BIOCONVERSION OF LIGNOCELLULOSIC MATERIAL INTO ETHANOL:

PRETREATMENT, ENZYMATIC HYDROLYSIS,

AND ETHANOL FERMENTATION

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BIOCONVERSION OF LIGNOCELLULOSIC MATERIAL INTO ETHANOL: PRETREATMENT, ENZYMATIC HYDROLYSIS, AND ETHANOL FERMENTATION

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VITA

Tae Hyun Kim, son of Ih Chool Kim and Young Ja Ahn, was born on October 1, 1969, in Seoul, Korea. He graduated from Kon Kuk High School in Seoul, Korea in 1988. He entered Han Yang University in Seoul, Korea the following spring. He graduated with the degree of Bachelor of Science in Chemical Engineering in February, 1994. He went on to work as an engineer for the LG Chemical Co. Ltd., in Chung Ju, Korea and then as a process engineer at Samsung Engineering Co. Ltd, Seoul, Korea. Following this professional experience, he entered the Graduate School at Auburn University in September 1999 to pursue the degree of Doctor of Philosophy in Chemical Engineering. He married Mina Park, daughter of Si Kyung Park and Kye Soon Jo, on July 20, 2001..

DISSERTATION ABSTRACT

BIOCONVERSION OF LIGNOCELLULOSIC MATERIAL INTO ETHANOL-PRETREATMENT, ENZYMATIC HYDROLYSIS, AND ETHANOL FERMENTATION

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Four different novel processes, ammonia recycle percolation (ARP), low-liquid ARP, two-stage (hot water-ARP) percolation, and soaking in aqueous ammonia (SAA) at room/moderate temperature, were investigated for pretreatment of lignocellulosic material (corn stover). These pretreatment methods were aimed at improving enzymatic hydrolysis and increasing the fermentability of the biomass.

The ARP process feeds aqueous ammonia into a flow-through reactor at high temperatures and high pressures. This method is highly effective in delignifying biomass, reducing the lignin content by 70–85%. FTIR (Fourier transform infrared spectroscopy) and lignin staining results verify the lignin removal by ARP process. The SEM (scanning electron microscope) pictures indicate that the biomass structure is deformed and its fibers are exposed by the pretreatment. ARP pretreatment increases the crystallinity index as the amorphous portion of biomass is removed. The crystalline structure of the biomass cellulose, however, is not changed by the ARP treatment.

Low-liquid ammonia treatment method reduced the liquid throughput to the level of 3.3 mL of liquid per gram of corn stover, leading to a shorter residence time and lower energy requirements. A high degree of delignification is not necessary to attain high enzymatic digestibility or high ethanol yield. An ethanol yield of 85% of the theoretical maximum was achieved using the low-liquid ARP treatment with SSF (simultaneous saccharification and fermentation) process.

The two-stage process combines the hot water and ARP treatments, using a flow-through (percolation) reactor. The first stage hot water processing removes hemicellulose and the second stage ARP performs the delignification. A high fractionation of the biomass was achieved using the two-stage treatment; resulting in 92–95% xylan hydrolysis and xylose yield of 83–86% with 75–81% lignin removal. The solid residue after two-stage treatment contained 78−85% cellulose.

A simpler alternative pretreatment process was also investigated. In this process, corn stover was soaked in 30% aqueous ammonia for 10 days at room temperature (SAA at room temperature) or in 15% for 12 hours at 60°C (SAA at moderate temperature), with no agitation under atmospheric pressure in a closed vessel. This process retains 85% of the xylan and removed 55–67% of the lignin. The treated corn stover was fermented to ethanol by the simultaneous saccharification and co-fermentation process using a recombinant *E.coli*. This organism utilized both glucan and xylan in the biomass, producing ethanol yield of 77.0–77.3% based on total glucan and xylan, and an ethanol concentration of 19.2−19.8 g/L . The advantage of this method is that the process is simple and yet provides high fermentability. The ethanol yield based on glucan alone was 113–116%, a clear indication that most of xylan is converted to ethanol during the SSCF.

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I. INTRODUCTION

For several decades, ethanol has been promoted as a promising alternative fuel for transportation. The use of fossil fuels has contributed to the buildup of carbon dioxide in the atmosphere; however ethanol is a clean-burning fuel that makes no net contribution to global warming because the carbon dioxide produced by the combustion of ethanol is consumed by plant growth which continues the carbon cycle balance in the nature.

Ethanol can be produced from inexpensive and abundant lignocellulosic biomass. It is important that we look for sources of lignocellulosic biomass from which ethanol can be produced at a reasonable cost with available resources. Corn stover is currently an abundant biomass resource that is widely available in the United States, with about 80–100 million dry t/year of corn stover being available based on recent estimates (Kadam and McMillan, 2003)

The biological conversion of ethanol from lignocellulosic biomass can be achieved by pretreatment, enzymatic hydrolysis and fermentation. The simultaneous saccharification and fermentation (SSF) process was first introduced in 1977, and it gives high yields and rates for ethanol production (Ghosh et al., 1984; Pemberton et al., 1980). Pretreatment is an essential step to improve the ethanol yield of SSF from a lignocellulosic biomass. Production of ethanol from lignocellulosic biomass is a very different system than that used for corn grain because carbohydrates are much more difficult to solubilize than the starch in grain (Gibbson et al., 1986). Lignocellulosic

material is very resistant to enzymatic breakdown, requiring pretreatment in order to enhance the susceptibility of the biomass to the enzyme. There have been numerous attempts made to enhance the enzymatic reaction, including methods such as steam/steam explosion (Fernandez-Bolanos et al., 1999; Mes-Hartree et al., 1984; Sawada et al., 1995; Schwald et al., 1988), grinding/milling (Caufield and Moore, 1974; Koullas et al., 1990; Matsumura et al., 1977; Puri, 1984; Sintsyn et al., 1991), hot water/autohydrolysis (Allen et al., 2001; Garrote et al., 2002; Váquez et al., 2001), acid treatment (Burns et al., 1989; Grethlein, 1985; Jacobsen and Wyman, 2000; Kim et al., 2001; Mok et al., 1992), alkali treatment (Ferrer et al., 2000; Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003; Tarkow and Feist, 1969), and other methods (McGinnis et al., 1983; Zheng et al., 1998). Aqueous ammonia has been used for the pretreatment because it has a number of desirable characteristics, such as its ability to swell cellulosic material and its selectivity for lignin. The ammonia recycled percolation process (ARP) has been investigated as a promising approach by our laboratory (Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003).

Pretreatment of biomass based on aqueous ammonia

Ammonia has a number of desirable characteristics as a pretreatment reagent. It is an effective swelling reagent for lignocellulosic materials and has a high selectivity for reactions with lignin over those with carbohydrates. It is one of the most widely used commodity chemicals, with about one-fourth the cost of sulfuric acid on a molar basis. Its high volatility makes it easy to recover and reuse. It is a non-polluting and non-corrosive chemical. One of the known reactions of aqueous ammonia with lignin is the cleavage of

C-O-C bonds in lignin, as well as the ether and ester bonds in the lignin-carbohydrate complex (LCC). Indications are that ammonia pretreatment selectively reduces the lignin content in a biomass. There are many advantages to removing lignin early in the conversion process, before it is subjected to biological processing, as lignin is believed to be a major hindrance in enzymatic hydrolysis (Chaing and Knight, 1960; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988). Lignin and its derivatives are toxic to microorganisms and inhibit enzymatic hydrolysis. Low-lignin substrates have improved microbial activity and enzyme efficiency, thus lowering the enzyme requirement.

We have previously investigated various pretreatment processes using a flow-through (percolation) reactor system in our laboratory. Among these is the ammonia recycled percolation (ARP) process, which we have studied for the pretreatment of various lignocellulosic biomass feedstocks, including hardwood (Yoon et al., 1995), herbaceous biomass (Iyer et al., 1996; Kim and Lee, 1996), and pulp mill sludges (Kim et al., 2000). Process modifications have also been attempted using additional reagents such as hydrogen peroxide (Kim et al., 2000; Kim and Lee, 1996).

The latest version of the ammonia-based pretreatment method was proposed by Kim and Lee (2003). This novel pretreatment method, which requires a long reaction time at a lower temperature (room temperature), was tested and compared to the results from previous trials using short reaction times at relatively high temperatures. In this method, corn stover is soaked in 30% aqueous ammonia for an extended period (10–60 days) at room temperature, without agitation, under atmospheric pressure in a closed vessel. Therefore, it is referred to as the “Soaking in Aqueous Ammonia (SAA) process”.

The room temperature treatment was attempted because of the reduced heat input needed during the treatment phase, and the low interaction of ammonia with hemicellulose. Retention of xylan is not a negative factor in pretreatment since it can be hydrolyzed by the xylanase activity normally embodied in “cellulase”.

However, this SAA at room temperature process encountered two main problems: the high solid-to-liquid ratio and very long treatment time (8–10 days) needed to achieve appreciable pretreatment effect. This would lead to a high operating costs and a large capital cost. In order to resolve the two main problems of the old SAA process, the “soaking in aqueous ammonia process at moderate temperature” was introduced. In this process, the corn stover is pretreated using the SAA process at moderate temperatures (40–90 °C) for a relatively short treatment time (4–24 h). The intent here was to reduce the reaction time to <1 day.

The primary purpose of this investigation was to assess the effectiveness of the ammonia based pretreatment process for corn stover. We were interested in verifying the changes in chemical composition and physical characteristics caused by the pretreatment and how those factors affect enzymatic digestibility and fermentability.

Objectives

The overall objective of this study is to improve the ethanol yield and enhance our understanding of the mechanisms involved in the pretreatment of biomass.

Specifically, the study aimed:

- To develop an economically viable and environmentally benign process for the pretreatment of biomass.
- To improve the yield in the enzymatic hydrolysis and fermentation step by investigating the factors affecting (inhibiting) the enzymatic hydrolysis step.
- To develop a method for efficient pentose fermentation into ethanol.

II. LITERATURE REVIEW

BIOETHANOL: ETHANOL AS FUEL

The United States and much of the world are faced with complex economic and environmental issues associated with energy use that must be addressed if we are to maintain and improve our lifestyles. The largest single portion (about 40%) of the energy used in the United States is petroleum-derived fuel, which makes up more than half of the imported energy (U.S. Department of Energy, 1995). The United States and many other countries are working to develop new sources of energy that would reduce oil imports and improve their strategic and economic strength (Wyman, 1996).

One fuel that has the potential to match the convenient features of petroleum at a low price is ethanol produced from lignocellulosic biomass resources-conveniently referred to as bioethanol. (Lynd et al., 1991).

Ethanol can be produced from inexpensive and abundant lignocellulosic biomass such as agricultural and forestry residues, wastepaper, a significant fraction of municipal solid waste, and woody and herbaceous energy crops grown as feedstocks for ethanol production. However, even though lignocellulosic biomass provides a low-cost resource, it is difficult to convert to ethanol (Wyman, 1996).

A promising approach is to break down the cellulose and hemicellulose chains, which comprise two-thirds to three-quarters of the biomass, into their component sugars and then ferment those sugars into ethanol. Ethanol from lignocellulosic biomass

(bioethanol) can now be produced at a cost competitive with the market price of ethanol derived from corn.

Ethanol is already an important element of transportation fuel production. About 12% of U.S. gasoline sold contains 8% to 10% ethanol as a fuel additive to boost octane and reduce carbon monoxide and other toxic air emissions. About 25% of U.S. gasoline now has a petroleum-derived additive that it would be advantageous to phase out because of water pollution concerns. Because ethanol can directly replace this additive, ethanol can also be used as an alternative fuel (typically in an 85% blend) to reduce the U.S.'s dependence on foreign oil (which currently supplies more than half the nation's petroleum supply). Making ethanol from the starch in corn grain already supports a $2 billion per year industry in the U.S. alone, with 55 ethanol plants providing jobs and economic stimulus to rural areas in the year 2000 and a dozen more planned for the future (NREL, 2001).

Bioethanol as a versatile fuel and fuel additive.

Ethanol can also be added to gasoline to reduce fossil fuel use, increase octane, and provide oxygen to promote more complete combustion and reduce exhaust emissions of carbon monoxide and unburned hydrocarbons. When reacted with isobutylene, ethanol forms ethyl tertiary butyl ether (ETBE), an emerging fuel additive that provides the same benefits when added to gasoline as direct ethanol addition while simultaneously reducing the vapor pressure of the mixture and the evaporative release of fuel compounds that contribute to ozone formation and smog (Wyman, 1996).

Environmental issues.

There is mounting concern about the buildup of carbon dioxide (CO_2) and other so-called greenhouse gases in the atmosphere, which could trap the heat that usually radiates from the earth, and cause global climate change (Houghton et al., 1990). The release of carbon dioxide is likely to be one of the largest contributors to this problem (NREL, 1994).

Total world carbon dioxide emissions from the consumption of fossil fuels increased by 11.4 percent from 1992 to 2001 (from 5.894 billion metric tons carbon equivalent in 1992 to 6.568 billion metric tons in 2001). The average annual growth rate of carbon dioxide emissions over the period was 1.2 percent. The United States, China, Russia, Japan, India, Germany, Canada, the United Kingdom, Italy, and South Korea were the world's ten largest sources of carbon dioxide emission from the consumption and flaring of fossil fuels in 2001, producing 64 percent of the world total. The United States was the largest producer of carbon dioxide from the consumption of petroleum in 2001, accounting for 24 percent of the world total. Japan was the second largest producer, followed by China, Russia, and Germany. Together, these four countries accounted for an additional 20 percent. (U.S. Department of Energy, 2001).

Bioethanol can be obtained from renewable sources with minimal emissions of carbon dioxide. Bioethanol production requires no fossil fuel input in the conversion step and ethanol is a clean-burning fuel that makes no net contribution to global warming because the crops/plants take it in during photosynthesis. In other words, its contribution of greenhouse gases to the atmosphere is minimal (Wyman, 1996). Adding ethanol into gasoline blends increases the oxygen content of the fuels and permits more complete

combustion of the hydrocarbons in the gasoline.

Because limited, if any, fossil fuel inputs are needed to produce bioethanol, and most of the carbon dioxide released during production and utilization of bioethanol can therefore be recycled to grow new biomass, the net release of CO_2 contributing to global climate change may be close to zero (Tyson et al., 1993).

CELLULOSE

Cellulose is the main constituent of wood. Approximately 40-45% of dry substance in most wood species is cellulose, located predominantly in the secondary cell wall (Sjöström, 1993). Cellulose molecules are completely linear and have a strong tendency to form intramolecular and intermolecular hydrogen bonds. Thus, bundles of cellulose molecules are aggregated together to form microfibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions. Microfibrils build up fibrils and, finally, cellulose fibers (Sjöström, 1993).

The free hydroxyl groups present in the cellulose macromolecule are likely to be involved in a number of *intra* and *inter* molecular hydrogen bonds which may give rise to various ordered crystalline arrangements (Hermans, 1949).

Cellulose chains are formed into microfibrils which constitute the basic framework of the cell, conveying a great resistance to tensile forces (Jarvis, 1984). Cellulose is a homopolysaccharide composed of β-D-glucopyranose units which are linked together by β–(1→4)-glycosidic linkages. Two adjacent glucose units are linked by elimination of one molecule of water between their hydroxylic groups at carbon 1 and

carbon 4. The cellobiose unit is the repeating unit of the cellulose chain, with a length of 1.03 nm (Fengel and Wegener, 1984).

Four principal allomorphs have been identified for cellulose: I, II, III and IV (Howsmon and Sisson, 1963). The natural form of cellulose, known as cellulose I or native cellulose, appears to be the most abundant form (Atalla and Van der Hart, 1984). Cellulose II is generally obtained by regeneration of cellulose from solution or by mercerization. This allomorph is known as "regenerated" cellulose. The transition from cellulose I to cellulose II is not reversible, implying that cellulose II is a stable form compared with the metastable cellulose I. Treatment with liquid ammonia or with certain amines such as ethylene diamine (EDA) allows the preparation of cellulose III either from cellulose I (which leads to the form cellulose III_I) or from cellulose II (which leads to the form III_{II}). Cellulose III treated at a high temperature in glycerol is transformed into cellulose IV (Chanzy et al., 1979).

LIGNIN

Lignin is the principal aromatic component of wood. The lignin molecule is a polymer with a DP (degree of polymerization) of 450–550, formed by the free radical, oxidative condensation of the three monomers, coniferyl alcohol, sinapyl alcohol and coumaryl alcohol, the formulae for which are shown in Fig. II-2 (Wayman and Parekh, 1990).

Lignin is a complex polymer of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. Lignin resists attack by most

microorganisms. Lignin is nature's cement, along with hemicellulose, which exploits the strength of cellulose while conferring flexibility.

Lignin as a product

Lignins are derived from an abundant and renewable resource: trees, plants, and agricultural crops. Lignins are nontoxic and extremely versatile in performance, qualities that have made them increasingly important in many industrial applications.

Industry first began to use lignins in the 1880's when lignosulfonates were used in leather tanning and dye baths. Since then, lignosulfonates have even found applications in food products, serving as emulsifiers in animal feed and as raw material in the production of vanillin, a widely used ingredient in food flavors, in pharmaceuticals and as a fragrance in perfumes and odor-masking products. Lignin is now used in literally hundreds of applications - impacting many facets of our daily lives.

Commercial lignin is currently produced as a co-product of the paper industry, separated from trees by a chemical pulping process. Lignosulfonates (also known as lignin sulfonates and sulfite lignins) are products of sulfite pulping. Kraft lignins (also called sulfate lignins) are obtained from the Kraft pulping process. Other delignification technologies use an organic solvent or a high pressure steam treatment to remove lignins from plants.

The lignin separated in the sulfite pulping process is projected to be used as a burn fuel in the pulping process with 26.3 MJ/OD kg of lignin (Saddler, 1993). As a clean uncontaminated lignin, it is also suitable for other applications, for example as a

binder, dispersant, emulsifier, or sequestrant (Adler, 1977; Northey, 1992; Sarkanen and Ludwig, 1971).

Because lignins are very complex natural polymers with many random couplings, their exact chemical structure is not known. Physical and chemical properties differ depending on the extraction technology. For example, lignosulfonates are hydrophilic and will dissolve in water, while and Kraft lignins are hydrophobic (will not dissolve in water). The usefulness of commercial lignosulfonates products comes from their dispersing, binding, complexing and emulsifying properties.

PRETREATMENT

The abundance, low cost and high carbohydrate content (70–80% carbohydrate, Table II-1), approximately equal to the starch content of corn and other grains, make biomass an attractive feedstock for enzymatic depolymerization and bioconversion to ethanol. Major difficulties in this conversion scheme are the heterogeneous composition of the polysaccharides in plant cell walls and the recalcitrant nature of the cellulosic part of the substrates. Cellulosic fibers are highly crystalline and thus very resistant to acid and enzyme-catalyzed hydrolysis (Grohmann, 1993).

Pretreatment is an essential element in the bioconversion of lignocellulosic substrates. It is required for efficient enzymatic hydrolysis of biomass because of the physical and chemical barriers that inhibit the accessibility of the enzyme to the cellulose substrate (Saddler, 1993). Lignocellulosic biomass is only partially digestible in its native form, often less than 20%, with the enzymatic hydrolysis proceeding at an extremely low rate (Dulap et al., 1976).

The biological production of ethanol from lignocellulosic biomass includes the following steps: biomass pretreatment, enzymatic hydrolysis, fermentation, product recovery and waste treatment. Pretreatment is a prerequisite step for the bioconversion of biomass to ethanol. The primary purpose of pretreatment is to make the cellulosic biomass amenable to the action of cellulase enzymes. Enzymatic hydrolysis can produce high yields of relatively pure glucose syrups without generating glucose degradation products, and utility costs are low since the hydrolysis occurs under mild reaction conditions (NREL, 1995).

The methodology used to achieve this goal varies widely depending on the specific application: treatments with various types of acids, alkaline treatments, steam treatment, and simple mechanical treatment. The net effect of the pretreatment also varies widely in terms of its physical and chemical characteristics.

Mechanical treatment

It has been reported that mechanical grinding/milling causes some improvement in the enzymatic digestibility (Caufield and Moore, 1974; Koullas et al., 1990; Matsumura et al., 1977; Puri, 1984; Sintsyn et al., 1991). However, milling/grinding is both energy and capital intensive, and hence unattractive on a large scale.

Ball milling not only decrystallizes model lignocelluloses but also reduces their particle size. It is possible that any benefits from ball milling generally attributed to lower crystallinity actually result from the smaller particle size (Chang and Holtzapple, 2000).

Irradiation and simple heating have also been used to break down lignocellulose. Electron radiation and ball milling are particularly effective in increasing the rate of

hydrolysis and yield of sugar under dilute acid saccharification conditions. They have the advantage of yielding a high bulk density of cellulose without the need for washing. However, they are both relatively sophisticated technologies that require considerable energy and have not yet been developed commercially.

Chemical treatment

Lignocellulosic feedstocks contain three main constituents, namely cellulose, hemicellulose, and lignin. Their various chemical treatments are usually designed to pretreat lignocellulosic biomass by removing lignin and hemicellulose, destroying the cellulose crystalline structure, and increasing the pore size and surface area.

Many kinds of pretreatment methods have been used to enhance the enzymatic reaction, including steam/steam explosion (Fernandez-Bolanos et al., 1999; Mes-Hartree et al., 1984; Sawada et al., 1995; Schwald et al., 1988), grinding/milling (Caufield and Moore, 1974; Koullas et al., 1990; Matsumura et al., 1977; Puri, 1984; Sintsyn et al., 1991), hot water/autohydrolysis (Allen et al., 2001; Garrote et al., 2002; Váquez et al., 2001), acid treatment (Burns et al., 1989; Grethlein, 1985; Jacobsen and Wyman, 2000; Kim et al., 2001; Mok et al., 1992), alkali treatment (Ferrer et al., 2000; Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003; Tarkow et al., 1955) and other methods (McGinnis et al., 1983; Zheng et al., 1998)

Considerable attention has been devoted to agents that will cause swelling of the cellulose and disrupt the crystalline structure. There are two ways in which this may occurs:

1. Intercrystalline swelling caused by uptake of water between the crystal units, which causes a reversible volume change of up to about 30 percent.
2. Intracrystalline swelling, which involves the penetration of the crystalline structure and can lead to unlimited swelling or complete solution of the cellulose.

Sodium hydroxide, amines, and anhydrous ammonia have been used for intercrystalline swelling. In Europe, during World War II, high concentrations (70–75 percent) of sulfuric acid or fuming hydrochloric acid and metal chelating solvents were used for intracrystalline swelling.

All the chemical pretreatment methods for improving enzymatic digestibility generates hydrolysates containing a mixture of sugars (i.e. hexose and pentose) and lignin. Usually the hydrolysate from the pretreatment/fractionation process requires detoxification, because the microorganisms (Jeffries, 1983) poorly withstand the inhibitory environment of lignocellulose-hydrolysates (Bjóling and Lindman, 1989; Fein et al., 2004; Hahn-Hägerdal et al., 1994; Sanchez and Bautista, 1988; Tran and Chambers, 1986; van Zyl et al., 1991; Watson et al., 1984). These factors increase the cost of pentose (xylose) fermentation. This is a disadvantage that any chemical treatment must overcome.

Alkali

Alkalis have been used for many years as a means of improving the texture of cellulose textiles (mercerization) and to improve the nutritive value of forage and forest residues for feeding ruminants. The treatment of cellulose-containing residues with low concentrations of alkali makes them considerably more susceptible to enzymatic and

microbiological conversion, which is particularly important for the alcoholic fermentation of these materials. Alkali treatment causes swelling, decreases the degree of polymerization, and the lignin content, and increases the surface area. Saponification (de-esterification) of intermolecular ester bonds has also been reported by many researchers (Fan et al., 1982; Feist et al., 1974). Steeping various straws in 1.5 percent sodium hydroxide for 24 hours can increase their ruminant digestibility from an initial 30-40 percent to 60-70 percent. This process was patented by Beckmann and used extensively in Europe during World Wars I and II.

Ammonia has also been used as a reagent for pretreatment, though in general the enhancement obtained is less than that of the sodium hydroxide treatment. Ammonia has a number of desirable characteristics as a pretreatment reagent. It is an effective swelling reagent for lignocellulosic materials and has a high selectivity for reactions with lignin over those with carbohydrates. It is also one of the most widely used commodity chemicals with about one-fourth the cost of sulfuric acid on a molar basis. Its high volatility makes it easy to recover and reuse and it is a non-polluting and non-corrosive chemical. One of the known reactions of aqueous ammonia with lignin is the cleavage of the C-O-C bonds in lignin as well as the ether and ester bonds in the lignin-carbohydrate complex (LCC). The enzymatic digestibility of lignocellulosic material is increased remarkably by ammonolysis, breaking the linkage between the lignin and hemicellulose, and increasing the surface area.

In our laboratory, we have previously investigated various pretreatment processes using a flow-through (percolation) reactor system. Among these is the ammonia recycled percolation (ARP) process which we have studied to pretreat various lignocellulosic

biomass feedstocks, including hardwood (Yoon et al., 1995), herbaceous biomass (Iyer et al., 1996; Kim and Lee, 1996), and pulp mill sludges (Kim et al., 2000). Process modifications such as the use of additional reagents, such as hydrogen peroxide (Kim et al., 2000; Kim and Lee, 1996), have also been tested.

Autohydrolysis/hot-water

The autohydrolysis reaction involves the formation of acids from the solubilization of acidic components in hemicellulose, such as acetic acid, formic acid, and glucuronic acid (McGinnis et al., 1983; Timell, 1967). Under hot-water treatment conditions, the hydronium ion initially causes xylan depolymerization and cleavage of the acetyl group. The autohydrolysis reaction then follows, in which the acetyl group catalyzes the hydrolysis of the hemicellulose (Casebier et al., 1969; Fernandez-Bolanos et al., 1999; Lora and Wayman, 1978).

The hydrolysis of glycosidic linkages in hemicellulose and the beta-ether linkages in lignin are catalyzed by acetic acid formed at high temperature from acetyl groups present in hemicellulose (autohydrolysis) (Fernandez-Bolanos et al., 1999).

When corncobs are contacted with water and heated, the hydronium ions coming from water auto-ionization cause both xylan depolymerization (to give xylooligomers and xylose) and cleavage of acetyl groups (to give acetic acid, which increases the hydronium concentration in the reaction medium). Under the operational conditions usually used in autohydrolysis (at temperatures below 230°C), cellulose is not significantly affected, whereas the lignin fraction can be depolymerized to give soluble compounds and a non-

soluble fraction with increased susceptibility towards further processing (Garrote et al., 2002).

Steam explosion

Steam explosion technology involves treating various biomass resources. In steam explosion the biomass is pressurized with high steam pressure for a certain period of time, (typically at 200–450 psig) for a period of 1–10 minutes and then explosively discharging the product to atmospheric pressure, which results in a sudden decompression. This explosive discharge changes the biomass into fibrous mulch by a combination of mechanical and chemical action. In the case of wood chips, the explosion causes defibrillation of chips into fiber bundles, and partial hydrolysis of cellulose, other carbohydrates, lignin and volatile components. Steam explosion of biomass is known to be a hydrolytic pretreatment, which increases enzyme and solvent accessibility of cellulose, renders biomass separable (by fractionation) into different components and raises the crystallinity of the cellulose component.

The ultrastructure of steam-exploded wood from the softwood *Pinus radiata D. Don* was examined by electron microscopy. Cellulose regions were shown to contain numerous pores greater than 2 nm, while lignin agglomerates did not contain such pores (Donaldson et al., 1988).

