AN ABSTRACT OF THE THESIS OF

Yun-Chin Chung for the degree of Doctor of Philosophy in Food Science and Technology presented on May 30, 1996. Title: Saccharification and Fermentation of Lignocellulosic Biomass using *Trichoderma reesei* Cellulases and *Saccharomyces cerevisiae*

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Michael H. Penner

The efficiency of cellulose hydrolysis under straight saccharification and simultaneous saccharification and fermentation (SSF) conditions was evaluated using three lignocellulosic materials (switchgrass, cornstover, and poplar), which had been pretreated with dilute sulfuric acid under conditions which optimized xylose concentrations in the prehydrolysate liquid. Yields of glucose, cellobiose and ethanol obtained from the pretreated feedstocks were measured over 168 hrs. The final theoretical conversions of cellulose from pretreated switchgrass, cornstover, and poplar in straight saccharification were 85-100% (average 94%), 84-100% (average 96%), and 75-100% (average 87%), respectively, while in SSF the conversions were 84-90% (average 87%), 91-96% (average 90%), 72%-82% (average 76%), respectively. The conversion rates of poplar in straight saccharification and SSF were significantly lower than those of switchgrass and cornstover. The effects of reaction parameters such as enzyme activity, cellulose availability, and yeast cell viability on the extent of hydrolysis in straight saccharification

and SSF were also studied. Results indicate that the lower glucose or ethanol yields associated with some of the poplar were due to the recalcitrant nature of its cellulose.

To compare accurately the efficiencies between straight saccharification and SSF, a direct method for determining the cellulose content of the feedstocks residues resulting from SSF experiments has been developed and evaluated. The method improves on classical cellulose assays by incorporating a yeast lysing enzyme to remove yeast glucans from the feedstocks residue prior to acid hydrolysis and subsequent quantification of cellulose derived glucose. A freeze-drying step was identified as necessary to render the SSF yeast cells susceptible to enzyme lysis. The method was applied to the analysis of the cellulose and yeast-glucan content of SSF residues from the three pretreated feedstocks. Cellulose assays employing the lysing enzyme preparation demonstrated relative errors up to 7.2% when yeast-associated glucan were not removed prior to analysis of SSF residues. Enzymatic lysis of SSF yeast cells may be viewed as a general preparatory procedure to be used prior to the subsequent chemical and physical analysis of SSF residues.

Saccharification and Fermentation of Lignocellulosic Biomass using *Trichoderma reesei* Cellulases and *Saccharomyces cerevisiae*

by

Yun-Chin Chung

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yun-Chin Chung, Author

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CONTRIBUTION OF AUTHORS

Dr. Michael H. Penner and Dr. Alan Bakalinsky were involved in design, analysis, and writing of each of manuscript. Dr. Andrew G. Hashimoto assisted in the interpretation of data.

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SACCHARIFICATION AND FERMENTATION OF LIGNOCELLULOSIC BIOMASS USING *Trichoderma reesei* CELLULASES AND *Saccharomyces cerevisiae*

CHAPTER 1

INTRODUCTION

The term "biomass" was introduced by Eugene Adam and relates to the quantity of all living/once living matter from the five biological kingdoms: plants, animals, fungi, protists, and monerans (Margulis, 1974). Lignocellulosic biomass is used with reference to the type of biomass that contains cellulose (cellulosic biomass), such as unused standing forests, crop residues, animal manure from confined livestock operations, and industrial and urban waste products. The major components of lignocellulosic biomass are cellulose, hemicellulose and lignin. In general, lignified plant cell walls, the largest biomass resource in the world, contain 30-45% cellulose, 20-30% hemicellulose and 20-35% lignin (Carrasco et al., 1994). Cellulose molecules are linear paracrystalline homopolymers made up of β-D-glucopyranosyl monomers linked 1,4. Hemicellulose is a class of branched amorphous molecules comprised primary of xylose, glucouronic acids, and deoxy sugars (Richmond, 1991; Landisch, 1988; Fan et al., 1980; Cowling, 1985). Lignin is a 3-dimensional, amorphous, aromatic polymer built up from phenol-propane subunits (Ladisch, 1988).

Lignocellulosic biomass is reportedly the most abundant renewable resource on the earth, the annual production being approx. $4X10^{10}$ t (Cought, 1975). The main

consideration in terms of the utilization of lignocellulosic biomass is to convert it into fuel products, such as ethanol and methane. Three principle factors continue to stimulate interest in using lignocellulosic biomass as an alternative energy source. First, the use of ethanol in gasoline is likely to be economically competitive in the near future if the price of petroleum continues to rise and the production of ethanol from agricultural and municipal wastes becomes more technologically advanced. Second, in terms of environmental considerations, ethanol is regarded as a clean fuel while petroleum based fuels are responsible for serious pollution problems. Third, biomass is a vastly underutilized renewable resource available in large amounts and the supplies of petroleum are continually being depleted (Klass, 1981; Reed, 1981; Robinson, 1980; Cheremisinoff, 1980).

The conversion of lignocellulosic biomass to ethanol and the use of ethanol in gasoline in the United States is currently technologically feasible. However, the technology for the production of biomass-derived ethanol must become more efficient if this fuel is to be economically competitive. In general, the processes used to convert biomass to ethanol include a "pretreatment" step to increase the reactivity of the feedstock, an enzymatic saccharification step to convert cellulose to glucose, and a fermentation step to convert the glucose to ethanol. The traditional process for converting pretreated lignocellulosic biomass to ethanol is a two-stage process called "separate hydrolysis and fermentation" (SHF) (Wyman et al., 1992). In SHF processes the cellulose is hydrolyzed by chemical or enzymatic methods in the first stage and the resulting glucose is subsequently fermented by microorganisms in the second stage. A

different approach is to use a one-stage process for saccharification and fermentation. A one-stage process was developed by Takagi et al. (1977) and is referred to as a "simultaneous saccharification and fermentation" (SSF) process. In SSF processes the pretreated feedstock is simultaneously incubated with the cellulolytic enzymes and a microbe capable of fermenting the reaction mixture glucose to ethanol. A major benefit of SSF processes over SHF processes is the reportedly higher specific activities of the cellulolytic enzymes in SHF processes due to the removal of the inhibitory saccharification products, glucose and cellobiose, via yeast fermentation (Abe and Takagi, 1991). An overview of biomass-to-ethanol processes is presented in Chapter 2 of this thesis.

Lignocellulosic biomass conversion efficiency in the SSF process is generally evaluated in term of the percentage of total cellulose converted to ethanol and/or the absolute amount of ethanol produced. Values based on the percent of total cellulose converted allow direct comparison of saccharification efficiencies between those process which do and those which do not include fermentation. An approach for estimating the amount of cellulose consumed in an SSF process is to directly measure the amount of residual cellulose remaining in the SSF system at the completion of the experiment. Although this sounds simple, there are several technical limitations associated with the methods currently available for measuring the cellulose content of SSF residues (Grohmann, 1993; Spindler et al., 1991; Wyman, et al., 1986). Chapter 3 of this dissertation presents an accurate alternative approach for measuring the cellulose content of SSF residues.

The efficiency of SHF and SSF processes depend strongly on the physical characteristics of the pretreated feedstock undergoing saccharification. These characteristics may be altered by different methods of pretreating the feedstock. A pretreatment method commonly used for this purpose is based on incubating the feedstock at elevated temperatures in the presence of dilute acid. Optimum dilute-acid pretreatment conditions, in terms of the hydrolysis and recovery of the xylan component of the feedstock, were previously determined in this laboratory for three lignocellulosic feedstocks: switchgrass, poplar, and cornstover (Esteghlalian et al., 1996). It was not clear from our previous work whether these "optimum" pretreatment conditions consistently provided pretreated solid residues that could be efficiently used in SHF and SSF processes. Chapter 4 of this dissertation summarizes a study which addresses this question. The study evaluates the SHF and SSF properties of pretreated poplar, switchgrass and cornstover feedstocks.

CHAPTER 2.

LITERATURE REVIEW

2.1. UTILIZATION OF LIGNOCELLULOSIC BIOMASS AS ENERGY RESOURCES

The term "biomass" was introduced by Eugene Adam and relates to the quantity of Biomass of all living matter from the five kingdoms in biology: plants, animals, fungi, protists, and monerans (Margulis, 1974). Lignocellulosic biomass is reportedly the most abundant renewable resource on the earth with an annual production of approx. $4X10^{10}$ tons (Coughlan, 1985). The major sources of lignocellulosic biomass include unused standing forests, crop residues, animal manures from confined livestock operations, and industrial and urban wastes products (Reed, 1981).

2.1.1 Composition of Lignocellulosic Biomass

The major components of lignocellulosic biomass are cellulose, hemicellulose and lignin. The relative amounts of these components very among the different sources of lignocellulosic biomass. For example, plant cell walls contain 30-45% cellulose, 20-30% hemicellulose and 20-35% lignin. Table 1 shows the major components from four lignocellulosic materials (Carrasco et al., 1994).

Table 1. The major components of four lignocellulosic materials (Carrasco et al., 1994).

	Composition, [a] %			
Substrate	Potential Glucose	Potential xylose	Lignin	Ash
Poplar wood	35.4	17	25.6	3.3
Pine wood (*P. pinaster*)	27.7	10.0	37.8	1.1
Wheat straw	35.8	26.8	16.7	11.3
Sweet sorghum bagasse	43.0	24.0	20.0	5.0

[a] Expressed in percentage based on dry weight of biomass.

2.1.1.1 Cellulose

Plant cell walls are differentiated into primary and secondary walls. The former is typical for dividing and rapidly growing cells. (Tarchevsky and Marchenko, 1991; Dekker, 1985; Brown, 1944). Cellulose fibrils are oriented approximately parallel to the long axis of the plant cell wall in the secondary layer (Dekker, 1985). Cellulose molecules are linear parallel crystalline homopolymers made up of β-1,4-linked D-glucopyranosyl monomers (Richmond, 1991; Fan et al., 1980; Cowling, 1975). Molecular weight measurements indicate that wood cellulose has a degree of polymerization (DP) corresponding to approximately 10,000 glucose units per molecule (Sjostrom, 1981). Cellulose may be considered as paracrystalline, having both crystalline and amorphous regions (Fan, et al., 1980). The overall crystallinity of the cellulose (defined as the relative amount of crystalline versus amorphous character) plays a very important role in dictating the rate and extent to which cellulose is degraded by cellulolytic enzymes. In general, celluloses of high crystallinity are much less reactive than celluloses of low crystallinity (Norkrans, 1950; Walseth, 1952). It is generally regarded that the amorphous region of cellulose is the first to be hydrolyzed by cellulolytic enzymes. The molecules in the amorphous regions are less ordered and, thus, more readily accessible to enzymes. The result being that the amorphous regions are more quickly degraded by microorganisms (Cowling, 1963).

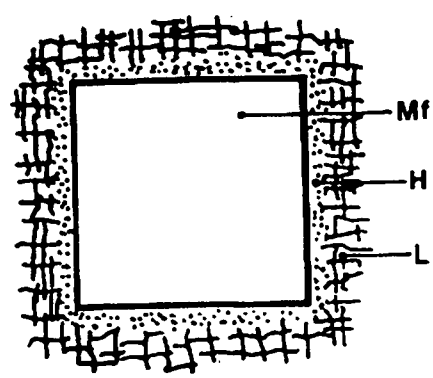


Figure 1. Schematic diagram of the ultrastructural of a cellulose microfibril (Mf), hemicellulose (H) and lignin (L) in the wood cell wall (Harada and Cote, JR., 1985).

2.1.1.2 Hemicellulose

The hemicelluloses are present in both the primary and secondary layers of the plant cell wall, where they are closely associated with lignin and cellulose (Dekker, 1985). Figure 1 is schematic diagram of the ultrastructure of a wood cell wall, showing cellulose, hemicellulose and lignin (Harada and Cote, JR., 1985). Hemicellulose is a class of branched amorphous molecules which contain high levels of xylose, glucuronic acids, and deoxy sugars (Ladisch, 1989). The main chains of the principal hemicelluloses are composed of 1,4 glycosidically linked β-D-xylopyranosyl units, with DPs ranging from 50 to 200 (Tarchevsky and Marchenko, 1991; Whistler and Daniel, 1985). The molecular chains of hemicellulose are shorter, more branched, and less regular than those of cellulose. Hemicellulose chains do not pack together in regular parallel bundles like cellulose (Tarchevsky and Marchenko, 1991). Hemicellulose can be rapidly hydrolyzed by microorganism because they are much less resistant to enzyme action than is cellulose (Mandels et al., 1974).

2.1.1.3 Lignin

Lignin is a 3-dimensional amorphous molecule, and is an aromatic polymer which is mainly built from phenol-propane subunits with an infinite molecular weight (Ladisch, 1989). The most important linkages in lignin appear to be alkyl-aryl ethers as well as alkyl-aryl and aryl-aryl carbon-carbon bonds (Sakakibara, 1991). Lignin is a very insoluble substance and, in contrast to cellulose and hemicellulose, is not broken down into smaller fragments by mild chemical processes. Together with hemicellulose, the lignin matrix encapsulates the cellulosic microfibrils in lignocellulosic biomass to form a protective sheath.

2.1.2 Utilization of Biomass

The utilization of biomass has become an interesting and important area of research. A major consideration for the utilization of biomass is to use this material as a source of energy. United States gross energy consumption is projected to be 163.4 quadrillion Btu by the year 2000, more than a twofold increase from 1974 consumption levels (Cheremisionoff, 1980). Supplies of natural gas and petroleum are being rapidly depleted and many of our coal deposits are environmentally unacceptable because of problems related to air pollution. The bioconversion of biomass to clean burning usable fuels is an alternative source of energy that is showing great promise for partially replacing conventional fuels (Klass, 1981; Reed, 1981; Robinson, 1980; Cheremisinoff, 1980). Research on energy production from biomass in the form of direct combustion of biomass, charcoal production, methane production and ethanol production have become popular .

Ethanol is probably the most widely used alcohol in automobile engines. In various parts of the world, ethanol is mixed with gasoline as a energy source for automobile engines. The use of ethanol in gasoline in the United States is technologically feasible; however, it has not been considered economically competitive with petroleum products in the past (Cheremisionoff, 1980). In recent years, alternative sources of fuel and chemicals have been pursued because the price of petroleum products continues to rise sharply and the availability of petroleum continues to decrease. Improved techniques for the production of ethanol from biomass have made ethanol a more economically competitive substitute for petroleum products in gasoline (Spindler et al., 1991; Wyman et al., 1992).

2.1.3 Dilute Acid Hydrolysis as a Pretreatment for Lignocellulosic Biomass

Some form of pretreatment, such as dilute acid hydrolysis, alkaline swelling, or steam explosion, is necessary to achieve reasonable rates for the enzymatic hydrolysis of biomass (Grethlin and Converse, 1991; Landisch, 1989; Chang et al., 1981; Dekker and Wallis, 1983; Grethlein, 1984; Millett et al., 1975). The pretreatment causes an opening of the cellulose matrix, thus allowing more rapid diffusion of enzymes into matrix pores (Grethlein et al., 1984; Grohmann et al., 1986). Acid based pretreatment processes are often the preferred pretreatments because of their high xylose yields, their relatively low operating temperatures, and the generation of few sugar and lignin degradation products (Wright, 1988). The effectiveness of dilute acid pretreatments for increasing the enzymatic susceptibility of the cellulose component of biomass can be rationalized on the basis of changes in the cellulose's crystallinity and available surface area.

2.1.3.1 Hemicellulose and lignin removal

Lignin and hemicellulose form a matrix around the cellulose fibrils, thereby physically blocking enzyme molecules from contacting the cellulose. It is therefore desirable to remove lignin and hemicellulose in order to increase the enzyme accessibility of the cellulose (Converse et al., 1989, Grethlen and Converse, 1984). Most of the hemicellulose is hydrolyzed during the dilute acid pretreatment process, which makes it soluble. The resulting xylose is removed by washing. The cellulose is more resistant to hydrolysis than the hemicellulose because of its crystalline structure (Cowling, 1963). After a proper pretreatment, the hemicellulose is largely

removed from the solid residue and the cellulose-lignin interactions are weakened (Andren, 1975).

2.1.3.2 Reduction in crystallinity

Dilute acid pretreatments also decrease the crystallinity of cellulose (Fan et al., 1987). Acid molecules are much smaller than cellulase enzymes and, thus, can more readily penetrate the macroscopic structure of cellulose. This means that acid molecules are capable to of penetrating into cellulose fibrils to disrupt the fibril's crystalline structure when enzymes can not (Ladisch, 1989; Mandels et al., 1974). The acid disruption of the fibril's crystalline structure means increased accessibility to enzymes in subsequent treatments. The increased accessibility is associated with higher rates of enzyme saccharification.

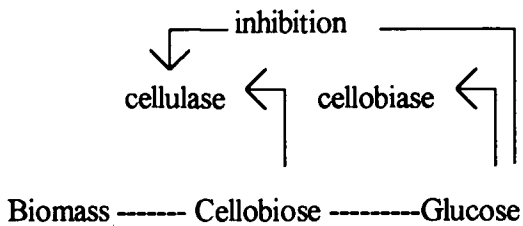
2.1.3.3 Increase in total pore volume and surface area to the enzyme

Pores are created in biomass during dilute acid pretreatments due to the removal of hemicellulose and the disruption of lignin-cellulose interactions. The total enzyme accessible surface area of the substrate is increased along with the increase in total pore volume (Grethlen and Converse, 1991). According to Grethlein (1984), the increase of surface area allows more enzyme molecules to associate with the cellulose, thus increasing hydrolysis rates.

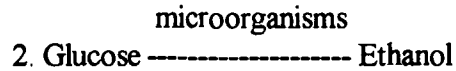
2.2 BIOCONVERSION TECHNOLOGY

Ethanol production from biomass involves the enzymatic saccharification of cellulose to glucose and the microbial fermentation of glucose to ethanol. The traditional bioconversion process of ethanol from biomass is "separate hydrolysis and fermentation" (SHF, Wright, 1988; Palnitkar and Lachke, 1990). The reactions of SHF can be described as a two-stage process:

STAGE ONE



STAGE TWO



In the first stage, the enzymatic saccharification stage, pretreated biomass (cellulose) is hydrolyzed into glucose through the use of enzymes (cellulase and cellobiase) in a batch reactor at controlled temperature and pH (Mitra and Wilke, 1974). After enzymatic saccharification, the crude glucose syrup is removed from the solids residue by filtration. The unreacted cellulose and the associated enzymes are recycled back to the reactor (Mitra and Wilke, 1974). In the second stage, the glucose filtrate is fermented to ethanol by microorganisms in a separate reactor.

The rate of enzymatic saccharification in the initial stage of these SHF processes is slowed due to product inhibition of the cellulolytic enzymes. An intermediate-product

(cellobiose) and the end-product (glucose) of enzymatic saccharification are inhibitors of cellulase and cellobiase activity, respectively (Blotkamp et al., 1978). This enzyme inhibition increases reaction times and makes the use of high enzyme loads unavoidable; both of which lead to increases in operating costs. In order to overcome these inhibitory effects in the SHF process, Takagi et al. (1977) developed a method called "simultaneous saccharification and fermentation" (SSF).

SSF is a one-stage process involving simultaneous enzyme saccharification and microbes fermentation. The reactions of SSF can be described simply as:

$$\text{Biomass} \xrightarrow[\text{microorganism}]{\text{enzymes}} \text{Ethanol}$$

SSF increases the rate of hydrolysis by continuously removing the inhibitors (glucose and, indirectly, cellobiose) of cellulase enzymes (Blotkamp et al., 1978; Spindler et al., 1990). As cellulose hydrolysis proceeds, the resultant glucose is fermented by an organism, thus preventing the build-up of this end product which would normally inhibit further hydrolysis (Wilke, 1976; Wright et al., 1988; Wyman et al., 1986; Blotkamp et al., 1978; Spindler et al., 1990; Wyman et al., 1992; Shah and Lee, 1992). The result of performing an SSF is that the cellulase enzymes are able to react with the substrate at their maximum rate for extended periods of time rather than being slowed by end-product inhibition. Elimination of these inhibitors from the reaction mixture allows the use of lower enzyme loads and, hence, lower operating costs. Other than lowering enzyme costs, SSF processes also eliminate expensive equipment because only one reactor is used for SSF processes instead of the two reactors required for SHF processes (Shah and Lee, 1992; Spindler et al., 1992; Szczodrak, 1988;

Wyman et al., 1986; Wilke, 1976; Wright et al., 1988). The SSF process also reduces the probability of contamination by unwanted organisms that are less ethanol tolerant than the microbes selected for fermentation (Wyman et al., 1992; Spindler et al., 1992; Wyman et al., 1986). Research directed toward further improvements in the economics of SSF processes can be grouped into three categories: enzyme production, microbial fermentation and process control.

2.3 FACTORS AFFECTING THE EFFICIENCY OF SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESSES

2.3.1 Enzymes

A major step in the conversion of cellulose to ethanol or other useful chemicals by SSF processes is the saccharification, which is the hydrolytic breakdown of cellulose to glucose catalyzed by cellulolytic enzymes. Several studies showed that the proper choice of enzymes is critical to the performance of the SSF process. A cellulase with well-balanced activities can result in improved SSF performance (Spindler et al., 1992; Schell et al., 1990; Wright, 1988;). In particular, the relative ratio of β-glucosidase-to-cellulase activity in the reaction mixture significantly affects the rate and yield of ethanol production (Spindler at el., 1992; Spindler et al., 1989; Wright, 1988; Wyman et al., 1986; Ghosh, et., 1982; Pemberton et al., 1980).

2.3.1.1 Selection of *T. ressei* mutants with higher β-glucosidase activity

Trichoderma ressei is known to be one of the best producers of cellulolytic enzymes, excreting high levels of cellulose solubilyzing activity into its culture medium (Szczodrak, 1988; Sternberg, 1976). However, extramycelial yields of β-D-glucosidase in culture filtrates of this fungus are low (Sternberg, 1976). The use of *T reesei* cellulase preparations low in β-glucosidase activity for the enzymatic saccharification of cellulose leads to a significant accumulation of cellobiose in the saccharification liquid phase. The accumulation of cellobiose in the reaction mixture decreases rates of cellulose saccharification because it is a strong inhibitor of cellulases (Szczodrak, 1989; Ghosh et al., 1982;). Problems associated with the accumulation of cellobiose in SSF reaction mixtures may be overcome by either selecting *T. ressei* mutants with higher β-glucosidase activity, supplementing the cellulase complex with a β-glucosidase from another source or selecting yeast mutants that are able of fermenting glucose and cellobiose simultaneously (Szczodrak,1989; Bailey et al., 1982).

Szczodrak (1989) used a hyper β-glucosidase mutant of *T. reesei* strain (F-522) in SSF studies on ethanol production from wheat straw. The mutant, V-7, showed about 6-times higher B-glucosidase activity than the parent strain (F-522). Ethanol yields from SSF processes using the mutant derived enzymes increased 1.5-fold and reaction times were shortened to 24 hr (48 hr for parent strain). No cellobiose could be detected in the SSF reaction mixtures containing the higher β-glucosidase activity.

2.3.1.2 Whole broth cellulase

In general, cellulase is produced inside fungal cells and secreted into the surrounding medium. After the production of cellulase is complete, the cells are separated from the broth, and the soluble enzyme is concentrated and sold. Acebal et al. (1988) have shown that as much as 50% of the B-glucosidase activity and 7% of the endoglucanase activity remained bound to the mycelia of *T. reesei* strain QM 9414. Thus, one possible method of improving the SSF performance characteristics of enzymes is to use whole cellulase culture broth (with cells) instead of culture filtrate (without cells), thereby using the enzyme retained in the cells. Takagi et al. (1977) reported as much as 25% improvement in ethanol yields when culture broth from cellulase-producing strains T.viride QM 9123 and T.viride QM 9414 were used in place of the corresponding culture filtrates in SSF experiments. Schell et al. (1990) reported that the use whole broth cellulase, in which a significant amount of the β-glucosidase remained associated with the cells, resulted in 8-25% higher ethanol yields than comparable SSF fermentations that used cellulase filtrates.

2.3.2 Selections of Microbial Strains

The enzymatic hydrolysis phase of the SSF process is known to be rate limiting (Spindler et al., 1988). Thus, it is desirable to maintain SSF conditions that are optimal for the cellulase enzymes and to efficiently remove cellobiose from the reaction mixture. Since *T. reesei* cellulolytic enzymes work optimally at temperatures ranging from 45-50°C, a temperature at which many yeasts fail to grow, it is important to identify microorganisms with the ability to ferment sugars at these relative high temperatures (Ballesteros et al., 1991;

Spindler et al., 1989_a; Szczdrak and Targonski, 1988; Spindler et al., 1988). It is also important to identify cellobiose-fermenting yeast, such as *Brettanomyces custersii*, that can efficiently remove cellobiose from the SSF reaction mixture (Spindler et al., 1992).

2.3.2.1 Selection of thermotolerant yeast strain

Several studies have focused on identifying and selecting thermo-tolerant yeast strains that can be used in SSF processes at >40°C. Various thermo-tolerant yeast strains have been found to produce ethanol from glucose at temperatures above 40°C; these includes *Kluyveromyces marxianus* (30-48°C) (Hughes et al., 1984; Hacking et al., 1984), *K. fragills* (37-45°C) (Hacking et al., 1984), *C. brassicae* and *S. urarum* (43°C) (Spindler et al., 1988). However, in a study to test the combinational effect of high temperature and ethanol toxicity to yeast, Szczodrak and Targonski (1988) found that *Fabospora fragilis* fermented glucose effectively at 46°C but did not actively grow or produce sufficient ethanol at 46°C. In their studies ethanol concentrations never rose above 40 g/L. Obviously, thermotolerance alone is not sufficient for the SSF process, yeast strains must also be capable of generating high amounts of ethanol at the elevated temperatures.

2.3.2.2 Mixed culture

The use of a mixed culture of cellobiose-fermenting yeast, *Brettanomyces clausenii*, along with the ethanol tolerant yeast, *Saccharomyces cerevisiae*, has been found to be a promising strategy to improve ethanol production rates in SSF processes (Spindler, 1991). In

general, *Saccharomyces cerevisiae*, a strong glucose fermenter with a fast rate of fermentation, has been found to perform well if the enzyme preparation is high in β-glucosidase, whereas a mixed culture of *B. clausenii* and *S. cerevisiae* provides better ethanol yields and overall rates of fermentation if the cellulase enzyme preparations are low in β-glucosidase (Spindler, 1991; Wyman et al., 1992).

2.3.3 Processing

2.3.3.1 Vacuum cycling

It is widely known that ethanol accumulation eventually inhibits the activity of the yeast that produce the ethanol. It is also reported that ethanol can inhibit the activity of cellulase enzymes (Ghosh, 1982; Takagi, 1984). The application of a vacuum to an SSF reaction medium allows the ethanol in the fermentation broth to evaporate at normal SSF operating temperatures, ~37°C. Ghose et al. (1984) have developed a process in which the fermentor broth is rapidly cycled between the fermentor and the flash vessel where ethanol is evaporated. Their results showed that SSF combined with continuous removal of ethanol by vacuum cycling increased ethanol yields by 50%.

2.3.3.2 Enzyme cycling

It is desirable to be able to recycle the enzyme used in SSF processes because enzyme costs are a major part of overall operating costs. As cellulose is hydrolyzed, the endo- and

exoglucanase components of cellulase are released into solution. Because of their high affinities to cellulose, the enzyme components can be recovered and reused by contacting the hydrolysate with fresh cellulose in a countercurrent flow process (Wyman, et al., 1986). The amount of enzyme that can be recovered in this way is limited because some of the cellulases, particularly β-glucosidase, remain attached to the lignin and unreacted cellulose components of the biomass. Enzyme is also thermally denatured under normal SSF conditions (Wright, 1988). Consequently, efficient SSF processes must be capable of functioning with enzyme recycling and the associated lower levels of β-glucosidase activity (Wyman, et al., 1986). This problem may be overcome by adding supplementary β-glucosidase to SSF reaction mixtures or by incorporating a yeast, such *as Brettanomyces clausenii*, which is capable of directly fermentating cellobiose(Ghosh et a;., 1982; Ghose et al., 1984; Wyman et al. (1986).

2.3.3.3 Couple xylose fermentation with cellulose fermentation in SSF process

In current practice, pretreated biomass is divided into two streams; a liquid stream containing xylose, furfual, acetic acid and other soluble components and a solid stream containing cellulose and lignin. The fermentation of cellulose is separated from the fermentation of xylose because the degradation products resulting from the dilute acid pretreatment, such as furfual and acetic acid, are toxic to *S. cerevisiae*, which is the predominant fermenting strain for the solid stream (Gaddy et al., 1992; Frazer and McCaskey, 1989; Tran and Chambers, 1985; Clark and Mackie, 1984; Fein et al., 1984; Banerjee et al., 1981; Leonard and Hajny, 1945). Researchers have been trying to integrate xylose fermentation with cellulose fermentation in SSF process to reduce the cost and the labor

associated with separating the solid stream (cellulose) from liquid stream (xylose) (Wright, 1988).

Two major areas of research related to SSF of the liquid xylose stream are the identification and characterization of xylose fermentors and the identification and minimization of toxic compounds in the xylose containing liquid. Only a few yeasts, *Pachysolen tannophilus*, *Canadida shehatae* and *Pichia stipitis*, are known to be capable of fermenting xylose to ethanol (Schneider et al., 1981; duPreeze and van, 1983; Dellweg et al., 1984; Jeffris, 1984). Palitkar and Lachke (1990) used *S. cerevisiae* (glucose fermenting strain) *and C. shehatae* (xylose fermenting strain) separately and also in coculture to evaluate the efficiency in coculture based SSF systems. The coculture system was shown to be more efficient than the isolated yeast strains acting independently. They reported a 30-38% and 10-13% increase in ethanol production under coculture SSF conditions as compared to the separate cultivation of *C. shehatae* and *S. cerevisae*, respectively.

Another difficulty in integrating xylose and cellulose fermentation is the presence of compounds toxic to *S. cerevisiae* in the xylose stream. These compounds are generated during the high temperature, dilute acid, pretreatment. Some of the toxins, such as furfural, are degradation products from xylose. Banerjee et al. (1981) found that furfural inhibited the activity of some glycolytic enzymes, particularly the dehydrogenase of *S. cerevisiae* , to limit fermentation. Gaddy et al. (1992) also demonstrated a decrease in metabolic rate and growth of *S. cerevisiae* due to the presence of furfural. Chung and Lee (1985) suggested increasing the size of the yeast inocula used to initiate the SSF fermentation as a means of overcoming inhibitor problems. It may also be possible to overcome problems of furfural inhibition by

inoculating with SSF mixture with furfural metabolizing yeast strain that can convert furfural to furfuryl alcohol. A strain of *S. cerevisiae* that is capable of catalyzing this reaction has been identified (DiazDe Villegas et al., 1992). There appear to be many inhibitors other than furfural that are associated with xylose in dilute acid prehydrolysate liquids. Further research is needed on the identification, mechanism of formation and means of removal of these yeast toxins.

CHAPTER 3

ANALYSIS OF BIOMASS CELLULOSE IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION PROCESSES

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3.1 ABSTRACT

A direct method for determining the cellulose content of biomass residues resulting from simultaneous saccharificaion and fermentation (SSF) experiments has been developed and evaluated. The method improves on classical cellulose assays by incorporating the enzymatic removal of yeast glucans from the biomass residue prior to acid hydrolysis and subsequent quantification of cellulose derived glucose. An appropriate cellulase-free, commercially available, yeast lysing enzyme preparation from *Cytophaga* was identified. A freeze-drying step was identified as necessary to render the SSF yeast cells susceptible to enzymatic lysis. The method was applied to the analysis of the cellulose and yeast-glucan content of SSF residues from three pretreated feedstocks; hybrid poplar, switchgrass and cornstover. Cellulose assays employing the lysing enzyme preparation demonstrated relative errors up to 7.2 % when yeast-associated glucans were not removed prior to analysis of SSF residues. Enzymatic lysis of SSF yeast cells may be viewed as a general preparatory procedure to be used prior to the subsequent chemical and physical analysis of SSF residues.

Key words: saccharification, fermentation, yeast, cellulose, lysing enzyme.

3.2 INTRODUCTION

Simultaneous saccharification and fermentation (SSF) is a process involving the simultaneous enzymatic saccharification of biomass cellulose and the microbial fermentation of the resulting glucose to ethanol. It has been reported that SSF is superior

to the analogous two-stage process, enzymatic saccharification followed by yeast fermentation, for the following reasons: (1) lower enzyme loads may be used since saccharification products which may inhibit cellulases, particularly glucose and cellobiose, are removed from the reaction mixture via yeast fermentation, (2) equipment costs are reduced due to the use of a single reactor and (3) contamination is less of a problem due to the relatively low pH (~5) and ethanol content of reaction mixtures (*1-3*).

Biomass conversion efficiency in the SSF process is generally evaluated in terms of the percentage of total cellulose converted to ethanol and/or the absolute amount of ethanol produced. Values based on the percent of total cellulose converted allow direct comparison of saccharification efficiencies between those processes which do not include fermentation. Generally, the amount of cellulose consumed in an SSF process is estimated by a summative analysis of the major products resulting from cellulose degradation (*4-8*). The major saccharification products being glucose and cellobiose; the major fermentation product being ethanol. The summative approach requires one to assume that an unknown amount of saccharification product, in terms of glucose equivalents, is used for yeast cell growth. Saccharification product that goes into cell growth is obviously not measured as either glucose, cellobiose or ethanol. It must generally be assumed that 5 to 10% of the total saccharification product is incorporated into new cell growth since the actual amount going to cell growth is very hard to quantify (*5, 9*). The summative approach suffers from the inaccuracy associated with this assumption. Further limitations of the summative approach are that the final value obtained for total cellulose consumed will reflect the

additive error from each of the separate product analyses, that minor products are not directly assayed and that it is labor intensive.

An alternative approach for estimating the amount of cellulose consumed in an SSF process is to directly measure the amount of residual cellulose remaining in the SSF system at the completion of the experiment. The measured amount of residual cellulose at time "t" is then subtracted from the amount of cellulose at time "0" to obtain the amount of cellulose that has been consumed in the SSF process through time "t". The approach is straight-forward and it avoids many of the problems associated with the summative approach. However, the analytical techniques necessary for the implementation of this more direct approach have not been satisfactorily developed. To measure the cellulose content of an SSF residue one typically hydrolyzes the cellulose containing residue with aqueous sulphuric acid and then quantitatively measures the glucose present in the hydrolysate. It is assumed that all of the glucose found in the hydrolysate resulted from the hydrolysis of cellulose. This assumption presents a problem for SSF systems because in some situations a significant portion of the glucose that is generated during the hydrolysis step may arise from the yeast cells associated with the SSF residue. Yeast cell walls contain a β-1,3 linked glucan polymer which is hydrolysed under the conditions used to hydrolyze cellulose (*10,11*). The presence of appreciable quantities of yeast derived glucose will obviously result in an overestimation of the amount of cellulose associated with the SSF residue.

Two different approaches have been used to correct for the problem of the presence of yeast associated glucans in SSF residues. In one approach, the yeast glucans

are prehydrolysed with 3% HCl and separated (washed) from the insoluble residue prior to the measurement of cellulose (*5,9,12*). This approach is based on the observation that yeast glucans are hydrolysed under less stringent conditions than those required for the complete hydrolysis of cellulose. However, this method tends to underestimate the cellulose content of SSF residues because the process used to hydrolyze the yeast cell wall polymers may also hydrolyze the more susceptible fractions of the biomass cellulose. Wyman et al. (*12*) indicated that roughly 5% of the cellulose component of bimass is susceptible to hydrolysis under the conditions employed for the acid-hydrolysis of yeast cell walls. A second approach that has been used to avoid quantification of yeast-derived glucans is to exhaustively hydrolyze the residual cellulose with cellulase enzymes in the absence of fermenting yeasts (*1*). In this case, at a specified time the active yeasts in an SSF mixture are poisoned with NaF, and excess of cellulase enzyme is than added to the reaction mixture, and the mixture is incubated until no further glucose is generated. The amount of glucose generated following the poisoning of the yeast cells is taken as proportional to the amount of residual cellulose in the SSF residue. This method avoids the hydrolysis of yeast glucans provided that the cellulase enzyme preparations employed for cellulose saccharification do not contain hydrolytic enzymes active toward yeast glucans. This method suffers from another limitation, however, in that it is likely that a fraction of the total cellulose component of a biomass sample will not be accessible for enzymatic digestion. Hence, the application of this method to SSF residues is likely to underestimate the residues actual cellulose content. The degree of underestimation is likely to be a function of the severity of the pretreatment the biomass has undergone prior

to the SSF process - since this in known to effect extents of cellulose saccharification (*13,14*).

