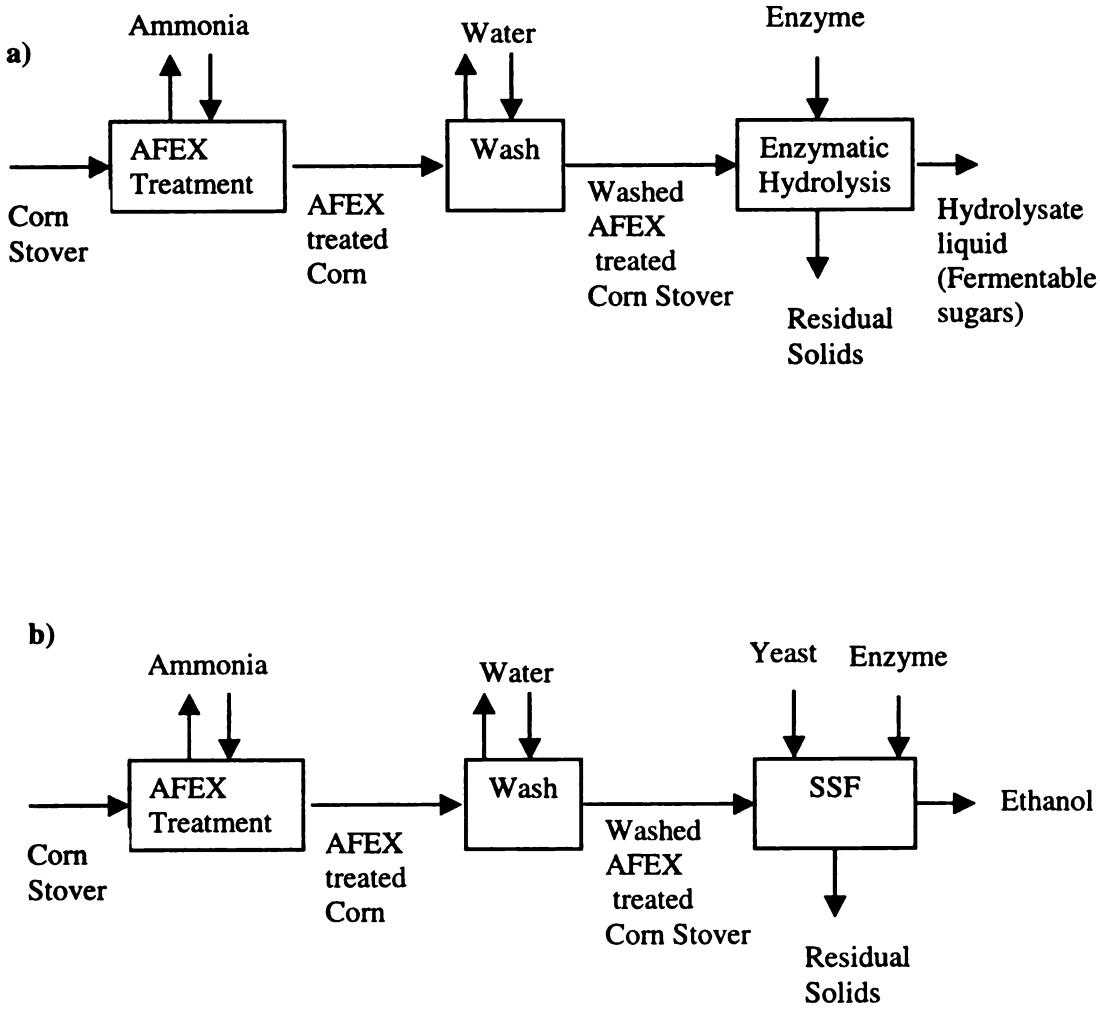
Ammonia Fiber Explosion of Corn Stover

Abstract

The potential of ammonia fiber explosion (AFEX) treatment for enhancing the enzymatic conversion of lignocellulosic biomass is well recognized. However, optimizing the process conditions and parameters such as ammonia loading, moisture content of biomass, temperature, and residence time are necessary for maximum effectiveness of this process.

In this research our focus was to identify the AFEX process conditions to maximize the enzymatic conversion of corn stover to fermentable sugars, which in turn would result in higher ethanol production. The effectiveness of the AFEX treatment was measured by the extent of enzymatic hydrolysis of the treated corn stover and also by the amount of ethanol produced during simultaneous saccharification and fermentation (SSF) from these samples. The stepwise schematic diagrams of these processes are illustrated in Figure 3.1 (a & b).

Figure 3.1. Stepwise schematic diagram of the process



Ammonia Fiber Explosion treatment

Following materials were used in this experiment:

Liquid anhydrous ammonia from AGA (Lansing, MI)

Dried and milled corn stover (with 10% moisture): provided by National Renewable Energy Laboratory (Golden, CO) (NREL).

Throughout this report ammonia to biomass ratio is presented as g of ammonia: g of dry biomass, and moisture content is presented as g of moisture/g of dry biomass (dry weight basis [dwb]).

Ammonia hazards

Ammonia as gas or liquid is highly soluble in water. The reaction between ammonia and water generates ammonia hydroxide, a caustic compound. This makes ammonia a contact hazard, as well as inhalation hazard. The gas or liquid can be absorbed by moisture of skin or eyes and cause caustic burns. Anhydrous liquid ammonia is also dangerous because of the high heat of vaporization, which may lead to skin freezing (Davis, 1987; Kirk-Othmer, 1992). Therefore all the AFEX treatments were carried out in fume hood, and safety glasses and heavy-duty gloves were worn during the experiment.

Description of the apparatus

A 300 ml stainless steel pressure vessel (PARR Instrument Co., IL), equipped with pressure gauge, feeding port, and exhaust ball valve was used as the reactor in this experiment. However, in order to have better control on the system, several modifications were made. To uniformly distribute ammonia in the vessel, three holes 1/16 inch of

diameter, one inch apart were drilled in the feeding tube. Another modification was to install a thermocouple well to monitor the temperature during the experiment. The well is located one half inch from the bottom of the vessel, between the center and the inner wall of the vessel. The arrangement of the feeding port, exhaust valve, and thermocouple on the lid, the whole assembly of AFEX unit, and the schematic diagram of the laboratory AFEX apparatus are shown in Figure 3.2, Figure 3.3, and Figure 3.4 respectively.

In order to load the vessel with the desired amount of ammonia, three precalibrated sample cylinders with different capacities (10, 20, and 30g of ammonia) were made (Figure 3.5). The anhydrous ammonia used was supplied in a 50lb cylinder equipped with a dip tube to ensure liquid delivery. In order to reduce the heating time, the liquid ammonia was preheated before loading. For this purpose a lecture bottle with 300g ammonia capacity, equipped with a dip tube, was filled from the main ammonia cylinder (50lb cylinder) and maintained at about 35°C in a water bath.

Figure 3.2. Detail design of AFEX vessel's lid

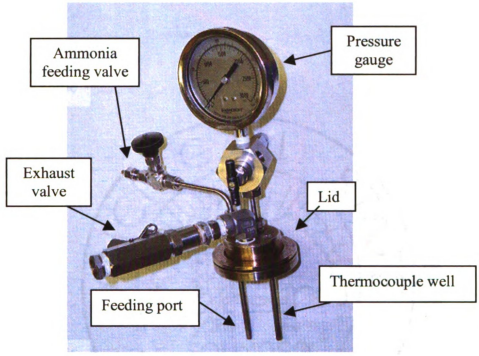


Figure 3.3. Whole assembly of AFEX unit.

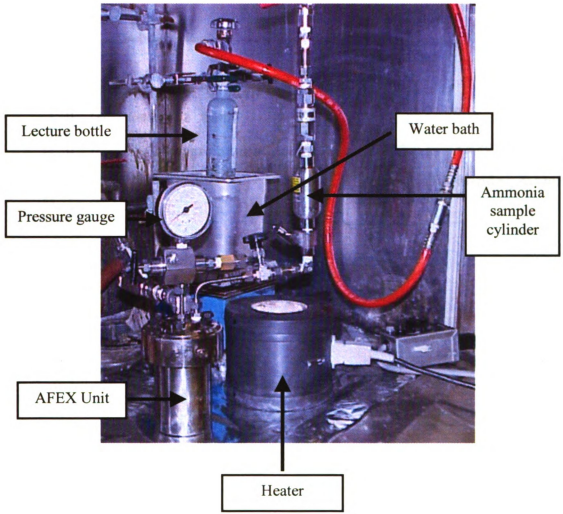


Figure 3.4. Schematic diagram of laboratory AFEX apparatus

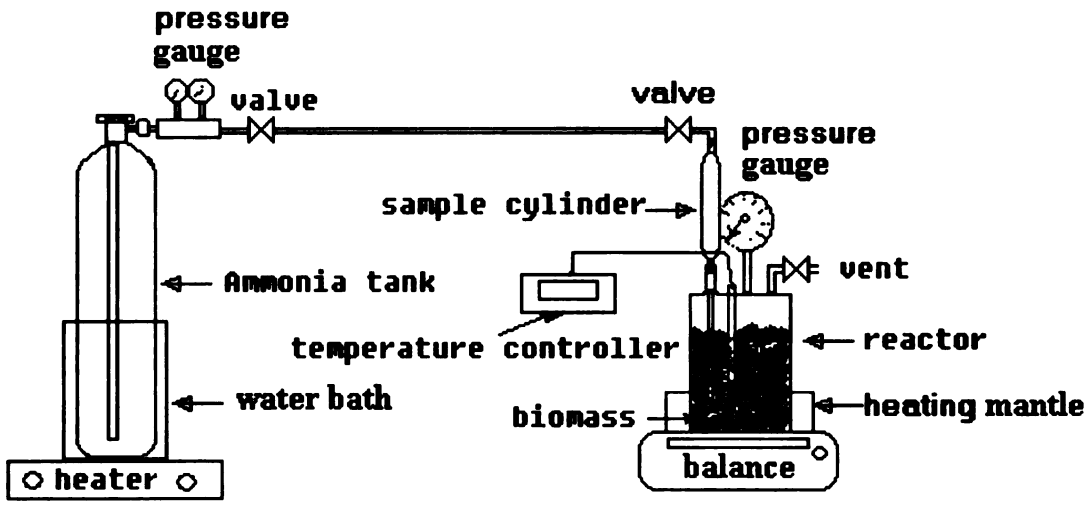
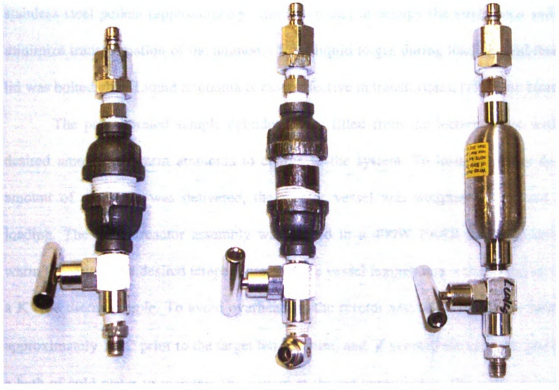


Figure 3.5. Ammonia sample cylinders.



Capacity: 10g of Ammonia Capacity: 20g of Ammonia Capacity: 30g of Ammonia

Experiment Procedure

To carry out the experiment, corn stover samples were moistened with distilled water to the desired moisture content and allowed to equilibrate for 30 minutes. The prewetted samples were placed in the pressure vessel. The vessel was topped up with stainless steel pellets (approximately 1mm diameter) to occupy the void space and thus minimize transformation of the ammonia from liquid to gas during loading, and then the lid was bolted shut. Liquid ammonia is most effective in transforming cellulosic biomass.

The precalibrated sample cylinders were filled from the lecture bottle with the desired amount of warm ammonia to charge to the system. To insure that the desired amount of ammonia was delivered, the reactor vessel was weighed before and after loading. The entire reactor assembly was placed in a 400W PARR heating mantle to warm the unit to the desired temperature, and the vessel temperature was monitored using a K type thermocouple. To avoid overheating, the reactor was taken out of the heater at approximately 10°C prior to the target temperature, and, if needed, the unit was placed in a bath of cold water to maintain the system at the set temperature. The pressure and the temperature were monitored and recorded throughout the experiment. After the experiment was completed the exhaust valve was rapidly opened to relieve the pressure and accomplish the explosion. The treated corn stover samples were removed and allowed to stand overnight in a fume hood to evaporate the residual ammonia. An example of an AFEX run recipe and the conditions during the run (temperature and pressure) is presented in Table 3.1.

Table 3.1. Conditions during an AFEX run

18 g of Untreated corn stover (containing 1.8 g of water)			
7.8 g of distilled water was added for 60% moisture (dry weight basis)			
16.5 g of liquid ammonia for 1:1 ammonia loading ratio; Ammonia temperature: 35°C			
Elapsed time, min.	Temperature, °C	Pressure, psig	Comment
0	50	90	Put in the heater
2	54	120	
4	58	150	
6	67	180	
8	74	205	
10	78	225	
12	80	240	Taken out of the
14	83	255	
16	86	260	
18	88	255	
20	91	245	
22	91	220	
23	91	195	Pressure released

Treatment of the corn stover with ammonia resulted in a considerable darkening of corn stover compare with untreated sample, but did not change the macroscopic appearance of the substrate (Figure 3.6). The treated samples were stored in sealed plastic bags in the refrigerator.

Figure 3.6. Physical appearance of AFEX treated and untreated corn stover.

The corn stover samples were treated under different process variables (temperature, moisture content, ammonia:biomass level, and time) to investigate interplay between AFEX treatment conditions and to optimize this treatment for corn stover. The various treatment conditions in AFEX pretreatment of the corn stover are summarized in Table 3.2.

Table 3.2. AFEX treatment conditions in corn stover treatment*

(g of NH_3: g of dry Biomass)	20% Moisture**	40% Moisture	60% Moisture
0.7:1	T=60°C	T=60°C	T=60°C
	T=70°C	T=70°C	T=70°C
	T=80°C	T=80°C	T=80°C
	T=90°C	T=90°C	T=90°C
1:1	T=60°C	T=60°C	T=60°C
	T=70°C	T=70°C	T=70°C
	T=80°C	T=80°C	T=80°C
	T=90°C	T=90°C	T=90°C
1.3:1	T=60°C	T=60°C	T=60°C
	T=70°C	T=70°C	T=70°C
	T=80°C	T=80°C	T=80°C
	T=90°C	T=90°C	T=90°C

* All run were kept at the target temperature for 5 min.

** Moisture content was calculated on dry weight basis.

Enzymatic Hydrolysis of AFEX-Treated Samples

Following materials were used:

Untreated corn stover as control (from NREL)

AFEX-treated corn stover

Cellulase enzyme (provided by NREL, CAS 9012-548, activity: 28 FPU/ml; the activity was measured based on NREL standard filter paper unit assay protocol, LAP-006)

β-glucosidase (Novozym 188) from Sigma (St. Louis, MO)

The following chemicals were provided from Sigma:

α-cellulose (Cat. # C-8002), tetracycline, cycloheximide, sodium citrate and pure sugars: glucose, xylose, mannose, galactose, cellobiose and arabinose.

Method

In this series of experiments we essentially followed the NREL standard biomass enzymatic hydrolysis protocol (LAP-009). All the NREL standard protocols can be obtained from the following website:

http://www.ott.doe.gov/biofuels/analytical_methods.html

Experimental Procedure

The pretreated corn stover along with the untreated sample was washed to remove glucose and inhibitors. The samples were squeezed through miracloth with 22-25μm pore size (Calbiochem, La Jalla, Ca), and then the wash water was subjected to sugar analysis using high performance liquid chromatography (HPLC). The samples must be washed until the glucose concentration in the water wash falls below 0.1 g/l. For all the samples the concentration of glucose and other monosaccharides (xylose, mannose,

galactose, and arabinose) in the wash water were either zero or less than 0.1 g/l after the first wash, which indicates that no monosaccharides are produced during the AFEX treatment. The water wash was kept in the freezer for further enzymatic hydrolysis to check for oligomers. The washed samples were stored in Petri dish (sealed with paraffin film) at 4°C for further analysis.

Total solids of the washed samples were determined according to NREL protocol (LAP-001). One gram of washed samples was dried at 105°C overnight; the percent of total solids on 105°C dry weight basis was calculated as follows (equation 3.1):

$$\% \text{ Total solids} = \frac{\text{weight of dried sample}}{\text{weight of sample before drying}} X100 \qquad \textbf{(3.1)}$$

Based on the glucan content of the corn stover (the composition of the corn stover sample was provided to us by NREL, Table 3.3), a sample equivalent to 0.25g of glucan on a 105°C dry weight basis was weighed and added to the 25ml glass vial. To each vial an appropriate amount of sodium citrate buffer, tetracycline and cycloheximide were added. An amount of distilled water was added to bring the total volume in each vial to 25ml after addition of the enzymes in the following steps. The solution and the biomass are assumed to have a specific gravity of 1.00g/ml. An example of an enzymatic hydrolysis recipe and related calculations are presented in Table 3.4.

The contents of the vials were warmed to 50°C in the incubator. Then the appropriate volume of cellulase equal to 15 or 60 FPU/g of glucan and the appropriate

volume of β-glucosidase equal to 40 IU/g of glucan were added to each vial. The vials were incubated at 50°C with gentle rotation (75 rpm) for a period of 168 hr.

Table 3.3. Composition of the Corn Stover (provided by NREL)

Component	Percentage (based on dry basis)
Glucan	36.1
Xylan	21.4
Arabinan	3.5
Mannan	1.8
Galactan	2.5
Lignin	17.2
Protein	4.0
Acetyl	3.2
Ash	7.1
Uronic Acid (est)	3.6
Non-structural Sugars	1.2
Total	101.6

Table 3.4. An example of an enzymatic hydrolysis recipe and related calculations

Washed sample (wet)	1.0482 g
Dried sample at 105°C	0.2972 g
Solid content	28.37 %
Sample loaded in vial for 0.25 g of glucan	2.44 g
Sodium citrate buffer (0.1M, pH 4.8)	1.25 ml
Tetracycline (10 mg/ml in 70% ethanol)	0.1 ml
Cycloheximide (10 mg/ml in distilled water)	0.075 ml
Cellulase (28 FPU/ml)	0.535 ml for 60 FPU or 0.134 ml for 15 FPU/g of glucan
β-glucosidase	0.05 ml for 40 IU/ g of glucan
Distilled water	20.95 ml
Total	25 ml

A 1ml aliquot was removed at each predetermined time interval (0, 3, 6, 24, 48, 72, and 168hr). The samples were centrifuged; the supernatants were filtered and subjected to sugars analysis using HPLC.

High Performance Liquid Chromatography (HPLC)

The sugars were analyzed in a BioRad (Richmond, CA) High Performance Liquid Chromatograph using an Aminex HPX87P column (HPLC Carbohydrates Analysis Column) at 85°C and a BioRad Deashing Cartridge as a guard column. The mobile phase used was degassed HPLC water at a flow rate of 0.6 ml/min. The injection volume used was 20 µL and the run time was 20 minutes.

Standard solutions of pure sugars: glucose, xylose, mannose, galactose, cellobiose and arabinose were individually run on the HPLC to determine their retention time and calibrate the system (calibration curves are presented in Appendix B).

Results and Discussion

To optimize the AFEX treatment conditions for maximum enzymatic conversion of corn stover, the corn stover samples were treated at different process conditions (e.g., temperature, moisture content, ammonia loading ratio, and residence time).

The effects of each variable on the conversion of glucan to glucose and xylan to xylose through enzymatic hydrolysis are illustrated in the following figures. All enzymatic hydrolysis experiments were performed in duplicate. The difference within each duplicate was from 0.2% to 6% (observed in 30 runs). In the results the glucan conversion to glucose and xylan conversion to xylose have been calculated as follows (equation 3.2 and 3.3):

$$\%\ \text{Glucan Conversion} = \frac{[\text{Glucose}] + 1.053[\text{Cellobiose}]}{1.111 f_g [\text{Biomass}]} \text{X}100\% \qquad \textbf{(3.2)}$$

$$\% \text{ Xylan Conversion} = \frac{[\text{Xylose}]}{1.136 f_x [\text{Biomass}]} \text{X}100\% \qquad \textbf{(3.3)}$$

where:

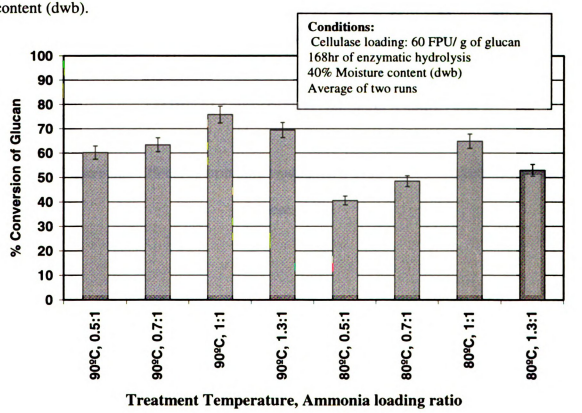
[Glucose]	glucose concentration in hydrolysates (g/L)
[Xylose]	xylose concentration in hydrolysates (g/L)
[Cellobiose]	cellobiose concentration in hydrolysates (g/L)
[Biomass]	Dry biomass concentration at the beginning of the fermentation (g/L)
f_g	Glucan fraction in dry biomass (g/g)
f_x	Xylan fraction in dry biomass (g/g)
1.111	Converts glucan to equivalent glucose
1.136	Converts xylan to equivalent xylose

Effects of ammonia to biomass ratio on the enzymatic hydrolysis of AFEX treated corn stover

Figure 3.7 and Figure 3.8 show the effect of ammonia to biomass ratio (0.5:1, 0.7:1, 1:1, and 1.3:1 g of NH_3:g of dry biomass) on the subsequent enzymatic hydrolysis of AFEX-treated corn stover at two temperatures (80 and 90°C) with different moisture contents (40% and 60% dwb). Glucan conversion increased with increasing ammonia loading at any temperature and moisture content and attained a maximum value at a mass ratio of 1:1 (ammonia:dry biomass). Xylan conversion also showed the same trend as glucan conversion in response to ammonia loading (Figure 3.9). Ammonia at this loading ratio provided maximum overall enhancement of reactivity during pretreatment. It is

known that ammonia can react with lignocellulose by ammonolysis of the ester crosslinks of some uronic acids with the xylan units and cleaving the bond linkages between hemicellulose and lignin (Wang *et al.*, 1967). However it is evident from Figures 3.7, 3.8, and 3.9 that further increases in ammonia loading decreased glucan conversion. It is possible that extra liquid ammonia plasticizes the cellulose and thereby reduces the disruptive effect of the pressure release (O'Conner, 1972). Based on these observations the ammonia ratio 1:1 is the optimum ammonia loading ratio for AFEX treatment of corn stover. Previous work also recognized the same ammonia ratio as the optimum ratio for AFEX treatment of corn fiber (Moniruzzaman *et al.*, 1997).

Figure 3.7. Effects of ammonia loading on glucan conversion at 40% moisture content (dwb).