Samples exploded in the explosion temperature or longer duration at a particular temperature resulted in a decrease in the thermal softening or melting temperature. which has been attributed to the loosening of the wood texture (Lonikar et al., 1985).

Acid

Acid hydrolysis is often used as a pretreatment because it can be adapted to suit a wide variety of feedstocks. Except in the case of strong hydrochloric acid hydrolysis, it is generally carried out at elevated temperatures (100 to 240 °C) for various lengths of time. However, at higher acid concentrations it can be carried out at temperatures as low as 30 °C. Although this is generally an inexpensive process, acid hydrolysis may also produce large quantities of degradation byproducts and undesirable inhibitory compounds at higher temperatures.

Closely related to crystallinity is the concept of accessible surface area. Burns et al. (1989) reported that the rate of enzymatic hydrolysis is a function of the surface area available to the cellulase enzyme. Another significant challenge in cellulose hydrolysis is the physical protection of cellulose provided by hemicellulose and lignin (Jacobsen and Wyman, 2000).

The best residues for enzymic hydrolysis and Simultaneous Saccharification and Fermentation (SSF) have been found to be produced by a higher temperature pretreatment using the Parr reactor. However, a large portion of the xylose fraction was degraded to furfural and glucose was degraded to HMF (Sanchez and Bautista, 1988; Lee et al., 1997).

ENZYMATIC HYDROLYSIS

Cellulose is a long chain of glucose molecules, linked to one another only with β-1-4 glycosidic bonds. The simplicity of the cellulosic structure, using repeated identical bonds, means that only a small number of enzymes are required to degrade this material.

The hydrolytic step is catalyzed by the synergistic action of three types of activities encompassed in an enzyme complex referred to as cellulase as shown in Fig. II-1 (Lonikar et al., 1985; Philippidis and Smith, 1995). These activities consist of:

1. Endoglucanase, which randomly attacks the cellulose chain to produce polysaccharides of shorter length.
2. Exoglucanase, which attaches to the non-reducing ends of these shorter chains and removes cellobiose moieties; and
3. β-Glucosidase, which hydrolyzes cellobiose and other oligosaccharides to glucose

Hemicelluloses are branched polymers of xylose, arabinose, galactose, mannose, and glucose. Hemicelluloses bind bundles of cellulose fibrils to form microfibrils, which enhance the stability of the cell wall. They also cross-link with lignin, creating a complex web of bonds which provide structural strength, but also challenge microbial degradation (Ladisch et al., 1983; Lynch, 1992).

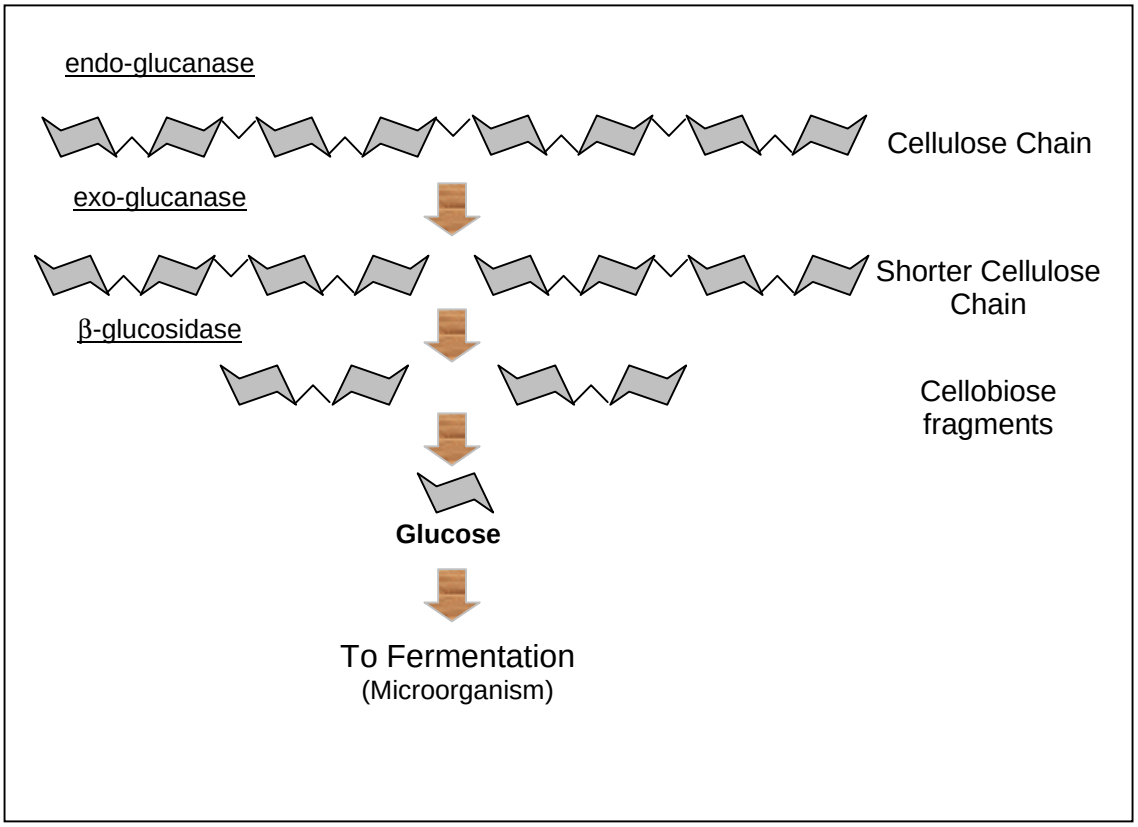


Fig. II-1. Enzymatic hydrolysis of cellulose

FACTORS AFFECTING ENZYMATIC HYDROLYSIS

Upon pretreatment, the biomass undergoes considerable changes in its physical properties and chemical composition. There are a number of factors that affect the digestibility of the lignocellulosic biomass because of its heterogeneous characteristics. Lignocellulosic material is very resistant to enzymatic breakdown.

A number of factors have also been suggested to affect enzymatic hydrolysis, including lignin (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988), hemicellulose (Schwald et al., 1988), biomass crystallinity (Caufield and Moore, 1974; Cowling and Kirk, 1976; Fan et al., 1980; Oji et al., 1977; Sasaki et al., 1979; Schwald et al., 1988), acetyl group (Chang and Holtzapple, 2000; Grohmann et al., 1989; Kong et al., 1992), particle size (Converse, 1993), degree of polymerization (Puri, 1984), surface area (Burns et al., 1989; Lee et al., 1995), and pore size (Grethlein, 1985; Knappert et al., 1980; Mooney et al., 1998). These will be examined in turn in this section.

Lignin

Lignin is believed to be a major hindrance for enzymatic hydrolysis (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988).

Lignin forms a solid seal around the cellulose micro-fibrils and exhibits limited covalent association with hemicellulose, preventing the enzyme from accessing the cellulose (Agosin et al., 1985; Chang and Holtzapple, 2000; Chesson et al., 1983a). Lignin and its derivatives are also toxic to microorganisms and inhibit enzymatic

hydrolysis. Low-lignin substrates have improved microbial activity and enzyme efficiency, and thus have a lower enzyme requirement.

Because lignin is the most recalcitrant component of the plant cell wall, the higher the proportion of lignin, the lower the bioavailability of the substrate. The effect of lignin on the bioavailability of other cell wall components is thought to be largely a physical restriction, with lignin molecules reducing the surface area available to enzymatic penetration and activity (Haug, 1993). Complete biomass delignification, however, is difficult because of its location within the deep cell wall (Timell, 1967), its hydrophobicity, its physical stiffness, the strong poly-ring bonds of C-O-C, C-C, and its tendency to recondense (lignin-carbohydrate complex) during delignification.

Hemicellulose

Many researchers have assumed that hemicellulose plays an important role in enzymatic reactions. Hemicellulose removal by acid treatment and autohydrolysis in the 1980's increased enzymatic hydrolysis of lignocellulosic material (Grethlein, 1980; Knappert et al., 1980). Over a substantial range of hemicellulose contents, enzymatic hydrolysis correlates well with the extent of hemicellulose removal (Chum et al., 1985; Grohmann et al., 1985; Schwald et al., 1988). It is generally believed that the removal of hemicellulose increases pore size, thus increasing the susceptibility of cellulose.

Knappert et al., have shown that the accessible water adsorbed by the cell wall of the fiber increases significantly due to the removal of hemicellulose. This change in internal void volume is primarily due to the increase in the pore size; the consequent

increase in accessibility for the enzyme would be significant (Cowling and Kirk, 1976; Knappert et al., 1980).

Crystallinity

One frequently cited property that affects enzymatic hydrolysis is biomass crystallinity. The crystallinity index (CrI) is strongly influenced by the biomass composition and type. It is very important for complete hydrolysis of cellulose to open up the crystalline structure of the cellulose and decrease the interaction between cellulose chains (Sasaki et al., 1979). For lignocellulosic biomass, the crystallinity index measures the relative amount of crystalline cellulose in the total solid. Although many researchers have indicated that crystallinity correlates inversely with digestibility, increased CrI after biomass pretreatment has been observed in many previous investigations (Chang and Holtzapple, 2000; Kasahara et al., 2001; Tanahashi et al., 1983). Tanahashi et al (1983) observed an increase in crystallinity of both pine and white birch upon steam explosion. Even though the CrI increased from about 50 to almost 70%, the hydrolysis rate was also greatly increased by the pretreatment.

Another report indicated that the crystallinity of cellulose in various lignocellulosic materials remained unchanged, irrespective of the structural changes caused by several pretreatments. However, a significant reduction in molecular weight, along with very large increases in the extent of enzymatic saccharification, were observed. It appears likely that the percentage crystallinity is therefore not the main determinant of the enzymatic degradation of cellulose (Puri, 1984). This conclusion was reinforced by

the finding that no correlation was evident between saccharification yields and the crystallinity index of low-lignin and low-hemicellulose samples (Schwald et al., 1988).

Acetyl groups

Xylan, the major hemicellulose present in hardwood, is extensively acetylated (Sjöström, 1993; Timell, 1967), with acetyl groups (OAc) amounting to about 3–5% of the wood substance (Browning, 1967). The acetyl group in corn stover is approximately 2–3%.

The xylan backbones in native cell walls are extensively acetylated, and experimental evidence indicates that xylans may be cross linked to lignin by ester and ether bonds (Erins et al., 1976).

The role of acetyl ester groups in the resistance of cell walls to enzymatic hydrolysis is less clear, since no direct investigations have been done, but indirect evidence points to their protective role (Agosin et al., 1985; Bacon et al., 1981; Bacon and Gordon, 1980; Chesson et al., 1983b; Morris and Bacon, 1977; Theander et al., 1981). Also, the chemical acetylation of carbohydrates in wood renders the wood resistant to enzymatic hydrolysis and microbial decay (Goldstein et al., 1961; Stamm and Baechler, 1960; Tarkow et al., 1955). Deacetylation has been found to increase swellability (Weimer et al., 1986) and the enzymatic digestibility of poplar wood and wheat straw (Grohmann et al., 1989; Kong et al., 1992).

Most pretreatments change biomass compositions, particularly the hemicellulose and lignin, at the same time as the deacetylation (Grohmann et al., 1989).

Particle size/Degree of polymerization

Dry grinding increases the amorphous character of cellulose, and greatly increases its in vitro digestibility. This size reduction increases the available surface areas of both the amorphous and crystalline cellulose (Caufield and Moore, 1974).

Studies of the hydrolysis of Kraft pulp indicated that larger average particle sizes of this substrate might be an inhibiting factor, since it was hydrolyzed more slowly than delignified RMP (refiner mechanical pulp) despite having a higher median pore width and lower lignin content (Mooney et al., 1998). Another study also found that particle size had little effect on biomass digestibility (Chang and Holtzapple, 2000), and the degree of polymerization of the residual cellulose after hydrolysis was slightly lower than for celluloses which were untreated (Walset, 1952).

Surface area/ Pore size

It is probable that the extent and/or rate of hydrolysis is significantly influenced by the surface area of the substrates and the degree of polymerization (DP) of the cellulose in combination with other structural features (Puri, 1984). Knappert et al. (1980) indicated that changes in internal void volume are primarily due to changes in the pore size region, and hence the increase in accessibility for the enzyme would be significant.

The specific surface area and the rate of hydrolysis are apparently not clearly related to enzymatic hydrolysis (Fan et al., 1980). The surface area of a biomass sample pretreated under typical conditions (175 °C, 1 h, 10 wt.% ammonia) was found to increase as compared with that of untreated biomass (Yoon et al., 1995). However, Burns

et al. (1989) reported that the surface area of pores too small to be accessible to the enzyme decreases more slowly, presumably because the substrate containing these small pores reacts only at the external surface.

FERMENTATION

Many organisms grow without using the electron transport chain. The generation of energy without the electron transport chain is called fermentation. This definition is the exact and original meaning of the term fermentation, although currently it is often used in a broader context (Shuler and Kargi, 1992).

Fermentable sugars, especially glucose, can be converted to other valuable products such as fructose, ethanol, numerous organic acids and many other products by the enzymatic hydrolysis and biochemical conversion of cellulosic substrates (Grohmann, 1993). Production of ethanol from lignocellulosic biomass is a very different system from the method used to extract it from corn because the carbohydrates are much more difficult to solubilize than the starch in grain (Gibbson et al., 1986). The processes commonly include:

SHF: In a typical bioconversion of corn stover, the cellulase production, enzymatic hydrolysis, and fermentation is performed sequentially in separate processes. This separated process is so-called Separate Hydrolysis and Fermentation (SHF).

DMC: Direct Microbial Conversion (DMC) combines all three processes (cellulase production. Cellulose hydrolysis, and fermentation) in one step. There are resulting cost savings because of the reduced number of vessels required. However, the

ethanol yields are rather low, several metabolic by-product are produced, and the organisms usually suffer from low ethanol tolerance.

SSF: Simultaneous Saccharification and Fermentation (SSF) combines cellulose hydrolysis and fermentation in one step. Because the glucose produced by the hydrolysis process is immediately consumed by the microorganism, only very low levels of cellobiose and glucose are observed in the reactor. This reduces cellulase inhibition, which in turn increases sugar production rates, concentrations, and yields, and decreases enzyme loading requirements.

Particularly, SSF is introduced in following section.

Simultaneous Saccharification and Fermentation (SSF)

Enzymatic reaction suffers from its products, cellobiose and glucose. One of the technologies addressing this problem is the SSF (Simultaneous Saccharification and Fermentation) process since the glucose is immediately consumed by microorganism as soon as it is produced. SSF is a process in which enzymatic hydrolysis and fermentation are carried out simultaneously in one reactor vessel.

The simultaneous saccharification and fermentation (SSF) process was first introduced in 1977, and it gives high yields and rapid rates for ethanol production (Ghosh et al., 1984; Pemberton et al., 1980). The SSF approach offers several advantages over the separate hydrolysis and fermentation (SHF) procedure where the substrate is either separated from residual solid or transferred without purification to the second vessel where the fermentation takes place. The SSF approach eliminates one reactor vessel and the need for a solid-liquid separation device. More importantly, it addresses one of the

most serious shortcomings of current method involving cellulolytic enzyme, i.e. its extreme sensitivity to end-product inhibition (Grohmann, 1993).

In the utilization of biomass, the enzymatic hydrolysis step is usually the critical step in the overall process since it is a slow reaction compared to microbial reactions. The enzymatic hydrolysis step is strongly inhibited by released sugar, especially cellobiose. The SSF of lignocellulosic materials thus has advantages over separate hydrolysis and fermentation (SHF) (Ghosh et al., 1982). Glucose released by the cellulase enzyme is simultaneously converted to the end product by the microorganism. Glucose inhibition on the enzyme reaction is therefore minimized. Other advantages include the potential for use of low enzyme loading, single vessel processing, and reduced potential for microbial contamination (Moritz and Sheldon, 1996).

However, the major disadvantage of the SSF approach is the loss of freedom in the adjustment of important parameters for hydrolysis and fermentation. The enzymes and microorganisms must also be matched with respect to pH, temperature etc. (Grohmann, 1993).

Xylose fermentation

The plant cell wall is made up of crystalline bundles of cellulose embedded in a covalently linked matrix of hemicellulose and lignin. Bioconversion of cellulosic material requires the saccharification of both hemicellulose and cellulose, because the pentose sugar D-xylose is the major carbohydrate component of hemicellulose in a wide variety of lignocellulosic biomass species (Table II-1). The ability to ferment xylose is an

important characteristic of microorganisms that are being considered for use in large-scale fermentation based hemicellulose conversion processes.

Throughout the pretreatment and enzymatic hydrolysis processes, the hydrolysate contains glucose, mannose, galactose, xylose, arabinose and cellobiose. Most organisms in nature easily convert glucose into ethanol, but this is not the case for xylose, mannose etc. Improved yields can be expected if the other hemicellulose, in addition to the cellulose, can be effectively fermented to ethanol (Ingram and Doran, 1995; Keller et al., 1998).

Fermentation of pentose sugar xylose into ethanol is essential for the economically feasible production of fuel ethanol from a biomass (Dien et al., 1998; Wright, 1988). Only in the last few decades have researchers been able to ferment pentose efficiently into ethanol by using either xylose-fermenting yeast or recombinant microorganisms (Bothast et al., 1996; McMillan, 1996). One of the most effective microorganisms currently available for fermentation of mixed sugar is ethanologenic *Escherichia coli* strain KO11 (Ohta et al., 2004). This strain ferments glucose, xylose, and arabinose with good ethanol yields and productivities and a high tolerance for common hydrolysate inhibitors such as acetate (Hahn−Hägerdal et al., 1994).

Two strains of the recombinant *E.coli* strains have been characterized in considerable detail: strain ATCC 11303 (pLOI297), in which the foreign genetic elements are plasmid-borne, and strain KO11 in which the foreign genetic elements are integrated into the host chromosome. An ethanologenic xylose-fermenting *Z. mobilis* strain has also recently been developed, but extensive information is not yet available on fermentation performance characteristics (Burchhardt and Ingram, 2004; Zhang et al., 1995).

In xylose fermentation, both yield and productivity are one to two orders of magnitude lower than in hexose fermentation. The major aims of the research in this field have therefore been to understand the limitations of the xylose fermentation, with a view to finding suitable organisms and process conditions, and to use modern genetic engineering techniques to improve yield and productivity (Saddler, 1993).

Ideally, a xylose fermenting microorganism should have the following properties: (i) the ability to use glucose and xylose simultaneously; (ii) the ability to ferment glucose and xylose at equally high fermentation rates; (iii) the absence of byproduct formation; (iv) the absence of inhibition by ethanol derived hydrolysates; (v) the ability to ferment at a low pH; (vi) the ability to ferment at high temperatures. So far, the recombinant bacteria have only the ability to ferment xylose and glucose a the same rate, although not simultaneously (Saddler, 1993).

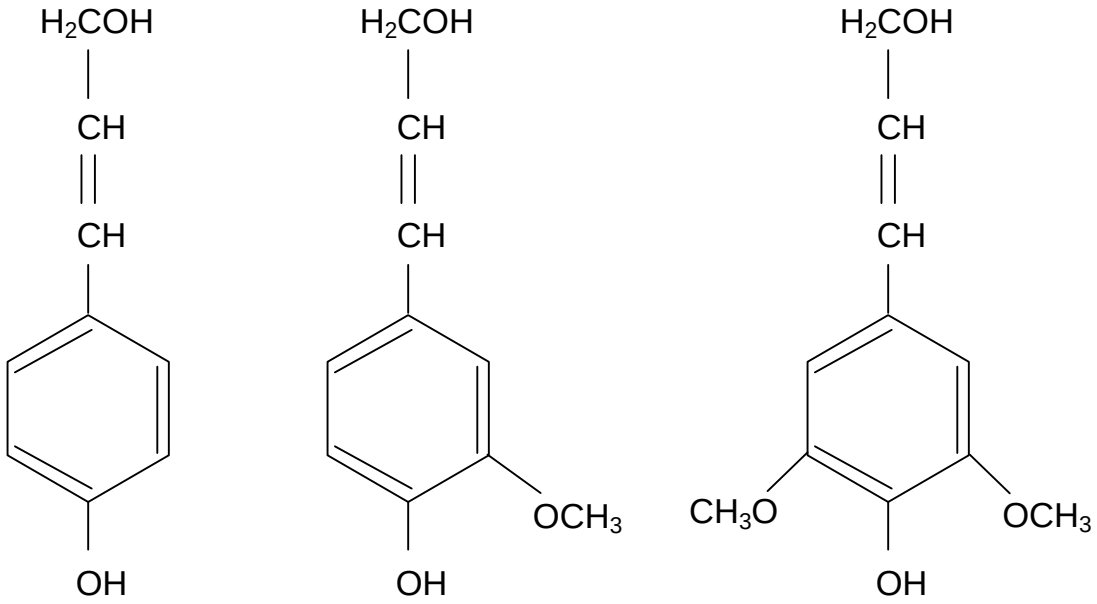
In metabolism; xylose is transported across the cell membrane whereupon it is converted to xylulose-5-phosphate (X-5-P). X-5-P is then converted to pyruvate by way of the pentose phosphate (PP) and Embden-Meyerhof-Parnas (EMP) or Enter-Doudoroff (ED) pathways (Fig. II-3 and Fig. II-4). Within the PP cycle, X-5-P is metabolized to glycolytic intermediates such as glyceraldehyde-3-phosphate and fructose-6-phosphate. These compounds are converted to pyruvate via the EMP (or ED) pathway, and pyruvate is converted to ethanol via an acetaldehyde intermediate by the sequential action of pyruvate decarboxylase and alcohol dehydrogenase (ADH) enzymes (Chaing and Knight, 1960; Horecker, 1962; Jeffries, 1983; Magee and Kosaric, 1985; Manderson, 1985; McCracken and Gong, 1983). In this scheme, a minimum of 3 moles of xylose are required to produce 5 moles of ethanol. The theoretical ethanol yield based on this

stoichiometry is 0.51 g-ethanol/g-xylose or 1.67 mol ethanol/mol xylose. Although the mass yield is identical to that for glucose fermentation (0.51 g/g), the molar yield is lower than that for glucose fermentation (2 mole ethanol/mol glucose). The reduced molar yield indicates poorer energetics for xylose fermentation compared to glucose. The overall Gibbs free energy change for the conversion of xylose to X-5-P is estimated to be about two-thirds of that for the analogous conversion of glucose to glucose-6-phosphate (Jeffries, 1990).

Table II-1. Percent dry weight compositions of various biomass

Feedstock	Glucan	Xylan	Galactan	Arabinan	Lignin	Mannan	Extractives	Ash
Tree Species								
Hybrid Poplar	48.6	14.6	0.3	0.3	21.8	0.5		0.7
Poplar	49.9	17.4	1.2	1.8	18.1	4.7		0.5
White Oak	43.6	18.0	0.4	2.4	23.2	2.9		0.6
Red Oak	43.4	18.9		1.9	25.8	2.7		0.4
Walnut	46.2	16.5		1.8	21.9	2.6		1.0
Maple	44.9	17.3		2.8	20.7	2.9		0.6
Paper								
Office Paper	68.6	12.4			11.3	7.8 *		
Herbaceous Species								
Corn stalks + cobs	36.4	18.0	1.0	3.0	16.6	0.6	7.3	9.7
Corn stover	40.9	21.5	1.0	1.8	16.7			6.3
Bagasse	40.2	21.1	0.5	1.9	25.2	0.3	4.4	4.0
Wheat straw	38.2	21.2	0.7	2.5	23.4	0.3	13.0	10.3
Rice straw	34.2	24.5			11.9		17.9	16.1
Switch grass	31.0	20.4	0.9	2.8	17.6	0.3	17.0	5.8

(Wyman, 1996)
* Including Ash
Blanks indicate that no data were available



p-Coumaryl alcohol Coniferyl alcohol Sinapyl alcohol

	Coumaryl alcohol	Coniferyl alcohol	Sinapyl alcohol
Spruce	14	80	6
Beech	8	48	44
Grasses	30	50	20

Fig. II-2. Three lignin monomers and ratios in the representative biomass

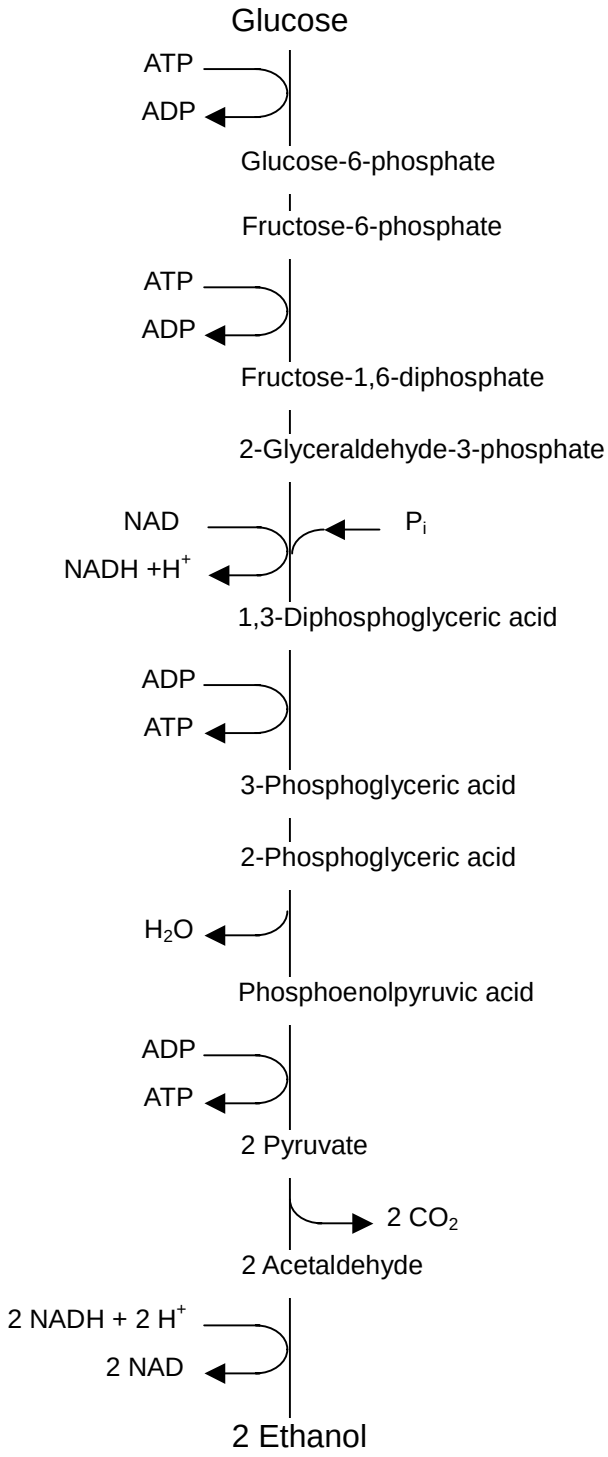


Fig. II-3. The Embden-Meyerhof-Parnas pathway of anaerobic conversion of glucose to ethanol
(Reproduced from Wayman and Parekh, 1990)

