BIOCONVERSION OF CORN STOVER INTO VALUE-ADDED CHEMICALS:

DILUTE SULFURIC ACID PRETREATMENT, XYLO-

OLIGOSACCHARIDES PRODUCTION, AND

LACTIC ACID FERMENTATION

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VITA

Yongming Zhu, son of Shixin Zhu and Cuiyun Ai, was born on July 23, 1973, in China. He attended Shaoyang County First High School in his hometown and graduated in July 1990. In September 1990, he entered Zhejiang University in in Hangzhou, China. He graduated with the degree of Bachelor of Science in Chemical Engineering in 1994, then he worked as a process engineer for the Dongting Nitrogen Fertilizer Co. Ltd., in Hunan, China for two years. He entered the Research Institute of Petroleum Processing of China, Beijing, in August 1996 to pursue the degree of Master of Science in Chemical Engineering. He graduated on April 1999 and worked in the same institute as a researcher. On August 2000, he entered the Graduate School at Auburn University to pursue the degree of Doctor of Philosophy in Chemical Engineering. He married Furong Zhou, daughter of Yanwen Zhou and Jufen Yan on October 01, 1999. They have one daughter, Rose and one son, Stanley.

DISSERTATION ABSTRACT

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In the first part of this dissertation, dilute-acid pretreatment of corn stover was investigated under high-solids condition using a percolation reactor. High-solids condition in a pretreatment process can lead to high productivity and low liquid throughput. The latter reduces the cost of process energy as well as the load of wastewater treatment. The effects of temperature, acid concentration, acid flow rate, and reaction time on the glucan digestibility and recovery of hemicellulose sugars were investigated. The experimental results were assessed to determine the optimum ranges of reaction conditions. The xylose yield was affected sensitively by the flow rate under a given reaction condition. This behavior appears to be related to sugar decomposition,

mass transfer resistance and the fact that acid is neutralized by the buffering components of the biomass. Further improvement of this process was sought with modifications in the operation mode of the percolation reactor. The reactor was preheated under atmospheric pressure to remove moisture that may cause autohydrolysis and subsequent sugar decomposition. In addition, liquid throughput was minimized to the extent that only one reactor void volume of liquid was collected. This was done to attain high xylose concentration. For the substrates treated under the optimum reaction and operating conditions, near quantitative glucan digestibility was obtained while the decomposition of carbohydrates was suppressed to an extremely low level. The digestibility tests done on the pretreated samples indicated that the digestibility is related to the extent of xylan removal.

The second part of this dissertation dealt with enzymatic production of xylo-oligosaccharides (XOs, also known as xylo-oligomers) from corn stover and cobs. The process started with a pretreatment known as soaking in aqueous ammonia (SAA). The pretreated feedstock containing digestible xylan was then directly subjected to selective enzymatic hydrolysis for the production of XOs. The complex substrate purification step required in the conventional method is not necessary in this process. This simplifies the overall process, and more importantly, improves the process economics. In the subsequent stage, fractionation and refining of XOs were accomplished by charcoal adsorption followed by ethanol elution, with XOs being collected in relatively high yields. Xylanolytic hydrolysis of the SAA treated corn stover has shown high digestibility of the remaining glucan. As a feedstock for production of XOs, corn cobs are superior to corn stover because of high xylan content and density. The high packing

density of corn cobs reduced water use in both SAA treatment and enzymatic hydrolysis, which eventually led to high XOs concentration.

In the last part of the dissertation, production of lactic acid by simultaneous saccharification and co-fermentation (SSCF) was investigated. The SAA treated and water washed corn stover was the substrate of this investigation. The microorganism used for the co-fermentation was *Lactobacillus pentosus* ATCC 8041 (CECT-4023). When the SSCF was conducted in batch mode based on 3% (w/w) glucan loading, the carbohydrates (both cellulose and hemicellulose) in the treated corn stover were effectively converted to lactic acid and acetic acid, the maximum lactic acid yield reaching 92 % of the theoretical maximum based on the total of carbohydrates (glucose, xylose, and arabinose). Small amount of acetic acid was also produced from the pentoses through the phosphoketolase pathway. The impacts of enzyme loading, inocula size, yeast extract concentration, and clarified corn steep liquor (cCSL) concentration were investigated in relation to lactic acid production following a statistical experimental design. The statistical analysis showed that enzyme and yeast extract were the most important factors affecting the final lactic acid yield. In contrast, the impact of inocula size was found to be insignificant. The response surface analysis indicated that cCSL could be used as a nitrogen source in place of yeast extract without loss of lactic acid yield. The product concentration was improved by operating the SSCF in fed-batch mode. The maximum lactic acid concentration in the fed-batch operation was 74.8 g/L. Further improvement of the lactic acid concentration was difficult to achieve due to severe product inhibition.

ACKNOWLEDGEMENTS

This dissertation is dedicated to my wife, Furong Zhou. During the past five years, her support and understanding have helped me continue to work on this research.

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I. INTRODUCTION

Agricultural residues represent an important segment of lignocellulosic materials, which are currently the only sustainable resource for fuel and chemical productions (Lynd et al., 1991; Wyman, 1994; Danner and Braun, 1999; Mosier et al., 2005). Every year, an estimated 200 million dry tons of agricultural residues was generated in the U.S. (Glassner et al., 1998), but most of this material was not effectively utilized. However, this situation is now changing due to the approaching depletion of fossil oils, and the competitive uses of starch materials as chemical feedstock and as food, as well as the increasing public concern about the environment. During the past decades, intensive research being conducted in this field has yielded significant progress. A good example of this is the production of bioethanol from wheat straw, which has moved from laboratory study to near-commercial stage (Foody and Tolan, 2004).

Corn stover is a mixture of crop residues that is comprised of corn stems, stalks, cobs and leaves. The amount of corn stover that can be sustainably collected in the U.S. is estimated to be 80-100 million dry t/yr, which is equivalent to more than 4.8 billion gal of ethanol per year (Kadam and McMillan, 2003). Owing to its sustainable abundance, corn stover has been considered to be one of the most promising feedstock for bioethanol production in the U.S. (Kadam and McMillan, 2003).

Corn stover is composed of 36-38% cellulose, 20-23% hemicellulose and 17-20% lignin. Cellulose and hemicellulose are carbohydrate polymers, which must be broken

down into low-molecular-weight sugars (basically monomers) so that they can be fermented by microorganisms. The cellulose fraction is, however, natively resistant to enzymatic breakdown. In order to render the cellulose amenable to enzymatic conversion, a pretreatment step is required. One of the most important pretreatments is dilute sulfuric acid hydrolysis, in which most of the hemicellulose is hydrolyzed and the glucan digestibility is significantly increased. Although this pretreatment has been shown to be effective for a variety of lignocellulosic materials (Torget et al., 1992; Nguyen et al., 1998), the study on it for corn stover is still insufficient. In view of this, the first part of this dissertation discussed dilute sulfuric acid pretreatment of corn stover using a percolation reactor that was operated under high-solids condition. High-solids condition is desirable for a pretreatment process because it can lead to high productivity and low liquid throughput, the latter reducing the process energy cost as well as the load of wastewater treatment. This pretreatment process was assessed in terms of glucan digestibility, the recovery of hemicellulose sugars and the sugar concentration in the hydrolyzate.

In the subsequent parts of this dissertation, bioconversion of corn stover/cobs into value-added chemicals was investigated. An alternative pretreatment method, soaking in aqueous ammonia (SAA), was used to enhance the enzymatic saccharification of corn stover/cobs. SAA pretreatment has demonstrated high selectivity toward delignification at low levels of carbohydrate removal (Kim and Lee, 2004). The treated biomass contains low lignin content, is highly digestible and is enriched in carbohydrates. These features make the SAA treated biomass suitable as a substrate for the production of a variety of sugar-derived chemicals. In this dissertation, two high-value products were produced

from SAA treated corn stover/cobs: xylo-oligosaccharides (XOs) and lactic acid. More detailed descriptions of these two processes are given below.

Production of xylo-oligosaccharides via enzymatic hydrolysis

XOs with low degree of polymerization (DP) can be used as a functional food because of the ability to effectively improve gastroenteritic performance. The conventional production of XOs was based on enzymatic hydrolysis of xylan that was extracted from lignocellulosic biomass. Since the extraction of xylan usually occurred simultaneously with the release of lignin and other contaminants, a complex purification step was required before the extracted xylan can be used as a substrate for enzymatic hydrolysis. However, the use of SAA treated biomass for XOs production can significantly simplify the process because the digestible xylan retains in the solids. The goal for this research was, therefore, to examine the feasibility of producing low-DP XOs from SAA treated corn stover/cobs through enzymatic hydrolysis.

Production of lactic acid via simultaneous saccharification and co-fermentation (SSCF)

Lactic acid finds various applications in the food, pharmaceutical, and cosmetic industries. At present, lactic acid is predominantly manufactured by the fermentation of starch-derived glucose or sucrose. Agricultural residues like corn stover will offer a competitive, less expensive feedstock for producing lactic acid. To make the process more economically viable, however, it is desirable that both the hexose and pentose sugars (mainly glucose and xylose) in the feedstock be converted into lactic acid. For this purpose, SSCF of SAA treated corn stover as a prospective new approach for lactic acid production was studied. A *Lactobacillus* strain, *L. pentosus* ATCC 8041, was selected for this process as it is able to co-ferment glucose and xylose (Bustos et al., 2004).

Objectives:

The following objectives were set for this dissertation,

1. To investigate the process performance of the dilute-sulfuric acid pretreatment of corn stover using a percolation reactor (flow-through type) for operation at high-solids condition;
2. To investigate the technical feasibility of employing SAA treated corn stover/cobs as substrates for enzymatic production of xylo-oligosaccharides; and
3. To develop a process for co-fermentation of both hexose and pentose sugars from SAA treated corn stover to produce lactic acid.

II. LITERATURE REVIEW

1. Structure and chemical composition of lignocellulosic biomass

Lignocellulosic biomass is comprised of three main components: cellulose, hemicellulose and lignin, the remainder being extractives and ash. Table 1 gives the typical composition of some agricultural lignocellulosics. It is notable that considerable differences exist between the compositions of different species.

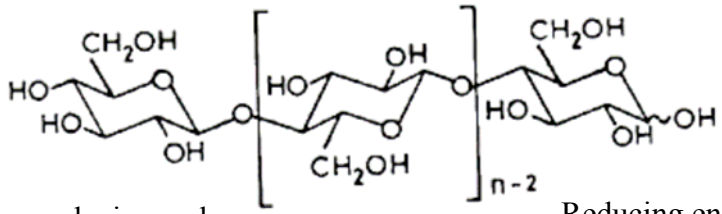
Cellulose is a linear molecule composed of repeating cellobiose units held together by β-1, 4-glycosidic linkages (Fig. 1a). The degree of polymerization of a cellulose molecule varies from 10,000 to 14,000 (Goldstein, 1981). Bundles of cellulose molecules form microfibrils, which build up into fibrils and, finally, to cellulose fibres. Hydrogen bonding occurs between linear molecules resulting in a strong microcrystalline structure (Sojorm, 1993). The compact structure of cellulose renders it a specific moiety that is relatively resistant to the attack of enzymes and chemicals.

Hemicellulose, an amorphous heterogeneous group of branched polysaccharides, surrounds the cellulose fibres and intrudes into cellulose through pores. Xylose, arabinose, mannose, glucose, glucoronic acid and galactose are the major sugar residues. The structure of agricultural lignocellulosic hemicellulose is characterized by a long, linear backbone of repeating xylose, with short, branched side chains composed of glucuronic groups, acetate and sugars (Fig. 1b). The composition of hemicellulose varies between species of agricultural lignocellulosic biomass (Table 2). Hemicellulose

Table 1 Composition of some agricultural lignocellulosic biomass (%, dry basis). (Saha, 2003)

	Cellulose	Hemicellulose	Lignin
Corn fibera	15	35	8
Corn cob	45	35	15
Corn stover	40	25	17
Rice straw	35	25	12
Wheat straw	30	50	20
Sugarcane bagasse	40	24	25
Switchgrass	45	30	12
Coastal bermuda grass	25	35	6

a. Cellulose



Non-reducing end Reducing end

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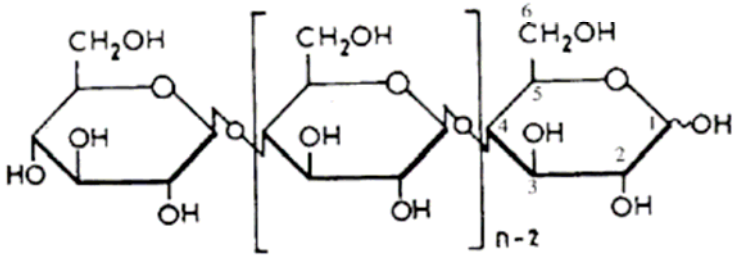


b. Corn fiber heteroxylan

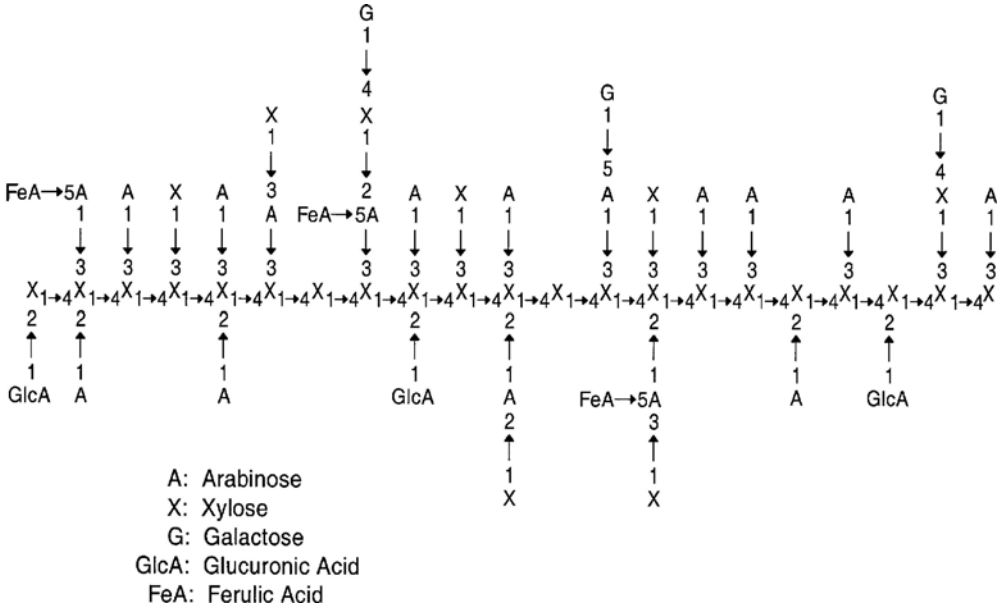


Fig. 1. Schematic structures of cellulose (a) and corn fiber heteroxylan (b) (Saulnier et al., 1995).

Table 2 Composition of different agricultural lignocellulosic hemicelluloses

	Rice bran neutral xylan	Wheat arabinoxylan	Corn fiber xylan
Xylose	46%	65.8%	48-54%
Arabinose	44.9%	33.5%	33-35%
Mannose		0.1%	
Galactose	6.1%	0.1%	5-11%
Glucose	1.9%	0.3%	
Anhydrouronic acid	1.1%		
Glucoronic acid			3-6%
Reference	Shibuya and Iwasaki, 1985	Gruppen et al., 1992	Saha and Bothast, 1999

hydrogen-bonds to cellulose microfibrils, thus forming a network that provides the structural backbone of the plant cell wall.

Lignin is a complex phenylpropanoid polymer that surrounds and strengthens the cellulose-hemicellulose framework. The main substituent of lignin, coniferyl alcohol (guaiacyl), sinapyl alcohol and hydroxycinnamyl alcohol, are polymerized in a random fashion by an enzyme-catalyzed dehydrogenative reaction (Kringstad and Lindstrom, 1984). The presence of lignin in some cell walls imparts additional strength and provides resistance against pests and diseases. It is believed that hemicellulose acts as a molecular bonding agent between the cellulose and lignin fractions (Torrie, 1991).

2. Dilute sulfuric acid pretreatment

The known factors contributing to the resistance of lignocellulosic biomass to cellulolytic hydrolysis are: the crystallinity of cellulose, its accessible surface area, the protection of cellulose by lignin, the heterogeneous character of the biomass particles, and cellulose sheathing by hemicellulose (Rydholm, 1965; Wenzel, 1970; Hsu et al., 1980; Hsu, 1996; Chang and Holtzapple, 2000). In principle, a pretreatment causing disruption of all or some of these barriers can facilitate the penetration of hydrolytic reagents and promote the hydrolysis of cellulose. Treatment of lignocellulosic biomass with dilute sulfuric acid has proven to be an effective method in this regard.

In general, acid-based pretreatment processes are effective in solubilizing the hemicellulose component of biomass (Elander and Hsu, 1994; McMillan 1992), while leaving most of the cellulose fraction intact (Lee et al., 1978). Such pretreatments remove the lignin-hemicellulose protective shield and render the remaining cellulose amenable to enzymatic conversion to glucose (Grohman et al., 1986). The effectiveness of dilute acid

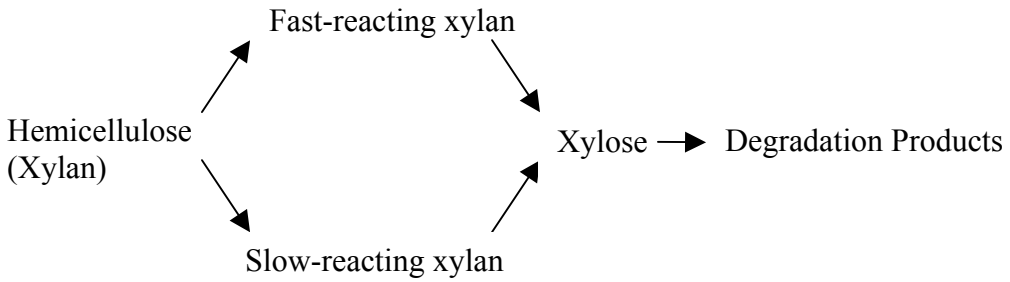
hydrolysis as a pretreatment has been verified experimentally (Grohman et al., 1985, 1986; Torget et al., 1988, 1990). In addition, a dilute acid pretreatment will eliminate the acid-recovery system, which seems to be essential for a pretreatment process using concentrated acid. As such, the dilute acid treatment of biomass aimed at hemicellulose hydrolysis has become a widely accepted pretreatment method for enzymatic hydrolysis (Schell et al., 1992; Nguyen et al., 1998; Schell et al., 2003).

2.1 Kinetics of hemicellulose hydrolysis

In most kinetic studies of hemicellulose hydrolysis, hemicellulose is classified in terms of two distinguishable fragments, i.e. fast- and slow-reacting hemicelluloses (Kobayashi and Sakai, 1956; Nee and Wen, 1976; Conner, 1984; Grohmann et al., 1986(1); Maloney et al., 1985; Maloney et al., 1986; Kim and Lee, 1987; Esteghlalian et al., 1997), although a few other models have treated the whole hemicellulose as a single (Garrote et al., 2001). The fraction of fast-reacting hemicellulose reported in the literature usually falls between 0.6-0.9 (Conner, 1984; Maloney et al., 1985; Kim and Lee, 1987; Eken- Chen et al., 1996; Saracoglu et al., 1998).

Since xylan is the major component of hemicellulose in hardwood and agricultural biomass, the overall hemicellulose hydrolysis of these biomass species can be presented as in terms of the xylan degradation (Fig. 2). The only difference between the two models in Fig. 2 is that Model 2 involves oligomers as intermediates while Model 1 does not. Model 1 applies to the situations where the rate of the oligomers-to-monomer reaction is so much faster than the rate of oligomers production that the latter can be omitted; and model 2 is best used for reactions under relatively mild conditions where buildup of oligomers cannot be neglected, for example in flow-through systems (Chen et

(a) Model 1



(b) Model 2

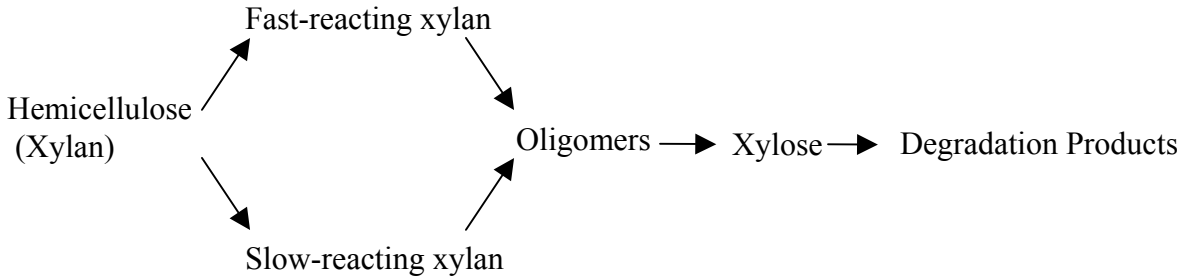


Fig. 2. Two types of kinetic models for hemicellulose hydrolysis (a) (Kim and Lee, 1987; Eken-Saracoglu et al., 1998); (b) (Chen et al., 1996; Jacobsen and Wyman, 2000)

al., 1996; Jacobsen et al., 2000). In either case, the reactions are assumed to be pseudo-homogeneous following a first-order dependence on reactant concentrations, with an Arrhenius temperature relationship for reaction rate constants:

$$k_i = k_{i0} \times A^{m_i} \times e \exp(-E_i/RT) \quad (1)$$

in which k_i is the reaction rate constant; k_{i0} is the pre-exponential factor; A is the concentration of acid (wt%); m_i is the power; and E_i is the activation energy. The concentration of acid (A in equation (1)) should be corrected to take into consideration the neutralizing capacity of the biomass. Neutralizing capacity, also called buffering capacity, is defined as the amount of acid neutralized by the reactive species present in the biomass structure (e.g., ash) per gram of dry biomass. The neutralization effect is not generally an issue in concentrated acid hydrolysis, but might be significant at conditions where a low liquid/solid ratio and dilute acid solution are used. The neutralizing capacity may vary significantly depending on the biomass species. For instance, Maloney et al. (1985) found that paper birch has a neutralizing capacity of 3.5 mg H_2SO_4/g dry wood, while Esteghlalian et al. (1997) reported the neutralizing capacities of corn stover, switch grass and poplar to be 43.7, 25.8 and 16.7 mg H_2SO_4/g dry biomass, respectively. In general, since agricultural residues contain higher ash contents than wood, they also have higher neutralizing capacities.

2.2 Reactors and processes for dilute acid pretreatment

2.2.1 Batch reactor

Batch reactors are frequently employed in kinetic studies of hemicellulose hydrolysis (Esteghlalian et al., 1997; Bhandari et al., 1984), of which tubular and Parr reactors are the two main types. They are operated in autoclave modes without pressure control since pressure is not a factor affecting the kinetics of dilute acid hydrolysis, which takes place in the liquid and solid phases. Before the reaction starts, the biomass is soaked in liquid to ensure uniform wetting. The reactor is then preheated to increase the temperature and thus initiate the hydrolysis.

Table 3 summarizes some data from the literature on dilute acid hydrolysis of hemicelluloses in various agricultural biomasses using batch reactors. The variables in these studies are acid concentration, temperature, reaction time, and liquid/solid ratio. Sometimes the effect of particle size is also considered. The maximum xylose yield usually occurs at higher temperatures and higher acid concentrations. It appears that in dilute acid hydrolysis of agricultural lignocellulosic biomass, a maximum xylose yield of 0.7 to 0.9 is available.