In this paper, we present an alternative direct approach for measuring the cellulose content of SSF residues. The method is based on the hydrolysis of yeast cell wall glucans with commercially available cellulase-free hydrolytic enzymes, then separation of the hydrolysed yeast glucans from the insoluble SSF residue and subsequent quantitative analysis of the cellulose-containing SSF residue. This approach to cellulose analysis circumvents the limitations associated with the direct approaches discussed above.

3.3 MATERIALS AND METHODS

3.3.1 Materials.

Hybrid poplar, switchgrass and a cornstover mixture were obtained from the National Renewable Energy Laboratory (Golden, CO, USA). A *Trichoderma reesei* cellulase preparation (59 FPU/ml, 57 β-glucosidase units/ml - units defined as described by Ghose,*15*) was obtained from Environmental Biotechnologies, Inc. (CA). *Saccharomyces cerevisiae* (D_5A), a SERI strain genetically derived from Red Star Brewers yeast, was used as the fermentation yeast (*1*). Yeast extract-peptone-dextrose (YPD) media was 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose, adjusted to pH 5.0. Lysing enzyme (from *Cytophaga* species, product # L9893), α-cellulose and the glucose analysis reagents (glucose oxidase/peroxidase) were purchased from Sigma Chemical (St. Louis, MO). The lysing enzyme preparation had 164 U/mg solid (656 U/mg protein) - a unit

being defined as the glucanase, protease and cell lytic activity on brewers yeast in 50 mM phosphate buffer, pH 7, at 37°C (1U is equivalent to that amount of enzyme that causes a 1% decrease in OD at 670μm in 1 min).

3.3.2 Dilute Acid-Pretreatment of Biomass Substrates

Dilute acid-pretreatment of biomass substrates was done in batch reactors as described by Fenske (*16*). Pretreatment conditions for the switchgrass, poplar and cornstover substrates were 1.2% H_2SO_4/180°C/0.5 min., 1% H_2SO_4/180°C/0.56 min, and 1.2% H_2SO_4/180°C/0.9 min, respectively. Following pretreatments, residual biomass was washed with distilled water until the filtrate was neutral. Washed solids were stored at 4°C until used in experiments.

3.3.3 Enzymatic Lysis of Yeast Cell Walls

Yeast cells used as substrate for the lysing assays were prepared by transferring a frozen stock culture (1 ml) into 50-ml YPD media for 12 hr and then transferring to 400-ml YPD and incubating at 38°C for 4 days. Cells were then harvested by centrifugation (1500 x g, 10min), washed with distilled water and freeze-dried. For standard assays, substrate (yeast cells, 30 mg dry cells; α-cellulose, 300 mg; whatman #1 filter paper, 250 mg) and enzyme were incubated in 10 ml of 50 mM sodium phosphate buffer, pH 7.0, at 37°C with orbital agitation. Enzyme loads varied as indicated in text. The influence of biomass on lysing enzyme activity was determined by supplementing standard assay

solutions containing 0.5 mg/ml lysing enzyme with biomass (pretreated or native) to 3%. The amount of glucan associated with a given quantity of yeast cells was determined by summing the total glucose equivalents resulting from the complete acid hydrolysis of all yeast glucans.

Cycloheximide (0.1%) and tetracycline (0.1%) were added to all reaction mixtures to prevent yeast growth and bacterial contamination. Following predetermined incubation times, yeast glucan hydrolysis was followed by measurement of soluble glucose. Disappearance of intact yeast cells was monitored microscopically at 400 to 1,000X.

The bioamss was pretreated at 10% solids (w/w) in a 0.6L Parr batch-type, stirred reactor. After dilute acid pretreatment, the slurries were washed with distilled water (1300ml water/30g biomass) in a large funnel with a Whatman No.5 filter paper. Solid residues were collected and stored in the refrigerator (about 4°C), and served as samples for this study.

3.3.4 Simultaneous Saccharification and Fermentation (SSF)

SSF conditions were essentially those outlined by Philippidis et al. (*17*). SSF was done in 250-ml Erlenmeyer flasks equipped with water traps, initiating the experiment with a 2:5 medium: flask volume ratio. Flasks were incubated at 38°C with orbital agitation (150 rpm). On initiation of the SSF experiment a typical sample contained 3% cellulose, 25 FPU /g cellulose of cellulase activity, 10% (v/v) yeast inoculum (log phase cells), in YPD medium to give a total liquid volume of 100 ml.

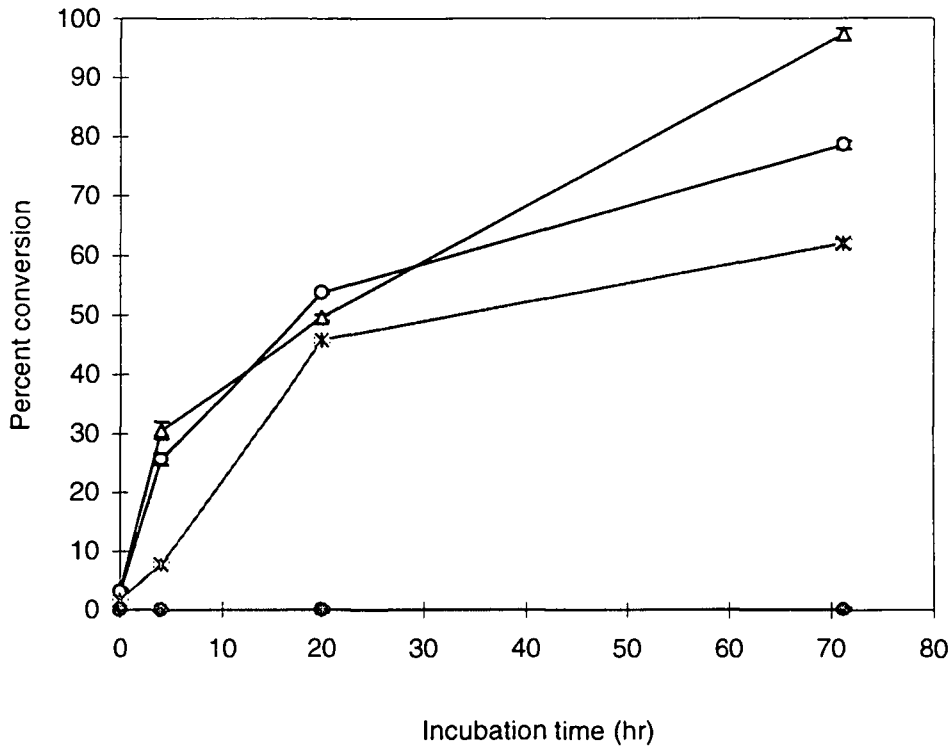
SSF incubations were terminated after seven days by immersion of the reaction flask in boiling water for 10 min. The SSF insoluble residue was then separated from the soluble phase by centrifugation and decantating and then washed repeatedly with distilled water. The washed SSF residue was then freeze-dried and stored desiccated until analyzed.

3.3.5 Cellulose in SSF residue

The freeze-dried SSF residue was assayed for total cellulose by two methods. In the first method a 300 mg sample was incubated at 30°C in 3 ml of 72% sulphuric acid for 2 hrs. The sample was then diluted to 4% sulphuric acid with distilled water and autoclaved for 1 hr. The resulting hydrolysate was assayed for glucose using a coupled glucose oxidase/peroxidase assay. The total glucose content of the hydrolysate was used to calculate the cellulose content of the SSF residue. This approach is widely used for the quantification of biomass cellulose (*16, 18-19*). The second method mimics the first with the exception that the SSF residue is treated with lysing enzyme for the removal of yeast glucans prior to the acid-hydrolysis step. Yeast cell glucans were solubilized by incubating 300 mg SSF residue in 10 ml lysing buffer (see above) containing 0.5 mg enzyme per ml for three days at 38°C. Solubilized glucans were separated from SSF biomass residue by centrifugation (1500 x g, 10 min) and decantating and repeated washing with distilled water.

3.4 RESULTS

Lysing enzyme preparations are a complex mixture of enzyme activities, including chitinases, proteases, deoxyribonucleases and the β-1,3-glucanases responsible for hydrolyzing cell wall glucans (*20*). These preparations may also posses significant cellulase (β-1,4-glucanase) activity (*21*). To be an effective enzyme preparation for the removal yeast glucans from biomass cellulose it is essential that the enzyme preparation display minimal cellulase activity. The hydrolytic activity of the Sigma *Cytophaga* lysing enzyme preparation on yeast cells, α-cellulose, filter paper and pretreated switchgrass is shown in Figure 2. The results are reported in terms of glucose generated from each substrate. The enzyme preparation's lack of activity on the two cellulose substrates and its demonstrated ability to hydrolyze yeast glucans make it appropriate for selective removal of yeast glucans from biomass cellulose. This result may not apply to lysing enzyme obtained from other species of the genus *Cytophaga* since some species of this genus can readily degrade cellulose (*22*). The Sigma preparation was capable of hydrolyzing essentially all (~97%) of the theoretically available glucan present in the yeast cells. The time course of yeast glucan hydrolysis is shown to be dependent on enzyme load, as expected. The data of Figure 2 is based on measured glucose, which is the endproduct for the complete hydrolysis of these glucans. However, complete hydrolysis is not required for separation of yeast glucans from insoluble biomass. Separation of yeast glucans from particulate biomass will occur when the yeast glucans, or their microenviroment, have been sufficiently modified to insure that they no longer pellet with the biomass residue during the centrifugation phase of the washing process (in this study



*Curves of filter paper (250 mg) + lysing enzyme (0.124 mg/ml), filter paper (250 mg) + lysing enzyme (0.5 mg/ml) and pretreated switchgrass (3%) + lysing enzyme (0.5 mg/ml) were Identical as the curve of cellulose (3%) + lysing enzyme (0.124 mg/ml)

Fig 2. Glucan conversion of dry yeast cells, α-cellulose and filter paper treated with lysing enzyme (0.5 mg/ml) in 50 mM phosphate buffer, pH 7, at 38°C and 150 rpm. Data points are means and error bars are standard deviations for two determinations from one reaction flask per treatment.

that corresponds to centrifugation at 1500 x g for 10 min). Thus, the 72 hr time point indicating complete conversion of yeast glucans to glucose (curve 1) represents an enzyme treatment appreciably more extensive than that required for separation of yeast glucans from biomass cellulose. In this regard, microscopic examination (at 400X) of reaction mixtures corresponding to curve 1 of Figure 2 showed that most of the yeast cells had been lysed after only a 0.5 hr reaction time and that all yeast cells had been lysed by 2 hrs.

The apparent hydrolytic activity of the *Cytophaga* lysing enzyme preparation decreased when authentic biomass substrates were included in reaction mixtures. This result is not likely due to the presence of traditional soluble inhibitors since the biomass substrates have been washed extensively. A more likely cause is the non-specific adsorption of lysing enzyme to the biomass substrate. Non-specific adsorption of other proteins and enzymes to lignocellulosic substrates have been reported (*23-25*). The effect of the presence of a lignocellulosic material on lysing enzyme activity is in part determined by the history of that particular material. For example, figure 3 shows that lysing enzyme activity was more sensitive to the presence of a pretreated switchgrass than to the presence of native switchgrass. The extent of this apparent "inhibition" is not large (figure 3), in each of the cases depicted in figure 3 greater than 80% of the theoretical yeast glucan had been converted to glucose at 48 hr of incubation. The higher rates of glucan hydrolysis reported in Figure 3, compared to those in Figure 2, are due to the use of higher enzyme loads in the figure 3 experiments. The different rates of glucan hydrolysis in reaction mixtures containing raw versus pretreated switchgrass illustrates that the minimum time required for incubation of lysing enzyme with an SSF residue, for purposes

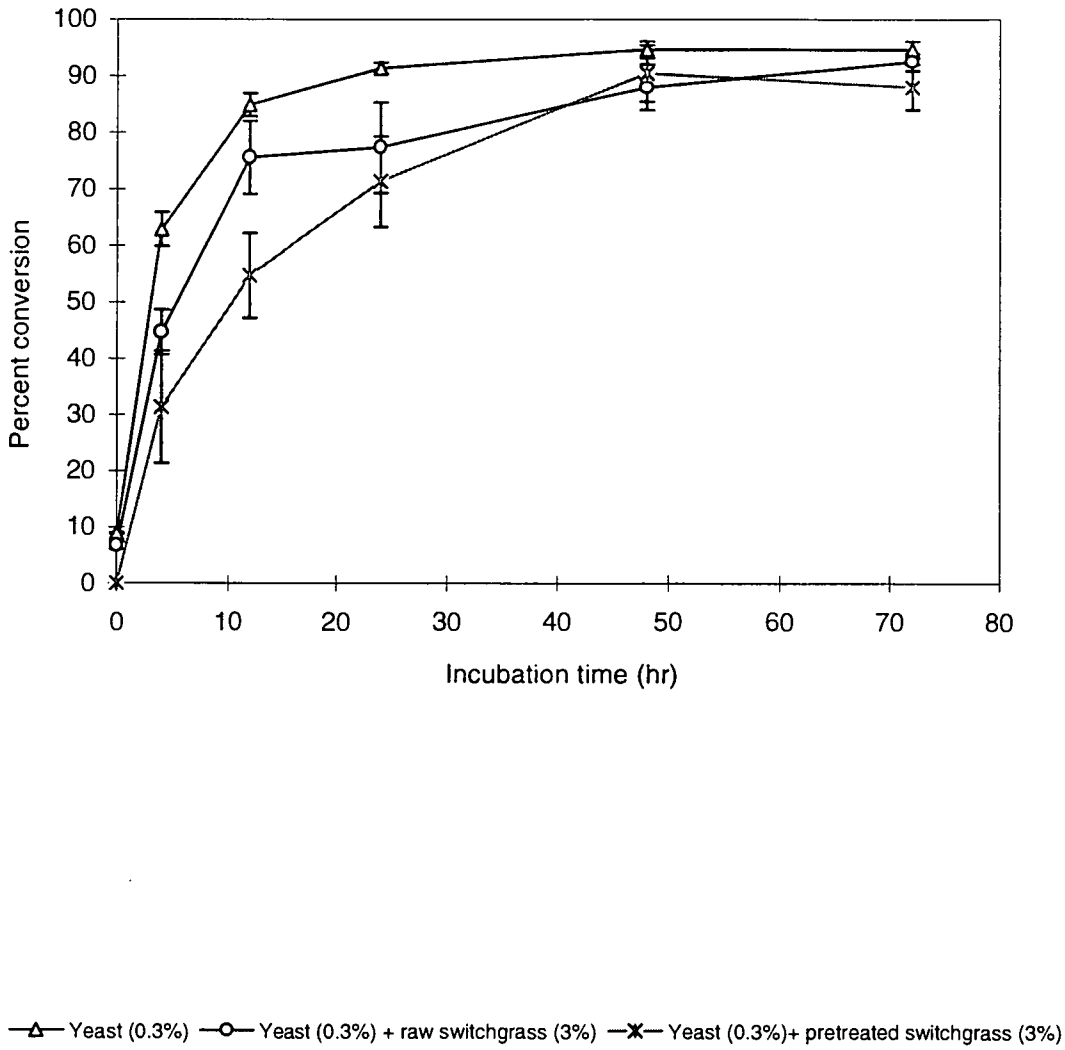


Fig 3. Glucan conversion of yeast cells with/without biomass treated with lysing enzyme (0.5 mg/ml) in 50 mM phosphate buffer, pH 7, at 38°C and 150 rpm. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment.

of separating yeast glucans from biomass cellulose, will be dependent on the identity and the history of the lignocellulosic substrate. Of course, the rate of cell lysis can be effectively increased for all substrates by the use of higher enzyme loads.

An effective strategy for determining the incubation time required to get efficient separation of yeast glucans from biomass cellulose is presented in Figure 4. In this experiment a predetermined amount of yeast cells are added to a series of flasks containing the test substrate. A chosen amount of lysing enzyme is then added and the samples are allowed to incubated under optimum conditions . Following appropriate incubation times, representative test samples are centrifuged to separate the solubilized yeast glucans from the biomass cellulose. The supernatant is then assayed for total glucose equivalents solubilized. This assay requires that solubilized oligomers and polymers be hydrolysed to monomeric glucose. The time course for solubilization of glucose equivalents should reach a maximum value, the maximum value corresponding to the known amount of glucan that was originally introduced into each flask as an integral part of the yeast cells. A representative plot for such an experiment is shown in Figure 5 for a pretreated switchgrass substrate. It is clear that under the conditions used for this assay essentially all of the yeast glucan could be separated from the biomass cellulose following 10 hrs of incubation.

The data of Figure 5 and the photomicrographs in Figure 6 illustrate that stock yeast cells, with or without previous freeze-drying, are readily lysed and the inherent glucans readily hydrolysed when these cells are incubated with lysing enzyme in the presence of biomass substrates. It was therefore interesting to find that yeast cells which

had actually been part of an SSF experiment were resistant to lysing enzyme if the duration of the SSF experiments exceeded 3 days (conclusion based on microscopic observation of intact yeast cells, data not shown). The resistance to lysing enzyme was observed even at enzyme loads of up to 3 mg/ml. Several modifications of the lysing reaction conditions were tested in an attempt to increase the susceptibility of SSF yeast cells to lysing enzyme. Addition of neutral proteins such as BSA and β-lactoglobulin (to decrease non-specific adsorption of enzyme), addition of reducing agents such as β-mercaptoethanol and dithiothreitol (to increase yeast cell accessibility*26*), and oven-drying prior to enzyme treatment all failed to increase yeast cell susceptibility. However, it was found that freeze-drying the SSF residue prior to enzyme treatment rendered the yeast cells susceptible to lysing enzyme. The photomicrographs of Figure 7 illustrate the success of this approach. In this case an SSF residue resulting from a 7 day experiment was freeze-dried prior to treatment with lysing enzyme. No yeast cells could be detected following a 24 hr incubation period with a relatively low enzyme load. Similarly, lysing enzyme was found to effectively degrade yeast cells in native and pretreated switchgrass, poplar and cornstover freeze-dried SSF residues. The indication is that this technique is applicable to a wide variety of biomass feedstocks. Based on these observations, our recommendation it that SSF residues be freeze-dried prior to treatment with lysing enzyme. The molecular basis for the increased susceptibility of freeze-dried yeast cells to lysing enzyme is not clear. It has been reported that freeze-thawing processes may alter the membrane structure of yeast cells and cell outlines (*27-29*); however, these papers did not report changes in cell wall structure. It is known that intact yeast cells (without prior

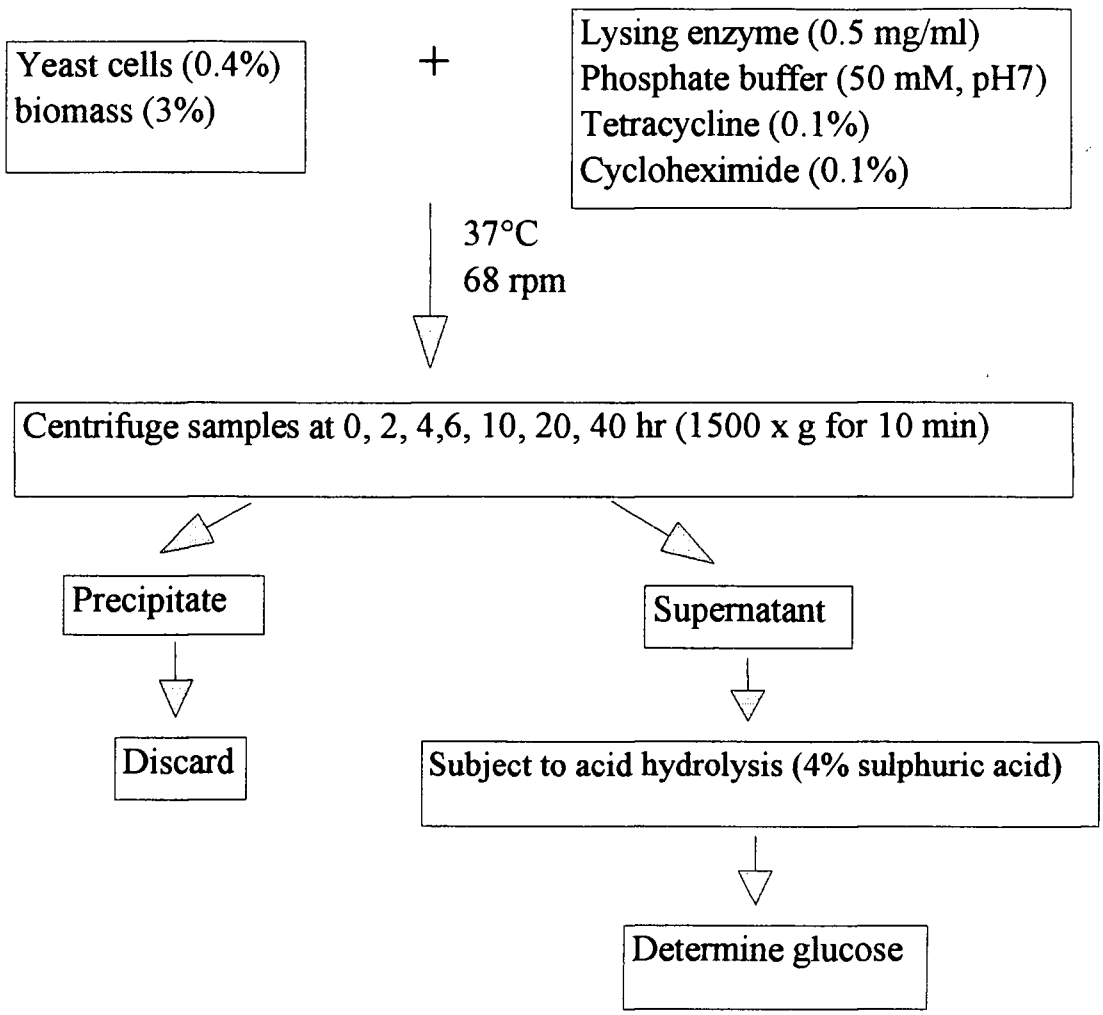


Fig 4. Protocol for determining the incubation time necessary for separation of yeast glucans from biomass cellulose.

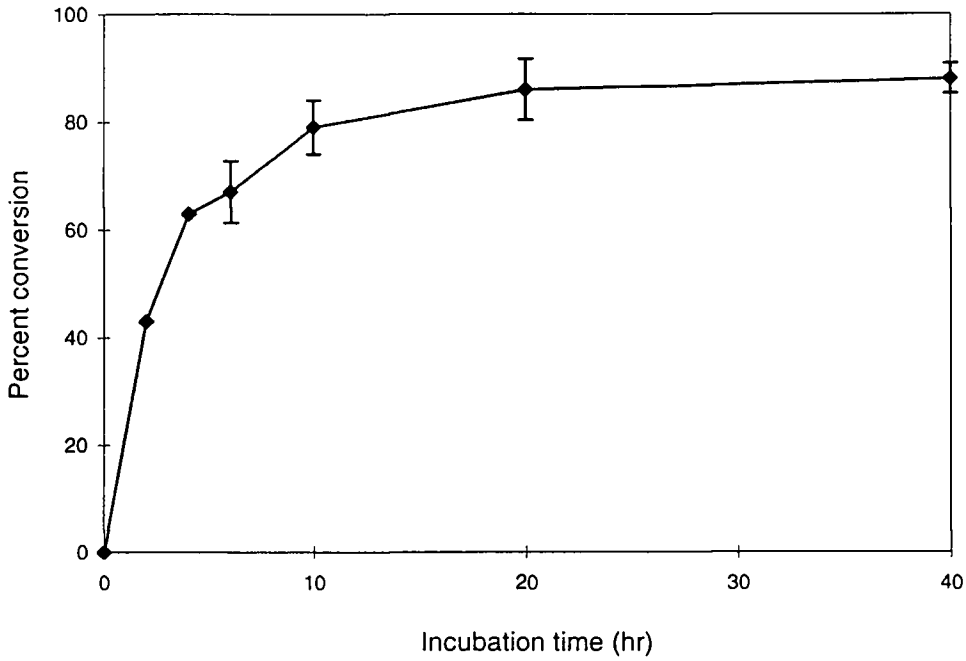


Fig 5. Glucan conversion of yeast cells by measuring soluble glucan. Data points are means and error bars are standard deviations for one determinations in each of two reaction flasks.

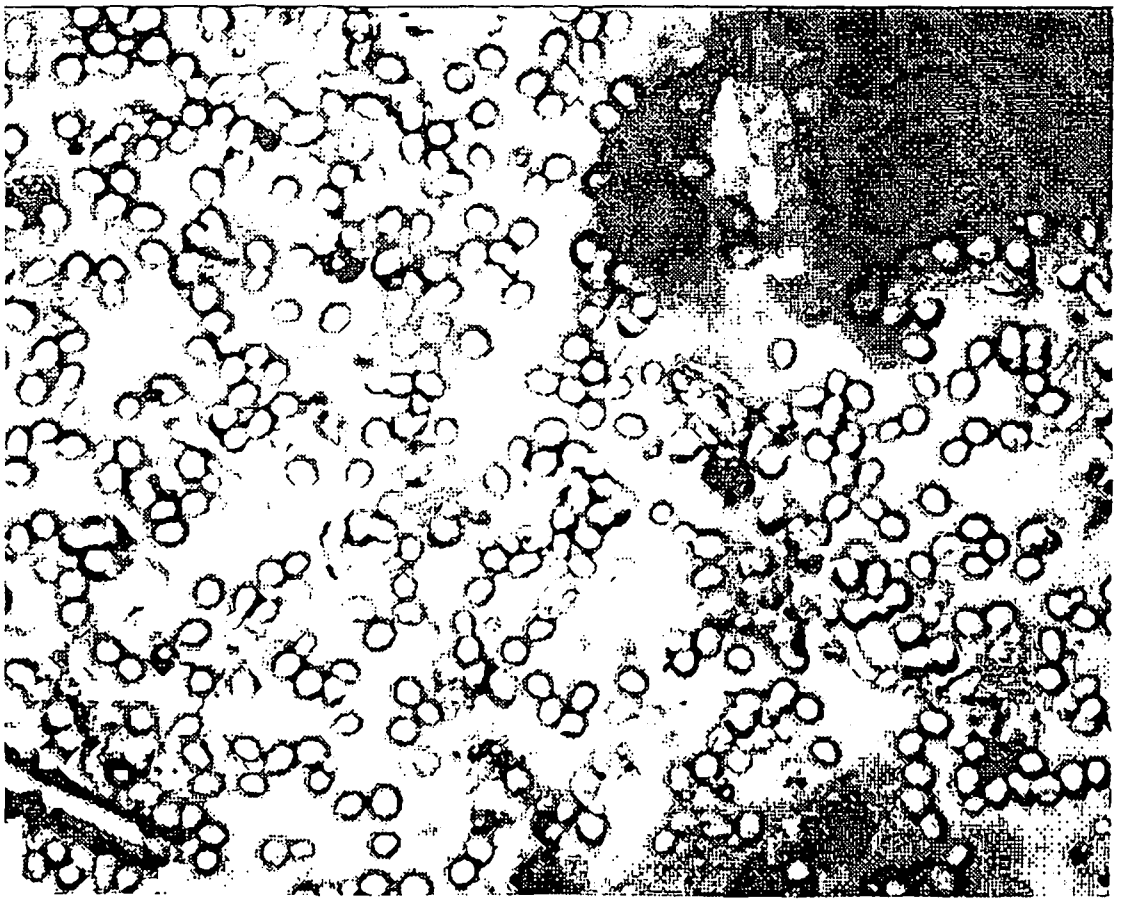


Fig 6a. Freeze-dried yeast cells (0.3%) with dilute acid pretreated switchgrass (3%) before treated with lysing enzyme monitored microscopically at 400 X.

Fig 6b. Freeze-dried yeast cells (0.3%) with dilute acid pretreated switchgrass (3%) after treated with lysing enzyme monitored microscopically at 400 X.

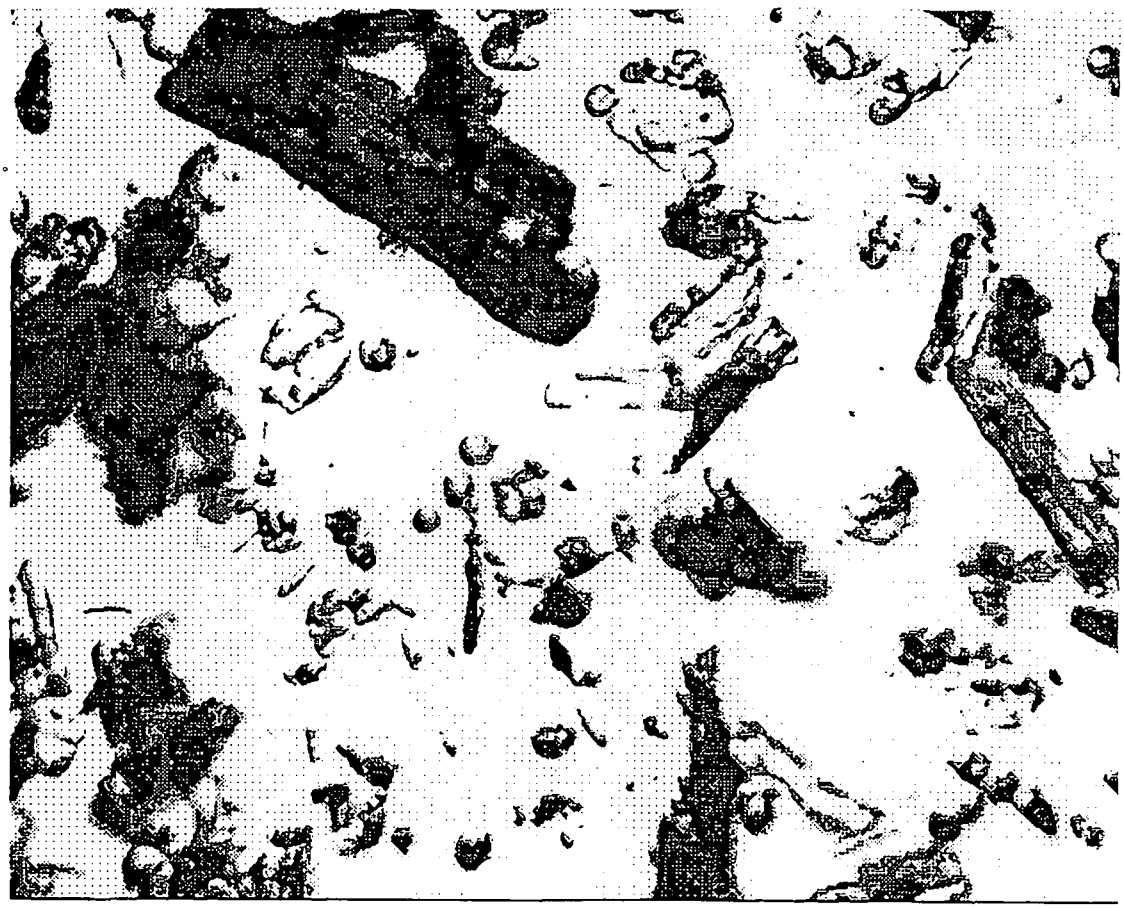


Fig 7a. SSF residue of dilute acid pretreated switchgrass before treated with lysing enzyme monitored microscopically at 400 X.

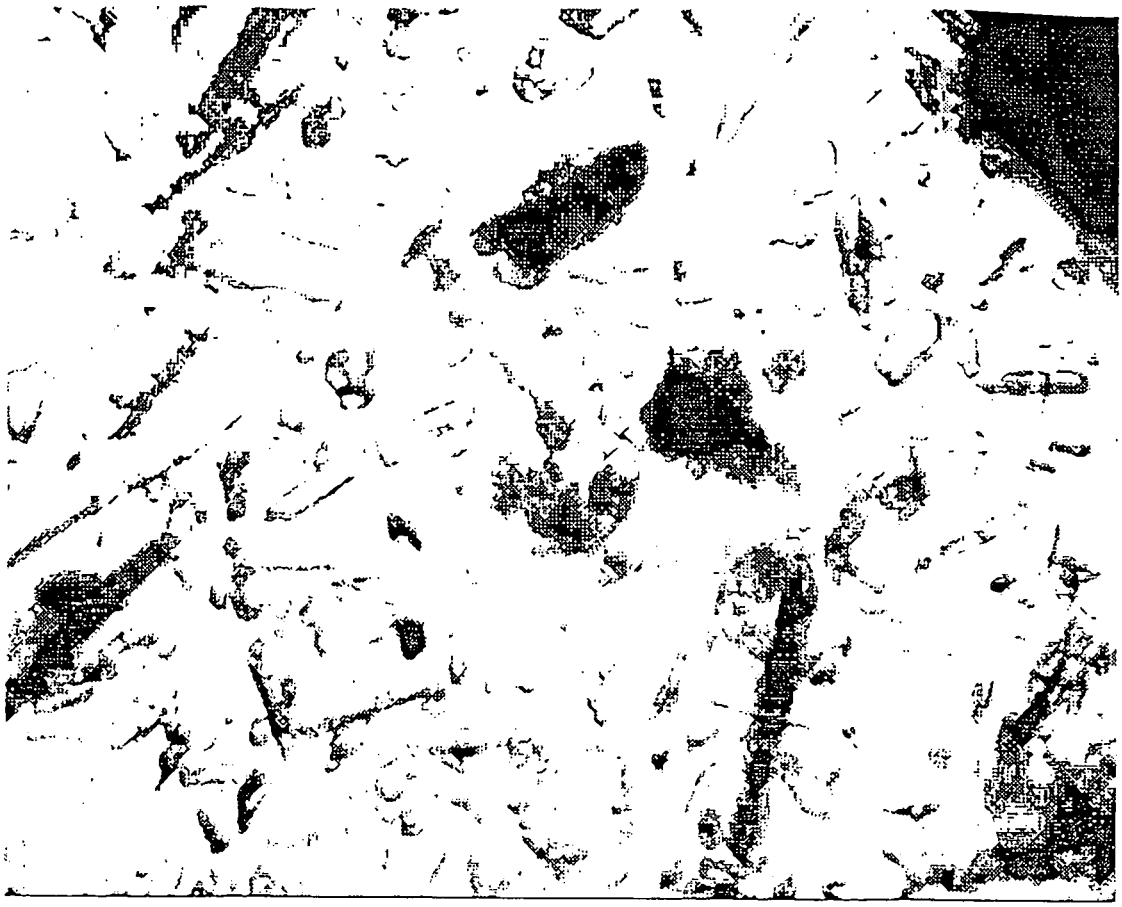


Fig 7b. SSF residue of dilute acid pretreated switchgrass after treated with lysing enzyme monitored microscopically at 400 X.

freeze drying) become more resistant to protoplasting enzymes when they are grown in the stationary phase and that their susceptibility to these enzymes can be increased by the addition of reducing agents (26, *30*). In this study, the yeast cells became resistant to lysing enzyme when ethanol yields had reached 70-75 % of maximum (equivalent to three days incubation, Figure 8). We found that reducing agents did not improve enzymatic lysis of these SSF cells unless the SSF residue was previously freeze-dried. Once the residue was freeze-dried, then the addition of dithiothreitol to the lysing reaction mixture did improve the efficiency of cell lysis.

The application of lysing enzyme to the analysis of the cellulose content of SSF residues from pretreated poplar, switchgrass and cornstover/corncob substrates is summarized in Table 2. The cellulose content of each SSF residue was measured with and without the inclusion of the enzymatic lysing step for the removal of yeast glucans. The actual amount of cellulose in the 7-day SFF residues was 773 mg (29% of original), 389 mg (14% of original) and 361 mg (12% of original) for poplar, switchgrass and cornstover, respectively. Failure to remove yeast associated glucans from the SSF residues prior to cellulose analysis resulted in a relative error of 4.1, 2.8 and 7.2% for the poplar, switchgrass and cornstover substrates, respectively. The error is always associated with an overestimation of the amount of cellulose remaining in the SSF residue. The magnitude of the error will increase as the number of yeast cells increase and the amount of residual cellulose decreases, as may be expected toward the end of an SSF experiment. The magnitude of the error is likely to increase in proportion to the effectiveness of the pretreatment that the feedstock undergoes prior to the SSF experiment, since the severity

of saccharification (*13,14*). The difference in the two measured cellulose values for a given substrate (measured with and without the use of lysing enzyme) is equivalent to the amount of yeast derived glucan in that SSF residue. The values generated in this study may be used by others to gauge the potential relevance of yeast glucans under their particular experimental conditions. The absolute amount of yeast-derived glucose equivalents associated with the poplar, switchgrass and cornstover residues following seven days SSF were 32, 11 and 26 mg, respectively.