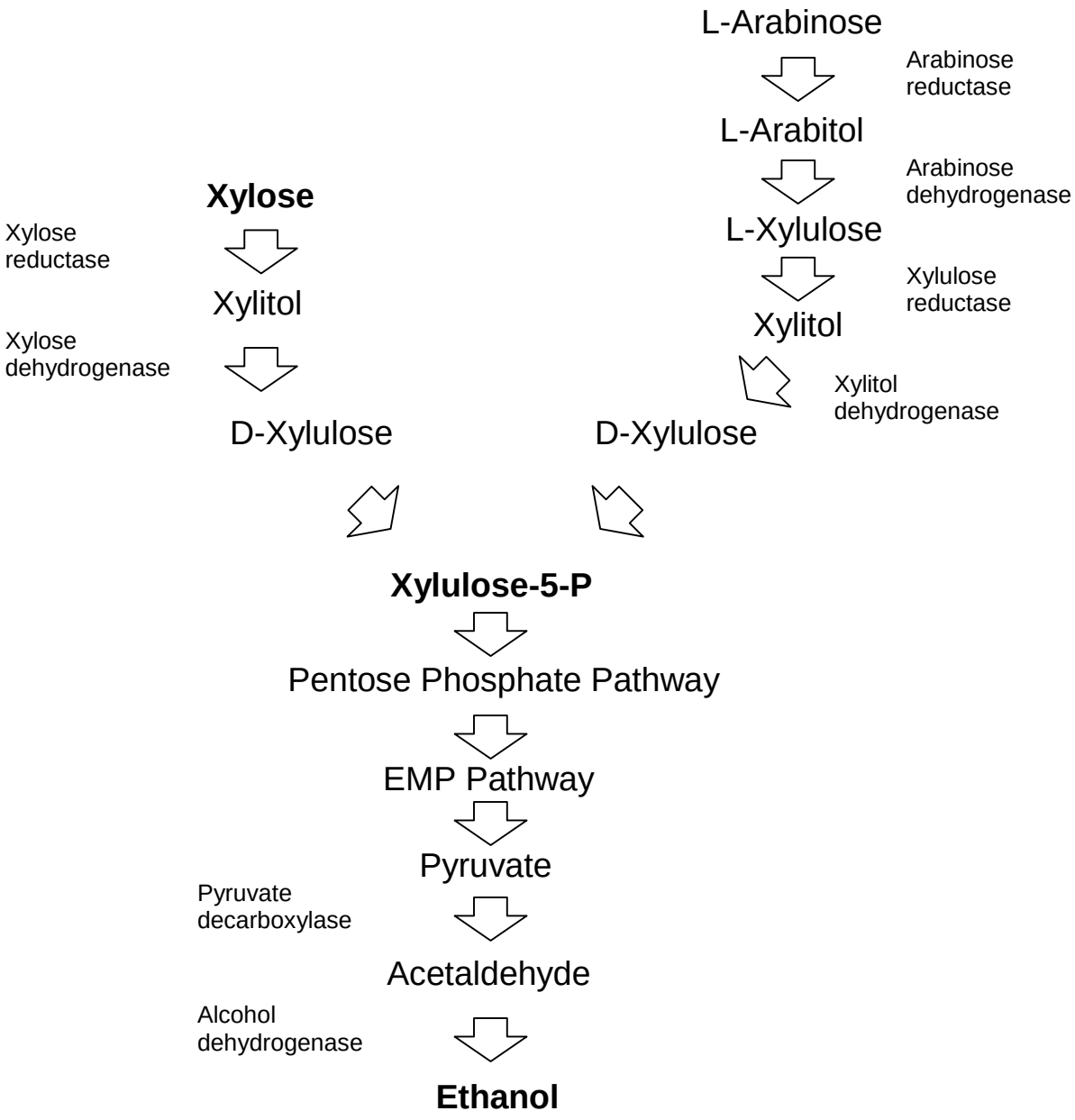


Fig. II-4. Pentose metabolism

III. PRETREATMENT OF CORN STOVER BY AQUEOUS AMMONIA

ABSTRACT

Corn stover was pretreated with aqueous ammonia in a flow-through column reactor, a process termed Ammonia Recycle Percolation (ARP). This method is highly effective in delignification of the biomass, reducing the lignin content by 70–85%. Most lignin removal occurred within the first 20 minutes of the process. Lignin removal by ARP was further confirmed by FTIR analysis and lignin staining. The ARP process solubilizes 40–60% of the hemicellulose but leaves the cellulose intact. The solubilized carbohydrate exists in oligomeric form. Carbohydrate decomposition during the pretreatment is insignificant. Corn stover treated for 90 minutes exhibited enzymatic digestibility of 99% with 60 filter paper unit (FPU)/g of glucan of enzyme loading, and 92.5% with 10 FPU/g of glucan. The digestibility of ARP treated corn stover is substantially higher than that of α-cellulose. The enzymatic digestibility is related with the removal of lignin and hemicellulose, perhaps due to the consequent increased surface area and porosity. The SEM pictures indicate that the biomass structure is deformed and its fibers are exposed by the pretreatment. The crystallinity index increases with pretreatment, reflecting removal of the amorphous portion of the biomass. The crystalline structure of the cellulose in the biomass, however, is not changed by the ARP treatment.

INTRODUCTION

Corn stover is one of the most promising renewable feedstocks for biological conversion to fuels and chemicals. Pretreatment is an essential element in the bioconversion of lignocellulosic substrates. Ammonia has a number of desirable characteristics as a pretreatment reagent. It is an effective swelling reagent for lignocellulosic materials and has a higher selectivity for reactions with lignin over those with carbohydrates. It is one of the most widely used commodity chemicals, with about one-fourth the cost of sulfuric acid on a molar basis. Its high volatility makes it easy to recover and reuse. It is a non-polluting and non-corrosive chemical. One of the known reactions of aqueous ammonia with lignin is the cleavage of C-O-C bonds in lignin, as well as the ether and ester bonds in the lignin-carbohydrate complex (LCC). Indications are that ammonia pretreatment selectively reduces the lignin content in biomass. There are many advantages to removing lignin early in the conversion process, before it is subjected to biological processing. Lignin is believed to be a major hindrance in enzymatic hydrolysis (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988). Lignin and its derivatives are toxic to microorganisms and inhibit enzymatic hydrolysis. Low-lignin substrates have improved microbial activity and enzyme efficiency, eventually lowering the enzyme requirements.

Complete biomass delignification, however, is difficult because of its location within the deep cell wall (Timell, 1967), its hydrophobicity and physical stiffness, its strong poly-ring bonds of C-O-C, C-C, and the tendency to recondense (lignin-carbohydrate complex) during delignification. A number of factors other than lignin are

also thought to affect enzymatic hydrolysis, including biomass crystallinity (Caufield and Moore, 1974; Cowling and Kirk, 1976; Fan et al., 1980; Polcin and Bezuch, 1977; Sasaki et al., 1979; Schwald et al., 1988), degree of polymerization (Puri, 1984), particle size (Converse, 1993), surface area (Burns et al., 1989; Lee et al., 1995), and pore size (Grethlein, 1985; Knappert et al., 1980; Mooney et al., 1998).

We have previously investigated various pretreatment processes using a flow-through (percolation) reactor system in our laboratory. Among them is the ammonia recycle percolation (ARP) process, which we have studied to pretreat various lignocellulosic biomass feedstocks including hardwood (Yoon et al., 1995), herbaceous biomass (Iyer et al., 1996; Kim and Lee, 1996), and pulp mill sludges (Kim et al., 2000). Process modifications have also been attempted using additional reagents, including hydrogen peroxide (Kim et al., 2000; Kim and Lee, 1996). The primary purpose of this investigation is to assess the effectiveness of the ARP pretreatment process for corn stover. We were interested in verifying the changes in chemical composition and physical characteristics caused by the pretreatment and how those factors affect enzymatic digestibility.

MATERIALS AND METHODS

Materials

Corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, CO). It was ground and screened. The fraction collected between 9–35 mesh was used in all experiments. The initial compositions of corn stover was determined to be: 40.19% glucan, 21.71% xylan, 2.61% arabinan, 0.29% mannan, 0.68% galactan, 18.53%

Klason lignin, 2.30% acid soluble lignin, 7.08% ash, 2.20% acetyl group, 2.90% protein, and 1.51% unaccounted for. α-Cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246). Cellulase enzyme, Spezyme CP, Lot 301-00348-257, was obtained from Genencor International Inc. (Palo Alto, CA). An average activity, as determined by NREL, is 31.2 FPU/mL. β-Glucosidase was purchased from Sigma (Cat. No. G-0395).

Experimental setup and operation of ARP

Figure III-1 shows the overall layout of the ARP apparatus. The system consists of a stock solution reservoir, pump, temperature-programmable oven, SS-316 column reactor (9/10 in. ID × 10 in. L, internal volume of 101.9 cm^3), and liquid holding tank. The reactor was operated in a flow-through mode, in which the liquid flows through a reactor column packed with biomass. The reactor system was pressurized by nitrogen at 2.3 MPa to prevent flash evaporation. In a typical ARP experiment, 15 g of biomass were packed into the reactor, soaked with ammonia solution and left overnight. The reaction was initiated by raising the reactor temperature in a forced-air convection oven. Approximately 15 minutes of preheating was necessary to reach the desired temperature. The reaction time was counted after the desired temperature was attained. All of the ARP experiments were run in duplicate.

Digestibility test

The enzymatic digestibility of corn stover was determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure (NREL, 2004).

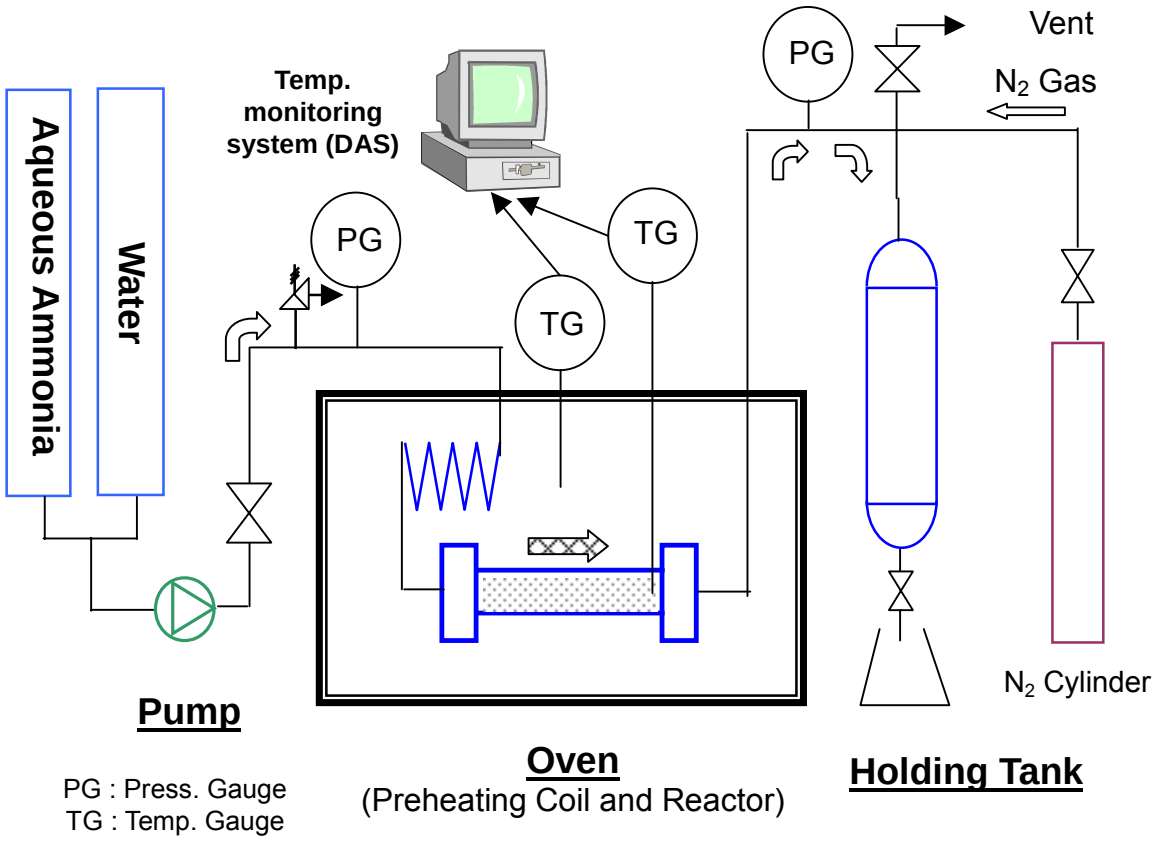


Fig. III-1. Schematic diagram of the ARP system

The corn stover feedstock was presoaked overnight with 15 wt.% NH_3 before being subjected to ARP experiment. Presoaking alone had a discernible effect on digestibility, raising it from 14.3% (untreated) to 35% for a presoaked sample with 10 FPU/g of glucan. The enzymatic digestibility tests were conducted as follows:

Reaction Conditions: 50°C, pH 4.8 (0.05 M sodium citrate buffer), 150 rpm shaker flask (New Brunswick Scientific, Model Innova 4080). The digestibility is defined as the percentage of theoretical glucose released after 72 h of incubation with cellulase enzyme.

Enzyme loading: 10–60 FPU of Spezyme CP/g of glucan supplemented with 37 IU of β-glucosidase (Sigma, G-0395).

Reactor: Screw-capped 250 mL Erlenmeyer flasks were filled with 100 mL of liquid and solid biomass containing one gram of glucan. The total solid mass in the reactor thus varied according to the glucan content in the treated/untreated biomass. The total glucose content after 72 h of hydrolysis was used to calculate the enzymatic digestibility. Untreated corn stover and α-cellulose were subjected to the same digestibility test as a control and a reference, respectively.

Analytical methods

The corn stover samples were analyzed for sugar, Klason lignin, and acid-soluble lignin following the procedures of NREL Chemical Analysis and Testing Standard Procedures (NREL, 2004). Each sample was analyzed in duplicate. The moisture content was measured by an infrared moisture balance (Denver Instrument, IR-30). Sugars were

determined by HPLC using a Bio-Rad Aminex HPX-87P column. For enzymatic digestibility, the glucose content was measured by an HPX-87H column.

Crystallinity index

Crystallinity of the corn stover samples was determined by X-ray diffraction using a diffractometer (Rigaku DMAX) operated at 40 kV and 200 mA. The diffraction spectra were taken by θ–2θ method. Duplicate samples were scanned at 1°/min from 2θ =10–30° with a step size of 0.01°. The water content retained in the sample was characterized by X-ray scattering, which has a maximum at 2θ=28°. The crystallinity index (CrI) was calculated according to the method of Segal et al. (1959), i.e. $(I_{002}-I_{18})/I_{002}]\times 100$, with the diffraction intensities, I_{002} at 002 peak position (2θ≈22.5°) and I_{18} at 2θ=18° (amorphous) (Lewin and Roldan, 1971; Segal et al., 1954; Segal et al., 1959).

Infrared spectroscopy

Infrared spectra were measured by Perkin Elmer FT-IR System 2000 with the Diffusive Reflection Accessory (Courtesy of Dr. Bruce Dale, Michigan State University). The spectra were obtained using 32 scans of the sample (no dilution), triangular apodization, a resolution of 4 cm^{-1} and an interval of 1 cm^{-1}.

SEM

The microscope pictures of the biomass samples were taken with a ZEISS DSM940 scanning electron microscope.

Lignin staining

Untreated corn stover and the ARP samples were stained for lignin detection using 10% phloroglucinol treated with HCl according to the procedure given by other researchers (Gahan, 1984; Waterworth, 1969). The lignin was indicated by a bright red color. The original color pictures were reprocessed to gray scale monotone pictures enhancing only the red-tone by Adobe Photoshop software. The darkness in the picture (Fig. III-9) thus indicates the mass concentration of lignin.

Statistical analysis

In order to test the significance of differences among the various treatments and the parameters tested, experimental results were subjected to a one-way analysis of variance (ANOVA) test using JMP software (SAS Version 4.0). Significance in differences was indicated at $p \leq 0.05$, with the number of replicates being 2 for all cases. SigmaPlot (Version 4.0, SPSS) was used for linear regressions where applicable.

Table III-1
Effect of reaction time on the composition in ARP treatment [1]

Time	Solid					Liquid		Total		Delign.	Digestibility [5]	
	S.R.[2]	K-Lignin[3]	A.S.L.[4]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan		60 FPU	10 FPU
[min]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100	18.5	2.3	40.2	21.7	0.0	0.0	40.2	21.7	0.0	21.2	14.3
		± 0.4	± 0.2	± 0.3	± 0.2			± 0.3	± 0.2		± 1.5	± 1.2
10	61.4	6.0	1.1	39.4	12.8	0.9	8.8	40.3	21.6	67.9	92.2	83.9
	± 2.5	± 0.4	± 0.2	± 0.4	± 0.2	± 0.1	± 0.3	± 0.5	± 0.5	± 2.2	± 2.4	± 2.9
20	58.5	3.6	1.0	38.6	11.0	1.1	11.0	39.7	22.0	80.5	92.0	88.3
	± 1.5	± 0.2	± 0.2	± 0.4	± 0.4	± 0.2	± 0.1	± 0.2	± 0.5	± 1.1	± 1.1	± 2.1
40	56.4	3.3	0.9	38.5	10.5	1.3	11.2	39.8	21.7	82.2	95.1	89.2
	± 2.1	± 0.2	± 0.2	± 0.4	± 0.1	± 0.2	± 0.1	± 0.2	± 0.0	± 1.0	± 2.1	± 0.4
60	55.2	3.0	0.8	37.8	9.7	1.6	11.7	39.4	21.4	83.9	94.4	88.3
	± 1.0	± 0.1	± 0.2	± 0.5	± 0.3	± 0.2	± 0.2	± 0.3	± 0.5	± 0.6	± 2.3	± 1.8
90	53.6	2.8	0.8	36.9	9.2	1.8	12.2	38.7	21.5	84.7	99.6	92.5
	± 1.1	± 0.2	± 0.2	± 0.6	± 0.3	± 0.2	± 0.2	± 0.8	± 0.5	± 1.1	± 2.5	± 2.0
α-Cellulose	100	0.0	0.0	91.4	4.0	0.0	0.0	91.4	4.0	0.0	93.4	71.7
				± 0.8	± 0.3			± 0.8	± 0.3		± 1.5	± 1.4

Notes. 1. All sugar and lignin content are based on the oven-dry untreated biomass. Values are expressed as mean and standard deviation. Pretreatment conditions: 15 wt.% of ammonia, 170°C, 5 mL/min of flow rate, 2.3 MPa

2. S.R.: solid remaining after reaction

3. Klason lignin

4. A.S.L. stands for acid soluble lignin during analysis

5. Conditions of Enzymatic hydrolysis: 72 h, 60 or 10 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.

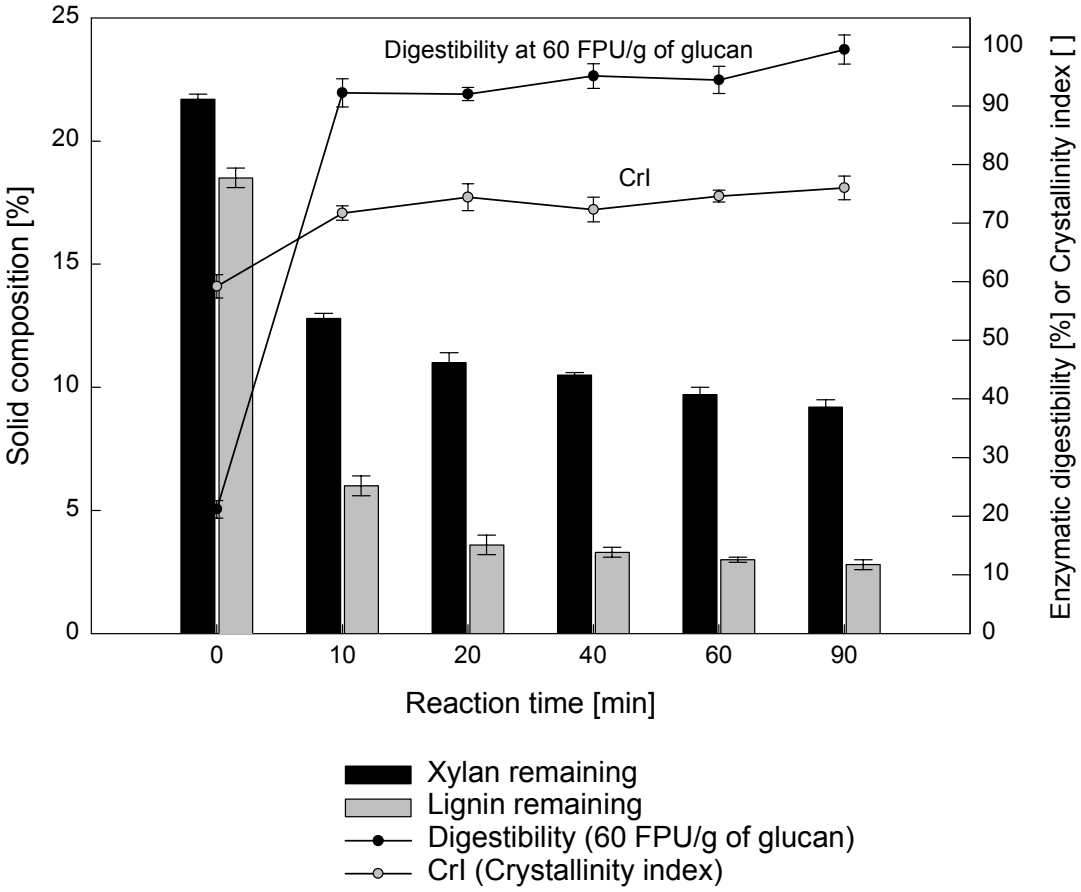


Fig. III-2. Effect of reaction time on solid composition, crystallinity index (CrI) and enzymatic digestibility in ARP [1]

Note. All sugar and lignin content are based on the oven-dry untreated biomass. Pretreatment conditions: 15 wt.% of ammonia, 170°C, 5 mL/min of flow rate, 2.3 MPa
Enzymatic hydrolysis conditions: 72 h, 60 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.

RESULTS AND DISCUSSION

Effect of ARP treatment on composition of corn stover

On the basis of our previous investigation and the results of preliminary experiments for this work, 170°C and 15 wt.% of ammonia concentration were chosen for the ARP treatment of corn stover (Iyer et al., 1996; Yoon et al., 1995). Table III-1 and Fig. III-2 summarized the compositional changes in the solid and liquid samples, along with their effects on enzymatic hydrolysis.

The most significant ($p \leq 0.05$) composition change is in the lignin. The ARP process removed 70–85% of the total lignin of the corn stover feedstock. The delignification reaction is rapid; 70% of the lignin was removed within 10 minutes of treatment.

About half of the xylan, the main component of hemicellulose, was also solubilized. The glucan content, however, remained relatively intact. ARP reduced the total solid mass by slightly less than half; the solid remaining (S.R.) was in the range of 53.6–61.4%. Table III-1 shows that the total glucan and xylan in the solid plus liquid is above 95% for glucan and near 100% for xylan. The biomass carbohydrates are thus well preserved in the ARP process, a very important benefit in a pretreatment process.

Table III-2. Composition of solid after pretreatment [1]

Composition	Untreated	ARP [2]	Dilute Acid [3]
S.R.[4]	100	53.6 ± 1.1	54.2 ± 0.7
K-Lignin[5]	18.5 ± 0.4	2.8 ± 0.2	12.5 ± 0.4
Glucan	40.2 ± 0.3	36.9 ± 0.6	37.6 ± 0.3
Xylan	21.7 ± 0.2	9.2 ± 0.3	1.3 ± 0.1

Note 1. Carbohydrate and lignin contents are expressed as wt.% of oven-dry untreated biomass. Values are expressed as mean and standard deviation..

2. ARP pretreatment conditions: 15 wt.% of ammonia, 170°C, 90 min, 5 mL/min of flow rate, 2.3 MPa.

3. Dilute acid pretreatment conditions: 0.07 wt.% of sulfuric acid, 180°C, 30 min, 5 mL/min of flow rate, 2.3 MPa, bed shrinking flow through reactor.

4. S.R.: solid remaining after treatment.

5. Klason lignin

Compositional changes and the enzymatic digestibility

Table III-1 summarizes the enzymatic digestibility of ARP-treated corn stover. The digestibilities were measured with two different cellulase loadings: 60 FPU/g of glucan and 10 FPU/g of glucan. Regardless of the treatment conditions, the digestibility of the pretreated biomass has significantly ($p \leq 0.05$) improved compared to that of the control (untreated biomass). The lowest digestibility of the treated biomass is 84% at 10 FPU/g of glucan, which occurred with the sample treated by ARP for 10 minutes. The digestibility of the control is only 14.3% at10 FPU/g of glucan. The digestibilities increase with treatment time and with enzyme loading. With 60 FPU/g of glucan enzyme loading, the digestibilities were all above 90%, the highest being 99.6%, which occurs with biomass treated for 90 minutes. The digestibility difference with regard to composition is more discernible with low enzyme loading (10 FPU/g of glucan) than with high enzyme loading (60 FPU/g of glucan) (Fig. III-3). The digestibilities of ARP-treated biomass are substantially higher than those of α-cellulose. The hydrolysis reaction profiles of Fig. III-3 also show that the ARP-treated samples have much higher initial hydrolysis rates than α-cellulose.

The composition and digestibility data of Table III-1 and Fig. III-2 collectively indicate that the lignin and hemicellulose contents in the biomass play an important role in the enzymatic hydrolysis reaction. The hemicellulose fraction of corn stover is in amorphous form. Lignin is closely associated with cellulose fibers and acts as a binder. They are both non-crystalline zone of the biomass. Their removal increases the surface area and porosity within the biomass, thus providing easier enzyme access to the cellulose. In theory, the rate of enzymatic hydrolysis is proportional to the surface area.

However, Yoon et al. (1995) found that ARP-treatment increased the BET surface area only by 50%, which does not explain the 7−10 folds increase in digestibility. Burns et al. (1989) provide a partial explanation: for substrates with pores too small to accommodate cellulase enzyme (2−4 nm), the reaction occurs on the external surface. It appears that the cellulase reaction mechanism that relates to porosity and surface area is quite complex and is not yet fully understood.

Because both lignin and xylan are reduced with the ARP treatment, it is unclear how each factor affects the enzymatic hydrolysis. To further verify this point, we conducted digestibility tests on corn stover samples treated differently. For these samples, the treatment used dilute acid (0.07% H_2SO_4, 180^{o}C) instead of aqueous ammonia. Compared to ARP, dilute-acid treatment removes most of the xylan, but only 20% of the total lignin in the biomass. The dilute-acid treated biomass is therefore basically xylan-free, whereas the ARP treated biomass is lignin-free. As shown in Fig. III-4, the enzymatic digestibilities of ARP sample (lignin-free) are 99.6% at 60 FPU/g of glucan of enzyme loading and 92.2% at 10 FPU/g of glucan loading, whereas those of dilute acid treated samples (xylan-free) are 89.9% with 60 FPU/g of glucan loading and 82.8% with 10 FPU/g of glucan loading.

The hydrolysis rate of ARP treated samples is also much higher than that of dilute-acid treated samples. The yield with 60 FPU/g of glucan reached 97% in 6 h and the yield with 10 FPU/g of glucan reached 90% in 24 h.

The fact that lignin hinders enzymatic hydrolysis of lignocellulose is well documented. Mooney et al. (1988) found that delignified refiner mechanical pulp (RMP) was enzymatically hydrolyzed more completely than untreated substrate and suggested

that steric hindrance from residual lignin may be a rate-limiting factor. Schwald et al. (1998) reported that the rate and extent of enzymatic hydrolysis of biomass correlated better with removal of alkali-insoluble lignin than with removal of xylan. It has also been reported that lignin content has the greatest impact on biomass digestibility, whereas the acetyl content has only a minor impact (Chang and Holtzapple, 2000).

According to Converse (1993), it is possible to disrupt the lignin shield without removing lignin. Although this makes cellulase more accessible, the enzymes adsorb on lignin, making the enzyme ineffective. Our findings agree with many previous studies that indicate lignin plays a larger role than hemicellulose as a resistance to enzyme reaction. It also explains why lignin-free corn stover shows higher digestibility than xylan-free biomass.