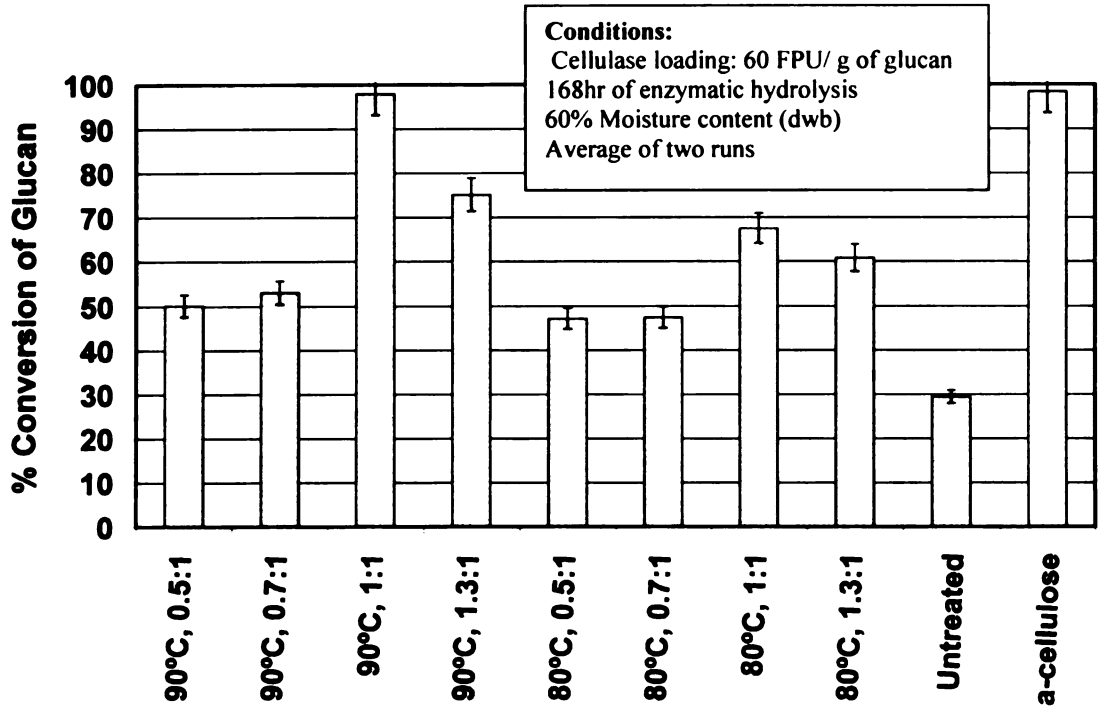
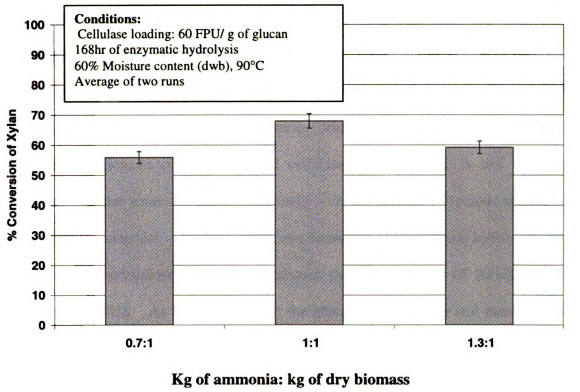


Figure 3.8. Effects of ammonia loading on glucan conversion at 60% moisture content (dwb).

Figure 3.9. Effects of ammonia loading on the xylan conversion at 60% moisture content (dwb).



Effects of moisture content on the enzymatic hydrolysis of AFEX treated corn stover

Effects of different moisture contents (20%, 40% and 60% dwb) on the subsequent enzymatic hydrolysis (after 168hr) are presented in Figure 3.10. Glucan conversion increased with increasing moisture content at any temperature. Even though at higher moisture contents ammonia is more diluted, but apparently the affinity of ammonia for biomass components (e.g., cellulose, hemicellulose) is still sufficiently strong so that the ammonia reacts adequately with the biomass. Previous studies have shown that the moisture in the biomass allows formation of ammonium hydroxide, which hydrolyzed hemicellulose and thereby enhance the overall effect of AFEX treatment (Dale *et al.*, 1985). As these data show, the glucan conversion is still increasing with moisture content so with this series of run we were not able to identify the optimum moisture content for AFEX treatment of corn stover. Therefore another set of the AFEX runs of corn stover with higher moisture contents were conducted. The results of these runs are presented later in this chapter.

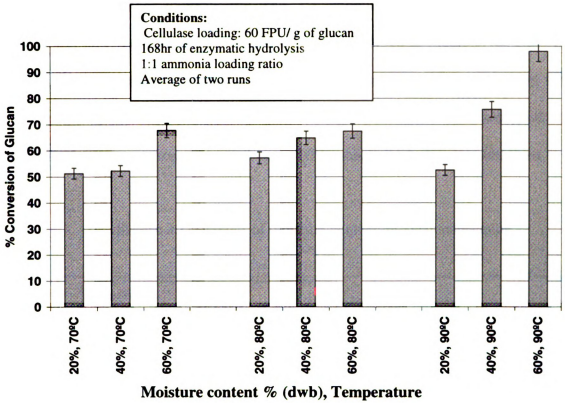
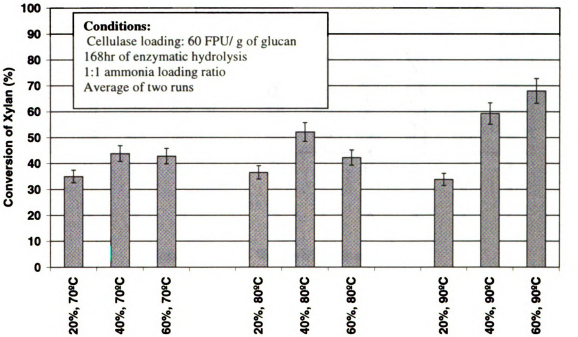


Figure 3.10. Effects of moisture content on glucan conversion.

Figure 3.11 summarizes the effects of different moisture contents on xylan conversion at different temperatures with 1:1 ammonia loading ratio. At a 90°C temperature, this figure shows the same trend as we observed in Figure 3.10. The xylan conversion was enhanced with increasing moisture content from 20, to 40, and to 60%. As these data demonstrate, at 70 and 80°C the xylan conversion was increased with increasing the moisture from 20 to 40% but at 60% moisture a decline was observed.

Figure 3.11. Effects of moisture content on xylan conversion



Effects of temperature on the enzymatic hydrolysis of AFEX treated corn stover

The effect of temperature on glucan conversion was explored at different ammonia loading levels and different moisture contents. The results are illustrated in Figure 3.12, 3.13, and 3.14.

Figure 3.12. Effect of temperature on glucan conversion at 0.7:1 ammonia loading

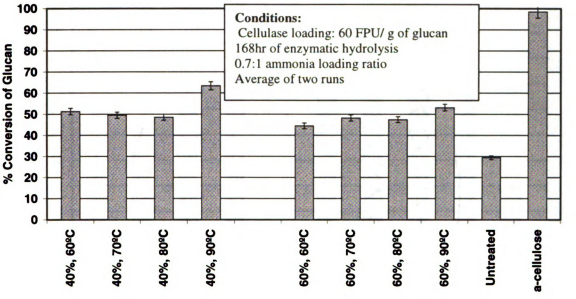


Figure 3.13. Effect of temperature on glucan conversion at 1:1 ammonia loading.

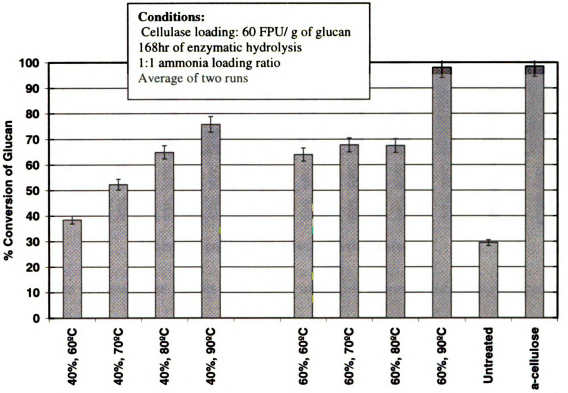
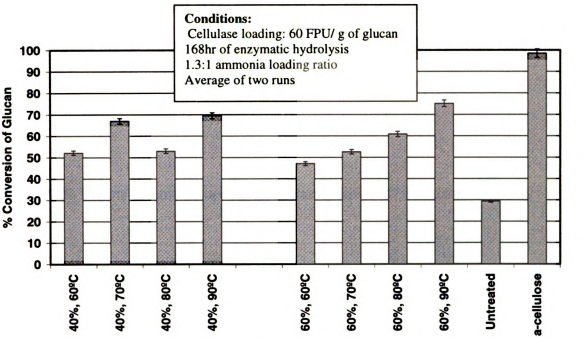


Figure 3.14. Effect of temperature on glucan conversion at 1.3:1 ammonia loading



Data in these three figures (3.12, 3.13, and 3.14) illustrate increasing temperature at any ammonia loading level dramatically enhanced the conversion of the glucan to glucose. Pretreatment temperature is a very important variable, it determines the amount of ammonia vaporized during the explosive flash, influences system pressure and affects the rate of reaction occurring. At higher temperature more ammonia vapors flash, thereby greater disruption of biomass fiber structure might be achieved. Also higher temperature accelerates the chemical reactions such as alkaline hydrolysis of hemicellulose. Figures 3.15, 3.16, and 3.17 show the effect of treatment temperature on the xylan conversion at different ammonia loading levels. With one exception, xylan conversion increased as the treatment temperature increased. At 60% moisture and 0.7:1 ammonia loading ratio the

xylan conversion was increased as the temperature rose to 70°C, but at higher temperature a slight decrease in xylan conversion was observed.

Since both glucan and xylan conversions are still increasing with temperature so we investigated higher temperature (100 and 110°C) to identify the optimum temperature for AFEX treatment of corn stover. The results are presented later in this chapter.

Figure 3.15. Effects of temperature on xylan conversion at 0.7:1 ammonia loading.

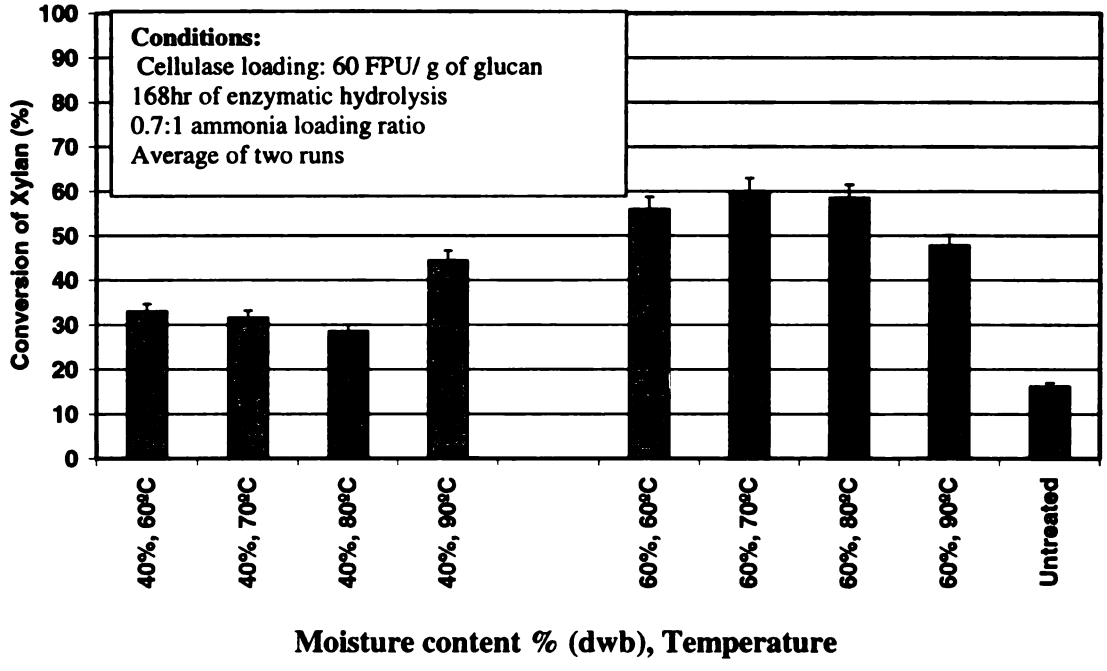


Figure 3.16. Effects of temperature on xylan conversion at 1:1 ammonia loading.

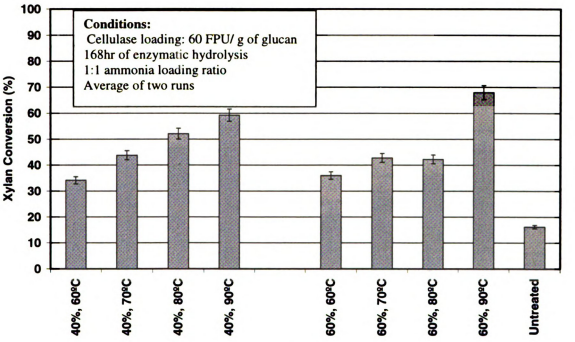


Figure 3.17. Effects of temperature on xylan conversion at 1.3:1 ammonia loading.

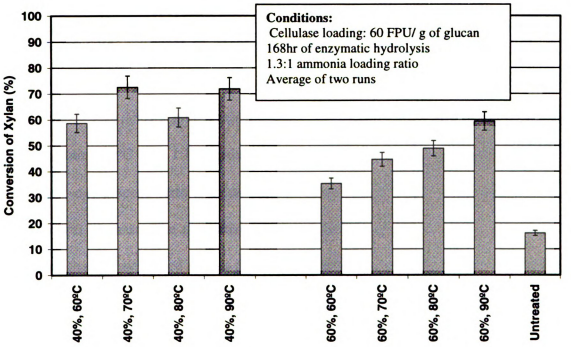


Figure 3.18 shows the hydrolysis profiles for selected AFEX-treated and untreated corn stover samples at enzyme loading levels of 60 FPU/g of glucan. In all cases, the curves that describe sugar production vs time have a similar shape. The AFEX-treated material showed a consistently higher degree of digestibility than the untreated sample. Figure 3.18 also shows that the initial rate of digestion of treated material was higher than untreated. Some of the AFEX treatments approximately double (almost triples in one case) the yield of glucose from cellulose under these conditions versus untreated corn stover.

Figure 3.19 shows glucose production vs time for one of our best AFEX runs (60% moisture, 90°C and 1:1 ammonia ratio), with the data normalized to the 3-day yield. The 3-day glucose yield is a convenient measure of enzymatic susceptibility of the AFEX treated biomass. The normalized curve allows determination of glucose yield at times other than 3 day. Based on this figure about 96% of the 3-day glucose was released in 24h and about 97% of glucose was released in 48 hr. These data demonstrate the high digestibility of the AFEX treated corn stover.

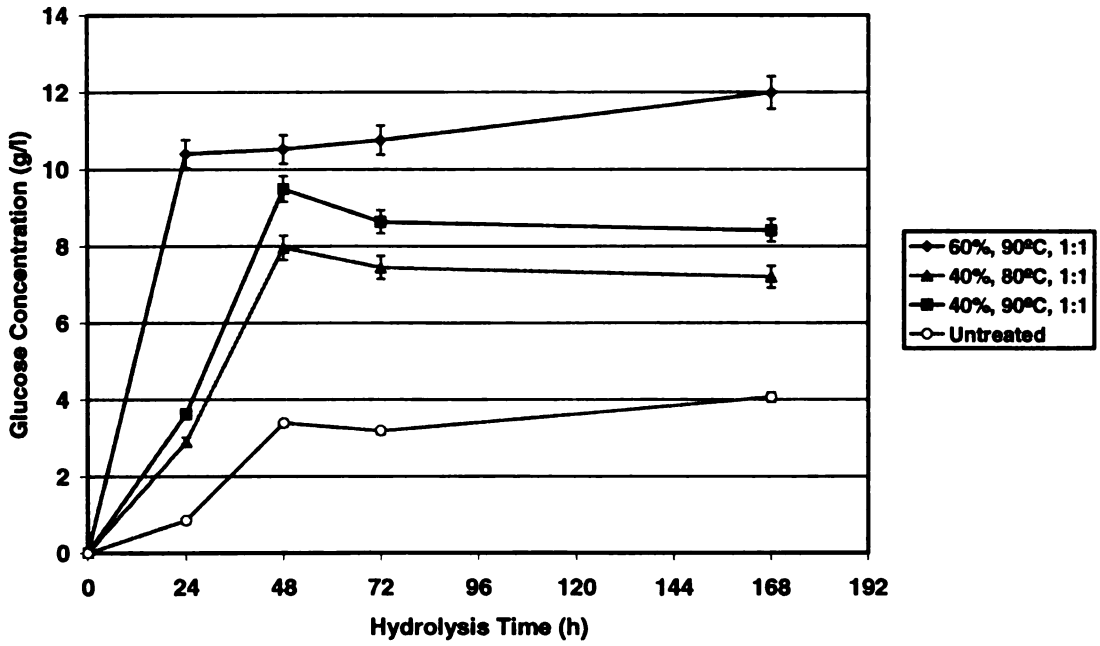


Figure 3.18. Glucose concentration vs hydrolysis time at enzyme loading of 60 FPU/g of glucan

Figure 3.19. Normalized hydrolysis profile for AFEX treated corn stover (60% moisture, 1:1 ammonia loading ratio, 90°C) at enzyme loading of 60 FPU/g of glucan

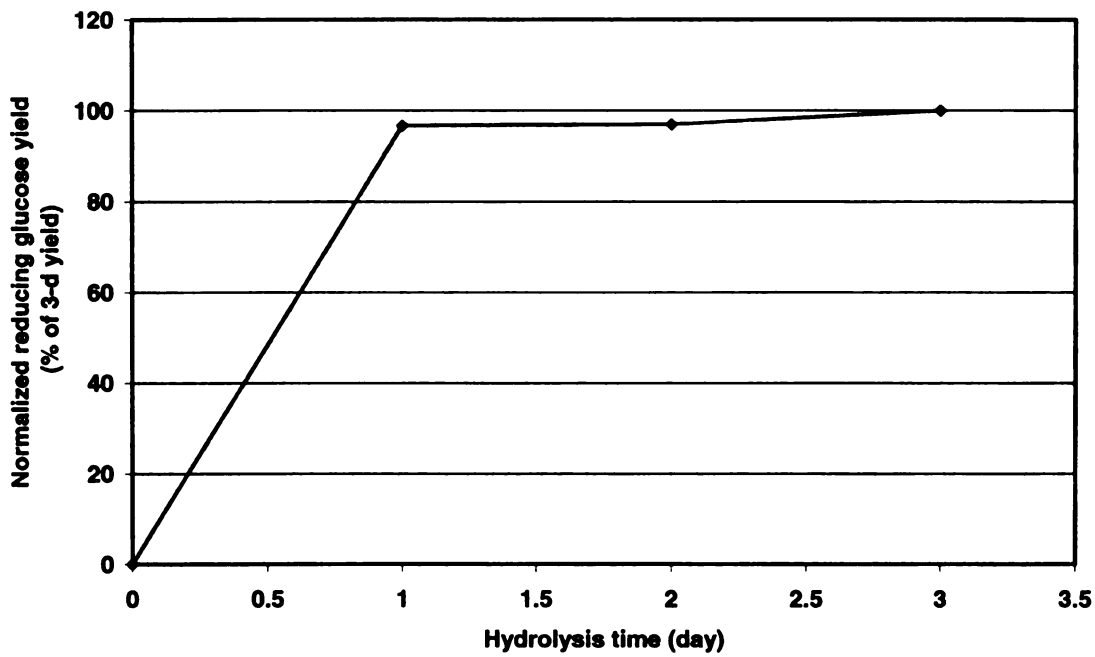


Figure 3.20 shows the xylose production profile for selected AFEX-treated and untreated corn stover samples at an enzyme loading level of 60 FPU/g of glucan. In all cases, AFEX-treated material showed a consistently higher degree of xylose production than the untreated sample. Figure 3.20 also shows that the initial rate of xylose production from treated material was higher than untreated. Some of the AFEX treatments approximately quadruple the yield of xylose versus untreated stover. These findings once again prove that the AFEX treatment has the ability to increase hemicellulose hydrolysis.

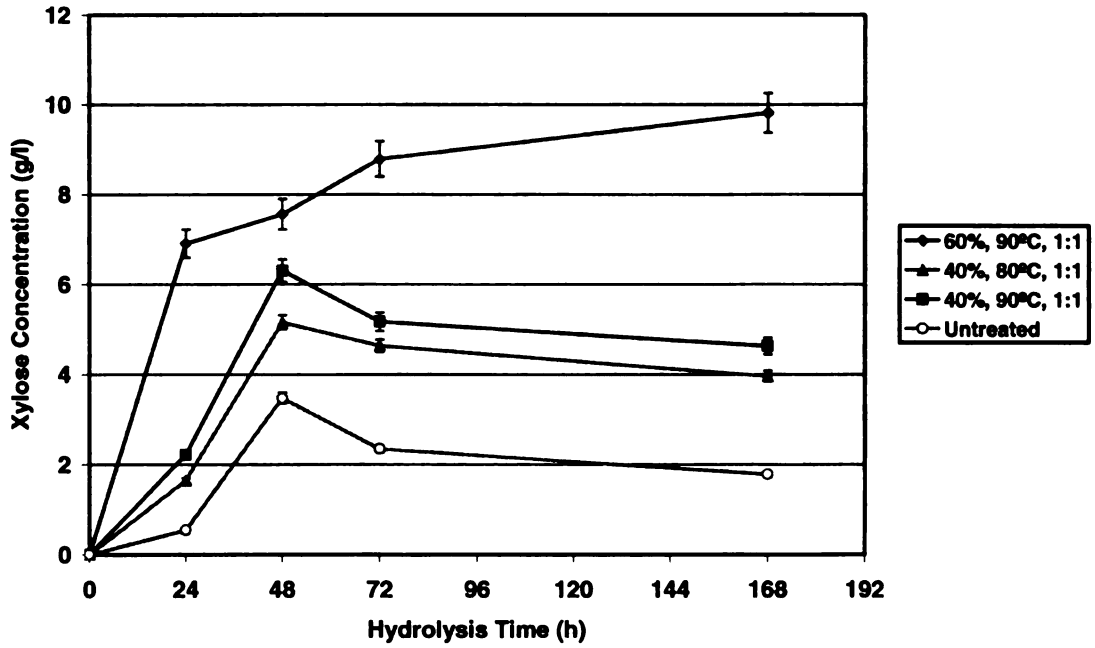


Figure 3.20. Xylose conversion vs hydrolysis time at enzyme loading of 60 FPU/g of glucan

AFEX treatment with higher temperatures, higher moistures, and longer treatment times

Since in our first trials of AFEX treatment of corn stover, both glucan and xylan conversion were increasing with temperature and moisture content, we were not able to top out on the treatment temperature and the moisture content. Therefore some AFEX runs with higher temperature (100 and 110°C), higher moisture (70 and 80% dry weight basis) and longer treatment times (10 and 15 min) all at 1:1 ammonia loading ratio were conducted to establish the optimum conditions for AFEX treatment of corn stover. The AFEX treatment conditions are summarized in Table3.5.

Table 3.5. AFEX treatment conditions in corn stover treatment*

Treatment time (min)	60% Moisture**	70% Moisture	80% Moisture
5	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C
10	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C
15	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C	T=90°C T=100°C T=110°C

* Ammonia loading ratio of 1:1 was used in all the runs.
** Moisture content was calculated on dry weight basis.

The AFEX treatment and the enzymatic hydrolysis were conducted as described before. The enzyme loading level of 15 FPU/g of glucan was used in the following enzymatic hydrolysis. The hydrolysis results are presented in following figures.

Figure 3.21 compares the effects of the different moisture contents on the glucan and xylan conversion, at 90°C treatment temperature and 1:1 ammonia loading ratio. As this figure shows further increases in moisture content after 60% (70 and 80% dry weight basis) did not improve either glucan or xylan conversion. Dilution of ammonia at higher moisture content may reduce the affinity of ammonia for biomass components (e.g., cellulose, hemicellulose). Figure 3.22 shows the effects of different moisture content on both glucan and xylan conversion at 100 and 110°C with 1:1 ammonia loading ratio. This figure also shows the same trend as we observed in Figure 3.21. Based on these data we selected 60% dry weight basis moisture content as the optimum moisture for AFEX treatment of corn stover.

Figure 3.21. Effects of moisture content on glucan and xylan conversion, all runs were kept at the set temperature for 5 min.

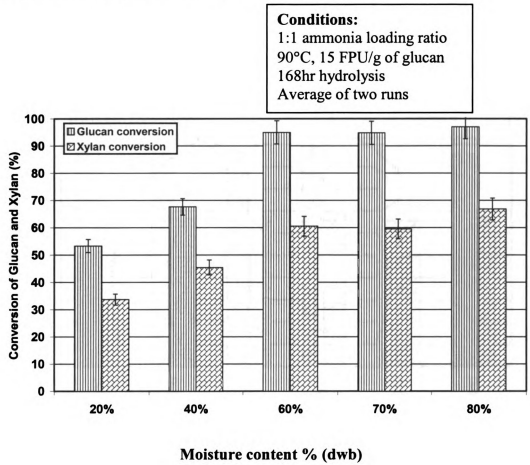


Figure 3.22. Effects of moisture content on glucan and xylan conversion, all runs were kept at the set temperature for 5 min.