The concept and application of using lysing enzyme to more accurately determine the cellulose content of SSF residues has been the primary focus of this paper. However, this same approach is valuable for other aspects of SSF residue analysis. For example, it is often of interest to follow changes in the physical properties of particulate substrates as SSF experiments progress. A technique that can be used to gather information on particle size and shape makes use of optical microscopy-image analysis systems. In using these systems it is sometimes necessary to remove yeast cells from the SSF residue so that the microscopic image to be analyzed is limited to the particulate substrate and not a mixture of substrate particles and yeast cells. Similarly, the accuracy of some of the techniques used for the measurement of residue surface area are dependent on the removal of yeast solids.

Table2. Cellulose remaining in SSF residues after 7 day SSF treatment

SSF residues	Glucan from cellulose without prior hydrolysis yeast (mg) [1]	Glucan from cellulose following removal of previously hydrolyzed yeast (mg)[2]	Glucan from yeast after SSF(mg)[3]	Overestimate of cellulosc remaining due to yeast associated glucan (%)[4]
Poplar	809 ± 5	773 ± 10	32	4.1
Switchgrass	400 ± 4	389 ± 12	11	2.8
Cornstover	387 ± 10	361 ± 1	26	7.2

[1]represents the glucan from cellulose and yeast.
[2]represents the glucan from cellulose.
[3]determined by subtracting column 3 from column 2.
[4]determined by dividing column 4 by column 3.

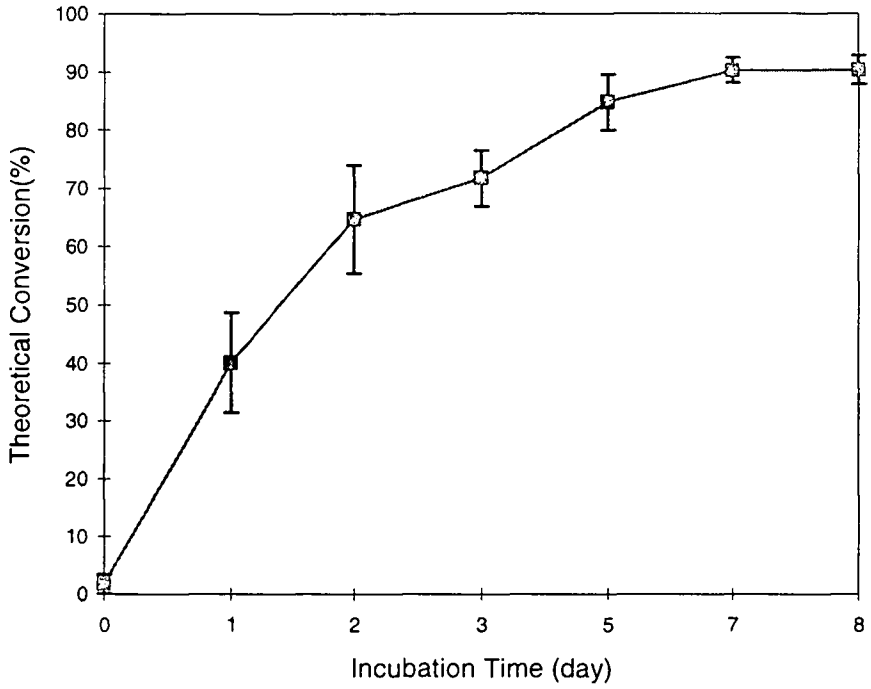


Fig 8. Ethanol Yield for dilute acid pretreated switchgrass under SSF process.

In conclusion, the commercially available lysing enzyme preparation from *Cytophaga* was found to be appropriate for use in removing yeast cell glucans from SSF residues, thus allowing the more accurate determination of the cellulose content of these residues. Lysing enzyme activity was shown to be effected by the presence of biomass substrates and, hence, an experimental protocol was suggested for the determination of the minimum incubation time necessary to insure the complete removal of yeast glucans from a given type of SSF residue. Yeast cells in the latter phases of SSF experiments were shown to be resistant to lysing enzyme. However, this resistance could be overcome by freeze-drying the SSF residue prior to enzyme treatment.

3.5 ACKNOWLEDGMENTS

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3.6 REFERENCES

1. Spindler, D. D., Wyman, C. E. and Grohmann, K. (1991), Appl. Biochem. Biotechnol. 28/29, 773-786.

2. Shan, M. M. and Lee, Y. Y. (1992), Appl. Biochem. Biotechnol. 34/35, 557-568.

3. Wyman, C. E., Spindler, D. D., and Grohmann, K. (1992), Biomass Bioenergy 3, 301-307.

4. Vinzant, T., Panfick, L., Nagle, N. J., Ehrman, C. I., Reynolds, J. B. and Himmel, M.E. (1994), Appl. Biochem. Biotechnol, 45/46, 611-626.

5. Spindler, D. D., Wyman, C. E. and Grohmann, K. (1989a), Biotechnol. Bioeng. 34,189-195.

6. Spindler, D. D., Wyman, C. E., Grohmann, K. and Mohagheghi, A. (1989b), Appl. Biochem. Biotechnol. 20/21, 529-540.

7. Mohagheghi, A., Tucker, M., Grohman, K., and Wyman, C. (1991), Appl. Biochem. Biotechnol. 33, 67-81.

8. Meyers, S. G. (1978), The American Institute of Chemical Engineers. 184(74), 79-84.

9. Spindler, D.D., Wyman, C.E., Mohagheghi, A. and Grohmann. (1988), Appl. Biochem. Biotechnol. 16/17, 279-293.

10. Kreutzfeldt, C. and Witt, C. C. (1991), in Saccharomyces, Tuite, M. F. and Oliver, S. G., eds., Plenum Press, New York, NY, pp. 5-58.

11. Fleet, G. H. (1991), in The yeasts, vol. 4, Rose, A. H. and Harrison, J. S., eds, Academic Press Inc., San Dieago, CA, pp.199-277.

12. Wyman, C. E., Spindler, D. D., Grohmann, K. and Lastick, S. M. (1986), Biotechnol. Bioeng. Symp. 17, 221-238.

13. Grethlein, H. E. and Converse, A. O. (1991), Bioresource Technol. 77-82.

14. Fan, L. T., Lee, Y. H. and Beardmore, D. H. (1980), in Proceeding of Bioconversion and Biochemical Engineering Symposium, vol. 1, Ghose, T. K., ed., Indian Inistitute of Technology, Hauzkhas, New Dalhi, Indian, pp. 233-259.

15. Ghose, T.K. (1988), Pure & Appl. Chem. 59(2), 257-268.

16. Fenske, J.J. (1994), Master thesis, Oregon State University, Corvallis, Oregon.

17. Philippidis, G. P., Smith, T. K. and Schmidt, S. L. (1993), SSF experimental protocols: Lignocellulosic biomass hydrolysis and fermentation.

18. Moore, W. E. and Johnson, D. B. (1967), Procedures for the chemical analysis of wood and wood products, USDA Forest Products Laboratory, Madison, WI.

19. Ehrman, C. I., and Himmel, M. E. (1994), Biotechnol. Techniques. 8(2), 99-104.

20. Tuite, M. F. and Oliver, S. G. (1991), in Saccharomyces, Tuite, M. F.and Oliver, S. G., eds., Plenum press, New York, NY, pp. 283-320.

21. Sigma Chemical Co. (1996), Product Information Catalog.

22. Stainer, R.Y., Adelberg, E.A., and Ingraham, J.L. (1976), The Microbial World, 4th ed., Prentice-Hall, Inc., NJ.

23. Tatsumoto, K., Baker, J. O., Tucker, M. P., Oh, K. K., Mohaghehi, A., Grohmann, K. and Himmel, M. E. (1988), Appl. Biochem. Biotechnol. 18, 159-173.

24. Suttcliffe, R. and Saddler, J. N. (1986), Biotechnol. Bioeng. Symp. 17, 749-762.

25. Sinitsy, A. P., Bungay, H. R. and Clesceri, L. S. (1983), Biotechnol. Bioeng. XXV, 1393-1399.

26. Sommer, A. and Lewis, A. J. (1971), J. Gen Microb. 68, 327-335.

27. Souzu, H. (1973), Cryobiology. 10, 427-431.

28. Van Steveninck, J. and Ledeboer, A. M. (1974), Biochim. Biophys. Acta. 352, 64-70.

29. Kruuv, J., Lopock, J. R. and Keith, A. D. (1978), Cryobiology. 15, 73-79.

30. Eddy, A. A. and Williamson, D. H. (1957), Nature. 179, 1252-1253.

CHAPTER 4

SACCHARIFICATION AND FERMENTATION OF LIGNOCELLULOSIC BIOMASS USING *Trichoderma reesei* CELLULASE AND *Saccharomyces cerevisiae*

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4.1 ABSTRACT

The efficiency of cellulose hydrolysis under straight saccharification and simultaneous saccharification and fermentation (SSF) conditions was evaluated using three lignocellulosic materials (switchgrass, cornstover, and poplar), which had been pretreated with dilute sulfuric acid under conditions which optimized xylose concentrations in the prehydrolysate liquid. The yields of glucose, cellobiose and ethanol obtained from the pretreated feedstocks were measured over 168 hrs. The final theoretical conversions of cellulose from pretreated switchgrass, cornstover, and poplar in straight saccharification were 85-100% (average 94%), 84-100% (average 96%), and 75-100% (average 87%), respectively, while in SSF the conversions were 84-90% (average 87%), 91-96% (average 90%), 72%-82% (average 76%), respectively. The conversion rates of poplar in straight saccharification and SSF were significantly lower than those of switchgrass and cornstover. The effects of reaction parameters such as enzyme activity, cellulose availability, and yeast cell viability on the extent of hydrolysis in straight saccharification and SSF were also studied. Results indicate that the lower glucose or ethanol yields associated with some of the poplar were due to the recalcitrant nature of its cellulose.

4.2. INTRODUCTION

Lignocellulosic biomass is the most abundant renewable resource on earth, with an annual production of approximate 4×10^{10} tons (Coughlan, 1985). It is readily available from agriculture (energy crops, agricultural residues), forestry (deciduous and coniferous woods), the pulp industry, and municipal wastes. A prime consideration for the

utilization of lignocellulosic biomass is for the production of fuel ethanol. The use of fuel ethanol in gasoline mixtures is likely to be economically competitive in the near future if the price of petroleum continues to rise and technological advances allow the price of ethanol to continue to decline. In terms of environmental considerations, ethanol is regarded as a clean fuel relative to gasoline due to its more complete combustion. Taken together, all of these factors indicate that we will see increases in the utilization of lignocellulosic biomass as a renewable source of alternative fuels (Klass, 1981; Reed, 1981; Robinson, 1980; Cheremisinoff, 1980).

The cellulose fraction of biomass has been the traditional focus of biomass-to-ethanol processing schemes. This is because cellulose is the primary polysaccharide of lignocellulosics and because it's hydrolysis product, glucose, is readily fermented by a number of well characterized microbes. The focus of modern biomass-to-ethanol schemes is no longer limited to the cellulose component of the biomass. To be economically feasible, modern biomass-to-ethanol processes must utilize all of the major polysaccharide components in these feedstocks. Hence, a general aim of research in this field is to identify processes which are capable of efficiently converting both the cellulose and the hemicellulose fractions of biomass into readily utilizable sugars. One such process is based on the dilute acid "pretreatment" of a feedstock, which hydrolyses the xylan (hemicellulose) component of the native feedstock, followed by an enzymatic saccharification "treatment" of the pretreated solids residue, which hydrolyzes the cellulose component to glucose. The result of this process is the acid catalyzed

production of a liquid xylose stream in the "pretreatment" step and the enzymatically catalyzed production of a liquid glucose stream in the "treatment" step.

The enzyme catalyzed hydrolysis of the cellulose component of native lignocellulosic feedstocks is inefficient, presumably because the cellulose fibrils of intact, lignified, cell walls are encased in a lignin-hemicellulose matrix (Sasaki, 1989; Harada and Cote, JR., 1985). A pretreatment, such as dilute acid hydrolysis, alkaline swelling, or steam explosion, is needed to disrupt this lignin-hemicellulose matrix and, thus, make the cellulose more accessible to cellulolytic enzymes (Tayor et al., 1983; Warwicker and Wright, 1967; Tanahashi et al., 1983; Sawada et al., 1987; Grethlein and Converse 1991). It has been repeatedly shown that the efficiency of enzymatic saccharification depends strongly on the physical characteristics of the lignocellulosic substrate. Important structural features include: (1) degree of hydration/water swelling, (2) cellulose crystallinity, (3) degree of polymerization, (4) enzyme accessible surface area, (5) pore size distribution and (6) particle size (Cowling and Kirk, 1976; Fan et al., 1980a, Fan et al., 1980b; Fan et al., 1987; Taylor et al., 1983; Focher et al., 1981; Lee and McCaskey, 1983; Tanaka et al., 1988; Dermoun and Belaich, 1988; Wong et al., 1988; Sinitsyn et al., 1991; Sasaki et al., 1979; Dunlap et al., 1976; Thompson and Chen, 1992; Cowling, 1975; Torget et al., 1990; Grohmann et al., 1985) . Among these features, cellulose crystallinity and enzyme accessible surface area are considered to be the most important structural parameters affecting enzymatic saccharification (Thompson and Chen, 1992; Fan et al., 1980b; Cowling, 1975). An inverse linear relationship has been demonstrated for the crystallinity of the cellulose compnent of a feedstock and it's susceptibility to enzymatic saccharification

(Dunlap et al., 1976; Sinitsyn, et al., 1991; Fan et al., 1980a). A direct linear relationship has been demonstrated between the enzyme accessible surface area of a feedstock and the susceptibility of it's cellulose component to enzymatic saccharification (Stone et al., 1969; Grethlein and Converse, 1991; Thompson and Chen., 1992). The chemical composition of native and pretreated feedstocks has also been shown to correlate with enzyme susceptibility. A positive relationship between rates and extents of cellulose saccharification from pretreated feedstocks and the extent of xylan dissolution from that feedstock in the dilute acid pretreatment prior to saccharification has been reported (Torget et al. 1990; Grohmann et al., 1985).

The enzymatic saccharification of cellulose in biomass-to-ethanol schemes is generally done by one of two processes. In the traditional "straight saccharification" process the washed, pretreated, feedstock is incubated with cellulolytic enzymes under conditions for optimum enzymatic activity, the resulting glucose stream is subsequently fermented to ethanol in a second stage. This traditional scheme is thus a two-stage process; first saccharification, then fermentation. Another approach is to use a one-stage process in which enzymatic saccharification and fermentation occur simultaneously. This approach is known as simultaneous saccharification and fermentation (SSF). In SSF processes the saccharification environment is adjusted for optimum ethanol production, not simply optimum enzyme activity. SSF processes usually employ lower temperatures (for yeast viability) and lower enzyme loads. SSF processes are often considered to have greatly industrial potential due to lower capital investments, high reaction rates and yields, and less problems with contamination (Spindler, et al., 1991; Shah and Lee, 1992; Wyman

et al., 1992). Several lignocellulosic feedstocks have been shown to be suitable substrates for SSF processes, including grasses, straws, softwoods, hardwoods, and paper mill by-products (Detroy et al., 1981; Saddler et al., 1983; Spindler et al., 1989; River et al., 1984; Walkinshaw et al., 1984; Saddler et al., 1982; Spindler et al., 1991; Wyman et al., 1992). Dilute acid pretreatments designed to hydrolyze the xylan fraction of lignocellulosic feedstocks are relatively low cost processes which successfully increase the reactivity of the feedstock's cellulose component (Wright, 1988; Grohmann et al., 1985). These pretreatments increase the reactivity of the feedstock's cellulose fraction while simultaneously generating a xylose rich liquid phase that is available for fermentation to ethanol. Xylose accounts for 30-60% of the potentially fermentable sugars in hardwood and herbaceous feedstocks (Wright, 1988) and, thus, represents a major source of fermentable sugar. Yeast species capable of fermenting xylose to ethanol include *Pachysolen tannophilus*, *Canadida shehatae* and *Pichchia stipis* (Schneider et al., 1981; duPreeze and Van, 1983; Dellweg et al., 1984; Jeffris, 1984). Dilute acid pretreatments have been identified which are capable of recovering over 80% of a feedstock's total xylose equivalents (Esteghlalian et al., 1996; Beck, 1993; Torget et al., 1990).

Optimum pretreatment operations must fully maximize xylose recovery and, simultaneously, generate a pretreated solids residue whose cellulose component is readily available for subsequent enzymatic saccharification. The primary focus of the majority of published studies has been to evaluate pretreatment parameters based primarily on their effect on the enzymatic saccharification of the resulting solids residue. In this study we have taken the opposite approach, evaluating the enzymatic saccharification and SSF

properties of the cellulose component of selected feedstocks that have been dilute acid pretreated under conditions chosen to maximize xylose yields in the prehydrolysate liquid. The pretreatment conditions corresponding to maximum xylose yields for the feedstocks used in this study were determined previously (Esteghlalian et al., 1996). An aim of the study was to determine whether pretreating feedstocks under conditions chosen for maximum xylose recovery would simultaneously generate prehydolyzed solid residues whose cellulose component is readily available for bioconversion to glucose and/or ethanol. In general, the data agrees with the concept that the rates and extents of cellulose saccharification correspond to the percentage of original xylan remaining in the prehydrolyzed solid and, thus, optimizing dilute acid pretreatments for maximum xylose yields in the liquid phase will generate pretreated solid residues appropriate for enzymatic saccharification.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Switchgrass, hybrid poplar and cornstover/corncob that had been air dried and milled to pass a 40 mesh screen were obtained from the National Renewable Energy laboratory (NREL, Golden, CO). A *Trichoderma reesei* cellulase preparation (59 FPU/ml and 57 β-glucosidase unit/ml with units defined as described by Ghose, 1988) was obtained from Environmental Biotechnologies, Inc. (Melon Park, CA). *Saccharomyces cerevisiae* (D_5A), a SERI strain genetically derived from Red Star Brewers Yeast, was

used as the fermentation yeast (Spindler et al., 1991). Yeast extract and peptone were purchased from Difco (Detroit, MI). Glucose and glucose oxidase/peroxidase (product # 510-A) and α-cellulose (product # C-8002) was purchased from Sigma Chemical Company (St. Louis, MO). Yeast extract-peptone-dextrose (YPD) media was 1% yeast extract, 2% peptone and 2% glucose, adjusted to pH 5.0, and 10X YP media was 10% yeast extract and 20% peptone, adjusted to pH 5.0.

4.3.2 Preparation of Dilute Acid Pretreated Solids

Biomass was pretreated at 10% solids (w/w) in a 0.6 L Parr batch-type, stirred reactor. After dilute acid pretreatment, the slurries were washed with distilled water (1300 ml water/30 g biomass) in a large funnel with a Whatman No.5 filter. Solid residues were collected and stored in the refrigerator (about 4°C), and served as samples for this study.

4.3.3 Straight Saccharification

The cellulose level of the dilute acid pretreated solid or raw biomass was adjusted to 1% with 50 mM sodium citrate (pH 5.0) buffer containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. After transferring the mixtures to a 20-ml glass scintillation vial, cellulase (60 FPU/g cellulose and 58 βGU/g cellulose) was added to the mixtures in a total 10 ml working volume. The vials were capped tightly and the reaction mixtures were incubated at 50°C with gentle rotation at 68 rpm for 8 days or until the

release of soluble sugar from the samples became negligible. At predetermined times, a 0.5 ml slurry of reaction mixture was withdrawn and boiled for 5 min. to terminate the reaction (Torget, 1993). The sample was centrifuged at 10,000 x g for 5 min. and glucose in the supernatant was quantified by the glucose oxidase/peroxidase assay (Liaw, 1994). Cellobiose in the supernatant was determined by incubation of the sample with cellobiase for 1 hr at 50°C followed by assay of the released glucose by the assay noted above (Liaw, 1994). The percentage of cellulose converted was taken as the glucose released upon cellulase and cellobiase treatment divided by the initial cellulose content (expressed as glucose following two stage-acid hydrolysis, Fenske, 1994) multiplied by 100. α-cellulose was used as a cellulose control in all enzyme saccharification experiments.

4.3.4 Simultaneous Saccharification and Fermentation (SSF)

A frozen yeast stock culture was added to a 125-ml baffled shake flask containing 50 ml of YPD and incubated at 38°C and 150 rpm for 8-12 hours. A 10% v/v inoculum from this culture was then transferred to a 250-ml baffled shake flask containing 1% yeast extract, 2% peptone and 5% glucose (pH 5.0) was incubated at 38°C and 150 rpm for an additional 8-12 hours. This culture served as inoculum for the SSF experiments.

The SSF reaction mixture was prepared aseptically in a 250 ml erlenmeyer flask fitted with a water trap and contained 1) 3% autoclaved cellulose derived from the dilute acid pretreated solid or raw biomass adjusted to pH 5.0 with NaOH and/or phosphoric acid, 2) 10 ml of autoclaved 10X YP, 3) 10 ml yeast inoculum, and 4) sterile-filtered cellulase (25 FPU/g cellulose and 25 βGU/g cellulose) in a final volume of 100 ml. Sterile

water was added to the water trap to minimize ethanol evaporation and to permit release of carbon dioxide. The reaction mixtures were incubated at 38°C and 150 rpm for a period of 8 days or until the production of ethanol in the reaction mixture became negligible (Philippidis et al., 1993). At predetermined times, 4 ml slurry were withdrawn aseptically and centrifuged at 10,000 x g for 5 min. The supernatants were then filtered through a 0.22 μM nylon membrane. Glucose in the supernatant was quantified by glucose oxidase/peroxidase assay (Liaw, 1994). Cellobiose in the supernatant was determined by incubation of the sample with cellobiase for 1 hr at 50°C followed by assay of the released glucose by the assay noted above (Liaw, 1994). Ethanol in the supernatant was measured by gas chromatography using a HP PoraPLOT Q 10 m, 0.53 mm capillary column (product # 19095P-Q01, Hawlett packard, Wilmington, DE) with a FID Detector. The internal standard for the ethanol determination was 1 g/L isopropanol. The percentage of cellulose converted was taken as the glucose released upon cellulase and cellobiase treatment plus ethanol fermented by yeast divided by the initial cellulose content (expressed as glucose following two stage-acid hydrolysis, Fenske, 1994) multiplied 100. As in the enzyme saccharification reactions, α-cellulose was used as a control in all SSF experiments. At the final day of fermentation (day 8), bacterial contamination was checked by plating 200 μl of the fermented slurries on yeast peptone dextrose (YPD) solid plates containing 0.03% cycloheximide and incubating the plates at 38°C for up to 3 days. No bacterial growth was observed in any of the SSF reaction flasks.

4.4 RESULTS AND DISCUSSION

4.4.1 Composition of Native and Pretreated Feedstocks

The compositional data for each of the native feedstocks used in this study are presented in Table 3. The values are presented in terms of the % by weight of the dry native feedstock. The macrocomponent composition of the three feedstocks are similar. The feedstocks ranged in lignin content from 20 to 29%. Carbohydrate accounted for approximately 60% of the dry weight of each feedstock; the glucan (apparent cellulose) content ranging from 32 to 40. Each of these feedstocks was pretreated with dilute sulfuric acid over a minimum of eight different pretreatment conditions. The pretreatment conditions were chosen based on models previously developed to optimize xylose yields from each of these feedstocks (Esteghlalian et al., 1996). The pretreatment conditions used for each feedstock and the corresponding compositional data for the pretreated samples are presented in Table 4, 5 and 6. Comparison of the compositional data of Tables 3 and Tables 4-6 shows that xylan is the predominant macrocomponent removed from the feedstocks during dilute acid pretreatments. In the course of this thesis the results from saccharification and SSF data will be interpreted with respect to the ratio of the percentage of cellulose to the percentage of xylan in the pretreated feedstock. The severity of the pretreatments can be assessed from this value, since xylans are relatively more susceptible to acid dissolution than glucans. In general, this ratio will tend to increase as the severity of the pretreatment increases.

Table 3. Composition of raw lignocellulosic biomass (percent dry weight)

Substrate	glycan (total)	glucan	xylan	galactan	arabinan	mannan	lignin (klason)	lignin (acid soluble)	ash	uronic acid	other
Switchgrass											
Mean	56.6	32.2	20.3	nd[a]	3.7	0.4	19.5	3.7	7.1	1.1	12.0
SEM[b]	nd	0.29	0.26	nd	0.04	0.02	0.3	0.03	0.06	0.01	nd
Cornstover/cob											
Mean	58.5	35.2	19.2	1.55	2.55	nd	18.24	2.41	12.56	nd	8.3
SEM	nd	0.51	0.24	0.06	0.04	nd	0.14	0.03	0.61	nd	nd
Poplar											
Mean	58.2	39.8	14.8	nd	1.2	2.4	26.9	2.2	1.3	2.4	9
SEM	nd	0.01	0.03	nd	0.02	0.04	0.026	0.06	0.02	0.13	nd

[a] Not determined.
[b] Standard error of mean.

Table 4. Composition of dilute acid pretreated switchgrass.

Pretreatment condition H_2SO4, temperature, time	Cellulose in PHS[a] (% dry weight)	Xylan in PHS (% dry weight)	Cellulose (%) / Xylose (%)
0.9%, 140°C, 15 min	47.8	nd[b]	nd
0.9%, 160°C, 5.0 min	51.0	nd	nd
0.9%, 180°C, 1.0 min	50.9	nd	nd
1.2%, 160°C, 1.0 min	48.6	nd	nd
0.6%, 180°C, 0.5 min	49.6	nd	nd
0.9%, 160°C, 3.0 min	49.3	nd	nd
0.9%, 180°C, 0.5 min	48.2	2.9	16.7
0.9%, 180°C, 0.5 min	51.5	nd	nd
0.9%, 180°C, 0.5 min	49.3	2.8	17.6
1.2%, 180°C, 0.5 min	49.7	2.2	22.6
1.2%, 180°C, 0.5 min	49.6	nd	nd
1.2%, 180°C, 0.5 min	51.4	1.5	34.3
1.2%, 180°C, 0.5 min	47.4	4.3	11.0

[a] Prehydrolysate solid
[b] Not determined

Table 5. Composition of dilute acid pretreated cornstover/corncob.

Pretreatment condition H_2SO4, temperature, time	Cellulose in PHS[a] (% dry weight)	Xylan in PHS (% dry weight)	Cellulose (%) / Xylose (%)
1.1%, 170°C, 2.1 min	66.8	9.6	7.0
1.2%, 160°C, 3.9 min	59.6	5.5	16.7
1.0%, 180°C, 1.2 min	65.6	5.1	16.2
0.9%, 180°C, 1.4 min	63.5	4.5	14.2
1.2%, 170°C, 1.9 min	61.9	5.1	12.1
1.0%, 170°C, 2.4 min	60.6	4.2	12.9
1.1%, 180°C, 1.0 min	61.7	3.8	11.9
1.2%, 180°C, 0.9 min	61.9	3.9	11.6

[a] Prehydrolysate solid

Table 6. Composition of dilute acid pretreated poplar.

Pretreatment condition H_2SO4, temperature, time	Cellulose in PHS[a] (% dry weight)	Xylan in PHS (% dry weight)	Cellulose (%) / Xylose (%)
1.2%, 170°C, 0.5 min	52.6	2.5	21.0
1.1%, 170°C, 1.3 min	53.1	1.3	40.8
1.2%, 170°C, 1.2 min	54.0	1.4	39.0
1.2%, 170°C, 2.0 min	54.4	1.3	42.0
1.2%, 180°C, 0.5 min	54.9	1.3	42.2
1.1%, 180°C, 0.53 min	53.8	1.6	34.0
0.9%, 180°C, 0.6 min	48.2	2.4	20.1
0.9%, 180°C, 0.6 min	53.9	nd	nd
0.9%, 180°C, 0.6 min	51.2	nd	nd
1.0%, 180°C, 0.56 min	52.0	nd	nd
1.0%, 180°C, 0.56min	54.8	nd	nd
1.0%, 180°C, 0.56min	53.8	2.3	23.6
1.0%, 180°C, 0.56 min	52.9	3.4	15.6

[a] Prehydrolysate solid
[b] Not determined

4.4.2 Saccharification and SSF of Native and Pretreated Feedstocks

The time courses for the saccharification of the switchgrass feedstocks and an α-cellulose reference standard are presented in Figure 9 and 10. The final glucose yield for each of the samples exceeded 85% of theoretical and seven of the eight treatments gave glucose yields above 90%. Theoretical glucose yields are based on the total amount of glucan contained in the pretreated feedstock. In the case of the native feedstock (without dilute acid pretreatment), less than 20% of the theoretically available glucose was in solution following 168 hrs of saccharification. The 12, 24 and 168 hr glucose yields for each of the substrates are presented in Table 7. The 12 hr glucose yields ranged from 65 to 80%, average of 73%, of the final values that were obtained at 168 hrs. In three cases, essentially 100% of the total glucose yield was obtained following 24 hrs saccharification. The principal products expected from the saccharification of cellulose are glucose and cellobiose (Philippidis et al., 1993; Spindler et al., 1992). Product analysis of these saccharification mixtures showed glucose as the only product of significance, suggesting that cellobiohydrolase activity was limiting relative to cellobiase activity under these saccharification conditions (see Figures 10). The relationship between both the 12 hr glucose yields and 168 hr glucose yields (as a % of the theoretical maximum) and the ratio of the cellulose-to-xylan content of the corresponding feedstocks is shown in figure 11. It was anticipated that the feedstocks having the higher glucan-to-xylan ratios would be more readily digested by the cellulolytic enzymes. This general trend was observed for both the rate of saccharification (12 hr values) and the overall saccharification yield (168 hr values). Stronger correlations would be expected for feedstocks covering a larger

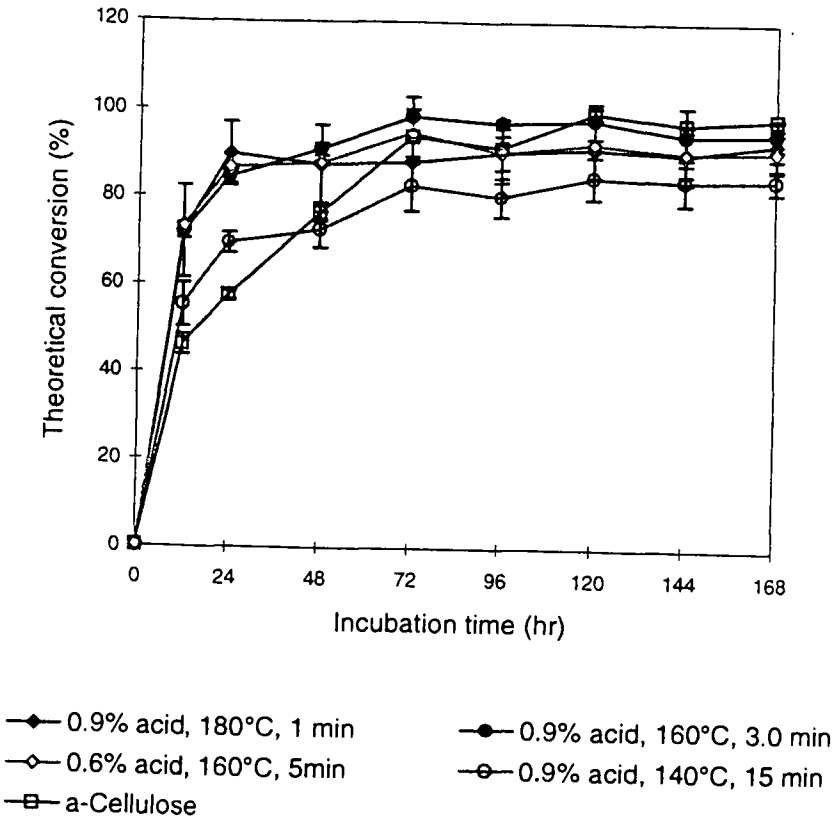

Figure 9a. Glucose yield of dilute acid pretreated switchgrass under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

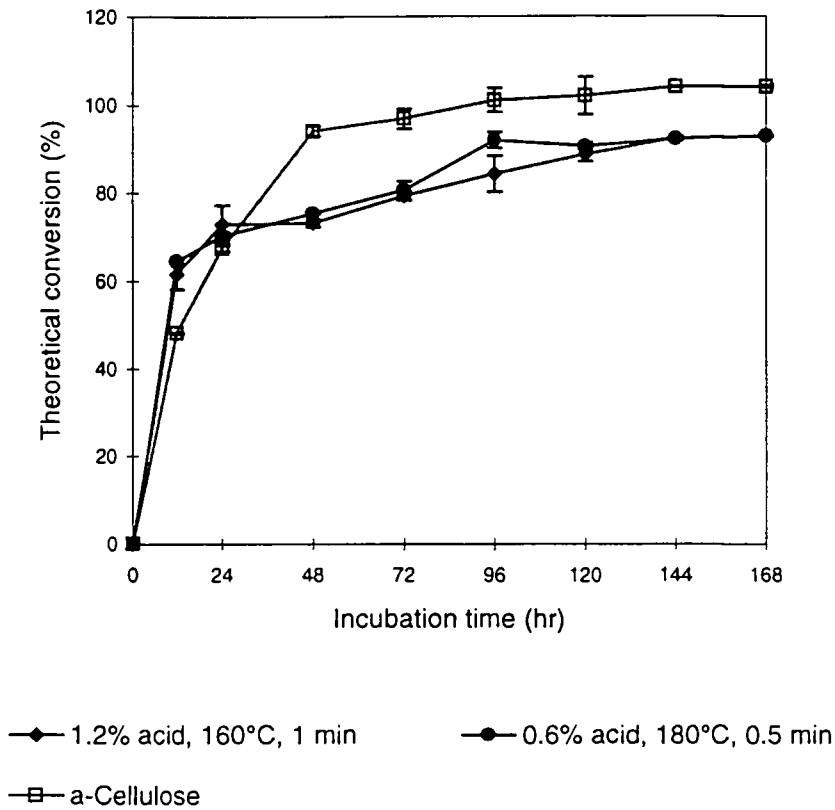

Figure 9b. Glucose yields of dilute acid pretreated switchgrass under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

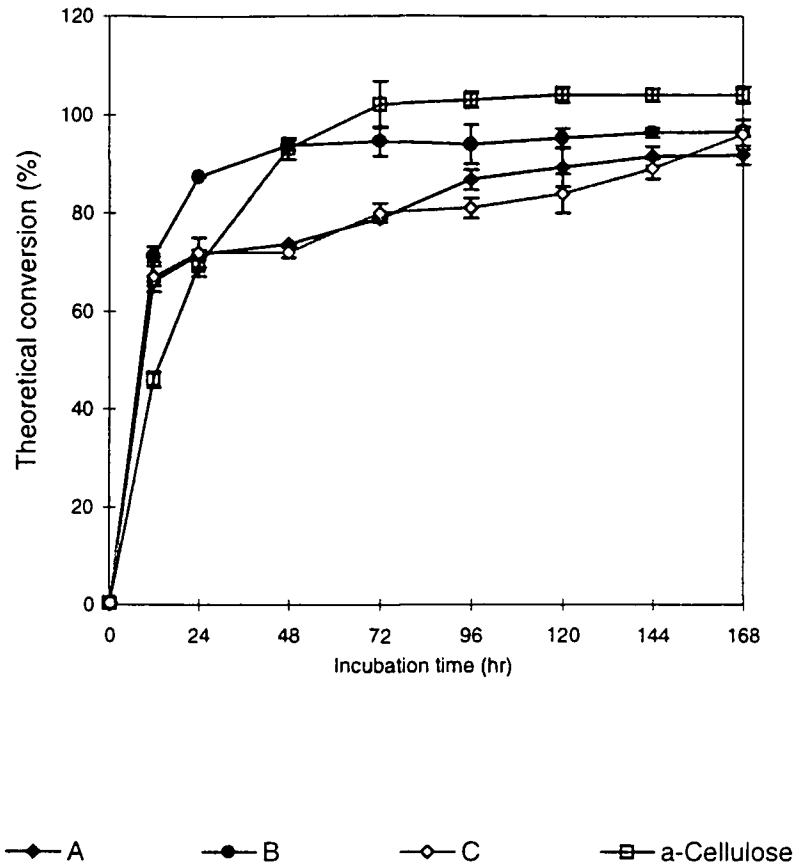

Figure 9c. Glucose yields of dilute acid pretreated switchgrass under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for curve A, B and C were pretreated individually with 0.9% sulfuric acid at 180°C for 0.5 minutes. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

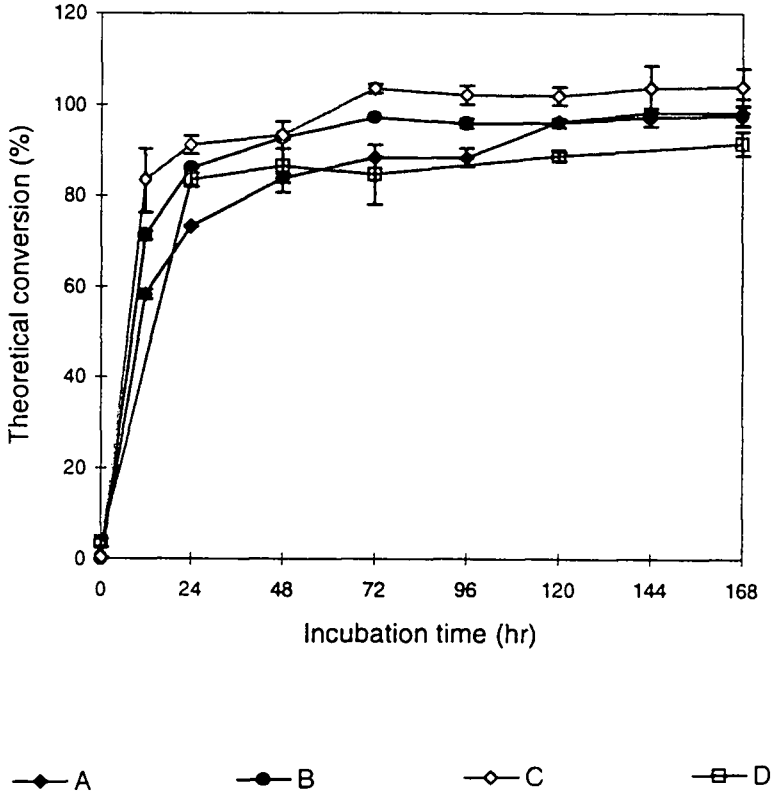

Figure 9d. Glucose yields of dilute acid pretreated switchgrass under straight saccharification processes. The pretreatment condition was the same for each samples (1.2% sulfuric acid at 180°C for 0.5 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

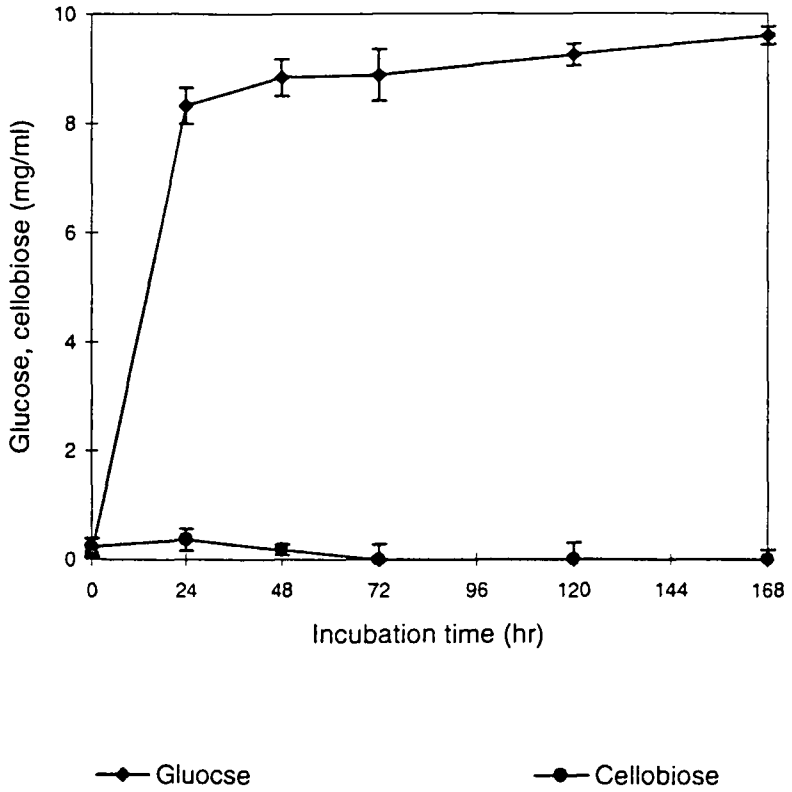

Figure 10. Glucose and cellobiose concentrations of dilute acid pretreated switchgrass under straight saccharification processes. The sample was pretreated with 0.9% sulfuric acid at 180°C for 0.5 minutes. Data points are means and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

Table 7. Comparative sugar yield from switchgrass following dilute acid pretreatments.