2.2.2 Cocurrent-flow (plug-flow) reactor

A plug-flow reactor (PFR) is the continuous version of a batch reactor. In a PFR, the liquid and the solids move through the reactor at the same velocity (Fig. 3). Therefore, a PFR can be modeled as a reactor that is composed of a number of small batch reactors with different residence times. Since there is no back mixing in the reactor, it can achieve a high product concentration. A PFR can be used either for kinetic studies

Table 3 Operating conditions and maximum xylose yields (total of xylose + oligomers) for the dilute-acid hydrolysis of various agricultural hemicellulose species

Feedstock	Reactor	Acid Conc. (w/w)	T /°C	L/S (w/w)	Time /min	Experimental maximum xylose yield	Reference
Corn stover	600 ml Parr reactor	0.6-1.2	140-180	10	0-60	0.84	Esteghlalian et al., 1997
Corn stover	500 ml Parr reactor	0.49-1.47	160-240	2	0-44	0.787	Bhandari et al., 1984
Wheatstraw	1000 ml Parr reactor	0.5-1.0	100-210	20	0-50	0.89	Ranganathan et al., 1985
Switchgrass	600 ml Parr reactor	0.6-1.2	140-180	10	0-60	0.88	Esteghlalian et al., 1997
Corn cob	Glass tube reactor (0.5 cm i.d.)	0.98-2.9	98-130	4	0-180	0.82	Eken-Saracoglu et al., 1998
Sunflower seed hull	Glass tube reactor (0.5 cm i.d.)	0.98-4.8	98-130	3	0-180	0.72	Eken-Saracoglu et al., 1998
Bagasse		0.3-4.0	80-150	3.6-15			Trickett and Neytzell -de Wilde,1982

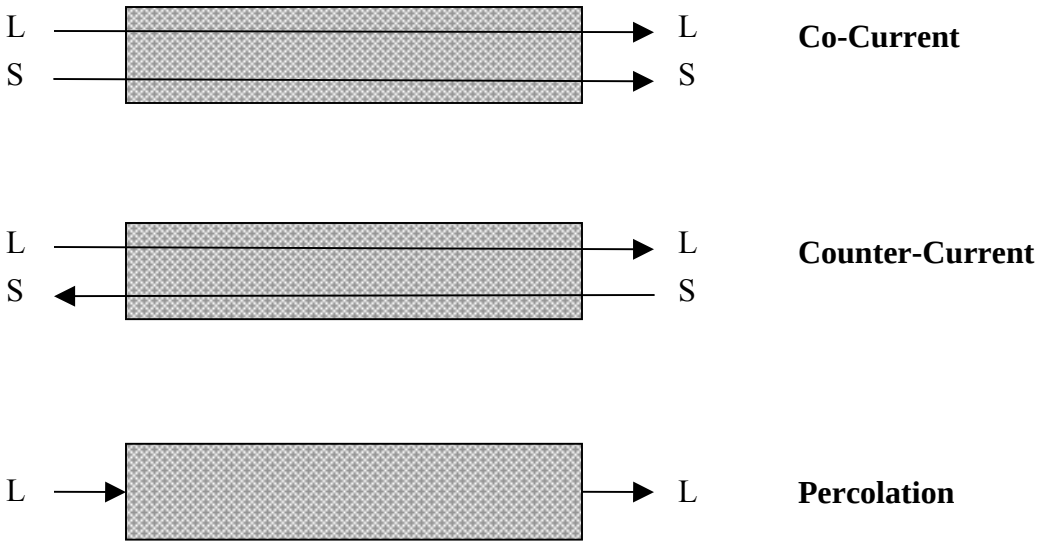


Fig. 3. Reactors used for biomass pretreatment.

(Converse et al., 1989; McParlandet al., 1982) or process investigations (Martinez et al., 1995; Nathan et al., 2003). The pretreatment reactor in the process development unit (PDU) at the NREL is essentially a PFR (Schell et al., 2003). Another pilot-scale PFR for biomass pretreatment is the snake-coil reactor used for corn fiber pretreatment (Ladisch et al., 2003).

2.2.3 Countercurrent-flow reactor

The countercurrent-flow reactor was first introduced by Song and Lee (1983) as a conceptual reactor for biomass treatment. In this type of reactor, the solids and the liquid are moving in opposite directions (Fig. 3). In the countercurrent reactor scheme, the major portion of the sugars is produced in the vicinity of the liquid outlet point. The stream then travels a relatively short distance before it is washed out of the reactor, thus reducing the time available for the sugar to decompose, and consequently raising the yield and sugar concentration. Burns et al. (1990) analyzed the acid hydrolysis of cellulosic slurries in a cyclone reactor. This reactor can be operated either in co-current or countercurrent mode. For the case of 250 ^{o}C and 1% acid, the model indicates an increase in the xylose yield from 91.6% in a plug flow reactor to 97.7% in a counter-current reactor. A pilot-scale continuous counter-current reactor is being tested at NREL, which combines the properties of multi-stage percolation and the so-called shrinking bed (Lee et. al., 1999). This prototype reactor employs a vertical broken flight screw as a moving-bed mechanism. The upward motion of the solids allows gravity-driven bed compaction within the reactor, and the liquid is driven down the reactor by exerting a positive pressure above the reactor pressure to overcome the frictional forces and forces

exerted by the solids moving upward. A mathematical model has been developed for this type of reactor by Lee et al. (2000).

2.2.4 Percolation reactor

A percolation reactor is a packed-bed flow-through reactor operated in unsteady mode, where the product concentration decreases with time. A conceptual sketch of this reactor is given in Fig. 3.

There are certain advantages in a percolation reactor in comparison to a straight batch reactor or a PFR. First, the sugar product is removed as it is formed. This provides an important benefit in that it reduces sugar decomposition. Second, a packed-bed reactor can be operated with a low liquid/solid ratio. Therefore, a relatively high concentration of the sugar product can be obtained. Third, unless the feedstock is in the form of extremely fine particles, the liquid product is separated as it leaves the reactor. No solid-liquid separation is necessary, as it would be in a batch reactor or a PFR. This reactor is also relatively easy to operate since it does not involve the use of a moving-bed mechanism inside the reactor (Lee et al., 1999).

In an attempt to obtain further improvements in the sugar recovery and sugar concentration, the operation of a percolation reactor with step changes in temperature has also been proposed (Kim et al., 1993; Chen et al., 1996). In this scenario, the low temperature works primarily on the fast-reacting hemicellulose fraction, and the subsequent high-temperature step works primarily on the slow-reacting hemicellulose. Since optimum reaction conditions are applied separately for the two hemicellulose fractions, the decomposition of hemicellulose sugars is reduced and the yield is enhanced. A further variation on the operation of a percolation reactor is the use of a two-stage

reverse-flow scheme (Fig. 4) (Kim et al., 1994; Torget et al., 1996; Chen et al., 1996). This combines the advantages of a step change in temperature and a counter-current operation.

2.3 Pretreatment at high-solids condition

For a thermo-chemical pretreatment process to be easily performed, a significant amount of water must be introduced into the reactor. The reasons are as follows: (1) biomass is highly water-absorbing and the chemical agents used as catalysts (e.g. sulfuric acid) cannot enter the biomass unless the biomass is fully wetted; (2) the void volume between biomass particles should be filled with liquid or steam to facilitate heat transfer; and (3) excess water is required to create an easily-deliverable slurry in a continuous pretreatment reactor, or an agitable slurry in a batch reactor.

There is no clear definition of a high solids condition. Previous study on this topic has shown that a solid content of above 10%, depending on biomass species, can be considered to be a high-solids level. A high solids condition is critical for the process economics because this substantially reduces the reactor size, limits chemical use, and lowers overall costs due to reductions in heating energy and water usage (Hsu et al., 1996; Kadam and Hsu, 1997). For example, the National Renewable Energy Laboratory (NREL) employed a dry solids loading of 35% for their estimation of ethanol production cost because ethanol production costs increase prohibitively with lower solids loadings (Hinman et al., 1992; Hsu et al., 1996). In other studies, Grohmann et al. (1986(2)) used a solids loading of 40% for pretreatment of aspen wood and wheat straw in a small (1/2" o.d. × 4" long) pipe reactor with indirect heating. Schell et al. (1992) used a loading of 30% solids for pretreatment of corn stover using open trays in a cylindrical reactor with

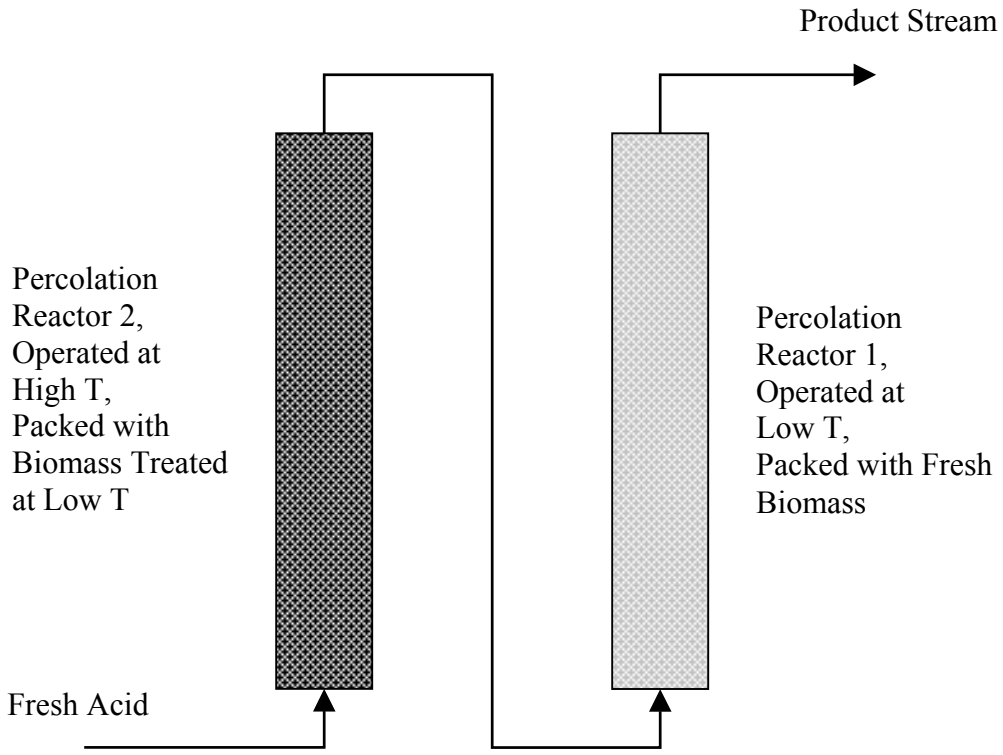


Fig. 4. Schematics of two-stage reverse-flow operation.

direct steam injection aided by indirect heating. Hsu et al. (1996) used a 100-L pilot-scale horizontal shaft mixer/reactor for dilute-acid pretreatment of lignocellulosic biomass at a solids loading of 10-15%, with heating either by steam injection into the reactor jacket or directly into the reactor. Shortly afterwards, a solids concentration of 40-50% using a 130-L reactor was reported by the same group (Kadam and Hsu, 1997). Recently, a pilot-scale process was developed with a reactor capacity of 1 t corn stover/d operated at 20 wt% solids concentration (Schell et al., 2003).

3. Enzymatic production of xylo-oligosaccharides

3.1 Background/Introduction

Xylo-oligosaccharides (XOs) are oligomers of xylose which are connected by a β-1,4-linkage. The low-DP XOs have shown to be able to promote the growth of beneficial bifidobacteria in the intestine (Suwa et al., 1999). It has also been reported that xylo-oligosaccharides ingestion can enhance calcium absorption (Toyoda et al., 1993). At the present time, the largest XOs manufacturer in the world is the Suntory Limited, Osaka, Japan, followed by several other producers in China.

XOs appear naturally in bamboo shoots, fruits, vegetables, milk and honey, but their production at an industrial scale is carried out through hydrolysis of xylan, which is one of the major components in a variety of lignocellulosic materials. The cleavage of β-linkages between xylose units in xylan results in a mixture of xylose, XOs and some heteropolysaccharides. To produce high-purity XOs products, the monosaccharides and high molecular mass carbohydrates must be removed from the XOs.

3.2 Enzymatic systems for xylan degradation

The preferred method for XOs production is enzymatic hydrolysis. Total biodegradation of xylan requires endo- and exo- β-1,4-xylanases , β-xylosidase, and several accessory enzymes, such as α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and β-coumaric acid esterase, which are necessary for hydrolyzing various substituted xylans. Table 4 lists the enzymes involved in the degradation of heteroarabinoxylan and their modes of action. The endo- and exo-xylanases attack the main chains of xylans, and β-xylosidase hydrolyzes XOs to xylose. The α-arabinofuranosidase and α-glucuronidase remove the arabinose and 4-O-methyl glucuronic acid substituents, respectively, from the xylan backbone. The esterases hydrolyze the ester linkages between the xylose units of the xylan and acetic acid (acetylxylan esterase) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase) and β-coumaric acid (β-coumaric acid esterase).

3.3 Production of xylo-oligosaccharides from lignocellulosic materials

Depending on the nature of the lignocellulosic material, polymers of xylose (xylan), arabinose (arabinan) or mannose (mannan) may account for a substantial portion of the hemicellulose, which can be substituted via ether or ester bonds (for example, with α-D- glucopyranosyl uronic acid or its 4-O-methyl derivative, acetyl groups and acids (Ebringerova and Heinze, 2000; Puls and Schuseil, 1993). Typical raw materials for XOs production are hardwoods, corn cobs, straws, bagasses, hulls, malt cakes and bran. Three different approachs have been used for XOs production from these feedstocks:

a. Enzyme treatments of native, xylan-containing lignocellulosic materils (LCM);

Table 4 Enzymes involved in the hydrolysis of complex heteroarabinoxylans (Saha and Bothast, 1999)

Enzyme	Mode of action
Endo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolyzes β -1,4-xylose linkages releasing xylobiose
β-Xylosidase	Releases xylose from xylobiose and short chain xylo-oligosaccharides
α -Arabinofuranosidase	Hydrolyzes terminal nonreducing a-arabinofuranose from arabinoxylans
α -Glucuronidase	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	Hydrolyzes feruloylester bonds in xylans
β-Coumaric acid esterase	Hydrolyzes β-coumaryl ester bonds in xylans

b. Chemical fractionation of a suitable LCM to isolate (or to solubilize) xylan, with further enzymatic hydrolysis of this polymer to XOs; and
c. Hydrolytic degradation of xylan to XOs by steam, water or dilute solutions of mineral acids.

The direct XOs production from xylan-containing LCMs must be carried out from a susceptible feedstock. For this purpose, XOs manufacture from the membranes of citrus fruit pulp by enzymatic methods has been reported by Takao and Yoshio (1996).

The production of XOs by combined chemical-enzymatic methods is shown in Fig. 5. Xylan (or soluble xylan fragments) can be obtained from LCMs by treatments with alkalis (for example, with solutions of NaOH, KOH, $Ca(OH)_2$, ammonia or a mixture of these compounds). The processing of xylan-containing LCM in alkaline media is favoured by the pH stability of this polymer, and the solubilized fraction can be recovered from liquors by further processing. In some cases, the raw material has been pretreated with oxidizing agents, salts or alcohols to remove lignin or pectic sustances. In case where the xylan has been solubilized in caustic liquors, precipitation with organic compounds (including acids, alcohols or ketones) allows the recovery of dissolved hemicelluloses and hemicellulose-degradation products. Once the xylan has been isolated or degraded to a soluble form, further DP reduction can be accomplished by hydrolysis with xylanases (Masayasu et al., 1993 (1), 1993 (2); Hiroyuki et al., 1995). For enzymatic production of XOs, enzyme complexes with low exo-xylanase and/or β- xyosidase activity are desired, in order to avoid the production of xylose. The enzymes can be directly added to the reaction media (Pellerin et al., 1991), immobilized (Suwa et al., 1999), or produced in situ by microorganisms (Cai et al., 1997). Using any of these

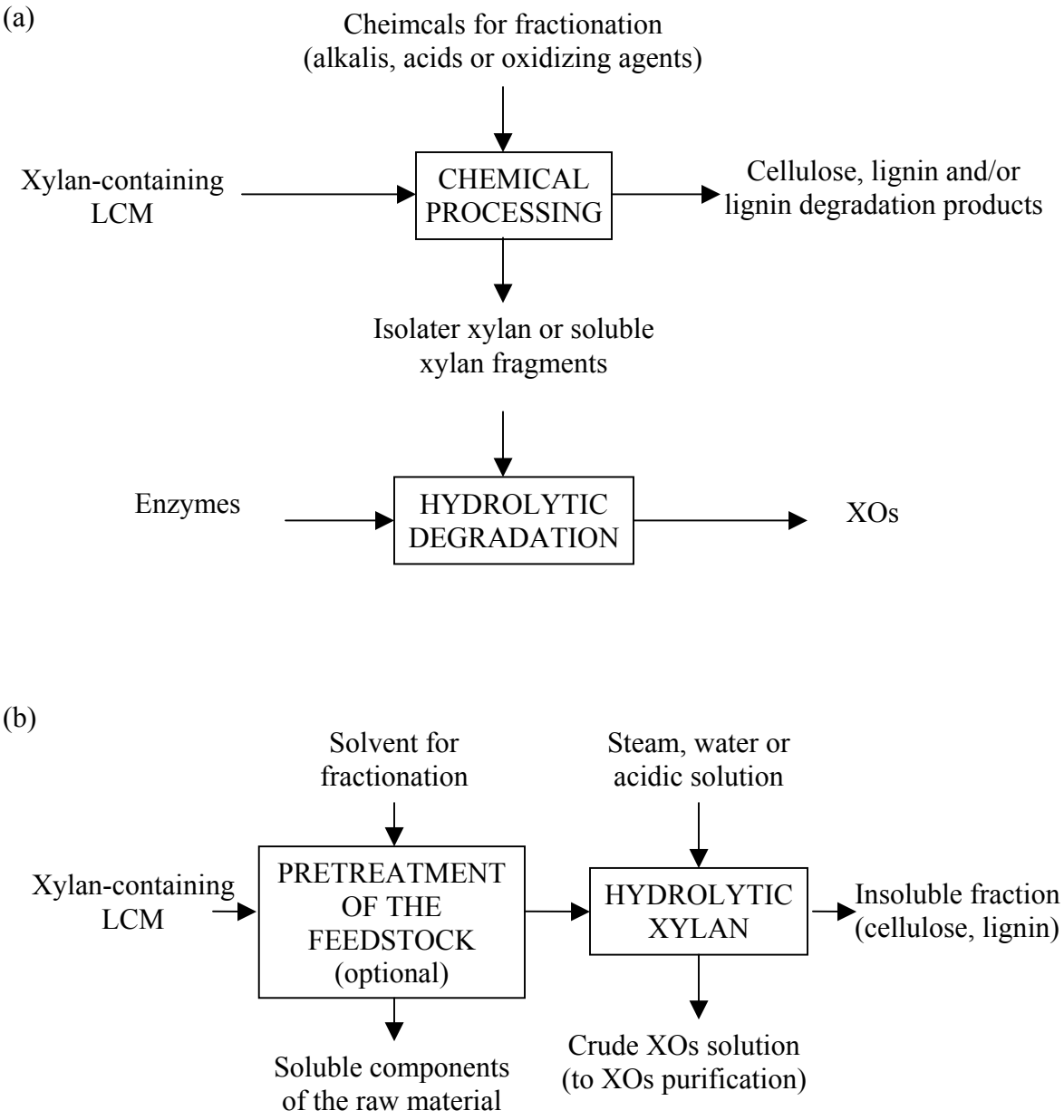


Fig. 5. Procedures for xylo-oligosaccharides manufacture (a) chemical-enzymatic methods; (b) hydronium-catalyzed process (Vazquez et al., 2000)

approaches, low-DP XOs can be produced. For food-related applications, the preferred DP range is 2–4 (Loo et al., 1999).

Alternatively, XOs can be produced from LCMs in a single step by reaction with steam or water through hydronium-catalysed degradation of xylan, according to the procedure known as autohydrolysis, hydrothermolysis or water prehydrolysis (see Fig. 5). In the first stages of the reaction, the catalytic species come from water autoionization, but side reactions (for example, cleavage of acetyl groups to give acetic acid) contribute to the generation of hydronium ions in the later reaction stages. The hydrolytic degradation of hemicelluloses is facilitated when an acid is externally added (acid prehydrolysis), but in this case the oligosaccharides behave as reaction intermediates and the main reaction products are monosaccharides. Besides the degradation of xylan, several side-processes occur in such treatments, including extractive removal, solubilization of acid-soluble lignin and neutralization of ash. All of these contribute to the presence of comparatively high concentrations of undesired, non-saccharide compounds in the liquors from hydrothermal processing. Because of this, the purification of XOs is of major importance.

3.4 Purification of crude xylo-oligosaccharides solutions

The purification of crude XOs solutions is a complex problem, particularly when they are produced by way of hydrothermal processing. A variety of strategies have been proposed for refining the crude liquors, in order to remove undesired compounds and/or to select XOs within a given DP range. Depending on the degree of purity desired, a sequence of several physicochemical treatments may be necessary (Schweiger, 1973; Sihtola, 1976).

Adsorption (using adsorbents such as activated charcoal, acid clay, bentonite, diatomaceous earth, aluminium hydroxide or oxide, titanium, silica and porous synthetic materials) has been widely used for the purification of XOs-containing liquors (Pellerin et al., 1991). In the first stage, the XOs are retained by the adsorbents, and the DP range of the fractions in the ethanol eluent depends on the alcohol concentration, allowing fractionation of the XOs on the basis of their molecular weight.

4. Lactic acid production

4.1 Introduction

Today, most of the commercial lactic acid was produced from fermentation of starch-derived glucose or sucrose. The fermentation of these sugars into lactic acid is well established in terms of both the microbiology and the process. To reduce the feedstock cost, considerable study has also focused on the fermentation of lignocellulosic carbohydrates for lactic acid production (McCaskey et al., 1994; Garde et al., 2002; Neureiter et al., 2004; Patel et al., 2004; Miura et al., 2004). This type of sugar source contains a wide range of hexoses and pentoses, and thus microorganisms capable of co-fermenting hexoses and pentoses are desired. In comparison to the fermentation of hexoses, fermentation of pentoses is difficult and the existing knowledge of the mechanism is comparatively scarce. Despite these deficiencies, considerable progress along these lines has still been made, especially through the application of advanced genetic technologies to develop new strains (Dien et al., 2002).

4.2 Microorganisms

Lactic acid bacteria are defined as gram-positive, non-motile, catalase-negative, non-sporulation, microaerophilic or anaerobic rods or cocci. They require carbohydrates as energy sources and produce lactic acid from glucose with a yield coefficient of more than 50% (w/w). The boundaries of the group have been subject to some controversy, but there has been general agreement that the genera *Lactobacillus, Leuconostoc, Pediococcus*, and *Streptococcus* form the core of the group (Salinen and Wright, 1993). The preferred species from the commercial point of view for the production of lactic acid belong to the genus *Lactobacillus*.