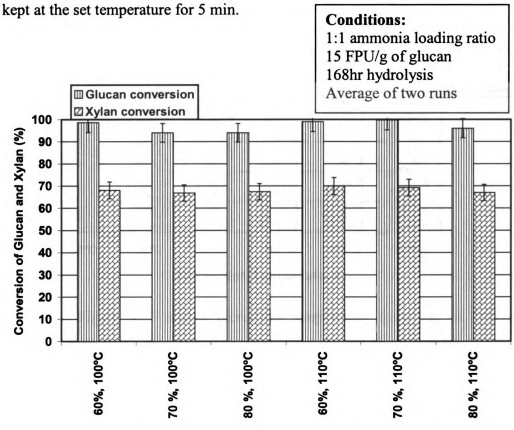
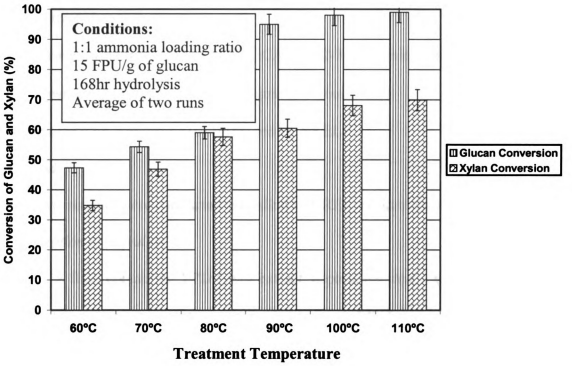


Figure 3.23. Effects of temperature on glucan and xylan conversion; all runs were kept at the set temperature for 5 min.



As Figure 3.23 shows increasing temperature from 90° to 100 and 110°C improved the conversion of glucan to glucose only by about ~ 2-3%. Apparently further increases in treatment temperature do not have much additional beneficial effect. The ultimate goal of the AFEX treatment is to increase the ethanol yield of the fermentation process by increasing the digestibility of the biomass. Therefore some of the above runs that showed higher glucan and xylan conversion were chosen for further SSF analysis. The best treatment temperature was selected based on the fermentation results, not just enzymatic hydrolysis results.

Simultaneous Saccharification and Fermentation (SSF)

Following materials were used:

Untreated corn stover

AFEX treated corn stover

Cellulase enzyme (provided by NREL, CAS 9012-548, activity: 28 FPU/ml)

β-glucosidase (Novozym 188) from Sigma (St. Louis, MO)

Saccharomyces cerevisiae D_5A (provided by NREL)

Method

SSF experiments were conducted according to NREL standard protocol (LAP-008).

Preparation of inoculum

A flask containing YPD (yeast extract, pepton, dextrose) was inoculated with one stock vial of *Saccharomyces cerevisiae* D_5A (provided by NREL) and incubated for 14hr in a rotary incubator shaker at 45°C and 130 rpm. Based on optical density (O.D. at 600nm) of the inoculum the amount of culture needed to inoculate the SSF flask for a starting O.D. of 0.5 was calculated.

Experimental procedure

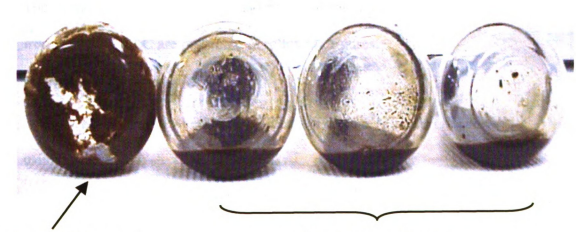
The pretreated corn stover along with the untreated sample was washed and squeezed through miracloth. The wet samples were kept in sealed plastic bags in refrigerator until next day for the SSF experiment. Total solids of the washed samples were determined according to NREL protocol (LAP-001).

All SSF experiments were performed in duplicate and all appropriate controls (SSF flasks without any substrate or with α-cellulose as the substrate) were included in the SSF experiment. The difference within each duplicate was from 2% to 7% (observed

in 20 runs). Each SSF flask was loaded with 6% w/w glucan, 1% w/v yeast extract, 2% w/v peptone, 0.05 M citrate buffer (pH 4.8), an appropriate amount of cellulase enzyme for 15 FPU/g of glucan and an appropriate amount of D_5A inoculum (starting O.D. 0.5). The SSF flasks were equipped with water traps to maintain anaerobic conditions. The SSF flasks were incubated at 45°C with gentle rotation (130 rpm) for a period of 168hr.

At each predetermined time interval (0, 3, 6, 24, 48, 72, 96 and 168hr) a 2ml aliquot was removed aseptically. The samples were centrifuged and the supernatants were filtered and subjected to sugars analysis using HPLC and to ethanol analysis using gas chromatography (GC). At the last time point, a sample from each SSF flask was streaked on an YPD plate to check for contamination. In this series of SSF experiments no contamination was observed. After 168 hr the physical appearance of SSF solid residues of the AFEX-treated corn stover samples was very different compared to that of untreated corn stover sample. After 168hr of SSF the AFEX-treated samples were mostly solubilized and had lost their rigid structure, while there still was considerable rigid solid in the untreated sample. These differences are illustrated in Figure 3.24.

Figure 3.24. Picture of SSF flasks after 168hr of fermentation.



Gas Chromatography (GC)

The fermentation samples were analyzed for ethanol by gas chromatography using model GC 17 (Shimadzu). The injection temperature was 240°C and the detector temperature was 255°C. The column was first maintained at 80 °C up to 3 min followed by a temperature program at 15°C/min up to 125 °C for 6 min. The carrier gas was helium and ethanol was used as external standard for calibration. The calibration curve is presented in Appendix B.

The fermentation samples were also analyzed for sugars by HPLC. The results obtained from GC and HPLC are presented in following figures.

Results and Discussion

The % theoretical ethanol yield or % cellulose (glucan) conversion was calculated using equation 3.4:

$$\%\ \text{Cellulose Conversion} = \frac{[Ethanol]_f - [Ethanol]_0}{0.51(f * [Biomass] * 1.111)} \text{X}100\% \qquad \textbf{(3.4)}$$

where:

$[Ethanol]_f$	Ethanol concentration at the end of the fermentation (g/L) minus any ethanol produced from the enzyme and medium
$[Ethanol]_0$	Ethanol concentration at the beginning of the fermentation (g/L) which should be zero
[Biomass]	Dry biomass concentration at the beginning of the fermentation (g/L)
f	Cellulose fraction of dry biomass (g/g)
0.51	Conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast.

1.111 Converts cellulose to equivalent glucose

As Figure 3.25 shows, throughout the SSF process, glucose produced by the cellulase enzyme was almost completely consumed by the yeast and converted to ethanol in all the selected runs (the high concentration of glucose at 0hr is due to YPD medium in the SSF flask). On the other hand, the concentration of xylose produced consistently increased throughout the SSF process (Figure 3.26). Our *Saccharomyces cerevisiae* D_5A yeast does not have the ability to utilize xylose and to convert it to ethanol. The data in Figure 3.25 also show similar results as we observed in our enzymatic hydrolysis, earlier in this chapter. These data show that in some cases the AFEX treatment approximately quadruples the yield of xylose versus untreated corn stover.

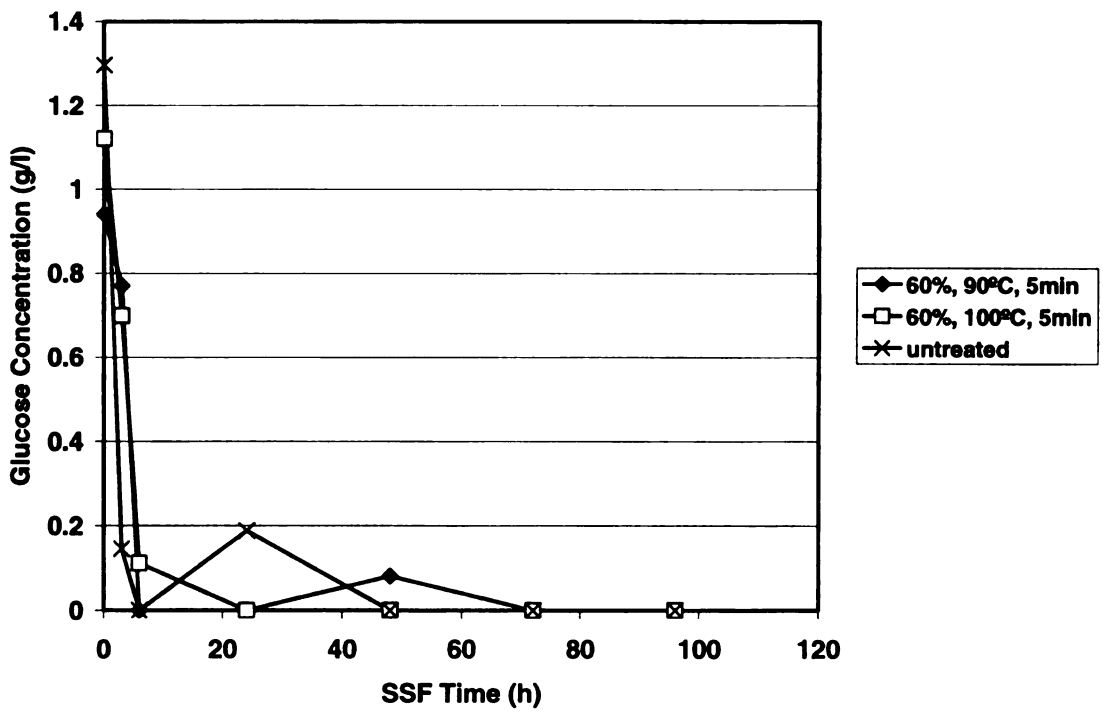


Figure 3.25. SSF profile for glucose concentration

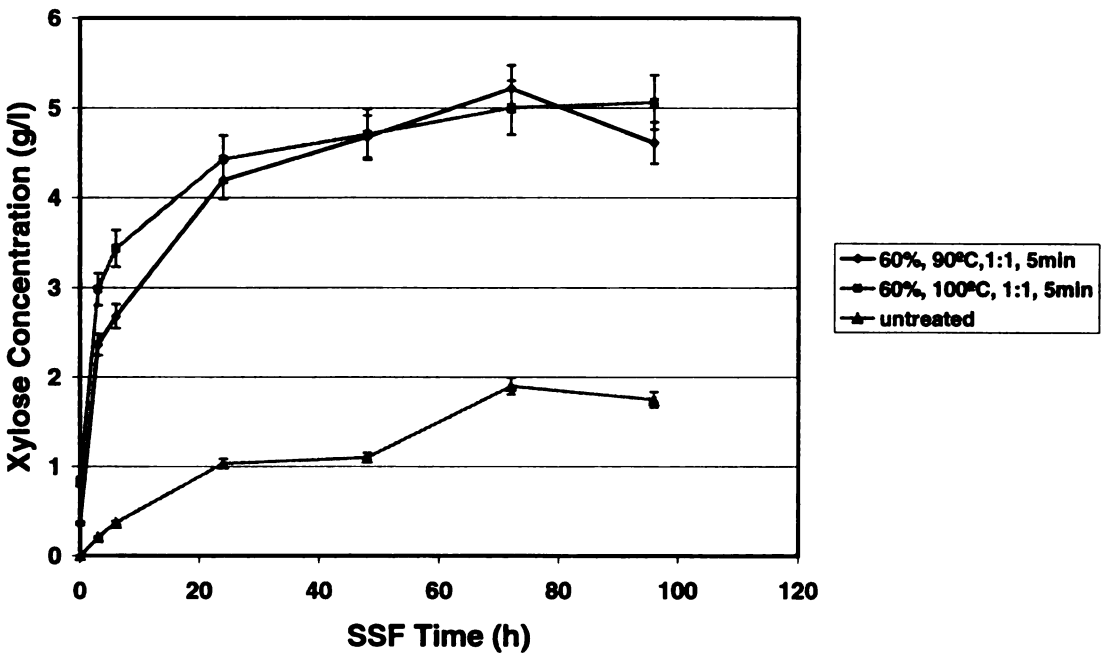


Figure 3.26. SSF profile for xylose concentration

Figure 3.26. shows the time profile of ethanol production during the SSF hydrolysis of AFEX-treated and untreated corn stover. The AFEX-treated samples consistently produced higher amount of ethanol throughout the entire course of the SSF process. The rate of ethanol production was quite rapid during the first 6 hr of the fermentation. All of the samples attained the maximum amount of ethanol after 96hr. As seen in Figure 3.27, run treated at 90°C, 1:1 ammonia loading ratio, and 60% moisture content produced more than twice as much as ethanol compared to the untreated sample. The data presented in Figure 3.28 also shows the AFEX treatment under these conditions had the highest ethanol yield as a percent of theoretical.

Even though the enzymatic hydrolysis of sample treated at 90°C, 1:1 ammonia loading ratio, and 60% moisture content showed lower glucan conversion compared to sample treated at 100°C, 1:1 ammonia loading ratio, and 60% moisture content, the results presented in figure 3.27and 3.28 indicate that this run produced higher amount of ethanol. The higher temperature may have produced some inhibitory material that affected the performance of the yeast and reduced SSF productivity. Since the main objective is higher production of ethanol, we selected 90°C as the best treatment temperature for AFEX treatment of corn stover.

Figure 3.27. Ethanol concentration vs. SSF time

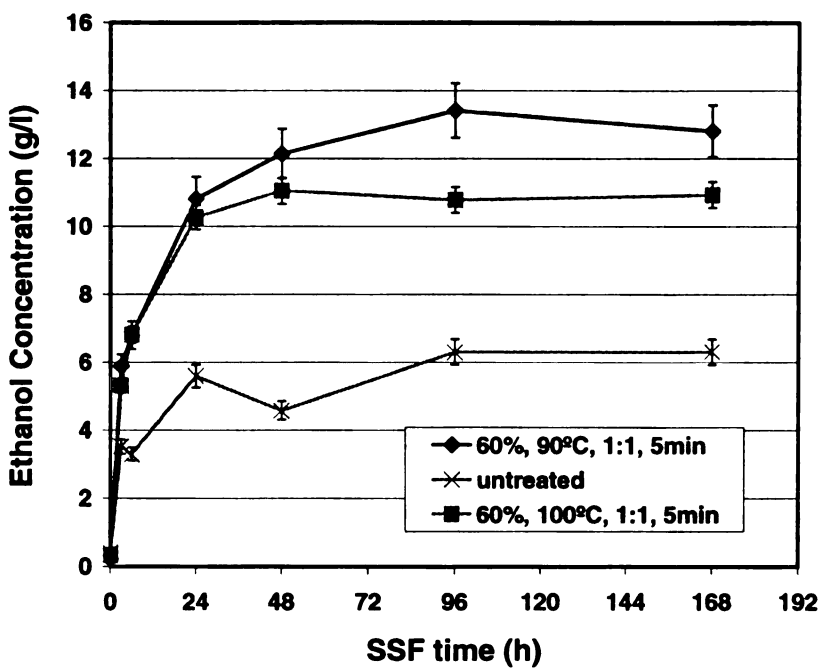
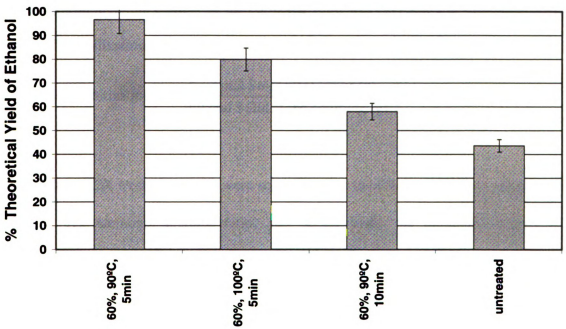


Figure 3.28. Yield of ethanol as percent of theoretical after 96 hr of SSF



Solubilization of corn stover during enzymatic hydrolysis

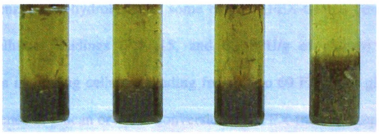
To investigate the solubilization of the AFEX treated and untreated corn stover, a series of the enzymatic hydrolysis vials were set up, using the selected AFEX treated samples and untreated sample with an enzyme loading of 60 FPU/g of glucan (as described above). The vials were incubated at 50°C with gentle rotation (75rpm) for a period of 168hr. No sample was taken during the entire period of 168hr. At the end of 168hr of hydrolysis the solid residues were collected by squeezing the samples through miracloth. The moisture content of the collected solid was calculated as described before. The % of solubilization was calculated according to equation (3.5).

$$\% \text{ Solubilization} = 1 - \left(\frac{\text{Total Solid after 168hr (g)}}{\text{Total Solid at the begining (g)}} \right) \qquad \textbf{(3.5)}$$

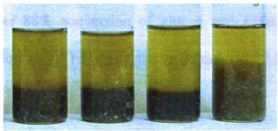
The AFEX treated samples were solubilized up to 66% (condition of sample: 60% moisture, 1:1 ammonia loading ratio, and 90°C) while the untreated sample was solubilized only 45%. The different amount of solubilization can easily be noticed by the appearance of the enzymatic hydrolysis vials after 168hr of hydrolysis. These differences are visible in Figure 3.29.

Figure 3.29. Pictures of AFEX-treated and untreated samples after treatment with 60 FPU/g of glucan; a) At 0 hr; b) After 168 hr of hydrolysis

a)



b)

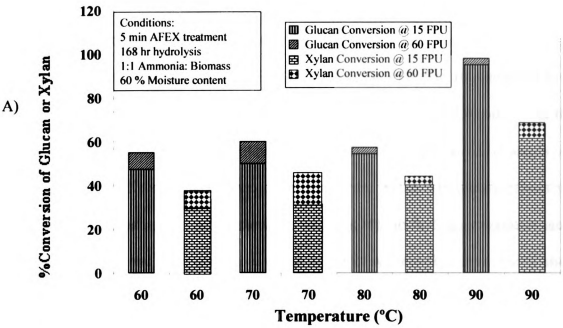


Different cellulase enzyme loading

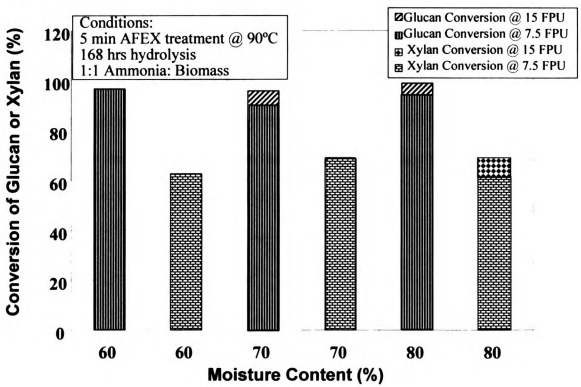
The cost of enzyme used for saccharification of cellulosic residues is dominant in the overall bioconversion process. One way to decrease this cost is to use lower amount of enzyme per kg of biomass in enzymatic hydrolysis. Therefore as part of our study we performed enzymatic hydrolysis on some of the AFEX-treated corn stover with three different cellulase loadings (7.5, 15, and 60 FPU/g of glucan). As Figure 3.30A demonstrates increasing cellulase loading from 15 to 60 FPU/g of glucan, did not make much difference in glucan or xylan conversion of AFEX-treated samples. These data also show that at higher temperature these differences are diminished, for example at 90°C, 1:1 ammonia loading, and 60% moisture content both 15 and 60 FPU result in almost the same amount of glucan and xylan conversion. Figure 3.30B presents the results of enzymatic hydrolysis of AFEX-treated corn stover at enzyme loading of 7.5 FPU/g of glucan compared with 15 FPU/g of glucan. These data show that the AFEX treatment makes possible over 88% conversion of glucan and xylan to glucose and xylose at enzyme loading as low as 7.5 FPU/g of glucan (equal to 2.75 FPU/ g of dry biomass).

Figure 3.30. Conversion of glucan and xylan vs treatment temperature A) at enzyme loading 15 and 60 FPU/g of glucan; B) at enzyme loading 7.5 and 15 FPU/g of glucan

A)



B)



Effects of longer AFEX treatment time

In an effort to explore the effects of longer treatment time on the hydrolysis results, a series of AFEX runs (all at 1:1 ammonia loading ratio) were conducted; the system was kept at the target temperature for 5, 10 or 15 min. The conditions of these runs and the effect of increasing the AFEX treatment time from 5 to 10 minutes or from 5 to 15min in glucan or xylan conversion are summarized in Tables 3.6 and 3.7 As these tables show increasing the treatment time in some cases had a positive effect and in some cases a negative effect on the conversion of glucan and xylan. Since these effects were very different in each set of AFEX runs, we were not able to find any correlation among the treatment time and other conditions of the AFEX runs. Therefore to be able to choose the best treatment time, we selected some of the runs, which showed greater glucan and xylan conversion with increasing time, for further SSF experiments. The results are shown in Figure 3.31 As seen in this figure all the runs (with one exception) with longer treatment time showed lower ethanol yield. However, the hydrolysis results of these runs all showed greater glucan and xylan conversion (Table 3.6 and 3.7). Perhaps during longer treatment times some inhibitory materials were produced which in turn reduced the yield of the fermentation. Based on these findings we selected 5 minutes as the best treatment time for the AFEX treatment of corn stover.

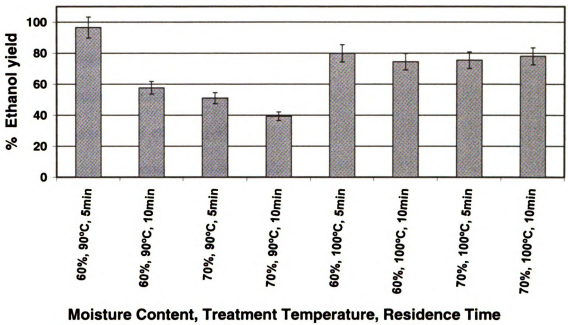
Table 3.6 Effect of increasing the AFEX treatment time from 5 to 10 minutes in glucose and xylose conversion (at 15 FPU, after 168hr).

Temperature	Sugar	Moisture Content		
		60 %	**70 %**	**80 %**
90°C	**Glucose Conversion**	-6.9 %	3.2 %	4.3 %
	Xylose Conversion	-4 %	4.97%	0.9 %
100°C	**Glucose Conversion**	4.14 %	15.7 %	-3.13 %
	Xylose Conversion	7.8 %	14.5 %	-4.8 %
110°C	**Glucose Conversion**	-2.9 %	26 %	23 %
	Xylose Conversion	13.6 %	25.8 %	23.2 %

Table 3.7 Effect of increasing the AFEX treatment time from 5 to 15 minutes in glucose and xylose conversion (at 15 FPU, after 168hr).

Temperature	Sugar	Moisture Content		
		60 %	**70 %**	**80 %**
90°C	**Glucose Conversion**	7.5 %	11.9 %	-4.7 %
	Xylose Conversion	4 %	13 %	-3.9 %
100°C	**Glucose Conversion**	21.6 %	0.0 %	12.4 %
	Xylose Conversion	19.2 %	2.66 %	6.9 %
110°C	**Glucose Conversion**	8.7 %	12.5 %	16.8 %
	Xylose Conversion	16 %	15.4 %	17.6 %

Figure 3.31. SSF results of some of the AFEX runs with longer residence time.



Conclusion

Our major findings and conclusions are as follows:

- Our AFEX unit works essentially as designed and gives results within the expected ranges based on previous studies.
- Even without mechanical mixing, ammonia distribution appears to be uniform in this 300 ml reactor, but there is substantial non-isothermal behavior, which is hard to avoid in a lab unit.
- Optimum conditions are quite broad. However, highest glucan and xylan conversion and ethanol yield from AFEX-treated corn stover were achieved at: 1:1 kg of ammonia / kg of dry biomass, moisture content 60% (dwb), temperature 90°C, and residence time 5 min.
- It appears possible, at least within some limits, to achieve similar hydrolysis results by increasing temperature while reducing ammonia levels, that is, we can "trade off" these two parameters.
- Increasing temperature and moisture content enhance the AFEX treatment.
- Enzymatic hydrolysis of the corn stover treated under optimal AFEX conditions showed almost 97% glucan conversion and 68% xylan conversion versus 29 % and 16% for untreated corn stover respectively (with enzyme loading of 60 FPU/ g of glucan).
- Lowering the cellulase loading from 60 FPU/ g of glucan to 7.5 or15 FPU/ g of glucan did not make appreciable differences in glucan or xylan conversion of AFEX treated corn stover. These results are very important in terms of process economics

- AFEX treatment doubled the amount of ethanol production compared to untreated corn stover in the SSF process (*S. cerevisiae* strain that we used in our SSF process does not utilize xylose).

Future Work

The AFEX treatment, unlike high temperature or acidic treatment produces significant amount of xylose. Existing cellulase mixtures have been developed to hydrolyze these acid/high temperature-treated lignocellulosic material and are probably not optimal for AFEX-treated materials. Therefore supplementing the enzymatic hydrolysis system with xylanase might help to achieve maximum hydrolysis of plant cell wall polymers.

Enzymatic hydrolysis of AFEX-treated material, produces large amount of xylose therefore, using microorganisms capable of utilizing xylose as well as glucose in the SSF process could increase ethanol production yield.