Pretreatment condition	cellulose (%) /xylan (%)	Sugar Yields (% of theoretical)		
H_2SO4, temperature, time		Glucose yield@ 12 hr	Glucose yield@ 24 hr	Glucose yield @ 168 hr
Raw switchgrass	1.6	13 ± 1 [a]	15 ± 1 [a]	18 ± 4 [b]
0.9%, 140°C, 15 min	nd	55 ± 5 [a]	69 ± 2 [a]	85 ± 3 [a]
0.9%, 160°C, 5.0 min	nd	73 ± 1 [a]	87 ± 0 [a]	91 ± 4 [a]
0.9%, 180°C, 1.0 min	nd	74 ±10 [a]	89 ± 7 [a]	93 ± 3 [a]
1.2%, 160°C, 1.0 min	nd	62 ± 3 [a]	73 ± 4 [a]	93 ± 0 [a]
0.6%, 180°C, 0.5 min	nd	64 ± 1 [a]	70 ± 1 [a]	93 ± 0 [a]
0.9%, 160°C, 3.0 min	nd	72 ± 1 [a]	84 ± 1 [a]	95 ± 3 [a]
0.9%, 180°C, 0.5 min	16.7	67 ± 3 [a]	72 ± 3 [a]	96 ± 2 [a]
0.9%, 180°C, 0.5 min	nd	71 ± 2 [a]	87 ± 0 [a]	97 ± 1 [a]
0.9%, 180°C, 0.5 min	17.6	66 ± 1 [a]	72 ± 1 [a]	92 ± 2 [a]
1.2%, 180°C, 0.5 min	22.6	58 ± 1 [a]	73 ± 0 [a]	98 ± 0 [a]
1.2%, 180°C, 0.5 min	nd	71 ± 1 [a]	87 ± 0 [a]	98 ± 2 [a]
1.2%, 180°C, 0.5 min	34.3	83 ± 7 [a]	91 ± 2 [a]	104 ± 4 [a]
1.2%, 180°C, 0.5 min	11	nd*	83 ± 2 [a]	91 ± 3 [b]

[a] Standard deviation of two reaction flasks from single experiment using individual pretreated sample.
[b] Standard deviation of three experiments using same batch pretreated sample.
* not determined

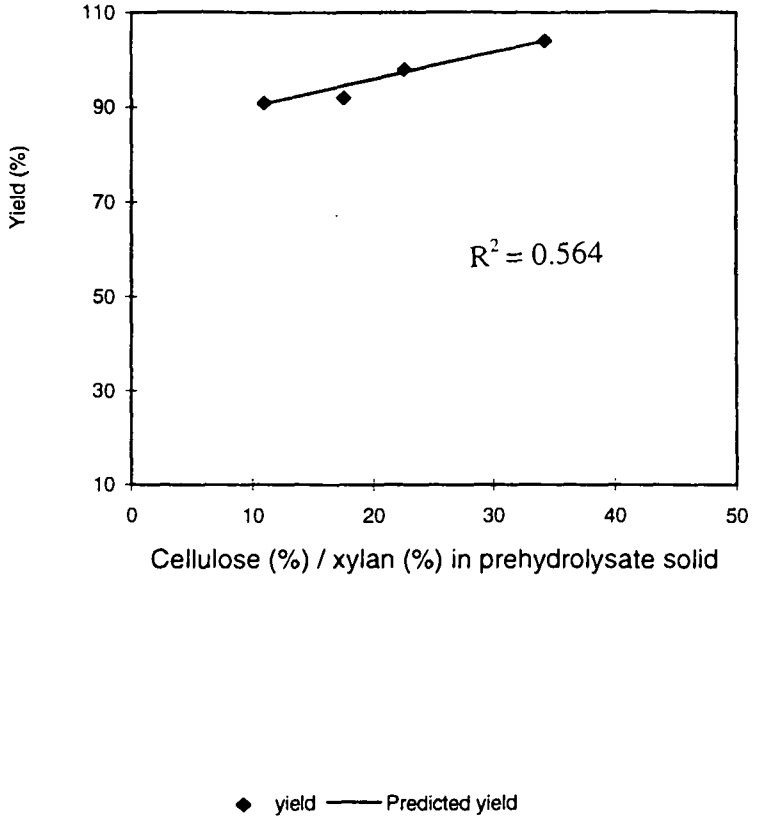

Figure 11a. Fit plot of the 12 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated switchgrass.

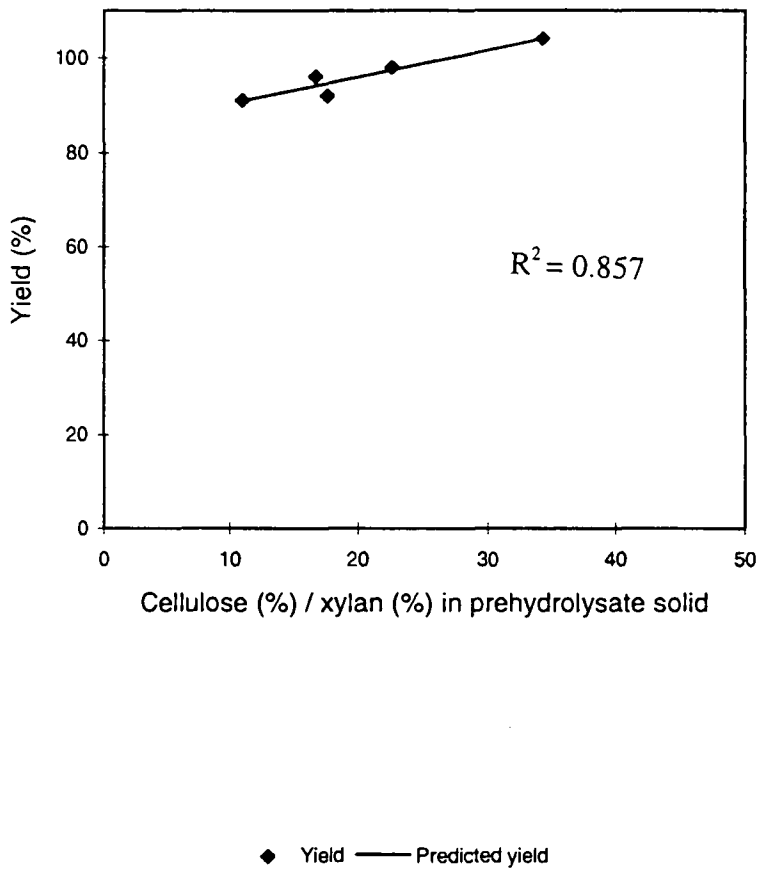

Figure 11b. Fit plot of the 168 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated switchgrass.

range of xylan contents (Grohman et al., 1985). Each of the pretreatments used in this study removed greater than 80% of the xylan originally present in the native feedstock, the objective being to study those pretreatments associated with maximum xylose yields.

Five switchgrass feedstocks, representing two different pretreatment conditions, were tested under SSF conditions. The time course of ethanol yields for each of the pretreated switchgrass feedstocks are presented in Figures 12 (absolute) and 13 (% of theoretical). Ethanol yields ranged from 84 to 90% of theoretical. When considering % of theoretical for ethanol, we have assumed that two moles of ethanol can be produced per each mole of glucose in the feedstock. This approach means that a 100% yield can never by achieved because a portion of the glucose consumed by the yeast will be used for cell maintenance and growth. Enzyme saccharification and glucose fermentation are occurring simultaneously in the SSF process and, hence, it is informative to monitor the saccharification products over the time course of the SSF experiment. These values are presented in Figures 14 and 15 for the switchgrass feedstocks. The presence of cellobiose in the initial phase of the SSF experiments suggests a limiting amount of β-glucosidase activity under SSF conditions. This is in contrast to what was observed under the straight saccharification conditions, as discussed above. The data also shows that in the early phase of the SSF experiment the rate of enzyme saccharification is greater than the rate at which the yeast cells can consume the saccharification products. However, little glucose, or cellobiose, can be detected three days into the experiment - suggesting that enzyme saccharification is now the limiting part of the process. The difference in the % cellulose conversion in straight saccharification experiments and SSF experiments for the

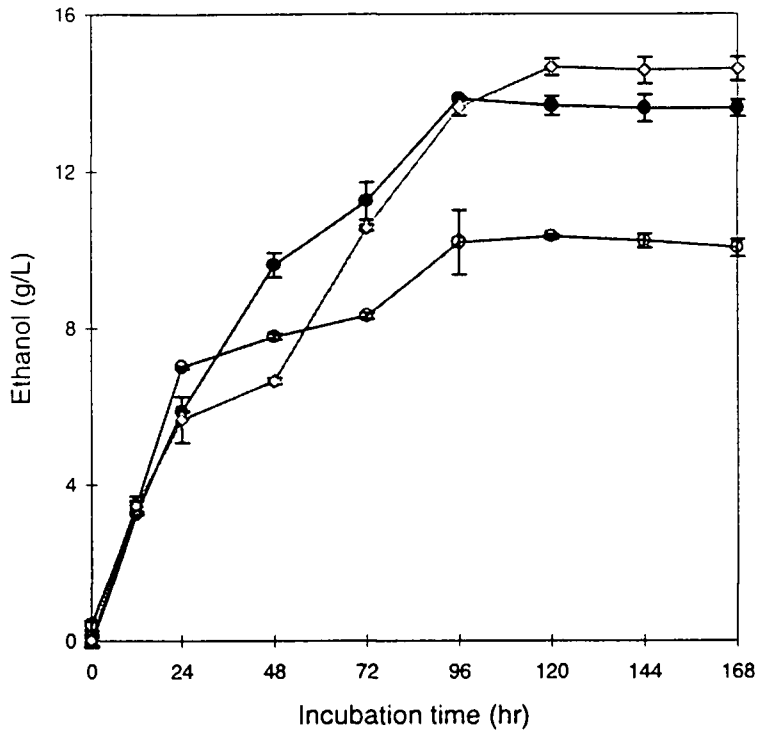

Figure 12a. Ethanol concentrations of dilute acid pretreated switchgrass under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.5 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

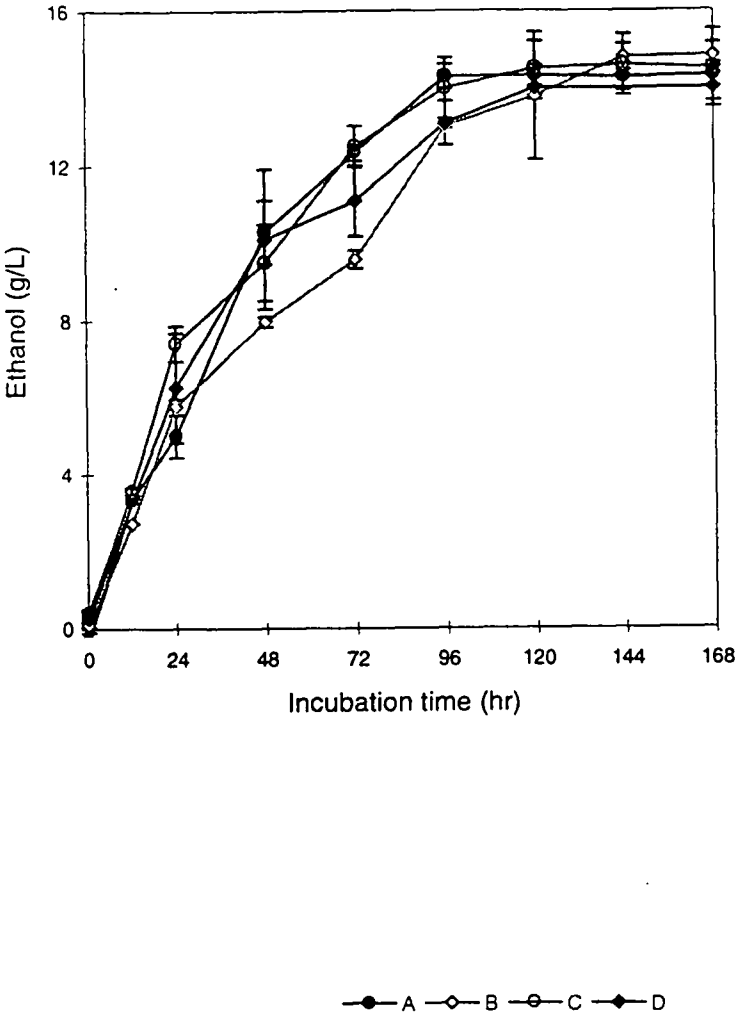

Figure 12b. Ethanol concentrations of dilute acid preteated switchgrass under SSF process. The pretreatment condition was the same for each samples (1.2% sulfuric acid at 180°C for 0.5 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

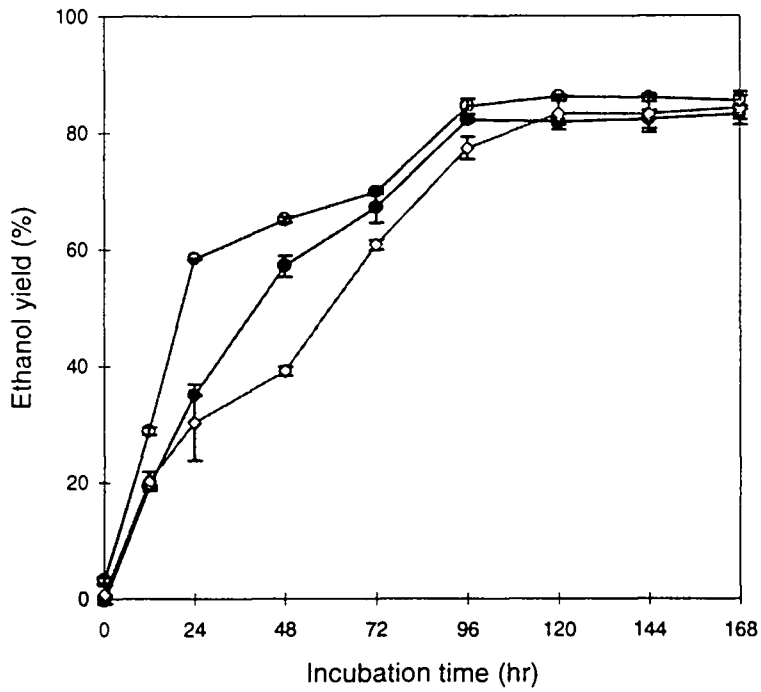

Figure 13a. Theoretical ethanol yields of dilute acid pretreated switchgrass under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.5 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

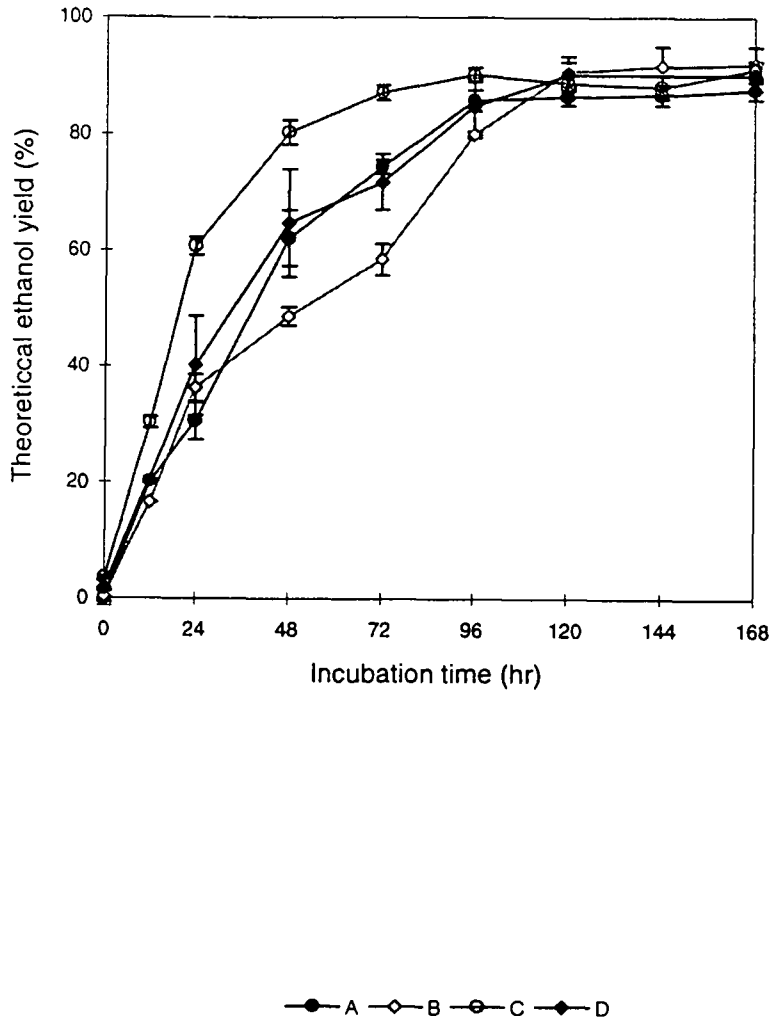

Figure 13b. Theoretical ethanol yields of dilute acid pretreated switchgrass under SSF processes. The pretreatment condition was the same for each samples (1.2% sulfuric acid at 180°C for 0.5 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

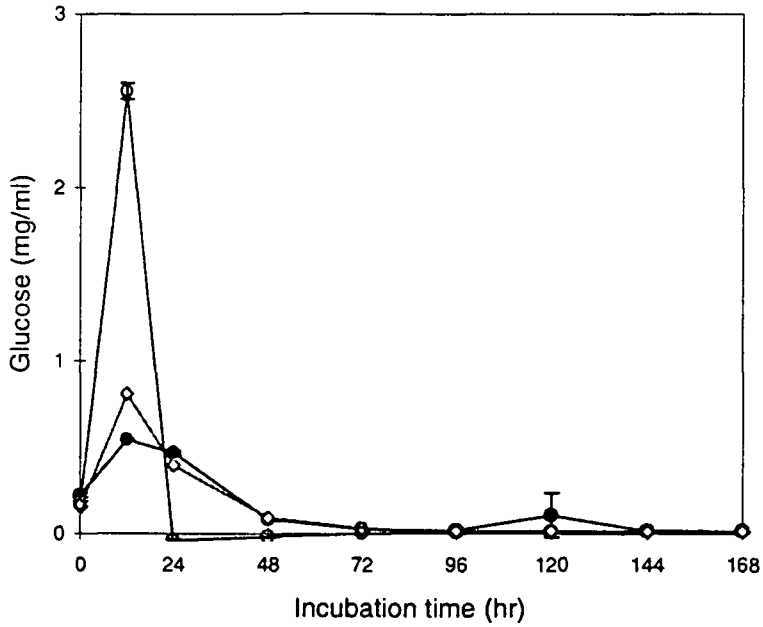

Figure 14a. Glucose concentrations of dilute acid pretreated switchgrass under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.5 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

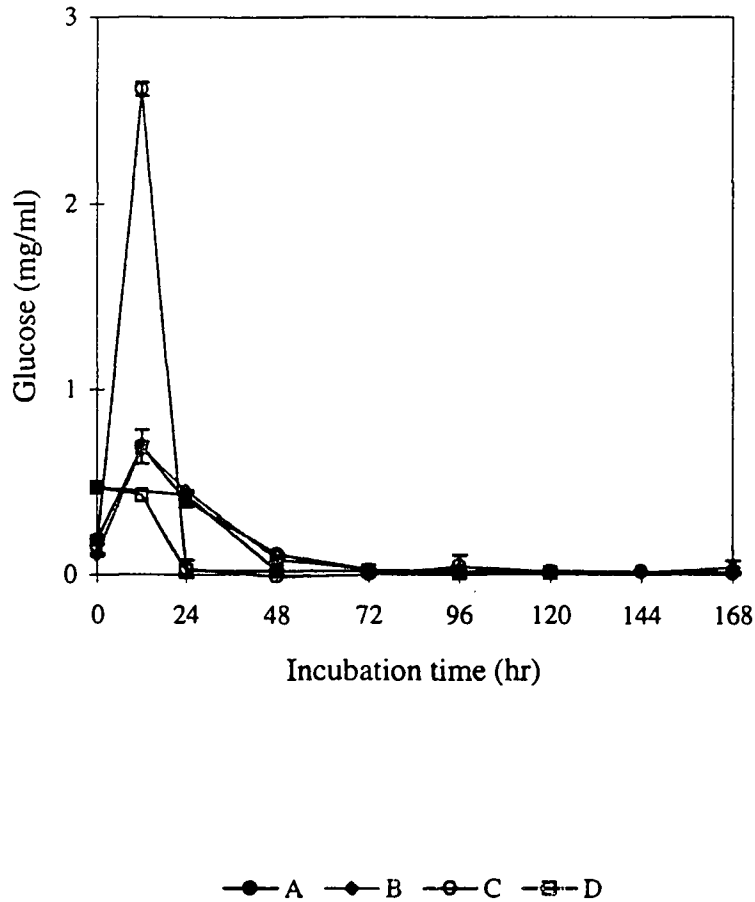

Figure 14b. Glucose concentrations of dilute acid pretreated switchgrass under SSF processes. The pretreatment condition was the same for each samples (1.2% sulfuric acid at 180°C for 0.5 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

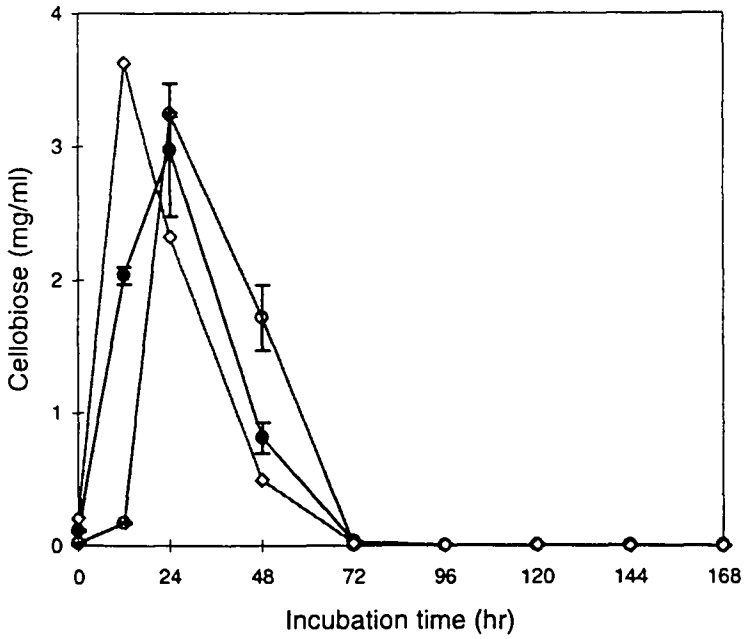

Figure 15a. Cellobiose concentrations of dilute acid pretreated switchgrass under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.5 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

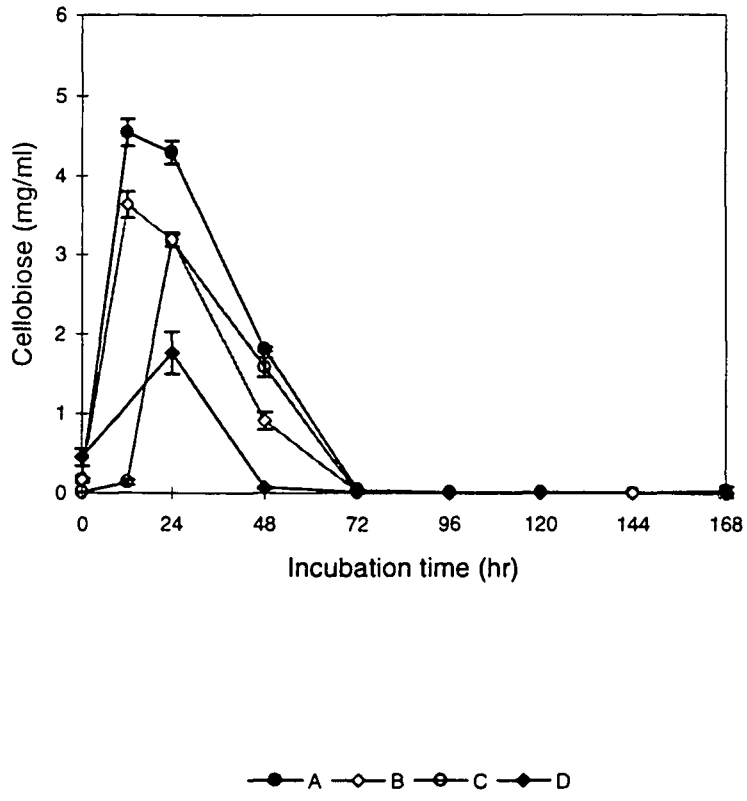

Figure 15b. Cellobiose concentrations of dilute acid pretreated switchgrass under SSF processes. The pretreatment condition was the same for each samples (1.2% sulfuric acid at 180°C for 0.5 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

switchgrass feedstocks are summarized in Table 8. In each case where the appropriate data is available, see footnote to Table 8, the % cellulose converted in the two types of experiments were essentially equal (< 5%).

The enzyme saccharification and SSF properties of pretreated cornstover feedstocks were analyzed in experiments analogous to those described for switchgrass. The enzyme saccharification time courses in terms of percent of theoretical yield, glucose concentrations and cellobiose concentrations are shown in Figures 16, 17 and 18, respectively. Saccharification yields at the completion of the 168 hr incubation period ranged from 84 to 100 %, with six of the eight feedstocks tested corresponding to theoretical yields above 95%. In contrast, the saccharification yield for raw cornstover under equivalent conditions is approximately 32%. The 12 hr yields for the pretreated feedstocks ranged from 75 to 92 % of theoretical, which corresponds to 85 to 95%, average of 90%, of the final values that were obtained at 168 hrs (Table 9). The high product yields at 12 hr and 24 hr saccharification times are consistent with the pretreated cornstover feedstocks being the most readily susceptible to enzyme saccharification. The product profiles show only minimal amounts of cellobiose in saccharification mixtures at 12 hrs, with glucose being the only detectable product beyond 12 hrs reaction time. The relationship between both the 12 hr glucose yields and 168 hr glucose yields (as a % of the theoretical maximum) and the ratio of the cellulose-to-xylan content of the cornstover feedstocks are shown in Figure 19. These figures indicate a general trend which suggests that those cornstover feedstocks with higher glucan-to-xylan ratios will have a more reactive cellulose component. SSF experiments with cornstover feedstocks also indicated

Table 8. Comparison of enzyme digestibility under straight saccharification and SSF porcess

Substrate	yield[a] saccharification	yield[b] SSF	celllulose converted SSF[c]	difference[d]	cellulose (%) / xylan (%) in PHS
Switchgrass	96 ± 2.0	84 ± 2.8	92	4	20.1
	98 ± 2.0	88 ± 1.5	94	4	23.6
	91± 3.0	90 ± 2.0	93	2	15.6

[a] = sum of saccharification products (glucose + cellobiose) / potential cellulose in pretreated solid
[b] = sum of fermentation products (ethanol + glucose + cellobiose) / potential cellulose in pretreated solid
[c] = 100% - residual cellulose after 8 day SSF
[d] = column 2 - column 4

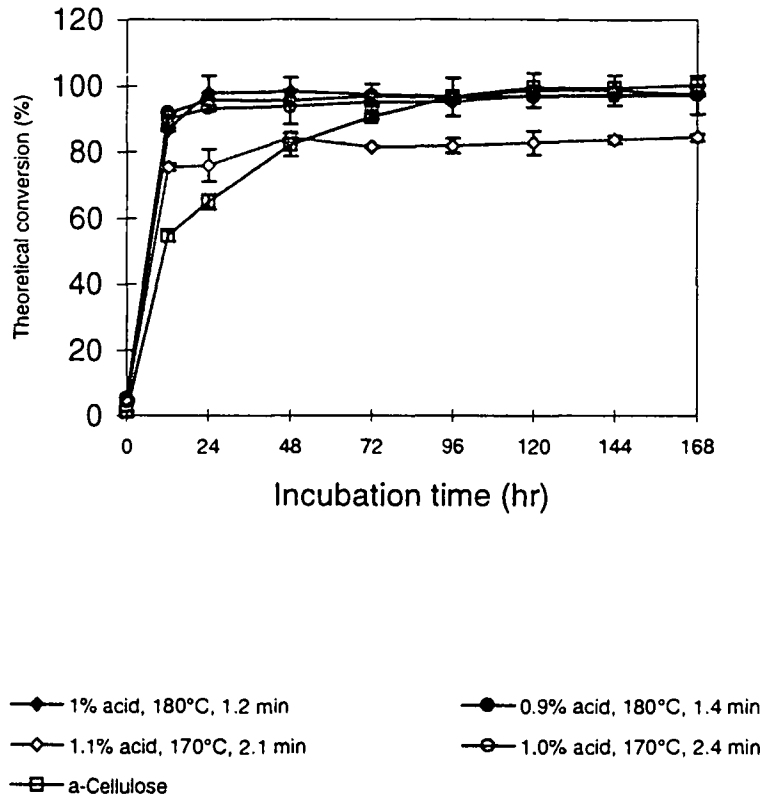

Figure 16a. Glucose yield of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

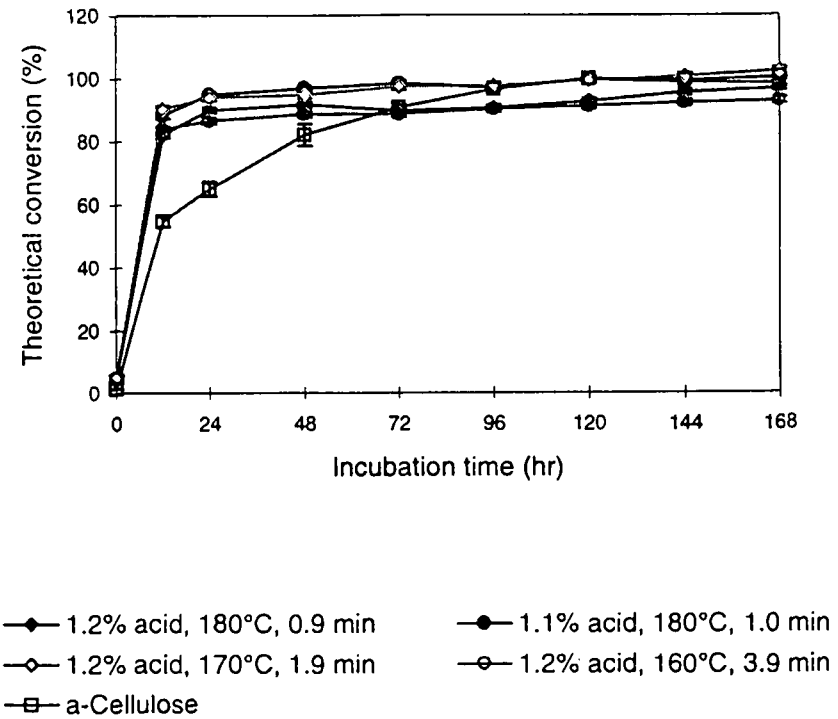

Figure 16b. Glucose yield of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

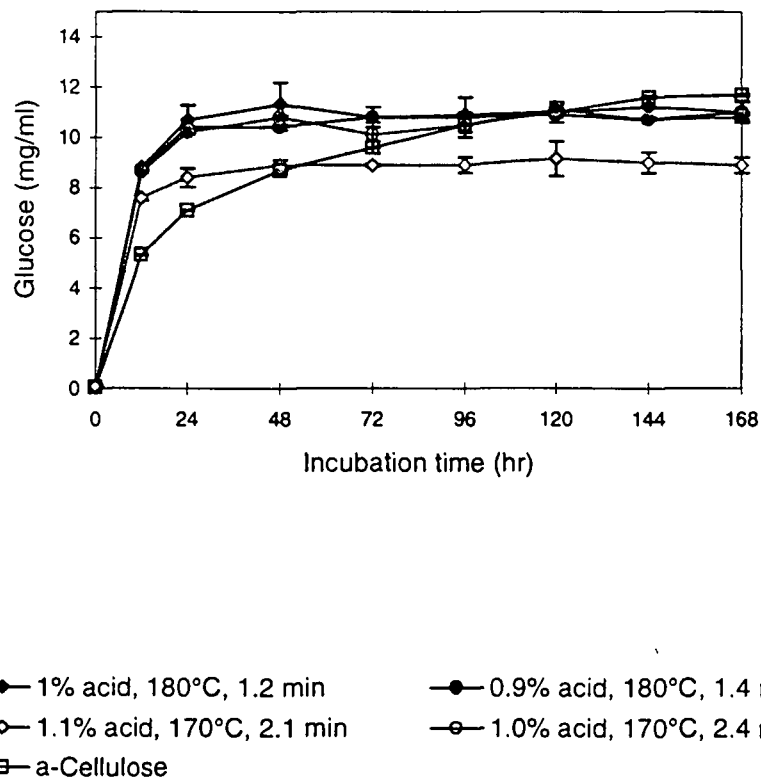

Figure 17a. Glucose concentrations of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

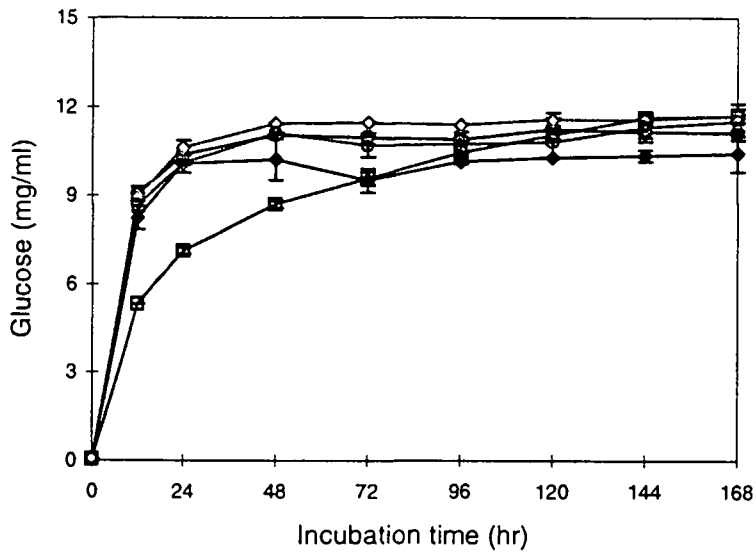

Figure 17b. Glucose concentrations of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

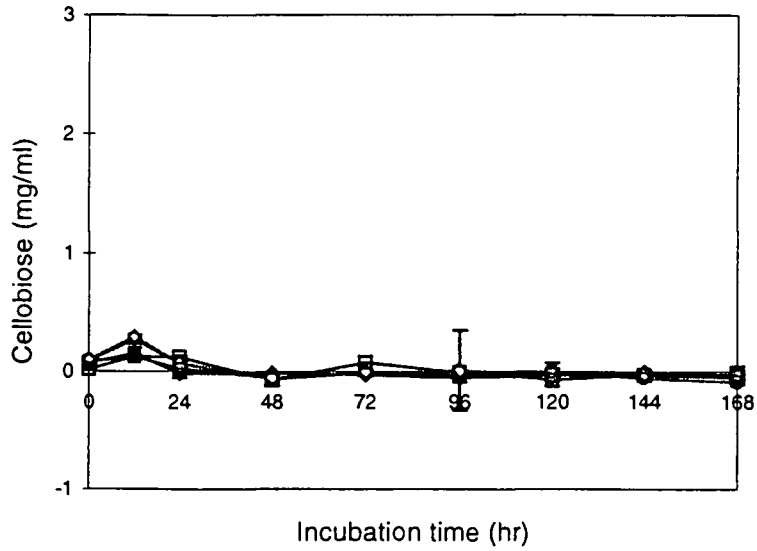

Figure 18a. Cellobiose concentrations of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

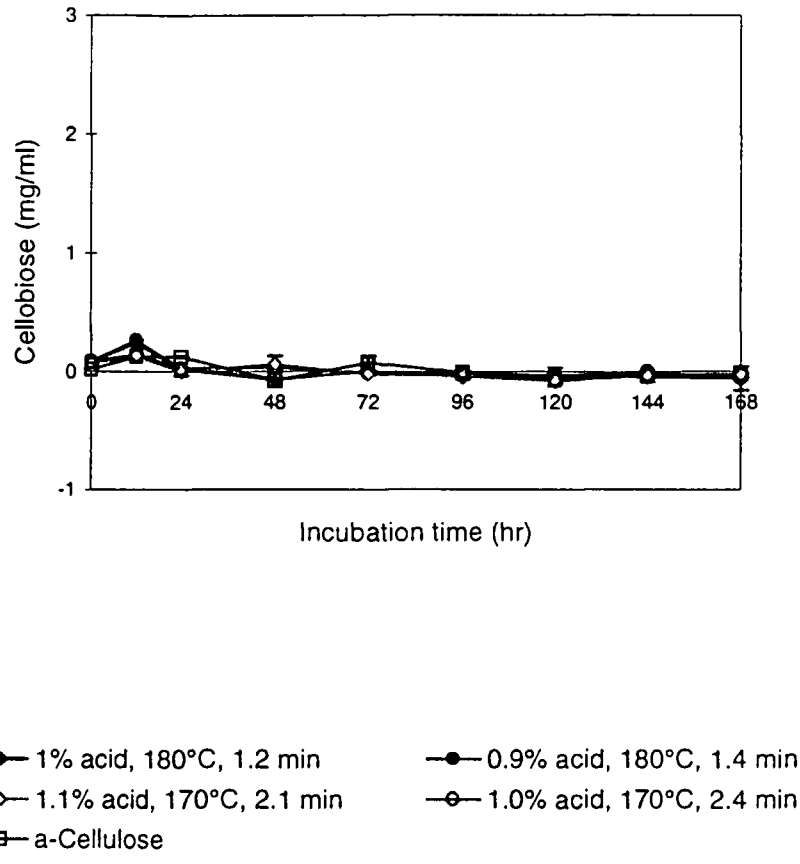

Figure 18b. Cellobiose concentrations of dilute acid pretreated cornstover/corncob under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

Table 9. Comparative sugar yield from cornstover/corncob following dilute acid pretreatments.