4.3 Metabolic pathways

Two important metabolic pathways are proposed for the fermentation of hexoses and pentoses in lactic acid bacteria (LAB): the Embden-Mayerhof-Parnas (EMP) pathway and the pentose phosphoketolase (PK) pathway. The two pathways are simplified on the left and right side of Fig. 6, respectively (Garde et al., 2002). Homofermentative LAB use the EMP pathway to assimilate hexose with a theoretical yield of two moles of lactate/one mole of glucose, while heterofermentative LAB use the PK pathway, generating a mixture of lactate, acetate, ethanol, and CO_2 from hexoses and pentoses. In practice, the yield of homolactic fermentation with glucose can easily reach above 0.9 g of lactate/g of glucose. The high yield represents an important incentive for the utilization of glucose as the feedstock for lactic acid production. An anaerobic condition is necessary for the LAB to use the EMP pathway. On the other hand, if oxygen exists or the LAB lacks the fructose-6-phosphate enzyme system, the assimilation of

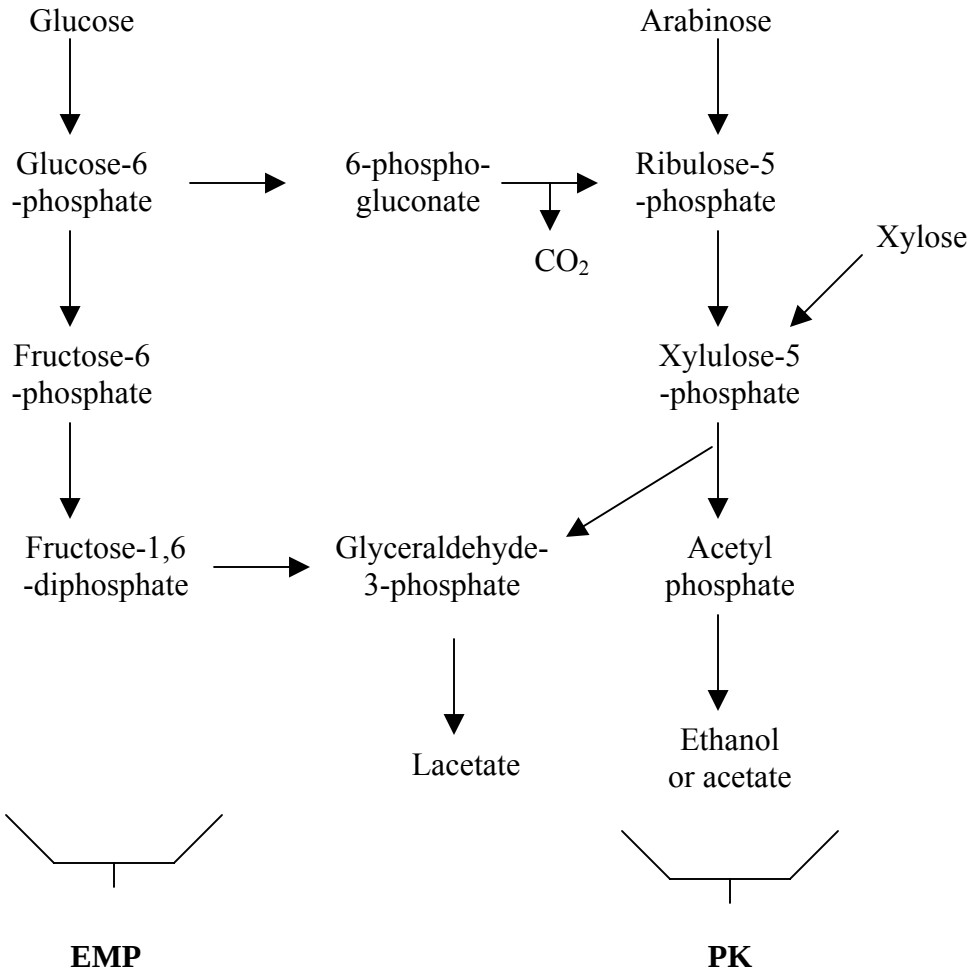


Fig. 6. Simplified illustration of the EMP and PK pathways for lactic acid fermentation (Garde et al., 2002)

hexose may shift to the PK pathway, resulting in a mixture of products with one mole of lactate/one mole of glucose.

According to the pathways in Fig. 6, the yield for pentose assimilation does not exceed 1 mole lactate /mol sugar (0.6g lactate/g xylose). However, some researchers have reported significantly higher values (Ishizaki et al., 1992, 1993; Iyer et al., 2000; Patel et al., 2004). For example, Iyer et al. (2000) reported a weight yield of 0.8 g lactic acid/g xylose, corresponding to a molar yield of 1.3, for the xylose fermentation using *Lactobacillus rhamnosus* ATCC 10863. This seems to indicate that the mechanism for pentose utilization by LAB is still unclear. As such, different pathways have been proposed to account for the high yield from xylose fermentation (Tanaka et al., 2002).

III. DILUTE ACID PRETREATMENT OF CORN STOVER USING A HIGH-SOLIDS PERCOLATION REACTOR

Abstract

Pretreatment of corn stover by dilute sulfuric acid was investigated using a laboratory percolation (flowthrough) reactor operated under high solids conditions. The effects of reaction conditions and operating parameters on the performance of percolation rector were investigated in order to identify the optimum range at which acceptable levels of yield and sugar concentration could be attained. It was demonstrated that 70-75% recovery of xylose and 6 –7% (w/w) xylose concentration were attainable. The high sugar concentration was obtained as a result of dense packing of dry corn stover and the low liquid throughput. Xylose was mostly unreacted, rather than decomposed. The glucan and the unreacted xylan in the treated corn stover were both effectively hydrolyzed by a "cellulase" enzyme preparation, which also exhibited some activity on xylan. The xylose yield was affected significantly by the flow rate under the same reaction time and conditions. This behavior appears to be related to sugar decomposition, mass transfer resistance and the fact that acid is neutralized by the buffering components of the biomass.

1. Introduction

Treatment of biomass by hot dilute sulfuric acid is a well-known pretreatment method. In this process, most of the hemicellulose and some of the lignin is removed. Since hemicellulose and lignin provide a shield to the cellulose fibers, removal of these substances increases the accessibility of cellulose to cellulase enzymes and, consequently, the rate and extent of enzymatic hydrolysis. Hemicellulose is a heteropolymer composed of a variety of sugar residues, such as glucose, xylose, galactose, arabinose and mannose. Since hemicellulose occupies approximately 20% of the total biomass, nearly half as much as the cellulose contribution, it is important to recover and utilize this portion of sugars.

A percolation reactor (packed-bed flowthrough type) was proven to work well for biomass pretreatment (Lee et al., 1978; Cahela et al., 1983; Kim and Lee, 1993; Chen et al., 1996). The unique feature of this reactor is that it permits concurrent discharge of sugar products during the reaction process, thereby suppresses sugar decomposition and improves the sugar yield. The percolation process also removes greater amounts of lignin than a batch process and can result in enhanced enzymatic digestibility of cellulose. In addition to the sugar yield and digestibility of cellulose, there is another important performance measure in pretreatment, i.e. the concentration of sugars produced in the process. A certain level must be attained for a pretreatment process to be economically feasible. The percolation reactor performs reasonably well in this regard, since it is a packed-bed reactor allowing a high solid/liquid ratio.

This research was focused on recovering hemicellulose sugar in high concentration and at the same time minimizing sugar decomposition. The xylose yield at

the pretreatment stage was not particularly concerned with because the unreacted hemicellulose can potentially be hydrolyzed enzymatically in the next step. Enzymatic hydrolysis of xylan is feasible because many cellulase preparations exhibit xylanase activity. The author explored the optimum reaction and operating conditions of the percolation reactor with these constraints in mind. It was also of interest to evaluate the treated corn stover for enzymatic digestibility of the glucan and the remaining xylan.

2. Experimental methods

2.1 Materials

Washed and dried corn stover was supplied by BioMass AgriProducts (Harlan, IA) and was knife-milled at NREL. It was screened and the fraction between 20-60 mesh was used as the feedstock for this work. It was stored at a temperature wherein the average moisture content was 6.6 % (w/w). The chemical composition of the corn stover was (w/w, dry basis): 36.8% glucan, 21.7 % xylan, 2.6 % arabinan, 0.68 % galactan, 0.3 % mannan, and 17.2 % lignin. The cellulase enzyme used for the digestibility was Spezyme CP (Genencor, Lot No. 301-00348-257). It was supplemented with β-glucosidase (Novozyme 188) to break down the cellobiose to glucose.

2.2 Analytical methods

The liquid samples were analyzed for sugars by HPLC operated with Bio-Rad Aminex HPX-87P column and a refractive index detector (Shodex, Model-71). The sugar contents in solids and the amounts of total sugars (monomers+oligomers) in the hydrolyzate were measured using the NREL standard analytical procedures No. 002 and 014, respectively (NREL, 1996). The sugars in the hydrolyzate contain high amount of

oligomers. The hydrolyzates were put through secondary hydrolysis (4% H_2SO_4, 121^oC, 1 hr) to determine the oligomer content. The enzymatic digestibility of the treated biomass was measured by the NREL standard analytical procedure No. 009 (NREL, 1996). The enzymatic digestibility test was conducted at 50^oC in a laboratory shaking incubator (150rpm) with working volume of 50mL in a 250-mL Erlenmeyer flask. The washed solid containing 0.5 g of glucan was added to the flask to attain 1% (w/v) of glucan. Two levels of enzyme dosage were applied: 15 FPU/g - glucan and 60 FPU/g - glucan of Spezyme CP supplemented with 30 CBU of Novozyme 188/g - glucan.

2.3 Experimental setup and operation

The percolation reactor system is described in Fig. 7. The reactor was constructed of Monel tubing (2.29cm ID x 9cm Length). Hastelloy C-276 tubing (1/8 in.) was used for connections and for the preheating coil. The reactor system had a provision to apply back pressure to 300 psig with N_2 gas. In the percolation reactor operation, 10.7 g of biomass (containing 10 g of dry solids) was packed into the tubular reactor with an internal volume of 37 cm^3. Under typical operating conditions, the reactor void fraction was 75% (v/v), thus the reactor was operated with 25% (v/v) solid concentration. The reactor and preheating coil were placed in a forced convection oven for temperature control. The reactor system was preheated to a desired temperature, at which point the dilute acid was pumped into the reactor through a preheating coil by a piston pump (SSI, Accuflow-Series III). The reaction temperature was monitored by a thermocouple inserted into the center of the reactor bed and at the end outlet section of the reactor. The reactor effluent (hydrolyzate) was quenched by a cooling heat exchanger connected immediately after the reactor.

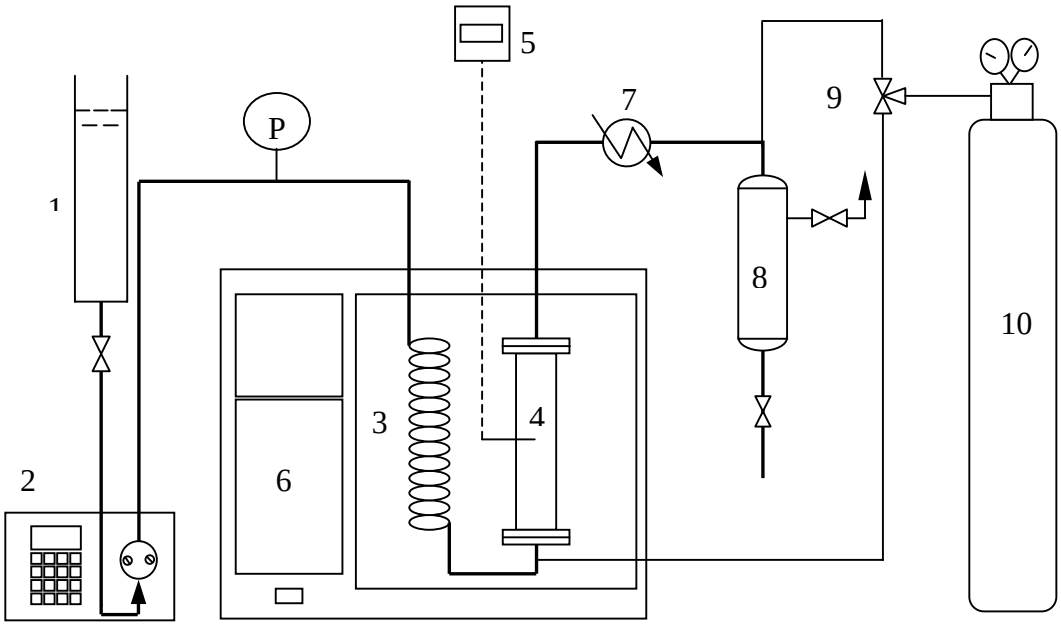


Fig. 7. Laboratory setup for percolation reactor system: 1, acid tank; 2, HPLC pump; 3, preheating coil; 4, percolation reactor; 5, thermometer; 6, GC oven; 7, heat exchanger; 8, product collecting tank; 9, three-way valve; 10, Nitrogen gas.

3. Results and discussion

3.1 Percolation reactor behavior

Fig. 8 shows the time courses of xylose production from the percolation reactor with variation of acid level. The amount of xylose expressed in this figure and later in this chapter should be interpreted as the total amount of xylose plus oligomers. The acid flow rate was 10 mL/min, corresponding to a liquid throughput of 1.0 mL /(gram of dry feedstock · min). The time zero was defined as the point when the first drop of liquid came out of the reactor. Since the reactor contained dry biomass, the reaction time represents that of the particles at the reactor exit point. The particles at the entrance of the reactor would have longer reaction time. The xylose production curves are similar in shape for all acid concentrations, with the xylose yield rapidly increasing early in the reaction and then leveling off thereafter. The effluent contained sugars both in monomeric and oligomeric forms, the latter accounting for 40-80% of the total. For all acid levels, the maximum xylose yields surpassed 90%. At the 4 min point, the yield of xylose increased from below 55% to above 86% as the acid level increased from 0.2% to 1%. It was found that the pH of the liquid coming out of the reactor was in the range of 2-3.5 for reaction times less than 2.5 minutes. This indicates that a large fraction of the acid was neutralized in the reactor due to the buffering components present in corn stover. This unusual situation is related to the high-solids condition applied in this work.

In theory, the first drop coming out of the reactor should have the highest concentration (Cahela et al., 1983), decreasing gradually as the reaction progresses. With longer reaction time, the overall cumulative sugar yield increases while the sugar product

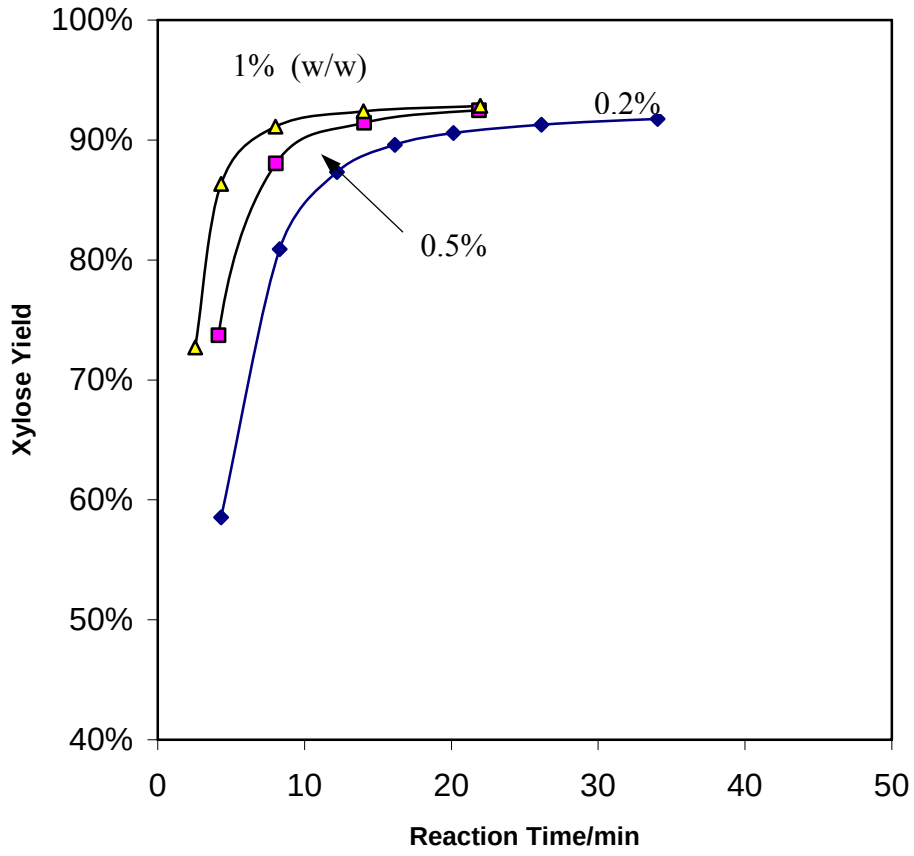


Fig. 8. Time course of xylose production at different acid concentrations: 180°C, acid flow rate of 10mL/min.

is diluted. The yield and sugar concentration, therefore, have an inverse relationship in a percolation reactor, as indicated in Fig. 9.

Figs. 10 and 11 present the effects of temperature on xylose production. Temperature shows a distinct positive effect on yield and concentration within the range of 160-180^{o}C. The effects of temperature and acid concentration are consistent with the previous kinetic models, in which the hydrolysis rate constant follows an Arrhenius relationship for temperature and increases proportionally with acid concentration raised to a certain exponent (Bhandari et al., 1984).

3.2 Mass balance of xylan

An interesting feature in Fig. 9 is the run in which the yield of xylose was 73% and the concentration 66 g/L (or 6.6 wt%). This occurred with 1% (w/w) acid, 180^{o}C, and 2.5 minutes of reaction time (recovery of 25 ml total fluid). Of the 27% unrecovered xylan, 9% was left unreacted, and 9% was left in the liquid trapped inside the reactor. The effective yield was then actually 82%, as the sugars trapped in the retained liquid were available for conversion in subsequent process steps. Only 7% of the xylan was unaccounted for, most likely due to decomposition. Most of the xylan decomposition was found to take place during the preheating stage, which was done under 300-psi nitrogen backpressure. It appears that the moisture content in the biomass under high temperature and pressure caused autohydrolysis and subsequent decomposition. The measured decomposition of xylan during preheating (180^{o}C, 25 minutes) was 4.8%, which accounts for the majority of the decomposition in the entire process. Some preliminary experiments have shown that the decomposition during preheating period could be reduced to almost zero when performed under atmospheric pressure. From this

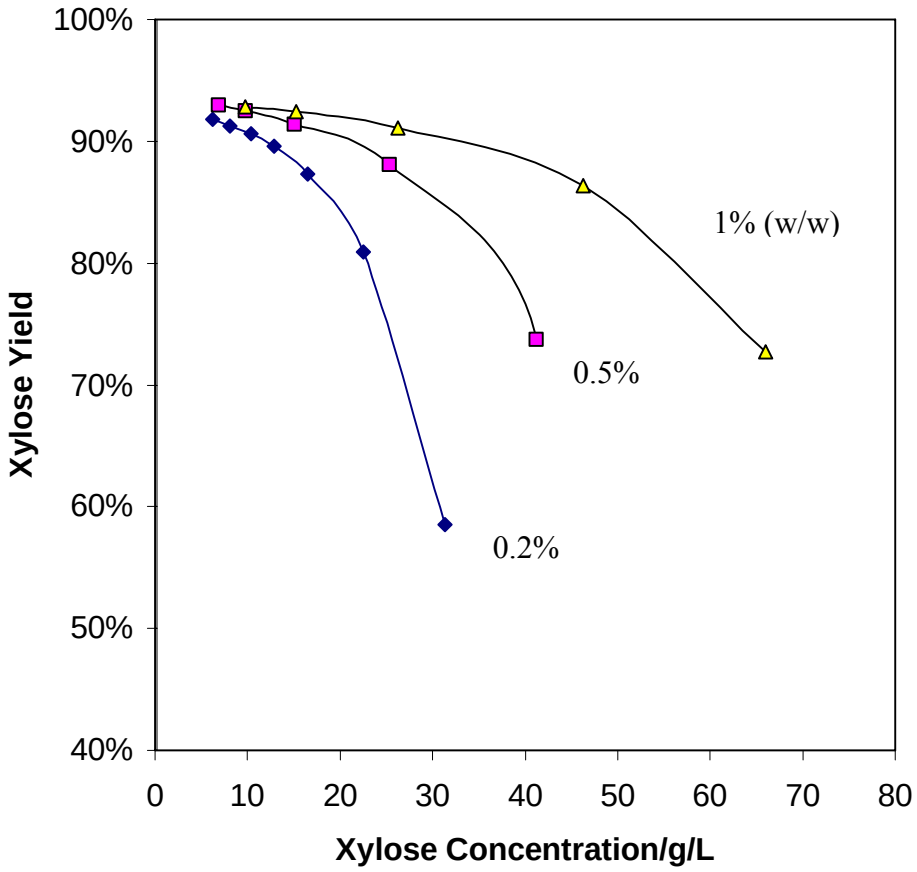


Fig. 9. Relationship between xylose yield and xylose concentration at different acid levels: 180^{o}C, acid flow rate of 10mL/min.

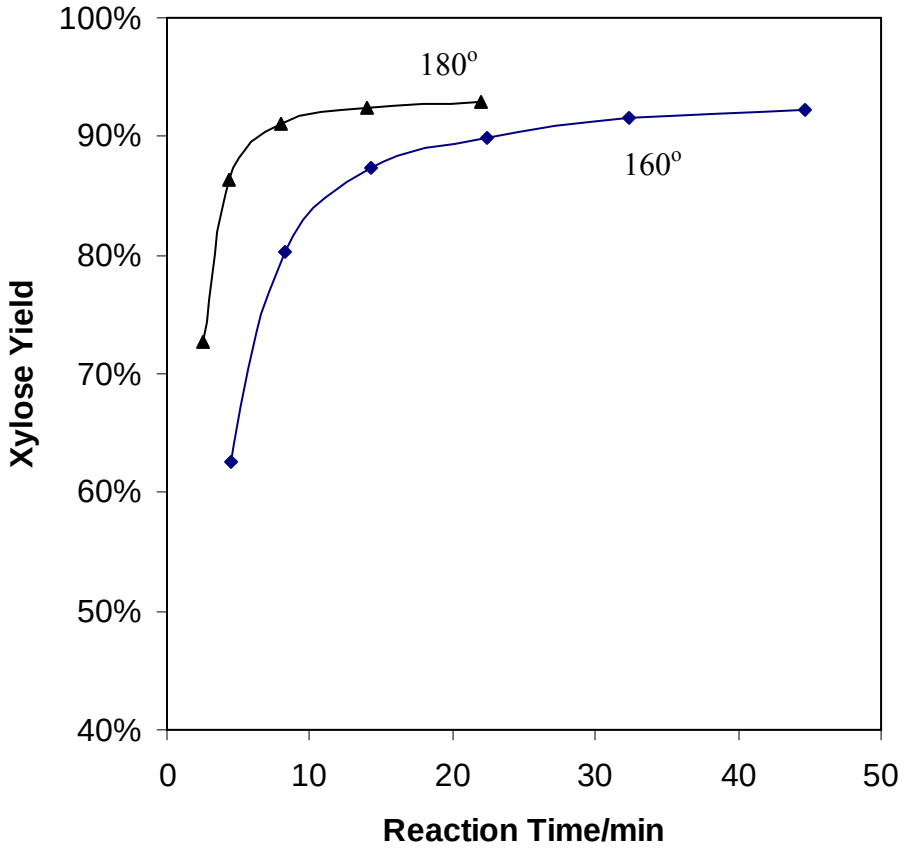


Fig. 10. Time courses of xylose production at 160 °C and 180°C: 1 wt% acid concentration, acid flow rate of 10mL/min.