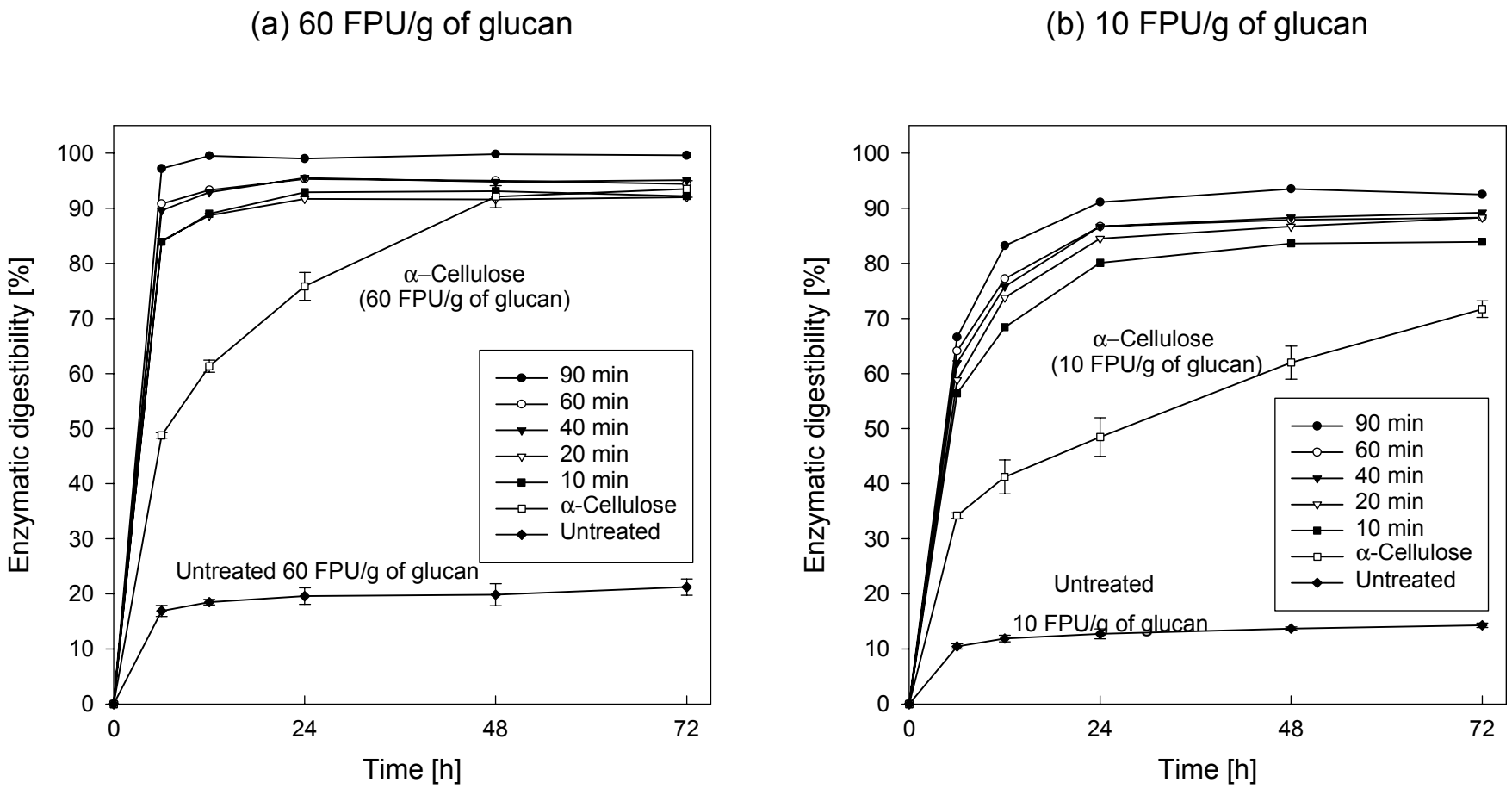


Fig. III-3. Enzymatic digestibility of ARP-treated samples [1]

Note 1. All sugar and lignin content are based on the oven-dry untreated biomass. Pretreatment conditions: 15 wt.% of ammonia, 170°C, 5 mL/min of flow rate, 2.3 MPa, All numbers in legend refer to reaction time (e.g. 10 min is 10 min ARP treatment)
Enzymatic hydrolysis conditions: 72 h, 60 or 10 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.
2. Standard error was shown only for α-cellulose and untreated samples. The other data points in the graph show the mean value (SE ≤ 2.5 %, n=2).

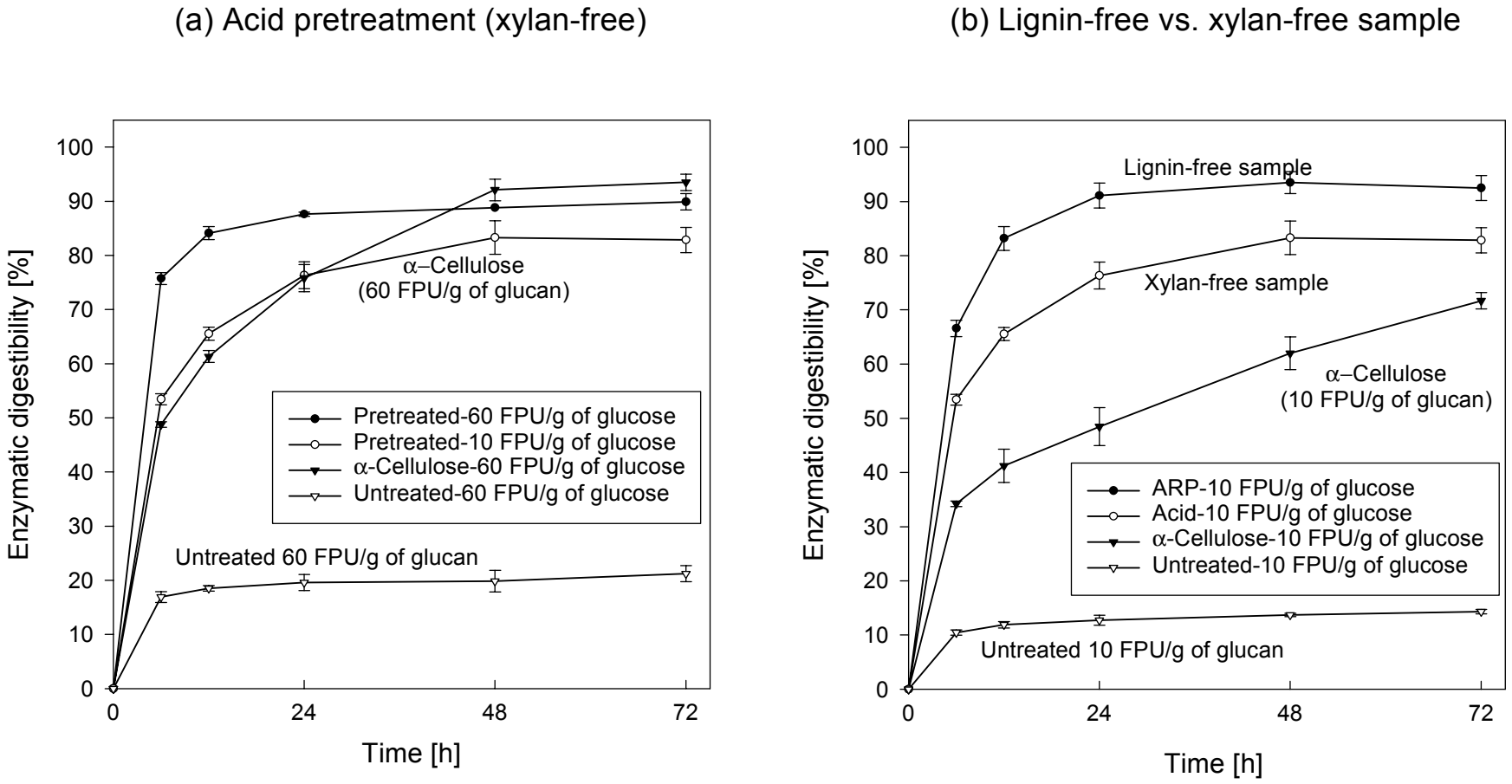


Fig. III-4. Comparison of xylan-free and lignin-free corn stover [1]

Note. All sugar and lignin content are based on the oven-dry untreated biomass.

ARP pretreatment conditions: 15 wt.% of ammonia, 170°C, 90 min, 5 mL/min of flow rate, 2.3 MPa. Dilute acid pretreatment conditions: 0.07 wt.% of sulfuric acid, 180°C, 30 min, 5 mL/min of flow rate, 2.3 MPa, bed shrinking flow through reactor.

Enzymatic hydrolysis conditions: 72 h, 60 or 10 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.

Crystallinity and digestibility

The chemical composition is obviously not the sole factor affecting enzymatic hydrolysis because the digestibility of pure α-cellulose is lower than that of pretreated corn stover. Physical properties and cellulose microstructure are among the potential factors influencing enzymatic hydrolysis. One frequently cited property is biomass crystallinity. We measured the X-ray diffraction pattern of treated, untreated biomass, and α-cellulose (Fig. III-5) from which the crystallinity indexes were determined. The diffraction patterns show that the crystallinity index (CrI) of corn stover actually increases with ARP treatment time (Fig. III-6). Figure III-2 shows the CrI of treated corn stover and the enzymatic digestibility data. The crystallinity index is strongly influenced by biomass composition. For lignocellulosic biomass, the crystallinity index measures the relative amount of crystalline cellulose in the total solid. ARP treatment mainly removes xylan and lignin - both of which are amorphous - so the CrI increases after ARP treatment. The increased CrI after biomass pretreatment has been observed in many previous investigations (Chang and Holtzapple, 2000; Kasahara et al., 2001; Tanahashi et al., 1983). It is uncertain, however, whether the cellulose structure in corn stover has been altered by the ARP treatment. In the XRD of Fig. III-5, the peak height of the corn stover line increases approximately in proportion with the cellulose content of the biomass. This indicates that the increased CrI after ARP treatment is primarily due to the removal of amorphous substances (Schwald et al., 1988), and is not due to changes in the basic crystalline structure of the cellulose. This agrees with the findings of Puri (1984), who found that the crystallinity of cellulose in various lignocellulosic materials remained

unchanged irrespective of the structural changes caused by several pretreatments. As the ARP treatment time increases both the crystallinity index and the digestibility, if one considers the CrI and digestibility data of Fig. III-6, the two seem to be related. However, the extent of the relationship is actually rather low, with the statistical coefficient of determination (R^2) being only 0.4889 (Fig. III-7). Moreover, this apparent relation is indirect because the increase of CrI is caused by the removal of amorphous substances in the biomass (lignin + xylan), which raises the enzymatic digestibility. We therefore find no direct relationship between the digestibility and the CrI.

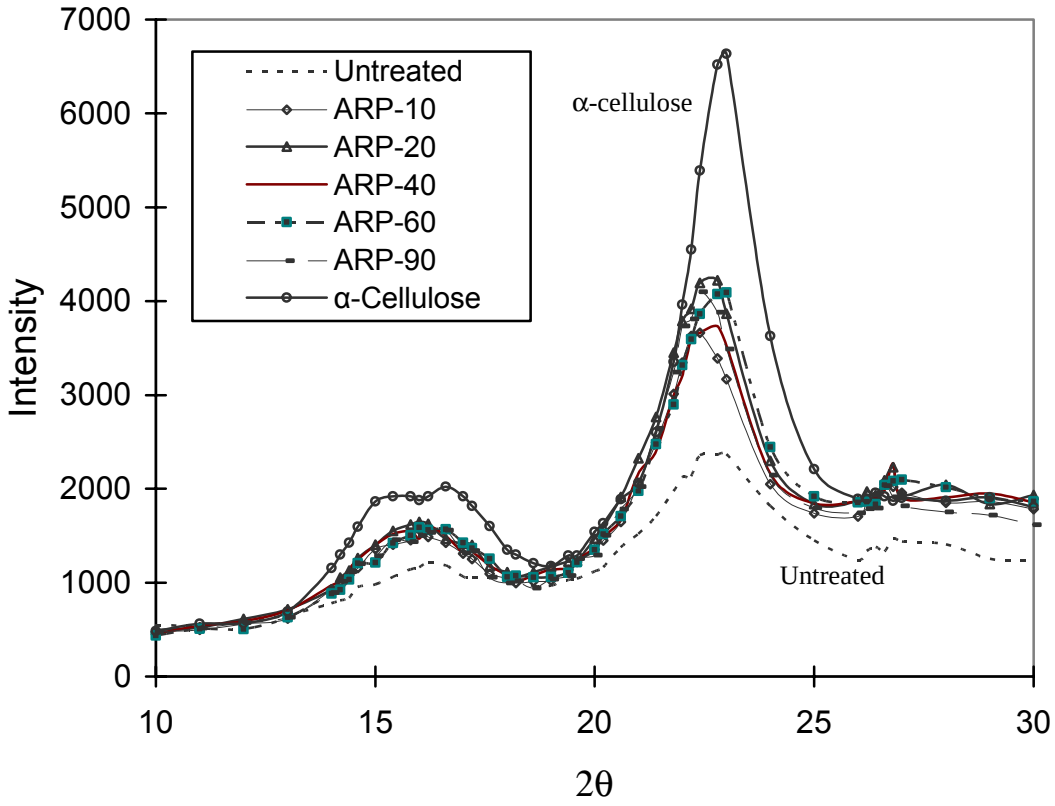


Fig. III-5. XRD diagram of ARP-treated samples [1]

Note 1. Number in legend indicates reaction time (e.g. ARP-10 is 10 min ARP treatment). Pretreatment conditions: 15 wt.% of ammonia, 170°C, 5 mL/min of flow rate, 2.3 MPa

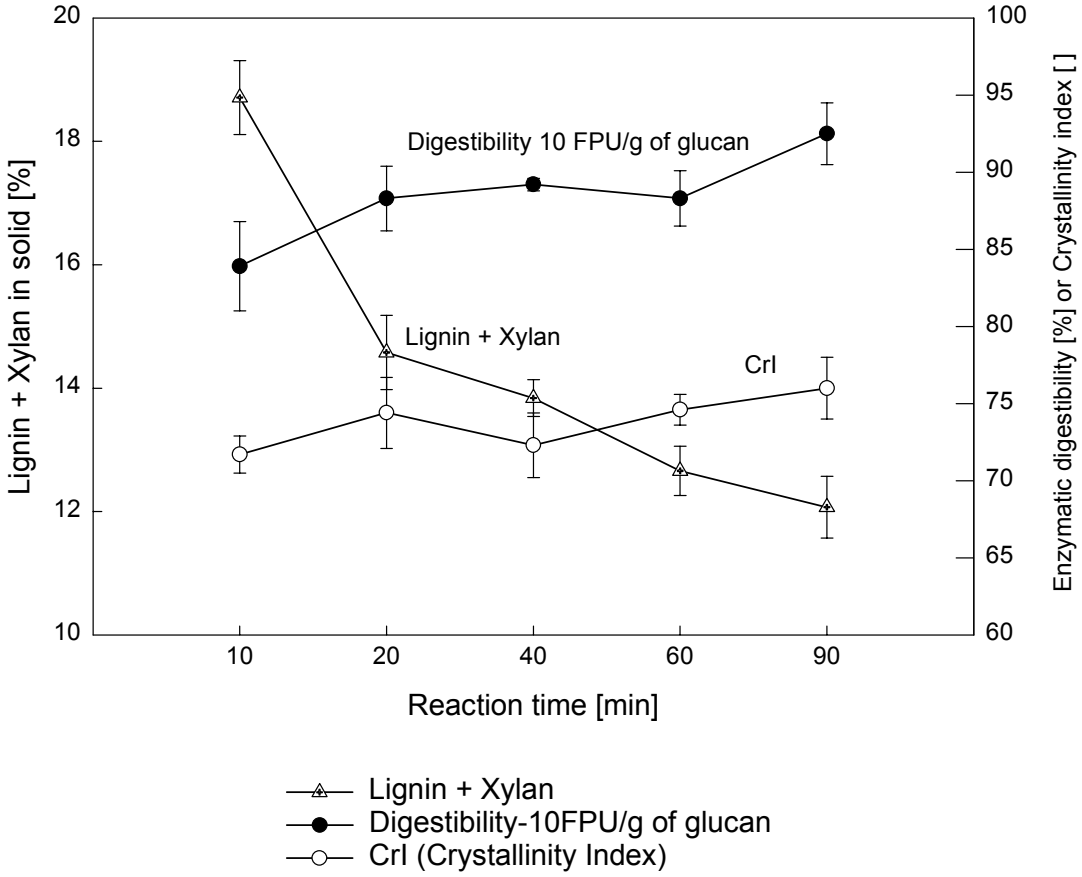


Fig. III-6. Profiles of amorphous region (lignin + xylan) over ARP reaction time, CrI, and enzymatic digestibility (10 FPU/g of glucan) [1]

Note. All sugar and lignin content are based on the oven-dry untreated biomass. Pretreatment conditions: 15 wt.% of ammonia, 170 °C, 5 mL/min of flow rate, 2.3 MPa
Enzymatic hydrolysis conditions: 72 h, 10 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.

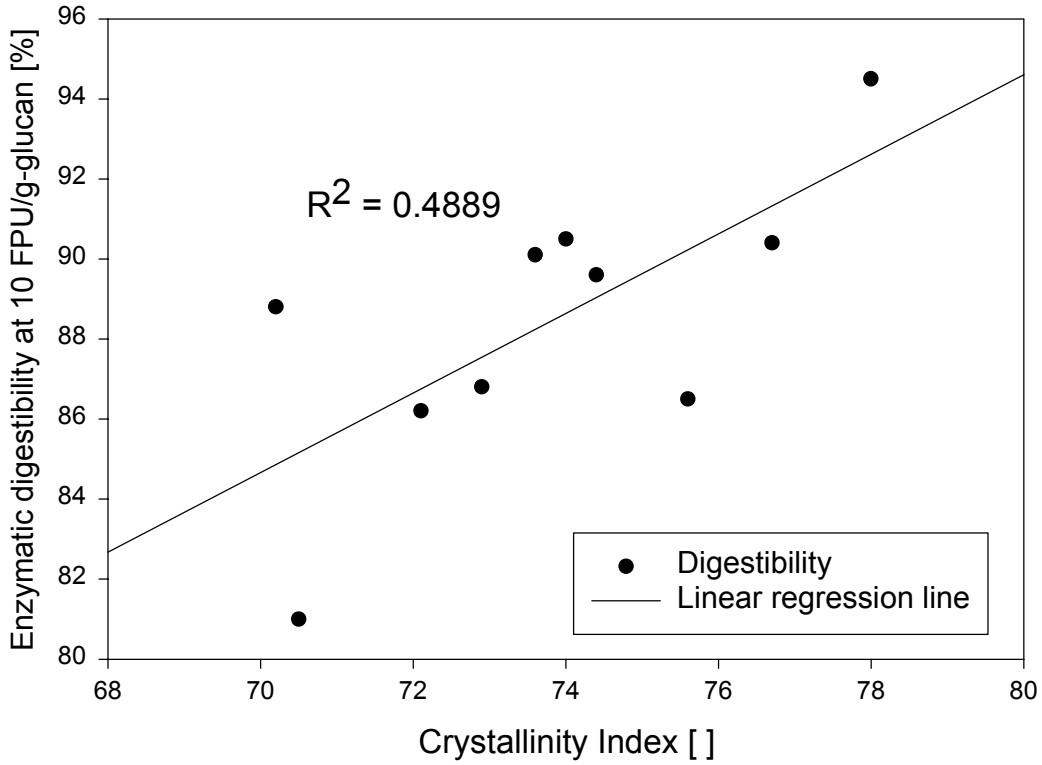


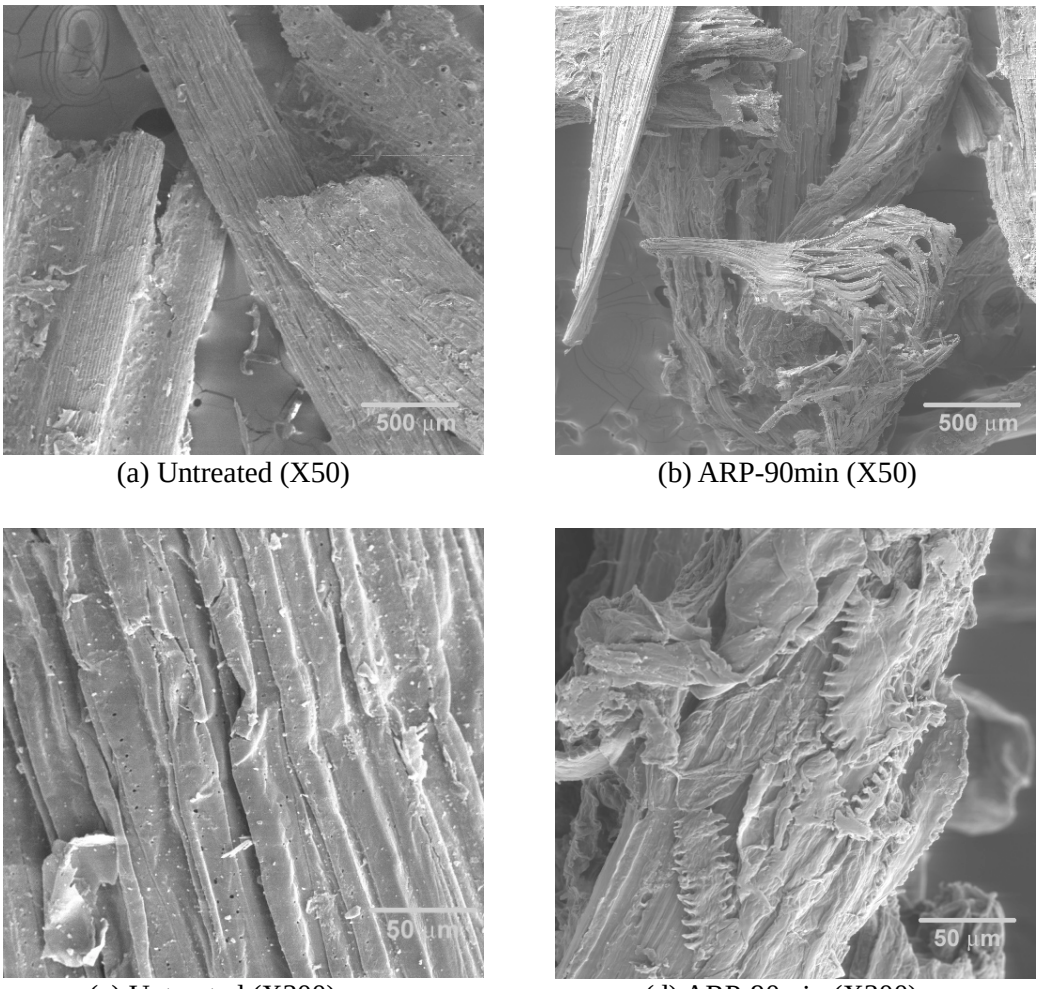
Fig. III-7. Effect of crystallinity index (CrI) on enzymatic digestibility in ARP [1]

Note. ARP pretreatment conditions: 15 wt.% of ammonia, 170°C, 10–90 min, 5 mL/min of flow rate, 2.3 MPa. Enzymatic hydrolysis conditions: 72 h, 10 FPU/g of glucan, pH 4.8, 50°C, 150 rpm. The fitted line indicates linear regressions (R^2=0.4889).

SEM and lignin staining

Because a large fraction of the xylan and lignin is removed by ARP treatment, we became interested in examining the physical changes in the biomass. For this purpose, we took SEM pictures of the treated and untreated biomass samples. Figure III-8 shows that significant morphological changes have indeed occurred. The untreated sample shows rigid and highly ordered fibrils (Fig. III-8-a, c), while fibers of the ARP-90min sample appear to be distorted (Fig. III-8-b, d). The micro-fibrils are also separated from the initial connected structure and fully exposed, thus increasing the external surface area and the porosity. By simply feeling the material with our hands, we found that the wet treated biomass is much softer than the wet untreated biomass.

Lignin staining was also conducted for the treated and untreated biomass (Gahan, 1984). Lignin staining tints the lignin with a red-colored indicator to give a visual indication of the lignin distribution in the solid biomass. Using computer software, the original color pictures were reprocessed to convert the red-tones into a gray scale picture (Fig. III-9). The intensity of the gray tone in each picture signifies the distinct delignification effect of the ARP process, confirming our Klason lignin analysis of Table III-1.

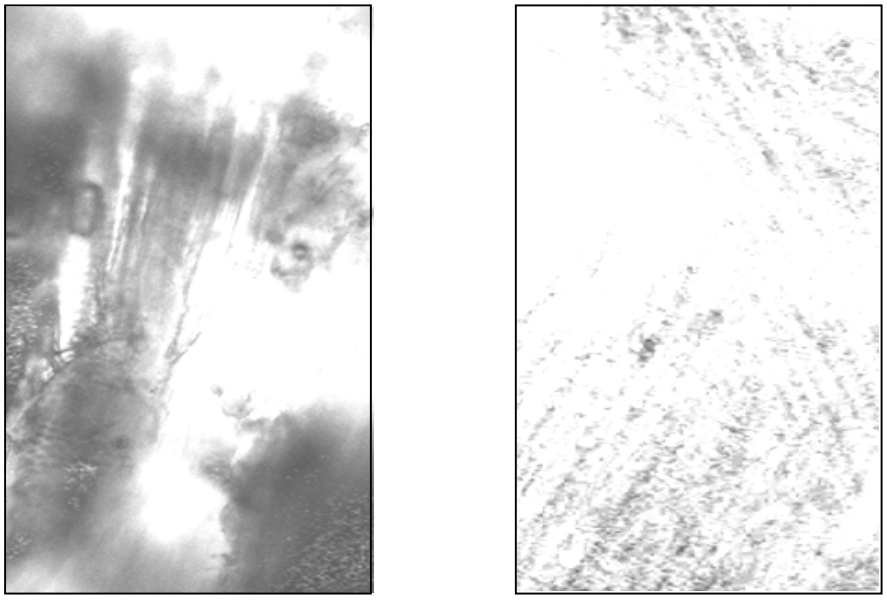


(a) Untreated (X50) (b) ARP-90min (X50)

(c) Untreated (X300) (d) ARP-90min (X300)

Fig. III-8. Scanning electron micrographs (SEM) of treated and untreated corn stover.

Note. For example; ARP 90 min (X50) indicates 90 min of reaction time by ARP and X50 of manification



(a) Untreated (b) ARP 90 min [2]

Fig. III-9. Distribution of lignin in treated and untreated corn stover. [1]

Note. 1. Red tone is selectively enhanced to gray scale by Adobe photoshop (magnification; X400).
2. Pretreatment conditions of (b): 15 wt.% of ammonia, 170°C, 90 min, 5 mL/min of flow rate, 2.3 MPa

FTIR

Infrared spectroscopy is frequently used for investigations of the structure of constituents and the chemical changes in lignocellulosic materials. In this study, diffuse reflectance infrared (DRIFT) spectra were measured to study the difference in their chemical structure, particularly lignin constituent. This task was performed at Michigan State University courtesy of Professor Bruce Dale and his coworkers. In his study of the chemical structure of wood, Pandey reported specific band positions of each constituent (1999). Corn stover, being a grass species, has two types of lignin (guaiacyl and syringyl lignin). According to the report, the lignin characteristic peaks were observed at 1218, 1268 (C-O of guaiacyl ring), 1315 (C-O of syringyl ring), and 1502–1600 cm^{-1} (aromatic skeletal vibration). Figure III-10 shows the FTIR spectra of three ARP-treated samples and untreated corn stover. The band intensities at all lignin peaks (1502–1600 cm^{-1}) of the untreated sample are higher than those of all ARP-treated samples. This again supports the delignification effect of the ARP pretreatment.