The cost of enzyme used for saccharification of lignocellulosic biomass is dominant in the overall bioconversion process; therefore, using lower cellulase (less than 7.5 FPU/g of glucan) deserves attention.

Chapter 4

Constructing Plasmid DNA containing Cellulase Genes

&

Effects of AFEX on the activity of the plant-produced heterologous cellulase

Abstract

A critical parameter affecting the economic feasibility of lignocellulosic bioconversion is the production of inexpensive and highly active cellulases in bulk quantity. A promising approach to reduce enzyme costs is to genetically transform plants with the genes of these enzymes, thereby producing the desired enzymes in plants themselves. Extraction and recovery of active proteins or releasing active cellulase from the plants during bioconversion could have a significant positive impact on overall lignocellulose conversion economics.

In an effort to produce cellulases in transgenic corn plant, a research group from the Crop and Soil Sciences Department (at Michigan State University) and I constructed several different plasmid DNAs to target these enzymes to different compartments (cytosol or chloroplasts) of transgenic corn plants.

We have also investigated the effects of AFEX pretreatment, employing a range of treatment temperatures, moisture contents and ammonia loadings on the activity of plant-produced heterologous cellulases. The plant materials included transgenic tobacco plants expressing E1 (endoglucanase from *Acidothermus cellulolyticus*). The E1 activity was measured in untreated and AFEX-treated tobacco leaves to investigate the effects of the treatment on the activity of this enzyme.

Literature Review

Enzymatic degradation of cellulose to glucose is generally accomplished by the synergistic action of three distinct classes of cellulases (Wilke *et al.*, 1983):

- Endo-1,4-β-glucanases (EC 3.2.1.4), which act randomly on the interior of the cellulose chain to generate new chain ends.
- Exo-1,4-β-D-glucanases or 1,4-β-D-glucan cellobiohydrolases (EC 3.2.1.91), which act on the nonreducing ends of the cellulose chain and liberate D-cellobiose from 1,4-β-glucans.
- β-D-glucosidases (EC 3.2.1.21), which act to release D-glucose units from cellobiose.

Many naturally occurring cellulose-degrading fungi and bacteria have been reported so far. More than 60 cellulase-producing fungi, representing the soft-rot, brown-rot and white-rot (Robson and Chambliss, 1989) and 46 unique bacterial producers of cellulases have been reported (Coughland and Ljungdahl, 1988). The most studied and well characterized cellulosic fungus is the filamentous soft rot fungus *Trichoderma reesei* which is also the most widely used in industrial cellulase system (Mandels and Sternberg, 1976). Another well characterized and useful cellulase source is *Acidothermus cellulolyticus* (Mandels and Sternberg, 1976).

The enzyme production step is important. Preliminary economic analysis shows that about 6% of the final ethanol cost in a simultaneous saccharification fermentation (SSF) process and 43.4% of final costs in a separate hydrolysis and fermentation (SHF) process can be attributed to enzyme costs (Hinman *et al.*, 1992). Therefore any effort to reduce cellulase costs can have a positive impact on the final cost of bioethanol. One way

to reduce this cost would be to find the most effective combination of cellulases and then try to produce these enzymes in transgenic plants in a large scale.

It is well established that cellulase is a multicomponent enzyme complex and that crystalline cellulose is hydrolyzed by synergism of cellulase components. The synergism of these enzymes has been investigated by many researchers. Different combinations of endoglucanases and exoglucanases isolated from heterologous systems show different levels of synergy (Thomas *et al.*, 1995). Therefore it is important to determine the best combination that produces the greatest synergistic effect and offers the highest amount of glucose production per unit of time at equivalent enzyme loading. The synergetic effect is defined as the ratio of glucose released when both enzymes are used versus the sum using equivalent amount of enzymes in separate individual reaction.

$$\text{Synergism Ratio} = (\text{glucose}_{\text{endo+exo}})/ (\text{glucose}_{\text{endo}} + \text{glucose}_{\text{exo}})$$

A ratio greater than 1 means a synergistic effect between the two enzymes. A values around 1 indicates no synergistic effect and a ratio less than 1 indicates some degree of interference between the two enzymes. Baker *et al.*, (1994) tested the synergistic effect of 16 different endo/exo binary pairs. Table 4.1 shows the four best synergistic pairs of the tested pairs. These results show that the most synergistic pair is the combination of cellobiohydrolase, CBHI, from *T. reesei* and endoglucanase, E1, from *A. cellulolyticus.* This observation highlights the potential value of using these enzymes in future recombinant systems and using the genes of these enzymes to transform plants.

Table 4.1. Best Synergistic Endo/Exo Cellulase Pairs Tested*

Endo/Exo Pair	Synergism Ratio
A. cellulolyticus E1 & *T. reesei* CBHI	2.74
T. fusca E5 & *T. reesei* CBHI	2.61
M. bispora rEndo A & *T. reesei* CBHI	2.04
T. neapolitana rEndo B & *T. reesei* CBHI	1.90

*Adopted from Baker *et al.* (1994)

The thermotolerant endoglucanase, E1, which is secreted from *A. cellulolyticus,* demonstrates temperature activity characteristics that are useful for high temperature saccharification and, like other bacterial cellulases, shows a high specific activity. The E1 enzyme has three domains a cellulose binding domain, a linker region and a catalytic domain. Prior studies have shown that the catalytic domain by itself is sufficient for efficient degradation of cellulose *in vitro* (Ziegelhoffer *et al.*, 2001).

Testing the effect of truncation of E1 enzyme (E1 with only catalytic domain) on the protein expression level, a research group from University of Wisconsin transformed tobacco plants with both truncated E1 (E1cd) enzyme and the whole E1 enzyme, and as the results show (Table 4.2) the expression level of E1cd was much higher than the expression level of E1 (Ziegelhoffer *et al.*, 2001).

Table 4.2. Maximum observed expression levels* (percent of total soluble protein) for E1and E1cd enzyme in transgenic tobacco.

Compartment	E1	E1cd
Cytosol	0.00065	0.014
Chloroplast	0.007	0.07
Apoplast	0.33	0.58

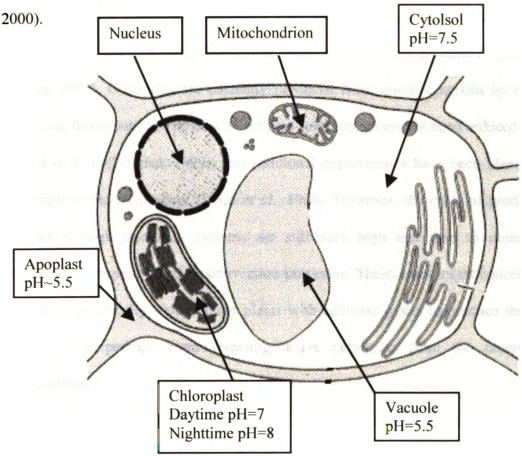
*Adopted from Ziegelhoffer *et al.*, 2001

There are many unfavorable codons (with respect to plants) in the linker region. It contains frequent proline residues, which are encoded by rare (in plant) CCG codons. In E1cd expression constructs a stop codon was introduced at the end of the catalytic domain, effectively removing the linker and the cellulose binding domains. This research group made series of plasmids to target E1 and E1cd enzymes to different cellular compartments of plant cells such as the apoplast, chloroplast, and cytosol. Their results showed that the expression level of E1 or E1cd in apoplast was much higher compared to the expression level of E1 in cytosol and chloroplast. These results are summarized in Table 4.2 (Ziegelhoffer *et al.*, 2001). In this study, the accumulation of E1 or E1cd did not affect normal growth of the plants

Expression of recombinant proteins in plant cells is dependent upon many factors such as transcriptional, posttranscriptional, and posttranslational factors (Dai et al., 2000). Besides factors influencing transcription and translation efficiency, recombinant protein accumulation as well as stability strongly depend on the compartment of the plant cell chosen for expression (Conard and Fiedler 1998). As mentioned above, Ziegelhoffer *et*

et al., 2001 showed that for E1 enzyme, subcellular targeting had significant influence on the expression level of this enzyme in tobacco plants. The abundance of mRNA in this study was very similar in tobacco plants transformed with either cytosol, chloroplast or apoplast constructs, therefore, the barrier to higher expression in the cytosol and chloroplast is post transcriptional. There is evidence that there are distinct molecular chaperon systems in targeted compartments to translocate or fold proteins (Boston *et al.*, 1996). The action of the unfolded protein with these molecular chaperon systems would yield different rates of protein folding. Therefore targeting the E1 to the apoplast is thought to promote correct protein folding leading to higher stability and accumulation. In addition to these factors the pH of each compartment also can play a very important role in the stability of enzyme. Each compartment of the plant cell has a different pH, therefore targeting the enzyme to the compartment with closer match to its favorable pH can improve the stability of the enzyme. As Figure 4.1 shows the apoplast pH is approximately 5.5 which is very similar to the ideal pH for E1, whereas the cytosol pH is 7.5 and chloroplast pH is 7 at daytime and 8 at nighttime (Sander and Bethke, 2000). These facts can indicate that the barrier to higher expression in cytosol and chloroplast might be because of their pH, which is much higher than the ideal pH for E1cd.

Figure 4.1. Different compartments in plant cell (adobted from Sander and Bethke, 2000).



Dai *et al.* (1999) have also successfully transformed tobacco plants with *T. reesei* exo-cellobiohydrolase I (*cbhI*) gene. In this study the accumulation of CBHI did not affect normal growth of the transgenic plants.

These successes have shown the feasibility of cellulase production in plants and are the important first steps in the development of crop plants as production system for cellulases.

Introduction

In ethanol production enzymatic hydrolysis of cellulose to glucose is a very attractive route, because nearly theoretical yields of glucose are possible (Wyman, 1999; Wyman, 1995). Cellulases are currently produced from microorganisms by expensive large-scale fermentation. The cost of enzyme-based processes has been reduced by about a factor of four (Wyman, 1999), and additional opportunities have been identified that may improve the technology (Lynd *et al.*, 1996). However, the costs of production of enzymes through microbial systems are still very high and tend to dominate the economics of enzyme-based bioconversion processes. These costs might conceivably be reduced by genetically transforming plants with cellulase genes to produce the desired enzymes, and perhaps even releasing active cellulases from the plants during bioconversion.

Transgenic plants are an attractive and cost-effective alternative to microbial systems for production of biomolecules (Goddijn, 1995). Advances in biotechnology are enabling plants to be exploited as bioreactors for the production of proteins, carbohydrates (Kidd and Devorak, 1994; Stark *et al.*, 1992), lipids (Grayburn *et al.*, 1992; Poirier *et al.*, 1995), and industrial enzymes (Austin *et al.*, 1994; Pen *et al.*, 1992; Pen *et al.*, 1993) in bulk quantities with minimal inputs of raw materials and energy. As this technology continues to grow and improve the production levels of biomolecules in plants, the development of downstream processing technology to extract and to recover these biochemicals will increasingly determine progress in this area.

With an ultimate goal of production of cellulases in transgenic corn plants, several different DNA plasmids were made through our collaboration with a research group from

the Crop and Soil Sciences department of Michigan State University. These plasmids were designed to target the cellulases to cytosol, chloroplast, or apoplast of transgenic corn plants.

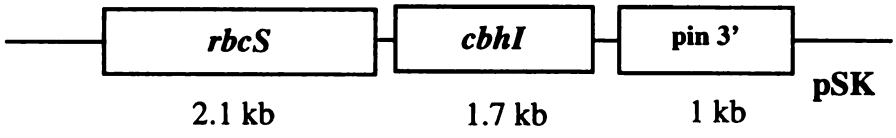
Since *A. cellulolyticus* endoglucanase (E1) and *T. reesei* exo-cellobiohydrolase I (CBHI) demonstrate a high synergistic activity, this pair seemed a good choice for initial studies of the effect of recombinant cellulase expression in plants. To test the effect of sub-cellular targeting on the accumulation of CBHI enzyme, *cbhI* was fused to *rbcS* for chloroplast-targeting, and for cytosol targeting the sequence encoding leader peptides of *cbhI* was removed. In all plasmids the expression of *cbhI* was driven by the rice *rbcS* promoter and terminated with either pin3' or nos3' region (Fig. 4.2). The plasmid structures and their features are summarized in Table 4.3.

Table 4.3. CBHI plasmid features

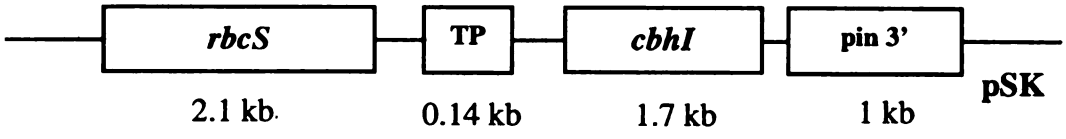
Plasmid	Construct	Plasmid features
pSMF13	rbcS-cbhI-pin3'	rbcS leaf-specific promoter driving cellulase cDNA of *T. reesei.* Expressing CBHI in cytosol.
pSMF14	rbcS-TP-cbhI-pin3'	The rbcS TP targets cellulase of *T. reesei* into maize chloroplasts.
pSMF15	rbcS-TP-syn-cbhI-pin3'	The rbcS TP targets modified cellulase of *T. reesei* into maize chloroplasts.

Figure 4.2. CBHI plasmid construct

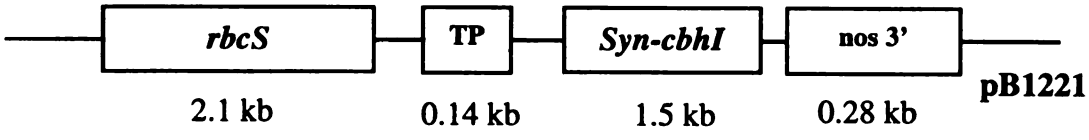
pSMF13



pSMF14



pSMF15



Abbreviations in consutructs:

rbcS = Full-length rice *rbcS* promoter region.

cbhI= Cellulase cDNA isolated from the *T. reesei.*

Syn-cbhI= Synthetic *cbhI* gene.

pin 3' = Potato protease inhibitor II gene 3' non-coding region.

nos 3' = 3' region from *Agrobacterium* nopaline synthase gene.

TP = rice *rbcS* transit peptide.

Plant proteins and enzymes produced in transgenic crops have very high value; therefore recovering and utilizing valuable bioactive plant proteins and enzymes in an overall process for producing fuels and chemicals from biomass might improve the economics of lignocelulosic conversion.

To process cellulase-containing transgenic plants, the following technical options can be envisioned:

1) Harvest the cellulase-containing transgenic plants while they are green and grind them in a suitable buffer to produce an enzyme concentrate, which can later be used for the enzymatic hydrolysis of pretreated biomass. Depending on the production level of this enzyme in transgenic plants, the need for externally added cellulases in the enzymatic hydrolysis step might be avoided or minimized. The debris also can be combined with other pretreated biomass to increase the production of fermentable sugars.
2) Harvest the cellulase-containing transgenic plants at the end of the growing season (dry), grind this material to release enzyme and then combine them with pretreated biomass for ethanol production.
3) Harvest the cellulase-containing transgenic plants at the end of the growing season, treat them with a biomass pretreatment technique to rupture cells to facilitate the release of enzyme, and then combine with additional pretreated biomass for enzymatic hydrolysis.

The success of any of these approaches depends on having a sufficiently high level of cellulase in the transgenic plant to make hydrolysis effective. Each of these options might find use under particular circumstances.

A high yield recovery of plant proteins and enzymes from biomass depends upon extensive cell maceration; the more cell wall that is disrupted the more protein will be recovered (Carroad *et al.*, 1981). Therefore a pretreatment that disrupts plant cells can be useful in protein recovery process.

An integrated pretreatment that improves the protein recovery and increases the conversion of cellulose and hemicellulose to fermentable sugars may significantly enhance the biomass process. Many pretreatments that increase the conversion of cellulose to fermentable sugars operate under harsh conditions that tend to degrade the sugars, proteins, and enzymes. However, the data presented in Appendix A have shown that under limited treatment conditions, the AFEX process not only increases the conversion of cellulose and hemicellulose to simple sugars, it also allows the recovery of plant proteins in their native functional form.

In this study we have explored the third option that was mentioned above, by investigating the potential of using AFEX treatment as an integrated pretreatment to release active cellulase from a transgenic plants while it increases the digestibility of the biomass.

Plasmid construction

Material and Methods

Molecular biology techniques were essentially those of Sambrook *et al.*, 1989 or those of commercial suppliers of enzymes and other products. In all of the CBHI-constructs, *cbhI* expression was driven by the rice rubisco small subunit (*rbcS*) promoter and terminated with pin3' or nos3' region. The CHBI-constructs were prepared as follows:

1. The plasmid pRR1, containing the rice *rbcS* (Yong *et al.*, 1987), was kindly provided by Dr. Ray Wu of Cornell University. The 2.1-kb *Eco*RI/*Eco*RV fragment containing *rbcS* was excised from pRR1. The excised fragment was inserted into the *Eco*R1/*Eco*RV site in the poly-linker of pBluescript II SK +/- (Figure 4.3) from Stratagene (La Jolla, CA) to use as a promoter in our CBHI constructs. This plasmid with *rbcS* promoter is called pSMF8 (Figure 4.4).

Figure 4.3. pBluescript II SK +/-

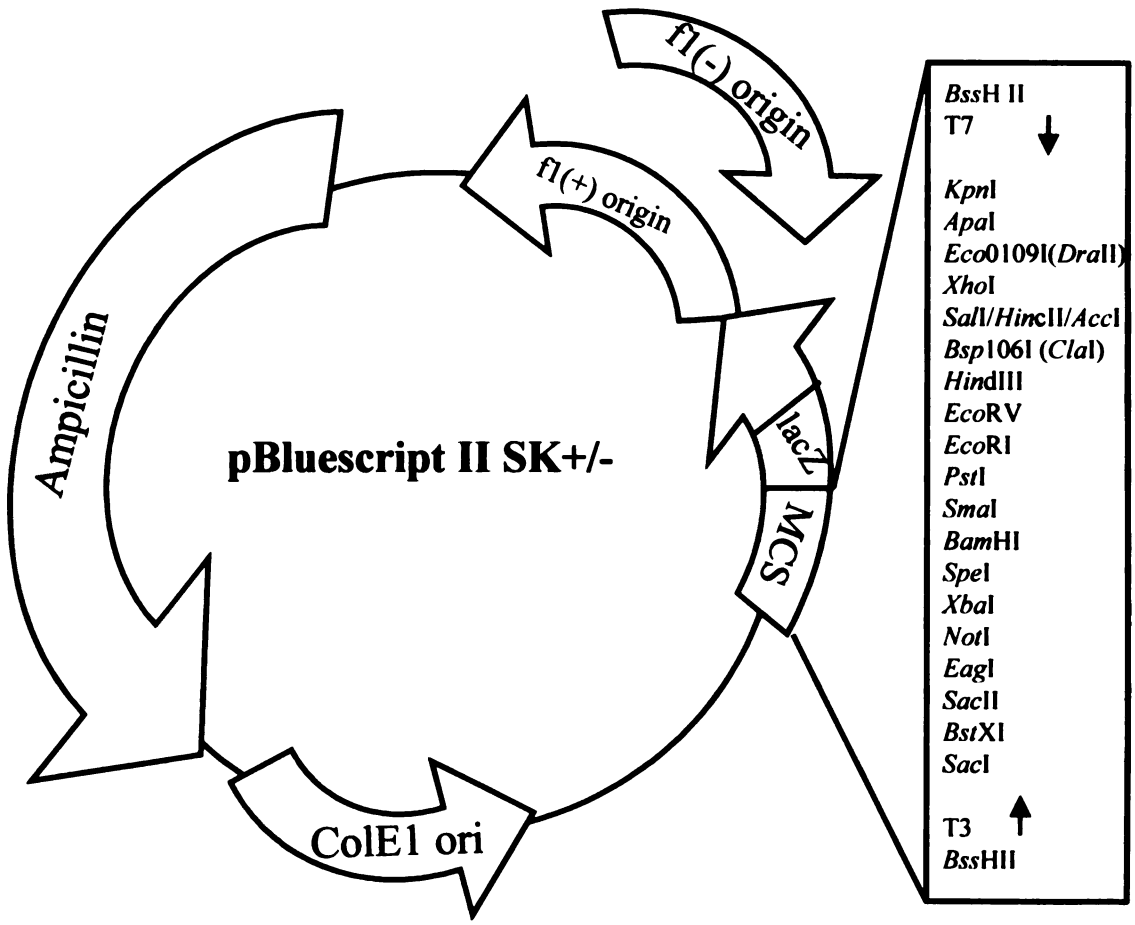
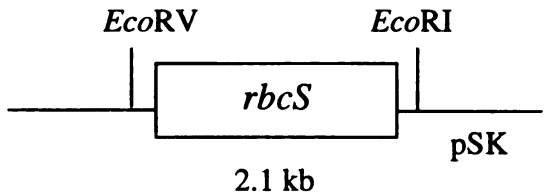
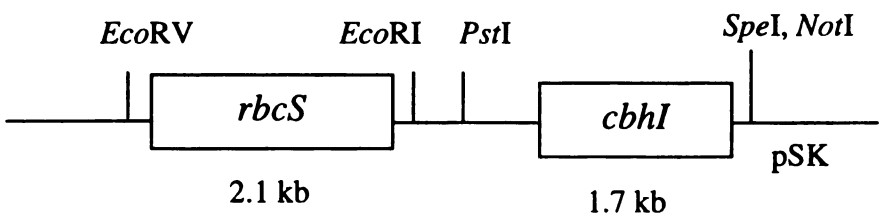


Figure 4.4. pSMF8 construct



2. The plasmid pB210-5A containing *cbhI* was obtained from the National Renewable Energy Laboratory (NREL) Golden, Colorado. To clone *cbhI* down stream of the *rbcS* promoter in pSMF8, the 1.7-kb *SalI/XhoI* fragment containing *cbhI* was cleaved from pB210-5A (Shoemaker *et al.*, 1983). The digested plasmid was electrophoresed in an agarose gel, and the 1.7 kb fragment was purified. The cleaved fragment was treated with DNA polymerase I (Klenow enzyme) to produce blunt ends. pSMF8 was digested with *Bam*HI (a unique site) and then blunt ends were generated for cloning *cbhI*. Then *cbhI* fragment was blunt-end ligated with pSMF8/ *Bam*HI and used to transform *Escherichi coli* XL1Blue bacterial cells. The transformed cells were plated on LB agar media containing ampicillin for selection of transformed cells. Colonies were picked and screened for ones containing the correct construct. This cloning yielded the plasmid pSMF9 (Figure 4.5).

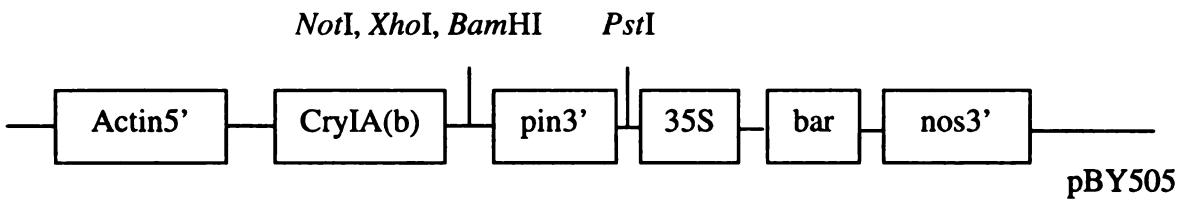
Figure 4.5. pSMF9 construct.



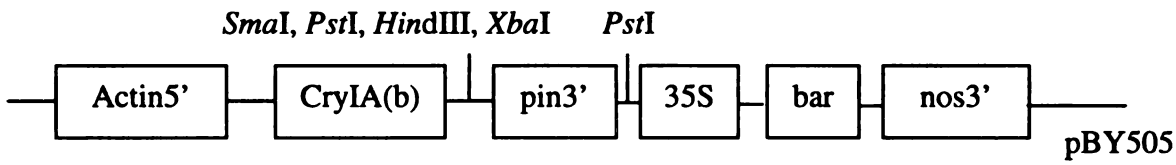
3. To complete the heterologous gene expression cassette, the pin3' transcription termination nucleotide sequence was inserted at the 3' end of *cbhI* in pSMF9. pin3' is the 3' region of the gene coding for protease inhibitor II from potato. This terminator was excised from plasmid pBR10-11, which was obtained from Dr. Ray Wu, Cornell University. To add pin3'gene to pSMF9 without disturbing the rest of the plasmid, we needed to excise pin3' as a *Pst*I fragment from plasmid pBR10-11. According to the pBR10-11 map (Figure 4.6) there is a *Pst*I site downstream of pin 3' sequence. To generate an additional *Pst*I site upstream of pin3'sequence, a 70 bp *NotI/Xho*I fragment containing multi- cloning site sequences from pSK vector was ligated with pBR10-11/ *NotI/Xho*I. pBR10-11 with additional multicloning sites was named pSMF11 (Figure 4.6). Then the pSMF11/*Pst*I fragment containing pin3' terminator sequences was cloned in pSK at *Pst*I site. This plasmid was named as pSMF12 (Figure 4.6). Then this pin 3' terminator was cleaved from pSMF12 with *Not*I enzyme as 1kb fragment and cloned downstream of *cbhI* in pSMF9. This construct was named pSMF13 (Figure 4.6). Using this construct for transformation should result the expression of *cbhI* in the cytosol.