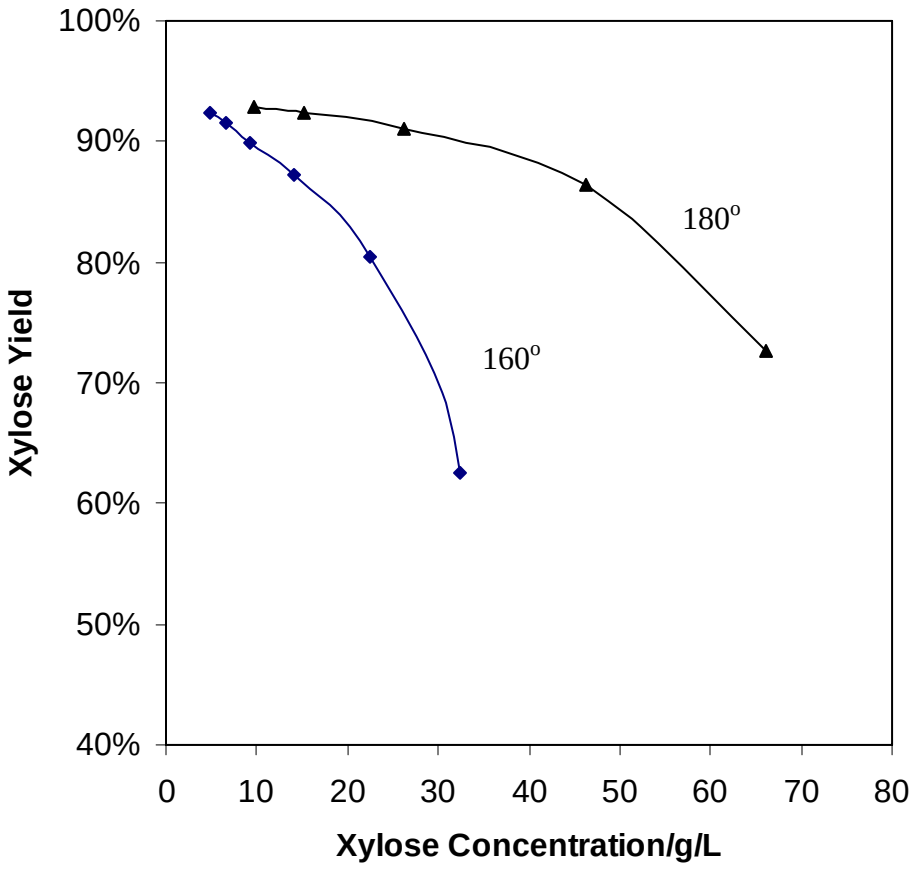


Fig. 11. Relationship between xylose yield and xylose concentration at 160°C and 180°C: 1% (w/w) acid, acid flow rate of 10mL/min.

observation, it is contended that when properly operated, dilute-acid percolation pretreatment can be carried out with negligible xylan decomposition.

One may consider further reducing liquid input to obtain even higher sugar concentration, which will then result in less xylose recovery. Assessment of the data suggests that it is possible to attain a 50% yield of xylose with 10% sugar concentration, which is close to the level where it can be used as a fermentation substrate without further concentration. The low yield of sugar during the pretreatment stage is not a detriment in this case because the balance of xylan mostly stays intact and unreacted, rather than being decomposed. The unreacted xylan can then be hydrolyzed enzymatically by the xylanase normally embodied in "cellulase". This may bring the overall xylose yield for the combined pretreatment and enzymatic process to a level economically feasible. It is also noteworthy that the enzymatically-produced sugar is non-toxic to microorganisms. The xylose trapped in the reactor after each run can be recycled into subsequent runs to retain a high sugar concentration. In some process scenarios, the entrained xylose-rich liquid can remain in slurry with the pretreated solids for use in a simultaneous saccharification and co-fermentation, allowing for fermentation of both xylose and enzymatically-released glucose using a single co-fermenting microorganism. It is also technically feasible to operate the percolation reactor such that no residual liquid remains in the reactor at the end of the run. This can be done by introducing the desired amount of liquid by pumping, and pushing it through the reactor with hot air. This would further reduce liquid input and increase the sugar concentration. This is similar to a new process concept that is currently being tested at NREL, namely high-solids pretreatment by Pneuma-Press® filtering operation (Elander, 2003).

Sugars other than xylose (glucose, arabinose, galactose and mannose) were also generated during the acid hydrolysis of corn stover hemicellulose. However, the glucose yield was much lower than that of xylose. As a reference point, in the run where 73% of xylose was recovered, with a concentration of 6.6% (w/w), the glucose yield was only 4.5%. The glucose produced in the pretreatment is believed to originate from the hemicellulose and amorphous cellulose.

3.3 Effect of flow rate on percolation reactor performance

The effects of acid flow rate on xylose yield and concentration in the percolation reactor are shown in Figs. 12 and 13. The temperature and acid concentration were fixed at 180^{o}C and 1% (w/w), respectively, for these runs. The acid flow rate showed significant positive effects for both concentration and yield over the range of 5-10 mL/min. The maximum xylose yields observed in the experiments were 83.5% and 92.9% for acid flows of 5mL/min and 10mL/min, respectively. The increase in yield was insignificant above a flow rate of 10 mL/min. The hydrolysis rate was also improved by an increase in acid flow rate, as indicated by the different slopes of the curves in Fig. 12. In the early phase of the reaction, taking the 3 min point as an example, 44% of the xylose yield was attained with a 5mL/min flow rate. This rose rapidly to 65 with a 10mL/min flow rate. No significant increase in the hydrolysis rate was observed for the flow rates above 10mL/min.

Fig. 13 presents the relationship between xylose yield and concentration at various acid flow rates. Again, the inverse relationship between the yield and the sugar concentration was displayed. The experimental run discussed earlier was also included:

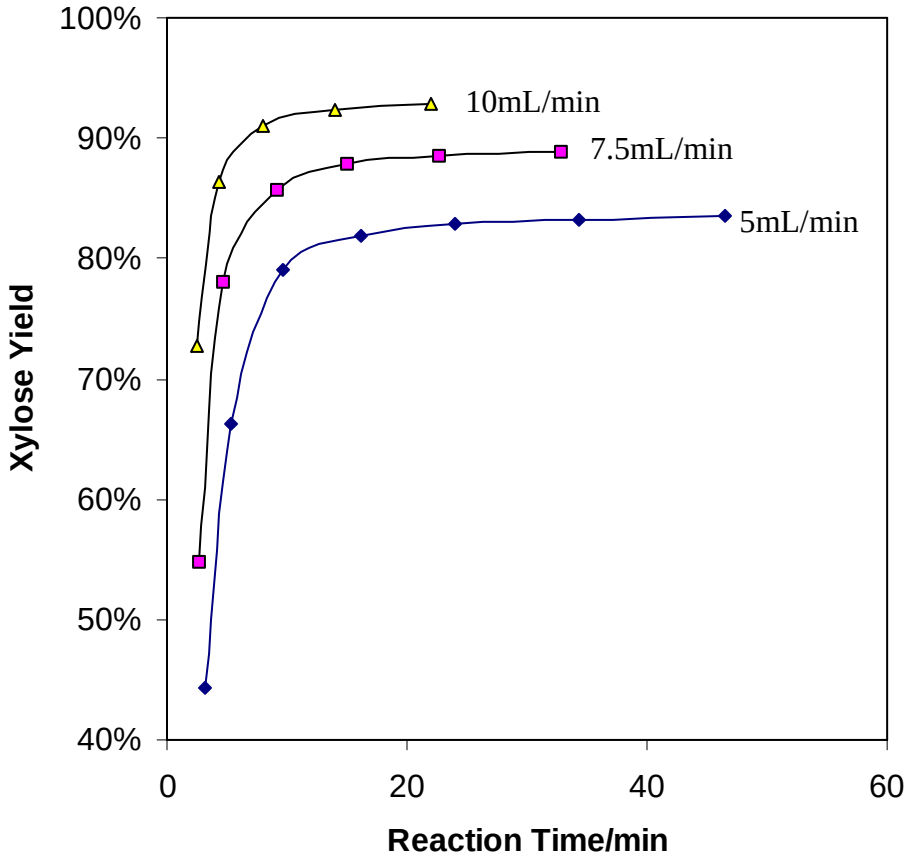


Fig. 12. Time courses of xylose production with various acid flow rates: 180^{o}C, 1 % (w/w) acid concentration.

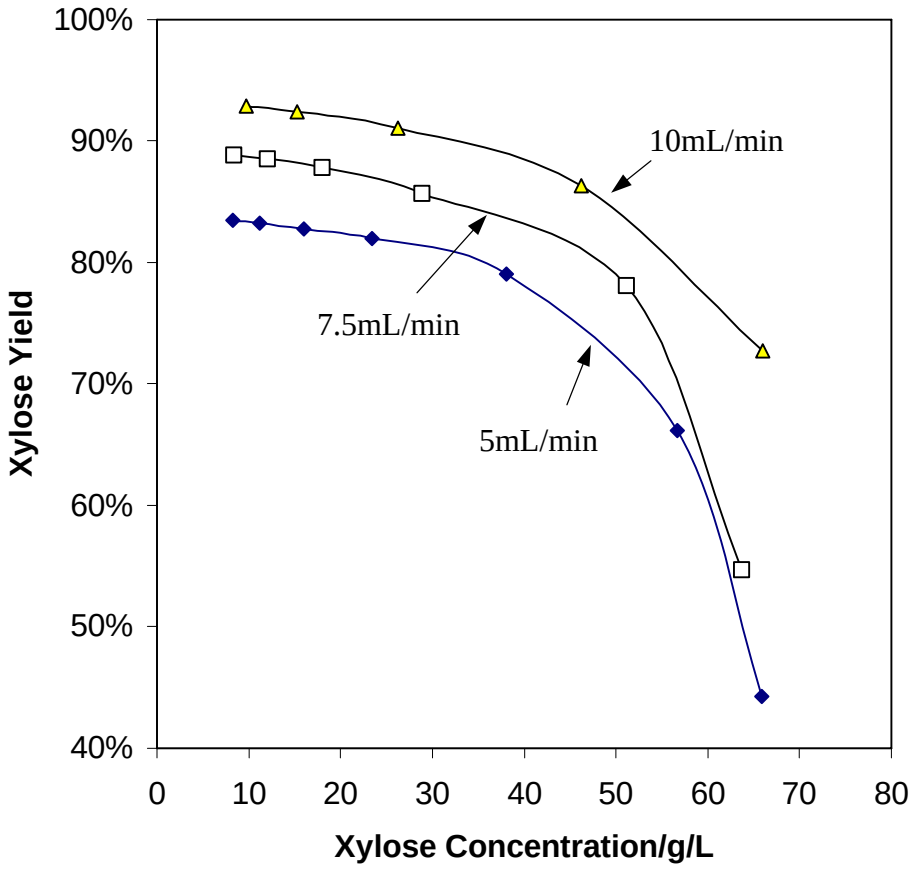


Fig. 13. Relationship between xylose yield and xylose concentration with various acid flow rates: 180^{o}C, 1% (w/w) acid concentration.

the highest xylose concentration (6.6%) was obtained at 10ml/min flow rate with 73% of xylose yield.

The rate of the hydrolysis reaction is dependent only on temperature and acid concentration. However, the extent of reaction is also dependent upon reaction time. From this viewpoint, the xylose yield profiles vs. reaction time should be constant for all flow rates, but obviously they are not (Fig. 12). One of the primary reasons for this behavior is the decomposition of sugars. The hydrolysis reaction occurs only in the solid phase whereas the decomposition reaction occurs only in the liquid phase. Therefore, the hydrolysis is controlled by clock time, but the decomposition is controlled by the residence time. Lower flow rates allow longer residence times, thus induces higher decomposition and lower xylose yields.

There are other factors that may also play roles in how the flow rate affecting the reactor performance. Liu and Wyman (2003) found that higher flow rates induce faster xylose (and lignin) removal in a percolation reactor, which is not explainable by traditional kinetics. They postulated that there might be a mass transfer effect in the reactor. This may partly explain the experimental results in this work due to the relatively low flow rates applied. The presence of buffering components in corn stover may also interfere with the reactor performance. It has been reported that corn stover has the neutralizing capacity of 43.7mg H_2SO_4/g dry substrate (Esteghlalian et al., 1997). As indicated earlier, the pH of the reactor effluents indeed rose to 2 to 3.5 for all the samples taken before 2.5 minutes, a drastic change of pH from the input pH of close to 1.0. This would add significantly to the effect of flow rate on reactor performance since the overall pH in the reactor is influenced by the flow rate: a low flow rate will induce a high pH

because a higher percentage of acid is consumed. It is believed that the strong influence of flow rate on the yield and concentration of the percolation reactor observed in this work is due to the combined effects of neutralization of acid by the buffering components, sugar decomposition and mass transfer.

3.4 Enzymatic digestibility of treated corn stover

To assess the efficiency of acid pretreatment with the percolation process, the standard enzymatic hydrolysis test was conducted for pretreated corn stover. The sample was taken from the run under the conditions of 1% (w/w) acid, 180^{o}C, 10mL/min flow rate, and 3 minutes. The reaction was stopped by nitrogen-gas-through quenching. The pretreated solids had the composition (w/w, dry basis) of 63.4% glucan, 2.4% xylan and 25.0 % Klason lignin.

Fig. 14 shows the enzymatic digestibility test profiles of the glucose for the pretreated corn stover. Untreated corn stover and α-cellulose were used as the control and reference substrates, respectively. The 96 h glucan conversion of untreated corn stover was only 28% and 23% for cellulase loadings of 60 and 15FPU/g-glucan, respectively. With the pretreated corn stover, much higher glucan conversions were observed after 96 h at both levels of enzyme loadings. It is evident that the high-solids dilute-acid percolation process is a very effective pretreatment method. The efficacy of the pretreatment can be further highlightened by the comparison of the profiles of treated corn stover with those of α-cellulose (Sigma, Lot No. 11K0246). Though quantitative glucan digestibility was also found with α-cellulose after 96 h, the initial glucan hydrolysis was substantially slower that that of pretreated corn stover. It is interesting to

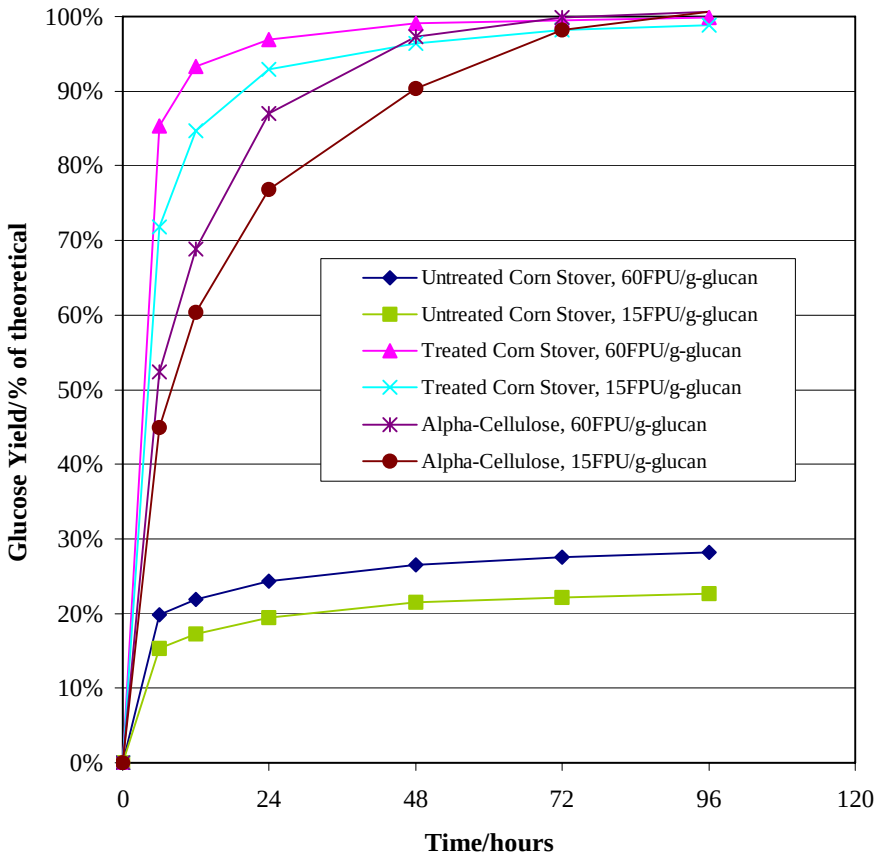


Fig. 14. Enzymatic digestibility of pretreated corn stover: pretreated with 1% (w/w) acid at 180^{o}C and with flow rate of 10mL/min, for 3 minutes, followed by nitrogen-gas-through quenching.

note that the pretreated substrate contained a high level of lignin (25% Klason lignin). Although lignin is regarded as one of the main factors controlling the enzymatic digestion, it appears that low-lignin in the substrate is not a necessary condition to achieve high-level digestibility in the case of dilute-acid pretreatment.

Fig. 14 also shows that most of the glucose from the pretreated corn stover was formed before 12 hours. Higher enzyme loading gave substantially faster hydrolysis in the early phase, as indicated by the significantly different glucose yields at 6 hours. The difference gradually diminished with time, giving little effect on the terminal glucan digestibility. It is also notable that the residual xylan, although only 2.4% after pretreatment, was hydrolyzed by the cellulase enzyme (data not shown). At 96 hours, 77% and 83% of xylan were hydrolyzed with 15 and 60 FPU/g-glucan of enzyme loadings, respectively.

4. Conclusions

Pretreatment of corn stover with dilute acid was investigated using a laboratory scale percolation reactor. The performance of the reactor was assessed in regard to recovery of hemicellulose sugars over a broad range of reaction and operating conditions. The results demonstrated that the percolation reactor could produce xylose with a 73% yield and 6.6% (w/w) concentration. Less than 10% of the total xylan was decomposed during this process. Retention of high sugar concentration was possible because the reactor could be operated with a low amount of liquid throughput (one reactor void volume, for example). The high solid-to-liquid ratio caused significant changes in the pH in the reactor due to neutralization by the buffering components of corn stover. This also amplified the effect of flow rate on the reactor performance. An enzymatic digestibility

test was performed for the treated corn stover. Quantitative conversion of glucan was observed with both 60 and 15FPU/g-glucan of enzyme loadings. The same test showed concurrent hydrolysis of the remaining xylan, with a resulting digestibility in the vicinity of 80%.

IV. OPTIMIZATION OF DILUTE ACID PRETREATMENT OF CORN STOVER USING A HIGH-SOLIDS PERCOLATION REACTOR

Abstract

In the previous chapter, it has been demonstrated that pretreatment of corn stover with dilute sulfuric acid can achieve high digestibility and efficient recovery of hemicellulose sugars with high yield and concentration. Further improvement of this process was sought in this work. A modification was made in the operation of the percolation reactor such that the reactor was preheated under atmospheric pressure to remove moisture that causes autohydrolysis. This eliminated sugar decomposition during the preheating stage and led to a considerable improvement in overall sugar yield. In addition, liquid throughput was minimized to the extent that that only one reactor void volume of liquid was collected. This was done to attain a high xylose concentration in the hydrolyzate. Optimum reaction and operating conditions were identified wherein near quantitative enzymatic digestibility was obtained with enzyme loading of 15FPU/g-glucan. With a reduced enzyme loading of 5 FPU/g-glucan, the enzymatic digestibility was decreased, but still reached a level of 92%. Decomposition of carbohydrates was extremely low, as indicated by the measured glucan and xylan mass closures (recovered sugar plus unreacted) which were 98% and 94%, respectively. The data obtained in this work indicate that the digestibility is related to the extent of xylan removal.

1. Introduction

In biomass conversion processes, the feedstock and enzyme have been identified as two primary cost factors (Nguyen and Saddler, 1991). For a pretreatment to be economically feasible, efficient sugar recovery and high enzymatic digestibility (especially at low enzyme loadings) must be attained. It is particularly important to minimize sugar decomposition in the case of dilute acid pretreatment. This not only improves the sugar yield but also has a beneficial effect on the subsequent bioconversion process by reduction of toxic components. It is well known that sugar decomposition generates various toxins, including hydroxymethyfurfural (HMF), furfural (Shah et al., 1984), and furans. The previous chapter of this dissertation has demonstrated that acid pretreatment of corn stover using a percolation reactor can provide high enzymatic digestibility, as well as efficient recovery of hemicellulose sugars. The primary objective of this study is to seek further improvement of this process. Improvements were sought from two different angles. One was to give further insight into the process over a broader range of reaction conditions. The other was to adjust the reactor operating conditions. In both cases, the focus of the improvement was on minimizing sugar decomposition, yet with an important constraint that a high glucan digestibility and acceptable levels of sugar concentration and yield in the pretreatment liquor are assured.

2. Experimental methods

2.1 Materials

Washed and air-dried corn stover was supplied by BioMass AgriProducts (Harlan, IA) and was knife-milled at NREL (National Renewable Energy Laboratory). It was

screened and the fraction between 2mm-20 mesh was used as the feedstock for this work. It was stored at refrigeration temperature, wherein the moisture content varied from 9% to 14% (w/w). The chemical composition (w/w, dry basis) of the corn stover was: 36.8% glucan, 21.7 % xylan, 2.6 % arabinan, 0.68 % galactan, 0.3 % mannan, and 17.2 % lignin. Alpha-cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246). The cellulase enzyme (Spezyme CP, Lot No. 301-00348-257) was provided by Genencor, Inc. through NREL. The average activity was 31.2 filter paper units (FPU) /mL. The β-glucosidase (Novozyme 188, Lot No 11K1088) was purchased from Sigma at an activity of 750 cellobiase units (CBU) /mL.

2.2 Analysis

The liquid samples were analyzed for sugars by HPLC equipped with Bio-Rad Aminex HPX-87P column and a refractive index detector (Shodex, Model-71). The total sugars (monomers+oligomers) in the hydrolyzate and the sugar contents in solid were measured following the procedures of NREL LAP No. 014 and 002 (NREL, 1996), respectively. The hydrolyzate liquors obtained contained significant amounts of oligomers and were subjected to secondary hydrolysis (4% H_2SO_4, 121^oC, 1 hr) to determine their oligomer content. The Klason lignin content in the solid was measured following the procedure of NREL LAP No. 003 (NREL, 1996).

2.3 Experimental setup and operation

The percolation reactor system was as described in the previous chapter of this dissertation. The operation of the reactor was initiated with the reactor preheating. After an initial series of experiments, it became apparent that the moisture retained in the

biomass was promoting autohydrolysis, consequently resulting considerable sugar degradation. Therefore, in the present experiments, the reactor was preheated under atmospheric pressure with the vent valve of the collection tank open. After 25 minutes of preheating, the reactor system reached the desired temperature (160-180 ^{o}C) and the biomass feedstock became totally moisture-free. The reactor was then immediately pressurized to 260 psi, at which point acid was pumped into the reactor (1"ID × 10"L) at a flow rate of 10 mL/min. The pretreatment was carried out until one reactor void volume (27 mL) of liquid had passed through the reactor. The total time of reactor operation was 5.7 minutes and the mean reaction time for the entire solids was estimated to be 4.4 minutes. Upon completion of the pretreatment reaction, the pump was stopped and the remaining reactor liquid was flushed out with nitrogen with concurrent cooling of the oven. Nitrogen was passed through the reactor by release of the reactor outlet pressure, enabling rapid quenching of reactor to occur. Liquid samples were collected at three different points: (1) the reactor effluent, which contained most of the hemicellulose sugars; (2) nitrogen-flush-out liquid (NFOL), which filled the reactor void volume, and was less concentrated than the effluent but contained considerable amount of sugars; and (3) liquid trapped in the solids (LTS), which stayed in the pores of the corn stover and was not flushed out by nitrogen. The sugars in the LTS were extracted with water in order to determine its constituents.

2.4 Enzymatic hydrolysis

The enzymatic digestibility of the treated biomass was measured by the procedure of NREL LAP No. 009 (NREL, 1996). The enzymatic digestibility test was conducted at 50^{o}C in a laboratory shaking incubator (150rpm) with working volume of 50mL in 250-

mL Erlenmeyer flasks. Washed solids containing 0.5 g of glucan were added to the flask to attain 1% (w/v) of glucan. Spezyme CP (cellulase) was supplemented with 30 CBU (cellobiase units) /g-glucan of Novozyme 188 (cellobiase) for the digestibility test. α-cellulose was used as the reference substrate.

3. Results and discussion

3.1 Recovery of hemicellulose sugars

A percolation (flow-through) reactor works well for the recovery of sugar since the sugar products in the reactor effluent are removed from the reactor as soon as they are produced, thereby minimizing decomposition (Lee et al., 1999). Table 5 presents the sugar recovery yields and mass closures. Taking the run with 1wt% acid and 170^{o}C as an example, 8.4% of xylan retained in solids after pretreatment, and hence 91.6% of xylan was removed, of which 85.6% was collected as xylose and xylo-oligomers, and only 6% was lost by decomposition. Xylan mass closures from these runs were all above 93% except for the two with 0.5wt% and 1wt% acids at 180^{o}C, in which the xylan mass closures were 89.8% and 85.2%, respectively. The glucan mass closures were consistently high throughout, with no more than 2% of glucose decomposition over the entire range of reaction conditions. Since only one reactor void volume of liquid was collected from the reactor, the sugars present in effluents were obtained in concentrated form. In the runs made with 1wt% acid, 69-75% of the total xylan was obtained in the effluents, which retained 6wt% of xylose concentration. If all the liquids (effluent plus NFOL and LTS) were recovered, the overall xylose yield would increase to 86%, but the concentration would decrease to 3wt%.