Pretreatment condition	cellulose /xylan (%)	Sugar Yields (% of theoretical)		
H_2SO4, temperature, time		Glucose yield@ 12 hr	Glucose yield@ 24 hr	Glucose yield @ 168 hr
Raw Cornstover/corncob	1.8	nd*	24 ± 1 [a]	32 ± 1 [a]
1.1%, 170°C, 2.1 min	7.0	75 ± 1 [a]	76 ± 5 [a]	84 ± 1 [a]
1.2%, 160°C, 3.9 min	16.7	84 ± 3 [a]	87 ± 3 [a]	93 ± 2 [a]
1.0%, 180°C, 1.2 min	16.2	87 ± 1 [a]	98 ± 5 [a]	97 ± 6 [a]
0.9%, 180°C, 1.4 min	14.2	92 ± 0 [a]	95 ± 0 [a]	97 ± 0 [a]
1.2%, 170°C, 1.9 min	12.1	90 ± 2 [a]	94 ± 3 [a]	102 ± 6 [a]
1.0%, 170°C, 2.4 min	12.9	90 ± 2 [a]	93 ± 1 [a]	97 ± 0 [a]
1.1%, 180°C, 1.0 min	11.9	88 ± 2 [a]	95 ± 1 [a]	98 ± 0 [a]
1.2%, 180°C, 0.9 min	11.6	82 ± 1 [a]	97 ± 6 [a]	97 ± 6 [a]

[a] Standard deviation of two reaction flasks from single experiment using individual pretreated sample.
* not determined

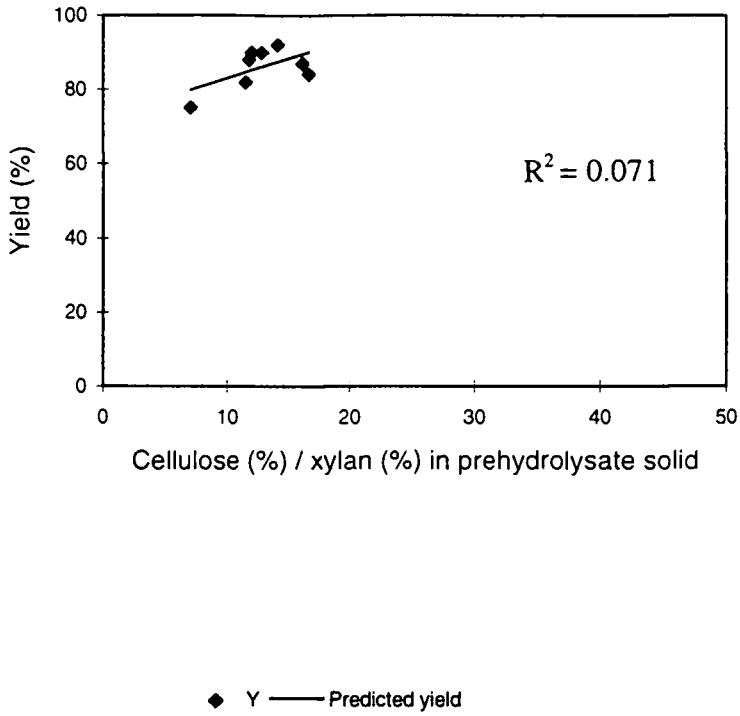

Figure 19a. Fit plot of the 12 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated cornstover/corncob.

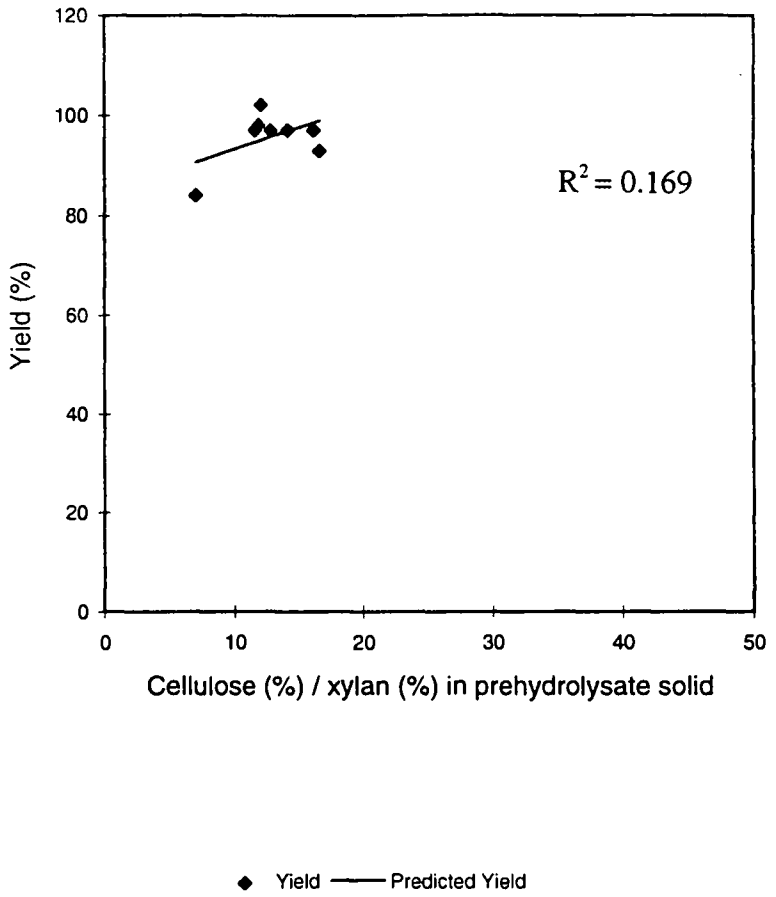

Figure 19b. Fit plot of the 168 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated cornstover/corncob.

that their cellulose component was readily available for enzymatic saccharification. SSF time courses for percent of theoretical cellulose converted, ethanol production, glucose concentrations and cellobiose concentrations are presented in Figures 20, 21, 22 and 23, respectively. Each of the pretreated feedstocks reached maximum ethanol yields, corresponding to an average of 90% of theoretical, within 3 days. Cellobiose was observed in the SSF cultures at 12 hrs and 24 hrs, with only low levels of glucose being present from 48 hrs on. The difference in the % cellulose conversion in straight saccharification experiments and SSF experiments for the cornstover feedstocks are summarized in Table 10. The % cellulose converted in the two types of experiments were essentially equal (< 5%), as was observed for the switchgrass feedstocks.

The enzyme saccharification and SSF properties of the pretreated poplar feedstocks were determined as for the other feedstocks. The enzyme saccharification time courses in terms of percent of theoretical yield and glucose and cellobiose concentrations are shown in Figures 24 and 25, respectively. The overall yields from the poplar feedstocks ranged from 76% to 100% of theoretical. Those values are in the same general range as those obtained for switchgrass and corn stover. However, it can be seen from the time courses and from the corresponding 12, 24 and 168 hr glucose yield data of Table 11 that the rate of glucose production is considerably slower for the poplar substrate relative to the two herbaceous feedstocks. On average, only 47% of the total glucose solubilized had been produced at 12 hrs, that is compared to 73% and 90% for the switchgrass and cornstover feedstocks, respectively. The relationship between the 12 hr and 168 hr glucose yields (as a % of the theoretical maximum) and the ratio of the cellulose-to-xylan content of the corresponding feedstocks is shown in Figure 26. In contrast to the other

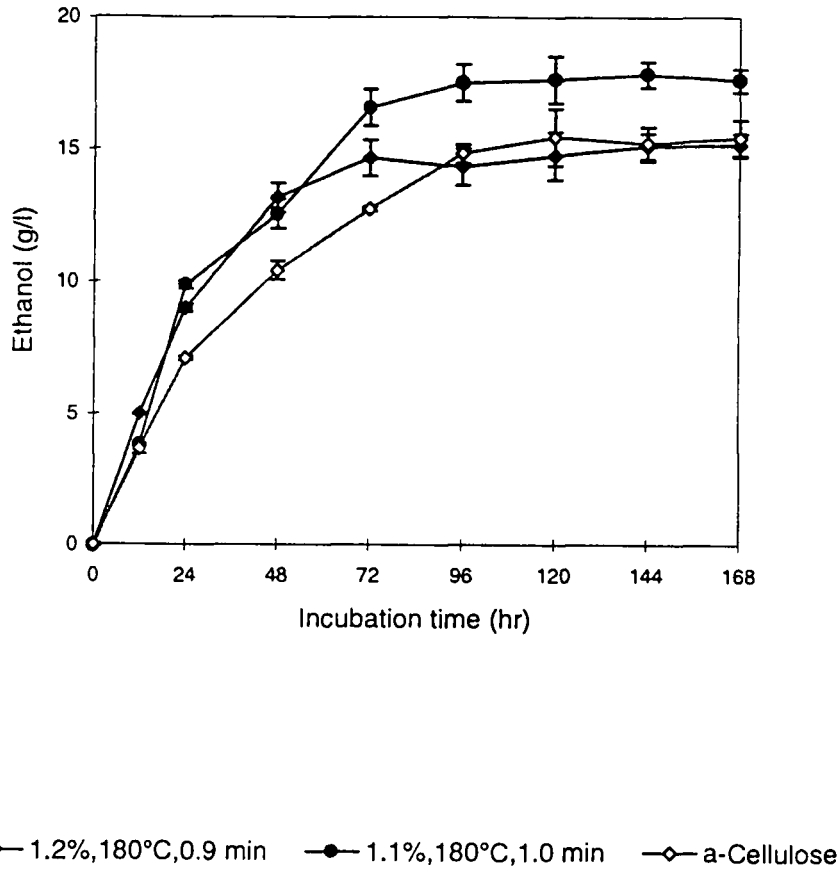

Figure 20. Ethanol concentrations of dilute acid pretreated cornstover/corncob under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

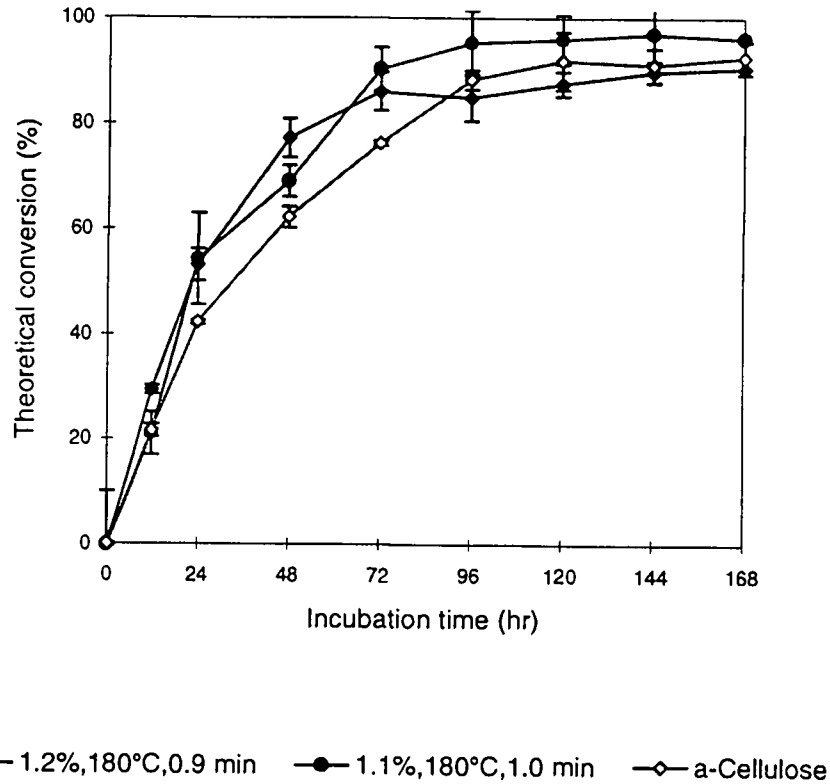

Figure 21. Theoretical ethanol Yield of dilute acid pretreated cornstover/corncob under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

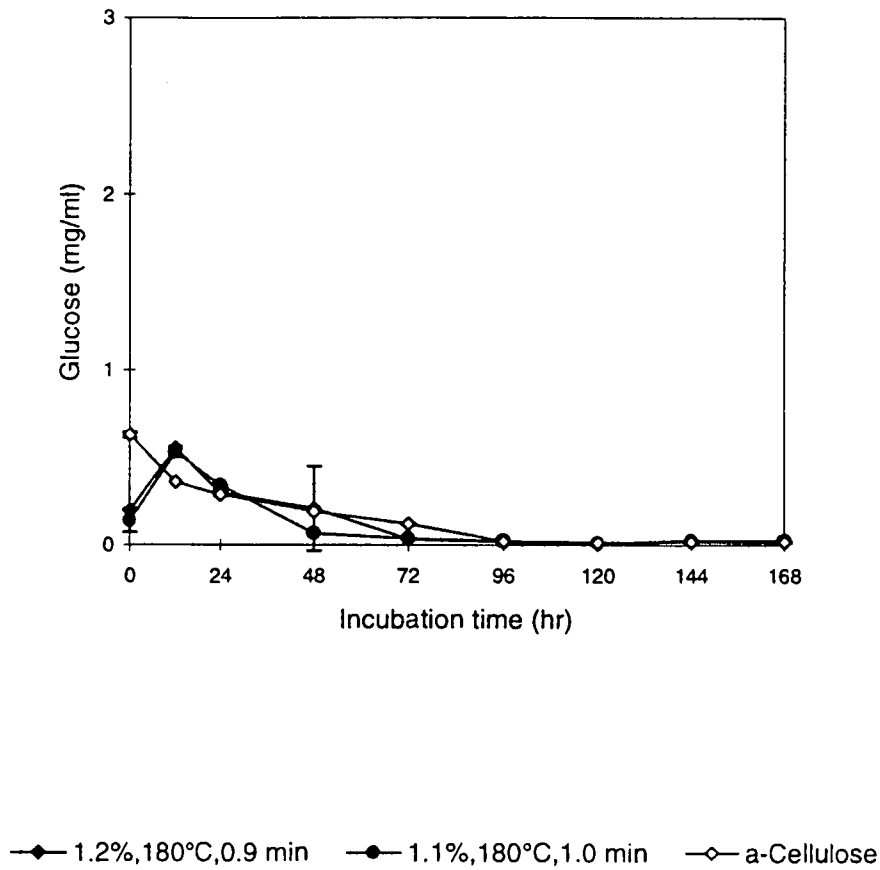

Figure 22. Glucose concentrations of dilute acid pretreated cornstover/corncob under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

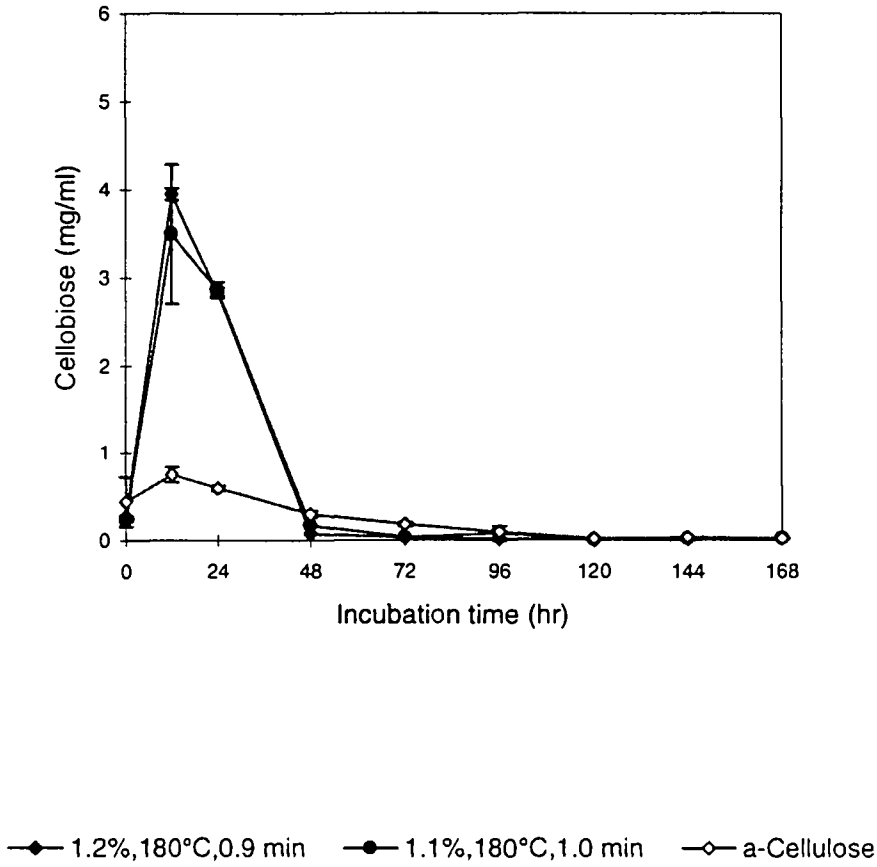

Figure 23. Cellobiose concentrations of dilute acid pretreated cornstover/corncob under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

Tab 10. Comparison of enzyme digestibility under straight saccharification and SSF porcess

Substrate	yield[a] saccharification	yield[b] SSF	difference[c]	cellulose (%) / xylan (%) in PHS
Cornstover	97 ± 5.5	91 ± 2	6	11.9
	98 ± 0.0	96 ± 5.1	2	11.6

[a] = sum of saccharification products (glucose + cellobiose) / potential cellulose in pretreated solid
[b] = sum of fermentation products (ethanol + glucose + cellobiose) / potential cellulose in pretreated solid
[c] = column 2 - column 3

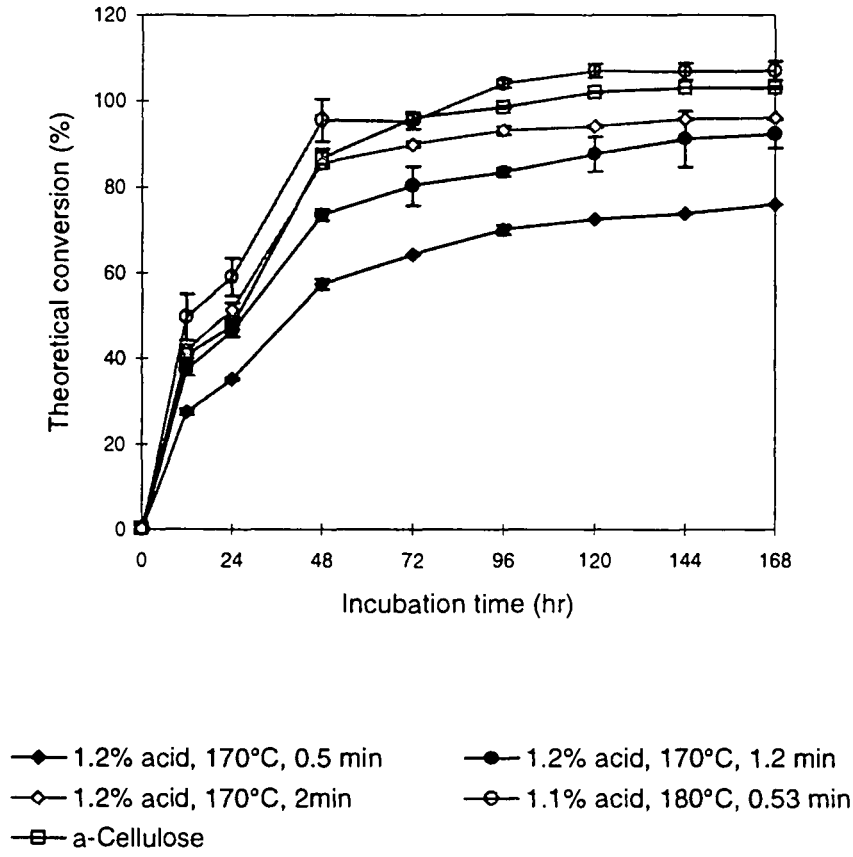

Figure 24a. Glucose yield of dilute acid pretreated poplar under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

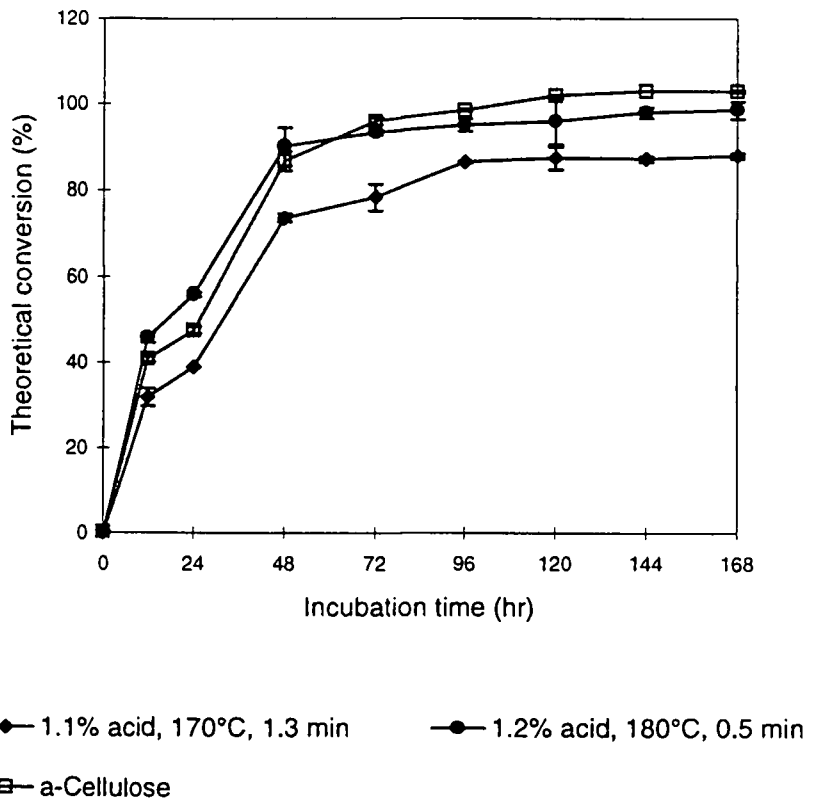

Figure 24b. Glucose yields of dilute acid pretreated poplar under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

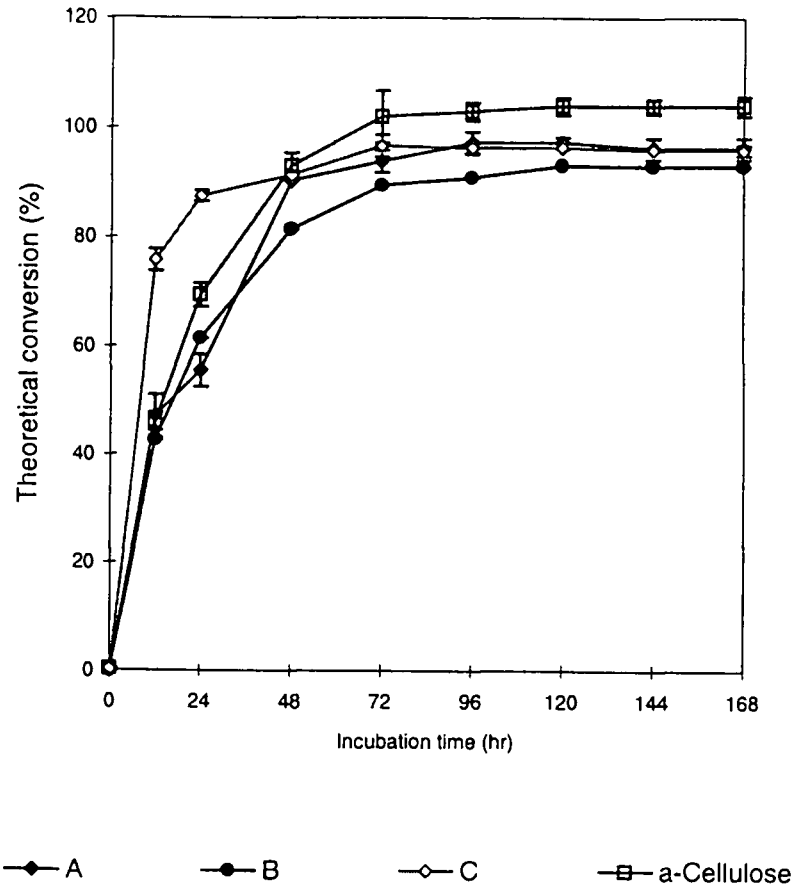

Figure 24c. Glucose yields of dilute acid pretreated poplar under straight saccharification processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for curve A, B and C were pretreated individually with 0.9% sulfuric acid at 180°C for 0.56 minutes. α-cellulose was used as a cellulose control. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

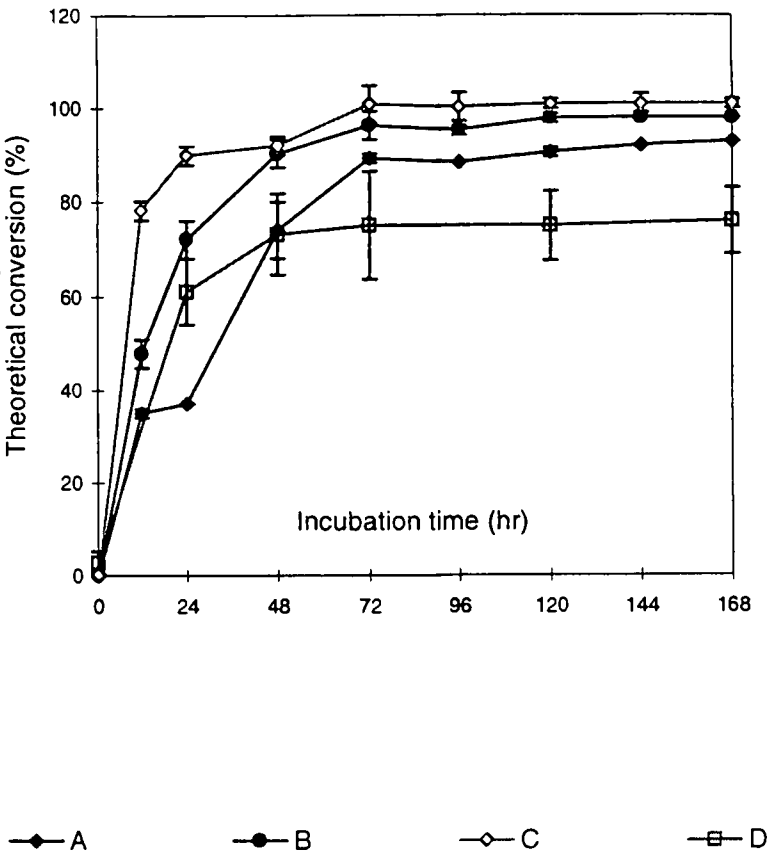

Figure 24d. Glucose yields of dilute acid pretreated switchgrass under straight saccharification processes. The pretreatment condition was the same for each samples (1.0% sulfuric acid at 180°C for 0.6 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

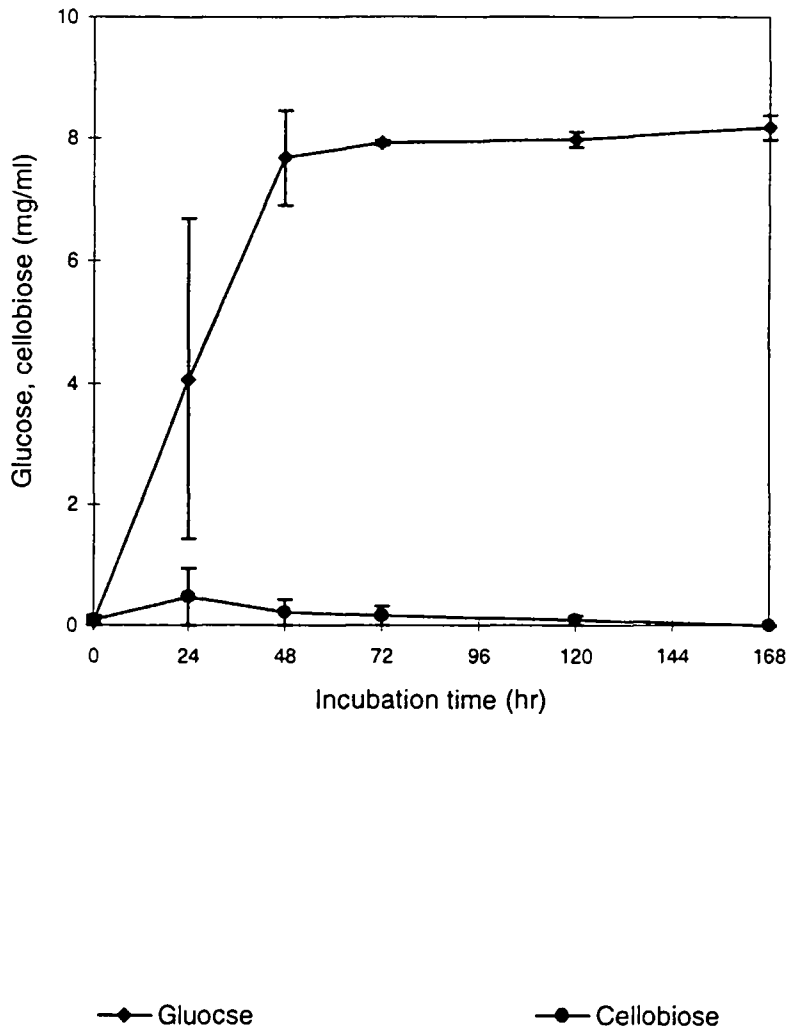

Figure 25. Glucose and cellobiose concentrations of dilute acid pretreated poplar under straight saccharification processes. The sample was pretreated with 1.0 sulfuric acid at 180°C for 0.6 minutes. Data points are means and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level of each reaction mixture was adjusted to 1% with 50 mM sodium citrate buffer (pH 5.0) containing 0.04% tetracycline and 0.03% cycloheximide as preservatives. The enzyme load was 60 FPU/g cellulose and 58 βGU/g cellulose. The reaction condition was 50°C with gentle rotation at 68 rpm.

Table 11. Comparative sugar yield from poplar following dilute acid pretreatments.