Figure 4.6. pBR10-11, pSMF11, pSMF12, and pSMF13 constructs

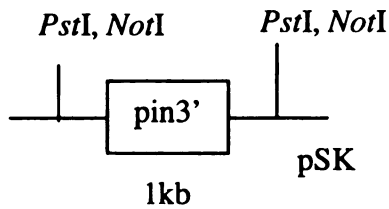
pBR10-11:



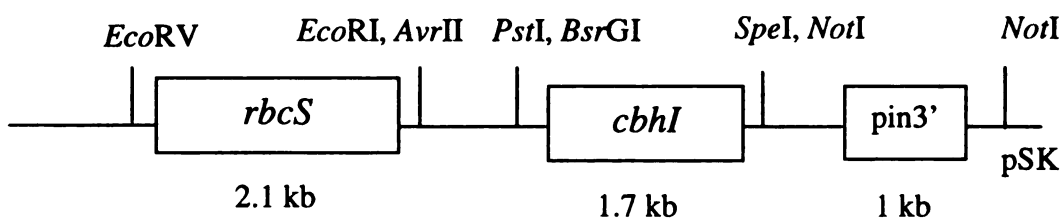
pSMF11:



pSMF12:

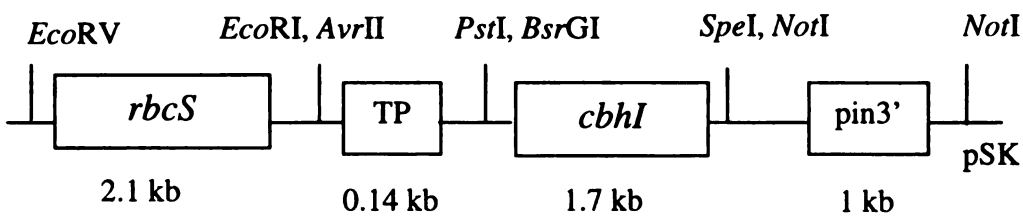


pSMF13:



4. To target the CBHI enzyme to the chloroplast we needed to insert a transit peptide (TP) sequence into the plasmid pSMF13 between the *rbcS* promoter and the *cbhI*. Transit peptide was isolated from *rbcS* sequence from pRR1. Since there were not any restriction sites inside the *rbcS* promoter to remove the transit peptide sequences, we amplified that region by PCR. The pSMF13 map shows there is a unique site *Avr*II at the end of the *rbcS* sequences (promoter) and there is a unique site *Bsr*GI available upstream of the first ATG sequences in the *cbhI*. Therefore, PCR primers were generated using sequences for these two sites and the TP sequences. The transit peptide was PCR-amplified and the product (145bp) was purified and confirmed by sequencing. This PCR-amplified TP was inserted in pSMF13 between the promoter and the *cbhI* as sticky ends using directional ligation using *Avr*II and *Bsr*GI restriction sites. This construct was named pSMF14 (Figure 4.7). This construct should result in chloroplast accumulation of CBHI.

Figure 4.7. pSMF14 construct



PCR Primers:

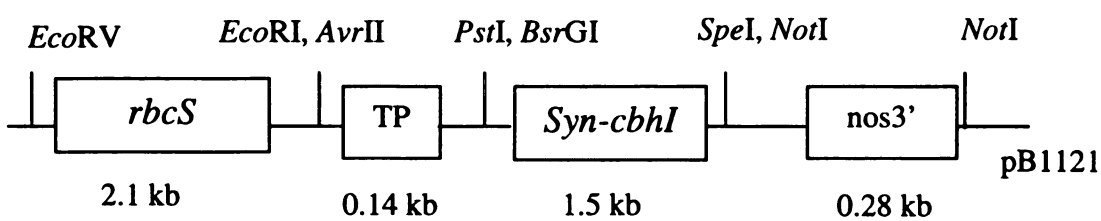
SP1F 5'CCGCCTAGGCGCATGGCCCCCTCCGT3'

SP3R 5'CGCTGTACACGCACCTGATCCTGCC3'

5. Dai *et al.*, (1999) have also tried to use *cbhI* from *T. reesei* to transform tobacco plants. Because of the relatively high difference between the GC content of tobacco and *cbhI*, to ensure higher expression of this gene in tobacco plants they codon-modified the original *cbhI*. The GC content in their synthetic *cbhI* (*Syn-cbhI*) is around 43%, which is much closer to tobacco GC content than the original *cbhI* with 55% GC content. But since the GC content of monocots (maize) is higher (~65%) than dicots (tobacco), the original gene with higher GC content is probably more suitable for maize (Murrary *et al.*, 1988). However, we have used their synthetic *cbhI* along with the original *cbhI* to compare their expression levels in maize plants.

Plasmid pZD408 containing *Syn-cbhI* was obtained from Dr. Dai. pZD408 was digested with *Nco*I and made blunt-ended. This blunt-ended vector was digested with *Hin*dIII to remove the CaMV35S promoter. The large fragment (4.5 kb) was isolated, and dephosphorylated. Plasmid pSMF14 was digested with *BSr*GI and its ends filled in with Klenows. Then this fragment was digested with *Hin*dIII. This removed *rbcS* promoter with TP from pSMF14 plasmid, which was then ligated with pZD408 fragment. The resulted clone was named pSMF15 (Figure 4.8). This plasmid should direct the synthetic CBHI to chloroplast.

Figure 4.8. pSMF15 construct.



Effects of the AFEX treatment on the activity of cellulase

Abstract

The potential of the AFEX process as a biomass treatment while preserving the proteins in their bioactive form, was explored as part of our research. We approached this goal by investigating the effects of the AFEX process on the activity of Rubisco in alfalfa plants. The results of this investigation are presented in Appendix A. Based on data presented in Appendix A, chapter 3 and previous work (De La Rosa *et al.*, 1994) we believe that, under a limited set of conditions, the AFEX treatment has the potential to be used as an integrated pretreatment to increase the digestibility of biomass, improve extraction of protein from biomass, and at the same time to preserve proteins in their active forms.

Thus, there is a legitimate reason to hope that industrial enzymes such as cellulases, produced in transgenic plants, can also survive the AFEX pretreatment and be recovered in their useable form during biomass refining. This possibility was explored in this chapter by treating transgenic tobacco plants, expressing cellulase, with the AFEX process.

Material and Methods

Plant material

Seeds for transgenic tobacco plants expressing E1cd (catalytic domain fragment of E1 endo-1,4-β-glucanase from *Acidothermus cellulolyticus*) were obtained from Dr. Sandra Austin-Phillips at the University of Wisconsin-Madison.

Figure 4.9 shows the plasmid that has been used by this group to transform tobacco plants to express E1cd in apoplast.

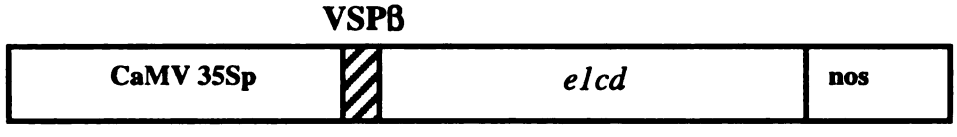


Figure 4.9. Schematic representation of E1cd expression cassette. CaMV35Sp: Cauliflower mosaic virus promoter; nos: The nopaline synthase transcription termination signal; *e1cd*: Catalytic domain of *e1*; VSPβ: Soybean vegetative storage protein β leader sequence to target the protein to apoplast (Ziegelhoffer *et al.*, 2001).

Transgenic plants were grown, harvested (leaves) and dried at Michigan State University greenhouses. As Figure 4.10 and Figure 4.11 show the expression of E1cd did not cause any obvious phenotypic effect in plants.

Nontransgenic tobacco plant Transgenic tobacco plant

Figure 4.10. Transgenic and nontransgenic tobacco plants grown in greenhouse

Figure 4.11. Transgenic tobacco plants growing in the greenhouse.

Identifying expressing E1cd plants

After seedlings developed their first true leaves, samples are removed for enzyme assay to identify transgenic plants.

Fifty milligrams of leaf samples were homogenized in grinding buffer (50 mM NaOAc, pH 5.5, 100 mM NaCl, 10% (v/v) glycerol, 0.5 mM ethylenediaminetetraacetic acid (disodium salt), 1 mM phenylmethylsulfonyl fluoride, 1 mg/l aprotinin, 1 mg/l leupeptin, 1 mg/l pepstatin) at a ratio of 2 µl per mg of sample (fresh weight). Samples were homogenized with the aid of a power drill using 1.5 ml microcentrifuge tubes and plastic pestles (Kontes). Soluble extract was recovered from insoluble debris after centrifugation at 15000 x g for 5 min. A solution of saturated ammonium sulfate was added to the extracts to achieve a final concentration of 2.7 M ammonium sulfate (about 70% saturation at 0°C). After incubation on ice for 30 minutes, the resulting precipitate was recovered by centrifugation at 15000 x g for 5 min. The ammonium sulfate pellet was resuspended in 5 µl of grinding buffer for each 2µl of starting crude extract.

Appropriate dilutions of plant extracts were assayed for E1cd activity using a 96-well plate format. The reaction buffer contains 50 mM NaOAc pH 5.5, 100 mM NaCl, 0.5 mM 4-methylumbelliferyl β-D-cellobioside (MUC) (from Sigma). MUC hydrolysis by E1/E1cd releases the fluorescent product, 4-methylumbelliferone (λex = 360 nm, λem = 465 nm). Each well contained 1 to 4µl of the sample to be assayed and 100 µl reaction buffer. Plates were covered with adhesive lids to prevent evaporation and incubated for 30 min at 65° C. The reaction was terminated by the addition of 100 µl of stop mix (0.15 M glycine, pH 10.0) and fluorescence was determined with a Spectra Max Gemini (Molecular Devices, Sunnyvale, CA) at 465 nm using an excitation wavelength of 360

nm. Fluorescence values were compared to values obtained with 12 to 240 pg of purified Elcd (kindly provided by Steven R. Thomas, National Renewable Energy Laboratory, Golden, Co), a range of enzyme concentrations that yields a linear response. A series of 4-methylumbelliferone standards (4 to 160 pmol) was also included. For all transgenic plant samples, Elcd activity was determined by subtracting the background contributed by W38 control extracts (done in parallel). The production level of Elcd in transgenic tobacco plants was up to 2.5 % of total soluble protein. The plants that were identified as expressing Elcd were grown to maturity, and their leaves were harvested, dried, and collected for AFEX treatment. Since each plant had different Elcd levels of expression, before AFEX treatment the dried leaves were ground and well mixed to insure the homogeneity of the samples.

Heat treatment of the transgenic tobacco plants expressing Elcd

The total moisture content of the dried transgenic tobacco plant material was measured as described in chapter 3. The samples were prewetted to the desired moisture content 30 min prior to each treatment. The prewetted samples were placed in the pressure vessel. The vessel was topped up with stainless steel pellets (approximately 1mm diameter) to occupy the void space, then the lid was bolted shut. The entire reactor assembly was placed in a 400W PARR heating mantle to warm the unit to the desired temperature. To avoid overheating, the reactor was taken out of the heater at approximately 10°C prior to the target temperature, and if needed the unit was placed in a bath of cold water to maintain the system at the set temperature. The pressure and the temperature were monitored and recorded throughout the experiment. The system was

kept in the target temperature for 5 minutes. Since there was not any ammonia in the system, no pressure increase was observed during the experiment. After the experiment was completed the heat-treated samples were removed and kept at 4°C until further analysis. Table 4.4 shows the summary of the conditions used in this set of experiments.

Table 4.4. Conditions used in heat treatment of transgenic tobacco plant

Temperature	Moisture content (% dry weight basis)
60°C	20
	40
	60
70°C	20
	40
	60
80°C	20
	40
	60
90°C	20
	40
	60

Table 4.5 shows the related calculations and the conditions of the system during one of the heat treatment runs of transgenic tobacco plant material.

10g transgenic tobacco plant material (containing 1g water)			
6g distilled water was added for 60 % moisture content (dry weight basis)			
Elapsed time, min	Temperature, °C	Pressure, psi	Comments
0	28	0	Placed in heater
2	36	0	
4	45	0	
6	62	0	Taken out of heater
8	69	0	
12	70	0	End of the experiment

Table 4.5. Conditions during heat treatment of transgenic tobacco plants.

Ammonia treatment of the transgenic tobacco plants expressing Elcd

The prewetted samples were placed in the pressure vessel. The vessel was topped up with stainless steel pellets to occupy the void space and then the lid was bolted shut. The precalibrated sample cylinders were filled from the lecture bottle with the desired amount of warm ammonia to charge the system. To insure that the desired amount of ammonia was delivered, the reactor vessel was weighed before and after loading. Without heating, the vessel was left in the hood for 10 min; during the course of the experiment

only 10 to 20 psi pressure increase was observed. Since during the experiment some of the liquid ammonia converted to gaseous ammonia, in all of the experiments the system temperature was decreased, in some of them down to 28°C. Table 4.6 shows the summary of the applied conditions in ammonia treatment of transgenic tobacco plants.

Ammonia loading g of NH_3: g of dry biomass	Moisture content (% dry weight basis)
0.5:1	20 40 60
0.7:1	20 40 60
1:1	20 40 60

Table 4.6. Conditions of ammonia treatment of transgenic tobacco plants

An example of related calculations and the conditions during the ammonia treatment is presented in Table 4.7.

Table 4.7. Conditions during one of the ammonia treatment runs

16 g of transgenic tobacco plant (containing 1.6g water)			
4.16 g distilled water was added for 40 % moisture content (dry weight basis)			
Temperature of NH_3 was 33°C; 10 g NH_3 was added for 0.7:1 ammonia level			
Elapsed time min	Temperature °C	Pressure psi	Comment
0	34	95	No heating
2	32	100	
4	31	105	
6	29	105	
8	28	100	
10	26	100	The pressure was released

AFEX treatment of transgenic tobacco plants expressing E1cd

The prewetted samples with desired moisture contents were placed in the pressure vessel and AFEX treated exactly as described in chapter 3. The treated samples were allowed to stand in a fume hood overnight to evaporate the residual ammonia, and then kept at 4°C for further analysis. The samples were AFEX treated under various conditions, which are summarized in Table 4.8.

Table 4.8. AFEX treatment conditions for transgenic tobacco plants

g of NH_3 : g of dry biomass	20 % Moisture	40 % Moisture	60 % Moisture
0.5:1	T= 60°C T=70°C T=80°C	T= 60°C T=70°C T=80°C	T= 60°C T=70°C T=80°C
0.7:1	T= 60°C T=70°C T=80°C	T= 60°C T=70°C T=80°C	T= 60°C T=70°C T=80°C

Measuring the activity of E1cd enzyme in transgenic tobacco plant after treatments

The treated and untreated samples were ground and sieved to 40 mesh prior to the E1cd activity assay. The activity was measured as described above.

Results and Discussion

Transgenic tobacco plants (dried leaves) were treated under different range of temperatures (Table 4.3) or under different levels of ammonia (Table 4.5) to assess the individual effect of each AFEX variable on the E1cd enzymatic activity. All the results are the mean of two replicates. The effects of the different temperatures are presented in Figure 4.12.

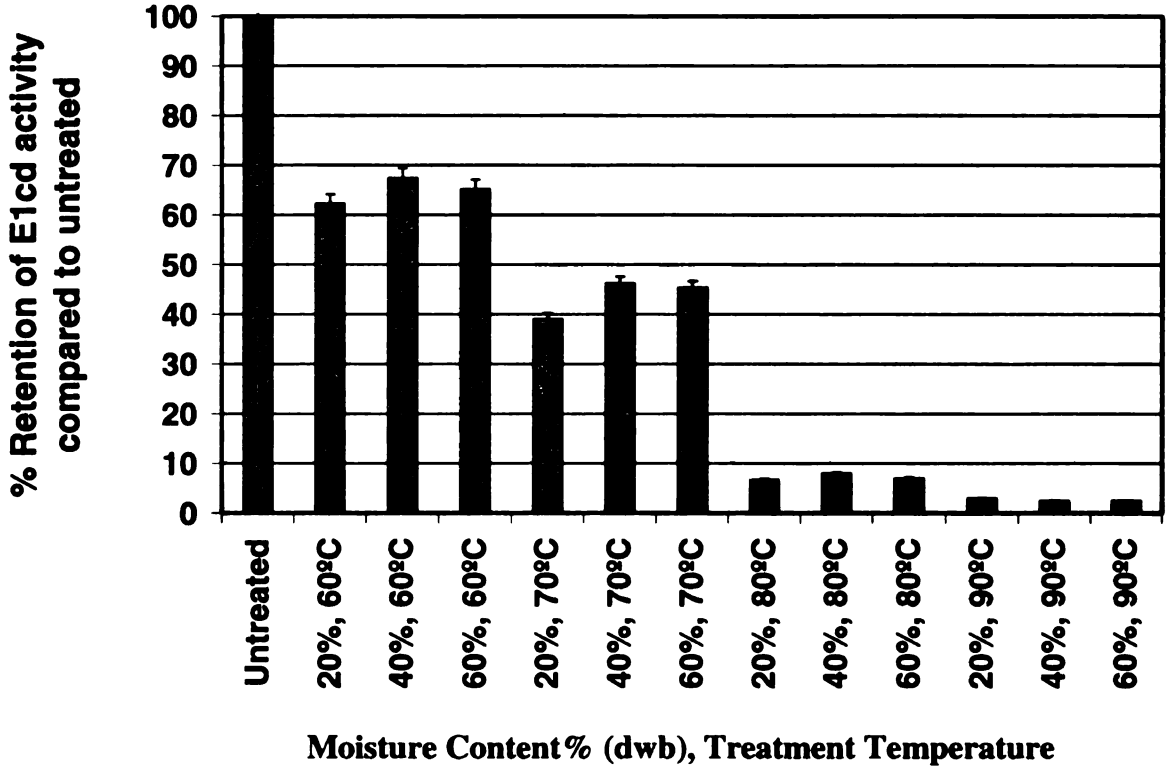


Figure 4.12. Effects of temperature on the activity of E1cd extracted from heat-treated transgenic tobacco plants.

Ziegelhoffer *et al.* (2001) tested the stability of apoplast-targeted Elcd in transgenic tobacco plants. In this study the apoplast-targeted Elcd enzyme extracted from tobacco plant along with the purified microbial Elcd were subjected to different temperatures (60°C-90°C) for 10 min. Both enzymes showed similar thermal stability throughout the experiment and as expected they both showed great heat resistance. Their results showed that at 60°C up to 95%, at 70°C up to 90%, at 80°C up to 80%, and at 90°C up to 40% of both enzyme activities were retained. But as seen in Figure 4.12, our heat stability test showed very different results from what Ziegelhoffer *et al.* (2001) reported. The Elcd extracted from transgenic tobacco plant treated at 60°C showed maximum 67% and at 70°C showed maximum 46% activity retention compared to Elcd extracted from untreated transgenic tobacco plant. The Elcd extracted from transgenic tobacco plant treated at 80°C and 90°C was almost totally inactivated. Based on these results and the previous study (Ziegelhoffer *et al.*, 2001), we believe that the Elcd extracted from transgenic plants is thermostable if it is heated in the extraction buffer (which has the enzyme's ideal pH 5.5) and its thermal stability drastically decreases if the expressing plants are heated directly.

The effect of ammonia on the activity of the E1cd enzyme was examined at different ammonia loading levels (Table 4.5) and the results are presented in Figure 4.13.

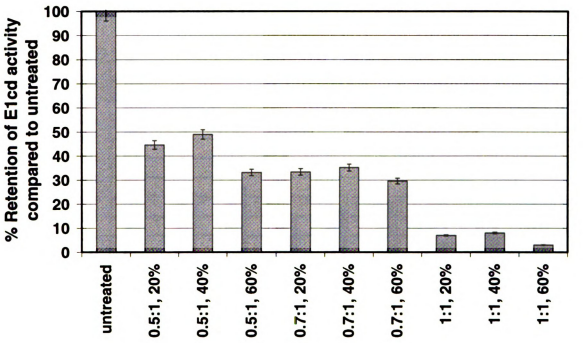


Figure 4.13. Effects of ammonia on the activity of E1cd extracted from ammonia-treated transgenic tobacco plants.

Figure 4.13 shows, at any moisture content, as the ammonia loading increases the percent of E1cd activity retention decreases and at 1:1 loading ratio the E1cd enzyme was almost totally inactivated. The highest percent of activity retention (49%) was observed at 0.5:1 ammonia loading with 40% of moisture.

The transgenic tobacco plants expressing E1cd were AFEX treated under different conditions (Table 4.7). The conditions of these experiments were selected based on the results of our heat and ammonia treatment of transgenic tobacco plants; we chose the conditions that showed higher enzymatic activity retention for E1cd. The results of these experiments are presented in Figure 4.14.

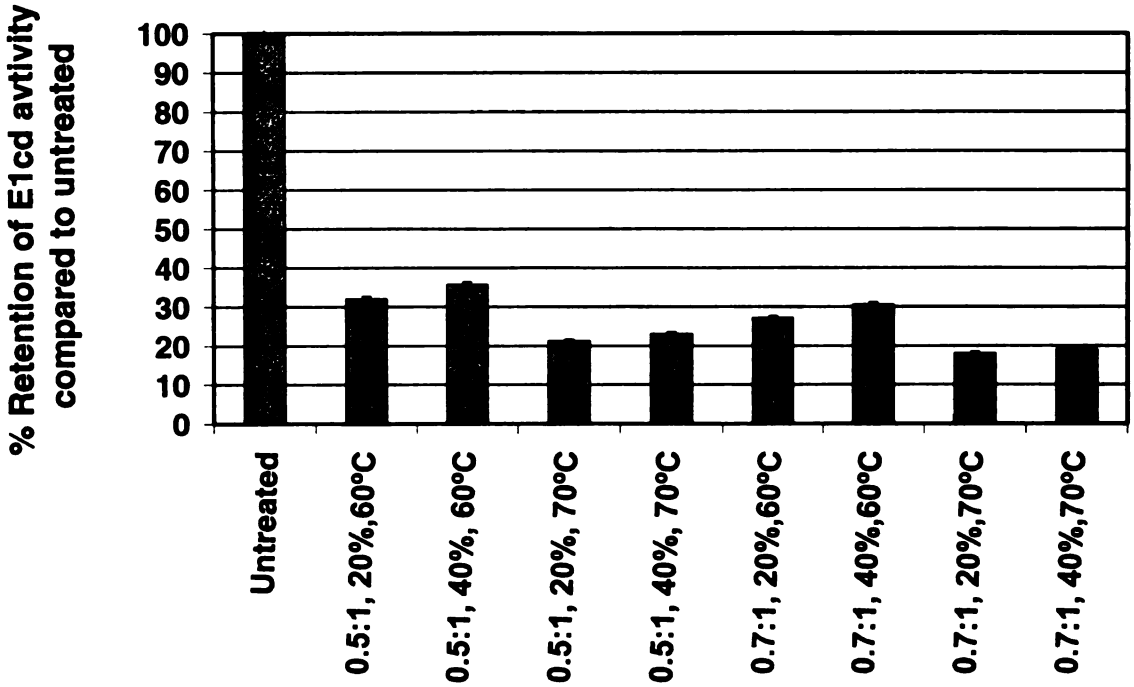


Figure 4.14. Activity retention of E1cd extracted from AFEX-treated transgenic tobacco plants.

E1cd enzyme showed a better survival rate with each heat or ammonia treatment (up to 67% and 49% respectively), but the combination of these conditions in AFEX treatment caused a drastic loss in the activity of E1cd extracted from AFEX-treated transgenic tobacco plants. The maximum observed activity retention in AFEX-treated

transgenic tobacco plant was only 35% at 60°C, 0.5:1 ammonia loading and 40% moisture.