Table 5 Sugar distribution in pretreatments and mass balance closure [a]

Pretreatment conditions[b]		Yield in effluent		Yield in NFOL[c]		Yield in LTS[d]		Remaining in solids		Mass balance closure	
Acid conc. /wt%	Temperature /°C	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucan	Xylan	Glucan	Xylan
0.2	160	2.7%	20.6%	2.0%	27.3%	0.4%	7.5%	96.1%	40.4%	101.2%	95.8%
	170	3.3%	29.8%	2.1%	29.4%	0.4%	6.8%	93.2%	29.1%	99.0%	95.0%
	180	3.9%	37.4%	1.6%	24.8%	0.4%	5.3%	93.8%	26.5%	99.7%	94.1%
0.5	160	4.5%	49.3%	2.0%	25.8%	0.4%	4.3%	92.2%	14.8%	99.2%	94.1%
	170	5.8%	62.5%	1.9%	18.1%	0.8%	4.2%	89.1%	8.5%	97.5%	93.3%
	180	5.1%	54.9%	1.8%	19.9%	0.6%	3.7%	90.7%	11.3%	98.4%	89.8%
1.0	160	6.0%	69.1%	1.8%	16.0%	0.6%	2.3%	92.3%	8.6%	100.7%	96.0%
	170	6.3%	73.3%	1.2%	10.1%	0.5%	2.2%	90.7%	8.4%	98.7%	94.0%
	180	6.5%	66.9%	1.8%	10.9%	1.4%	1.9%	88.4%	5.6%	98.0%	85.2%

[a] Yields are expressed as the total of sugar monomers and oligomers.

[b] Acid flow rate = 10ml/min. Reaction time = 4.4 minutes

[c] NFOL = nitrogen flush out liquid

[d] LTS = liquid trapped in solid

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3.2 Enzymatic digestibility

Pretreatment of corn stover by dilute acid has been investigated by a number of different researchers. Knappert et al. (1980) carried out dilute acid pretreatment of corn stover in a continuous flow reactor with a solid concentration of 5%, applying a fixed residence time of 0.22min. Quantitative glucan digestibility was achieved after 48 hours for a substrate treated with 0.9wt% acid and 216^{o}C, using an enzyme loading of 26FPU/g-glucan (calculated from the table data). Um et al. (2003) pretreated corn stover in a batch reactor operated with 2% (w/v) sulfuric acid at 121^{o}C for 120 min. They reported 80% digestibility with 40FPU/g-glucan enzyme loading. However, the most intense research along this line has been conducted at the National Renewable Energy Laboratory (NREL). In the early stages of their research, a continuous bench-scale cylindrical reactor operated at high solid concentration (20-30 wt%) was used for corn stover pretreatment. With an enzyme loading of 40IU/g solids (or 80.3 IU/g-glucan), the five-day enzymatic digestibility was reported to be 96% for a substrate treated with 1wt% sulfuric acid at180^{o}C for 10 minutes. Later, a pilot-scale process was developed with a reactor capacity of 1 t corn stover/d operated at 20wt% solids concentration (Schell et al., 1992). Over a range of conditions (165-195 ℃, 0.5-1.4 wt % acid, and 3-12 min), the highest cellulose conversion yields (digestibility) were 80-87%. Recently, Schell et al. (2004) reported that cellulose digestibility of 96% was also achievable for corn stover pretreated with the same reactor system under optimized reaction conditions.

In the present work, the enzymatic digestion of corn stover was carried out at a comparatively low enzyme level (no higher than 15FPU/g-glucan). The enzymatic digestibilities of the glucan in the untreated and treated corn stover are listed in Table 6.

Table 6 Summary of sugar recovery and 96-h enzymatic digestibility of treated corn stover[a]

Pretreatment conditions[b]		Accountability		96-h Enzymatic digestibility of glucan
Acid concentration /wt%	Temperature /^{o}C	Glucan	Xylan	
0.2	160	101.2%	95.8%	64.6%
	170	99.0%	95.0%	61.8%
	180	99.7%	94.1%	78.9%
0.5	160	99.2%	94.1%	85.3%
	170	97.5%	93.3%	90.6%
	180	98.4%	89.8%	93.4%
1.0	160	100.7%	96.0%	93.4%
	170	98.7%	94.0%	99.1±0.2%
	180	98.0%	85.2%	98.8±0.1%

[a] Enzymatic hydrolysis conditions: 1%(w/v) glucan concentration, 15 FPU/g-glucan cellulase, pH 4.8, 50^{o}C.

[b] Acid flow rate was hold at 10ml/min and reaction time at 4.4 minutes.

For corn stover pretreated with 1% acid, 160-180^{o}C, the 96-h digestibilities were in the range of 92% -98% using an enzyme loading of 15FPU/g-glucan. The enzyme loading was further reduced to 10 and 5 FPU/g-glucan for the substrate treated with 1wt% acid and 170^{o}C. As shown in Fig. 15, reduction of the enzyme loading resulted in a significant decrease in the initial enzymatic hydrolysis rate and a slight decrease in the digestibility. For the extremely low enzyme loading of 5 FPU/g-glucan, the 96-hour glucan digestibility was 92%. The digestibilities observed in this work are higher than or close to those reported by other groups (Knappert et al., 1980; Um et al., 2003) taking the enzyme loading into consideration, and are very comparable to those reported by Schell et al. (2003), who obtained 87% of cellulose conversion (digestibility) for pretreated corn stover employing 15 FPU/g-cellulose in an SSF (simultaneous saccharification and fermentation) test.

The digestibility of glucan appears to be proportional to the degree of xylan removal (Fig. 16). This agrees with an observation by Um et al. (2003) that the removal of xylan improved the exposure of the cellulose to enzyme attack, and consequently, the digestibility. Although lignin is generally considered to be an important factor hindering the access of enzyme to cellulose (Kim et al., 2003; Chang and Holtzapple, 2000; Holtzapple et al., 1992; Gould, 1984; Wu and Lee, 1997), high digestibilities have been achieved for some of the pretreated corn stover samples in this work regardless of their high lignin content. This implies that xylan and lignin are parallel factors affecting the enzymatic digestion. The removal of either xylan or lignin somehow makes the biomass more amenable to the enzymatic digestion. Acid treatment also resulted in structural changes to the corn stover, as shown by the SEM (Scanning Electron Microscopy)

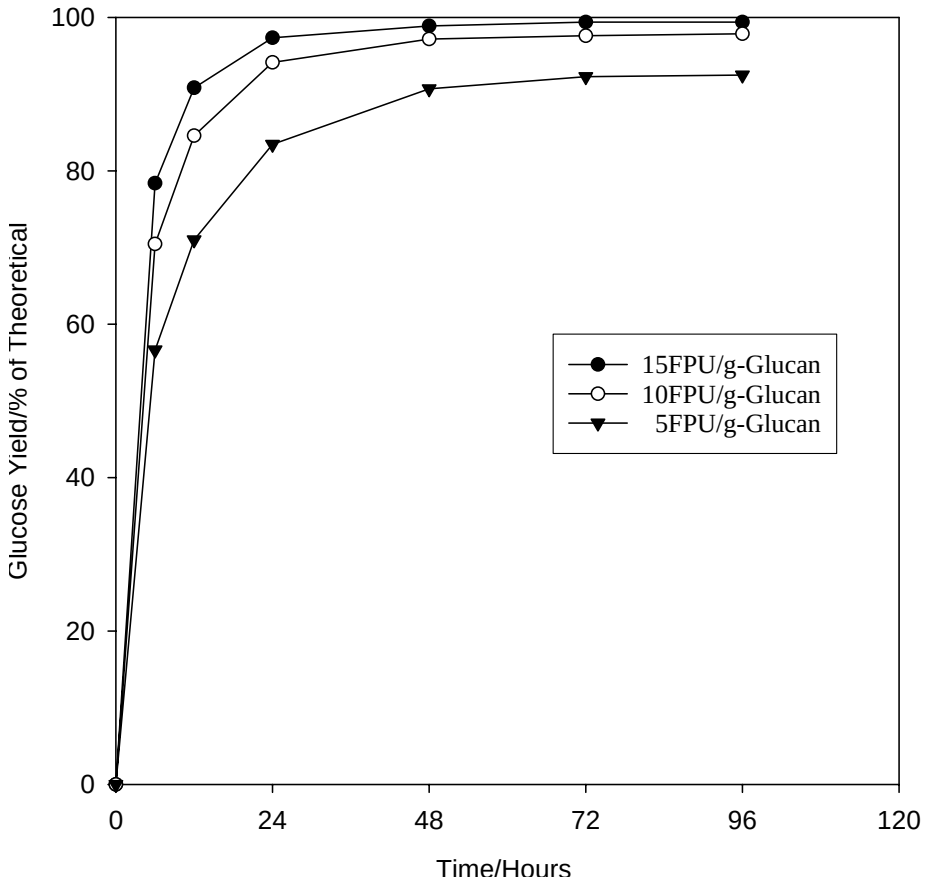


Fig. 15. Enzymatic hydrolysis of pretreated corn stover with various enzyme loadings. Pretreatment conditions: 1% acid, 10mL/min flow rate, 170^{o}C, 4.4 minutes.

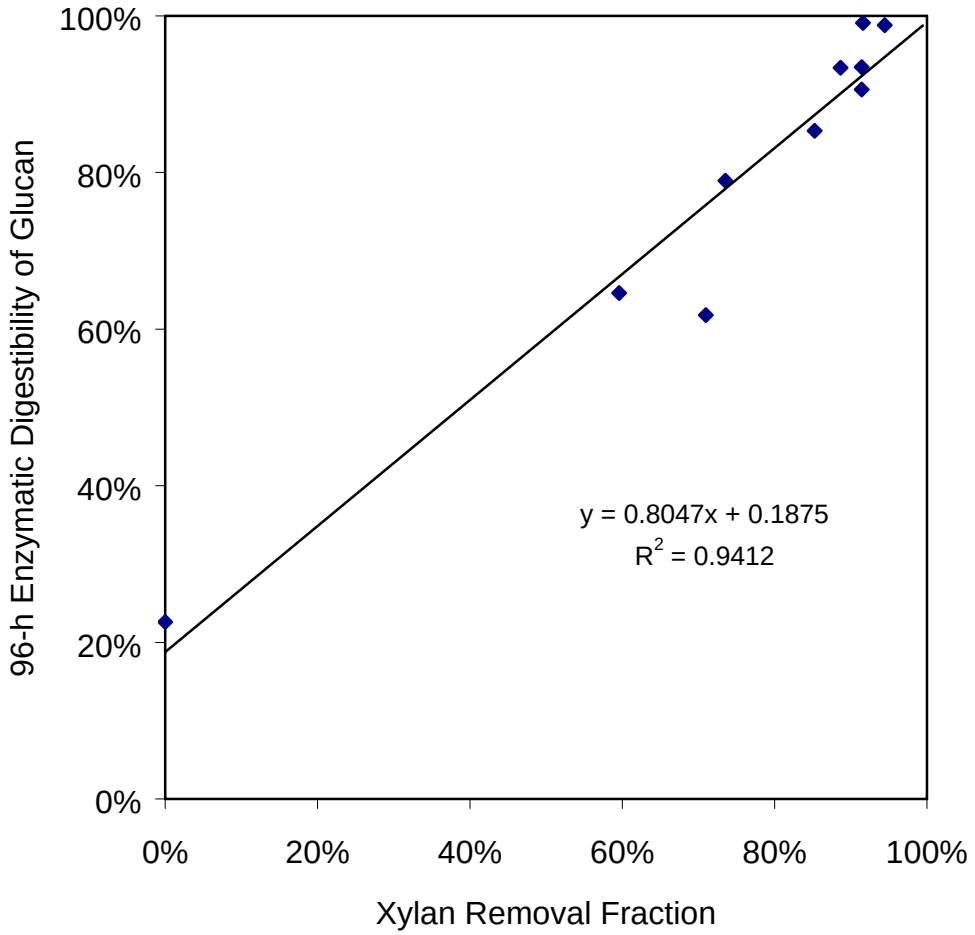


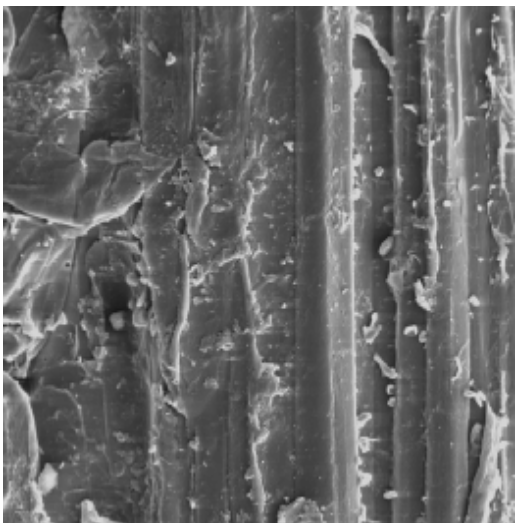
Fig. 16. Correlation between digestibility and xylan removal

pictures in Fig 17. It is evident that dilute acid treatment increased the surface area and the porosity of the corn stover, and consequently, the enzyme accessibility to cellulose. This observation agrees with an earlier finding by Grethlein (Grethlein, 1985).

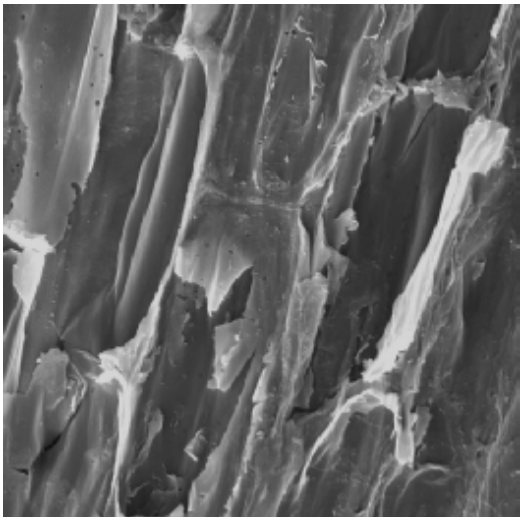
3.3 Evaluation of the pretreatment

Table 6 summarizes the digestibility of the treated corn stover and the sugar recovery under various pretreatment conditions. The data indicates that pretreatment with 1wt% acid and 170^{o}C is a near optimum condition. Under this particular pretreatment, 94% of xylan and 98.7% of glucan were accounted for, and quantitative enzymatic digestibility was obtained. Pretreatment at 180^{o}C with 1 wt% acid level also achieved a quantitative enzymatic digestibility. However, 15% xylose decomposition occurred in this run, indicating that the reaction condition was too severe. In the low severity runs (0.2 wt% acid), high glucose and xylose accountabilities were maintained, but the digestibilities were significantly lower.

There are two important factors to be considered in the pretreatments in regard to the fact that the biomass in the reactor was not uniformly treated. First, because only small amount of liquid (approximately one reactor void volume) passed through the reactor, the pretreatment time for the solids varied considerably depending on the longitudinal position of the reactor, with the reaction time of the biomass at the reactor inlet being nearly double that of the biomass at the outlet. Second, the acid was continually consumed as the liquid moved along the reactor because of the presence of buffering components in the corn stover. The pH value thus varied significantly from the reactor inlet (pH<1.0) to the outlet (pH=2~3). Therefore, the corn stover solids at different longitudinal positions were treated with different severities. This may lead to a



Untreated Corn Stover



Percolation Process, 1% Acid, 170^{o}C, 4.4 min

Fig. 17. Scanning electron micrographs of untreated and treated corn stover (500X)

non-uniform reactive situation in the reactor and, in consequence, considerably different composition among the solids remaining in the reactor. The composition data for the pretreated solids presented in this work was the average value. To overcome these potential problems, different strategies may need to be applied in reactor design and operation. A two-stage reverse-flow simulated counter-current reactor is one alternative (Lee et al., 1999).

There is also a concern as to whether acid impregnation into the biomass was a factor influencing the rate of the overall pretreatment process. In this work, corn stover was moisture-free prior to acid introduction. The acid then penetrated the particles through the pores for the reaction to occur. Since the corn stover was treated only for a short period of time and the reaction and impregnation occur simultaneously, it is uncertain whether the biomass attained a uniformly wet condition by the end of the pretreatment. Nonetheless, the pretreatment process appeared to progress very well, as shown by the near complete removal of xylan and the high digestibilities obtained. It is therefore conclude that the acid penetration is not a factor that significantly affects the pretreatment.

4. Conclusion

Dilute acid pretreatment of corn stover with a percolation reactor was investigated over a broad range of reaction conditions. The optimum conditions were identified to be 1wt% acid, 170^{o}C and 4.4 minutes (the average reaction time for the reactor particles). The optimum pretreatment run resulted in a 73% of xylose yield and a 6wt% xylose concentration. With recovery of trapped liquid, the xylose yield increased to 86%, but the xylose concentration decreased to 3 wt%. The treated and washed corn stover attained

near quantitative digestibility with an enzyme loading of 15 FPU/g-glucan at 1wt% solids concentration. With a reduced enzyme loading of 5 FPU/g-glucan, the digestibility was 92%. Decomposition of sugars was less than 6%, as 98% of the glucan and 94% of the xylan were accounted for. The high yield of sugar was attributed to the unique characteristics of the reactor (flow-through type) and the adjustment of the reactor operation (preheating under atmospheric pressure and quick quenching with nitrogen flush). Acid impregnation was not a factor significantly that affected the pretreatment process for the particle size used in this work. The digestibility of corn stover correlated directly with the extent of xylan removal.

V. ENZYMATIC PRODUCTION OF XYLO-OLIGOSACCHARIDES FROM CORN STOVER AND CORN COBS TREATED WITH AQUEOUS AMMONIA

Abstract

In the conventional enzyme-based method for xylo-oligosaccharides production, xylan was first isolated from the biomass solids by extraction with a strong alkaline solution or other chemicals, which forms a liquid intermediate containing soluble xylan. Since this intermediate is heavily contaminated with various extraneous components, a costly purification step is applied before the isolated xylan is subjected to enzymatic hydrolysis.

In this study, a novel process was investigated for producing food-grade xylo-oligosaccharides from agricultural biomass (corn stover and cobs). This process starts with pretreatment of feedstock in aqueous ammonia, which yields delignified and xylan-rich substrate. Since xylan remains in solid form after pretreatment, water washing is all that is required before enzymatic hydrolysis of this material. The complex step of purifying soluble xylan from contaminant is essentially eliminated. This reduces the process cost.

Refining of xylo-oligosaccharides to food-grade is accomplished by charcoal adsorption followed by ethanol elution. Xylanolytic hydrolysis of the pretreated corn

stover yielded glucan-rich residue that is easily digestible by cellulase enzyme. The digestibility of the remaining glucan reached 86% with enzyme loading of 10 FPU/g-glucan. As a feedstock for xylo-oligosaccharides production, corn cobs are superior to corn stover because of high xylan content and high packing density. The high packing density of corn cobs reduces water input and eventually raises the product concentration.

1. Introduction

Xylo-oligosaccharides (XOs) with a low degree of polymerization (DP) have been proven to promote proliferation of bifidobacteria, beneficial microorganisms in human intestine (Modler, 1994; Suwa et al., 1999; Yu et al., 2002). The demand of XOs as a food additive has shown rapid growth over the last two decades (Crittenden and Playne, 1996; Vazquez et al., 2000). Xylo-oligosaccharides are not foreign for human consumption as they exist in natural plants and food substances including bamboo shoots, fruits, vegetables, milk and honey. XOs can be produced by autohydrolysis of hemicellulose in lignocellulosic biomass (Vazquez et al., 2000; Vazquez et al., 2001; Kabel et al., 2002). In this case, relatively mild conditions are applied because XOs can easily be converted to monomer (xylose). The hydrolyzates from those processes contain a variety of undesirable components, such as soluble lignin, lignin- and sugar-degradation products, organic acids, and ash. Extensive downstream purification is, therefore, required.

Alternatively, XOs can be produced by enzymatic hydrolysis. Of the three major types of xylanases (endo-xylanase, exo-xylanase, and xylosidase), endo-xylanase and exo-xylanase are the ones responsible for production of XOs. Certain natural plant materials can be digested directly by endo-xylanase (Takao and Yoshio, 1996), but such feedstock is scarce, not available in large enough quantities for commercial production. In most natural lignocellulosic biomass, xylan exists mainly as xylan-lignin complex (Watanabe et al., 1993; Gubitz et al., 1998; Yang et al., 2001), and becomes resistant to enzyme attack. For this reason, current commercial processes are carried out in two stages: alkaline extraction of xylan from lignocellulosic biomass followed by enzymatic

hydrolysis of the dissolved xylan (Suwa et al., 1999; Yu et al., 2002). Because the alkaline extract is heavily contaminated, a complex purification process must be applied to the crude xylan before the enzymatic conversion. The situation is about the same as for the xylan produced by autohydrolysis. Recently, wood pulp has been investigated as feedstock for enzymatic production of XOs (Izumi et al., 2002). In this process, the washed wood pulp was hydrolyzed with hemicellulase or xylanase to give a xylooligosaccharide-lignin complex, which is further treated with acid or heat to generate XOs.

A pretreatment method based on soaking in aqueous ammonia (SAA) under moderate severity reaction condition for extended period (10-15% NH_3, 65-90^oC, 12-24 hours, for example) has been investigated in our laboratory. This method was found to be very efficient as a pretreatment for corn stover. The unique features of the SAA are that most of the lignin is removed and all of the cellulose and most of the xylan is retained in solid after treatment. A considerable portion of the ash is also dissolved in this treatment. The SAA treated corn stover is, therefore, clean, carbohydrate-rich, and amenable for enzymatic digestion. It is well suitable for enzymatic production of XOs. The primary goal of this research is to assess the feasibility of producing low-DP XOs by enzymatic conversion of the SAA treated corn stover and corn cobs. It is also of interest to evaluate the reacted glucan-rich residue as a feedstock for enzymatic saccharification since glucan constitutes the most significant carbohydrate fraction in biomass.

2. Experimental methods

2.1 Materials

Corn stover supplied by NREL was stored at 5℃. The moisture content was 9-14 %. Corn cobs was a kind gift of Andersons, Inc, Maumee, Ohio. It has a supplier's coding of 2040 WC, which is "milled woody portion of corn cobs". It has a moisture content of 8-10 %. The composition of 2040 WC as determined by the supplier is: 47.1% cellulose, 37.3% hemicellulose, 6.8% lignin and 1.2% ash. Endo-xylanase, extracted from *Thermomyces lanuginosus*, was purchased from Sigma-Aldrich (catalog # X2753, lot # 100K1359). It has manufacturer's nominal activity of approximately 2500 xylanase units per gram. The cellulase Spezyme CP (Genencor) was supplied by NREL. The cellulolytic activity as determined by NRELwas 30 FPU (filter paper units) per mL. Activated carbon powder was purchased from Sigma-Aldrich (catalog # C7606, batch # 073K0037).

2.2 Aqueous ammonia treatment (SAA treatment)

The treatment was conducted in a 250 mL autoclave. Heating and temperature controls were done in a GC oven (Varian Model 3700). Twenty grams of dry corn stover and 200 g of 15% ammonia solution (or in the case of corn cobs, 28 g dry corn cob particles and 75 g of 15% ammonia solution) were placed into autoclave for each treatment. The treated materials were washed with deionized water until the pH became neutral. The washed corn stover and corn cobs were then subjected to composition analysis and used as the substrate for xylo-oligosaccharides production.