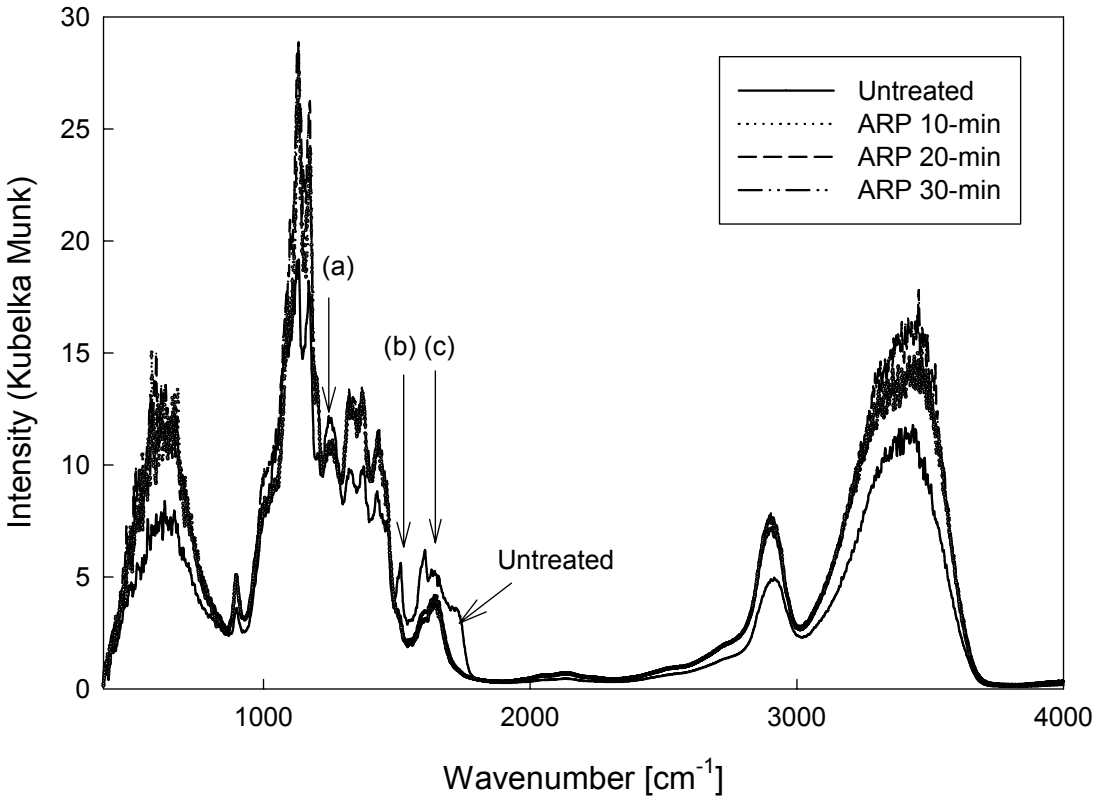


Fig. III-10. FTIR spectra of various ARP-treated samples

Note. Number in legend indicates reaction time (e.g. ARP-10min is 10 min ARP treatment)
Pretreatment conditions: 15 wt.% of ammonia, 170°C, 5 mL/min of flow rate, 2.3 MPa
a. IR band of C-O in guaiacyl or syringyl ring
b. IR band of aromatic skeletal vibration + C=O stretching
c. IR band of aromatic skeletal vibration

CONCLUSIONS

Pretreatment of corn stover by aqueous ammonia is highly effective in enhancing enzymatic digestibility and reducing lignin content. The ARP process removes 70–85% of the total lignin and solubilizes 40–60% of hemicellulose, but retains more than 95% of the cellulose. The carbohydrates of corn stover are well preserved during the process, with total accountability being above 95% for all pretreatment conditions. Longer ARP treatment resulted in more delignification, as well as higher enzymatic digestibility. Most delignification occurred during the first 20 minutes of treatment. The enzymatic digestibility of ARP-treated corn stover yielded near quantitative enzymatic digestibility at 60 FPU/g of glucan, and 92.5% digestibility at 10 FPU/g of glucan. The corn stover treated for 10 minutes with ARP exhibited higher digestibility than α-cellulose. Although the digestibilities were measured at 72 h, over 80% sugar yields were attained within the first 24 h of the enzymatic reaction. Comparison of digestibility data between the ARP samples and dilute-acid treated samples indicated that lignin removal is more effective in enhancing the digestibility than hemicellulose removal. Enzymatic hydrolysis of corn stover correlates well with the extent of removal of total amorphous substances. Increased surface area and porosity appear to be the primary reason for the enhanced enzymatic hydrolysis rate and extent of reaction. SEM pictures of the ARP-treated samples confirm that surface area increases. The crystallinity index of corn stover rises after ARP treatment mainly due to removal of amorphous substances. There is no indication that the crystalline structure of the glucan content of the biomass is changed because of ARP treatment.

IV. PRETREATMENT OF CORN STOVER BY THE LOW-LIQUID AMMONIA PERCOLATION PROCESS

ABSTRACT

A pretreatment method using aqueous ammonia was investigated. This process uses a flow-through packed column reactor (or percolation reactor), and reduces the liquid throughput to the level of 2.0–4.7 mL of liquid per gram of corn stover. It is thus termed the Low-liquid ammonia recycle percolation (ARP) process. The shorter residence time of the reaction, the reduced energy needed, and the fact that it retains a higher fraction of xylan than does conventional ARP are its main advantages. Lignin removal was 59–70%, and the xylan remaining was 48–57%. With proper operation of low-liquid ARP (170 ºC, 10 min with 3.3 mL of 15 wt.% ammonia per gram of corn stover), 95%, 90%, and 86% of enzymatic digestibility were achieved with 60, 15, and 7.5 FPU/g-glucan, respectively. The xylanase activity was not high, bringing the digestibility of xylan to the same level as that of glucan.

In the SSF (simultaneous saccharification and fermentation) test using *S. cerevisiae* (NREL-D_5A), the high ethanol yield (84–85% of the theoretical maximum on the basis of the glucan content in the treated corn stover) was achieved using low-liquid ARP pretreatment with 3% and 6% w/v glucan loading.

In the SSCF (simultaneous saccharification and co-fermentation) test using recombinant *E.coli* (KO11), both the glucan and xylan in the solid were effectively

utilized, giving an overall ethanol yield of 110% of the theoretical maximum based on glucan. With the xylooligomer in the hydrolysate, however, relatively low digestibility of 60% for xylooligomer in ARP hydrolysate and ethanol yield of 56% for ARP-treated corn stover plus hydrolysate were obtained based on glucan. ARP hydrolysate thus appears to inhibit the microorganism activity in the SSCF.

An economic analysis of this process was performed, and the projected minimum ethanol-selling price was found to be $1.43/gallon.

INTRODUCTION

For the last several decades, the use of ethanol has been on the rise as a novel fuel for transportation. The use of fossil fuels has contributed to the buildup of carbon dioxide in the atmosphere, but ethanol is a clean-burning fuel that makes no net contribution to global warming as the carbon dioxide produced by the combustion of ethanol is consumed by the growing raw material (the balance of the carbon cycle).

It is also important that we look for new sources of cellulosic biomass from which ethanol can be produced at a reasonable cost with available resources. Corn stover is currently a most abundant biomass that is widely available in United States. About 80–100 million dry t/year of corn stover are available based on recent estimates (Kadam and McMillan, 2003).

Production of ethanol from lignocellulosic biomass is quite different from the process used for corn because the carbohydrates are much more difficult to solubilize than starch in grain (Gibbson et al., 1986). Lignocellulosic material is very resistant to enzymatic breakdown. This brings up the need for pretreatment, which enhances the susceptibility of the biomass to the enzyme. There have been numerous efforts to enhance the enzymatic reaction, including steam/steam explosion (Fernandez-Bolanos et al., 1999; Mes-Hartree et al., 1984; Schwald et al., 1988; Sawada et al., 1995), grinding/milling (Caufield and Moore, 1974; Koullas et al., 1990; Matsumura et al., 1977; Puri, 1984; Sintsyn et al., 1991), hot water/autohydrolysis (Allen et al., 2001; Garrote et al., 2002; Lora and Wayman, 1978; Váquez et al., 2001), acid treatment (Burns et al., 1989; Grethlein, 1985; Jacobsen and Wyman, 2000; Kim et al., 2001; Mok et al., 1992), alkali treatment (Ferrer et al., 2000; Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003;

Tarkow and Feist, 1969; Yoon et al., 1995), and other methods (McGinnis et al., 1983; Zheng et al., 1998).

Aqueous ammonia has been used for pretreatment because it has a number of desirable characteristics, such as its ability to swell cellulosic material and its high selectivity for lignin. The ammonia recycle percolation process (ARP) has been investigated by our laboratory (Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003), and its process flow diagram is shown in Fig. IV-1. The amount of liquid throughput, reaction time, ammonia concentration, and reaction temperature are the primary factors influencing the reactions occurring in the ARP. An economic analysis of this process indicated that the amount of liquid throughput is one of the major cost items, since it is directly related to the process energy cost. A modification of the original ARP was made in which the amount of liquid input and the residence time was minimized, with the resulting process being referred to as low-liquid ARP. A continuous reaction is the ideal process for biomass pretreatment. In order to achieve continuous pretreatment using aqueous ammonia, 10–20 min of reaction time should be feasible. We found that the liquid input and residence time could be reduced to 3.3 mL/g-biomass and 10 min without adversely affecting the overall performance of the ARP.

Our purpose in this study was to find the most economical pretreatment condition and to evaluate its effectiveness in terms of the bioprocess and bioconversion steps. The digestibility test and SSF experiments were performed for substrates treated by low-liquid ARP, and the ultimate yields of ethanol were determined.

MATERIALS AND METHODS

Materials

Air-dried ground corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was screened to a nominal size of 9–35 mesh. The initial composition of corn stover, as determined by NREL, was: 36.1 wt.% glucan, 21.4 wt.% xylan, 3.5 wt.% arabinan, 1.8 wt.% mannan, 2.5 wt.% galactan, 17.2 wt.% Klason lignin, 7.1 wt.% ash, 3.2 wt.% acetyl group, 4.0 wt.% protein, and 3.6 wt.% uronic acid. α-Cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246). Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL. The average activity of the enzyme, as determined by NREL, was 31.2 filter paper unit (FPU)/mL. The activity of β-glucosidase (Novozyme 188 from Novo Inc., Lot No. 11K1088) was 750 CBU/mL.

Simultaneous saccharification and fermentation (SSF)

The fermentation microorganism used for SSF was *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A). The growth media was YP medium, which contained 1% yeast extract (Sigma Cat. No. Y-0500) and 2% peptone (Sigma Cat. No. P-6588).

Simultaneous saccharification and co-fermentation (SSCF)

Recombinant *Escherichia coli* ATCC® 55124 (KO11) was employed for SSCF tests. LB medium (Sigma Cat. No. L-3152) was used for KO11, which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

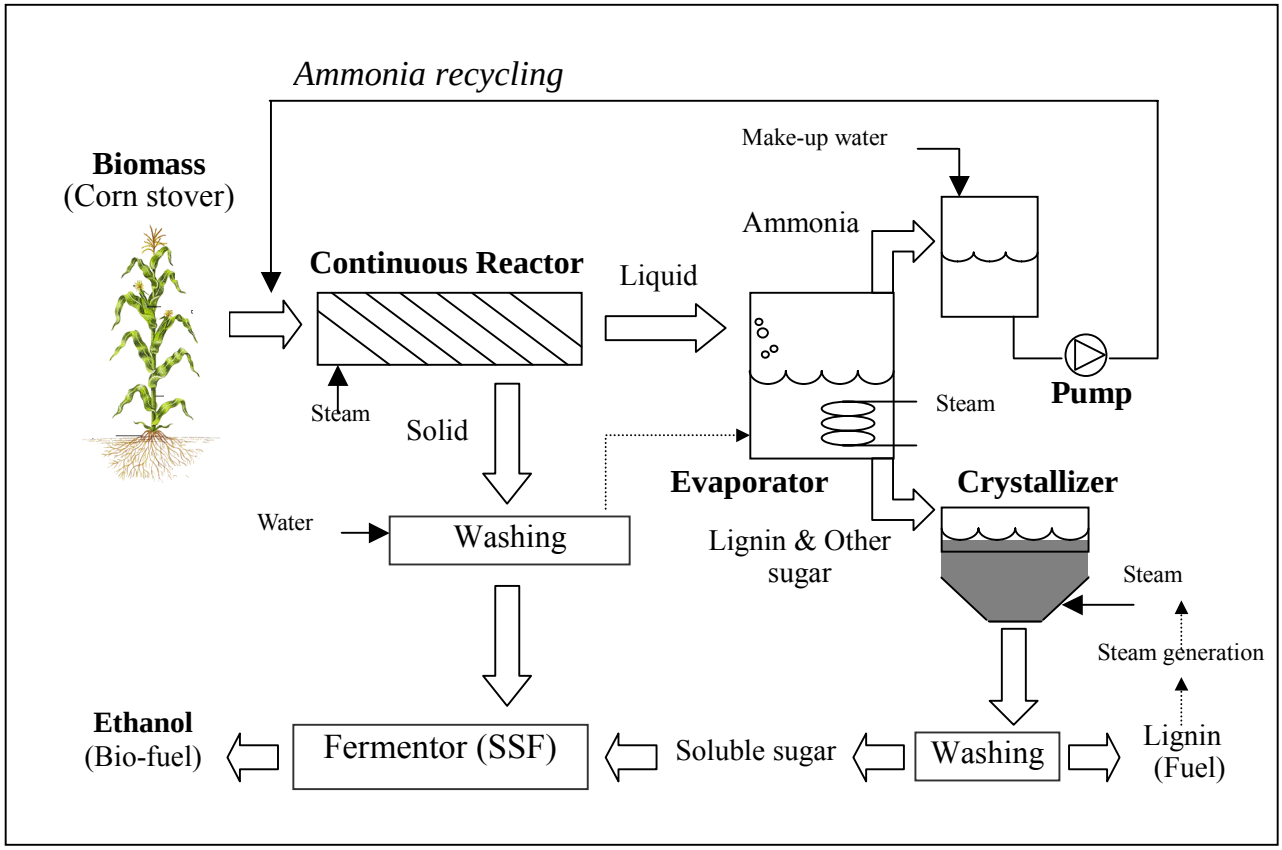


Fig. IV-1. ARP (Ammonia Recycle Percolation) process diagram

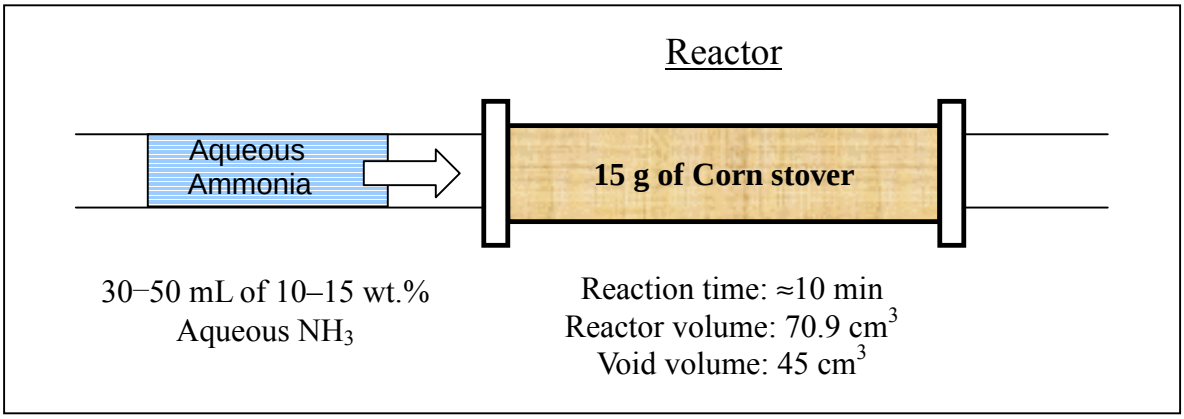


Fig. IV-2. Schematic diagram of low-liquid ARP experiment

Experimental setup and operation

A schematic diagram of the low-liquid ARP experiment is shown in Fig. IV-2, which also describes the reaction and operating conditions. The system consists of a stock solution feeding part, a pump, an oven (Varian 3700), and a liquid holding tank (not shown in the figure). A metering piston pump delivered aqueous ammonia to the reactor. The reactor (70.9 cm^3 of internal volume) was constructed out of SS-316 tubing with an I.D. of 9/10 inches. A 1.0 L SS304 cylinder was used as the receiver tank. In the low-liquid ARP experiment, 15 g of dry biomass sample was packed into the reactor with no presoaking step. The oven was preheated for 25 min., and 2.5 MPa of N_2-backpressure was applied to the reactor system before reaction startup. The specified volume of aqueous ammonia flowed through the system, followed by water. After completion of the reaction, the reactor was cooled down.

Digestibility test

The enzymatic digestibility of corn stover was determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure (NREL, 2004). The conditions of the enzymatic digestibility tests were 50°C and pH 4.8 (0.05 M sodium citrate buffer) in a shaker bath agitated at 150 rpm. Enzyme loadings of 15 and 60 FPU of Spezyme CP/g-glucan supplemented with 30 CBU of β-glucosidase (Novozyme 188, Sigma Cat. No. C-6150)/g-glucan were used. The initial glucan concentration was 1% (w/v) in 100 mL of total liquid. The 250 mL screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (New Brunswick Scientific, Innova-4080). Samples were taken periodically at appropriate sampling times

(6, 12, 24, 48, and 72 h) and analyzed for glucose, xylose, and cellobiose content using HPLC. Total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α-Cellulose and untreated corn stover were subjected to the same procedure as a reference and control, respectively.

Simultaneous saccharification and fermentation (SSF)/co-fermentation (SSCF)

Spezyme CP (Genencor, Lot No. 301-00348-257) was used as cellulase enzyme. It was supplemented with β-glucosidase, Novozyme 188 (Novo Inc., Sigma Cat. No. C6150, Lot No. 11K1088). A 250 mL Erlenmeyer flask was used as the bioreactor. It was shaken in the incubator shaker (New Brunswick Scientific, Innova-4080) at 38 °C with 150 rpm. Into 100 mL working volume of liquid, treated corn stover samples were added such that the glucan content becomes 3% w/v. α-Cellulose was subjected to the same procedure as a control. The SSF/SSCF runs were performed with buffer without external pH control, starting at pH 5.0/7.0 at the beginning of the fermentation and gradually decreasing to 4.5/6.0 at the end. The loading of cellulase enzyme was 15 FPU/g-glucan, and that of β-glucosidase was 30 CBU/g-glucan.

The ethanol yield was calculated as follows:

$$\text{Ethanol yield}[\%\ \text{of theoretical maximum}] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor} \times 0.511} \times 100$$

Note. Sugar is interpreted as glucose in SSF or glucose plus xylose in SSCF.

Analytical methods

The solid samples were analyzed for sugar and Klason lignin following the procedures specified in NREL Chemical Analysis and Testing Standard Procedures (NREL, 2004). Each sample was analyzed in duplicate. The moisture content was measured by an infrared moisture balance (Denver Instruments, IR-30). The sugars in the liquid samples were determined after secondary acid hydrolysis to account for the oligomer contents. The conditions of the secondary hydrolysis were 4 wt.% sulfuric acid and 121 ºC for one hour. Sugars were determined by HPLC using a Bio-Rad Aminex HPX-87P column. For the SSF test, an HPX-87H column and YSI 2300 Glucose/Lactate analyzer (for rapid analysis of glucose in prepared initial inoculum) measured the ethanol and glucose content. A refractive index detector was used in conjunction with HPLC.

Statistical analysis

A mean value and a standard deviation were calculated using JMP software (SAS Version 5.0). SigmaPlot (Version 8.0, SPSS) was used to plot the results.

RESULTS AND DISCUSSION

Effect of ammonia concentration and flow rate

Two levels of ammonia concentration (10 and 15 wt.%) were evaluated for the pretreatment. Table IV-1 summarizes the compositional changes in the solid and liquid samples, and their effects on enzymatic hydrolysis. The results indicate that the low-liquid ARP removes basically lignin, but also induces the solubilization of about half of the xylan. The low-liquid ARP process removed 62−70 % of the total lignin. Table IV-1

shows that the accountability of carbohydrate (total glucan and xylan in the solid and liquid combined) is above 95% for glucan and xylan. The biomass carbohydrates are thus well preserved in the ARP process. Table IV-1 shows that the digestibilities of the treated samples are 85–90% with 15 FPU/g-glucan. The digestibilities of 15 wt.% NH_3-treated samples were slightly higher than those of 10 wt.% NH_3-treated samples.

The enzymatic hydrolysis results are presented in Fig. IV-3-(a) showing 15 FPU/g-glucan enzyme loading profiles. Enzymatic hydrolysis occurs rapidly, completing most of the hydrolysis (80-90% of the 72 h digestibilities) within the first 12 hours. For all conditions tested, the hydrolysis rates of ARP treated samples were much faster than for α-cellulose.

Effect of ammonia throughput

The effect of the liquid throughput was investigated. In this experiment, three different liquid throughputs (2.0, 3.3, and 4.7 mL of 15 wt.% ammonia per gram of corn stover) were applied at 170°C. The results are summarized in Table IV-2.

The amount of liquid throughput is one of the critical economic factors in the ARP. A modification of the original ARP was made in which the amount of liquid input and the residence time were minimized. As shown in Table IV-2, the lignin removal (59% delignification) at 2.0 mL ammonia input per g biomass was somewhat lower than those (70% delignification) with higher liquid throughput. The 72-h digestibilities with 15 FPU/g-glucan for all three different liquid throughputs were in the range of 87–90%. It was thus concluded that the liquid input and residence time could be reduced to 3.3 mL/g of biomass and 10 min without adversely affecting the overall performance of the ARP.

Enzymatic hydrolysis results are presented in Fig. IV-3-(b) for 15 FPU/g-glucan enzyme loading profiles.

Low-liquid ARP at low temperature

The effect of temperature on the low-liquid ARP was investigated over a temperature range of 110–170 °C. Four different temperatures (110, 130, 150, and 170°C) were applied maintaining the liquid throughput at 3.3 mL of 15 wt.% NH_3 per gram of corn stover. Table IV-3 summarizes the compositional changes in both the solid and the liquid, along with the digestibility with three different enzyme loadings (60, 15, and 7.5 FPU/g-glucan). Xylan remaining in the treated solid increased as the temperature was lowered. Treatment at higher temperatures raises the equipment costs, as well as the operating costs. If the treatment at lower temperature shows an overall performance as good as that of conventional ARP, the process at lower temperature would be preferred.

Low-liquid ARP treatment at 150°C rendered 88% of the digestibility, close to the 90% obtained for the 170°C-treated sample, with 15 FPU/g-glucan. However, the digestibility of the 150°C-treated sample (77%) was much lower than that of the 170°C-treated sample (86%) with 7.5 FPU/g-glucan enzyme loading. Although its digestibility with 7.5 FPU/g-glucan (77%) was lower than that of the 170ºC low-liquid ARP (86%), low-liquid ARP treatment at 150ºC can be considered as a potential pretreatment temperature replacing 170ºC. The trials at other temperatures (110, 130 °C) resulted in fairly low digestibilities. With treatment at 170°C, about 50% of xylan was solubilized. The data in Table IV-3 clearly indicate that more lignin and xylan were removed at higher temperatures, but higher enzymatic digestibilities were also achieved.

Enzymatic hydrolysis results are presented in Fig. IV-4-(a) and (b), showing 15 and 7.5 FPU/g-glucan enzyme loading profiles, respectively.

From Table IV-3, the digestibilities of residual xylan in the 110–170°C treated samples were about 58–78% with 15 FPU/g-glucan of enzyme loading. It is obvious that there is a substantial xylanase activity in the "cellulase", Spezyme CP. However, the xylanase activity is not high enough to bring the digestibility of xylan to the same level (70–88%) as that of glucan in all conditions. From Table IV-3, it is apparent that the xylan digestion by enzymes is related to the glucan digestibility.

Table IV-1
Effect of flow rate and ammonia concentration on composition and digestibility (170°C, 3.3 mL of NH_3 liquid throughput per gram of corn stover) [1]

Flow rate	Solid				Liquid		Total		Digestibility [4]	
	S.R.[2]	Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	60FPU	15FPU
[mL/min]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100	17.2	36.1	21.4	-	-	36.1	21.4	21.2	15.7
10 wt.% of Ammonia										
2.5	59.8	6.6	36.1	10.9	0.5	10.2	36.6	21.1	94.7	85.1
5.0	58.1	5.1	35.0	9.6	0.5	11.0	35.5	20.6	99.7	87.7
7.5	59.2	5.1	35.9	10.9	0.5	9.7	36.4	20.6	94.1	86.8
15 wt.% of Ammonia										
2.5	58.5	6.4	35.9	10.7	0.5	10.6	36.4	21.3	95.2	86.2
5.0	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	95.3	90.1
7.5	57.0	5.6	35.7	10.1	0.8	10.5	36.5	20.6	97.2	90.0

Note. 1. Data in the table are based on the oven dry untreated biomass; pretreatment conditions: 170°C, 3.3 mL of 10 or 15 wt.% NH_3 liquid throughput per gram of corn stover.
2. S.R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 60 or 15 FPU/g-glucan, pH 4.8, 50°C, 150 rpm

Table IV-2
Effect of aqueous ammonia (15 wt.%) throughput on composition and digestibility [1]

Liquid	Solid				Liquid		Total		Digestibility [4]	
Throughput.	S.R.[2]	Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	60FPU	15FPU
[mL/g solid]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100	17.2	36.1	21.4	-	-	36.1	21.4	21.2	15.7
2.0	62.1	7.1	36.1	11.4	0.8	9.2	36.9	20.6	93.8	87.5
3.3	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	95.3	90.1
4.7	56.9	5.1	35.8	10.0	0.5	9.8	36.3	19.8	97.8	88.4

Note. 1. Data in the table are based on the oven dry untreated biomass; pretreatment conditions: 170°C, 2.0−3.3 mL of 15 wt.% NH_3 liquid throughput per gram of corn stover.
2. S. R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 60, 15 or 7.5 FPU/g-glucan, pH 4.8, 50°C, 150 rpm

Table IV-3
Effect of temperature on composition and digestibility [1]

Reaction	Solid				Liquid		Total			Digestibility [4]		
Temp.	S.R.[2]	Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan		60FPU	15FPU	7.5FPU
[°C]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]		[%]	[%]	[%]
Untreated	100	17.2	36.1	21.4	-	-	36.1	21.4		21.2	15.7	13.8
170	57.5	5.1	35.6	10.3	0.5	10.1	36.1	20.4	G	95.3	90.1	85.9
									X	85.5	77.9	71.2
150	60.4	6.3	35.8	12.3	0.5	8.5	36.3	20.8	G	96.9	88.0	77.1
									X	86.0	73.6	61.8
130	66.2	6.4	36.1	15.1	0.4	6.5	36.5	21.6	G	95.5	79.6	64.7
									X	87.6	67.2	58.2
110	76.1	9.2	36.2	18.5	0.3	3.1	36.5	21.6	G	82.4	69.8	53.4
									X	72.2	57.9	46.4

Note. 1. Data in the table are based on the oven dry untreated biomass; pretreatment conditions: 110–170°C, 3.3 mL of 15 wt.% NH_3 liquid throughput per gram of corn stover.
2. S. R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 60 or 15 FPU/g-glucan, pH 4.8, 50°C, 150 rpm; G (glucan digestibility), X (xylan digestibility)

SSF of low-liquid ARP-treated corn stover

Low-liquid ARP-treated solid samples were prepared under the optimum conditions of 170ºC, 3.3 mL of 15 wt.% ammonia per gram of corn stover liquid input, 5 mL/min flow rate, and 10 min reaction time. The glucan content of the pretreated corn stover was 62%. The α-cellulose was tested along with the corn stover as a control, which is 92% of glucan content. The ethanol yield and glucose concentration in the SSF operation were monitored. Two different glucan loadings (3% or 6% w/v of glucan) were applied. All procedures were as specified in the NREL Chemical Analysis and Testing Laboratory Analytical Procedures (NREL, 2004).