Summary and Future work

Our research collaborator (Dr. Sticklen's group from Michigan State University) has used plasmids pSMF13, pSMF14, and pSMF15 for maize transformation to target CBHI to different organelles of maize plants. In addition to these plasmids they have also used a series of plasmid containing the *elcd* (provided by Dr. Austin-Phillips) to target Elcd to the cytosol, chloroplast, or apoplast. The transformed plants are still in the regeneration step and have not been tested yet. But based on the prior work (Ziegelhoffer *et al.* (2001)) we are expecting to observe similar results in terms of subcellular targeting effects on enzyme expression level, which is higher in apoplast than in the cytosol or chloroplast.

As we mentioned before, the pH of each compartment might have a significant effect on the stability of any particular heterologous protein targeted to each of these organelles. Considering this fact, one can suggest that one way to optimize the expression of any foreign proteins in transgenic plants is to direct them to the organelle with a pH most similar to their ideal pH.

Vacuoles are very conspicuous organelles with pH around 5.5. In some plants the vacuole may occupy as much as 95% of the plant cell volume (Spremulli, 2000). Since the ideal pH for both E1 and CBHI is around 5.5, directing these enzymes to vacuoles and comparing these results with prior work could provide important insights that can help to improve the production of these enzymes in transgenic plants.

Originally, pretreatment techniques were developed for various end uses of lignocellulosic biomass with emphasis on ethanol production. The pretreatments have been used to enhance enzymatic hydrolysis of biomass to increase ethanol production and improve lignocellulose conversion. Advances in biotechnology are enabling plants to become economically important systems for producing heterologous proteins such as cellulases. Therefore expanding the application of biomass pretreatment in releasing and recovering these proteins could have a significant impact on biorefinery economics. In this research we examined the potential of AFEX treatment in this regard. The data show that the maximum Elcd activity retention in AFEX-treated transgenic tobacco plant (under 60°C, 0.5:1 ammonia loading and 40% moisture conditions) was only 35%, and the glucan conversion of the corn stover treated under the same conditions was only 47% of theoretical yield with 60 FPU/ g of glucan of cellulase (chapter 3). Based on these findings, it is our opinion that AFEX pretreatment is not a suitable option for releasing cellulase from transgenic plants. Considering the fact that other biomass pretreatments (e.g. steam explosion, acid treatment) operate under even harsher conditions, it is reasonable to postulate that none of these pretreatments are suitable choice for this purpose. Using biomass pretreatment for releasing cellulase from transgenic plants, was only one our three technical options; therefore, exploring the potential of other options (mentioned in the introduction) deserves attention in future work.

Chapter 5

The pea (*Pisum sativum* L.) rbcS transit peptide directs the *Alcaligenes eutrophus* polyhydroxybutyrate enzymes into the maize (*Zea mays* L.) chloroplasts

Heng Zhong, Farzaneh Teymouri, Brad Chapman, Shahina Bano Maqbool, Robab Sabzikar, Yahia El-Maghraby, Bruce Dale and Mariam B. Sticklen, (2003). Plant Science In Press

Abstract

With a concept of producing polyhydroxybutyrate (PHB), a biodegradable thermoplastic polymer, in maize chloroplasts, over 200 independent transgenic maize plants were produced. Five different constructs pPHBA, pPHBB, pPHBC and pBY520 or pJS101 were used in transformation experiments. PHB constructs contained *phb* genes from *Alcaligenes eutrophus* fused with the pea rbcS transit peptide plus the first 24 amino acids of the mature rbcS protein. Plasmids BY520 and JS101 contained the selectable marker *bar* gene linked with abiotic stress-related gene either *hva1* or *mtld*. Southern blots on putative transgenic plants confirmed that the transgenic plants harbor transgenes from all five constructs. Transcription of transgenes was confirmed by northern blot analyses. Western blot analyses using total cellular protein and protein from isolated chloroplasts confirmed that all three genes produced their corresponding enzymes, and all PHB enzymes were targeted into maize chloroplasts. Immunofluorescent localization on both transgenic and non-transgenic maize chloroplasts treated with PHBC-antiserum also confirmed the targeting of PHBC enzyme to the chloroplasts of transgenic maize.

Key words: Chloroplasts; Maize transformation; PHB; rbcS - transit peptide

Abbreviation: Rubisco = ribulose 1-5, bisphosphate carboxylase

1. Introduction

Polyhydroxybutyrate (PHB), first characterized in *Alcaligenes eutrophus*, is a biodegradable polymer, which can be completely degraded by enzymatic activities in the soil [1]. There are three enzymes needed for production of PHB polymer. These enzymes include 3-ketothiolase (PHBA), acetoacetyl-CoA reductase (PHBB), and polyhydroxybutyrate synthase (PHBC). Genes encoding these three enzymes were isolated from *A. eutrophus* and were transferred to the nuclear genome of *Arabidopsis thaliana* [1], where granules of PHB were produced in the cytoplasm of leaf mesophyll cells of this plant. However severe growth inhibition and yield reduction were observed in this research. In a follow up experiment, Nawrath et al. [2] used the pea rbcS transit peptide and the first 24 amino acid coding sequence for targeting all three PHB enzymes into the *A. thaliana* chloroplasts. In this follow up experiment, the PHB polymer was produced at a high level in the chloroplast without any reduction in plant growth and/or seed yield.

Use of transit peptide (TP) of Rubisco small subunit (rbcS) has become a routine practice to transport proteins into chloroplasts of transgenic plants. Corbin et al. [3] used the transit peptide from the *Arabidopsis* 1A Rubisco small subunit gene plus transit peptidase cleavage site to target cholesterol oxidase into chloroplasts of transgenic tobacco plants. In another event Comai et al. [4] used the pea rbcS transit peptide plus the first 24 amino acid coding sequence for targeting the 5-enolpyruvyl 3-phosphoshikimate (EPSP) protein into the chloroplasts of transgenic tobacco plants. Although literatures have shown that the transit peptide of pea is sufficient to direct foreign polypeptides both *in vitro* and *in vivo* in dicot [5,6] and monocot [7] plants, Comai et al. [4] showed that

chloroplast uptake also requires the first 24 amino acids of the mature rbcS protein in addition to the transit peptide. In all of the above experiments, genes were transferred into the plant nuclear genome while enzymes were targeted into the chloroplasts.

Since, the chloroplast genome has limited coding capacity, many chloroplast proteins are synthesized in cytoplasm by cytoplasmic ribosomes in the form of larger precursors. The precursors consist of an amino terminal extension referred to as a transit peptide and a carboxyl terminal portion. The import of the protein from cytoplasm to chloroplasts is initiated via binding of the precursor to a proteinaceous receptor followed by translocation across the membrane via an energy requiring mechanism. Inside the chloroplast, a stromatic protease cleaves the transit peptide, leaving the mature protein [8]. The most abundant protein in the chloroplast is Rubisco. The large subunit of Rubisco is encoded in chloroplast DNA but the small subunit is a nucleocytoplasmic product that is imported post translationally into the chloroplast [9].

Over the past few years progress in plastid transformation technology has showed the feasibility of chloroplast transformation. Chloroplast transformation opens the possibility of expressing multiple genes or entire biosynthetic pathway in a single transformation event [10]. Plastid transformation technology in higher plants was first developed for tobacco plants [11]. But it has been shown that extending this technology to other species especially important crops such as maize is still challenging [12].

Here, we chose to transform PHB constructs (PHBA, PHBB and PHBC) into maize nuclear genome and targeted the PHB enzymes inside the chloroplast. This is the first report about targeting of the PHB enzymes into a monocot chloroplast using a dicot

transient peptide plus the first 24 amino acids of the Rubisco small subunit mature protein.

2. Materials and methods

2.1. Plant materials

Honey N Pearl sweet corn genotype was used in our transformation experiments on the basis of its best response *in vitro* regeneration ability and fertility following our previous work [13,14].

Shoot tip clumps were obtained *in vitro* from the apical meristems of 7-day-old maize seedling as described [13]. Shoot tips with one to three visible leaf primordia were selected under a stereomicroscope (Carl Zeiss, Germany) for microprojectile bombardment. To obtain a better exposure of shoot meristems to the bombardment, leaf primordia were physically removed and discarded. These explants were placed in a circular area of about 1.5 cm diameter in a Petri dish containing phytagel-solidified culture medium prior to bombardment.

2.2. Plasmids

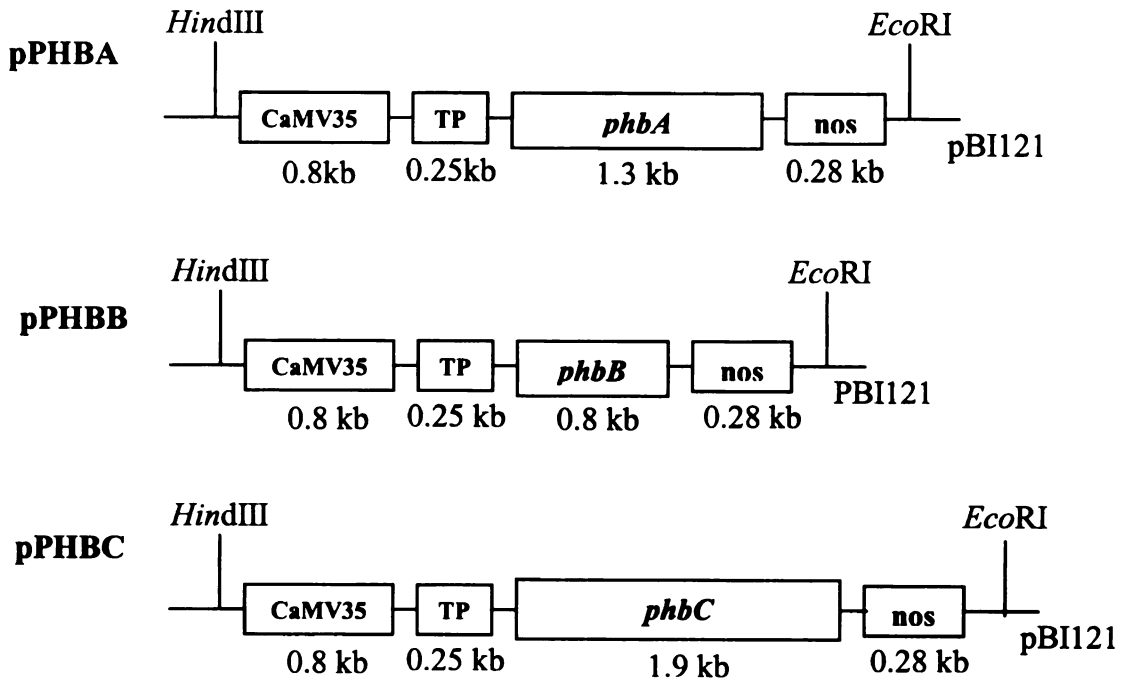
Three plasmids: PHBA, PHBB and PHBC were used to transfer *phb* genes into maize shoot apical meristems (Fig. 1). The expression of all three genes were driven by the CaMV35S promoter and terminated with nos 3' region [1]. Each of these plasmids was co-transformed with either pBY520 or pJS101 individually. The plasmid BY520

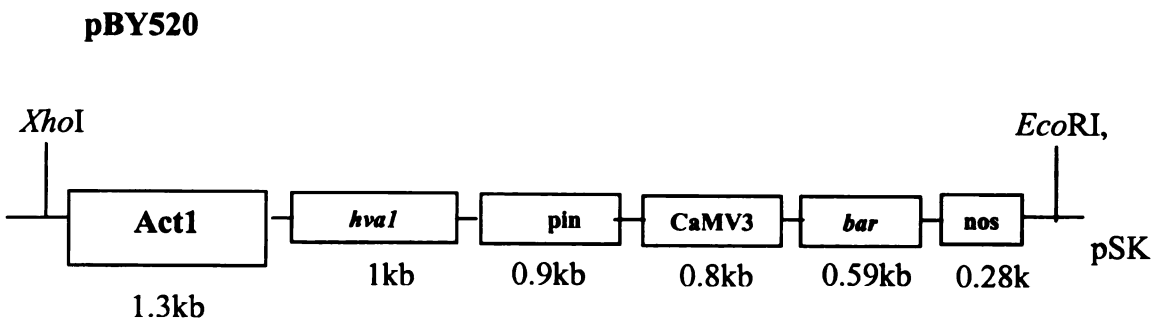
contains selectable marker *bar* gene and abiotic stress-related *hva1* gene [15]. The plasmid JS101 contains the *bar* gene and another abiotic stress-related *mtlD* gene [16]. In both plasmids, *bar* gene expression was driven by the CaMV35S promoter and terminated with nos 3' region [17]. In pBY520 and pJS101, the *hva1* and *mtlD* coding regions were driven by a region of 1.3 kb upstream of the rice Act1 translation codon and terminated with pin II 3' region.

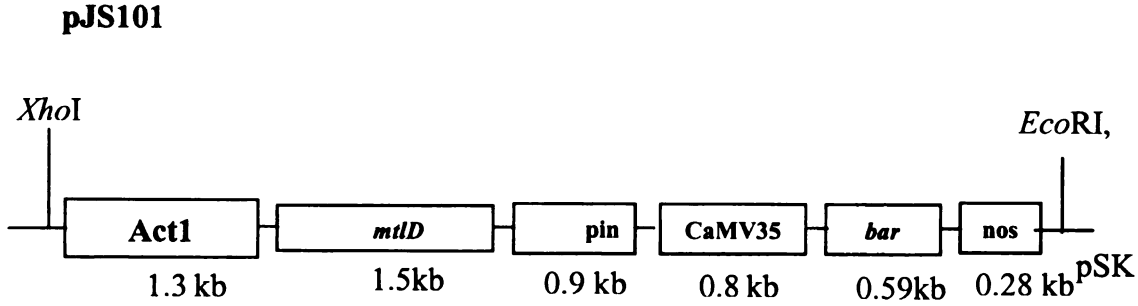
Abbreviation in Figure 1:

CaMV35S: Cauliflower Mosaic Virus 35S promoter; TP: Transit peptide of the small subunit of Rubisco of pea plus the first 24 amino acids of rbcS mature protein; *phbA*: gene of *Alcaligenes eutrophus* encoding the 3-ketothiolase; *phbB:* gene of *Alcaligenes eutrophus* encoding the acetoacetyl-C0A reductase; *phbC*: gene of *Alcaligenes eutrophus* encoding the PHB synthase; Act1: rice Act1 gene promoter, which includes the 5' intron region; *bar*: phosphinothricin acetyl transferase (selectable marker/herbicide resistance) gene; *hva1*: LEA3 gene from barley; *mtlD*: mannitol 1-P dehydrogenase gene from *E.coli*; pin: potato protease inhibitor II gene 3' non-coding region; nos: 3' region from *Agrobacterium* nopaline synthase gene.

Figure 1. Plasmid constructs used in maize transformation.







2.3. Microprojectile bombardment

Plasmid DNA was precipitated onto tungsten particles following the protocol described previously [14]. The bombardments were carried out using a biolistic particle acceleration device (PDS 1000/He, Du Pont,Wilmington, DE) under a chamber pressure of 26 mm of Hg at different distances. The distance between the rupture disc to the macrocarrier was 1.5 cm, to the stopping screen was 2.0 cm and to the target was 6.5 cm. The meristems were bombarded twice with 1.0 μm DNA coated tungsten particles as described [14] with a density of 150 μg per shot and 1550 psi acceleration pressure.

2.4. Selection and regeneration of transformants

Bombarded shoot tips were cultured on the MS medium [18] containing 2 mgl^{-1} N6-Benzyladenine (BA) and 0.5 mgl^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) for 4 weeks. The clumps then were divided and subcultured on the same medium supplemented with 3 mgl^{-1} of glufosinate ammonium for selection for 4 weeks. The green clumps were selected, divided and subcultured on the same medium containing 5 mgl^{-1} glufosinate ammonium at 4-week intervals. Plant regeneration was obtained by transferring the selected shoot-tip clumps to MS medium containing 0.5 mgl^{-1} BA, 0.5 mgl^{-1} Indole-3-butyric acid (IBA), and 10 mgl^{-1} glufosinate ammonium. Regenerated shoots (5-10 cm tall) were rooted on MS medium containing 1 mgl^{-1} IBA and 10 mgl^{-1}

glufosinate ammonium. Plantlets were transferred to pots containing a soil mixture of 1:1 (v/v) peat:perlite and grown to maturity in a green house.

2.5. DNA isolation, PCR and Southern blot analyses

Genomic DNA from glufosinate ammonium resistant plantlets was extracted as described [19]. PCR analyses were carried out using specific primers for *phbA*, *phbB* and *phbC*. The sequences of the primers were as follows: for *phbA*, forward primer 5′-GCGTCAAGCCGGAGCAGGTG-3′, reverse primer 5′-GATCGTGGCCAGCGGGG TCAGG-3′; for *phbB*, forward primer 5′-CCGGCGGCATGGGTGGTATC-3′, reverse primer 5′-CGGCCTTGGCGGTGGAGTAGTTG-3′; for *phbC*, forward primer 5′-CTGC CGCGTTCTACCTGCTCAATG-3′ and reverse primer 5′-CACCGTATGTCCC TGCTC CACCAC-3′. The DNA was subjected to PCR using a GeneAmp PCR system (Perkin Elmer, Norwalk, CT) for 3 min at 94 °C following by 40 cycles of 30 s at 94 °C, 1 min at 62 °C and 1 min at 72 °C and ending with 5 min at 72 °C. Amplified DNA was electrophoresed in a 0.8% agarose gel and visualized under UV.

A modified C-TAB method [20] was used to isolate high molecular weight DNA from control as well as putative transgenic plants. Southern blot hybridization was performed as described [21]. Twenty μg of plant DNA from each sample was restriction digested overnight with either *Hin*dIII alone or in combination with *Eco*RI. DNA was electrophoresed and transferred onto a piece of pre-wetted Hybond N^+ nylon membrane

(Amersham, Piscataway, NJ). Gene-specific probes were generated using *Eco*RI/*Sma*I digest of pPHBA, pPHBB and pPHBC to isolate fragments containing *phbA*-, *phbB*- and *phbC*- coding sequences. Restriction fragment was purified using the QIAquick kit (QIAGEN, Valencia, CA), and labeled with α-[^{32}P]-dCTP using Rad Prime labeling kit (GIBCO, Rockville,MR) according to the manufacturer's instructions. Membranes were hybridized at 68 °C overnight using specific probes and analyzed by autoradiography on Kodak X-OMAT film at –80 °C.

2.6. RNA isolation, northern blot analyses

Total RNA was isolated using Extract-A-Plant™ RNA Isolation Kit (Clontech, Palo Alto, CA) and treated with DNAase I (Gibco) to eliminate any traces of genomic DNA.

For northern blots, 20 µg of RNA were fractionated in 1.2% agarose-formaldehyde denaturing gels and blotted onto Hybond-N nylon membranes (Amersham) as described [22]. Gene specific probes used for Southern analyses were also used for northern hybridization.

2.7. Protein isolation and western blot analyses

Total leaf protein extraction: One gram of fresh leaf tissue from each sample was pulverized in liquid nitrogen and extracted in 0.4 ml of protein extraction buffer (Phosphate-buffered saline (PBS), 10% glycerol, 10 mm DDT). The concentration of

total soluble protein in each sample was measured according to Bradford [23] using bovin serum albumin as standard.

Extraction of Chloroplast protein: The intact chloroplasts were isolated as described [24]. Intact chloroplasts were lysed for protein extraction by resuspension in 40 µl of deionized water and incubated for 3 min at 4°C. Then 40 µl of 2X protein extraction buffer was added. The concentration of total soluble protein in each sample was measured according to Bradford [23] using bovin serum albumin as standard.

Seventy µg of total leaf protein or chloroplast protein from each sample were loaded side by side in SDS polyacrylamide gels lanes. The electrophoresed gels were transferred to the nitrocellulose membrane (Amersham) using Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The membranes were incubated with primary antibody raised against each of the PHB enzymes (kindly provided by Dr. Poirier) in recommended dilution and then with alkaline phosphatase conjugated anti-rabbit IgG (Sigma, St. Louis, MO) as the secondary antibody following by washing steps according to standard procedures [22]. Protein bands were visualized in alkaline phosphotase substrate buffer (Promega).

Transgenic *Arabidopsis* (homozygous) plants, expressing *phbA*, *phbB*, and *phbC* genes (provided by Dr. Somerville) were used as positive controls in our western blots. Proteins from these plants were isolated as described by Nawrath et al. [2].

2.8. Immunofluorescent localization and visualization of the PHB enzymes in chloroplasts

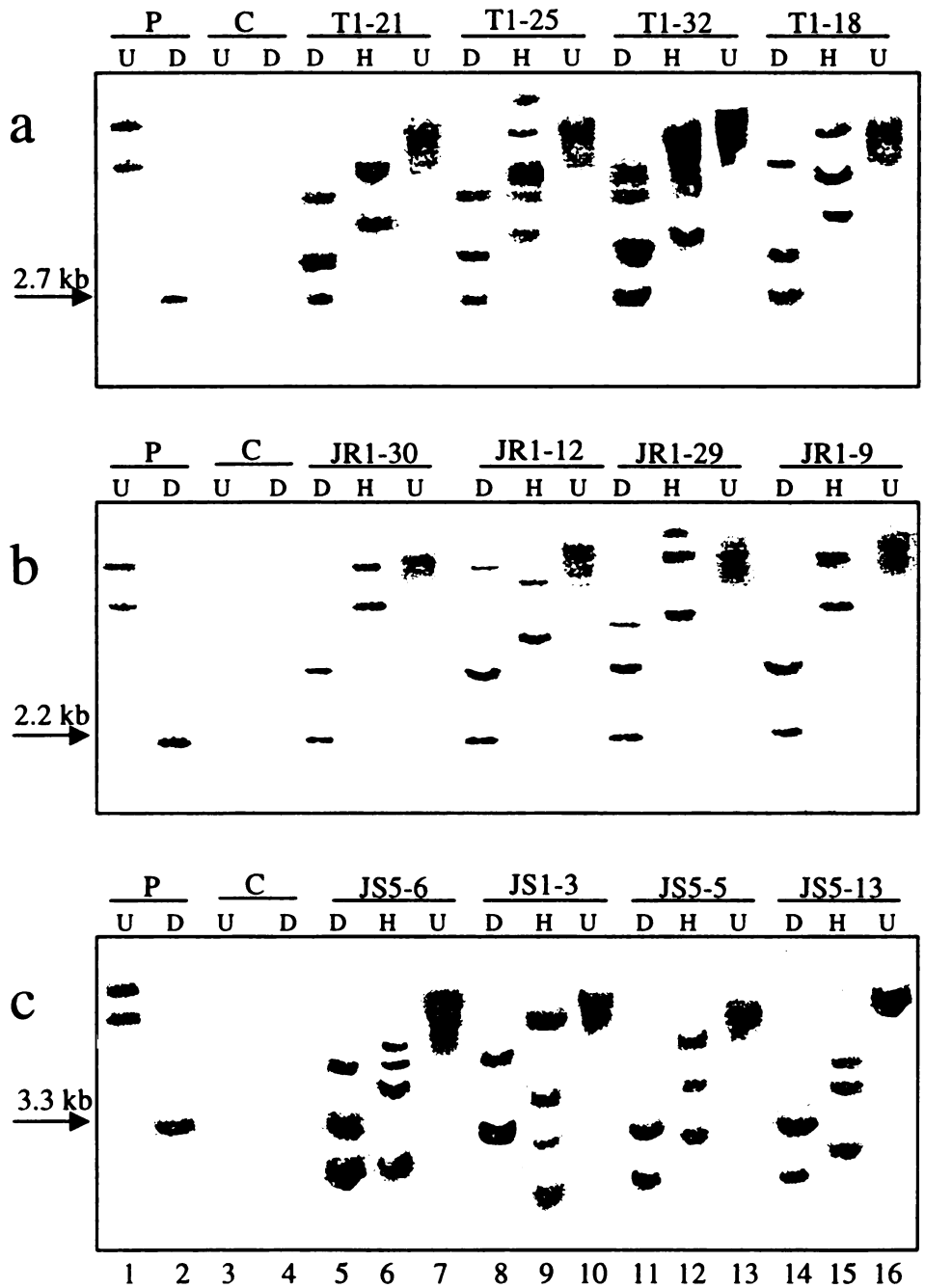
Intact chloroplasts of non-transgenic and transgenic plants were isolated as described [24]. The chloroplasts were resuspended in 200 µl of cold buffer B (0.3 M sucrose, 50 mM Tris- HCL, 20 mM disodium EDTA, pH 8.0) and incubated with the primary antiserum raised against the PHBC enzyme for 3 h, then centrifuged at 1000g for 5 min. The pellet was washed with buffer B and incubated with Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) as the fluorescent probe for 2 h. After centrifugation at 1000g for 5 min, the pellet was washed with buffer B and pelleted again then the pellet was resuspended in 50 µl of buffer B. Ten µl of the suspension was loaded on a glass slide and left for 5 min to dry out. A drop of Prolong Antifade (Molecular Probes) was added to the dry sample, and then the sample was covered with a coverslip. The slides were viewed with a Zeiss 210 confocal laser-scanning microscope with a filter to detect Alexa fluor 488.