Pretreatment condition	cellulose (%) / % xylan (%)	Sugar yields (% of theoretical)		
H_2SO4, temperature, time		Glucose yield@ 12 hr	Glucose yield@ 24 hr	Glucose Yield @ 168 hr
Raw poplar	2.7	nd*	7.0 ± 1 [a]	10 ± 2 [a]
1.2%, 170°C, 0.5 min	21.0	27 ± 1 [a]	35 ± 0 [a]	75 ± 0 [a]
1.1%, 170°C, 1.3 min	40.8	32 ± 2 [a]	39 ± 0 [a]	87 ± 1 [a]
1.2%, 170°C, 1.2 min	39.0	37 ± 1 [a]	46 ± 2 [a]	92 ± 0 [a]
1.2%, 170°C, 2.0 min	42.0	42 ± 5 [a]	51 ± 4 [a]	95 ± 2 [a]
1.2%, 180°C, 0.5 min	42.2	46 ± 1 [a]	55 ± 1 [a]	99 ± 2 [a]
1.1%, 180°C, 0.53 min	34	50 ± 4 [a]	59 ± 4 [a]	107 ± 4 [a]
0.9%, 180°C, 0.6 min	20.1	47 ± 3 [a]	55 ± 3 [a]	96 ± 2 [a]
0.9%, 180°C, 0.6 min	nd	43 ± 0 [a]	61 ± 0 [a]	93 ± 0 [a]
0.9%, 180°C, 0.6 min	nd	76 ± 2 [a]	87 ± 1 [a]	96 ± 1 [a]
1.0%, 180°C, 0.56 min	nd	48 ± 3 [a]	72 ± 5 [a]	98 ± 0 [a]
1.0%, 180°C, 0.56min	nd	35 ± 1 [a]	37 ± 0 [a]	93 ± 0 [a]
1.0%, 180°C, 0.56min	23.6	78 ± 2 [a]	89 ± 2 [a]	101 ± 1 [a]
1.0%, 180°C, 0.56 min	15.6	nd	61 ± 7 [a]	79 ± 4 [b]

[a] Standard deviation of two reaction flasks from single experiment using individual pretreated sample.
[b] Standard deviation of three experiments using same batch pretreated sample.
* not determined

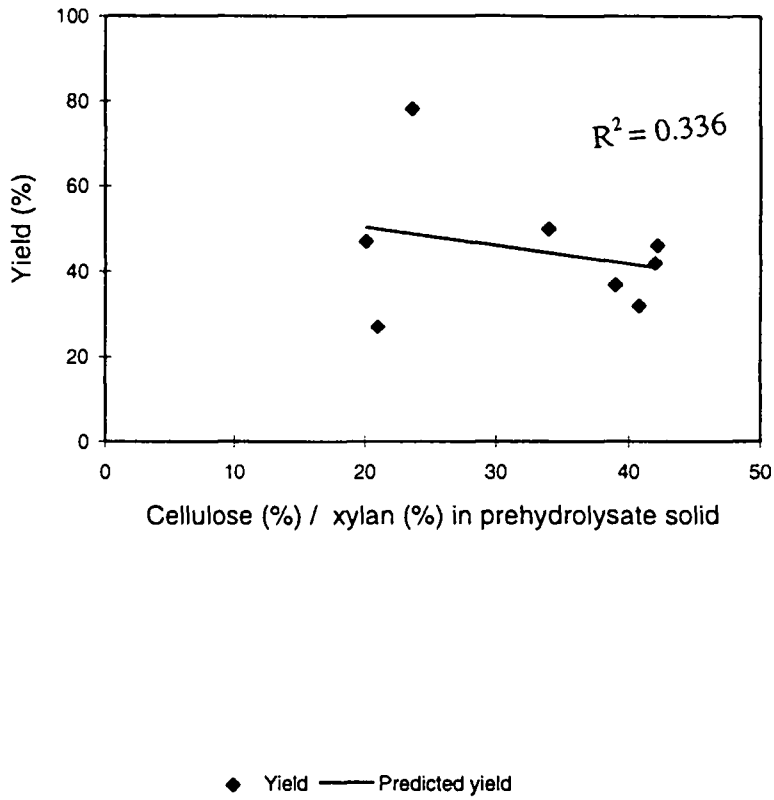

Figure 26a. Fit plot of the 12 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated poplar.

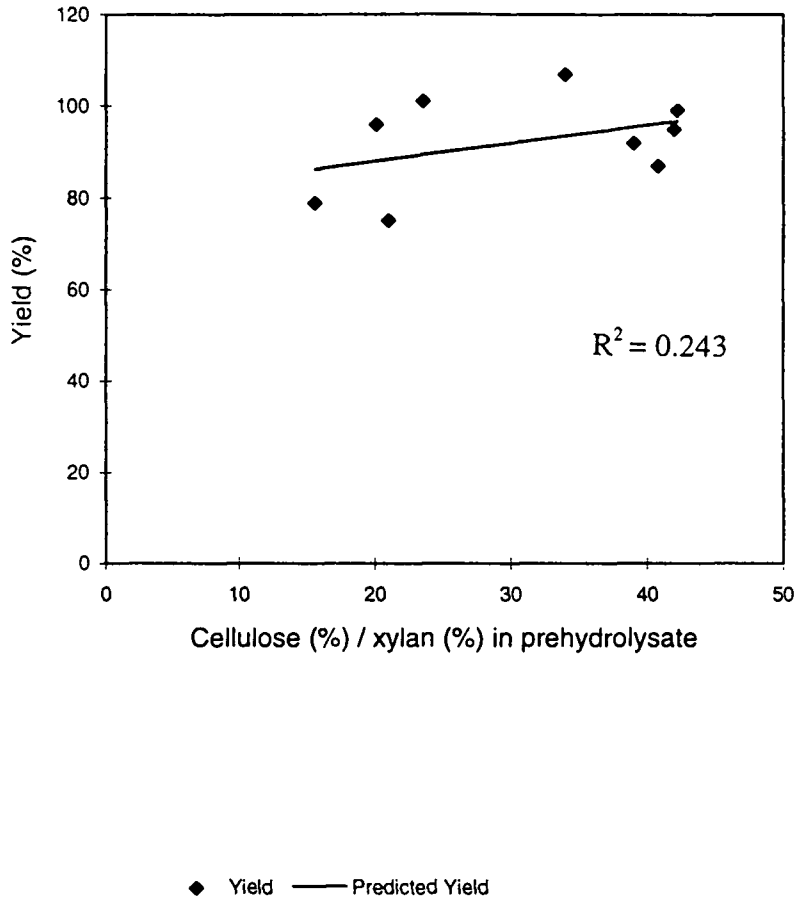

Figure 26b. Fit plot of the 168 hr glucose yield under straight saccharification versus the ratio of the cellulose-to-xylan content of the dilute acid pretreated poplar.

feedstocks, the pretreated poplar substrates showed no discernible correlation between saccharification parameters and glucan-to-xylan ratios. The SSF experiments with the pretreated poplar substrates also showed a slower rate of ethanol production compared to the other feedstocks (Figure 27). Maximum ethanol levels were not obtained until approximately seven days of SSF. Maximum yields, in terms of percent of theoretical, were also relatively lower for the poplar feedstocks (Figure 28). Maximum extents of conversion of cellulose to ethanol averaged only 76 % of theoretical for the poplar feedstocks, compared to over 90% for all of the other feedstocks. At the completion of the 7 day SSF incubation ethanol concentrations were significantly ($p<0.05$) lower for pretreated poplar than for the other substrates. The low rates of cellulose-to-ethanol conversion in SSF experiments with poplar were reflected in lower cellobiose and glucose levels in poplar reaction mixtures (Figure 29 and 30). The 12 hr cellobiose spike observed in SSF experiments with poplar was approximately one-half the size of those observed with the other feedstocks. The differences in the % cellulose converted in straight saccharification experiments and SSF experiments for the poplar feedstocks were larger than that seen with the other feedstocks (Table 12). Two of the three poplar feedstocks had cellulose conversion differences of 12 % and 14%, the higher conversion occurring in the straight saccharification experiments. The lower conversions in the SSF experiments are based on actual amounts of cellulose remaining in the SSF reaction mixtures and, thus, are not attributable to changes in yeast metabolism (changes that may explain discrepancies when "cellulose converted" is based on the summation of metabolic products).

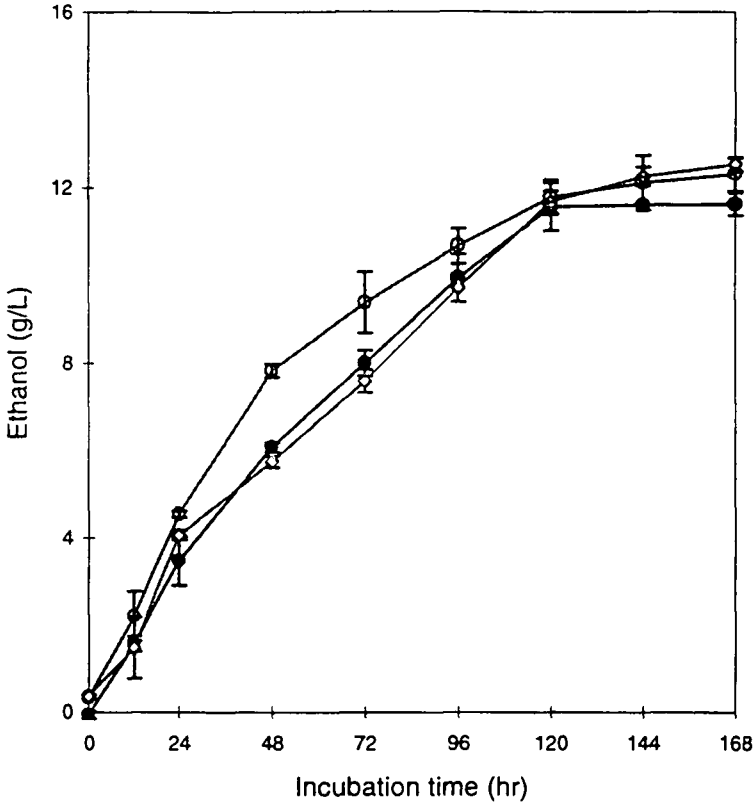

Figure 27a. Ethanol concentrations of dilute acid pretreated poplar under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.6 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

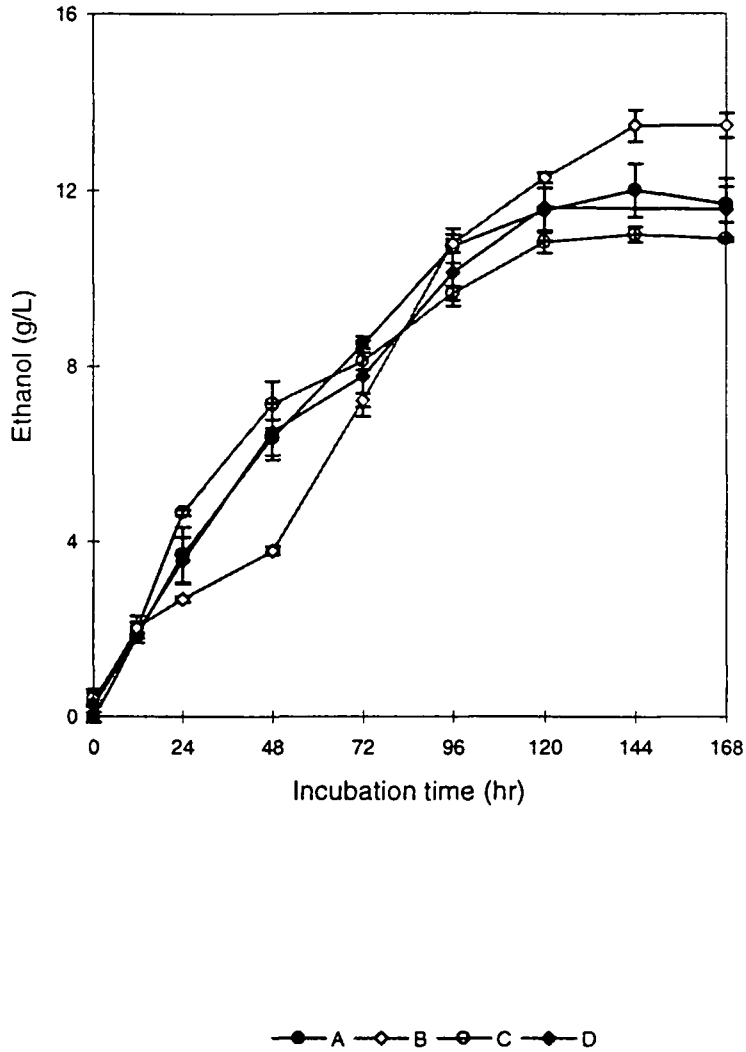

Figure 27b. Ethanol concentrations of dilute acid pretreated poplar under SSF processes. The pretreatment condition was the same for each samples (1.0% sulfuric acid at 180°C for 0.56 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

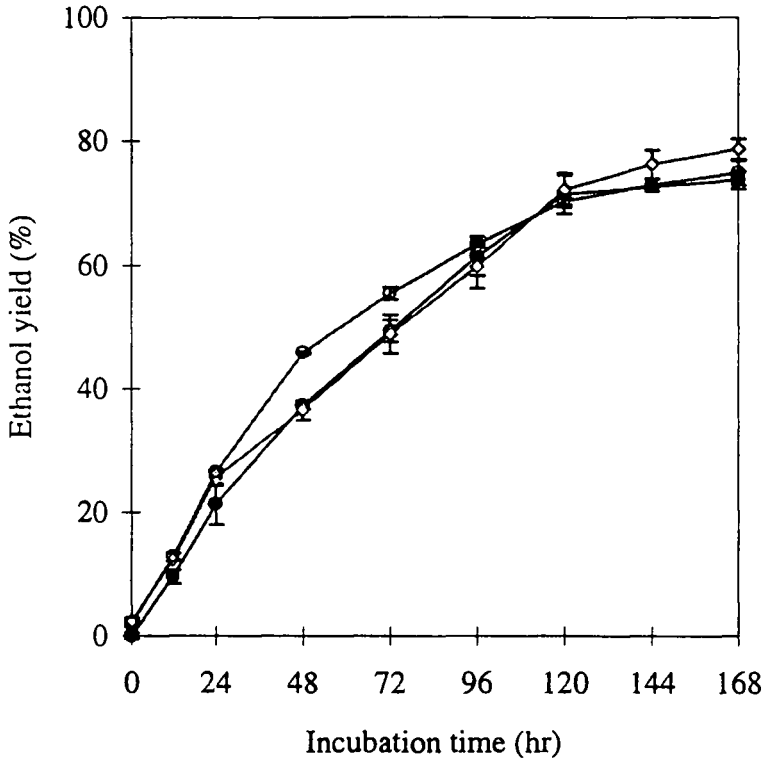

Figure 28a. Theoretical ethanol yields of dilute acid pretreated poplar under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.6 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

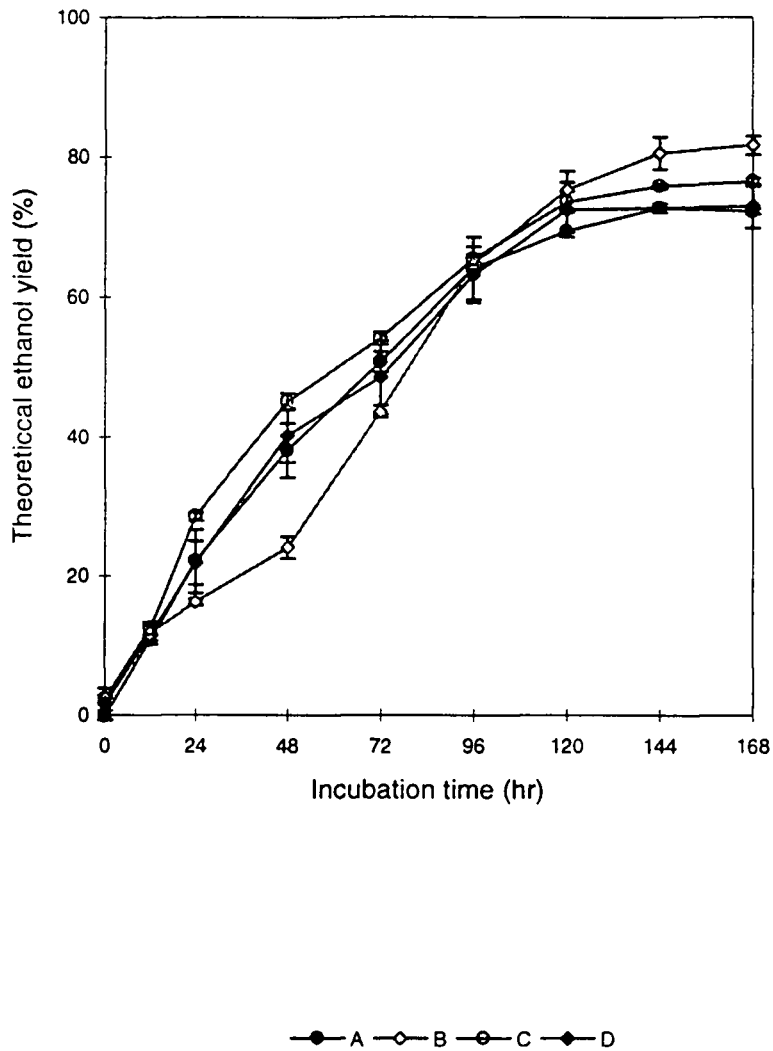

Figure 28b. Theoretical ethanol yields of dilute acid pretreated poplar under SSF processes. The pretreatment condition was the same for each samples (1.0% sulfuric acid at 180°C for 0.56 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

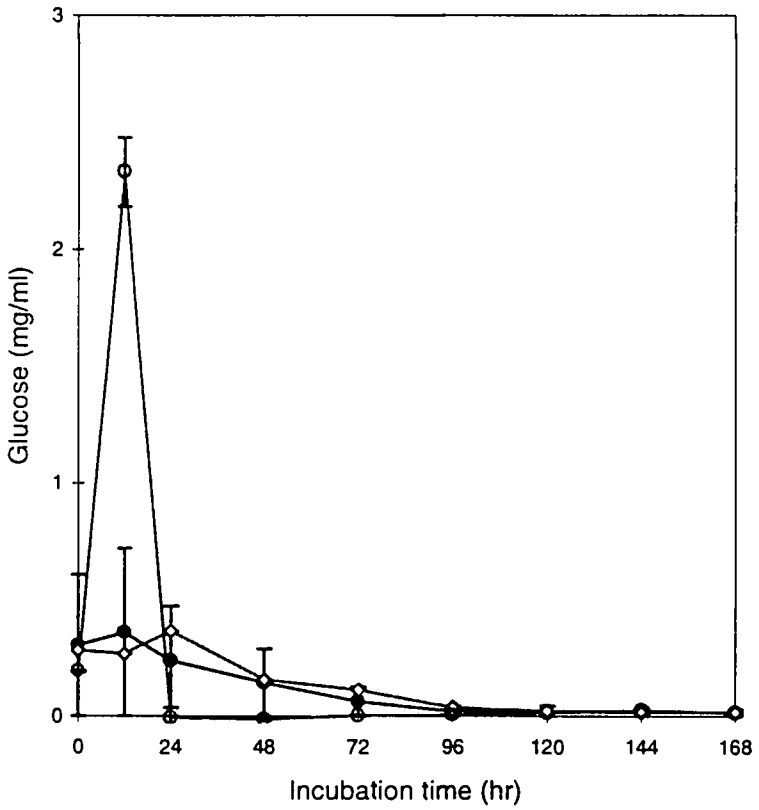

Figure 29a. Glucose concentrations of dilute acid pretreated poplar under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.6 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

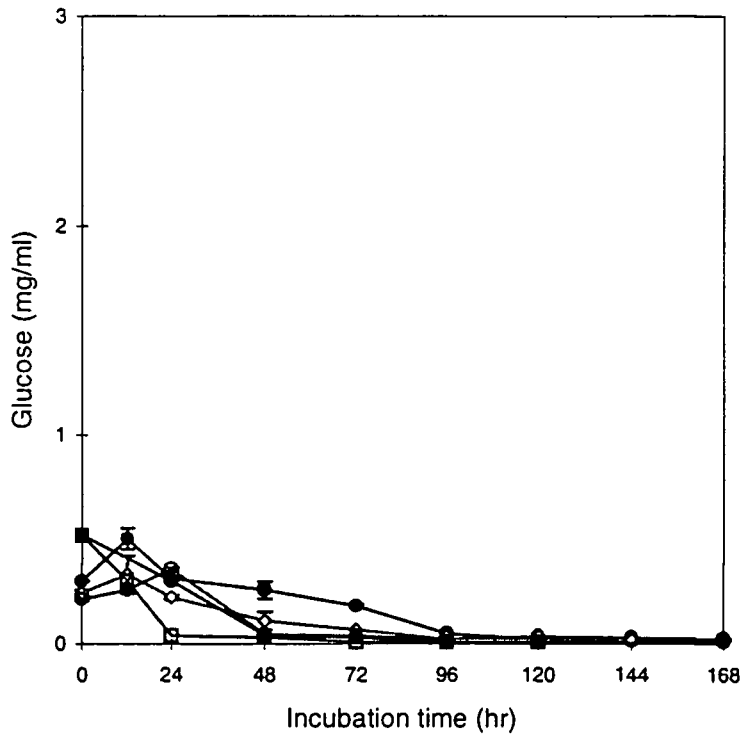

Figure 29b. Glucose concentrations of dilute acid preteated poplar under SSF processes. The pretreatment condition was the same for each samples (1.0% sulfuric acid at 180°C for 0.56 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

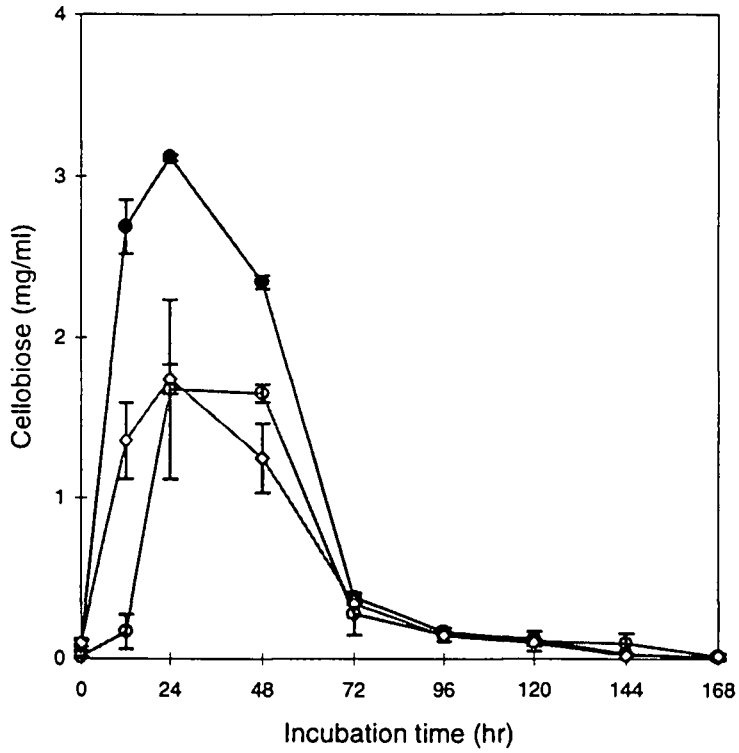

Figure 30a. Cellobiose concentrations of dilute acid pretreated poplar under SSF processes. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample. The samples for each curve were pretreated individually with 0.9% sulfuric acid at 180°C for 0.6 minutes. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

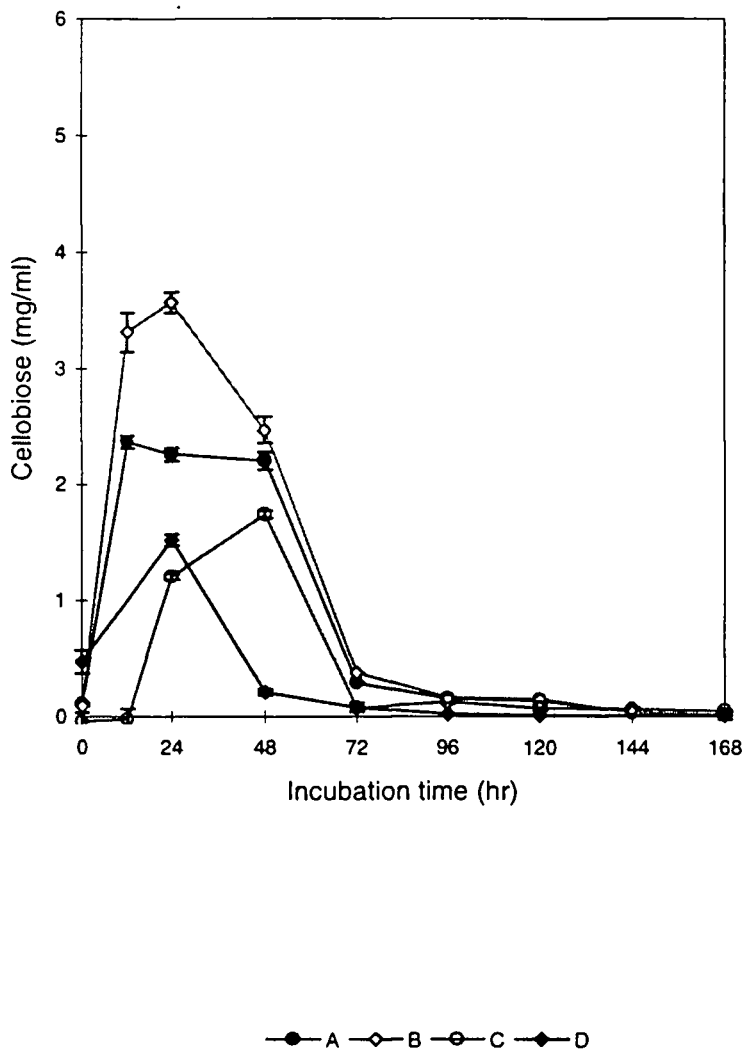

Figure 30b. Cellobiose concentrations of dilute acid pretreated switchgrass under SSF processes. The pretreatment condition was the same for each samples (1.0% sulfuric acid at 180°C for 0.56 minutes). Curve A, B and C are means and error bars are standard deviations for two determinations in each of two reaction flasks per sample using individual pretreated sample. Curve D is mean and error bars are standard deviations for three experiments using same batch pretreated sample. The cellulose level and pH of each reaction mixture was adjusted to 3% and 5.0, respectively. The enzyme load was 25 FPU/g cellulose and 25 βGU/g cellulose and the yeast inoculum was 10% volume of reaction mixture. The reaction condition was 38°C with gentle agitation of 150 rpm.

Table 12. Comparison of enzyme digestibility under straight saccharification and SSF porcess

Substrate	yield[a] sacchairification	yield[b] SSF	celllulose converted SSF[c]	difference[d]	cellulose (%) / xylan (%) in PHS
Poplar	96 ± 0.1	78 ± 1.7	84	12	20.1
	101 ± 0.3	82 ± 1.3	87	14	23.6
	79± 4.0	73 ± 3.0	80	1	15.6

[a] = sum of saccharification products (glucose + cellobiose) / potential cellulose in pretreated solid
[b] = sum of fermentation products (ethanol + glucose + cellobiose) / potential cellulose in pretreated solid
[c] = 100% - residual cellulose after 8 day SSF
[d] = column 2 - column 4

4.4.3 Enzyme Load and Temperature Effects on The Rates and Extents of Cellulose Saccharification

The apparent difference in the extents of cellulose conversion in SSF and straight saccharification experiments with some of the pretreated poplar feedstocks (Table 12) raised the question of whether changes in reaction mixture enzyme loads and/or temperature affect the enzyme accessibility of the feedstock's cellulose component. To answer this question a pretreated poplar, pretreated switchgrass and an α-cellulose standard were used in saccharification experiments which compared rates of saccharification at four different enzyme load/temperature combinations. The two enzyme loads tested were 60 FPU/g cellulose ("E", as used in straight saccharification experiments) and 25 FPU/g cellulose ("e", as used in SSF experiments). The two temperatures tested were 50°C ("T", as used in straight saccharification experiments) and 37°C ("t", as used in SSF experiments). The four enzyme load/temperature combinations tested with each feedstock were E/T, E/t, e/T and e/t. The time courses for glucose production from the poplar, switchgrass and α-cellulose feedstocks under each of these conditions are shown in Figures 31, 32 and 33, respectively. Time courses corresponding to treatments E/T and e/t show saccharification rates under enzyme/temperature conditions identical to those used in straight saccharification and SSF experiments, respectively. The rate of saccharification of each of the feedstocks was dependent on both the enzyme load and the temperature of reaction mixtures; as is most obvious when comparing the early time points (12 and 24 hr) within each figure. Conditions corresponding to those used in SSF experiments (e/t) were always associated with the

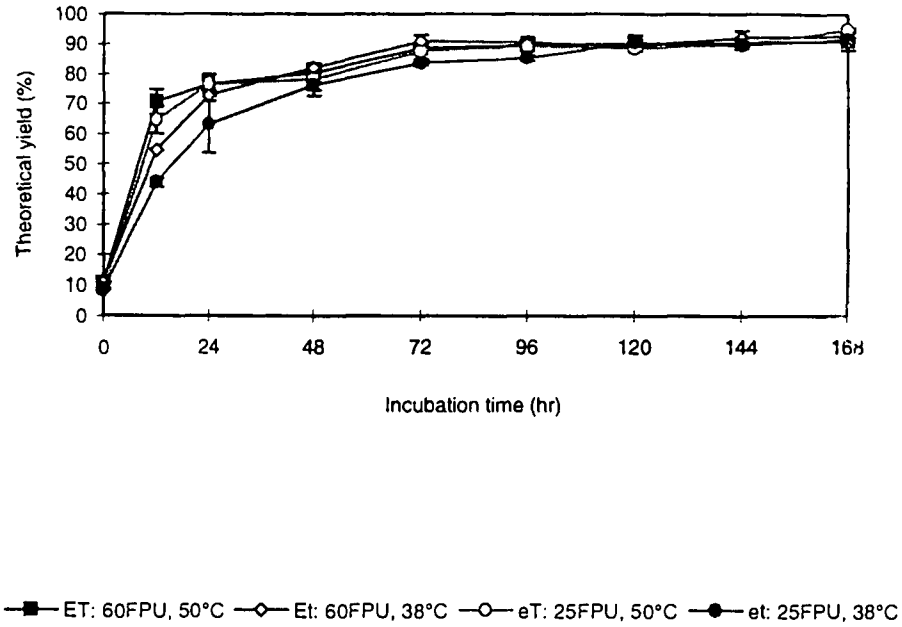

Figure 31. Theoretical glucose yields of dilute acid pretreated switchgrass for straight saccharification under different enzyme load and temperature conditions. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment.

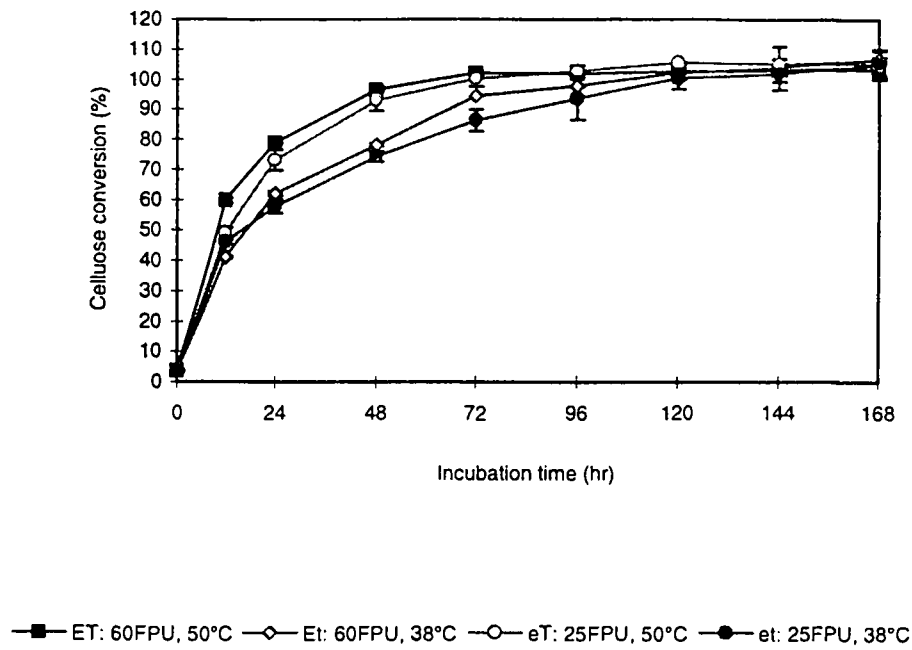

Figure 32. Theoretical glucose yields of α-cellulose for straight saccharification under different enzyme load and temperature conditions. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment.

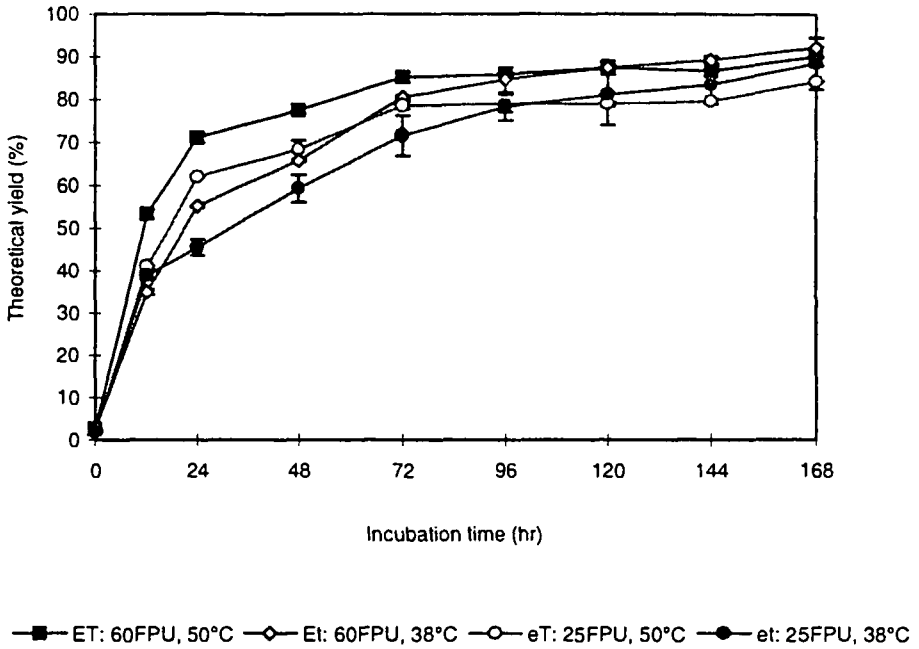

Figure 33. Theoretical glucose yields of dilute acid pretreated poplar for straight saccharification under different enzyme load and temperature conditions. Data points are means and error bars are standard deviations for two determinations in each of two reaction flasks per treatment.

slowest rates of glucose production, while those corresponding to straight saccharification experiments (E/T) were always associated with the fastest rates of glucose production. The 168 hr glucose yields resulting from saccharification under each of the four experimental conditions were not significantly different for any of the feedstocks tested ($p>0.05$). The similarity in the final glucose yields from each of the treatments indicates that the enzyme accessibility of these feedstocks is not inherently dependent on enzyme load or reaction temperature, at least not over the range of enzyme loads and temperatures employed in these experiments. This result argues against the concept that discrepancies in the percentage of cellulose converted in SSF and straight saccharification experiments can be summarily explained by differences in the enzyme accessibility of the cellulose component of the feedstocks under different saccharification conditions.

4.4.4 Parameter Changes in Saccharification and SSF Experiments

A pretreated poplar feedstock, a pretreated switchgrass feedstock, the corresponding native feedstocks, and an α-cellulose standard were evaluated in paired saccharification and SSF experiments to see how selected reaction parameters changed over the course of these experiments. Tables 13 and 14 summarize enzyme activity, cellulose availability, and yeast cell viability data taken at the initiation and termination of saccharification and SSF experiments, respectively. "Enzyme activities" in Tables 13 and 14 correspond to the amount of cellulase activity that could be detected in the aqueous phase of the reaction mixture. The difference between the amount of enzyme activity (filter paper units) added to a reaction mixture and the amount detected in the aqueous

phase provides an indication of the amount of enzyme adsorbed to the substrate (Suttcliffe and Saddler, 1986; Tatsumoto et al.,1988). The pretreated feedstocks and α-cellulose had significantly lower soluble enzyme activities than the enzyme control which contained no biomass; thus, suggesting the adsorption of cellulase by all of the substrates. Throughout the course of both SSF and saccharification experiments the pretreated poplar substrate had the highest levels of adsorbed enzyme. However, the rates of saccharification (in saccharification experiments) and ethanol production (in SSF experiments) were lower for pretreated poplar than for either the pretreated switchgrass or the α-cellulose substrates. The obvious conclusion is that the amount of adsorbed enzyme is not directly correlated with rates of saccharification for different feedstocks. A rational for this is that cellulase enzymes associate with the lignin component of lignocellulosic materials (or in other catalytically nonproductive modes), thus making them unavailable for cellulose saccharification. Decreases in soluble enzyme activity may be the result enzyme inactivation as well as enzyme adsorption. The "enzyme control" experiments in Tables 13 and 14 show that approximately 56 % of the total enzyme activity present at the initiation of the experiment was lost over eight days at saccharification temperatures while less than 10 % of initial enzyme activity was lost over the same time period at the lower temperatures used in SSF experiments. The overall conclusion from the enzyme activity measures is that enzyme is adsorbed to each of the feedstocks at the initiation of the experiment and that active enzyme could be detected in both saccharificaton and SSF reaction mixtures at the conclusion of the respective experiments. The extreme reduction in the rate of cellulose dissolution (>95%) in the later phases of saccharification and SSF

Table 13. Theoretical glucose yield, residue celluose and cellulase activity in straight saccharification experiment.