The ethanol yield data for low-liquid ARP-treated corn stover and α-cellulose are presented in Fig. IV-5. With an initial loading of 3% w/v glucan, the ethanol yield of treated corn stover reached 84% of the theoretical maximum (14.3 g/L) at 96 h (Fig. IV-5-a). At the 24 h point, the ethanol yield of the pretreated sample had reached 78%. At this point, the ethanol yield of corn stover was substantially higher than the 56% yield of α-cellulose (Fig. IV-5-a). The ethanol yield using 6% w/v glucan loading is presented in Fig. IV-5-(b). The ethanol yield of low-liquid ARP-treated corn stover gave about the same level of ethanol yield, 84% at 120 h, with a 3% w/v glucan loading case. The glucose level in the broth dropped to almost zero after 12 h for 3% w/v glucan loading and 24 h for 6% w/v glucan loading. The profile of the ethanol yield was similar to that obtained with α-cellulose. The ethanol conversion within the 24 h period was faster with 3% w/v glucan loading. The 12-h and 24-h ethanol yields of 3% w/v glucan loading were 65% and 78%, respectively, while those of 6% w/v glucan loading were 21% and 60%, respectively. The 12 h and 24 h glucose concentrations of 3% w/v glucan loading were

7.7 g/L and 4.9 g/L, respectively, while those of 6% w/v glucan loading were 11.7 g/L and 13.5 g/L respectively. Higher glucan loading (6% w/v glucan loading) also yielded 84% of ethanol, indicating that a higher level of glucan loading may be feasible without adversely affecting the enzymatic hydrolysis because the yeast eliminates the glucose inhibition from the hydrolysis step in the SSF.

SSCF of low-liquid ARP-treated corn stover and hydrolysate

Simultaneous saccharification and co-fermentation (SSCF) of low-liquid ARP-treated corn stover (under optimum conditions) and α-cellulose was performed using the recombinant *E.coli* ATCC® 55124 (KO11). Low-liquid ARP-treated corn stover contains about 61% of glucan and 18% of xylan based on weight, which means about 38 g/L of the total carbon source in 3% w/v glucan loading fermentation. Pentose, mainly in the form of xylan corn stover, presents in low-liquid ARP-treated corn stover as about 18% of the total treated solid, which *S. cerevisiae* does not ferment to ethanol. However, a microorganism in SSCF, recombinant *E.coli*, is capable of fermenting hexose and pentose into ethanol.

Fig. IV-6 presents the ethanol yield (based on glucan) for various substrates in the SSCF. Maximum ethanol concentration was 19.4 g/L, which corresponds to 89% of the maximum theoretical yield based on total input glucan and xylan. This was substantially higher than the 16.6 g/L obtained for α-cellulose.

For the utilization of sugar in the hydrolysate, the hydrolysate from the low-liquid ARP process was collected and subjected to the rota-evaporation to remove the ammonia. The pH was adjusted be < pH 2.0, and then 72 h settling time was applied for the

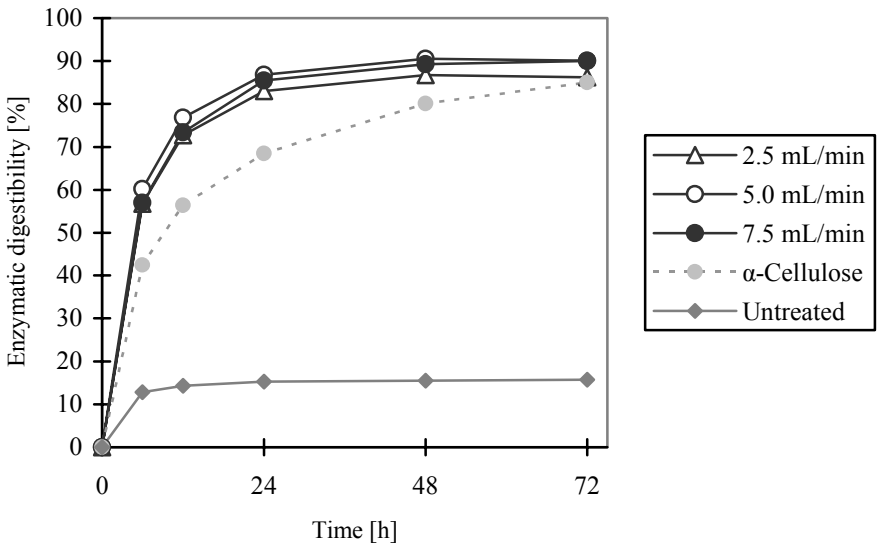
hydrolysate until the lignin was precipitated. The precipitated lignin was separated using centrifugation, which removed approximately 80% of original soluble lignin from the ARP liquor. During the lignin precipitation, it was inevitable that about 20−30% of xylan was precipitated along with the lignin. The low-liquid ARP process generally removes approximately half of the xylan, but preserves the glucan in the corn stover. Thus, the hydrolysate contains mainly xylan. The treated hydrolysate was added into the reactor with low-liquid ARP-treated corn stover and fermented by recombinant *E.coli*. For the treated-corn stover with ARP liquor, maximum ethanol concentrations were reached in approximately 96 h (9.5 g/L), at 56% of the maximum theoretical yield based on glucan and 34% based on glucan and xylan. This was substantially lower than the concentrations of 16.6 g/L obtained for α-cellulose and 19.4 g/L from the SSCF using the treated-solid only. Adding hydrolysate resulted in substantially lower yield than treated solid along case.

The lower yield with hydrolysate can be attributed to the microbial inhibitors that are formed during the hydrolysate preparation process. During pretreatment, various compounds which are inhibitory to microorganisms are formed or released. Those compounds limit efficient utilization of the soluble sugar for ethanol production using fermentation (Palmqvist and Hahn-Hägerdal, 2000a; Palmqvist and Hahn-Hägerdal, 2000b).

Hydrolysis of xylose oligomers by cellulase enzyme (Spezyme CP) was also investigated using the ARP effluent containing 2.1 g/L of xylose equivalent. The digestibility test results are summarized in Fig. IV-7. The digestibility of the soluble xylose oligomer is about 60% with 30 FPU/g-xylan of enzyme loading. An important

point is that the digestibility reaches its maximum within 6 h. In the previous digestibility test for xylan in the solid sample, about 70% of digestibility was achieved by cellulose (Table IV-3). It appears that the cellulase enzyme carries out the hydrolysis of the soluble xylose oligomers to monomer comparably to the level of xylan in the solid. We speculate that the limited hydrolysis yield is due to the structure of the oligomers, wherein a part of it is not hydrolyzed by the xylanase contained in the Spezyme CP. The low enzymatic digestibility of soluble xylose oligomers may also contribute to the lower ethanol yield in the SSCF.

(a) Flow rate vs. Digestibility at 15 FPU/g-glucan



(b) Liquid throughput vs. Digestibility at 15 FPU/g-glucan

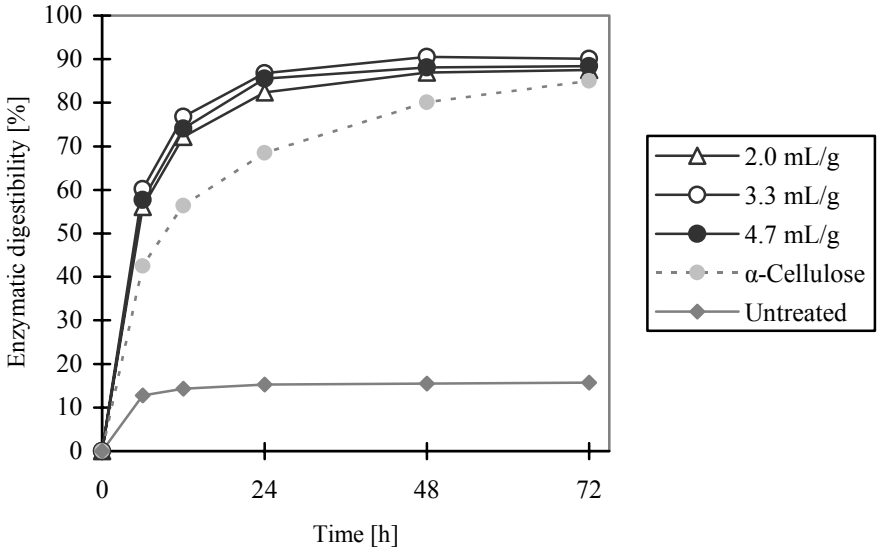
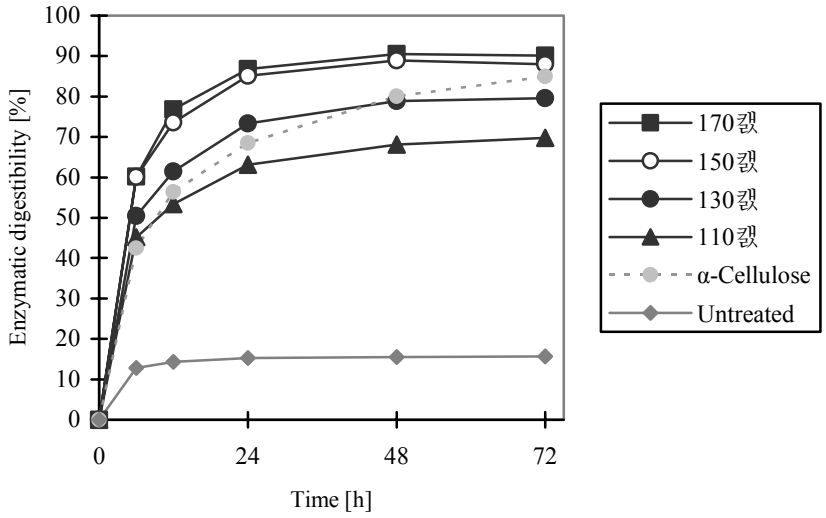


Fig. IV-3. Enzymatic digestibility of low-liquid ARP-treated samples at different enzyme loadings

Note. Pretreatment conditions: 170°C, (a) 2.5−7.5 mL/min of flow rate, 3.3 mL of 15 wt.% NH_3 throughput per gram of corn stover, (b) 5 mL/min of flow rate, 2.0; enzymatic hydrolysis conditions: 72 h, 15 FPU/g-glucan, pH 4.8, 50°C, 150 rpm.

(a) 15 FPU/g-glucan enzyme loading



(b) 7.5 FPU/g-glucan enzyme loading

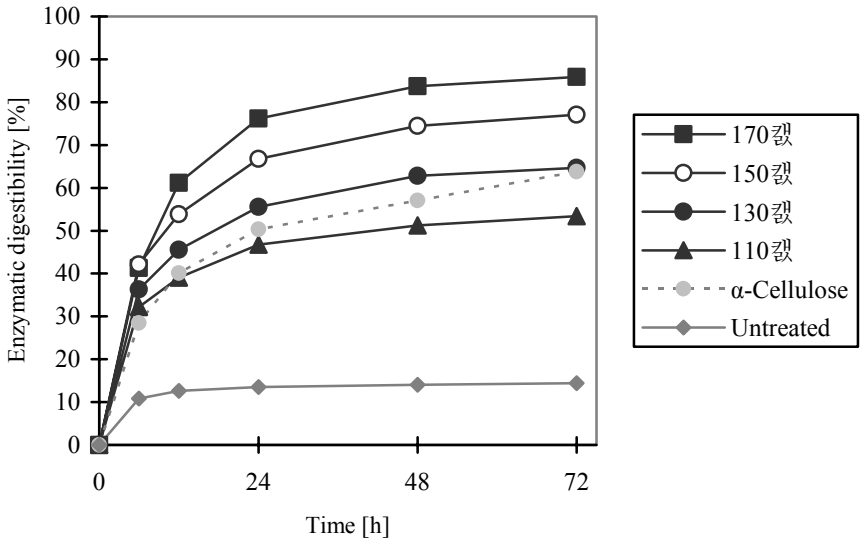
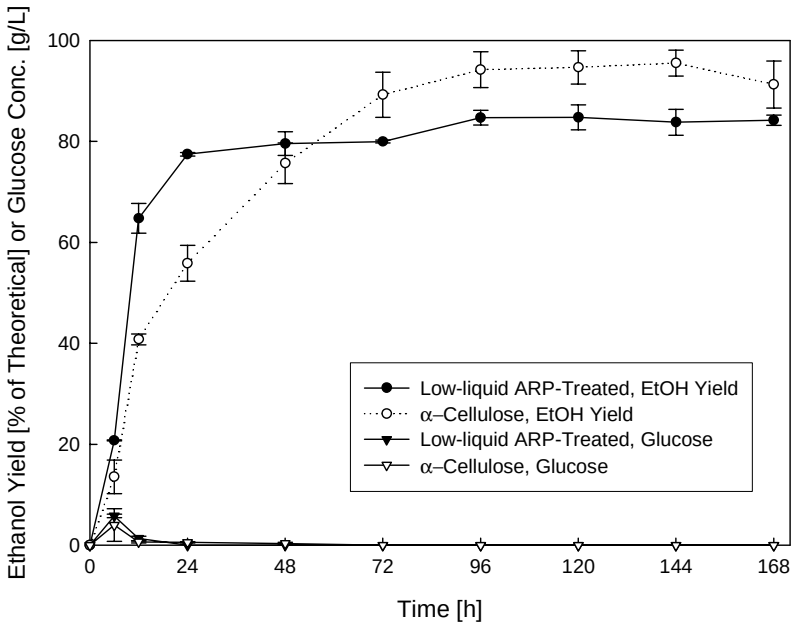


Fig. IV-4. Enzymatic digestibility of low-liquid ARP-treated samples at different enzyme loadings

Note. Pretreatment conditions: 110–170°C, 3.3 mL of 15 wt.% NH_3 throughput per gram of corn stover, 5.0 mL/min of flow rate; enzymatic hydrolysis conditions: 72 h, 15 or 7.5 FPU/g-glucan, pH 4.8, 50°C, 150 rpm.

(a) 3% w/v of glucan loading



(b) 6% w/v of glucan loading

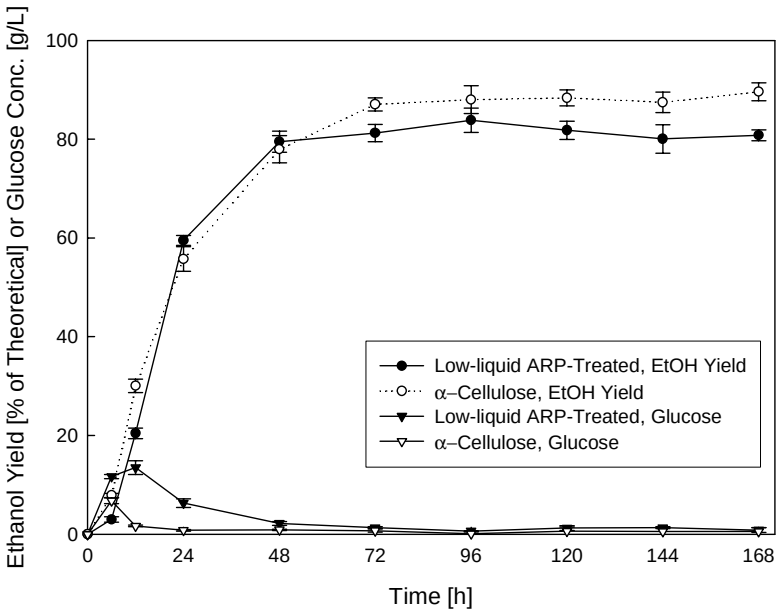


Fig. IV-5. Ethanol yield of low-liquid ARP-treated samples using SSF

Note. Pretreatment conditions: 170°C, 3.3 mL of 15 wt.% NH_3 throughput per gram of corn stover, 5.0 mL/min of flow rate; SSF test conditions: 15 FPU/g-glucan enzyme loading, D_5A yeast in YP medium, pH 5.0, 38°C, 150 rpm; (a) n=2 for low-liquid ARP, n=4 for α-cellulose (b) n=2 for low-liquid ARP, n=2 for α-cellulose.

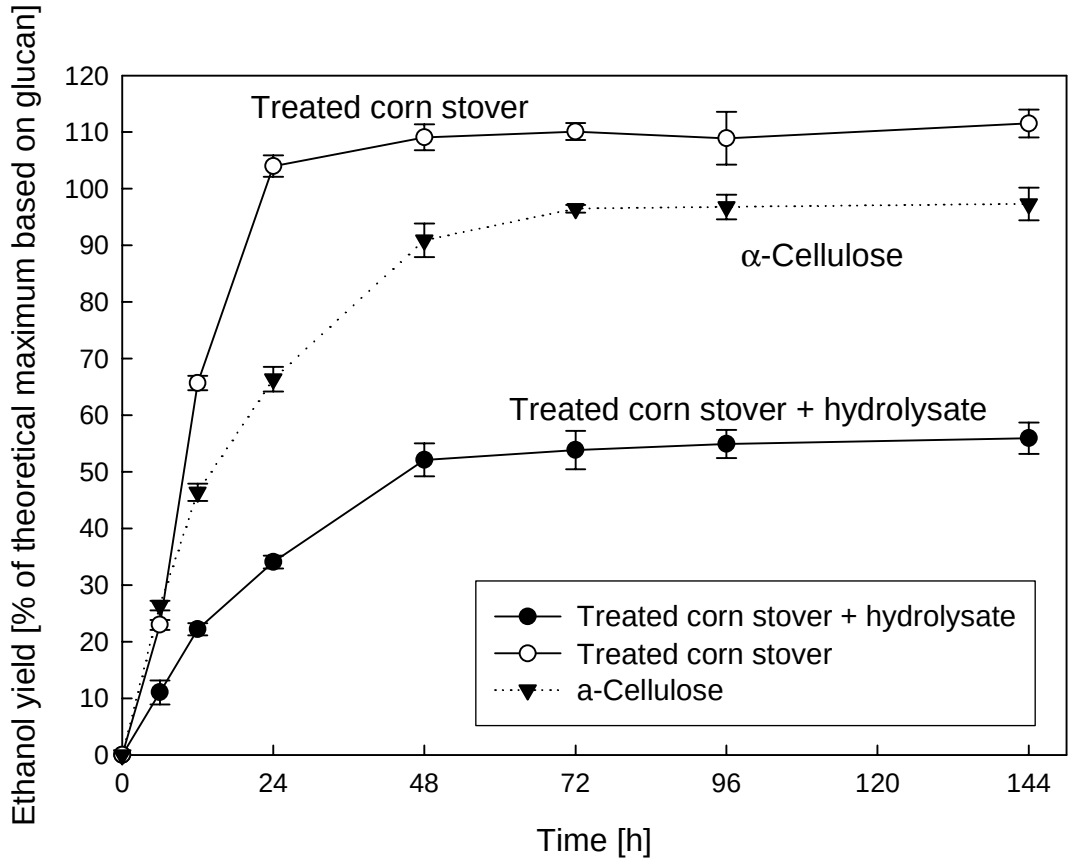


Fig. IV-6. Ethanol yield of low-liquid ARP treated samples using SSCF

Note. Pretreatment conditions: 170°C, 3.3 mL of 15 wt.% NH_3 throughput per gram of corn stover, 5.0 mL/min of flow rate.
SSF test conditions: *Escherichia coli* ATCC® 55124; substrate: 3% w/v glucan loading/100 mL reactor, 15 FPU of cellulase/g-glucan; 30 CBU of β-glucosidase/g-glucan; LB medium (0.5% of Yeast extract, 1% of Tryptone); anaerobic condition; 38˚C, pH=7.0, 150 rpm; n=3 for 'low-liquid ARP-treated corn stover' and 'α-cellulose', n=2 for 'low-liquid ARP-treated corn stover + hydrolysate'.

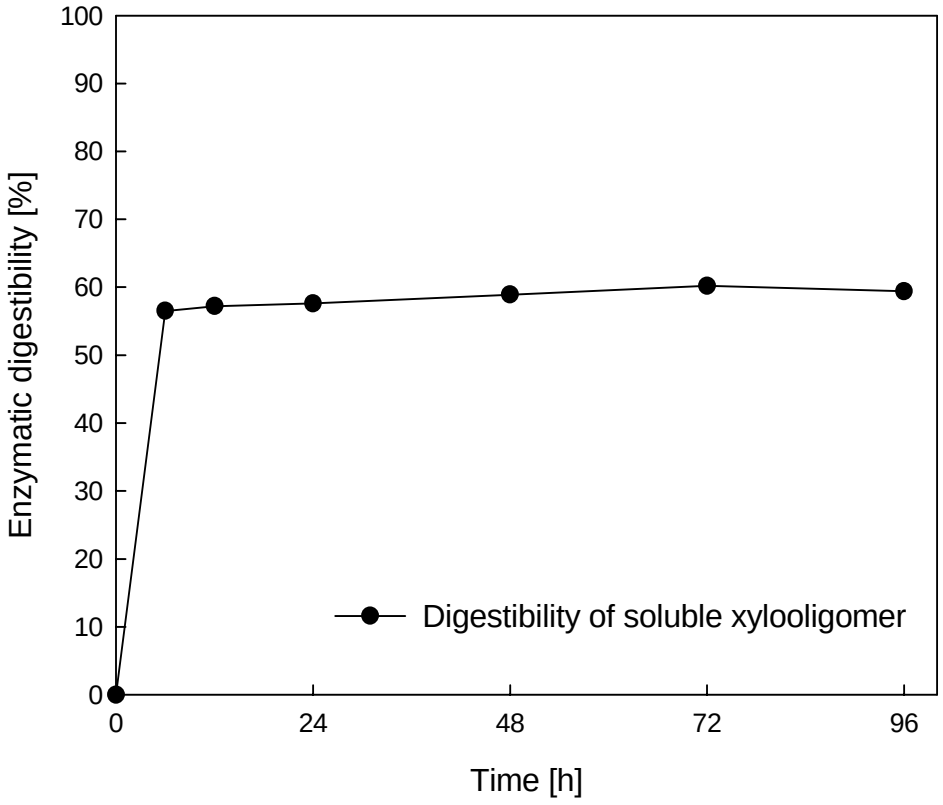


Fig. IV-7. Enzymatic digestibility of soluble xylooligomers in the low-liquid ARP hydrolysate

Note. Reaction conditions: 170°C, 3.3 mL of 15 wt.% NH_3 throughput per gram of corn stover, 5.0 mL/min of flow rate; Enzymatic hydrolysis conditions: 72 h, 60 FPU/g- xylooligomer, pH 4.8, 50°C, 150 rpm.
Treatment of hydrolysate for lignin precipitation: adjust pH to 1.8 for >72 h, then centrifuge.

Review of economic aspects

Significant further research and development are needed before large-scale implementation because the ARP model is still tentative in some areas. However, a preliminary economic analysis for a corn stover-to-ethanol process using low-liquid ARP pretreatment was performed. To this end, a process concept incorporating a combined evaporator and crystallizer was developed based on the experimental results, and the schematic diagram of the process is shown in Fig. IV-1. The economic analysis results are summarized in Table IV-4.

Aspen Plus Version 10.1-0 Build 56 was used to calculate the material and energy balance. This analysis was performed based on an ethanol production rate of 54.1 million gallons per year. The fermentation used in the model uses simultaneous saccharification and co-fermentation (SSCF) technology based on a recombinant *Zymononas mobilis* strain to convert glucose and xylose directly into ethanol. Ethanol is recovered from the dilute aqueous broth using a combination of conventional distillation and dehydration using adsorption on molecular sieves. The lignin recovered in the low-liquid ARP pretreatment, the unconverted corn stover and the cell mass from fermentation are burned and the heat released is used to produce steam. Some of the steam is used for process needs; the rest is used to generate electrical power. Power in excess of process needs is sold.

According to the economic analysis by NREL (Table IV-4), the projected minimum ethanol-selling price (MESP) is $1.43/gallon. The total project investment is projected at $205.4 million. Direct fixed capital for the low-liquid ARP pretreatment amounts to $28.3 million. The low-liquid ARP process has a slightly lower capital

requirement than conventional dilute acid pretreatment (not shown in Table IV-4). Overall yield of the low-liquid ARP pretreatment is 70 gal/dry US ton. The low-liquid ARP pretreatment does not require a neutralization process, while the high capital cost for pretreatment ($28.3 million) is necessary because a high pressure and temperature rating reactor system is required for the ARP system. Although the low-liquid ARP pretreatment has higher steam requirements as compared to the conventional dilute acid pretreatment, the high operating costs can be partially offset by exporting the electricity ($7 million) generated by the lignin that is sent directly to boiler fuel.

Table IV-4. Ethanol production process engineering analysis

Minimum Ethanol Selling Price $1.43 per gallon

Ethanol production (million gallons/year)	54.1
Ethanol yield (gal/dry US ton feedstock)	70
Internal rate of return (after-tax)	10%
Feedstock cost ($/dry US ton)	$35

Capital Costs		**Operating Costs**	
Feed handling	$7,300,000	Feedstock	$26,500,000
Pretreatment	***$28,300,000***	CSL	$1,600,000
Neutralization/conditioning	$0	Cellulase	$7,500,000
SSCF	$12,600,000	Other raw materials	$3,900,000
Others	$70,200,000	Waste disposal	$1,000,000
Total equipment cost	$118,400,000	Electricity	-$7,000,000
Added costs (%TPI=42%)	$87,000,000	Others (fixed costs, depreciation, ROI, tax)	$43,900,000
Total Project Investment	$205,400,000		

(Courtesy of NREL, Golden, CO) (All values in 1999$)

CONCLUSION

Low-liquid ARP is an effective pretreatment method that enhances the enzymatic digestibility and fermentability of corn stover to the level achieved by conventional ARP. The advantages of this process are that is faster, requires a shorter residence time of liquid and consumes less energy than conventional ARP. The short residence time (10 min with 3.3 mL of 15 wt.% ammonia per gram of corn stover) of liquid required in this process makes it feasible to construct a continuous process. low-liquid ARP removed 70% of the original lignin and 47% of xylan under optimum conditions (170ºC, 10 min of reaction time, and 3.3 mL of 15 wt.% ammonia per gram of corn stover). The glucan content, however, remained intact.

Higher temperatures and higher ammonia concentrations resulted in higher enzymatic digestibility. The digestibilities of low-liquid ARP-treated corn stover (under optimum conditions) with 60, 15, and 7.5 FPU/g-glucan were 95%, 90.1%, and 86% respectively. The same digestibility test based on Spezyme CP "cellulase" produced hydrolysis yield of 86%, 78, and 71% for residual xylan in treated samples with the same enzyme loadings (60, 15, and 7.5 FPU/g-glucan, respectively).

In the standard SSF test (3% w/v glucan loading) using yeast, the ethanol yield of low-liquid ARP-treated corn stover using optimum pretreatment conditions reached 85% of the theoretical maximum at 96 h based on glucan. Higher glucan loading (6% w/v glucan loading) also yielded 84% of ethanol yield based on glucan.