3. Results

Over 5,000 plantlets containing a total of 240 independent transformation events resistant to glufosinate ammonium were recovered from maize meristem clumps bombarded with one of the three *phb* genes, the *bar* selectable marker gene and either *mtlD* or *hva1* genes.

The PCR analysis on glufosinate ammonium resistant selected plantlets indicated the presence of the *phb* genes (*phbA*, *phbB* and *phbC*) in the analyzed transgenic plants (data not shown). Further, confirmation on *phb* genes integration into maize genome was made by Southern blot analyses (Fig. 2). Southern blots using DNAs digested with *Hin*dIII revealed the integration of 3-4 copies of *phb* genes (Lane H; Fig. 2) and the DNAs digested with *Hin*dIII-*Eco*RI confirmed the presence of complete *phb* gene cassettes into the maize genome. (Lane D; Fig. 2).

Figure 2. Southern blots of independent transgenic maize lines containing *phbA* (a), *phbB* (b) and *phbC* (c) genes. P: plasmid DNA: a) PHBA, b) PHBB and c) PHBC; C: control (untransformed maize plant); U: undigested; D: digested with *Hin*dIII/*Eco*RI; H: digested with only *Hin*dIII; lanes 5-16: independent transgenic maize lines.



Northern blot analyses confirmed the presence of mRNA for the *phb* genes at detectable levels in the Southern blot positive transgenic lines (Fig. 3). Western blot analyses confirmed the PHBA, PHBB and PHBC enzymes syntheses as the result of translation of the *phbA, phbB and phbC* genes (Fig. 4). Visual comparison was performed between the total protein isolated from chloroplasts (lane C; Fig. 4) and total protein from the whole leaf tissue (lane T; Fig. 4) based on the band intensity of each of the PHB enzymes. The whole-leaf protein and the chloroplast protein extracts from each transgenic plant showed that the level of the PHB enzymes in the chloroplast extracts were much higher than the PHB enzymes in the whole-leaf tissue extracts (Fig. 4). Immunofluorescent localization on both transgenic and non-transgenic maize chloroplasts treated with the PHBC-antiserum also confirmed the targeting of the PHBC enzyme to the chloroplasts of transgenic maize (Fig. 5).

Figure 3. Northern blot analyses of independent transgenic maize lines containing: *phbA* (a), *phbB* (b) and *phbC* (c) genes. C: untransformed maize plant; lanes 1-6: independent transgenic maize lines.

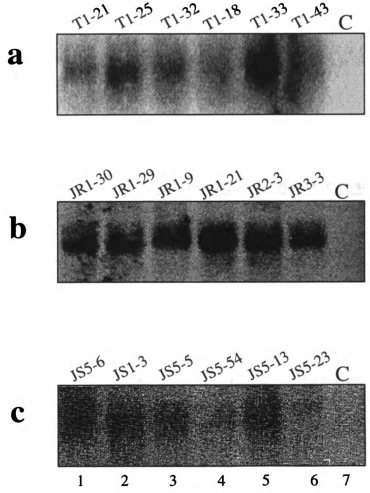


Figure 4. Immunoblot analyses of independent transgenic maize lines expressing: PHBA (a), PHBB (b) and PHBC (c). M: marker; P: positive protein (transgenic Arabidopsis); C: chloroplast protein; T: total leaf tissue protein; lanes 2-5: independent transgenic maize lines.

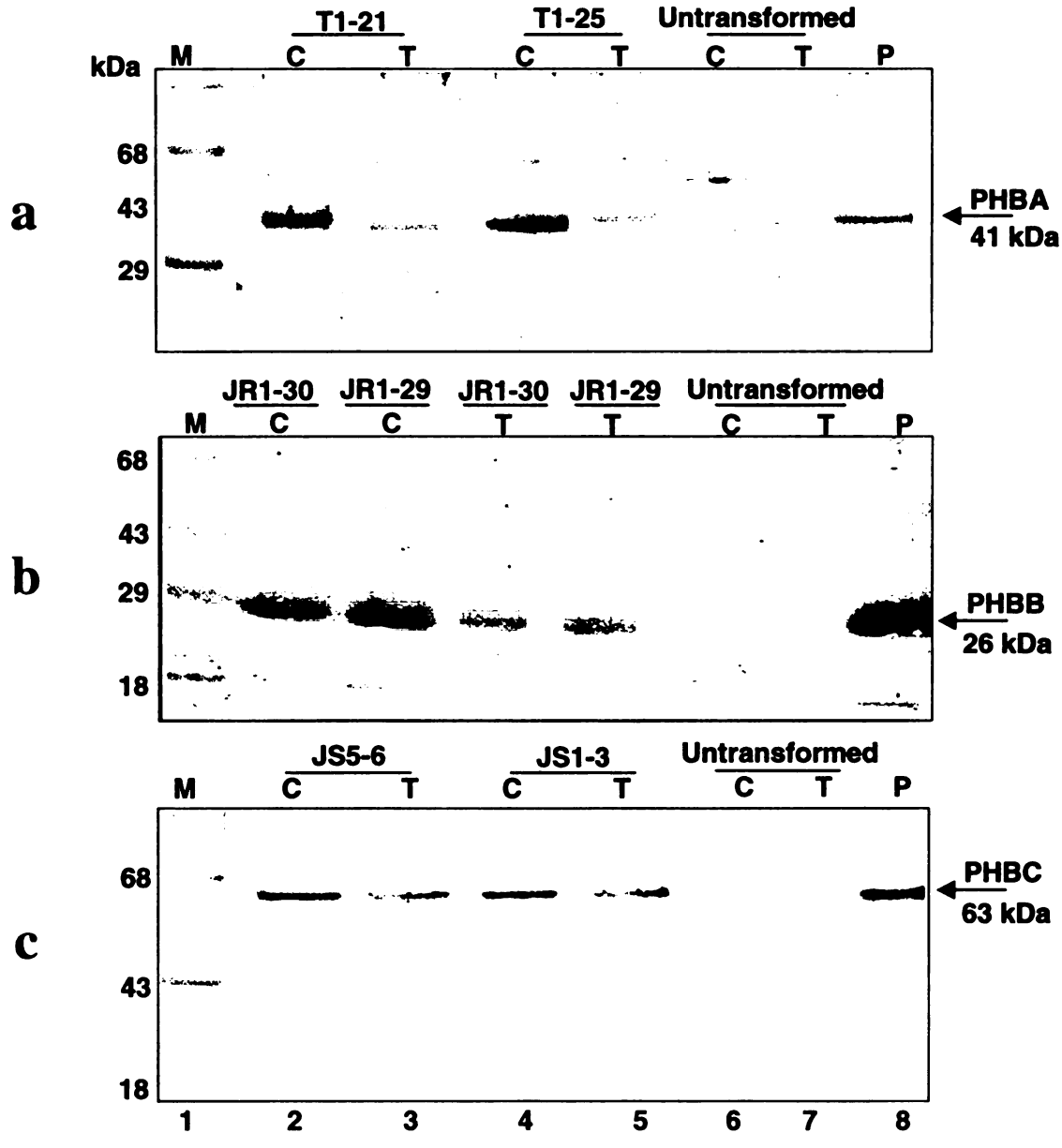
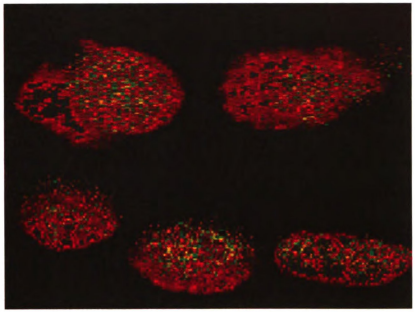
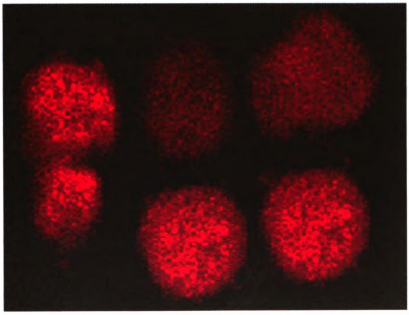


Figure 5. Immunofluorescent localization of PHBC enzyme: Chloroplasts of transgenic maize transformed with *phbC* gene and treated with PHBC-antiserum (a); Chloroplasts of control untransformed maize plants treated with PHBC-antiserum (b). (Please note that green dots are showing the PHBC enzyme, red shows chloroplast, and yellow dots are the overlaps of PHB enzymes with grana (contains chlorophyll) of chloroplasts).

a



b



4. Discussion

Here, we have expanded the application of the maize meristem transformation system and the *bar* gene selection scheme formulated in our laboratory, which proved to be effective in linked to unlinked co-transformation of maize [14,25].

It has been shown that the best way to optimize a metabolic pathway in plants is to direct the foreign proteins to organelles where substrate is most available [26]. Acetyl Co-A is the substrate needed in the pathway of the PHB synthesis. Since, there is a high amount of acetyl-coA substrate in chloroplast [2], we chose to direct the PHB enzymes into maize chloroplasts.

We used a transit peptide to target the PHB enzymes into the maize chloroplasts. As expected, chloroplasts of the transgenic plants showed higher level of the PHB enzymes accumulation as compared to the PHB accumulation in whole-leaf tissue (Fig. 4). The localization of the PHBC enzyme in chloroplasts was confirmed using immunofluorescent microscopy (Fig. 5).

According to Young et al. [27], the similarity between dicot and monocot rbcS transit peptides is very low (24-38%). But on the other hand, the similarity between the first 24 amino acids of rbcS mature protein of dicot and monocot is relatively high (66-79%). The result of our report suggests that in this chloroplast protein targeting, the low similarity (only 21%) between rbcS transit peptide of pea and maize may not be as important as the role of their high similarity (79%) in their first 24 amino acids of rbcS mature protein (Table 1; Table 2).

Table 1. Comparison of rbcS transit peptide sequence of Maize and Pea

Plant	Amino acid sequence	Total
Maize[a]	M A P T V M M A S S A T A V A P F Q G L K S	22
Pea[b]	M A S - M I S S S A V T T V S R A S R G Q S	22
Maize	A A S L P V A R R S T R S L G N V S N G G R	44
Pea	A A V A P F G G L K S M T G F P V - K K V N	44
Maize	I R C	47
Pea	T D I T S I T S N G G R V L C	59

[a] [27]
[b] [28]

Table 2. Comparison of the first 24 amino acid of Rubisco small subunit sequence in Maize and Pea

Plant	Amino acid sequence	Total
Maize[a]	M Q V W P A Y G N K K F E T L S Y L P P L S T D	24
Pea[b]	M Q V W P P I G K K K F E T L S Y L P P L T R D	24

Since the production of the PHB biodegradable plastic for commercial use in microbes is very costly [1], a promising alternative to this is to produce PHB polymer in transgenic plants. We chose maize because it is one of the major U.S. crops, a C-4 plant with large chloroplasts, and a more rapid rate of photosynthesis than C-3 species.

The targeting of the PHB enzymes in maize using the pea rbcS transit peptide is our first report towards the production of the PHB biodegradable plastic in maize. The crossbreeding of the transgenic plants for combining the three *phb* loci into a single maize genotype followed by backcrossing of the three *phb* loci into elite open-pedigree maize inbred lines is in progress.

Acknowledgement

We wish to thank Dr Chris Somerville for providing the PHB constructs and seeds of transgenic *Arabidopis*, Dr. Ray Wu for pBY520 and pJS101 constructs, and Dr. Yves Poirier for the gift of polyconal antiserum to each of the PHB enzymes. We would also like to thank Dr. Joan Whallon for viewing the chloroplast slides by confocal laser-scanning microscope and Dr. Barbara Sears for the review of this manuscript.

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Appendix A

Effects of Ammonia Fiber Explosion (AFEX) on activity of Rubisco

Effects of Ammonia Fiber Explosion (AFEX) on activity of Rubisco

Abstract

Ammonia fiber explosion treatment has shown a great potential to be used in integrated processing that allows more protein extraction from biomass as well as increasing the digestibility of biomass compared to untreated material. It would be beneficial to determine whether AFEX treatment affects the native functional properties of the extracted proteins. In this research we have chosen ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme from alfalfa and tobacco plant as our model protein to investigate the effects of AFEX treatment on the functional properties of protein. These effects were evaluated by measuring and comparing the activity of Rubisco enzyme in AFEX treated and untreated alfalfa and tobacco samples.

Introduction

Production of fuel and chemicals from lignocellulosic biomass such as agricultural crops and residues, has received a great deal of attention. These lignocellulosic materials have also been investigated as potential animal feedstuffs. A main obstacle in utilizing lignocellulosics for either feedstuffs or fuel/chemical production is the relatively unreactive nature of cellulose. Many biomass pretreatments have been used to increase the digestibility of the biomass to improve feed quality and also increase the yield of fermentable sugars in biomass enzymatic hydrolysis (Weaver, 1998; Gollapalli, 2001). In addition to cellulose, hemicellulose and lignin, biomass contains protein. Protein is potentially the most valuable fraction of biomass (De La Rosa

et al. 1994); therefore it provides an important byproduct credit in biomass refining. However, little attention has been focused on protein recovery. Plant proteins are comparatively fragile component of biomass; their potential value can be decreased by harsh processing steps. A fully integrated biomass process, which improves protein recovery while preserving protein native functional properties and increasing the conversion of cellulose and hemicellulose to fermentable sugars may significantly enhance biomass process economics. Unfortunately most of the biomass pretreatments operate under harsh conditions (e.g., steam explosion and various acid processes) that tend to degrade the proteins. However, the AFEX process operates under relatively mild conditions and has shown good potential for protein recovery from herbaceous crops with simultaneous conversion of cellulose and hemicellulose to simple sugars (De La Rosa *et al.* 1994). However, the quality of the recovered protein from AFEX treated biomass, in terms of their native functional properties such as enzyme activity has never been examined.

The predominant protein in leaf tissue is Rubisco, the key enzyme in photosynthesis and photorespiration. Rubisco catalyses the initial reactions of both the reductive pentosephosphate and photorespiration cycles. Rubisco is the most abundant soluble protein in the chloroplast and probably the most abundant protein on Earth (Malkin and Niyogi, 2000). Therefore, in this report we have chosen Rubisco as our plant protein model to investigate the potential of AFEX treatment in recovering bioactive protein from biomass. This potential was evaluated by measuring and comparing the activity of Rubisco enzyme in AFEX treated and untreated alfalfa and tobacco samples.

Material and Methods

Plant material

Dried alfalfa plant materials (harvested in north central Kansas in spring 2001) were provided by Wenger manufacturing Company. Plant materials were AFEX treated as described in chapter 3. The treated samples were left in a fume hood overnight to evaporate the residual ammonia. The next day the treated and untreated samples were ground and sieved to 40 mesh to ensure well mixed samples. Samples were kept in sealed plastic bag in the refrigerator for further analysis.

In addition to alfalfa, we also studied Rubisco in tobacco plants. Tobacco plants were grown, harvested and dried at the Crop and Soil Sciences greenhouses at Michigan State University. The dried tobacco leaves were treated by the AFEX process and stored as described above. The AFEX process conditions in alfalfa and tobacco treatment are summarized in Table A.1.

Table A.1. Summary of AFEX treatment conditions for alfalfa and tobacco plants

In all the runs ammonia loading ratio was 1:1

Sample#	Temperature	Moisture Content (%)	Plant material
1			Untreated Alfalfa
2	60°C	60	Alfalfa
3	70°C	60	Alfalfa
4	80°C	60	Alfalfa
5	90°C	60	Alfalfa
6			Untreated Tobacco
7	60°C	60	Tobacco
8	70°C	60	Tobacco
9	80°C	60	Tobacco
10	90°C	60	Tobacco

Chemicals

All the required chemicals for Rubisco extraction, Rubisco activity and Enzyme Linked Immunosorbent (ELISA) assay were provided from Sigma (St. Louis, MO)

Rubisco activity assay

Rubisco was extracted from plant materials according to Metodiev and Demirevska-Kepova (1992) with some modifications and Rubisco activity was measured as the rate of ^{14}C incorporation into acid-stable products in a 1 min assay at 25°C according to Catt and Millard, (1988).

Fifty milligram of AFEX-treated and untreated alfalfa samples were ground to a fine powder with mortar and pestle. The powder was homogenized with the aid of a power drill using 1.5 ml microcentrifuge tubes and plastic pestles (Kontes), in 4 ml extraction buffer (0.1 M Tris-HCl (pH 7.8), 0.1 mM EDTA, 1.5% polyvinyl-pyrrolidone-40T, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfonylfuoride (PMSF)) for one minute. The extraction buffer was prepared to be CO_2-free by gently boiling the distilled water for 15 min prior to addition of reagents, all the solutions were also bubbled with nitrogen gas to make sure the system is O_2-free. The homogenate was filtered through two layers of cheesecloth. The extract was brought to 37% saturation with solid ammonia sulfate (226g/liter of extract) and after standing for 1 hour was centrifuged at 9000 g for 30 min. The supernatant solution was brought to 50% saturation with solid ammonia sulfate (92.5g/liter). After centrifugation as described above, the precipitate was dissolved in 1 ml of extraction buffer. The extracted Rubisco was used in activity assay and ELISA assay. The concentration of total soluble protein in each sample was measured according to Bradford (1976) using bovin serum albumin as the standard. Total

activity was measured by adding an appropriate volume of each sample (equal to 50μg of total protein) to a sufficient volume of the activation mixture consisting of 100 mM $NaHCO_3$ and 200 mM $MgCl_2$ to bring to a final concentration to 10 mM $NaHCO_3$ and 20 mM $MgCl_2$. The mixture was incubated for 15 min at 25°C to activate any unactivated Rubisco. Then 50 μl of activated enzyme solution was added to 450 μl of assay buffer consisting of 0.1 M Tris-HCl (pH 8), 20 mM $MgCl_2$, 1 mM EDTA, 13mM $NaH^{14}CO_3$ and 0.5 mM ribulose biphosphate at 25°C. The reaction was terminated after 60 s with 100 μl of 2 N HCl. The samples were dried then acid-stable ^{14}C-radioactivity determined with a 1500 TRI CARB liquid scintillation counter (PACKARD, Downer Grove, IL). The activity was determined based on the comparison of the readings with the standard curve that was made for different concentration of $NaH^{14}CO_3$.

ELISA assay

An enzyme linked immunosorbent assay (ELISA) has been used to measure Rubisco concentration. The assay relied upon Rubisco in crude extract of plant material competing with pure Rubisco for a solid phase antibody, so the antigen was detected by a decrease in reaction product over a high background. ELISA method provides accurate and easy determination of the quantity of Rubisco in plant leaves (Catt and Millard, 1988).

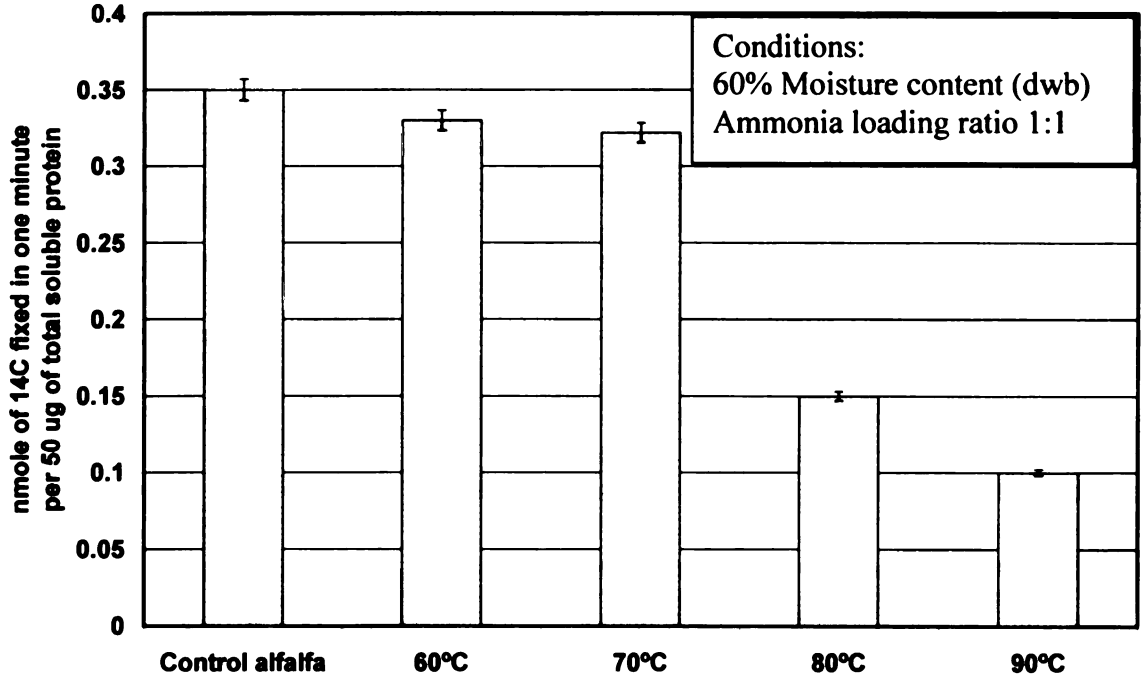
In this study ELISA assay was conducted as described by Catt and Millard, (1988). The Rabbit anti-Rubisco antiserum used in this assay was provided by Dr. Sticklen, Michigan State University. The amount of Rubisco in the leaf extract was calculated using a standard curve, which was plotted using pure Rubisco (from Sigma) diluted in PBS.

Result and Discussion

In all the experiments, untreated samples were used as controls and were run parallel to the other samples. The results are demonstrated in the following figures; all data are the mean of two replicates. In these figures the activity is reported as the amount of ^{14}C incorporation into acid-stable products in 1min.

Figure A.1 presents the activity of Rubisco in 50μg of total soluble protein extracted from AFEX treated alfalfa samples along with the untreated sample.

Figure A.1. Rubisco activity in AFEX-treated alfalfa



As this figure demonstrates, for the first two runs slight decreases (5 and 8%, respectively) were observed in Rubisco activity. But as the temperature rose to 80 and 90°C the activity dropped by 57 and 72%, respectively. These large drops in activity might be caused by both high temperature and the longer time that the AFEX unit had to be heated to reach to those high temperatures. Apparently these factors have damaged the protein.

As the result of the intense disruptive effect of the AFEX treatment on the cell walls of plant, the AFEX treatment has been able to improve the protein recovery from plant material (De La Rosa *et al.*, 1994). Therefore, to properly integrate above results we had to distinguish between increased protein extraction and stability of the activity. In fact, part of the observed activity in Figure A.1 might be due to the presence of a larger amount of Rubisco in the 50μg of total soluble protein that were subjected to the activity assay, and not an indication of the Rubisco activity retention. To clarify this issue we measured the amount of Rubisco in the samples by the ELISA method and then an appropriate volume of the leaf extract to equal 25μg of Rubisco was subjected to the activity assay. Even for the AFEX-treated samples that the retention of the Rubisco activity was very low we were able to measure the amount of the Rubisco by ELISA assay, apparently enough of the 3 dimensional structure of Rubisco had remained to react with ELISA assay.

As Figure A.2 shows, 25μg of Rubisco extracted from alfalfa treat at 60°C, and 70°C (with 60% moisture and 1:1 ammonia loading) lost about 12% and 24% of the activity respectively compared to 25μg of Rubisco extracted from untreated alfalfa. Whereas, the activity assay of 50μg of total protein extracted from the same samples showed only 5 and 8% lost of activity respectively compared to the 50μg of total protein from untreated sample (Figure 1). These differences clarified that in fact, part of the observed activity in Figure 1, was due to the presence of a larger amount of Rubisco in the 50μg of total soluble protein and actual percentage of Rubisco activity retention for alfalfa samples treated at 60°C or 70°C with 60 % moisture, 1:1 ammonia loading ratio were about 88% and 76%, respectively.