2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis was conducted in 250 mL Erlenmeyer flasks, which were placed in a laboratory shaking incubator (150rpm) with temperature control. Solid substrates (treated or untreated corn stover/corn cobs, α-cellulose) were mixed with citrate buffer to reach a total volume of 100 mL. The substrates and buffer were sterilized at 121℃ for 30 minutes before being placed in the incubator. Enzyme was added after the flask content reached the desired temperature. Addition of cellulase enzyme (Spezyme CP) was done on the basis of the glucan content of the substrate, while the addition of endo-xylanase enzyme was done on the basis of dry weight of substrate.

2.4 Purification of xylo-oligosaccharides

The hydrolyzate from the xylanolytic hydrolysis was centrifuged at 3800 g for 10 minutes. Activated carbon powder was added into supernatant liquid with loadings varying in the range of 1 - 10% of the liquid weight. The flask was placed in a room-temperature incubator shaker at 200rpm for 30 minutes to stabilize the carbohydrate-carbon adsorption. The mixture was suction filtered with a 50 mL Pyrex crucible filter and washed with 4×50 mL distilled and deionized water. The XOs-enriched carbon cake thus obtained was eluted by ethanol twice in succession: 2×50 mL 15% ethanol and 2×50 mL 30% ethanol. Where necessary, the third elution was applied with 50% ethanol. The eluates were concentrated by vacuum rotoevaporation and freeze-dried to recover the product in solid form.

2.5 Analysis

The liquid samples were analyzed for sugars by HPLC operated with Bio-Rad Aminex HPX-87P column and a refractive index detector. The HPLC was operated at 85 with a flow rate of 0.55 mL/min. Sugar oligomers in the hydrolyzate were determined by the increase of sugar monomers after secondary hydrolysis (4% H_2SO_4, 121°C, 1 h) following the NREL Standard Analytical Procedure No. 014 (NREL, 1996). The sugar, acetyl and lignin contents in the solids were measured by NREL Standard Analytical Procedure No. 002 (NREL, 1996). The glucan digestibility of the treated biomass was determined by NREL Standard Analytical Procedure No. 009 (NREL, 1996).

3. Results and discussion

3.1 Aqueous ammonia treatment

The details of the SAA treatment procedure and the results on corn stover were reported by Kim and Lee (2005). Table 7 shows the effect of SAA treatment on the composition of corn stover. It is clearly seen that the lignin content (both acid-soluble and -insoluble lignin) decreased significantly after treatment, whereas the sugars were largely retained (96.1% of glucan and 73.6% of xylan being recovered in treated and washed solids). These data support the idea of using the SAA treated corn stover as a substrate for enzymatic production of XOs.

Table 7 Composition of untreated and SAA treated corn stover [a] (% [w/w], dry basis of samples) and recovery of sugars and solids after washing

	Untreated Corn Stover	Ammonia Treated Corn Stover	% Recovery
Glucan	36.8	54.4	96.1
Xylan	22.0	24.9	73.6
Galactan	0.68	1.0	95.6
Arabinan	3.5	3.1	57.6
Mannan	0.7	0.6	55.7
Insoluble Lignin	17.2	6.1	23.0
Acid-Soluble Lignin	3.2	1.6	32.5
Acetyl	3.2	0	0
Ash	7.5	7.9	68.5
Total	94.8	99.6	65.0[b]

[a] SAA treatment conditions: 15% ammonia, L/S=10, 90^{o}C, 24h.

[b] Solid remaining.

3.2 Optimal conditions for enzyme activity and hydrolysis

A series of experiments were carried out to determine the optimal pH and temperature for the enzymatic activity. The SAA treated corn stover used as the substrate. The enzyme activity was expressed as the combined molar yields of xylose and XOs. From the results shown in Figs. 18 and 19, pH 5 and 40-50 ^{o}C were the optimal range applicable for the endo-xylanase enzyme (X2753). These findings are in agreement with the optimal ranges reported for endo- and exo-xylanase enzyme complexes (Vazquez et al., 2001; Cai et al., 2000; Frederick et al., 1981).

In order to evaluate the efficiency of the enzyme complex for the digestion of xylan, three enzyme loadings (w/w) were applied at pH 5 and 50 ^{o}C (Fig. 20). The combined molar yield of xylose and XOs increased with the increase of enzyme loading within the range of 0.01 to 0.16. However, the efficiency of the enzyme (sugar yield/enzyme loading) decreased with the increase of enzyme loading. Considering both the sugar yield and the cost of enzyme, the enzyme/solids ratio of 0.04 (w/w) was selected in subsequent experiments.

Fig. 21 presents the effect of substrate concentration on the total molar yield of xylose and XOs. No significant change of yield was found within the solids concentration of 2% - 5% (w/w). Attempts to further increase the solid concentration to 10% (w/w) failed because of the difficulty of agitating the medium. Obviously the solids concentration appropriate for digestion of SAA treated corn stover with xylanase is less than 10% (w/w). Usually a fed-batch operation is a solution for this type of problem. However, it is questionable whether it will work in this case because hydrolysis of xylan does not alter the structure of cellulose fibers, which constitute the framework of the

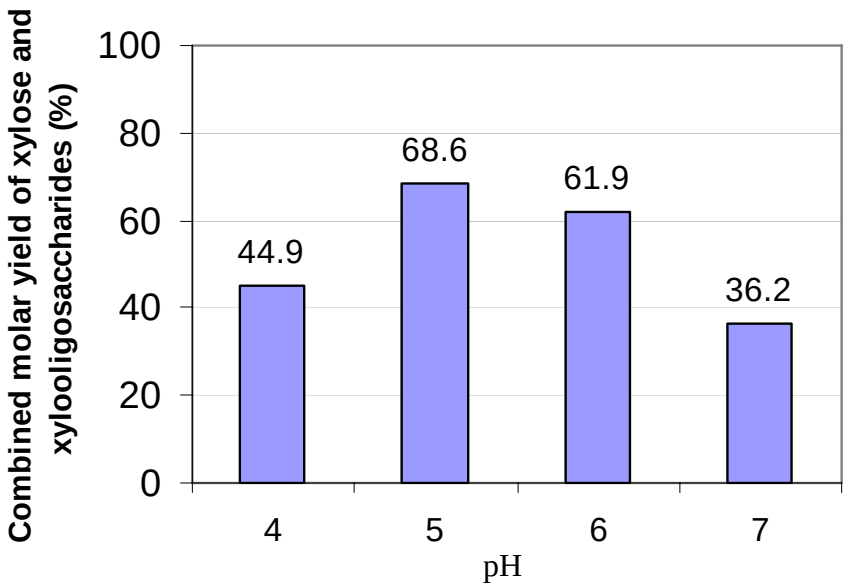


Fig. 18. Effect of pH on the combined molar yield of xylose and xylo-oligosaccharides from hydrolysis of SAA treated corn stover at 50^{o}C after 96 h. Enzyme complex: endo-xylanase X2753; enzyme loading: 0.04g / g solids; SAA treatment conditions: 15% ammonia, 90^{o}C, L/S =10, 24h;

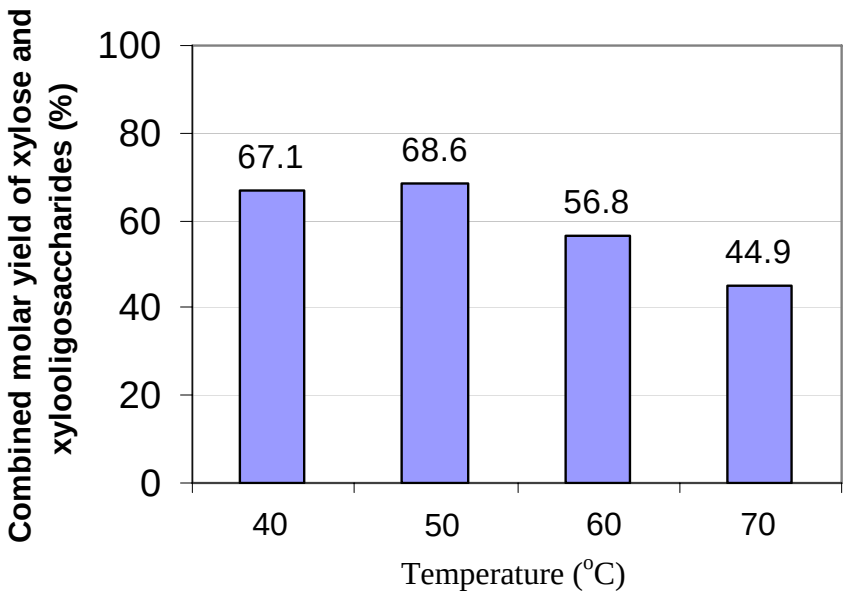


Fig. 19. Effect of temperature on the combined yield of xylose and xylo-oligosaccharides from hydrolysis of SAA treated corn stover at pH=5 after 96 h. Enzyme complex: endo-xylanase X2753; enzyme loading: 0.04g / g solids; SAA treatment conditions: 15% ammonia, 90°C, L/S =10, 24h;

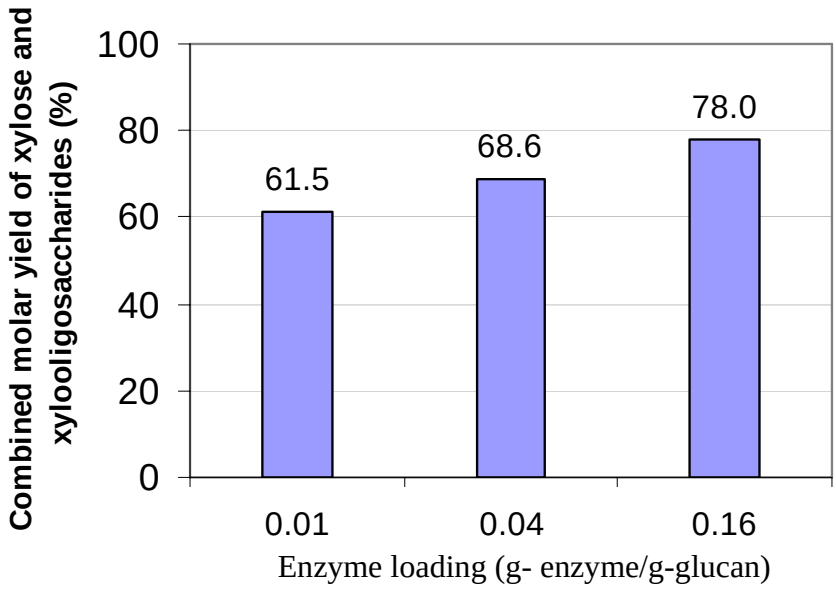


Fig. 20. Effect of enzyme loading on the combine molar yield of xylose and xylo-oligosaccharides from hydrolysis of SAA treated corn stover at pH=5 and 50^{o}C after 96 h. Enzyme complex: endo-xylanase X2753; SAA treatment conditions: 15% ammonia, 90^{o}C, L/S =10, 24h;

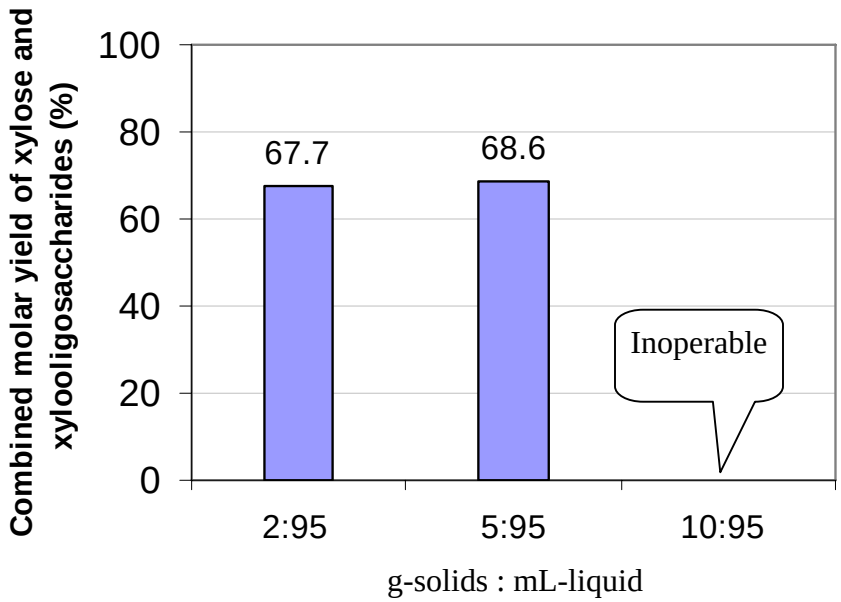


Fig. 21. Effect of substrate loading on the combined molar yield of xylose and xylo-oligosaccharides from hydrolysis of SAA treated corn stover at pH=5 and 50^{o}C after 96 h. Enzyme complex: endo-xylanase X2753; enzyme loading: 0.04g enzyme/ g solids; SAA treatment conditions: 15% ammonia, 90^{o}C, L/S =10, 24h.

lignocellulosic biomass. As a proof, it was noticed that the shape and rigidity of the SAA-treated corn stover solids were not changed significantly after 96 h of digestion. In subsequent experiments, the enzymatic hydrolysis of SAA treated corn stover for XOs production was conducted under pH=5, 50^{o}C, 0.04 g X2753 endo-xylanase/g-solids, solids loading 5% (w/w).

3.3 Characterization of xylo-oligosaccharides

Hydrolysis of xylan in SAA treated corn stover with endo-xylanase generated a liquid product containing XOs with a wide distribution of DP. As shown in the chromatogram of Fig. 22, the hydrolyzate is composed mainly of XOs with DP2 and DP4+. Small amount of xylose and arabinose was also detected. It appears that portion of the XOs of DP4+ exists in the form of heteropolysaccharides (i.e. arabinoxylan), as indicated by appearance of sugars other than xylose in the secondary hydrolyzates (Table 8). Nonetheless, no negative effects of these heteropolysaccharides were reported pertaining to their use as food additives.

3.4 Improvement of xylan digestibility by SAA treatment

The positive effect of delignification on the cellulose digestibility is well known. However, very little study, if any, was focused on the influence of delignification on xylan digestibility. This is primarily because few of the previous pretreatment methods lead to the concurrence of extensive lignin removal and large xylan retention. In other words, the selectivity towards delignification was usually very low in most pretreatment methods. However, the SAA pretreatment provided a unique substrate for this study. As shown in Table 8, when the corn stover was treated with 15% ammonia at 90℃ for 24

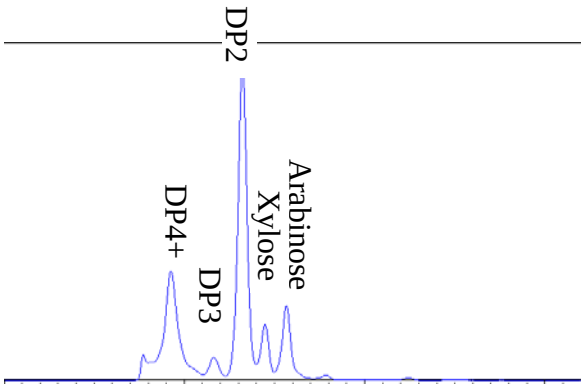


Fig. 22. Xylo-oligosaccharides from enzymatic hydrolysis of SAA-treated corn stover. Hydrolysis conditions: 5% (w/w) solids, pH=5, 50^{o}C, 150rpm, 72 h. Enzyme complex: Endo-xylanase X2753; Enzyme loading: 0.04g / g-solids; SAA treatment conditions: 15% ammonia, 90^{o}C, L/S=10, 24h.

Table 8 Composition of xylanolytic hydrolyzate from SAA treated corn stover (g/L)

	Monosaccharide	Oligosaccharides	Total
Glucose	-	-	1.488
Xylose	1.147	8.754	9.901
Arabinose	0.078	1.072	1.15

Hydrolysis conditions: 5% (w/w) solids, pH=5, 50^{o}C, and 72 h. Enzyme complex: Endo-xylanase X2753; Enzyme loading: 0.04g / g-solids; SAA treatment conditions: 15% ammonia, 90 ^{o}C, L/S=10, 24h.

hours, the Klason lignin was reduced from 17.2% to 8.2%, whereas the percentage of xylan increased slightly from 22.0% to 24.96%. The increase in percent xylan is due to the weight decrease after pretreatment.

Table 9 compares the digestibilities of corn stover xylan with and without delignification. The SAA treatment increased the xylan digestibility from 14.0% to 66.1%. This improvement is in line with the increase of cellulose digestibility upon delignification. However, the fates of cellulose and xylan in the SAA treatment are different. In lignocellulosic substrates, cellulose exists as homopolymers, which are surrounded by a complex of lignin and hemicellulose. The removal of lignin facilitates the transport of cellulase enzyme to the cellulose surface, but leaves the cellulose chains intact. However, in the ammonia treatment, some of the hemicellulose-lignin linkages are disrupted, and some of the side chains attached to the xylose backbone are removed. Ammonia treatment brings about significant changes to the hemicellulose structure. Despite these differences, the increased substrate accessibility by enzymes remains as the common factor responsible for increased digestibility in both cases.

3.5 Separation of xylo-oligosaccharides using different HPLC columns

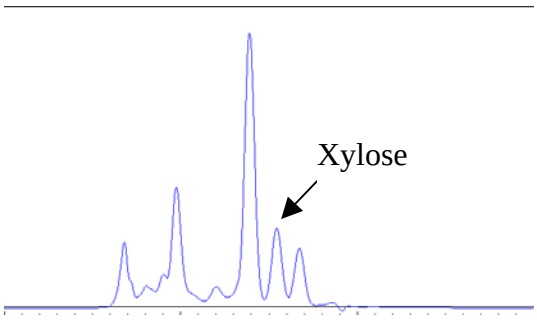
Hydrolysis of xylan with endo-xylanase yielded a number of XOs with varying degrees of polymerization. Separation of these XOs was examined using two different types of HPLC columns (42 C and 87 P), both purchased from Bio-Rad Laboratories, Inc., CA. The resultant chromatograms are shown in Fig. 23. In comparison to the results obtained with the 87 P, the 42 C column showed more efficient separation for the XOs components with relatively high degree of polymerization (DP above 5). However, for low-DP components, the degrees of separation for both columns are almost the same.

Table 9 Xylan digestibility of untreated and SAA treated corn stover

	Untreated Corn Stover	SAA Treated Corn Stover
Xylan Digestibility	14.0%	66.1%

Note: Xylan digestibility is expressed as the formation of xylose and xylo-oligosaccharides. Hydrolysis conditions: 5% (w/w) solids, pH=5, 50^{o}C, and 72 h. Enzyme complex: Endo-xylanase X2753; Enzyme loading: 0.04g / g-solids; SAA treatment conditions: 15% ammonia, 90^{o}C, L/S=10, 24h.

(A)



(B)

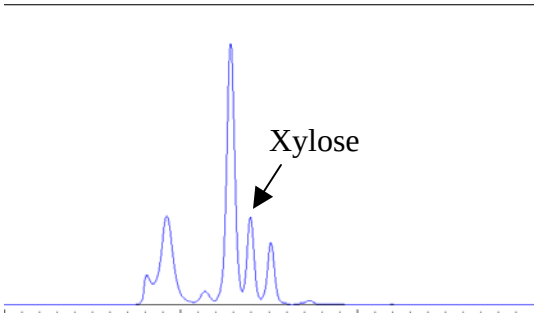


Fig. 23. Separation of xylo-oligosaccharides on the Bio-Rad 42 C (A) and 87P (B) columns. Samples are hydrolyzates from xylanolytic digestion of SAA treated corn stover. The SAA treatment conditions were: 15% ammonia, 90^{o}C, L/S =10, 24h. The digestion conditions were: 5% solids loading, 0.04 g X2753 xylanase/g solids, pH=5, 50^{o}C, 150 rpm.

Since this study is focused on the production of low-DP (preferentially DP 2-4) XOs and the maintenance of a 87 P column is more convenient by the author's experience, Bio-Rad 87 P column was selected for analysis.

3.6 Refining and fractionation of xylo-oligosaccharides

Hydrolysis of SAA pretreated corn stover with endo-xylanase produces hydrolyzates composed mainly of XOs (Table 8). It is necessary to eliminate the impurities such as soluble lignin and ash in the hydrolyzate to bring it to food-grade. Also, it is desirable to remove the sugar monomers and high-DP products to give a low-DP XOs product. For this purpose, the XOs was treated with charcoal (carbon) adsorption and ethanol elution. The carbon adsorption takes advantage of the different interactions between the XOs and carbon (Pellerin et al., 1991). The higher the DP, the stronger the adsorption to charcoal. For example, xylose (DP 1) has little interaction with carbon showing no adsorption on it. It is therefore removed from the XOs-carbon complex by water washing. Small amount of XOs are also lost in water washing stage before the ethanol elution, the actual amount depending on DP and carbon loading. As shown in Table 10, significant loss of XOs occurs with carbon-to-hydrolyzate ratio of 0.01 (w/w). Increasing the carbon to hydrolyzate ratio to 0.05 (w/w) markedly reduced the XOs loss thus increasing the yield of it. Further increase of the carbon-to-hydrolyzate ratio to 0.1 (w/w) essentially eliminated XOs loss as evidenced by negligible amount of XOs being detected in the water eluate. Most of the XOs adsorbed on charcoal were recovered with 15% ethanol elution. The remaining XOs were recovered by 30% ethanol elution. Although very low in quantity, the products recovered from the 50% ethanol elution were comprised of only high-DP XOs. In all practical sense, 30% ethanol elution

Table 10 Fates of xylo-oligosaccharides in carbon-ethanol refining [a]

Carbon/Hydrolyzate (w/w)	0.01	0.05	0.1
XOs loss by water washing	Significant	Moderate	Small
XOs yield from 15% ethanol elution	5.9%	21.3%	34.5%
XOs yield from 30% ethanol elution	2.5%	15.6%	15.9%
XOs yield from 50% ethanol elution	Not done	Not done	4.4%
Total XOs yield from ethanol elution	8.4%	36.9%	54.8%

[a] Yields are molar yields on the basis of xylan in SAA treated corn stover

is sufficient for recovery of the XOs of interest. Knowing that the xylan digestibility is 66.1% (Table 9), the recovery of XOs from the solubilized xylan is estimated to be more than 70% when 30% ethanol elution is applied with carbon/hydrolyzate of 0.1 (w/w). These data also indicate that successive elution with different ethanol levels may be used as a tool, if needed, to further fractionate the XOs into products of different DP. The final XOs retained pure-white color after freeze-drying.

3.7 Glucan digestibility after xylanase treatment

Corn stover, after the SAA treatment, turned into a carbohydrate-rich product. Subsequent hydrolysis of the SAA treated corn stover by xylanase generated a glucan-rich substrate. As shown in Table 11, the glucan content in the corn stover rose to 69.6% after hydrolysis by endo-xylanase, which is almost twice that of untreated corn stover. The residue remaining after XOs production is a byproduct suitable for further bioconversion. The enzymatic digestibility of this substrate is summarized in Fig. 24. For the digestibility test, the moisture of the treated solids was reduced (to 79.2%) by squeezing. The solids, however, were not sterilized so that endo-xylanase enzyme remains active along with the cellulase enzyme. It is conceivable that the combined action of cellulase and xylanase may not deteriorate the digestibility of glucan. As shown by the curves in Fig. 24, the glucan digestibility of the SAA treated corn stover maintained at a considerably high level (86% after 120 h) after xylan removal by endo-xylanase. This means that the xylanase treated corn stover can continue to serve as an excellent substrate for further saccharification.