In the SSCF test, recombinant *E.coli* (KO11) utilized both the glucan and xylan in the solid effectively, converting them into ethanol giving 89% (19.4 g/L) of the theoretical maximum based on total input glucan and xylan. Adding the xylooligomer in

the hydrolysate, however, reduced the digestibility to 60% and the ethanol yield to 56% based on glucan. ARP liquor appears to inhibit the microorganism activity in the SSCF.

The projected minimum ethanol-selling price achievable using this process is $1.43/gallon. The ethanol production process engineering analysis thus shows that the ethanol fuel process using low-liquid ARP is economically feasible.

V. FRACTIONATION OF CORN STOVER BY HOT-WATER AND AQUEOUS AMMONIA TREATMENT

ABSTRACT

A two-stage percolation process was investigated for pretreatment and fractionation of corn stover. The two-stage process is consisted of hot water treatment followed by treatment with aqueous ammonia, both applied in a flow-through (percolation) reactor. The first stage processing removed the hemicellulose removal while the second stage accomplished delignification. The conditions needed to achieve a satisfactory level of biomass fractionation and acceptable enzymatic hydrolysis were identified in terms of reaction temperature, flow rate (retention time) and reaction time for each stage. With proper operation of this two-stage treatment, fractionation of biomass was achieved to the extent that the xylan fraction was hydrolyzed with 92–95% conversion, and recovered with 83–86% yields; and the lignin removal reached 75–81%. The remaining solid after the two-stage treatment contained 78–85% cellulose. The two-stage treatments enhanced the enzymatic digestibility to 90–96% with 60 FPU/g of glucan, and 87–89% with 15 FPU/g of glucan. The composition and digestibility data indicate that the lignin content in the biomass is one of the major factors controlling the enzymatic digestibility.

INTRODUCTION

Corn stover is currently regarded as the most promising biomass resource in the U.S., with 60–80 million tons/yr available for conversion into fuels and chemicals (Kadam and McMillan, 2003). Production of corn stover roughly equals the mass of corn kernels (DOE, 2001). Pretreatment is one of the key elements in the bioconversion of this biomass. It is required for efficient enzymatic hydrolysis of biomass because of the physical and chemical barriers that inhibit the accessibility of the enzyme to the cellulose substrate (Saddler, 1993). Among the known chemical barriers are lignin, hemicellulose (Schwald et al., 1988), and acetyl group (Chang and Holtzapple, 2000; Grohmann et al., 1989; Kong et al., 1992). The physical factors of biomass include its crystallinity (Caufield and Moore, 1974; Cowling and Kirk, 1976; Fan et al., 1980; Polcin and Bezuch, 1977; Sasaki et al., 1979; Schwald et al., 1988), surface area (Burns et al., 1989; Lee et al., 1995), and degree of polymerization (Puri, 1984) and have also been known to influence the enzymatic hydrolysis. Among these factors, lignin has long been considered as a major impeding factor (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988). The main chemical bonds found in lignin are C-O-C and C-C linkages. Moreover, the existence of covalent bonds between lignin and carbohydrates has also been verified by various researchers (Karlsson, 1997; Nikitin et al., 1971; Polcin and Bezuch, 1977), thus forming what are known as “lignin-carbohydrate complex (LCC)”. These researchers further postulated that there are three different types of linkages (ether, ester, and glycosidic linkages) between lignin and carbohydrates, the ester linkage being the most prevalent and stable. A number of researchers have suggested that early removal of lignin would

eliminate the interaction between lignin and cellulase enzyme making the enzymatic hydrolysis more efficient (Converse, 1993; Dulap et al., 1976; Millet, 1974; Van Soest, 1969).

Fractionation of biomass into the three main biomass constituents is a concept being developed as a means to improve the overall biomass utilization. When separated from the biomass, hemicellulose may find broader acceptance for chemicals, fuel, and food applications. The lignin separated in the process is projected to be usable as a fuel, with 26.3 MJ/oven dry kg of lignin (Saddler, 1993). If available in the form of clean uncontaminated lignin, it is also suitable for other applications, including as a binder, dispersant, emulsifier, or sequestrant (Adler, 1977; Lin and Lebo, 1995; Northey, 1992; Sarkanen and Ludwig, 1971).

In our laboratory, the ammonia recycled percolation (ARP) method has been investigated as a pretreatment method. It utilizes aqueous ammonia as the pretreatment reagent. This method has been proven to give high a degree of delignification while retaining most of the cellulosic component in the biomass. However, one of the problems associated with this process is that a substantial amount of xylan is also removed along with the lignin (Iyer et al., 1996; Kim et al., 2000; Kim and Lee, 1996; Yoon et al., 1995; Yoon, 1998). The partial removal of xylan makes the overall process complicated if all of the xylan content is to be utilized. To alleviate this problem, we have devised a two-stage process in which a hot water treatment is followed by ARP. This processing scheme is designed to separate hemicellulose sugars in the first stage and lignin in the second stage. The remaining solid thus contains mostly cellulose. Upon completion of this process, one can anticipate a near complete fractionation of biomass,

In this study, the proposed two-stage percolation process was investigated to assess its effectiveness as a method of pretreatment as well as a fractionation scheme. A broad range of reaction and operating conditions was examined in order to identify the optimum range of the process parameters that would allow for satisfactory pretreatment and fractionation of corn stover.

MATERIALS AND METHODS

Materials

Air-dried ground corn stover was supplied by National Renewable Energy Laboratory in Golden, CO. The corn stover was screened to a nominal size of 9–35 mesh. The initial composition of the corn stover, as determined by NREL, was: 37.5 wt.% glucan, 20.8 wt.% xylan, 2.7 wt.% arabinan, 0.8 wt.% mannan, 1.6 wt.% galactan, 17.6 wt.% Klason lignin, 6.7 wt.% ash, 2.2 wt.% acetyl group, 2.9 wt.% protein, 3.6 wt.% uronic acid and 3.6 wt.% unaccounted for. α-Cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246). Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257) was obtained from NREL. An average activity of the enzyme, as determined by NREL, was: 31.2 filter paper unit (FPU)/mL. Activity of β-glucosidase (Sigma Cat. No. G-0395) was 12.0 IU/g.

Experimental setup and operation

A schematic diagram of the reactor setup is shown in Fig. V-1. The reaction and operating conditions are summarized in Fig. V-2. The system consisted of a stock solution reservoir, pump, temperature-programmable GC oven (Varian 3700), spring-loaded reactor, and liquid holding tanks, #1 and #2, which also served as the backpressure vessel. Aqueous ammonia was pumped using a metering piston pump to the reactor. The reactor (101.9 cm^3 of internal volume) was constructed out of 10 inches of SS-316 tubing with an I.D. of 9/10 inches. A 2.25 L and a 1.0 L SS304 cylinder were used as the receiver tanks for the first and second stage treatments. In the ARP experiment, 10 g of dry biomass sample was packed into the reactor and soaked with ammonia solution overnight. The

oven was preheated for 16–17 min. and 2.5 MPa of N_2 backpressure was applied to the reactor system before reactor startup. After completion of the first stage, temperature shifting was done over a 10-min. period. A typical temperature profile for the two-stage processing is shown in Fig. V-3. In the second series of runs, the biomass feedstocks were subjected to sequential two-stage treatment without intermittent sample taking. At the beginning of the second stage (ARP), the output effluent was switched into the second receiving tank by a 3-way valve. At the completion of the run, the reactor was flushed with water to remove the residual sugar and ammonia trapped in the treated biomass. The wet solids discharged from the reactor were separated into two portions. One was dried in a moisture analyzer for measurement of weight loss, and subjected to composition analysis. The other was used for the enzymatic digestibility test.

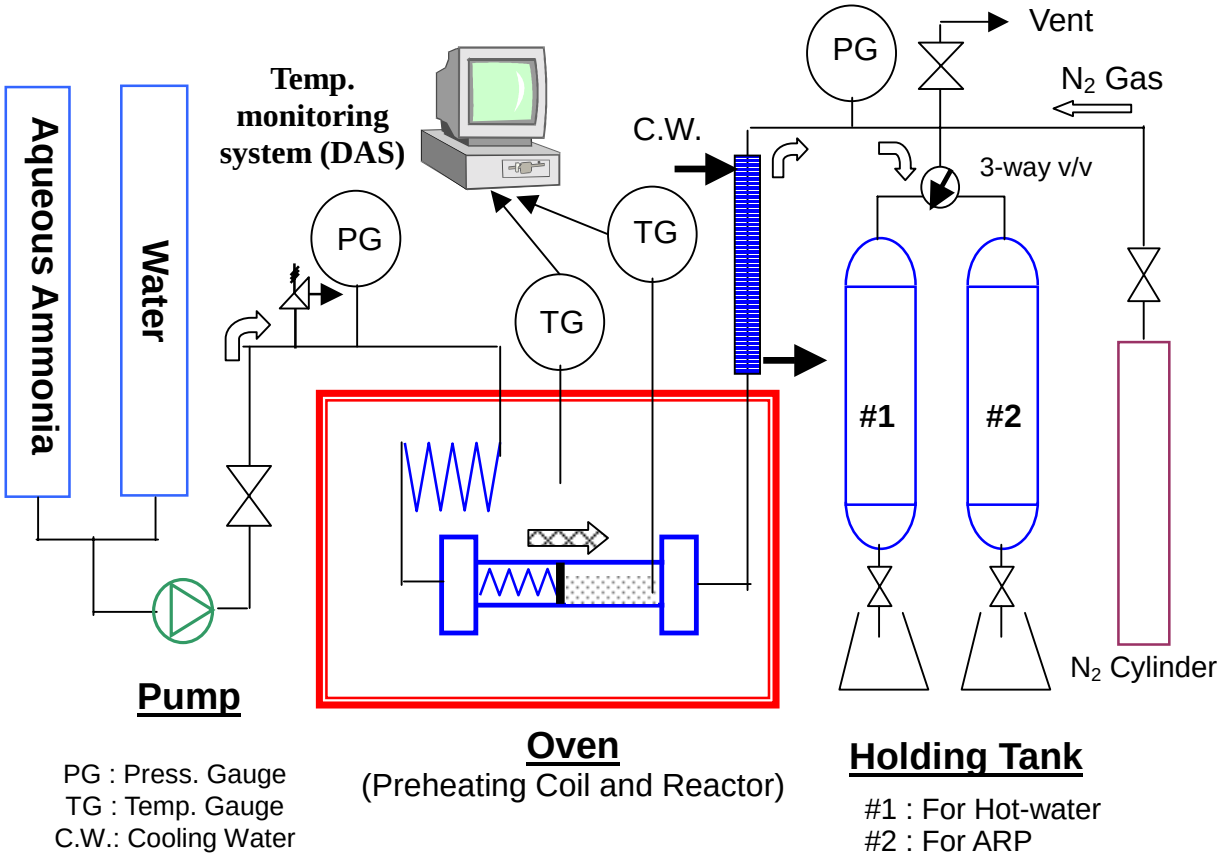


Fig. V-1. Experimental set-up of two-stage percolation

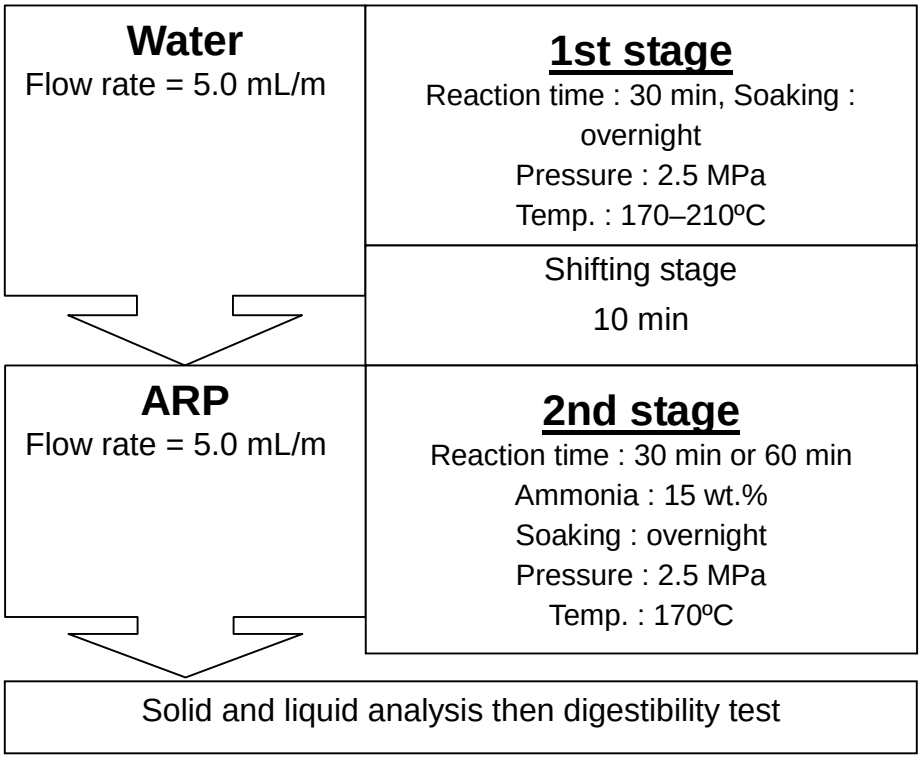


Fig. V-2. Experimental conditions

Note. Cellulase enzyme (Spezyme CP, Lot 301-00348-257, activity: 31.2 FPU), 60 or 15 FPU/g of glucan, β-glucosidase supplement (Sigma, Cat No.G-0395, 30 IU/g glucan), pH 4.8, 50°C, 150 rpm.

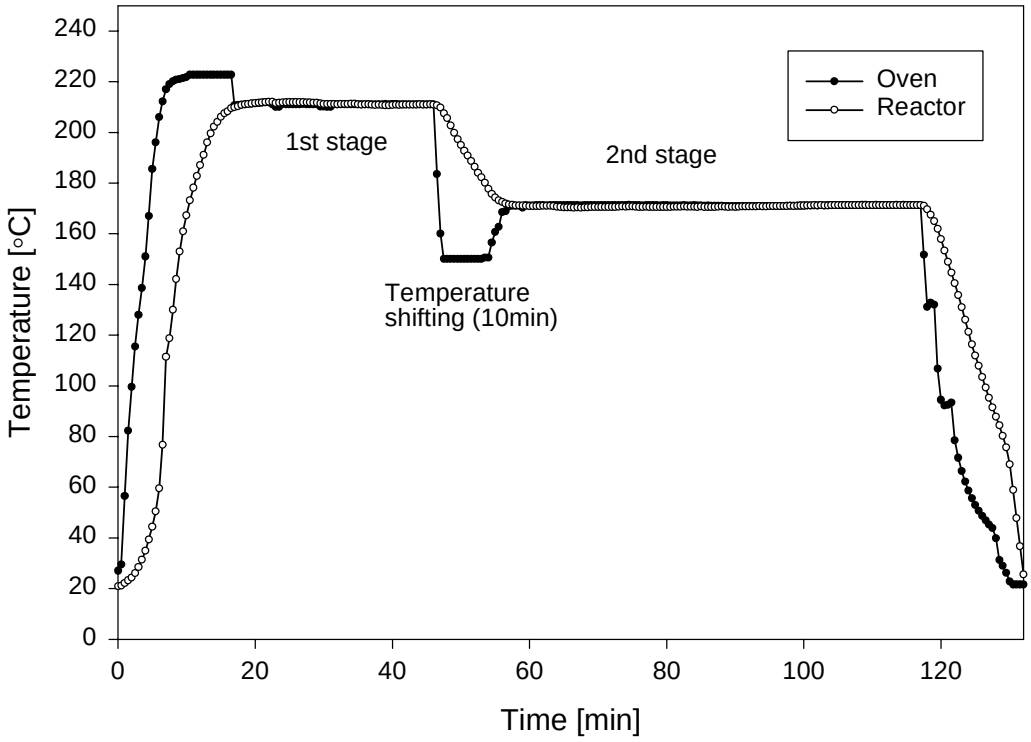


Fig. V-3. Temperature profile of a typical two-stage treatment.

Digestibility test

The enzymatic digestibility of corn stover was determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure (NREL, 2004). Enzymatic digestibility tests were conducted at 50°C and pH 4.8 (0.05 M sodium citrate buffer) on a shaker bath agitated at 150 rpm. Enzyme loadings of 15 and 60 FPU of Spezyme CP/g of glucan supplemented with 37 IU of β-glucosidase (Sigma Cat. No. G-0395) were used. The initial glucan concentration was 1% (w/v) based in 100 mL of total liquid. The 250 mL screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (NBS, Innova-4080). Samples were taken periodically at appropriate sampling intervals (6, 12, 24, 48, and 72 h) and analyzed for glucose and cellobiose content using HPLC. The total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α-Cellulose and untreated corn stover were put through the same procedure as a reference and control, respectively.

Analytical methods

The solid samples were analyzed for sugar and Klason lignin following the procedures of NREL Chemical Analysis and Testing Standard Procedures (NREL, 2004). Each sample was analyzed in duplicate. Sugars were determined by HPLC using a Bio-Rad Aminex HPX-87P column, and YSI 2300 Glucose/Lactate analyzer (for some of the digestibility hydrolysate samples). A refractive index detector was used with the HPLC. The sugars in the liquid samples were determined after secondary acid hydrolysis in order to account for the oligomer contents. The conditions of the secondary hydrolysis were 4 wt.% sulfuric acid and 121ºC for one hour.

Scanning electron microscope (SEM)

A scanning electron microscope (Zeiss-DSM940) was used to image the biomass samples.

RESULTS AND DISCUSSION

Hot-water treatment

Under hot-water treatment, the hydronium ion initially causes xylan depolymerization and cleavage of acetyl group. The autohydrolysis reaction then follows, in which the acetyl group catalyzes the hydrolysis of the hemicellulose (Casebier et al., 1969; Fernandez-Bolanos et al., 1999; Lora and Wayman, 1978). For the hot-water treatment, the effects of flow rate were first investigated to identify a reasonable range of operating conditions. In the first series of experiments, three different flow rates (2.5, 5.0, and 7.5 mL/min) were applied at 180^{o}C, keeping the reaction time constant at 30 min (Table V-1). In the second series, four different flow rates (1, 2.5, 5.0, and 7.5 mL/min) were applied for 75 mL of total liquid throughput. For example, 75 min reaction time was needed for the 1 mL/min run, but only 10 min reaction time for the 7.5 mL/min run (Table V-2).

The first series was performed to test the effects of flow rate, and the second series for determination of the optimal flow rate to maximize the xylan yield. In hot-water treatment, the xylan is recovered mostly in the form of soluble xylose oligomers. Table V-1 shows that xylan yield from the hot water treatment generally increases as the flow rate increases, with the highest xylan yield occurring at 7.5 mL/min. However, this also resulted in lower sugar concentration and high liquid throughput, which raises the

processing costs. From the results of the second series of runs (Table V-2), it became clear that a reaction time of 30 minutes is required to attain substantial conversion of the xylan. Based on the above results, 2.5 and 5.0 mL/min with 30 min reaction time were selected as conditions to be pursued further.

The hot-water-only pretreatment was then tested at five or six different temperatures covering 170– 220ºC and applying flow rates of 2.5 and 5.0 mL/min. The composition data obtained after these treatments are summarized in Table V-3. The xylan and lignin remaining in the solid after the treatments generally decreased as the temperature increased. Solubilization of xylan was 53–71% with 2.5 mL/min, and 58–86% with 5.0 mL/min of hot water. The data also indicate that reaction temperatures above 190 ºC are required for efficient solubilization of xylan,

The glucan content was well preserved. The accountability of glucan (glucan content in the solid plus that in liquid) was 97% for both flow rates. However, the accountability of xylan above 180ºC at 2.5 mL/min was less than 80%, indicating that a substantial amount of the xylan decomposed under those conditions. The xylan yield in liquid at 2.5 mL/min was lower than that at 5 mL/min for all temperatures. Hot water treatment at 190–200ºC and 5 mL/min attained a xylan recovery of 85%. The flow rate of 5 mL/min was, therefore, selected for the subsequent experiments.

Lignin removal in the hot-water-only treatment was in the range of 22–50%. We have observed some unusual behavior in that Klason lignin of the treated sample decreases with temperature up to a certain point, then rises again above that temperature (between 200 and 210ºC with 2.5 ml/min., and 210 and 220ºC with 5.0 ml/min). This behavior appears to be connected with other lignin-related reactions.

Table V-1
Effect of flow rate on composition in the hot-water-only treatment at 180°C[1]

Flow	Solid				Liquid		Total		Yield in Liquid	
rate	S.R.[2]	Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan
[mL/m]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100.0	17.6	37.5	20.8	-	-	37.5	20.8	-	-
2.5	63.1	12.1	36.5	4.5	0.8	13.9	37.3	18.5	2.0	67.0
5.0	58.1	11.8	35.9	4.3	1.3	15.6	36.8	19.9	3.4	74.9
7.5	58.6	11.9	36.6	4.0	1.2	17.4	37.8	21.5	3.1	83.8

Note. 1. Data in the table are based on the oven dry untreated biomass; Pretreatment conditions:, 30 min, 2.5 MPa; •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor..
2. S.R. stands for solid remaining after reaction.
3. Klason lignin.
4. The data in the table show the mean value (n=2; SE<0.3% for K-lignin, SE<0.8% for S.R., SE<0.3% for Glucan and Xylan in solid and liquid, SE : standard error).

Table V-2
Effect of flow rate and reaction time on composition in the hot-water-only treatment at 180°C [1]

Flow	Reaction	Solid				Liquid		Total		Yield in Liquid	
rate	time	S.R.[2]	Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan
[mL/m]	[min]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	-	100.0	17.6	37.5	20.8	-	-	37.5	20.8	-	-
1.0	75	61.8	14.7	37.4	3.5	0.4	14.3	37.8	17.8	1.1	68.9
2.5	30	63.1	12.1	36.5	4.5	0.8	13.9	37.3	18.5	2.0	67.0
5.0	15	67.2	14.7	37.3	7.5	0.6	12.4	37.9	20.0	1.6	59.8
7.5	10	72.0	15.5	37.9	10.4	0.4	9.1	38.3	19.5	1.0	43.9

Note. 1. Data in the table are based on the oven dry untreated biomass; Pretreatment conditions:, 2.5 MPa; •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor.2. S.R. stands for solid remaining after reaction

3. Klason lignin.

4. The data in the table show the mean value (n=2; SE<0.3% for K-lignin, SE<0.8% for S.R., SE<0.3% for Glucan and Xylan in solid and liquid, SE: standard error).

Table V-3
Effect of temperature on composition in hot-water-only pretreatment [1]

Temp.	Solid				Liquid		Total		Yield in liquid		Digestibility[4]	
	S.R.[2]	K-Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	60FPU	15FPU
[°C]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100	17.6	37.5	20.8	-	-	37.5	20.8	-	-	21.2	16.1
2.5 mL/min												
170	69.1	13.1	36.9	8.0	0.9	11.9	37.8	19.9	2.3	57.4	-	-
180	63.1	12.1	36.5	4.5	0.8	13.9	37.3	18.5	2.0	67.0	-	-
190	57.9	10.9	36.3	1.9	1.0	14.9	37.3	16.8	2.6	71.4	-	-
200	56.6	10.3	35.8	1.5	1.1	13.8	36.9	15.3	2.8	66.4	-	-
210	56.9	13.6	35.4	1.0	1.2	11.1	36.5	12.1	3.0	53.5	-	-
5.0 mL/min												
170	65.4	13.5	36.8	8.0	1.0	12.1	37.5	20.1	2.7	58.4	59.1	47.5
180	58.1	11.8	35.9	4.3	1.3	15.6	36.8	19.9	3.4	74.9	73.9	62.7
190	55.0	11.3	36.3	2.6	1.5	17.9	37.4	20.5	4.1	86.0	86.8	71.6
200	53.0	10.3	36.2	1.4	1.9	17.7	37.8	19.1	5.1	85.3	90.9	76.6
210	51.0	8.8	35.7	1.1	2.0	17.6	37.3	18.7	5.3	84.7	93.6	88.9
220	50.5	10.1	34.0	0.1	2.8	13.2	36.5	13.3	7.5	63.3	95.0	93.3

Note. 1. Data in the table are based on the oven dry untreated biomass. Pretreatment conditions: 2.5 or 5.0 mL/min, 30 min, 2.5 MPa •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor.
2. S.R. stands for solid remaining after reaction.
3. Klason lignin;
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 60 or 15 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.
5. The data in the table show the mean value (n=2; SE<0.2% for K-lignin, SE<0.9% for S.R., SE<0.3% for Glucan and Xylan in solid and liquid, SE<2.0% for digestibilities, SE: standard error).

As the hot water travels through the flow-through reactor, the lignin released into the liquid may undergo side reactions at high temperatures. Similar phenomena were also observed in the ensuing experiments and will be discussed separately in the next section.

The 72-h digestibilities of the samples treated at various temperatures are shown Fig. V-4. Here again, an interesting result is observed in that there is a sharp rise of digestibility with 15 FPU/g of glucan between 200 and 210ºC. This seems to be related to the lignin content in the solid samples because the difference in the lignin removal between these two temperatures is higher than the difference observed for the other temperature intervals. Hot-water treatment at 220ºC gave the highest digestibility, although the lignin value increased from that of 210ºC. This is yet another interesting point to which additional discussion is devoted in the next section. If the hot-water-only treatment is to be used as a pretreatment method, then 210–220ºC is the region that deserves further investigation because of the high digestibilities obtained under these conditions.

Hot-water-ARP treatment

The two-stage percolation process was tested for fractionation and pretreatment of corn stover. In this process, the hot water treatment was followed by ARP treatment. Figure V-2 summarizes the experimental procedure and conditions. Five different temperatures covering the range 170–210^{o}C were applied in the hot-water treatment stage.

In the second series of runs, the biomass feedstocks were subjected to the sequential two-stage treatment without intermittent sample taking. The conditions of the ARP treatment were: 170^{o}C, 15 wt.% NH_3, 30 min or 60 min. Again, the intent here was

to recover hemicellulose in the first stage by autocatalytic hydrolysis and then remove lignin in the second stage, with the process being performed in a single reactor, successively. The results are summarized in Table V-4.

The composition of the 30-min and 60-min ARP-treated samples indicates that ARP removes primarily lignin, but causes a slight decomposition of xylan. Glucan is well protected during the ARP treatment. Lignin removal after 60-min ARP was 70–80%, which is much higher than that of 30-min ARP (53–66%). The digestibilities after the two-stage treatment with 60-min ARP were 94–96% with 60 FPU/g of glucan, and 79–87% with 15 FPU/g of glucan. The digestibilities of the 60-min ARP samples were substantially higher than for the 30-min ARP samples. The glucan hydrolysis rates for the two different enzyme loadings are shown in Fig. V-5.

Generally the delignification and digestibility increase with the temperature of the hot-water treatment, although this trend was reversed in the 180–210^{o}C range (Table V-4). As the temperature increased above 180^{o}C, the Klason lignin increased, and the 15-FPU fluctuated corresponding to the lignin content. It is unlikely that the delignification reaction suddenly decreases at a certain reaction temperature, so the increased Klason lignin may be linked to other lignin-related reactions. A search of the literature indicated that lignin undergoes condensation and repolymerization, turning into insoluble substances (Genco et al., 1997; Lora and Wayman, 1978; Xu and Lai, 1999). Lignin may also bond to cellulose at high temperatures (Karlsson, 1997). These secondary reactions form complexes that are not hydrolyzed by concentrated sulfuric acid during the carbohydrate analysis procedure. Since it is included in the Klason lignins, it would thus appear as if the delignification rate was reduced at high temperatures. Figure V-6 shows

the relation between the lignin content in the solid and the digestibility for hot-water-ARP treatment samples.