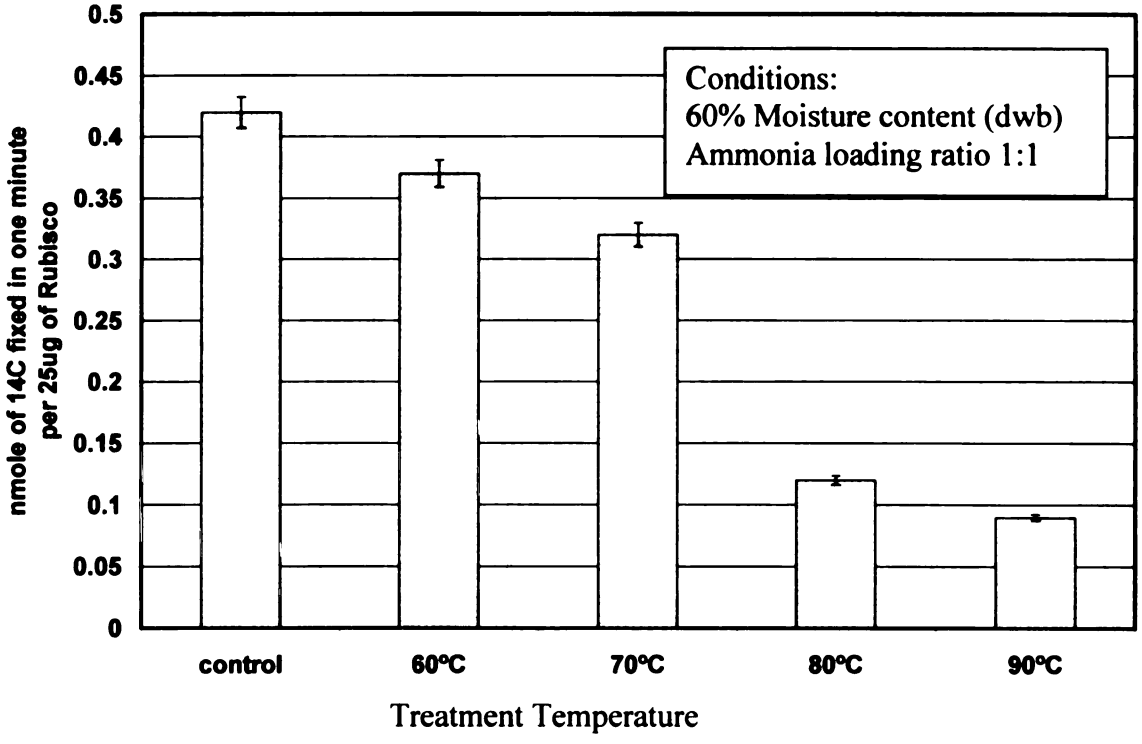
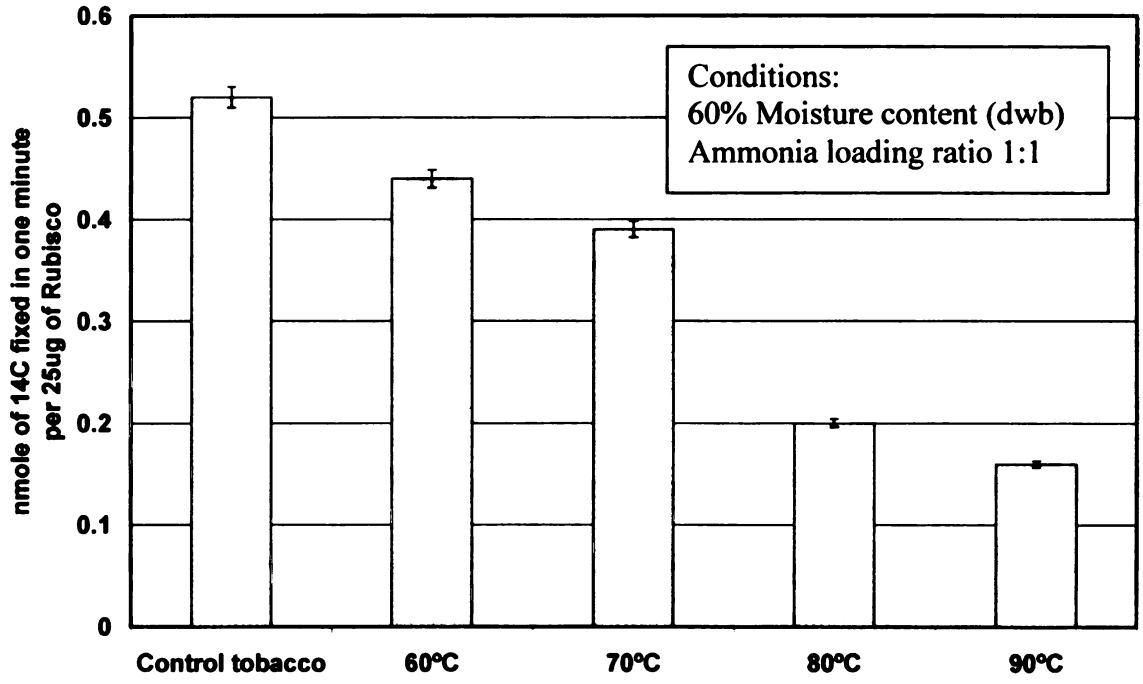


Figure A.2. Activity of 25 μg of Rubisco from AFEX-treated alfalfa.

Figure A.3 presents the activity of 25μg of Rubisco extracted from AFEX treated and untreated tobacco samples. This figure also shows the same trend as observed in Figure A.2, indicating that higher percentage of Rubisco activity survived at lower temperature vs higher temperature.

Figure A.3. Activity of Rubisco in AFEX-treated tobacco



Conclusion

Assuming 100% activity for untreated sample, the percentage of Rubisco activity retention for alfalfa samples treated at 60°C or 70°C with 60 % moisture, 1:1 ammonia loading ratio was about 88% and 76%, respectively. The enzymatic hydrolysis results (after 168hr with 60 FPU/g of glucan) of corn stover treated at the same conditions showed 64% and 68% glucan conversion respectively versus 29% glucan conversion for untreated corn stover (chapter 3). Based on the results of this chapter, chapter 3 and previous work (De La Rosa *et al.*, 1994) we believe that the AFEX treatment, under at least some conditions, has potential as an integrated pretreatment to increase the digestibility of the biomass, to improve extraction of protein from the biomass and at the same time to preserve plant protein in its bioactive form.

Thus there is a legitimate reason to hope that industrial enzymes such as cellulases, which produced in transgenic plants, can also survive the AFEX pretreatment, and be recovered in their useable from during biomass refining. This possibility was explored in chapter 4, by treating transgenic tobacco plants, expressing cellulase, with ammonia fiber explosion process.

Appendix B

HPLC and GC calibration curves

High Performance Liquid Chromatography (HPLC)

The sugars were analyzed in a BioRad (Richmond, CA) High Performance Liquid Chromatograph using an Aminex HPX87P column (HPLC Carbohydrates Analysis Column) at 85°C and a BioRad Deashing Cartridge as a guard column. The mobile phase used was degassed HPLC water at a flow rate of 0.6 ml/min. The injection volume used was 20 μL and the run time was 20 minutes.

Standard solutions of pure sugars: glucose, xylose, mannose, galactose, cellobiose and arabinose were individually run on the HPLC to determine their retention time (Table B.1) and calibrate the system.

Table B.1. Retention time of sugars on the HPLC

Sugars	Retention time, min
Cellobiose	10.416
Glucose	12.766
Xylose	13.866
Galactose	14.65
Arabinose	15.8
Mannose	16.73

Figure B.1. Calibration curve for celloboise

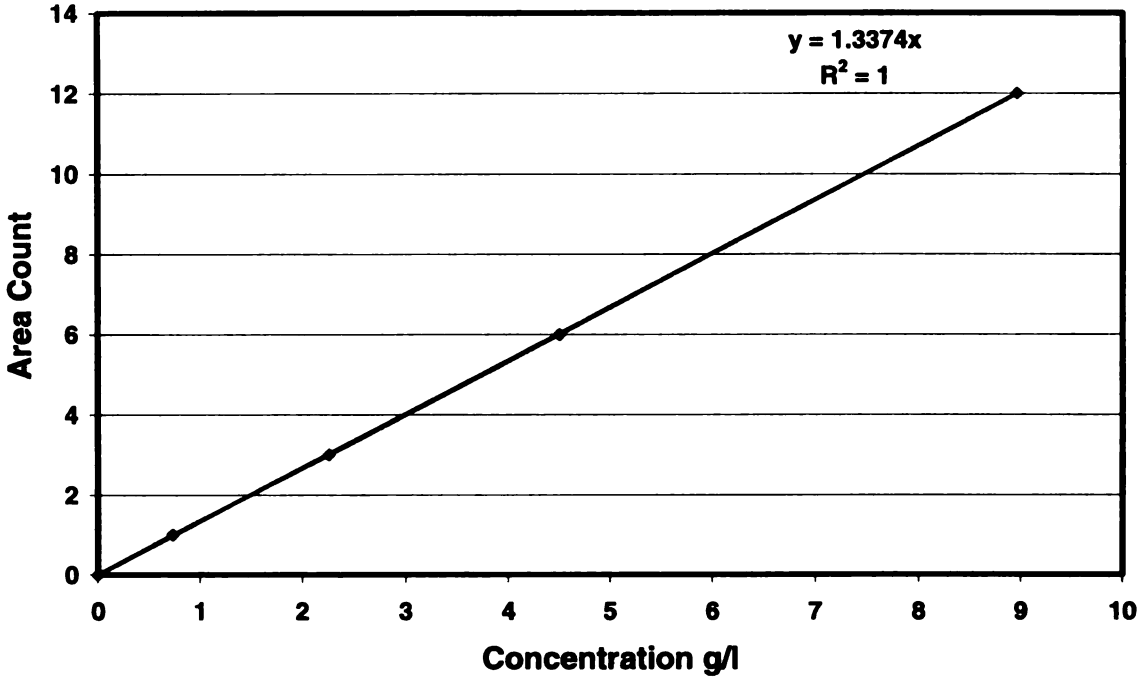


Figure B.2. Calibration curve for glucose

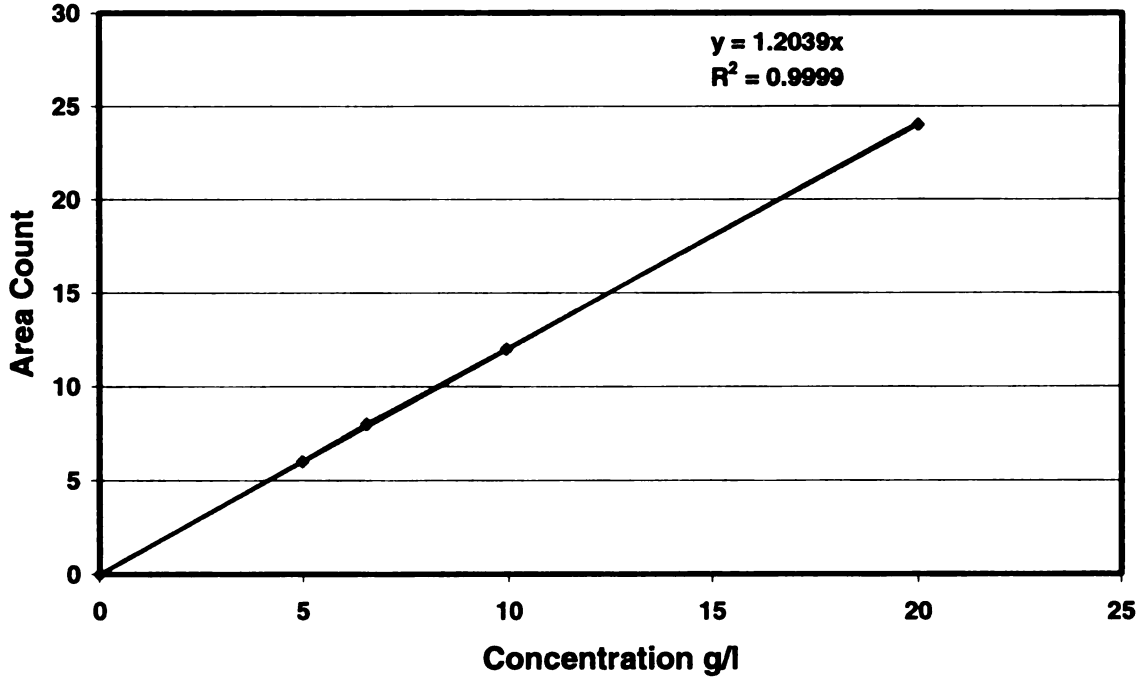


Figure B.3. Calibration curve for xylose

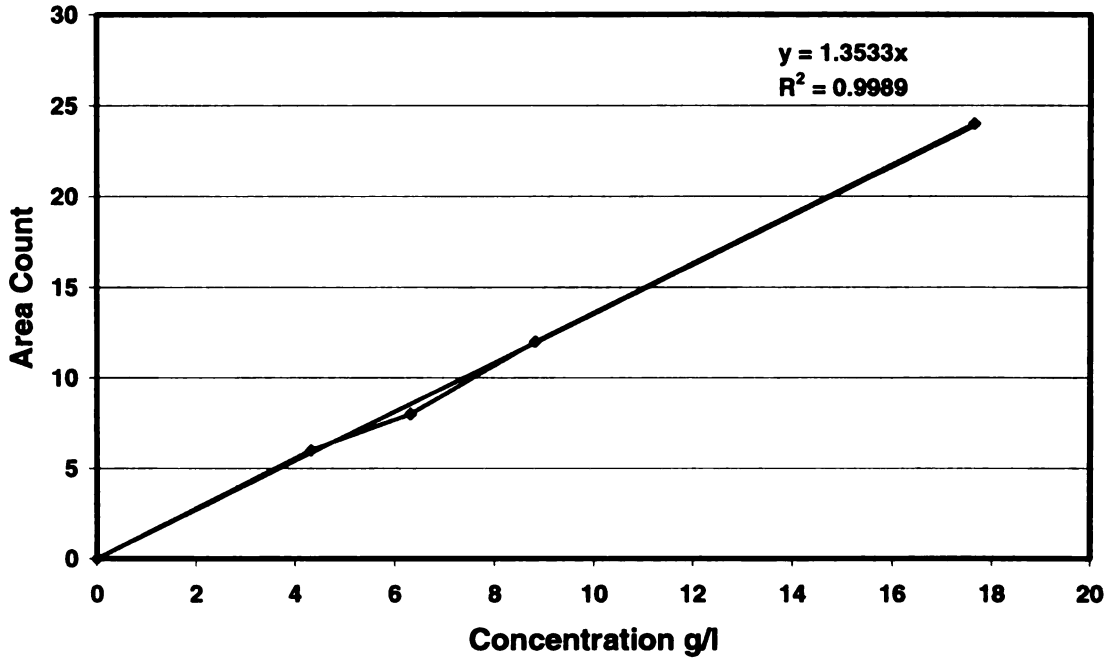


Figure B.4. Calibration curve for galactose

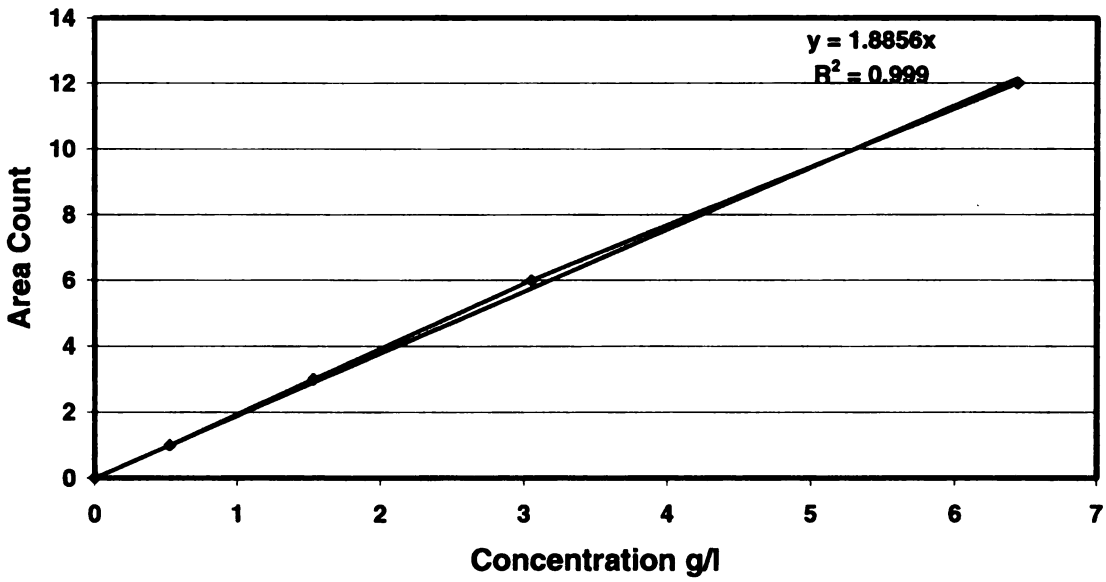


Figure B.5. Calibration curve for arabinose

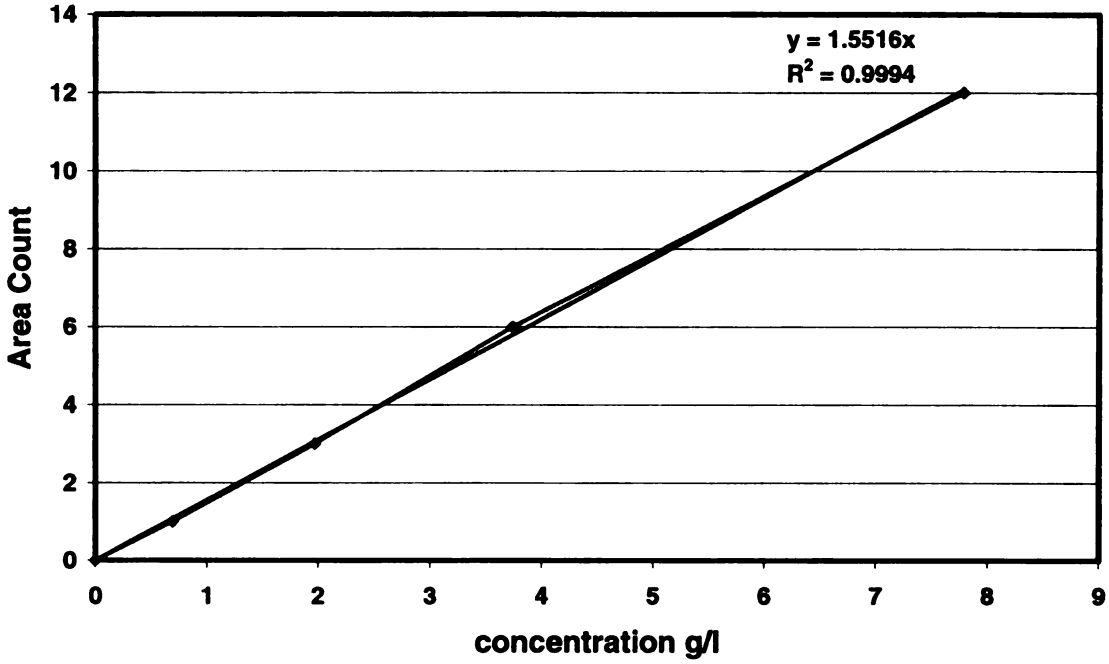
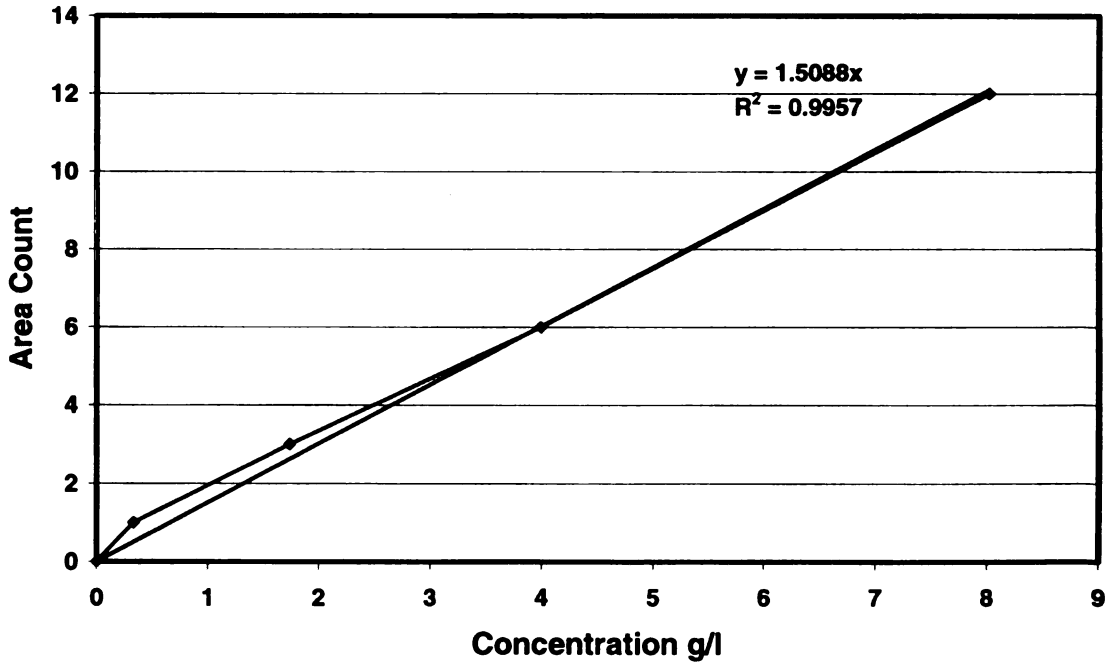


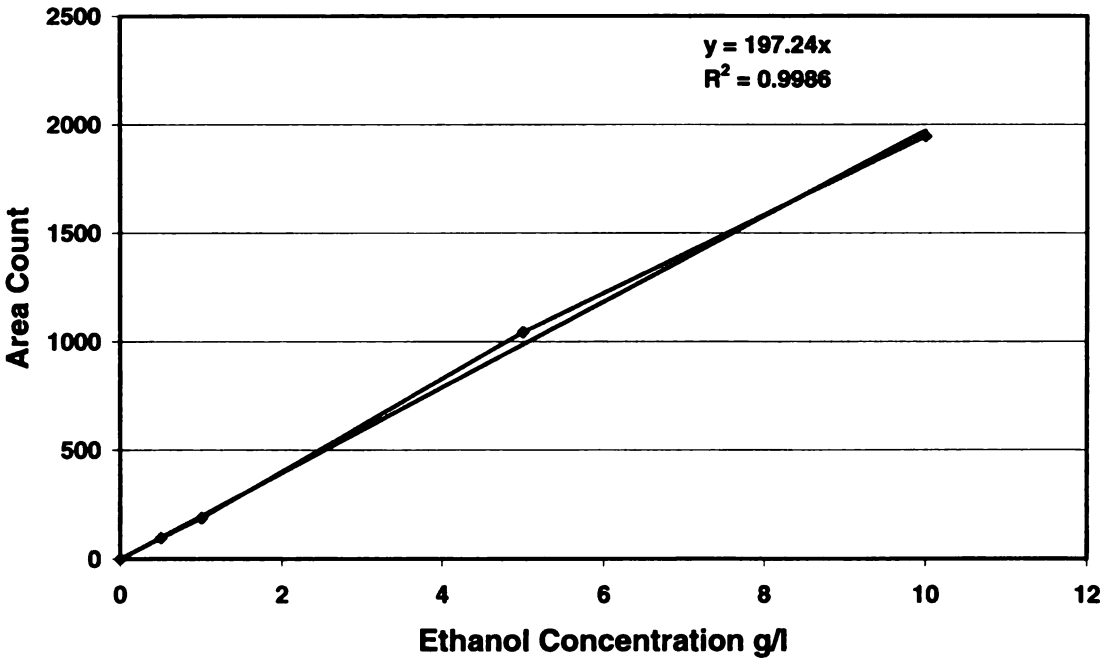
Figure B.6. Calibration curve for mannose



Gas Chromatography (GC)

The fermentation samples were analyzed for ethanol by gas chromatography using model GC 17 (Shimadzu). The injection temperature was 240°C and the detector temperature was 255°C. The column was first maintained at 80 °C up to 3 min followed by a temperature program at 15°C/min up to 125 °C for 6 min. The carrier gas was helium and ethanol was used as external standard for calibration (Figure B.7).

Figure B.7. Calibration curve for ethanol (retention time was 1.66min)



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