Table 11 Composition of SAA-treated corn stover after xylanolytic hydrolysis (% [w/w], dry basis of sample)

	SAA-treated Corn Cobs
Glucan	69.6
Xylan	11.9
Arabinan	1.4
Mannan	0.7
Acid Insoluble Lignin	6.4
Acid Soluble Lignin	1.3
Acetyl	0

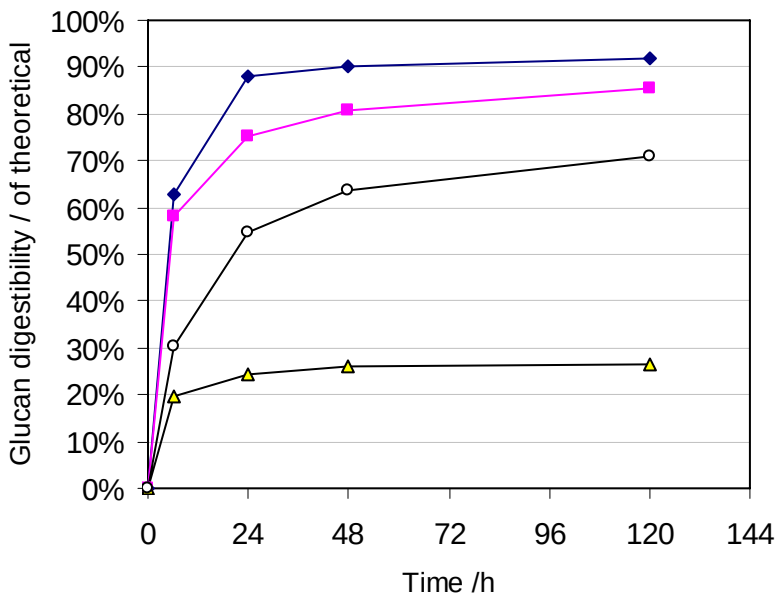


Fig. 24 Glucan digestibility of untreated, SAA-treated and SAA-treated xylanase-digested corn stover and *α*-cellulose. All the substrates except the xylanase-digested SAA-treated corn stover were sterilized at 121°C for 30 minutes before enzyme addition. Digestibility test conditions: 1 wt% glucan loading, 10 FPU/g-glucan, pH = 4.8, 50°C, 150 rpm. Δ untreated corn stover; ♦ SAA treated corn stover; ■ SAA-treated xylanase-digested corn stover; ○ α-cellulose.

3.8 Xylo-oligosaccharides production from corn cobs

Corn cobs were also evaluated as a substrate for XOs production. The procedures were almost identical to those of corn stover. The SAA treatment conditions for corn cobs were 15% ammonia, 60^{o}C,L/S 2.8 and 48 h. It is noticeable that a significantly lower liquid-to-solid ratio was applied for the corn cobs (only 2.8) since they have a much higher bulk density than corn stover. High solid loading is beneficial in that it lowers energy input as well as wastewater.

SAA treatment of corn cobs yielded a superior substrate for saccharification. As shown in Table 12, the total sugar content reached 92.5%, the total lignin level went down to 5.6%, and the ash content was reduced to 1%. The following hydrolysis of the treated and washed corn cobs using endo-xylanase gave a fairly clear hydrolyzate. Table 13 shows the concentrations and yields of XOs in the hydrolyzate for both untreated and SAA treated corn cobs. The yield increased by a factor of four after SAA treatment and the XOs concentration reached 1.57%. The SAA treated corn cobs can be loaded more densely into the hydrolysis reactor than corn stover (Table 14). The XOs concentration rose to 4.7% with 15% solids loading. The product inhibition by XOs to the xylanase appears to be insignificant since the XOs yield decreased slightly with increase of solid loading from 5% to 15%. The HPLC chromatogram of XOs from the hydrolysis of SAA treated corn cobs is very similar to that from the SAA treated corn stover (data not shown). Xylose accounted for approximately 10% (w/w) of the hydrolyzed xylan, the rest being XOs.

4. Conclusions

SAA treatment of corn stover and corn cobs resulted in delignified and xylan-enriched substrates. They are highly susceptible to enzymatic hydrolysis by endo-xylanase. The hydrolyzates contained predominantly xylo-oligosaccharides with a small amount of xylose and other components. Refining of the xylo-oligosaccharides can be accomplished by carbon adsorption of oligomers followed by ethanol elution. Under optimal treatment conditions, xylose was totally removed and the xylo-oligosaccharides were obtained in good yield. Removal of xylan from the SAA treated corn stover caused a slight decrease in the digestibility of the remaining glucan, but still retained a level above 85% with 10 FPU/g-glucan. For xylo-oligosaccharides production, corn cobs are a more suitable feedstock than corn stover because corn cobs possess higher xylan content and greater bulk density.

Table 12 Composition of SAA-treated corn cobs [a]

	% dry basis
Glucan	50.4
Xylan	37.0
Galactan	1.2
Arabinan	3.9
Total Sugars	*92.5%*
Acetyl	0
Acid Insoluble Lignin	3.6
Acid Soluble Lignin	2.0
Ash	1.0
Total Components	*99.1*

a SAA treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60 ^{o}C, 48h.

Table 13 Xylo-oligosaccharides production using untreated and SAA-treated corn cobs

	Untreated	Treated
Concentration in hydrolyzate (g/L)	4.11	15.74
Molar yield (% of xylan)	21.1	80.5

Note: xylanolytic hydrolysis conditions: 5% solids loading, 0.04 g-xylanase/g-solids, pH=5, 50 ^{o}C, 150 rpm, 72h. SAA treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60 ^{o}C, 48h.

Table 14 Enzymatic production of xylo-oligosaccharides from SAA treated corn cobs using different solids loadings

Solids loading	5%	10%	15%
Concentration in liquor (g/L)	15.74	30.02	47.18
Molar Yield (%)	80.5	72.7	71.8

Note: enzymatic digestion conditions: 0.04 g-xylanase/g-solids, pH=5, 50 ^{o}C, 150 rpm, 72h. SAA treatment conditions: 15% aqueous ammonia, L/S = 2.8, 60 ^{o}C, 48h.

VI. PRODUCTION OF LACTIC ACID FROM AQUEOUS AMMONIA-TREATED CORN STOVER VIA SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTATION

Abstract

Production of lactic acid by simultaneous saccharification and co-fermentation (SSCF) of aqueous ammonia-treated corn stover was investigated. The cellulase and lactic acid bacteria used for this study were Spezyme CP and *Lactobacillus pentosus* ATCC 8041 (CECT-4023). When the SSCF was conducted in batch mode based on 3% (w/w) glucan loading, the carbohydrates in the treated corn stover were effectively converted to lactic acid and acetic acid, the maximum lactic acid yield reaching 0.92 of theoretical, on the basis of total fermentable sugar content (glucose, xylose, and arabinose). Small amount of acetic acid was also produced in the process. The impacts of four major process variables of SSCF (enzyme loading, inocula size, yeast extract concentration, and clarified corn steep liquor [cCSL] concentration) on lactic acid production were examined using statistical experimental design. The statistical analysis showed that enzyme and yeast extract were the most important factors affecting the final lactic acid yield. In contrast, the impact of inocula size was found to be insignificant. The response surface analysis indicated that cCSL could be used as a nitrogen source replacing yeast extract without adversely affecting the lactic acid yield. The product concentration was improved by operating the SSCF in fed-batch mode. The

maximum lactic acid concentration attainable by fed-batch operation was 74.8 g/L. Further improvement of the lactic acid concentration was difficult due to severe product inhibition.

1. Introduction

Lactic acid is a commodity chemical with applications in food industry, cosmetics, pharmaceuticals, and plastics. Presently, its commercial production is largely based on microbial fermentation of starch-derived glucose or sucrose (Litchfield, 1996). With the concern on feedstock cost, the use of lignocellulosic materials (LCM) as an inexpensive carbon source for lactic acid production has been pursued (Parajo et al., 1997; Garde et al., 2002; Neureiter et al., 2004). The most important component in LCM is cellulose, complete hydrolysis of which leads to glucose. The conventional fermentation technology thus can be conveniently integrated with cellulose hydrolysis for lactic acid production (Iyer and Lee, 1999; Lee et al., 2004; Naveena et al., 2005).

Second to cellulose, hemicellulose represents the other important carbohydrate fraction in LCM. Hemicellulose is a heteropolymer composed of a variety of sugar units such as glucose, xylose, galactose, arabinose, and mannose. For a lignocellulosic conversion process to be economically viable, it is very important to utilize both cellulose and hemicellulose fractions effectively. One of the approaches for this purpose is called simultaneous saccharification and co-fermentation (SSCF), in which both cellulose and hemicellulose are hydrolyzed and the resultant soluble sugars (hexose and pentose sugars in monomeric and/or oligomeric forms) are simultaneously fermented to form desired products.

Intensive research has been performed on SSCF production of ethanol (McMillan et al., 1999; Sedlak and Ho, 2004; Kim and Lee, 2005), but very few studies were conducted on lactic acid production using this technique. The key to the success of a SSCF process lies in the microorganism, which should possess the capability to

efficiently utilize both hexoses and pentoses. Although a few researches have been conducted to develop new strains for lactic acid co-fermentation (Dien et al., 2001, 2002; Patel et al., 2004, 2005), one of the most common species used to this point is *Lactobacillus pentosus* (McCaskey et al., 1994; Perttunen et al., 2001; Bustos et al., 2004). As a facultatively hetero-fermentative species, *L. pentosus* ferment hexose (glucose) through Embden-Meryerhof-Parnas (EMP) pathway under anaerobic conditions giving lactic acid as the sole product (homo-fermentation), and uses phosphoketolase (PK) pathway for pentoses (xylose and arabinose) assimilation generating equal moles of lactic acid and acetic acid (hetero-fermentation) (Garde et al., 2002). Therefore, this species have the theoretical yield of two moles lactic acid per mole of hexose or one mole lactic acid per mole of pentose during anaerobic fermentation.

The present study discussed SSCF of ammonia pretreated corn stover for lactic acid production using *L. pentosus* bacteria ATCC 8041. Corn stover is a lignocellulosic agricultural residue that is abundantly available in the US. In this work, corn stover was soaked in aqueous ammonia to remove the lignin and improve the accessibility of carbohydrates to enzyme before used as the SSCF substrate. This aqueous ammonia treatment has been referred to as SAA pretreatment (Kim and Lee, 2005). The SAA treated corn stover was rich in carbohydrates, highly susceptible to enzyme attack, and contained low quantity of lignin. These features rendered the pretreated corn stover suitable as a saccharification substrate. The microorganism *L. pentosus* ATCC 8041 was originally introduced from the Spanish Collection of Type Cultures (Valencia, Spain; No. CECT-4023). It has been reported to work well for the co-fermentation of glucose, xylose

and arabinose in hemicellulose hydrolyzate from trimming wastes of vine shoots (Bustos et al., 2004).

The primary objective of this study was to develop a SSCF process for effective production of lactic acid from SAA treated corn stover. Statistical experimental design and response surface method were employed as a tool to analyze the significance of the variables in the SSCF process and provide useful information for the process optimization. Use of fed-batch technique was attempted as a means to improve the product concentration.

2. Materials and methods

2.1 Feedstock and chemicals

Corn stover was supplied by the National Renewable Energy Laboratory (NREL), Golden, CO, and stored at 5℃. The moisture content was 9-14 %. The chemical composition of the feedstock was (w/w, dry basis): 36.8% glucan, 21.7 % xylan, 2.6 % arabinan, 0.68 % galactan, 0.3 % mannan, and 17.2 % lignin. Ammonia hydroxide (30%, w/w) and MRS broth were purchased from Fisher Scientific Co.; while yeast extract, corn steep liquor (containing approximately 50% [w/w] solids), and agar were purchased from Sigma Co. The corn steep liquor was centrifuged at 3823 g for 20 min to separate the solids, and the supernatant -clarified corn steep liquor (cCSL) - was used as a nutrition supplement in the SSCF. The separation and marketing of the solids as animal food has been proposed as an approach to improve the wet milling process economics (Lawford and Rousseau, 1997).

2.2 Aqueous ammonia treatment (SAA pretreatment)

SAA treatment was conducted in a 600 ml stainless steel autoclave. Heating and temperature control were achieved by using a GC oven (Varian Model 3700). The treatment conditions are: liquid to solids ratio of 10 (w/w; air-dried corn stover containing 40 g of solids soaked in 400 g of 15% [w/w] ammonia solution), 90℃, 24 h. The treated materials were washed with de-ionized water until the pH became neutral. The washed corn stover was transferred onto a piece of cheesecloth, wrapped, and squeezed with hand to remove the majority of the free water. By this means the moisture content of the pretreated corn stover was reduced to 69-71%, and the recovery of the corn stover solids was 65% (w/w) on the basis of the untreated solids. The dewatered solids are then subjected to composition analysis and used as the substrate for SSCF experiments. The composition of the treated solids was (w/w, dry basis): 54.4% glucan, 24.9 % xylan, 3.1% arabinan, 1.0% galactan, 0.6 % mannan, and 7.7 % lignin.

2.3 Enzyme

The enzyme used in the SSCF experiments was Spezyme CP cellulase (Genencor Co.). The cellulolytic activity (filter paper units [FPU]/ml) was determined using the NREL Standard Analytic Protocol 007 (NREL, 1996). This enzyme has proved capability of hydrolyzing both cellulose and hemicellulose in lignocellulosic materials (Refer to Chapter III).

2.4 Inocula preparation

The *L. pentosus* ATCC 8041 (CECT-2043) strain was grown aerobically on plates composed of 5.5% (w/v) MRS broth and 1% (w/v) agar at 37^{o}C for 36h in the presence of

10% (v/v) carbon dioxide. A fresh colony was transferred to 10 ml of 5.5% (w/v) MRS medium that was placed in a 20 ml glass tube. The headspace of the test tube was filled with 10% (v/v) of carbon dioxide, capped and placed in an incubator, which was set at 37^{o}C without shaking. After 12 h of growth, 1 ml of the medium was transferred to 100 ml of 5.5% (w/v) MRS medium in a 250 ml Erlenmeyer flask. The headspace of the flask was also filled with 10% (v/v) of carbon dioxide and incubated at the same condition for 12h, and then the medium was used as inocula for SSCF experiments. The dry cell mass of the inocula was determined to be 2.1-2.3 g/L. All the medium and solutions were sterilized at 121 ^{o}C for 10 min before inoculation.

2.5 SSCF in batch mode

SSCF batch experiments were carried out anaerobically in 250 ml Erlenmeyer flasks with a working volume of 100 ml. An Innova 4080 incubator shaker (New Brunswick Scientific Co.) was used to control the temperature (37^{o}C) and provide agitation (150 rpm). The addition of substrate (SAA pretreated and water washed corn stover) was based on 3 g of glucan input. The pH of the fermentation media was automatically controlled by addition of 6 g calcium carbonate. The predetermined pretreated corn stover, calcium carbonate, yeast extract, and cCSL were added into the flasks and sterilized together at 121^{o}C for 10 min. After the flasks cooled down, inocula and enzyme were added. The flasks were then flushed with sterile nitrogen, capped, and placed in the incubator to start the SSCF. Samples were taken at given times, and after sampling, the flasks were flushed with nitrogen again to maintain the anaerobic environment.

2.6 SSCF in fed- batch mode

The assays of fed-batch SSCF were run in duplicates. The start of the fed-batch experiments was generally identical to that of the batch experiments with the only exception that double amount of calcium carbonate (12 g) was added for pH control. In each of the feeding operations, SAA pretreated and water washed corn stover containing 3 g glucan was fed into each of the flasks. Together added was 2 ml of diluted Spezyme CP enzyme having a specific activity of 7.5 FPU/ml. Therefore, the enzyme loading was maintained at 5 FPU/g-glucan throughout the experiments. The flasks were flushed with nitrogen before being placed back into the incubator shaker.

2.7 Statistical experimental design and result analysis

A central composite design (Box and Draper, 1987) with eight star points and four replicates in the center point was used to identify the significance of the variables to lactic acid yield. The experimental runs were carried out in randomized order. The four independent variables were enzyme loading (X1), inocula size (X2), yeast extract concentration (X3), and cCSL concentration (X4); and their respective levels (uncoded and coded) were listed in Table 15. The result assessment was carried out by using the SAS 9.1 ADX program (SAS Institute Inc., Cary, NC, USA). The response surface analysis was conducted to examine the feasibility of substituting the inexpensive nitrogen source (cCSL) for the expensive one (yeast extract) in SSCF.

2.8 Analysis

Vials containing slurry samples from SSCF flasks were boiled for 5 min to denature the enzyme and kill the cells. The boiled slurry samples were centrifuged at

Table 15 Factors and levels in statistical experimental design

Factor	Label	Levels				
		-2	-1	0	1	2
X1	Enzyme loading (FPU/g-glucan)	2.5	5	7.5	10	12.5
X2	Inoculum (v/v)	1%	2%	3%	4%	5%
X3	Yeast extract (w/v)	0	0.2%	0.4%	0.6%	0.8%
X4	cCSL (w/v)	0	0.5%	1.0%	1.5%	2.0%

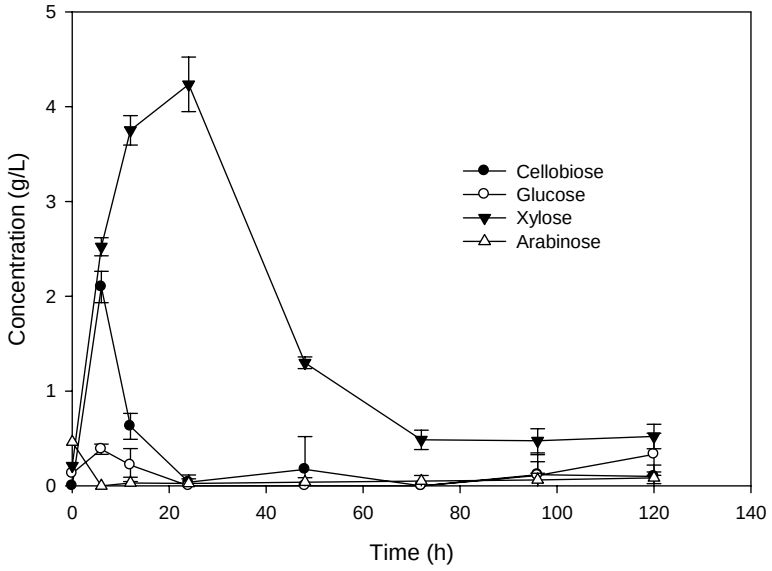
60,000 g for 5 min, and the supernatant was taken for analyses of sugars and acids by HPLC operated with Bio-Rad Aminex HPX-87H column and a refractive index detector. The HPLC was operated at 65 ^{o}C with a flow rate of 0.55 mL/min. The carbohydrate, acetyl and lignin contents in the solids were measured by following NREL Standard Analytical Procedure (NREL, 1996).

3. Results and discussion

3.1 Formation of sugars and acids in SSCF process

Fig. 25 shows the time courses of sugars and acids in a replicated SSCF assay, which was conducted in batch mode with 7.5FPU/g-glucan, 3% (v/v) inoculum, 0.4% (w/v) yeast extract, and 1% (w/v) cCSL. This assay represented the center point in the statistical experimental design (Table 15). The presence of sugars and acids at time zero were introduced from the additions of yeast extract, cCSL, and inocula. Glucose and arabinose remained at low levels (less than 0.5 g/L) throughout, indicating that these sugar species were efficiently assimilated after being generated (Fig. 25 a). The curve of cellobiose showed the similar pattern except that before 6h, cellobiose accumulated up to 2g/L due to the insufficient cellobiase activity of the hydrolysis enzyme and the lack of cellobiose-assimilating capability of this microorganism. The assimilation patterns of these three sugars (glucose, arabinose, and cellobiose) in this study resembled those in most SSF or SSCF processes, wherein sugars were converted so rapidly that the hydrolysis became the control step (Rivas et al., 2004; McMillan et al., 1999). However, the assimilation of xylose was rather slow compared to those of glucose and arabinose. As the curve indicates, the concentration of xylose increased almost linearly before 12 h

(a)



(b)

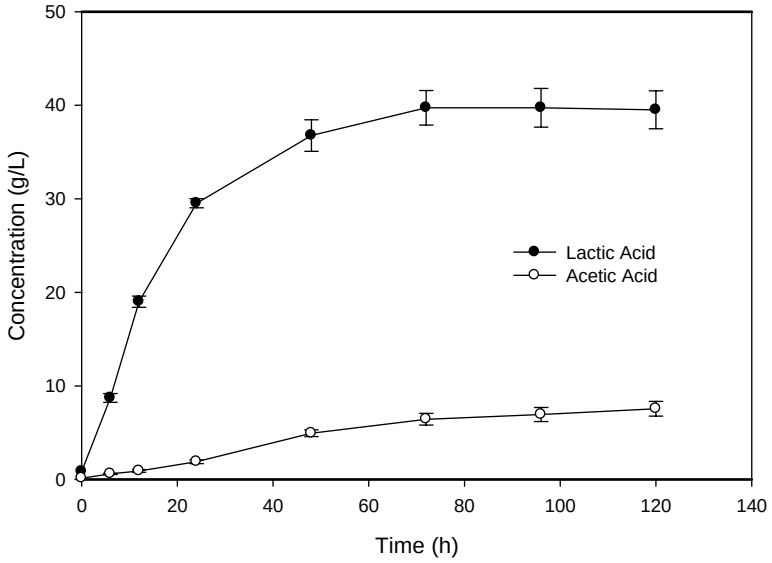


Fig. 25. Concentrations of sugars (a) and acids (b) in simultaneous saccharification and co-fermentation of aqueous ammonia treated corn stover. Average data from four replicates with standard deviations are presented. SSCF conditions: 3% (w/v) glucan loading, 7.5FPU/g-glucan, 3% (v/v) inocula, 0.4% (w/v) yeast extract, 1% (w/v) cCSL, 37 ^{o}C, pH 5.7, 150 rpm.

and further increased up to 4.2g/L before it started to decrease. Coinciding with the switch, the production of lactic acid decelerated; whereas the accumulation of acetic acid accelerated (Fig. 25 b). This is an indication that glucose (and probably arabinose as well) had been depleted and xylose assimilation became the predominant reaction, because the assimilation of xylose forms acetic acid in addition to lactic acid while the assimilation of glucose under anaerobic conditions gives only lactic acid (Garde et al., 2002).

Table 16 shows the yields of lactic acid and acetic acid (of theoretical maximum) under different SSCF conditions, which are defined by the central composite design and represented with coded factors. Since galactose and mannose occupied very small percentages relative to glucose, xylose, and arabinose, only the latter three were used as the base for yield calculations. The yields were calculated after deducting the contributions of acids at time zero, and assuming the assimilations of hexose (glucose) and pentoses (xylose and arabinose) respectively uses EMP and PK pathways. The data indicates that the final (120 h) lactic acid yield fell between 0.79-0.92. And it can be seen that the lactic acid yield can easily reach above 0.9 under most of the reaction conditions in this study. Table 16 also shows that the acetic acid yield varied between 0.7 and 1.2, depending on the reaction conditions. Some of the acetic acid yields exceeded 1.0 probably because, although carefully controlled, some oxygen still remained in the flasks. This could make part of the glucose assimilation take PK pathway, leading to the production of acetic acid in addition to lactic acid (Litchfield, 1996).