Sample	Pretreatment	Cellulose converted (%)			Cellulase activity(mg glucose/ml sample)[4]	
	Acid,temperature,time	Straight saccharification[1]	Additional saccharification[2]	Residue[3]	day 0	day 8
Poplar	!%, 180°C, 0.56min	79.1 ± 3.8	2.3 ± 2.2	18.8 ± 3.0	1.5 ± 0.3	0.4 ± 0.0
Raw poplar		9.9 ± 2.1	2.3 ± 0.4	90.9 ± 8.5	4.5 ± 0.6	1.6 ± 0.2
Switchgrass	1.2%, 180°C, 0.5 min	91.4 ± 3.0	1.7 ± 2.1	9.4 ± 1.8	2.0 ± 0.4	0.5 ± 0.0
Raw Switchgrass	18.0 ± 4.1	3.7 ± 0.2	76.5 ± 7.1	4.6 ± 1.3	1.8 ± 0.5	
Cellulose		98.9 ± 2.7	2.4 ± 3.6	ND[5]	3.5 ± 0.3	1.9 ± 0.1
Enzyme Control					5.0 ± 0.2	2.2 ± 0.2

[1] Theroretical glucose yield in straight sacchaification (50°C, 60FPU/g cellulose).
[2] Theroretical glucose yield in additional sacchaification (50°C, 60FPU/g cellulose).
[3] Digested saccahrification residue by sulfuric acid and generated gluocse was divided by the initial cellulose content multiplied 100..
[4] Measured filter paper activity in reaction mixture of straight saccharification at day 0 and day 8.
[5] Not able to detect due to little amount of sample was left.

Table 14. Theoretical ethanol yield, residue cellulose , cellulase activity and yeast viability in SSF experiment

Sample	Pretreatment	cellulose converted (%)			Cellulase activity(mg glucose/ml sample)[4]		Yeast viability (CFU/ml sample)[5]	
	Acid,temperature,time	SSF[1]	Additional saccharification[2]	Residue[3]	day 0	day 8	day 0	day 8
Poplar	!%, 180°C, 0.56min	73.0± 3.2	4.7 ± 2.9	15.5 ± 3.3	3.1 ± 0.0	2.7 ± 0.2	7.3E +07	1.7E +05
Raw poplar		6.8 ± 0.7	1.5 ± 0.0	92.4± 4.0	5.9± 0.2	4.3 ± 0.1	7.3E +07	1.3E +06
Switchgrass	1.2%, 180°C, 0.5 min	90.3 ± 2.5	0.7 ± 0.1	6.5 ± 3.9	4.0 ± 0.1	3.8 ± 0.1	7.3E +07	7.1E +04
Raw Switchgrass	24.0 ± 4.3	1.0 ± 0.0	79.8± 3.3	6.1 ± 0	4.8 ± 0.5	7.3E +07	1.2E +05	
Cellulose		91.2 ± 1.2	1.0 ± 0.2	ND[6]	6.6 ± 1.3	5.9 ± 0.3	7.3E +07	2.9E+ 04
Enzyme control					6.8	6.3		
Enzyme + yeast control					7.2	5.8	7.3E +07	6.5E +05

[1] Theroretical ethanol yield in SSF (38°C, 25FPU/g cellulose).
[2]. Theroretical glucose yield in sacchaification (50°C, 60FPU/g cellulose).
[3]. Digested saccahrification residue by sulfuric acid and generated gluocse was divided by the initial cellulose content multiplied 100.
[4]. Measured filter paper acitivty in reasction mixture of SSF at day 0 and day 8.
[5]. Measured colony form unit by plating serious dilution in yeast dextrane medium.
[6]. Not able to detect due to little amount of sample.

experiments is not likely due to limited amounts of enzyme activity in the reaction mixtures.

The number of viable yeast cells was also determined at the initiation and termination of SSF experiments (Table 14). In all cases, SSF reaction mixtures contained viable cells at the end of the eight day reaction period. This result is consistent with earlier graphs showing that SSF reaction mixtures contained negligible amounts of residual sugar (cellobiose and glucose) in the later phases of SSF experiments. The low residual sugar and high yeast viability of all of the SSF reaction mixtures implies that the lower ethanol yields associated with some of the poplar feedstocks were due to inefficient enzyme saccharification, and not to inefficiencies in the subsequent fermentation of saccharification products.

The enzyme activity and viable yeast counts discussed above suggest that the primary factor that limits the utilization of the cellulose remaining in a feedstock at the termination of saccharification and SSF experiments is the recalcitrant nature of that cellulose. This recalcitrant nature may be the result of the cellulose's limited accessibility to enzymes or due to its resistance to hydrolysis in the presence of enzymes. To test this reasoning the feedstocks remaining at the completion of SSF and saccharification experiments were washed, resuspended in buffer, and then incubated in the presence of high activities of fresh cellulase enzyme. Following an extended reaction period, the amount of saccharification product was determined (see "additional saccharification" in Tables 13 and 14) and the residual cellulose in the insoluble feedstock was determined (see "residual" in Tables 13 and 14). The results are consistent with the idea that the cellulose

component of the residual feedstock remaining at the end of SSF and saccharification experiments is recalcitrant to enzymatic saccharification. The "additional saccharification" values for all of the feedstocks were minor (<4%), and these values correspond to reaction mixtures in which potential inhibitors (glucose and cellobiose) have been removed. The amount of recalcitrant cellulose associated with a feedstock is obviously dependent on the pretreatment history of the feedstock, as the native samples in this study had far greater amounts of unavailable cellulose than the corresponding pretreated feedstocks.

The conclusions reached in these experiments are in agreement with a recent publication which tested potential limiting factors for their role in decreasing ethanol production rates in SSF experiments (Philippidis et al., 1995). The response of SSF reaction mixtures to supplementation with cellulose, glucose and/or enzyme led to the conclusion that the progressive decline in the rate or ethanol production in SSF experiments was caused mainly by a certain fraction of the cellulosic content of the biomass remaining inaccessible to cellulolytic enzymes. The results of Tables 1 3 and 14 support this conclusion. The concept of unavailable cellulose is well accepted in biomass-to-ethanol processes, as it is the basis for essentially all pretreatments used to prepare lignocellulosic substrates.

4.4.5 Potential Approaches for Improving Saccharification and SSF Efficiency

Saccharification products, glucose and cellobiose, were observed in the early phases of all SSF experiments, irregardless of the feedstock used. The build-up of glucose in SSF reaction mixtures suggests that the rate of saccharification exceeds the rate at

which the yeast can metabolize the generated glucose. This condition was observed for the first 24 hrs of all SSF experiments, a time period over which at least 50% of the total ethanol was produced. This result suggests that the limiting factor for rates of ethanol production in the early phase of SSF experiments is the rate of yeast fermentation. The way to increase rates of ethanol production in this phase of SSF experiments is thus to increase rates yeast fermentation. A simple approach that should be successful is simply to increase the size of the initial inoculum. It is possible that rates of yeast fermentation could also be increased by further optimizing growth conditions or by adding more cellulase enzyme. This latter approach would only be successful if the glucose concentrations currently found in SSF systems were below that required for saturation of yeast metabolism. Approximately 24 hrs into the SSF experiments the reaction mixtures became essentially devoid of either glucose and cellobiose. Thus, from this time on the rate of ethanol production appeared limited by the rates of cellulose saccharification. To increase rates of ethanol production in this phase of the study it would be most important to increase the cellulolytic activity of the reaction mixture. This may be accomplished by increasing the amounts of cellulase enzyme in the preparation if the system is not already saturated with enzyme. If the system is already saturated with enzyme, then improvements in cellulose accessibility and/or reactivity are required.

Comparing the product profiles from SSF and saccharification experiments shows that there is a higher accumulation of cellobiose in SSF reaction mixtures compared to that in the straight saccharification mixtures. The reason for this difference is not known for sure, but a simple explanation is that the higher enzyme load used in saccharification

experiments compared to SSF experiments (60 FPU versus 25 FPU per gram cellulose) provides the relatively higher cellobiase activities needed for conversion of all cellobiose to glucose. This rational must assume that the relative increases in total cellulase (saccharifying) activity and β-glucosidase (cellobiase) activity are not proportional, even though the relative increase in the number of moles of each enzyme are identical. This may be the case due to the possibility of saturating the surface of the insoluble cellulose substrate. Since β-glucosidase acts on a soluble substrate, it is expected that increases in the rate of this reaction will be strictly proportional to the amount of enzyme present in the reaction mixture. Cellobiose is an important intermediate product in these reaction mixtures because it is a known inhibitor of cellulose saccharification (Wyman et al., 1992; Spindler et al., 1989; Szczodrak, 1989; Ghosh et al., 1982). It is likely that increases in the rates of glucose production during the first 24 hrs of SSF experiments, with any of the feedstocks tested, may be obtained by further supplementing SSF reaction mixtures with β-glucosidase. Additional β-glucosidase is expected to increase glucose concentrations directly by increasing the rate at which cellobiose is hydrolyzed to glucose and, indirectly, by increasing overall rates of saccharification via the lowering of inhibitor (cellobiose) levels. This rational is in agreement with previous studies showing that β-glucosidase levels can significantly effect rates of ethanol production in SSF experiments (Spindler et al., 1990; Wyman et al., 1992).

4.4.6 Comparing Results From this Study with Previously Published Data

A comparison of the results obtained in this study with other researchers is shown in Tables 15 and 16. The final yields of poplar and cornstover/corncob in SSF process were similar to the data reported by Wyman et al. (1992). However, we obtained higher ethanol yields in pretreated switchgrass in the SSF experiments in this study. In straight saccharification, we also obtained higher final yields in pretreated switchgrass and cornstover/corncob samples, particularly in switchgrass 20-40% increase was found. This indicates that the optimum pretreatment conditions used in this study appear to be reasonably good for switchgrass and cornstover samples in both SSF and straight saccharification process, however, for poplar the pretreatment conditions may not be optimum conditions for SSF and straight saccharification. The results shown by Wyman et al (1994) indicate a slightly higher final yield in SSF than straight saccharification. However, our data did not show the same trend due to a higher amount of enzyme was used in the straight saccharification process than in SSF process. It was also noted that using the optimum pretreatment conditions (higher acid concentration and higher temperature), we can significantly reduce the reaction time of pretreatment and the amount of enzyme. Thus, finding optimum pretreatment conditions for various biomass samples not only allow us to increase the final yield but also allow us to reduce the cost associated with the pretreatment and the subsequent SSF process.

Table 15. Comparison of literature results of final yields in SSF for lignocellulosic biomass.

Substrate	Pretreatment H_2SO_4, temperature, time	Cellulase Activity FPU/ g cellulose	B-glucanase activity BGU/ g cellulose	SSF day	cellulose %) / xylan (%)	Yield %	Authors
Hybrid poplar	1.0%, 180°C, 0.56 min	25	25	8	16	73	
Hybrid poplar	1.0%, 180°C, 0.56 min	25	25	7	24	82	
Hybrid poplar	0.9%, 180°C, 0.6 min	25	25	7	20	78	
Yellow poplar	0.73%, 160°C, 30 min	25	25	8	17	78	Vinzant et al., 1994
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	26	0	8	46	61	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	26	0	8	144	47	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	26	0	8	16	51	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	52	0	8	46	80	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	52	0	8	144	62	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	52	0	8	16	76	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	26	52	8	46	84	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	26	52	8	144	62	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	26	52	8	16	79	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	52	52	8	46	89	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	52	52	8	144	72	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	52	52	8	16	84	Spindler et al., 1991
Switchgrass	0.45%, 140°C, 60 min	26	0	8	29	52	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	52	0	8	29	61	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	26	52	8	29	58	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	52	52	8	29	63	Spindler et al., 1990
Switchgrass	1.2%, 180°C, 0.5 min	25	25	8	11	90	
Switchgrass	1.2%, 180°C, 0.5 min	25	25	7	34	92	
Switchgrass	1.2%, 180°C, 0.5 min	25	25	7	23	88	
Switchgrass	0.9%, 180°C, 0.5 min	25	25	7	17	84	
Switchgrass	0.9%, 180°C, 0.5 min	25	25	7	18	85	
Comstover	0.45%, 140°C, 60 min	26	0	8	18	59	Wyman et al., 1992
Comstover	0.45%, 140°C, 60 min	52	0	8	18	84	Wyman et al., 1992
Comstover	0.45%, 140°C, 60 min	26	208	8	18	86	Wyman et al., 1992
Comstover	0.45%, 140°C, 60 min	52	208	8	18	92	Wyman et al., 1992
Comstover/cob	1.2%, 180°C, 0.9 min	25	25	7	12	91	
Comstover/cob	1.1%, 180°C, 1.0 min	25	25	7	12	96	

Table 16. Comparison of literature results of final yields in straight saccharification for lignocellulosic biomass.

Substrate	Pretreatment H_2SO_4, temperature, time	Cellulase Activity FPU/ g cellulose	B-glucanase activity BGU/ g cellulose	saccharification day	cellulose (%) / xylan (%)	Yield %	Authors
Hybrid poplar	1.0%, 180°C, 0.56 min	25	25	8	16	79	
Hybrid poplar	1.0%, 180°C, 0.56 min	25	25	7	24	101	
Hybrid poplar	0.9%, 180°C, 0.6 min	25	25	7	20	96	
Hybrid poplar (NE 388)	0.5-0.65%, 160C, 20 min	42	4.9	4		82	Torget et al., 1991
Hybrid poplar (N-11)	0.5-0.65%, 160C, 10 min	42	4.9	4		80	Torget et al., 1991
Poplar (NE 388)	0.45-0.5%, 160C, 10 min	42	4.9	4		95	Torget et al., 1990
Poplar (N-11)	0.45-0.5%, 160C, 5 min	42	4.9	4		95	Torget et al., 1990
Poplar	0.41%, 200C, 3.6-12.7 sec					85	Knappert et al., 1981
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	26	0	8	46	48	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	26	0	8	144	28	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	26	0	8	16	30	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	52	0	8	46	72	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	52	0	8	144	47	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	52	0	8	16	55	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	26	52	8	46	53	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	26	52	8	144	38	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	26	52	8	16	36	Spindler et al., 1991
Populus maximowiczii x nigra	0.45%, 140°C, 60 min	52	52	8	46	75	Spindler et al., 1991
Populus trichocarpa x deltoides	0.45%, 140°C, 60 min	52	52	8	144	51	Spindler et al., 1991
Populus tremuloides	0.45%, 140°C, 60 min	52	52	8	16	51	Spindler et al., 1991
Populus *trichocarpa*	0.49%, 170C, 60 min			4		97	Chum et al., 1988
Populus *tremuloides*	0.49%, 165C, 150 min			4		86	Chum et al., 1988
Switchgrass	0.45%, 140°C, 60 min	26	0	8	29	45	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	52	0	8	29	61	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	26	52	8	29	46	Spindler et al., 1990
Switchgrass	0.45%, 140°C, 60 min	52	52	8	29	62	Spindler et al., 1990
Switchgrass	1.2%, 180°C, 0.5 min	25	25	8	11	91	
Switchgrass	1.2%, 180°C, 0.5 min	25	25	7	34	104	
Switchgrass	1.2%, 180°C, 0.5 min	25	25	7	23	98	
Switchgrass	0.9%, 180°C, 0.5 min	25	25	7	17	96	
Switchgrass	0.9%, 180°C, 0.5 min	25	25	7	18	92	
Cornstover	0.45%, 140°C, 60 min	26	0	8	18	64	Wyman et al., 1992
Cornstover	0.45%, 140°C, 60 min	52	0	8	18	84	Wyman et al., 1992
Cornstover	0.45%, 140°C, 60 min	26	208	8	18	80	Wyman et al., 1992
Cornstover	0.45%, 140°C, 60 min	52	208	8	18	89	Wyman et al., 1992
Cornstover/cob	1.2%, 180°C, 0.9 min	25	25	7	12	97	
Cornstover/cob	1.1%, 180°C, 1.0 min	25	25	7	12	98	

4.5 REFERENCE

Beck, M. J. (1993), in Bioconversion of forest and agricultural plant residues. Saddlaer J. N. Saddler, ed., pp 211-229. C. A. B., International, Wallingford, Oxon, UK.

Cheremisinoff, N.P., Cheremisinoff, P.N., and Ellerbusch, F. (1980), Biomass--Applications, technology, and production. Marcek Dekker, Inc., New York.

Coughlan, M.P. (1985), Biochem. Soc. Trans. 13: 405-406.

Cowling, E. B. (1963), in Advances in enzymic hydrolysisof cellulose and related materials. Reese, E. T., ed., Pergamon press Inc. New York, NY, pp.1-32

Cowling, E. B. (1975), Biotechnol. & bioeng. Symposium. No.5, 163-181.

Dellweg, H., Rizzi, M., Methner, H., and DeBus, D. (1984), Biotechnol. Lett. 5, 357.

Detroy, R.W., Lindenfelser, L.A., Sommer, S., and Orton, W.L. (1981), Biotechnology and Bioengineering. 23: 1527-1535.

Dermoun, Z, and Belaich, J. P. (1988), Appl. Microbiol. Biotechnol. 27, 399-404.

Dunlap. C. E., Thomson, J., Chiang, L. C. (1976), AIChE Symp. Ser. 158, 58.

du Preez, J. C. , van der Walt, J. P. (1983), Biotechnol. Lett. 5, 357-362.

Esteghlalian, A., Hashimoto, A. G., Fenske, J. J. and Penner, M. H. (1996), Bioresource Technol. In review.

Eley, M.H., Guinn, G.R., and Bagchi, J. (1995), App. Biochem. Biotechnol. 51/52:387-397.

Fan, L. T., Lee, Y. H. and Beardmore, D. H. (1980a), Biochem. Bioeng. 22, 177-199.

Fan,L. T., Lee, Y. H. and Beardmore, D. H. (1980b), in Advances biochemical engneering, Fiechter, A., eds., Springer-Verlag inc., Berlin Heidelberg, NY. pp.101-117.

Fan, L. T., Gharpury, M. M., and Lee, Y. H. (1987), in Cellulose hydrolysis. Springer-Verlag, Berlin Heidelberg, New York.

Fenske, J.J. (1994). Master thesis, Oregon State University.

Focher, B., Marzetti, M., Cattaneo, M., Beltrame, P. L. and Carnti, P. (1981), J. Appl. Polym. Sci. 26, 1989-1993.

Ghosh, P., Pamment, N.B., and Martin, W.R.B. (1982), Enzyme Microb. Technol. 4: 425-430.

Grethlein, H.E. and Converse, A.O. (1991), Bioresource Technology. 77-82.

Grohmann, K., Torget, R. and Mimmel, M. (1985), Biotechnol. Bioeng. Symp. No. 15, 59-80.

Grohman, K., Wyman, C.E., and Himmel M.E. (1992), in "Emerging technologies for materials and chemicals from biomass, ACS Symposium series No. 476", Rowell, R.M., Schultz, T.P., and Narayan, R., eds., American Chemical Socie. pp. 354-392.

Harada, H. and Cote,JR., W. A. (1985), in Biosynthesis and biodegradation of wood components. Higuchi, T., ed., Academic press, Inc., Orlando, FL, pp.505-533.

Jeffries, T.W. (1984), Enzyme Microb. Technol. 6, 254.

Klass, D. L. (1981), in Fuels from biomass and wastes, Klass, D. L. and Emert, G. H., eds., Ann arbor science publishers, Inc., Ann Arbor, MI, pp. 1-41.

Lee, Y. Y. and McCaskey, T. A. (1983), Tappi. 66, 102-107.

Liaw, E. T. (1994), Ph.D. Thesis, Oregon State University.

Pilippidis, G. P., Smith, T. K. and Schmidt, S. L. (1993), SSF experimental protocols: lignocellulosic biomass hydrolysis and fermentation. NREL standard precedure No. 008.

Pilippidis, G. P. and Smith, T. K. (1995), Appl. Biochem. Biotechnol. 51/52, 117-124.

Reed, T.B. (1981), in Biomass gasification principle and technology. Reed, T.B., ed., Noyes data corporation, Park Ridge, NJ, pp.3-25,

Rivers, D.B., Gracheck, S.J., Woodford, L.C., and Emert, G.H. (1984), Biotechnology and Bioengineering. 26: 800-802.

Robinson, J. S. (1980), in Fuels from biomass, Robinson, J. S., ed., Noyes data corporation, Park Ridge, NJ, pp,1-2.

Saddler, J.N., Mes-Hartree, M., Yu, E.K.C., and Brownell, H.H. (1983), Biotechnology and Bioengineering Symosium. 13: 225-238.

Saddler, J.N., Hogan, C., Chan, M.K.H., and Louis-Seize, G. (1982), Canadian Journal of Microbiology. 28: 1311-1319.

Sasaki T. (1989), in Biomass handbook, Kitani, O and Hall, C. W., eds., Godrdon and Breach Science Publishers, Cooper station, NY. pp. 345-354.

Sasaki, T., Tanaka, T., Nanbu, N., Sato, Y. and Kainuma, K. (1979), Biotech. Bioeng. 21, 1031-1042.

Sawada, I., Kuwahara, M., Nakamura, Y. and Suda, H. (1987), Int. Chem. Eng. 27, 686-693.

Schneider, H., Wang, P.Y., Chan, Y.K., and Maleszka, R. (1981), Biotechnol. Lett. 2, 89.

Shan, M.M. and Lee, Y.Y. (1992), Applied Biochemistry and Biotechnology. 34/35: 557.

Sinitsy, A. P., Gusakov, A. V. and Vlasenko, E. Y. (1991), Appl. Biochem. Biotechnol. 30, 43-95.

Spindler, D.D., Wyman, C.E., Grohmann, K., and Mohagheghi, A. (1989), Appl. Biochem.and Biotech.20/21: 529-540.

Spindler, D. D., Wyman, C. E., Grohmann, K. and Mohagheghi, A. (1990), Appl. Biochem Biotechnol. 24/25, 275-286.

Spindler, D. D., Wyman, C. E. and Grohmann, K. (1991), Appl.Biochem and Biotechnol. 28/29, 773-786.

Spindler, D. D., Wyman, C. E., Grohmann, K. and Philippidis, G. P. (1992), Biotechnol. Lett.. 14(5), 403-407

Suttcliffe, R. and Saddler, J. N. (1986), Biotechnol. Bioeng. Symp. 17, 749-762.

Szczodrak, J. (1989), Biotechnol. Bioeng. 33:1112-1116.

Taylor, M. G., Deslandes., Y., Blum, R. H., Marchessault, R. H., Vincendon, M. and Saint German, J. (1983), Tappi. 66, 92-96.

Tanahashi, M., Takada, S., Aoki, T., Goto, T., Higuchi, T. and Hanai, S. (1983), Wood research. 69, 36-51.

Tanaka, M., Ikesaka, M., Matsuno, R. and Converse, A. (1988). Biotechnol. Bioeng. 32, 698-706.

Tatsumoto, K., Baker, J. O., Tunker, M. LP. , Oh, K. K., Mohagheghi, A., Grohmann, K. and Himmel, M. E. (1988), Appl. Biochem. Biotechnol. 18, 159-173.

Thompson, D. N. and Chen, H. C. (1992), Bioresource Technology. 39, 155-163.

Torget, R., Werdene, P., Himmel, M. and Grohmann, K. (1990), Appl. Biochem. Biotechnol. 24/25, 115-126.

Torget, R. 1993. Enzymatic saccharification of lignocellulosic biomass. NREL standard procedure No. 009.

Walkinshaw, J.W., Sladek, K.J., and Eberiel, D.T. 1984. Technical feasibility of enzymatic production of alcohol at existing paper mills. TAPPI Journal 67: 104-105.

Warwicker, J. O. and Wright, A. C. (1967), J. Appl. Polym. Sci. 11, 659-671.

Wong, K. K., Deverell, K. F., Mackie, T. A., Claek, T. A. and Donaldson, L. A. (1988), Biotechnol. Bioeng. 31, 447-456.

Wright, J. D. (1988), Chem. Eng. Prog. 84(8), 62-74.

Wyman, C.E., Spindler, D.D., Grohmann, K. 1992. Simultaneous saccharification and fermentation of several lignocellulosic feedstocks to fuel ethanol. Biomass and Bioenergy. 3: 301-307.

BIBLIOGRAPHY

Acebal, C., Castillon, M.P., and Estrada, P. (1988), Biotech. Biochem. 10,1.

Andren, R. K. Mandel, M. H. M. and Medeiros, J. E. (1975), Appl. Polym. Symp., 28, 205-219.

Bailey, R.B., Benitez, T., and Woodward, A. (1982), Appl. Environ, Microbiol. 44, 631.

Ballesteros, I., Ballesteros, M., Cabanas, A., Carrasco, J., Martin, C. and Negro, M. J. (1991), Appl. Biochem. Biotechnol. 28/29, 307-315.

Banerjee, N., Bhatnagar, R., and Viswanathan, L. (1981), Eur. J. Appl. Microbiol. Biotechnol. 11 (4), 226-228.

Beck, M. J. (1986), Biotechnol. Lett. 5,513-516.

Beck, M. J. (1993), in Bioconversion of forest and agricultural plant residues. Saddlaer J. N. Saddler, ed., pp 211-229. C. A. B., International, Wallingford, Oxon, UK.

Blotkamp, P.J., Takagi, M., Pemberton, M.S., and Emert, G.H. (1978), AICHE Symp., Ser 74, 85-90.

Brown, H. P. (1944), in Wood chemistry, Wise, L. E., ed., Reinhold publishing corporation, New York, NY, pp. 3-53.

Carrasco, J. E., Saiz, Ma. C., Navarro, A., Soriano, P. Saez, F., and Martinez, J. M. (1994), Appl. Biochem. Biotechnol. 45/46, 23-34.

Chang, M. M., Chao, T. Y. and Tsao, G. Y. (1981), Adv. Biochem, Eng. 20,15-42.

Cheremisinoff, N.P., Cheremisinoff, P.N., and Ellerbusch, F. (1980), Biomass--Applications, technology, and production. Marcek Dekker, Inc., New York.

Chung, I.S. and Lee, Y.Y. (1985), Biotech. Bioeng. 27, 308-315.

Clark, T. A. and Mackie, K. L. (1984), J. Chem. Technol. Biotechnol. 34, 101-110.

Converse, A. O., Kwarteng, I. K., grethlein, H. E., and Ooshima, H. (1989), Appl. Biochem. Biotech. 20/21, 63-78.

Cowling, E. B. (1963), in Advances in enzymic hydrolysisof cellulose and related materials. Reese, E. T., ed., Pergamon press Inc. New York, NY, pp.1-32

Cowling, E. B. (1975), Biotechnol. & bioeng. Symposium. No.5, 163-181.

Dekker, R. F. H. and Wallis, A. F. A. (1983), Biotechnol. Bioeng. 25, 3027- 3048.

Dekker, R. F. H. (1985), in Biosynthesis and biodegradation of wood components, Higuchi, T., ed., Academic press, Inc., Orlando, FL, pp.505-533.

Dellweg, H., Rizzi, M., Methner, H., and DeBus, D. (1984), Biotechnol. Lett. 6, 395-400.

DiazDe Villegas, M.E., Villa, P., Guerra, M., Rodriguez, E., Redondo, D., and Martinze, A. (1992), Acta Biotechnol. 12, 351.

duPreez, J.C. and van der Walt, J.P. (1983), Biotechnol. Lett. 5, 357.

Eddy, A. A. and Williamson, D. H. (1957), Nature. 179, 1252-1253.

Ehrman, C. I., and Himmel, M. E. (1994), Biotechnology Techniques. 8(2), 99-104.

Eley, M.H., Guinn, G.R., and Bagchi, J. (1995), Appl. Biochem. Biotechnol. 51/52, 387-397.

Fan, L. T., Lee, Y., and Beardmore, D. H. (1980), in Advances in biochemical engineering. Fiechter, A., ed., Springer-Verlag Inc. Berlin, Heidelberg, NY, pp.101-117.

Fan, L. T., LLee, Y. H. and Beardmore, D. H. (1980), in Proceeding of Bioconversion and Biochemical Engineering Symposium, vol. 1, Ghose, T. K., ed., Indian Inistitute of Technology, Hauzkhas, New Dalhi, Indian, pp. 233-259.

Fan, L. T., Gharpuray, M. M. and Lee, Y. H. (1987), in Celllulose hydrolysis. Springer-Verlag, Berlin, New York.

Fein, J. E., Tallim, S. R. and Lawford, G. R. (1984), Can. J. Microbiol. 30, 683-690.

Fenske, J.J. (1994). Master thesis, Oregon State University.

Franklin, W.E. and Franklin, M.A. 1992. EPA J. 18 (3), 7-13.

Fleet, G. H. (1991), in The yeasts, vol 4, Rose, A. H. and Harrison, J. S., eds, Academic Press Inc., San Dieago, CA.

Frazer, F. R. and McCaskey, T. A. (1989), Biomass. 18, 31-42.

Ghose, T.K., Roychoudhury, P.K., and Ghosh, P. (1984), Biotechnol. Bioeng. 26, 377-381.

Ghose, T.K. (1988), Pure & Appl. Chem. 59(2), 257-268.

Ghosh, P., Pamment, N.B., and Martin, W.R.B. (1982), Enzyme Microb. Technol. 4, 425-430.

Grethlein, H. E. (1984), Pretreatment for enchanced hydrolysis of cellulosic biomass. Biotech. Advs. 2, 43-62.

Grethlein, H. E., Allen, D. C., and Converse, A. O. (1984), Biotechnol. Bioeng. 26, 1498-1505.

Grethlein, H. E. and Converse, A. O. (1991), Bioresource technology. 77-82.

Grohmann, K., Torget, R. and Himmel, M. (1986), Biotech. Bioeng. Symp. 17, 135-151.

Grohman, K. (1993), in Bioconversion of forest and agricultural plant residues. Saddler, J.N., ed., C.A.B. International, Wallingford, Oxon, UK, pp.183-209.

Hacking, A.J., Taylor, J.W.F., and Hanas, C.M. (1984), Appl. Microbiol. Biotechnol. 19, 361-363.

Harada, H. and Cote,JR., W. A. (1985), in Biosynthesis and biodegradation of wood components. Higuchi, T., ed., Academic press, Inc., Orlando, FL, pp.505-533.

Hughes, D.B., Tudroszen, N.J., and Moye, C.J. (1984), Biotechnol. Lett. 6, 1-6.

Jeffries, T.W. (1984), Enzyme Microb. Technol. 6, 254-258.

Klass, D. L. (1981), inFuels from biomass and wastes, Klass, D. L. and Emert, G. H., eds., Ann arbor science publishers, Inc., Ann Arbor, MI, pp. 1-41.

Kreutzfeldt, C. and Witt, C. C. (1991), in Saccharomyces, Tuite, M. F. and Oliver, S. G., eds., Plenum Press, New York, NY, pp. 5-58.

Kruuv, J., Lopock, J. R. and Keith, A. D. (1978), Cryobiology. 15, 73-79.

Landisch, M. R. (1989), in Biomass handbook. Kitani, O. and Hall, C. W. ,eds., Gordon and breach science publishers. New York, pp.434-451.

Liaw, E. T. (1994), Ph.D. Thesis, Oregon State University.

Leonard, R. H. and Hajny, G. J. (1945), Industrial and Engineering Chemistry. 37, 390-395.

Margulis, (1974), Envol. Biol. 7, 45-78.

Meyers, S. G. (1978), The American of Chemical Engineers. No.184, vol 74, 79-84.

Mandels, M., Hontz, L., and Nystrom, J. (1974), Biotechnol. Bioeng. 16, 1471-1493.

Mitra and Wilke. (1972), J. Ferment. Technol. 50(12), 914-916.

Millett, M. A. Baker, A. J., and Satter, L. D. (1975), Biotechnol. Bioeng. Symp. 5,193-219.

Morimoto, S., Matsuo, M., Azuma, K., Oshima, T., and Sinskey, A.J. (1986), Jl Ferment. Technol. 64, 219.

Mohagheghi, A., Tucker, M., Grohmann, K. and Wyman, C. (1992), Appl. Bioch. Biotechnol. 33, 67-81.

Moore, W. E. and Johnson, D. B. (1967), Procedures for the chemical analysis of wood and wood products, USDA Forest Products Laboratory, Madison, WI.

Nprkrans, B. (1950), Physiologia Plantarum. 3, 75-78.

Palnitkar, S.S. and Lachke, A.H. (1990), Appl. Microbiol. Biotechnol. 151-158.

Pemberton, M.S., Brown, R. D. Jr. and Emert, G.H. (1980), Can. J. Chem. Eng. 58, 723-729.

Philippidis, G. P., Smith, T. K. and Schmidt, S. L. (1993), SSF experimental protocols: Lignocellulosic biomass hydrolysis and fermentation.

Phlipiddis, G. P., Spindler, D. D. and Wyman, C. E. (1992), Appl. Bioceh. Biotechnol. 35/35, 543-556.

Reed, T.B. (1981), in Biomass gasification principle and technology. Reed, T.B., ed., Noyes data corporation, Park Ridge, NJ, pp.3-25,

Richmond, P. A. (1991), in Biosynthesis and biodegradation of cellulose. Haigler, C. H. and Weimer, P. J. eds., Marcel dekker, Inc., New York. p p.5-23.

Robinson, J. S. (1980), in Fuels from biomass, Robinson, J. S., ed., Noyes data corporation, Park Ridge, NJ, pp,1-2.

Sakakibara, A. (1991), in wood and cellulose chemistry. Hon, D. N. S. and Shiraishi, N. Eds., Marcel Dekker,Inc., New York, pp113-176.

Schell, D.J., Hinman, N.D., Wyman, C.E., and Werdene, P.J. (1990), Appl. Biochem. Biotechnol. 24/25, 287-297.

Schneider, H., Wang, P.Y., Chan, Y.K., and Maleszka, R. (1981), Biotechnol. Lett. 2, 89.

Shan, M. M. and Lee, Y. Y. (1992), Appl. Biochem.Biotechnol. 34/35, 557-568.

Sigma catalog. (1996), Sigma Chemical Company, St. Louis, MO.

Sinitsy, A. P., Bungay, H. R. and Clesceri, L. S. (1983), Biotech. Bioeng, Vol XXV, 1393-1399.

Sjostrom, E. (1981), in Wood chemistryy, findamentals and applications. Academic press. pp.49-67.

Sommer, A. and Lewis, A. J. (1971), J. Gen Microb. 68, 327-335.

Souzu, H. (1973), Cryobiology. 10, 427-431

Spindler, D.D., Wyman, C.E., Mohagheghi, A. and Grohmann, K. (1988), Appl. Biochem. Biotechnol. 16/17, 279-293.

Spindler, D. D., Wyman, C. E. and Grohmann, K. (1989a), Biotechnol. Bioeng., 34, 189-195.

Spindler, D. D., Wyman, C. E., Grohmann, K. and Mohagheghi, A. (1989b), Appl. Biochem Biotechnol. 20/21, 529-540.

Spindler, D. D., Wyman, C. E., Grohmann, K. and Mohagheghi, A. (1990), Appl. Biochem Biotechnol. 24/25, 275-286.

Spindler, D. D., Wyman, C. E. and Grohmann, K. (1991), Appl.Biochem and Biotechnol. 28/29, 773-786.

Spindler, D.D., Wyman, C.E., and Grohmann, K. (1991), Appl. Biochem. Biotechnol. 24/25, 275.

Spindler, D. D., Wyman, C. E., Grohmann, K. and Philippidis, G. P. (1992), Biotechnol. Lett.. 14(5), 403-407.

Stainer, R.Y., Adelberg, E.A., and Ingraham, J.L. (1977), General Microbiology, 4th edition, MacMillan Press LTD

Sternberg, D. (1976), Appl. Environ. Microbiol., 31, 648-654.

Suttcliffe, R. and Saddler, J. N. (1986), Biotech. Bioeng. Symp. 17, 749-762.

Szczodrak, J. (1989), Biotechnol. Bioeng. 33, 1112-1116.

Szczodrak, J. and Targonski, Z. (1988), Biotechnol. Bioeng. 31, 300-303.

Takagi, M., Abe,S., Suzuki, S., Emert, G.H., and Yata, N. (1977), Proceedings Bioconversion Symposium, IIT, Delhi, India, pp.551-571.

Takagi, M. (1984), Inhibition of cellulase by fermentation products. Biotenol. Bioeng. Vol XXVI, 1506-1507.

Tarchevsky, I. A. and Marchenko, G. N. (1991), in Cellulose: biosynthesis and structure, Backinowki, L. A. and Chlenov, M. A., translated, Springer-Verlag Berlin, Heidelberg, New York, pp.9-31.

Tatsumoto, K., Baker, J. O., Tucker, M. P., Oh, K. K., Mohaghehi, A., Grohmann, K. and Himmel, M. E. (1988), Appl. Biochem. Biotech. 18, 159-173.