Fig. 26 depicts the average yields of lactic acid and acetic acid from all the 28 runs at different time intervals. Apparently, the average lactic acid yield leveled off after 72 h, while the acetic acid yield continuously increased even after 120 h. The reason for

Table 16 Statistical experimental design and acid yields (of theoretical maximum)

Coded levels				Lactic acid							Acetic acid						
X1	X2	X3	X4	6h	12h	24h	48h	72h	96h	120h	6h	12h	24h	48h	72h	96h	120h
-1	-1	-1	-1	0.11	0.22	0.40	0.66	0.74	0.82	0.87	0.04	0.08	0.11	0.21	0.38	0.58	0.72
-1	-1	-1	1	0.11	0.28	0.53	0.77	0.88	0.89	0.90	0.05	0.10	0.17	0.47	0.72	0.85	0.95
-1	-1	1	-1	0.13	0.38	0.61	0.80	0.83	0.82	0.83	0.06	0.16	0.38	0.77	0.94	1.03	1.13
-1	-1	1	1	0.11	0.38	0.62	0.78	0.85	0.86	0.87	0.07	0.13	0.32	0.70	0.84	0.89	0.95
-1	1	-1	-1	0.14	0.27	0.52	0.73	0.82	0.86	0.90	0.07	0.10	0.18	0.43	0.65	0.79	0.86
-1	1	-1	1	0.15	0.32	0.56	0.71	0.76	0.77	0.80	0.09	0.15	0.26	0.60	0.92	1.10	1.21
-1	1	1	-1	0.16	0.40	0.61	0.77	0.85	0.83	0.83	0.08	0.17	0.39	0.73	0.95	1.02	1.19
-1	1	1	1	0.17	0.42	0.64	0.78	0.87	0.86	0.87	0.08	0.15	0.37	0.70	0.92	0.95	1.05
1	-1	-1	-1	0.12	0.26	0.49	0.70	0.78	0.81	0.85	0	0.03	0.06	0.24	0.47	0.66	0.80
1	-1	-1	1	0.13	0.33	0.60	0.76	0.89	0.91	0.91	0	0.04	0.10	0.37	0.69	0.89	0.97
1	-1	1	-1	0.13	0.45	0.70	0.86	0.89	0.90	0.91	0.03	0.11	0.30	0.71	0.82	0.83	0.89
1	-1	1	1	0.13	0.48	0.74	0.89	0.92	0.88	0.90	0.03	0.11	0.35	0.76	0.93	0.96	1.18
1	1	-1	-1	0.15	0.30	0.52	0.73	0.8	0.86	0.91	0.03	0.05	0.05	0.25	0.48	0.66	0.77
1	1	-1	1	0.17	0.38	0.64	0.79	0.85	0.89	0.90	0.06	0.11	0.17	0.41	0.69	0.89	1.01
1	1	1	-1	0.21	0.52	0.73	0.82	0.89	0.89	0.89	0.09	0.15	0.39	0.75	0.89	1.03	1.07
1	1	1	1	0.20	0.53	0.78	0.91	0.93	0.92	0.92	0.08	0.15	0.44	0.82	0.89	0.89	0.98
-2	0	0	0	0.11	0.26	0.42	0.59	0.68	0.74	0.80	0.07	0.11	0.27	0.50	0.66	0.87	0.98
2	0	0	0	0.19	0.46	0.73	0.84	0.89	0.88	0.90	0.05	0.14	0.35	0.65	0.84	0.98	1.13
0	-2	0	0	0.09	0.35	0.62	0.76	0.91	0.84	0.90	0.02	0.10	0.22	0.64	0.92	0.91	1.03
0	2	0	0	0.20	0.45	0.68	0.89	0.87	0.90	0.85	0.09	0.15	0.33	0.80	0.96	1.08	1.25
0	0	-2	0	0.08	0.17	0.33	0.62	0.71	0.72	0.79	0.02	0.04	0.06	0.07	0.16	0.25	0.34
0	0	2	0	0.15	0.48	0.72	0.87	0.88	0.91	0.86	0.08	0.15	0.45	0.80	0.91	1.00	1.08
0	0	0	-2	0.16	0.36	0.59	0.68	0.82	0.81	0.86	0.08	0.13	0.25	0.60	0.88	0.91	0.99
0	0	0	2	0.15	0.43	0.69	0.88	0.89	0.84	0.86	0.07	0.12	0.30	0.74	1.02	1.09	1.24
0	0	0	0	0.18	0.41	0.65	0.78	0.79	0.80	0.79	0.09	0.12	0.30	0.76	1.01	1.10	1.19
0	0	0	0	0.17	0.41	0.64	0.84	0.90	0.90	0.92	0.07	0.15	0.23	0.65	0.80	0.84	0.92
0	0	0	0	0.15	0.40	0.63	0.79	0.88	0.89	0.88	0.06	0.10	0.26	0.66	0.90	0.98	1.09
0	0	0	0	0.16	0.38	0.64	0.83	0.88	0.90	0.89	0.07	0.11	0.26	0.70	0.91	0.99	1.11

Note: the assimilations of glucose and xylose are assumed to follow the EMP and PK pathways, respectively.

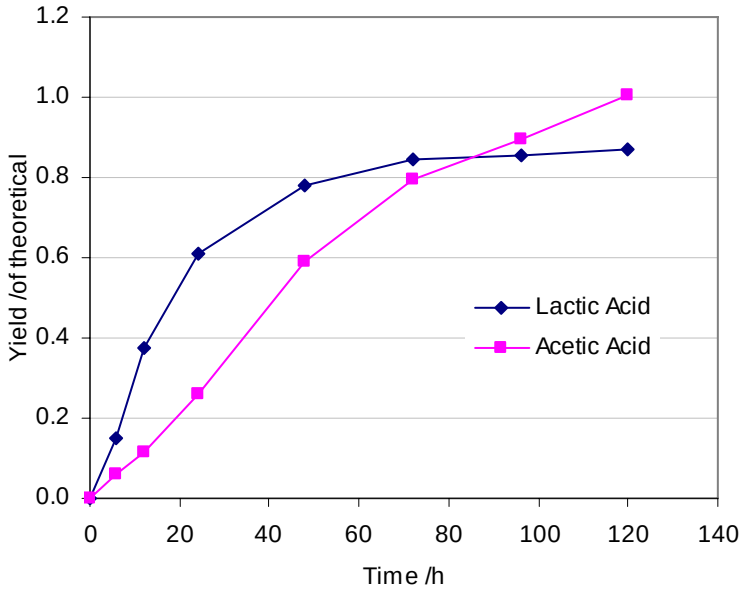


Fig. 26. Average yields of lactic acid and acetic acid from 28 runs at different time intervals. The assimilations of hexose (glucose) and pentose (xylose and arabinose) are assumed to follow the EMP and PP pathways, respectively.

the discrepancy is unclear at this point. It is postulated that under high lactate/acetate concentrations, the metabolic pathway of xylose assimilation shifted significantly such that the acetic acid became the primarily, if not the sole, product of the fermentation. Since lactic acid is the product of interest in this study, the data obtained at 72h is taken for response surface study.

3.2 Statistical analysis and response surface

The data associated with lactic acid yield was analyzed, and the estimates of the main effects and the interactions of the factors are listed in Table 17. The significances of the factors are represented by the probability levels (p values). Of the four factors under investigation, enzyme loading (X1) and yeast extract concentration (X3) are the most important factors responsible for lactic acid production, followed by cCSL concentration (X4) and inoculum size (X2). Besides the main effects, the quadratic term of enzyme loading (X1) also played an important role in lactic acid production. It is notable that the inoculum size was insignificant for final lactic acid yield despite the fact that it affected the lactic acid productivity during the early stage (<24h). This implies that the inocula addition for the SSCF process can be maintained at a rather low level.

In general, lactic acid bacteria are nutritionally fastidious. Costly nitrogen sources like yeast extract and peptone have been commonly provided for lactic acid bacteria to grow fast and function effectively (Mercier et al., 1992; Hujanen and Linko, 1996; Nancib et al., 2001). In order to improve the process profitability, a number of efforts have focused on using inexpensive nitrogen sources to replace the expensive ones. Corn steep liquor has been proposed as an alternative cost-effective nitrogen source for a range of cells (Amartey and Jefferies, 1994; Lawford and Rousseau, 1997; Rivas et al., 2004;

Table 17 Coefficient estimates of polynomial models (using coded levels) for lactic acid yield and significance examination.

Term	12h			24h			48h			72h		
	Estimate	Std error	t value	Estimate	Std error	t value	Estimate	Std error	t value	Estimate	Std error	t value
X1	0.040408	0.002464	16.3964[a]	0.055415	0.006664	8.315467[a]	0.040398	0.009114	4.432355[a]	0.031773	0.008461	3.755105[b]
X2	0.022379	0.002464	9.080788[a]	0.017545	0.006664	2.63269[c]	0.011352	0.009114	1.245449	-0.003163	0.008461	-0.37387
X3	0.07632	0.002464	30.96807[a]	0.081493	0.006664	12.22858[a]	0.052529	0.009114	5.763356[a]	0.035861	0.008461	4.238344[a]
X4	0.020053	0.002464	8.136795[a]	0.030001	0.006664	4.501869[a]	0.029809	0.009114	3.270491[b]	0.020496	0.008461	2.422365[d]
X1*X1	-0.00944	0.002464	-3.82926[b]	-0.01531	0.006664	-2.29738[c]	-0.020952	0.009114	-2.29878[c]	-0.016618	0.008461	-1.96404[d]
X1*X2	0.003612	0.003018	1.196645	-0.002035	0.008162	-0.24932	0.004215	0.011163	0.377627	1.62E-05	0.010363	0.001562
X1*X3	0.013821	0.003018	4.578959[a]	0.015459	0.008162	1.894112	0.014918	0.011163	1.336404	0.006814	0.010363	0.657579
X1*X4	0.00314	0.003018	1.040322	0.007216	0.008162	0.884107	0.009693	0.011163	0.868298	0.007069	0.010363	0.68215
X2*X2	0.000212	0.002464	0.086155	0.003968	0.006664	0.595405	0.006501	0.009114	0.71331	0.009067	0.008461	1.071636
X2*X3	-2.20E-05	0.003018	-0.00727	-0.008185	0.008162	-1.00283	-0.007012	0.011163	-0.62817	0.008594	0.010363	0.8293
X2*X4	0.000964	0.003018	0.319473	-0.002577	0.008162	-0.3157	-0.001427	0.011163	-0.12781	-0.015373	0.010363	-1.48345
X3*X3	-0.01763	0.002464	-7.15521[a]	-0.026139	0.006664	-3.92241[b]	-0.012758	0.009114	-1.39975	-0.014194	0.008461	-1.67758
X3*X4	-0.0109	0.003018	-3.61063[b]	-0.016209	0.008162	-1.98593	-0.007115	0.011163	-0.6374	-0.007789	0.010363	-0.75163
X4*X4	-0.00017	0.002464	-0.07025	0.002656	0.006664	0.398492	-0.005246	0.009114	-0.57559	0.00112	0.008461	0.132371
R^2		0.9913			0.9554			0.8555			0.7992	

[a] p<0.001; [b] p<0.01; [c] p<0.05; [d] p<0.1

Tellez-Luis et al., 2003). Lawford et al. (1997) reported that in the SSCF biomass-to-ethanol process using recombinant *Zymomonas*, yeast extract could be entirely replaced with corn steep liquor without affecting the growth and fermentation performance of the microorganism. Patel et al. (2004) reported that in the lactic acid fermentation of hemicellulose hydrolyzate obtained from acid hydrolysis of sugar cane bagasse, a theoretical yield of 89% was attainable by using 0.5% corn steep liquor as the only organic nitrogen source for a thermotolerant acidophilic *Bacillus* sp.

In this study, clarified corn steep liquor (cCSL) was tested as a nutrition supplement for *L. pentosus* ATCC 8041. Fig. 27 shows the response surface representing the 72-h lactic acid yield as a function of yeast extract and cCSL concentrations. The lactic acid yield increased linearly with both yeast extract and cCSL concentration. The almost linear effect of yeast extract concentration on lactic acid production by *L. casei* has been reported by Hujanen and Linko (1996) using the same statistical analysis method. Further examination of the response surface shows that yeast extract can be replaced by cCSL, with an estimated ratio of 1:5 (g yeast extract/g cCSL), to achieve equivalent lactic acid yield.

3.3 Fed-batch experiment

Lactic acid concentration is a key factor affecting the costs of the downstream recovery process. In this study, the concentration of lactic acid was improved by using fed-batch technique. In accordance with the statistical analysis and taking into consideration of the costs, the enzyme loading was set at 5 FPU/g-glucan and inoculum size 1% (v/v). The yeast extract was maintained at a low level of 0.2% (w/v); while cCSL was allowed at a comparatively high level (2% [w/v]) to compensate for the low yeast

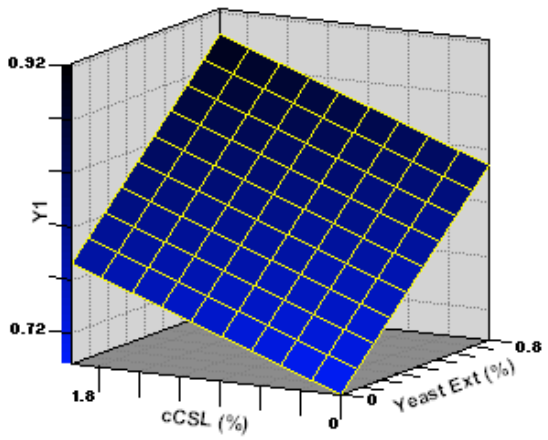


Fig. 27. Response surface representing lactic acid yield (Y1) as a function of yeast extract and clarified corn steep liquor (cCSL) concentrations. Enzyme loading: 5 FPU/g-glucan; inoculum size: 1% (w/w).

extract concentration. Four batches of SAA treated and washed corn stover was fed into the vessels at 0, 36, 72, and 108 h, resulting in a cumulative glucan addition of 12 g. The time intervals for substrate additions were chosen in such a manner that the previous batch of solids were well liquefied by observation. Proportionally added was cellulase enzyme, the level of which in the SSCF media was maintained at 5 FPU/g-glucan.

Shown in Fig. 28 (a) are the concentrations of lactic acid and acetic acid, as well as the lactic acid productivity in the fed-batch experiment. The abrupt drops at certain points (36, 72, and 108 h) were due to the feeding operations, which suddenly increased the working volume of the SSCF medium. The maximum lactic acid concentration achieved in the experiment was 74.8 g/L. Lactic acid increased fast before 72 h with an average productivity of more than 0.7 g/l · h, indicating that the microorganisms exerted high activity at the early stage. This value is comparable to that reported by Bustos et al. (2004) in the fermentation of hemicellulose hydrolyzate from vine-trimming waste, which was 0.800 g/l · h after 24 h. On the other hand, the productivity of lactic acid in this study rapidly reduced with time. For example, at 108 h, the productivity decreased to 0.38 g/l · h, and further decreased to 0.05 g/ l · h at 288 h. It is unlikely that the decrease in productivity resulted from the lack of available carbon source in the fermentation medium, as evidenced by the accumulations of sugars in the reaction (Fig. 28 (b)). Also, the agar plates verified that the cells remained high viability at the end of the operation (data not shown). Therefore, it is concluded that the drastic decrease in the lactic acid productivity was attributed to the strong inhibition of the elevated concentration of lactate/acetate anions to the lactic acid bacteria. The inhibitory effect of lactic acid (lactate ions) on *lactobacillus* strains has been well documented. Iyer and Lee (1999)

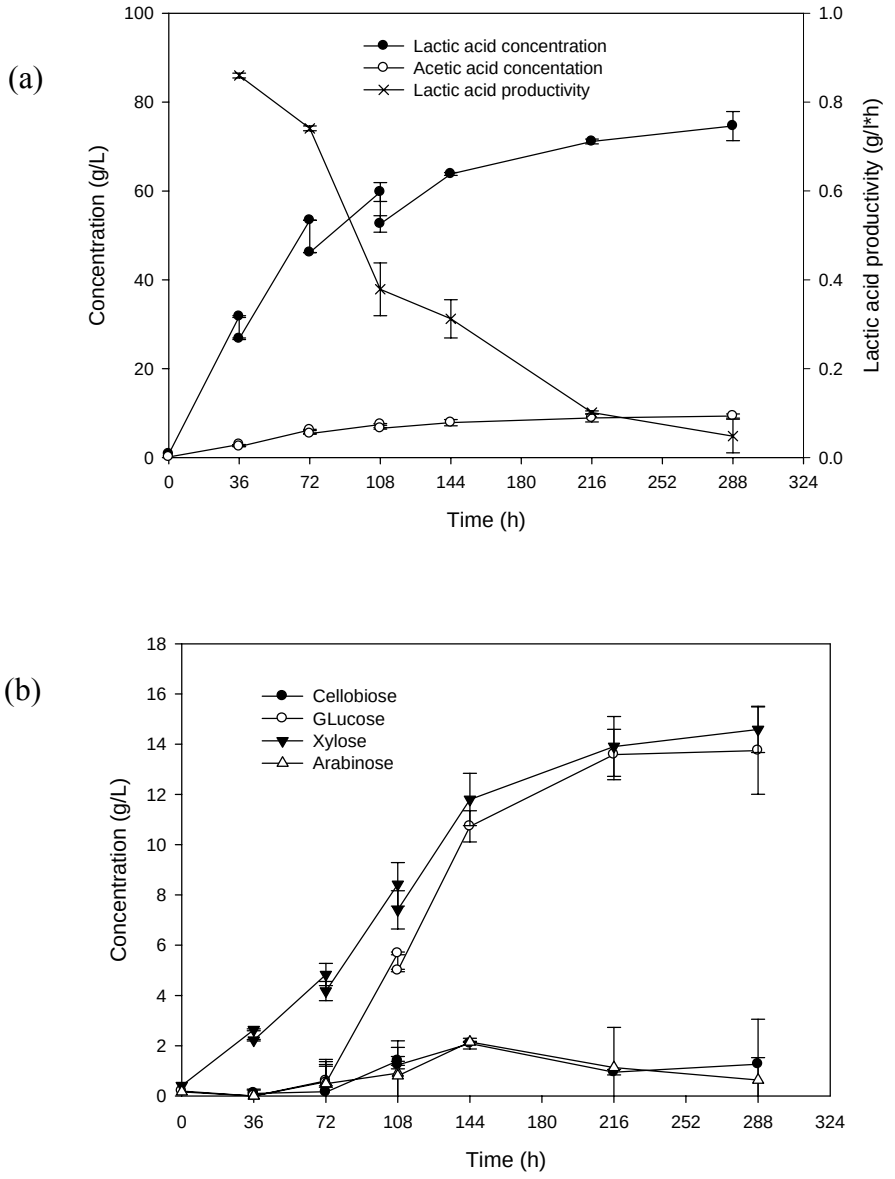


Fig. 28. Sugar concentrations (a), acid concentrations and lactic acid productivity (b) in fed-batch SSCF experiments. Average data from duplicates with standard deviations are presented.

reported that the *L. delbrueckii* NRRL-B445 strain was strongly inhibited when lactic acid reached 65g/L. Bustos et al. (2005) observed marked inhibition to *L. pentosus* ATCC 8041 by lactic acid as the lactic acid concentration reached up to 46.0g/L.

In addition, Fig. 28 (a) shows comparatively low acetic acid concentration throughout the experiment, indicating that the fermentation of xylose was not efficient. The yields of lactic acid and acetic acid at 288 h were only 0.65 and 0.51, respectively. If the complete utilization of carbohydrates is desirable, the inhibitory effect of the acid products must be taken into consideration before substrate additions. As an illustration, another series of fed-batch experiment was conducted with reduced substrate addition. In this experiment, SAA treated and water washed corn stover containing 6 g of glucan (versus 12 g of glucan in the earlier fed-batch SSCF) was evenly added in two batches, one at time zero and the other at 36 h. The results are summarized in Fig. 29. Unlike in the earlier fed-batch SSCF, the xylose concentration decreased at the late stage of this experiment, although at a fairly low rate as compared with that of batch SSCF (Fig. 25 (a)). At the end of the run (144h), the lactic acid concentration reached 61.8 g/L, and the acetic acid 8.8 g/L. In comparison to the earlier fed-batch experiments, both lactic and acetic yields obtained in this experiment were remarkably higher (0.81 and 0.80 vs. 0.65 and 0.61), indicating that the inhibition was much less pronounced..

4. Conclusions

This study showed that SAA pretreated corn stover is a suitable substrate for lactic acid production. The cellulose and hemicellulose fractions in the pretreated corn stover are effectively converted to lactic acid by simultaneous saccharification and co-fermentation (SSCF). The maximum lactic acid yield was above 90% of theoretical

maximum on the basis of all available fermentable sugars. The significance of enzyme, inocula, yeast extract, and cCSL (clarified corn steep liquor) for lactic acid production were examined using statistical experiment design. It was found that the concentrations of enzyme and yeast extract are the two most significant factors affecting the lactic acid yield. Impact of inocula size was insignificant within the range of study (from 1% to 5% [w/w]). The response surface study indicates that the yeast extract can be replaced by the less expensive substitute, cCSL, without affecting lactic acid yield. Acetic acid was also produced in the process mainly as a result of pentose assimilation (xylose and arabinose) through phosphoketolase (PK) pathway. The fed-batch operation increased the lactic acid product concentration to 74.8 g/L. Due to product inhibition, further improvement of the product concentration was difficult. Given the low feedstock price, the use of corn stover as the feedstock may be a viable option for lactic acid production.

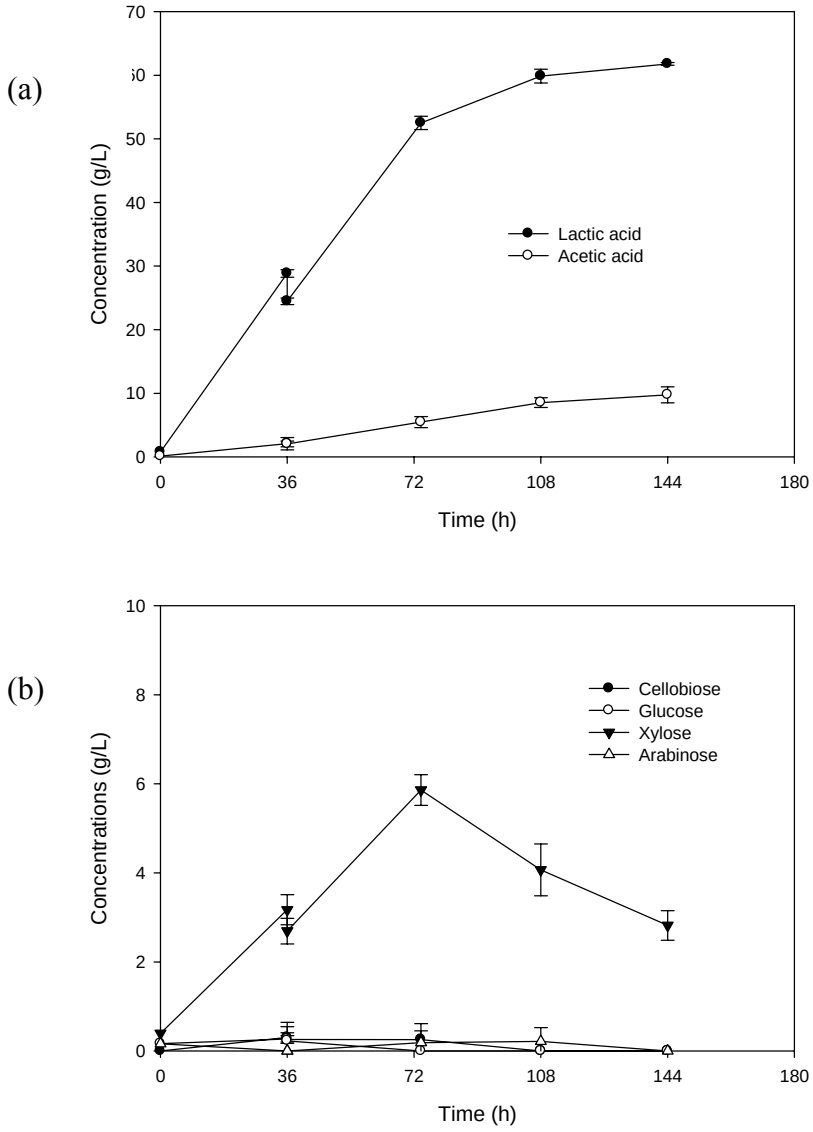


Fig. 29. Profiles of sugar concentrations (a) and acid concentrations (b) in fed-batch SSCF experiments with reduced substrate addition. Average data from duplicates with standard deviations are presented.

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