Tran, A. V. and Chambers, R. P. (1985), Biotechnology Letters. 7, 431-436.

Tuite, M. F. and Oliver, S. G. (1991), Biochemical techniques. In: Saccharomyces,Tuite, M. F. and Oliver, S. G., eds., Plenum press, New York. pp.283-320.

Van Steveninck, J. and Ledeboer, A. M. (1974), Biochim. Biophys. Acta. 352, 64-70.

Vinzant, T., Panfick, L., Nagle, N. J., Ehrman, C. I., Reynolds, J. B. and Himmel, M. E. (1994), Appl. Biochem. Biotechnol, 45/46, 611-626.

Walseth, C. S. (1952), Tappi. 35, 233-238.

Whistler, R. L. and Daniel, J. R. (1985), in Food Chemistry, Fennema, O. R., ed., Marcel Dekker, Inc., New york, pp. 69-137.

Wilke, C. R., Yan, R. D. and von Stockar, U. (1976), Biotechnol. Bioeng. Symp. 6, 55

Wright, J. D. (1988), Chem. Eng. Prog. 84(8), 62-74.

Wright, J. D., Wyman, C. E. and Grohmann, K. (1988), Appl. Biochem. Biotechnol. 17, 75-90.

Wyman, C. E., Spindler, D. D. and Grohmann, K. (1992), Biomass and Bioenergy. 3(5), 301-307.

Wyman, C. E., Spindler, D. D., Grohmann, K. and Lastick, L. (1986), Biotechnol. Bioeng. Symp. 17, 221-238.

Wyman, C. E., Spindler, D. D., and Grohmann, K. (1992), Biomass and Bioenergy. 1992, 3, 301-307.

APPENDICES

APPENDIX A

As mentioned in Chapter 3, lysing enzyme was used to successfully remove yeast cells from the freeze-dried residues of SSF experiments with switchgrass, cornstover, and poplar. It was noted that yeast cells present in SSF residues that had not been freeze-dried were resistant to lysing enzyme. It is not clear why the freeze-drying process makes the SSF cells more susceptible to enzymatic hydrolysis. This discovery was not made until after several other methods had been tested for their ability to increase the susceptibility of SSF yeast cells to lytic enzyme. The methods tested included: (1) increase lysing enzyme load, (2) addition of neutral proteins, (3) oven-drying the SSF residue prior to enzyme treatment, (4) addition of reducing agents, (5) regrow SSF residue yeast. None of these methods appeared to increase the susceptibility of SSF yeast cells to lytic enzyme. In the following paragraphs I summarize each of the tested methods.

Increase Lysing Enzyme Load. My study showed that increase lysing enzyme in pure yeast cell solution could increase the efficiency of the enzyme to digest yeast cells. I suspected that some of lysing enzyme added to the SSF residue was adsorbed by the SSF residue, so increase lysing enzyme load may be necessary to digest yeast cells in the residue. 3 mg/ml lysing enzyme was added to 3% SSF residue (after seven days' SSF process) with 50 mM phosphate buffer (containing 0.1% tetracycline and 0.1% cycloheximide, pH 7.0) and with/without dithiothreitol (served as a reducing agent), and

incubated at 37°C for 24 hrs. Results indicated that the yeast cells remained intact in the SSF residue even in the presence of supplemental lysing enzyme.

Addition of Neutral Proteins. I suspected that the lysing enzyme used to digest the yeast cells in the SSF residue was adsorbed by the SSF residue itself, making it unavailable for the lysis of yeast cells. The use of neutral proteins, such as BSA and b-lactoglobulin, to interact with the SSF residue was proposed to prevent the interaction between lysing enzyme and the residue. 0.5 and 1.0 mg/ml BSA or b-lactoglobulin was added to 3% SSF residue (after seven days' SSF process) with 50 mM phosphate buffer (containing 0.1% tetracycline and 0.1% cycloheximide, pH 7.0) , then incubated the mixture over night. In the following day, 0.5 mg/ml lysing enzyme was added to the mixture and incubated at 37°C for 24 hrs to digest the yeast cell wall. The result was that yeast cells remained intact even though BSA or b-lactoglobulin were added to the lysing reaction mixture.

Oven-drying the SSF Residue Prior to Enzyme Treatment. I expected that oven-drying could collapse the pores of the SSF residue and reduce the residues surface area, thereby decreasing the extent of interaction with lysing enzyme. Less interactions would make available more lysing enzyme to interact with the yeast cells in the SSF residue. In this experiment, the SSF residue was pre-dried in oven at 50°C for 3 days, then 0.5 mg/ml lysing enzyme was added to the oven-dried SSF residue with 50 mM phosphate buffer (containing 0.1% tetracycline and 0.1% cycloheximide, pH 7.0), then incubated the

mixture at 37°C for 24 hrs. The result was that the yeast cells that were contained in the oven dried SSF residue were still resistant to the lysing enzyme.

Addition of Reducing Agents. It has been reported that the use of reducing agents, such as dithiothreitol and b-mercaptoethanol, can change the structure of yeast cell wall by breaking down the disulfide bond in cell wall, and thus make yeast cell more susceptible to the attack of lysing enzyme. 10 mM and 100 mM dithiothreitol or b-mercaptoethanol was added to 3% SSF residue (after seven days' SSF process) with 50 mM phosphate buffer (containing 0.1% tetracycline and 0.1% cycloheximide, pH 7.0) , then incubated the mixture overnight. The following day 0.5 mg/ml lysing enzyme was added to the mixture and incubated at 37°C for 24 hrs to digest the yeast cell wall. The result of this test was that the yeast cells treated with reducing agents were still resistant to lysing enzyme.

Regrow SSF Residue Yeast. I suspected that the yeast cells in the SSF residue may reach stationary phase and become resistant to the attack of lysing enzyme. I suspected that if the yeast cells were provided with more nutrients, and allowed to go into log phase of growth, then they may become more susceptible to lysing enzyme. To test this the SSF residue was incubated with YPD medium (pH 5.0) at 37°C for 4, 8, 12 hrs, then the pH was adjusted to 7.0 with NaOH. 0.5 mg/ml lysing enzyme were then added into the mixture and incubated at 37°C for 24 hrs. The result of this experiment was that the yeast cells were still resistant to lysing enzyme even after the addition of more nutrients.

APPENDIX B

Simultaneous saccharification and fermentation (SSF) and straight saccharification experiments were a major part of this study. This appendix is included in the thesis because it provides information on the type of data that can be expected in SSF and saccharification experiments. For example, in figure 34 I have included three randomly selected saccharification runs. Each of the runs was done on a different day. The two curves in each plot are the results from duplicate flasks run side-by-side. The data indicates that the straight saccharification experiments are very reproducible when the same sample is analyzed in duplicate in the same experiment. It is possible that more variability would have been noticed had earlier time points been collected, since maximum yields were obtained very early in the time courses. Data analogous to that in Figure 34 is presented in Figure 35 for SSF experiments. Again, the data indicates that simultaneous duplicate SSF runs are quite reproducible. Considering the three plots of figure 35, there is really only one relatively large deviation in the duplicate points, and that is the 48 hr time point depicted in figure 35b. The conclusion from the data in figures 34 and 35 is that one can expect duplicate runs in straight saccharification and SSF experiments to be relatively reproducible, indicating that within experiment variation is not a major concern.

Figures 36a and 36b contain three independently obtained straight saccharification time courses for a single pretreated switchgrass substrate and an α-cellulose substrate, respectively. Figures 37a and 37b contain analogous time courses for the same substrates in SSF experiments. It is important to note that each plot contains three time courses that were obtained in different experiments (different days) using the same feedstock sample.

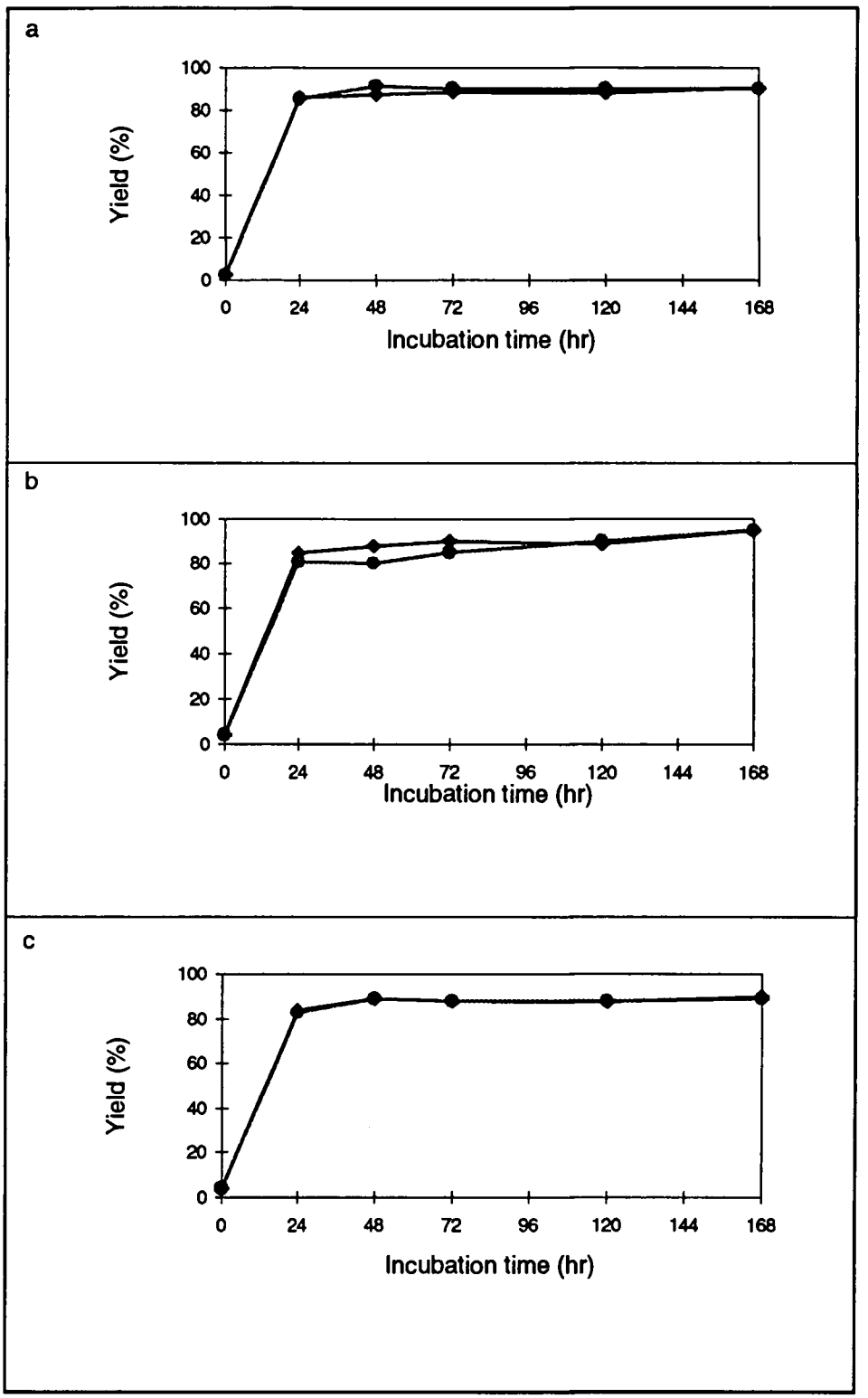

Fig 34. Reproducibility of straight saccharification from two reaction flasks performed at the same time with the same pretreated sample.

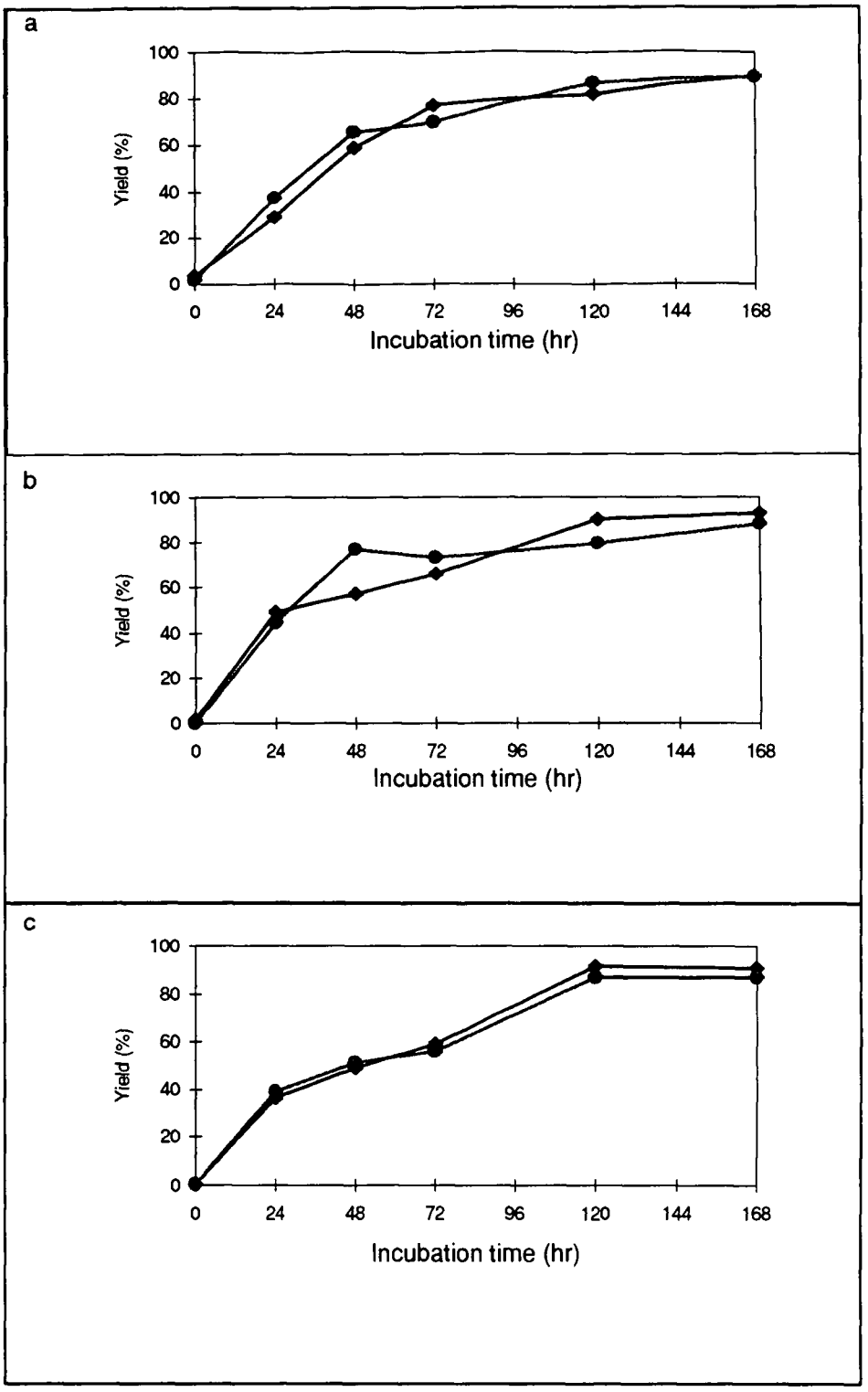

Fig 35. Reproducibility of SSF from two reaction flasks performed at the same time with the same pretreated sample.

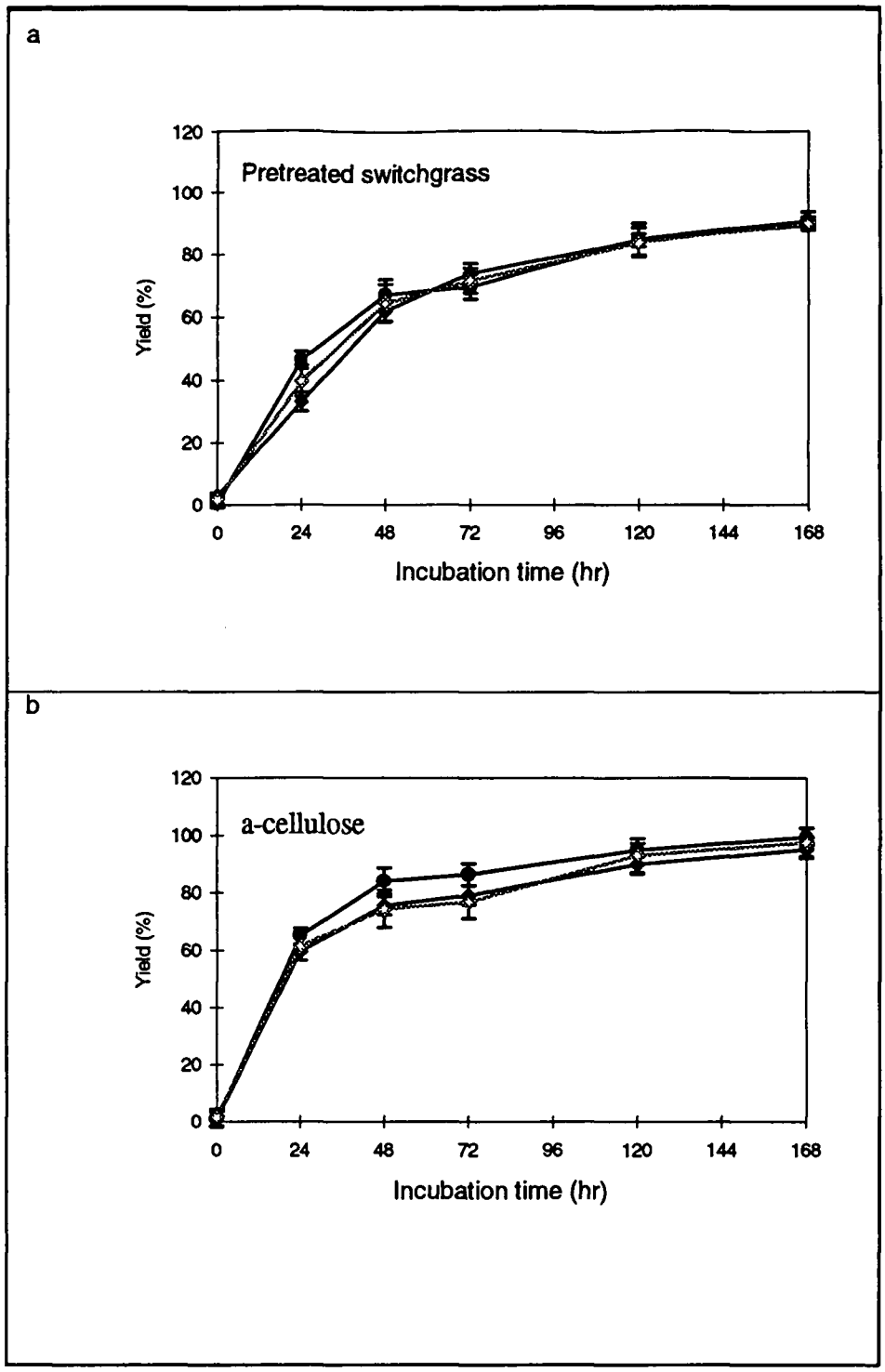

Fig 36. Reproducibility of straight saccharification from three experiments with the same pretreated sample.

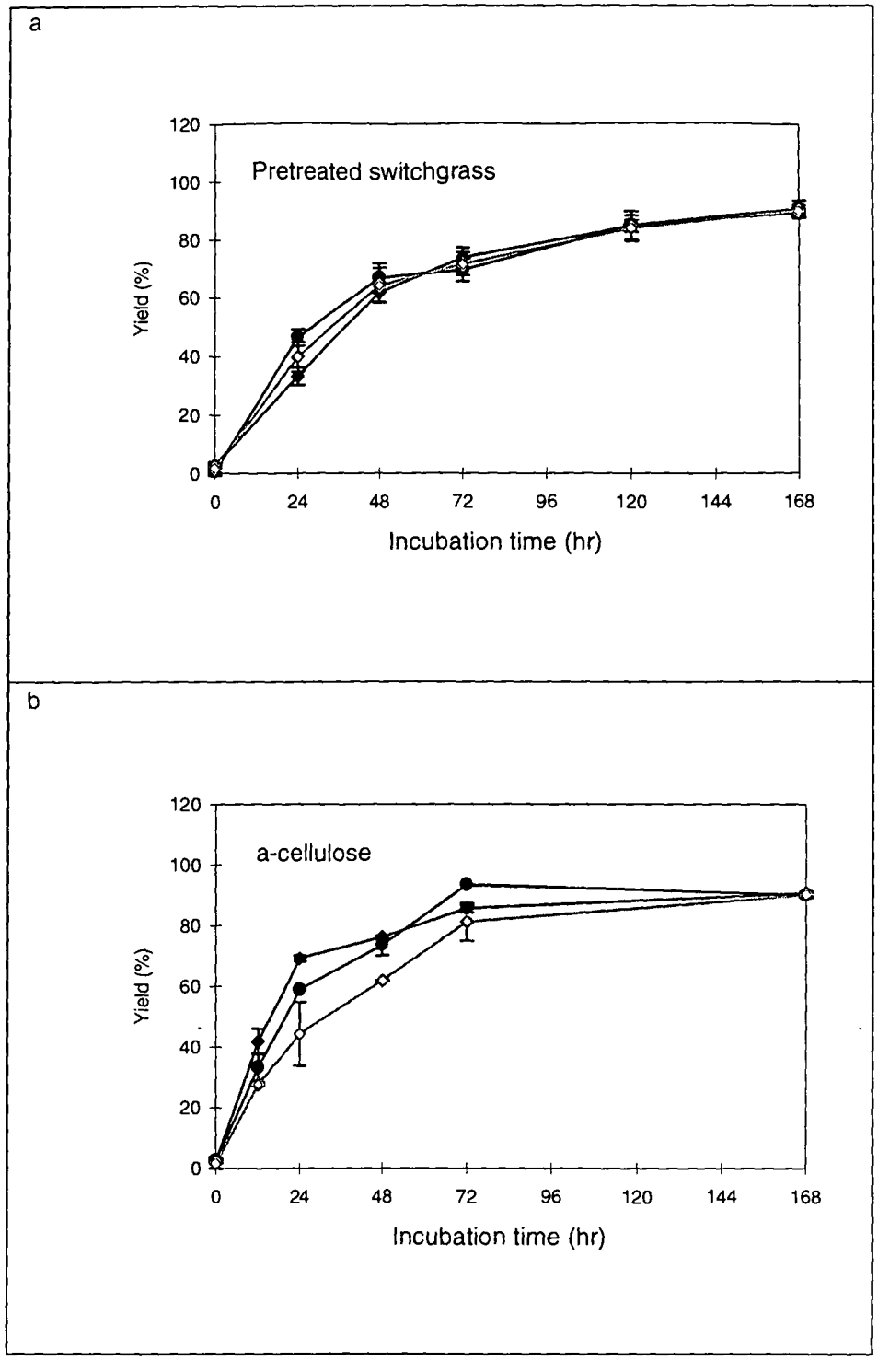

Fig 37. Reproducibility of SSF from three experiments with the same pretreated sample.

Each individual time course is the mean of duplicate runs (analogous to the duplicate runs shown in figures 34 and 35). Hence, these figures provide an example of the type of reproducibility that can be expected if a single feedstock is analyzed on different occasions. The plots indicate that independent saccharification runs (figure 36) are more reproducible than independent SSF runs (figure 37). However, the variability associated with both of these experimental techniques (straight saccharification and SSF) was relatively low.

Figure 38 contains three independently obtained straight saccharification time courses for a three distinct pretreated switchgrass substrates. Each of the substrates was pretreated under identical conditions on different days. Hence, the variance associated with the three time courses in figure 38 is a function of the sum of the variance associated with straight saccharification runs and feedstock pretreatment. Analogous curves for the same substrates in SSF experiments are presented in figure 39. The straight saccharification runs of figure 38 show much more variability than those of figures 34 and 36. The implication being that the relatively large increase in the variability associated with the curves of figure 38 is a result of irreproducible pretreatments, not due to high variability in the SSF phase of the experiment. If the high variability were due to problems in the saccharification protocol, then the high variability of figure 38 would likely also be detectable in the time courses of figures 34 and 36. Comparison of the time courses in figures 35, 37 and 39 allow one to draw the same conclusion for the SSF experiments. However, the proportion of the total variance that can be attributed to irreproducible

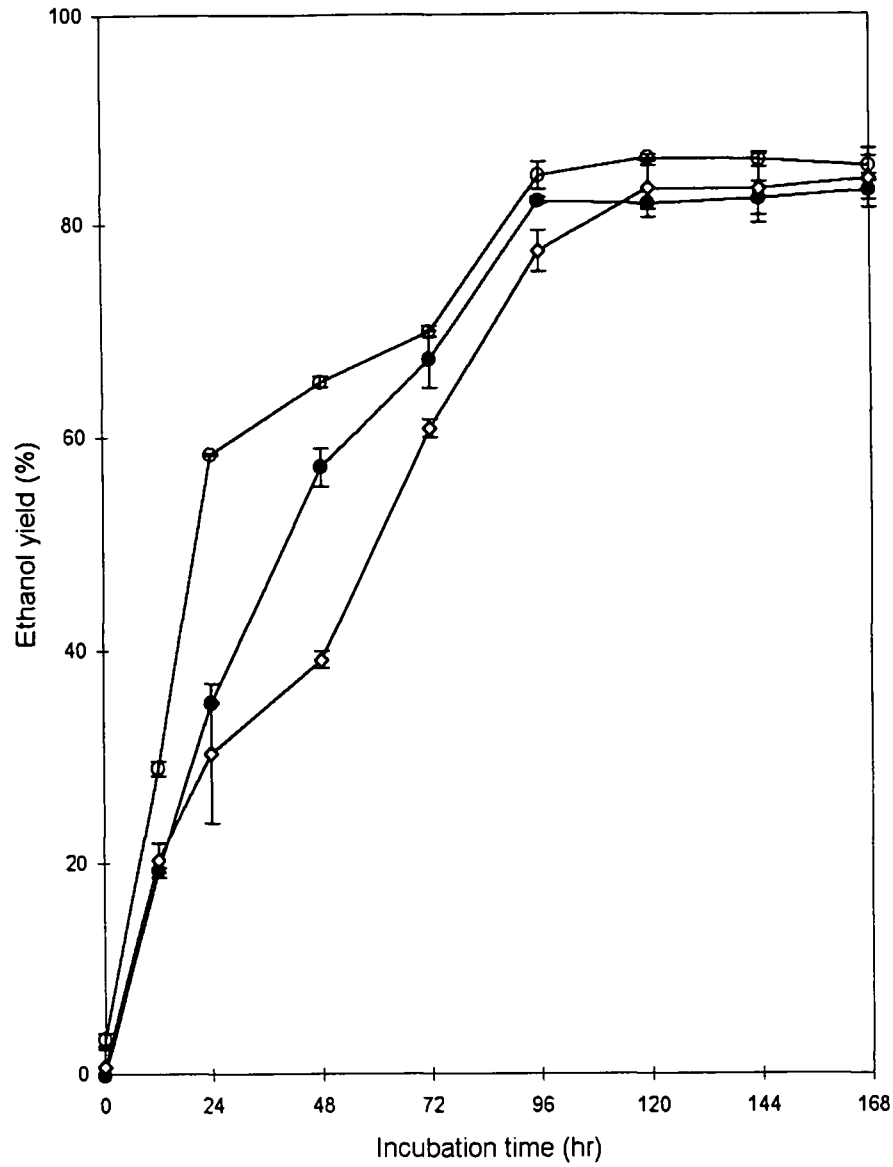

Fig 38. Reproducibility of straight saccharification from three experiments with samples from three different batches pretreated indivudually under the same conditions.

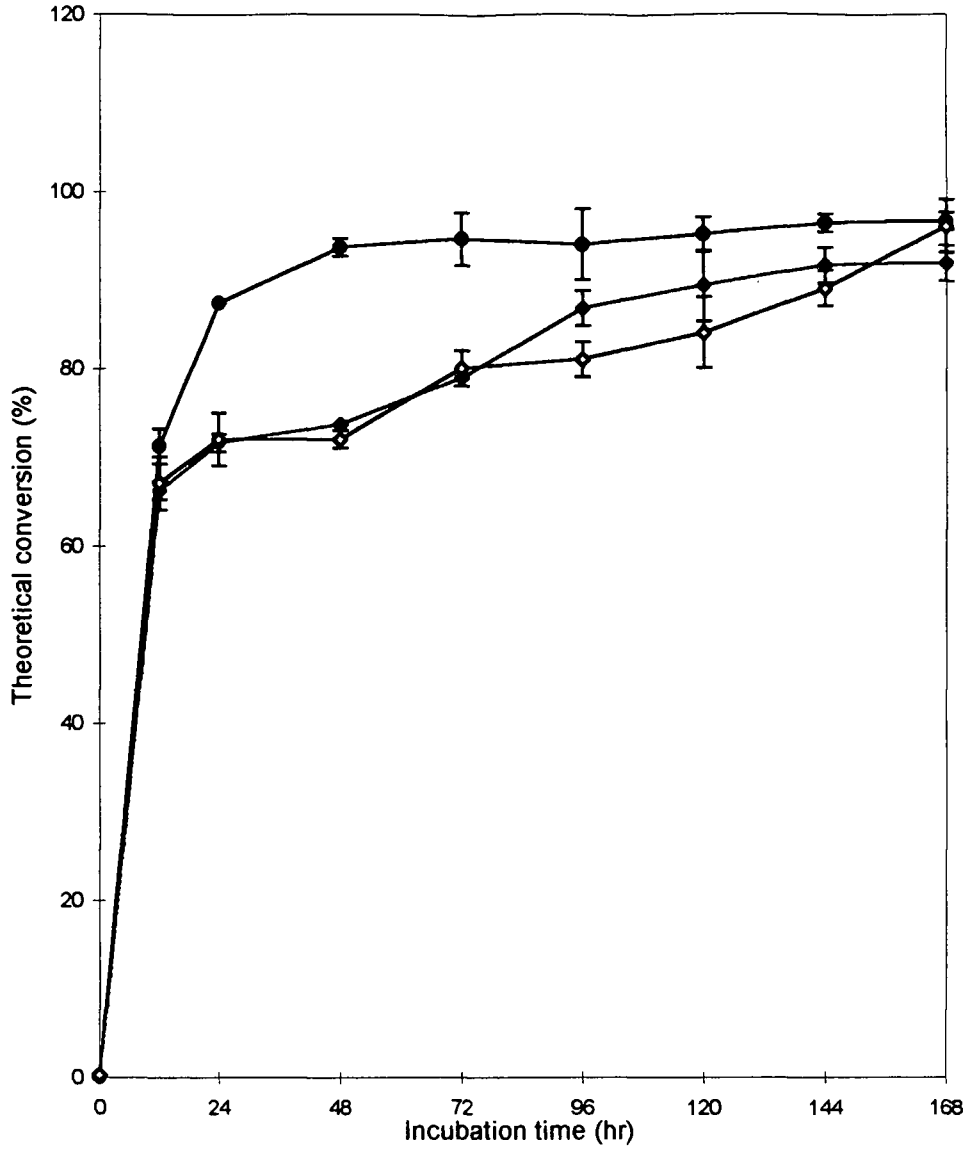

Fig 39. Reproducibility of SSF from three experiments with samples from three different batches pretreated indivudually under the same conditions.

pretreatments seems to be somewhat lower for the SSF experiments than for the straight saccharification experiments.

APPENDIX C

In order to compare the overall time courses of the theoretical yield of pretreated switchgrass, cornstover and poplar in saccharification and SSF, the Michaelis-Menten model, $Y = aX / (X + b)$, was used to depict the overall trend of the data. This nonlinear model can be further linearized by taking the reciprocal of both sides and results $1 / Y = A / X + B$ (where $A = b / a$; $B = 1 / a$), so parameter A and B can be obtained by using simple linear regression. The R^2 valuses of the linear model for most data sets were in the range of 0.91-0.99 indicating that the model was appropriate for fitting the theoretical yield of all pretreated feedstocks in both saccharification and SSF. The ranges of Parameter A and B for all data sets in this study were found to be 0.015 - 0.947 and 0.005 - 0.011, respectively. It was noted that increase in parameter A alone within its range decreases the initial slope and the plateau value of the model, so parameter A can be used to describe the initial reaction rate of the theoretical yield of feedstocks. However, increase in parameter B alone within its range decrease the plateau values but not the overall shape of the model, so parameter B offered little information regarding to the initial rate of the theoretical yield of feedstocks. As shown in table 17, cornstover had the highest initial rate in saccharification (indicated by having lowest parameter A value), followed by switchgrass, then poplar had the lowest initial rate. In SSF, cornstover under 1.2% acid, 180°C, and 0.9 min. treatment condition had the highest initial rate, however, in 1.1% acid, 180°C, and 1.0 min treatment condition there was no significant difference between cornstover and switchgrass. Again, poplar had the lowest initial rate in SSF.

Table 17. A comparison of the parameters of the model, $1 / Y = A / X + B$, used to depict the time couses of the theoretical yields of dilute acid pretreated switchgrass, cornstover and poplar in saccharification and SSF (where Y is the theoretical yield in unit of % and X is the reaction time in unit of hr).

Substrate	Pretreatment	Parameter A		Parameter B	
	acid, temperature, time	Saccharification	SSF	Saccharification	SSF
Switchgrass	0.9%, 180°C, 0.5 min	0.049 ± 0.006^{ax}	0.413 ± 0.015^{ay}	0.011 ± 0.000^{ax}	0.009 ± 0.001^{ay}
	1.2%, 180°C, 0.5 min	0.052 ± 0.002^{ax}	0.434 ± 0.016^{ay}	0.010 ± 0.000^{bx}	0.007 ± 0.001^{ay}
Cornstover	1.2%, 180°C, 0.9 min	0.019 ± 0.004^{bx}	0.296 ± 0.018^{by}	0.011 ± 0.000^{ax}	0.008 ± 0.001^{ay}
	1.1%, 180°C, 1.0 min	0.015 ± 0.001^{bx}	0.466 ± 0.045^{ay}	0.010 ± 0.000^{bx}	0.005 ± 0.002^{ay}
Poplar	0.9%, 180°C, 0.6 min	0.105 ± 0.006^{cx}	0.943 ± 0.031^{cy}	0.010 ± 0.000^{bx}	0.006 ± 0.001^{ay}
	1.0%, 180°C, 0.56 min	0.114 ± 0.006^{cx}	0.947 ± 0.015^{cy}	0.009 ± 0.000^{bx}	0.007 ± 0.001^{ay}

^{abc} Means in the same column followed by the different superscripts were significantly different ($p < 0.05$)

^{xy} Means in the same row followed by the different superscripts were significantly different ($p < 0.05$)

The results indicated that the initial reaction rate of saccharification and SSF depend strongly on the chacteristic of pretreated feedstocks, which depend on the types of feedstocks and the pretreatment conditions. Higher parameter A values were found in SSF of all feedstocks under different pretreatment condtions then that in saccharification indicating that the initial rate of SSF of all feedstocks were lower then that in saccharification.

The final yields of pretreated feedstocks in saccharification and SSF are shown in table 18. All three feedstocks under different pretreatment conditions showed similar final yields in saccharification, however, in SSF the final yields depended on the types of feedstocks. It was found that pretreated poplar had significantly ($p < 0.05$) lower final yield then pretreated switchgrass or cornstover in SSF. In the same feedstock, different pretreatment conditions used in this study affected only the final yields of switchgrass in SSF due to different acid concentrations. It was also noted that the final yields of pretreated switchgrass and poplar in SSF were significantly ($p < 0.05$) lower than that in saccharification, however, no significant difference in the final yields of pretreated cornstover.

Table 18. Theoretical final yield of dilute acid pretreated switchgrass, cornstover and poplar.

Substrate	Pretreatment	Theoretiacl final yield (%)	
	acid, temperature, time	Saccharification	SSF
Switchgrass	0.9%, 180°C, 0.5 min	94.8 ± 2.6^{ax}	84.2 ± 1.2^{by}
	1.2%, 180°C, 0.5 min	97.8 ± 5.1^{ax}	90.2 ± 1.9^{cy}
Cornstover	1.2%, 180°C, 0.9 min	96.7 ± 5.5^{ax}	90.5 ± 2.0^{bcdx}
	1.1%, 180°C, 1.0 min	98.2 ± 0.0^{ax}	96.1 ± 5.1^{dx}
Poplar	0.9%, 180°C, 0.6 min	95.1 ± 1.8^{ax}	75.9 ± 2.6^{ay}
	1.0%, 180°C, 0.56 min	92.0 ± 11.2^{ax}	75.9 ± 4.3^{ay}

[abc] Means in the same column followed by the different superscripts were significantly different ($p < 0.05$)

[xy] Means in the same row followed by the different superscripts were significantly different ($p < 0.05$)