The digestibility in this region fluctuates in concert with the lignin content in a manner that indicates the digestibility is inversely related to the lignin content. How the low level lignin affects the digestibility is unknown, however. We hypothesize that the soluble lignin, as it interacts with cellulose, modifies the cellulose surface structure enough to interfere with the cellulase action. The solubilized lignin may also interact with the soluble glucose at high temperatures, as suggested by Xiang and Lee, which would also reduce the digestibility numbers (2001).

The optimum operating conditions for the two-stage process, on the basis of fractionation and digestibility, are: 190°C, 5.0 mL, 30 min for hot-water treatment and 170°C, 5.0 mL, 60 min for ARP pretreatment. After this two-stage processing is complete, 92% of the xylan has been hydrolyzed, of which 83% can be recovered, and 75% of delignification has been achieved. The treated biomass contains 78% glucan, 3.6% xylan and 9.8% Klason lignin. The digestibility of the hot-water-ARP treated sample is 94% with 60 FPU/g-glucan and 85% with15 FPU/g of glucan.

Table V-4
Effect of temperature on composition in hot-water-ARP pretreatment [1]

Temp.	Solid				Liquid		Total		Yield in liquid		Digestibility[4]	
	S.R.[2]	K-Lignin[3]	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	Glucan	Xylan	60FPU	15FPU
[°C]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	100	17.6	37.5	20.8	-	-	37.5	20.8	-	-	21.2	16.1
Hot-water-30 min of ARP												
170	53.3	8.3	35.9	5.3	1.5	13.9	37.4	19.2	3.9	66.9	82.1	60.7
180	49.5	6.0	35.4	3.8	1.3	15.4	36.7	19.2	3.5	74.0	90.1	76.1
190	47.7	6.0	35.4	2.3	1.7	18.0	37.3	20.3	4.6	86.5	88.7	73.0
200	45.8	6.1	34.1	1.5	1.9	17.4	36.0	18.9	5.1	83.5	94.0	73.0
210	44.2	6.8	33.8	0.7	2.4	15.8	35.2	16.5	6.4	76.0	97.5	77.5
Hot-water-60 min of ARP												
170	47.7	3.7	35.7	3.7	1.5	14.5	37.2	18.2	3.8	69.6	96.3	87.0
180	47.0	3.8	34.8	2.7	1.8	15.5	36.6	18.2	4.7	74.4	95.9	87.1
190	44.9	4.4	34.9	1.6	1.6	17.4	36.5	19.0	4.3	83.4	93.6	84.8
200	44.7	5.7	33.2	1.2	2.1	17.5	35.2	18.7	5.5	84.0	94.5	79.6
210	43.2	5.4	33.2	0.7	2.3	15.4	35.4	16.2	5.9	74.2	94.7	82.7

Note. 1. Data in the table are based on the oven dry untreated biomass; Pretreatment conditions: 5.0 mL/min, 30 min, 2.5 MPa; ARP-15 wt.% of ammonia, 5.0 mL/min, 60 min, 2.5 MPa; •All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor.

2. S.R. stands for solid remaining after reaction.;

3. Klason lignin;

4. Digestibility at 72 h, Enzymatic hydrolysis conditions: 60 or 15FPU/g of glucan, pH 4.8, 50°C, 150 rpm

5. The data in the table show the mean value (n=2; SE<0.1% for K-lignin, SE<0.8% for S.R., SE<0.2% for Glucan and Xylan in solid and liquid, SE<2.0% for digestibilities, SE: standard error).

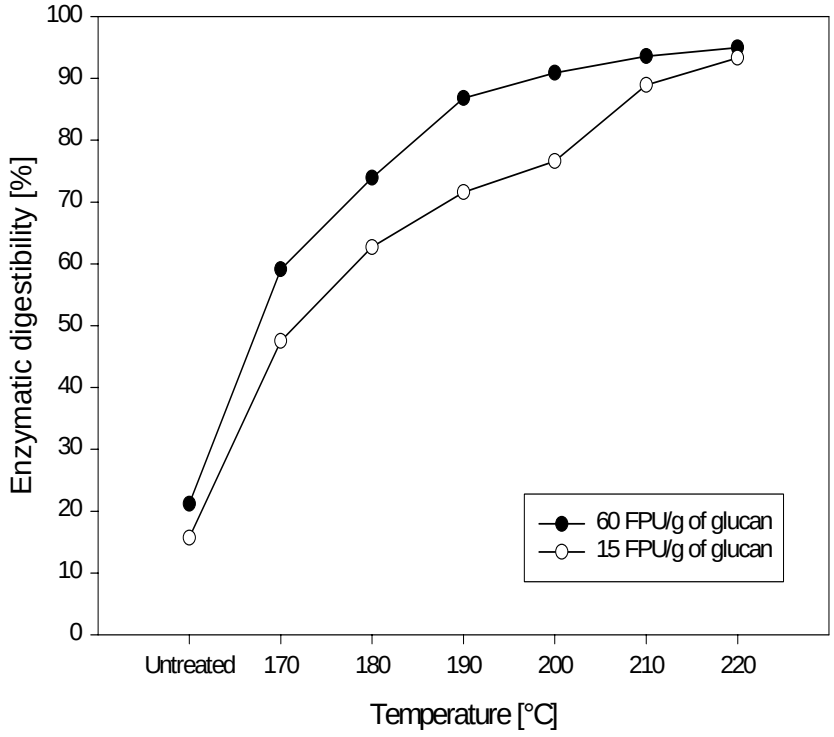


Fig. V-4. Effect of temperature on enzymatic hydrolysis in hot-water only treatment.

Note. 1.Pretreatment conditions: 5.0 mL/min, 30 min, 2.5 MPa; All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor;
2. Digestibility is the percentage at 72 h.
3. Enzymatic hydrolysis conditions: 60 or 15 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.
4. The data points in the graph show the mean value (SE<2.0%, n=2).

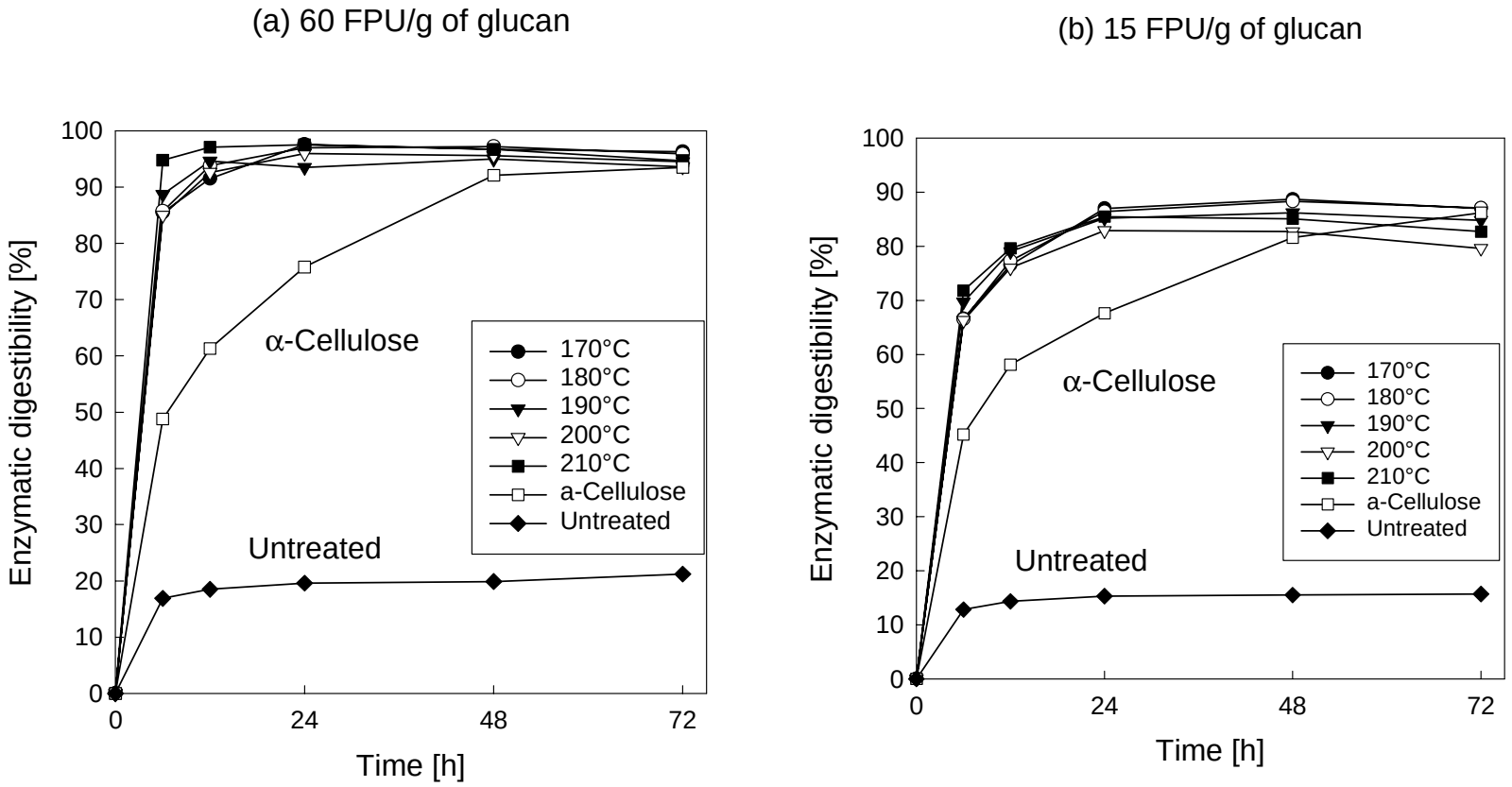


Fig. V-5. Enzymatic digestibility of hot-water-ARP treated-samples under different enzyme loadings.

Note 1. All sugar and lignin contents are based on the oven-dry untreated biomass. Pretreatment conditions: hot-water; 5.0 mL/min, 2.5 MPa; ARP; 170°C, 15 wt.% NH_3, 5.0 mL/min, 2.5 MPa

2. Enzymatic hydrolysis conditions: 72 h, 60 or 15 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.

3. The data points in the graph show the mean value (SE<2.0%, n=2).

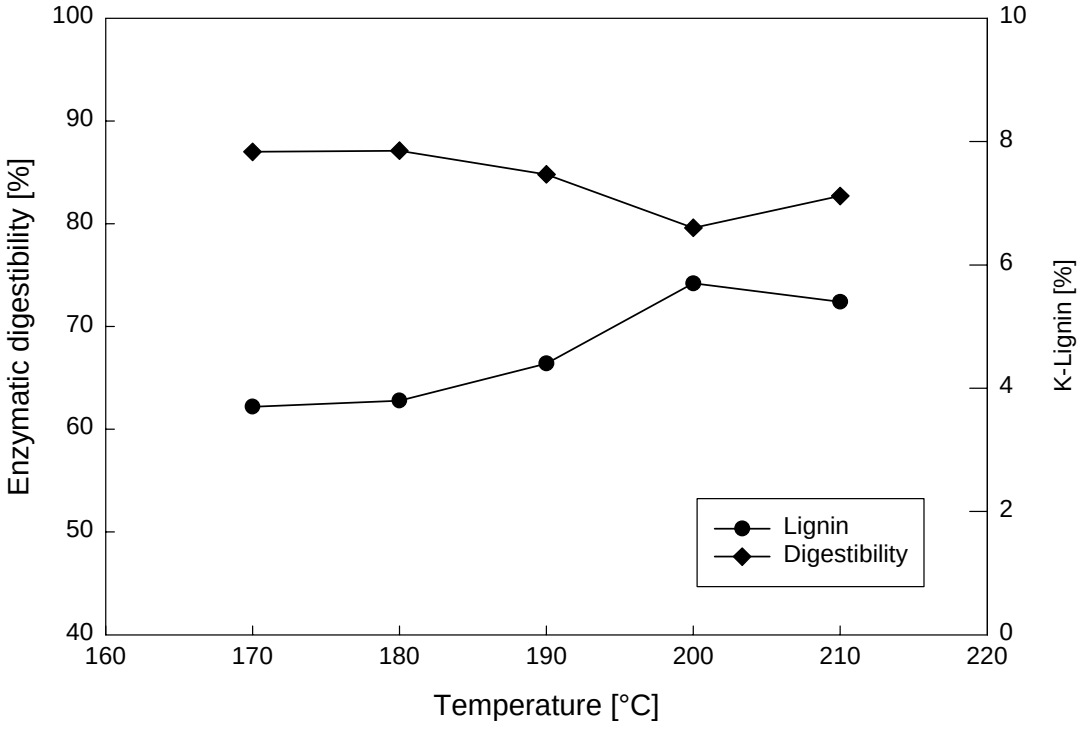


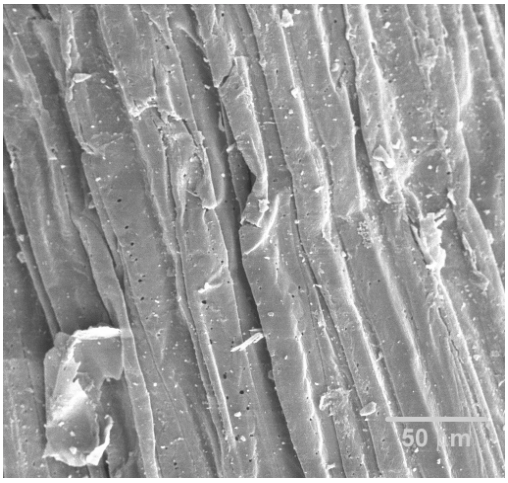
Fig. V-6. Relationship between lignin and digestibility for a two-stage treated solid sample.

Note 1. Pretreatment conditions (hot-water-ARP): hot-water; 5.0 mL/min, 30 min, 2.5 MPa; ARP; 15 wt.% of ammonia, 5.0 mL/min, 60 min, 2.5 MPa; All reactions are carried out in a Bed-Shrinking Flow-Through (BSFT) Reactor.

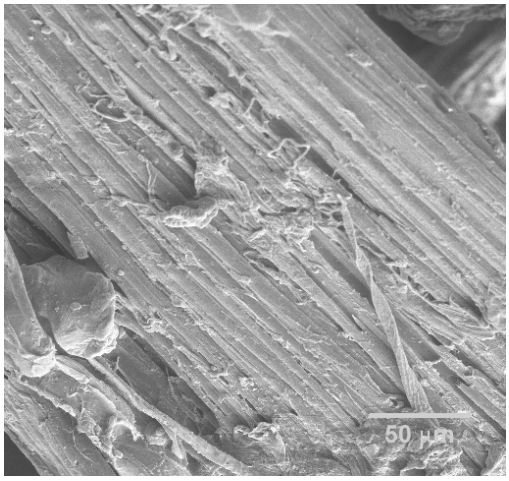
2. Enzymatic hydrolysis conditions: 72 h, 60 or 15 FPU/g of glucan, pH 4.8, 50°C, 150 rpm.
3. The data points in the graph show the mean value (SE<2.0%, n=2 for digestibility; SE<0.2%, n=2 for lignin).

SEM

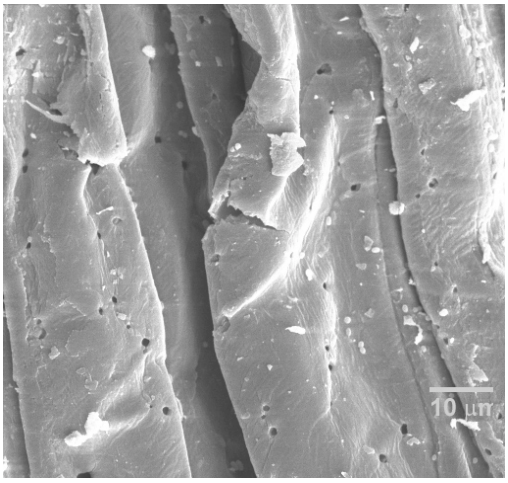
SEM pictures were taken of the treated and untreated samples. Figure V-7 shows that the two-stage treatment altered the biomass structure significantly. The untreated sample shows rigid and highly ordered fibrils (Fig. V-7-a, d), while the fibers of the treated samples are visibly separated from the initial connected structure and fully exposed (Fig. V-7-b, c, e, and f). It seems likely that the surface area and the porosity have also increased. Simply by touching material, we could feel that the wet treated biomass was much softer than the wet untreated biomass.



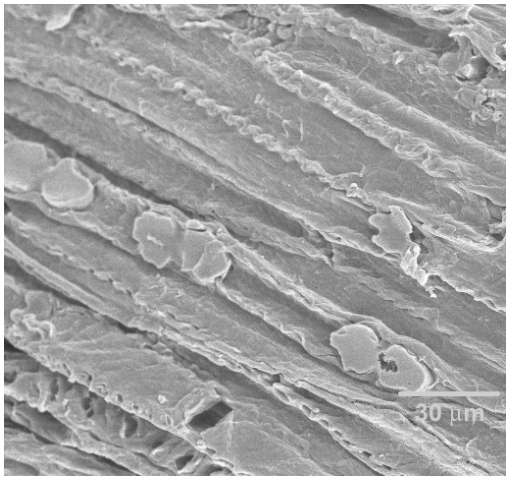
(a) Untreated (x300)



(b) Water-ARP (x300)



(c) Untreated (x1000)



(d) Water-ARP (X500)

Fig. V-7. Scanning electron micrographs (SEM) of treated and untreated corn stover.

CONCLUSIONS

Two-stage percolation processes were used to effectively fractionate corn stover into three main constituents: cellulose, xylan, and lignin. The optimum reaction conditions for fractionation were found to be: 190°C, 5.0 mL/min, 30 min for hot-water treatment section and 170°C, 15wt.% NH_3, 5.0 mL/min, 60 min for the ARP treatment section. Under these conditions, 83% of soluble xylan recovery and 75% of delignification were achieved. The treated residue contained 78% glucan, 3.6% xylan, and 9.8% lignin. This high glucan material may be suitable for use as a filler fiber in papermaking and in other value-added products. The enzymatic digestibilities of the two-stage treated residue were 94% with 60 FPU/g-glucan and 85% with 15 FPU/g of glucan. This compares to the digestibilities of 87% and 72% of the samples obtained from the hot-water-only treatment. Although there is a noticeable improvement of digestibility for the two-stage processing over the one-stage (hot-water-only), the proposed two-stage processing is more useful as a fractionation process than as a method of pretreatment since there are other one-stage pretreatment methods that are simple but equally effective. Composition and digestibility data for the treated corn stover indicate that the lignin is one of the prime factors controlling the enzymatic digestibility. Hot-water treatment above 210°C induces lignin recondensation and/or lignin-carbohydrate bonding.

VI. PRETREATMENT OF CORN STOVER BY SOAKING IN AQUEOUS AMMONIA

VI-I. SOAKING IN AQUEOUS AMMONIA AT ROOM TEMPERATURE

ABSTRACT

Soaking in aqueous ammonia (SAA) was investigated as a pretreatment method for corn stover. In this method, a feedstock is soaked in aqueous ammonia over an extended period (10–60 days) at room temperature. This is done without agitation under atmospheric pressure. Treatment of corn stover by SAA removes 55–74% of the lignin, but retains nearly 100% of the glucan and 85% of the xylan. The SAA treatment of corn stover achieved enzymatic digestibilities comparable to those obtained using high temperature aqueous ammonia treatments such as Ammonia Recycled Percolation. The xylan remaining in the corn stover after SAA was hydrolyzed, along with glucan, by cellulase enzyme due to the presence of xylanase in "cellulase". The SSA treated corn stover was further evaluated by simultaneous saccharification and fermentation (SSF) and by simultaneous saccharification and co-fermentation (SSCF). In the standard SSF test using *S. cerevisiae* (NREL-D_5A), an ethanol yield of 73% was obtained on the basis of the glucan content in the treated corn stover.

Xylose accumulation in the SSF appears to inhibit the cellulase activity of glucan, limiting the yield of ethanol. In the SSCF test using recombinant *E.coli* (KO11), both the

glucan and xylose were effectively utilized, giving an overall ethanol yield of 77% of the theoretical maximum based on glucan and xylan. With the SSCF results, the fact that the xylan fraction is retained is a desirable feature in pretreatment since the overall bioconversion can be carried out in a single step without separate recovery of xylose from the pretreatment liquid.

INTRODUCTION

Most pretreatment methods designed to improve enzymatic digestibility generate hydrolysates containing a mixture of sugars and lignin. Soluble lignin present in the pretreatment liquid is known to inhibit the enzymatic hydrolysis and bioconversion processes (Chang and Holtzapple, 2000; Cowling and Kirk, 1976; Dulap et al., 1976; Lee et al., 1995; Mooney et al., 1998; Schwald et al., 1988). Hydrolysates of common pretreatment processes also contain various other toxic components that create an inhibitory environment in which microorganisms cannot sustain their viability required for efficient bioconversion (Bjóling and Lindman, 1989; Fein et al., 2004; Hahn-Hägerdal et al., 1994; Sanchez and Bautista, 1988; Tran and Chambers, 1986; van Zyl et al., 1991; Watson et al., 1984). In order to utilize these soluble sugars, the contaminated hydrolyzates must be cleaned and detoxified before they are subjected to bioconversion. This is an untested and troublesome unit process and undoubtedly a significant cost factor. The underlying reason for this is because the pretreatment methods developed to this point are performed under acidic and/or high temperature conditions severe enough to make the biomass susceptible to enzymatic reactions.

We have investigated a pretreatment method based on aqueous ammonia (Iyer et al., 1996; Kim and Lee, 1996; Kim et al., 2003). In this method, aqueous ammonia is used in a flow through reactor at a relatively high temperature (160–180°C). While this process renders a high degree of delignification, it also requires high-energy input because of the high temperature and relatively high liquid throughput. In this process, about half of the xylan is removed along with the lignin, which complicates xylan recovery in the downstream processing. The primary intent of this work was to seek an

alternative pretreatment process that can alleviate these problems. The approach we have taken is to apply reaction conditions mild enough to prevent the formation of toxic byproducts. One feasible approach is to use low temperature alkaline treatment. To this end, a room temperature treatment with aqueous ammonia was attempted because of the reduced heat input needed during the treatment phase, and also to reduce the interaction of ammonia with hemicellulose. Retention of xylan is not a negative factor in pretreatment since it can be hydrolyzed by the xylanase activity normally embodied in "cellulase". Room temperature treatments with various alkaline reagents have previously been attempted for pretreatment of lignocellulosic biomass with varying degrees of success (Morris and Bacon, 1977b; Oji et al., 1977; Streeter and Horn, 1982).

In this study, a pretreatment method based on aqueous ammonia with a longer reaction time at room temperature was tested. This is done in a closed vessel without agitation under atmospheric pressure. It is thus termed the Soaking in Aqueous Ammonia (SAA) process. The focus of this work is to evaluate the overall effectiveness of the SAA process as an alternative pretreatment. The effects of reaction parameters on the composition and the digestibility of remaining glucan and xylan were among the items investigated. The reaction parameters of interest were the solid-to-liquid ratio, the reaction time and the ammonia concentration. As a method of evaluation for SAA, the simultaneous saccharification and fermentation (SSF) was included. In the case of SAA, utilization of the xylan fraction is important since most of it is retained after treatment. A proper test of the SAA process should therefore include a bioconversion where both the glucan and xylan are converted. As such, we have chosen a simultaneous saccharification and co-fermentation (SSCF) using recombinant *Escherichia coli* (strain KO11), one of

the most effective ethanologenic microorganisms currently available to the public for fermentation of mixed sugars (Hahn−Hägerdal et al., 1994; Ohta et al., 2004).

MATERIALS AND METHODS

Materials

Air-dried ground corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, CO). The corn stover was screened to a nominal size of 9–35 mesh. The initial composition of the corn stover, as determined by NREL, was 36.1 wt.% glucan, 21.4 wt.% xylan, 3.5 wt.% arabinan, 1.8 wt.% mannan, 2.5 wt.% galactan, 17.2 wt.% Klason lignin, 7.1 wt.% ash, 3.2 wt.% acetyl group, 4.0 wt.% protein, and 3.6 wt.% uronic acid. α-Cellulose was purchased from Sigma (Cat. No. C-8200, Lot No. 11K0246).

Cellulase enzyme, Spezyme CP (Genencor, Lot No. 301-00348-257), was obtained from NREL. The average activity of the enzyme, as determined by NREL, was: 31.2 filter paper units (FPU)/mL. The activity of the β-glucosidase (Novozyme 188 from Novo Inc., Lot No. 11K1088) was 750 CBU/mL.

Simultaneous saccharification and fermentation (SSF)

The fermentation microorganism used for SSF was *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A). The growth media was YP medium, which contained 1% yeast extract (Sigma Cat. No. Y-0500) and 2% peptone (Sigma Cat. No. P-6588).

Simultaneous saccharification and co-fermentation (SSCF)

Recombinant *Escherichia coli* ATCC® 55124 (KO11) was employed for SSCF tests. LB medium (Sigma Cat. No. L-3152) was used for KO11, which contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloroamphenicol.

Experimental setup and operation

Corn stover was treated with 29.5 wt.% of aqueous ammonia in screw-capped laboratory bottles at room temperature for 1–60 days. Solid-to-liquid ratios ranging from 1:2 to 1:15 were applied. After soaking, the solids were separated by filtering, washed with DI water, and subjected to the enzymatic digestibility tests. Klason lignin, carbohydrate content, and digestibility were determined by following NREL Standard Procedures (NREL, 2004).

Digestibility test

The enzymatic digestibility of corn stover was determined in duplicate according to the NREL Chemical Analysis and Testing Standard Procedure (NREL, 2004). The conditions of the enzymatic digestibility tests were 50°C and pH 4.8 (0.05 M sodium citrate buffer) in a shaker bath agitated at 150 rpm. Enzyme loadings of 15 and 60 FPU of Spezyme CP/g-glucan supplemented with 30 CBU of β-glucosidase (Novozyme 188, Sigma Cat. No. C-6150)/g-glucan were used. The initial glucan concentration was 1% (w/v) based in 100 mL of total liquid. The 250 mL screw-capped Erlenmeyer flasks containing the enzyme hydrolysis preparations were placed in an incubator shaker (New Brunswick Scientific, Innova-4080). Samples were taken periodically at appropriate

sampling times (6, 12, 24, 48, and 72 h) and analyzed for glucose, xylose, and cellobiose content using HPLC. Total released glucose after 72 h of hydrolysis was used to calculate the enzymatic digestibility. α-Cellulose and untreated corn stover were put through the same procedure as a reference and control, respectively.

Simultaneous saccharification and fermentation (SSF)/co-fermentation (SSCF)

Spezyme CP (Genencor, Lot No. 301-00348-257) was used as cellulase enzyme. It was supplemented with β-glucosidase, Novozyme 188 (Novo Inc., Sigma Cat. No. C6150, Lot No. 11K1088). A 250 mL Erlenmeyer flask was used as the bioreactor. It was shaken in the incubator shaker (New Brunswick Scientific, Innova-4080) at 38°C and a rate of 150 rpm. Into 100 mL working volume of liquid, treated corn stover samples were added such that the glucan content becomes 3% w/v. α-Cellulose was put through the same procedure as a control. The SSF/SSCF runs were performed with a buffer and with no external pH control, starting at pH 5.0/7.0 at the beginning of the fermentation and gradually decreasing to 4.5/6.0 at the end. The loading of the cellulase enzyme was 15 FPU/g-glucan, and that of β-glucosidase was 30 CBU/g-glucan.

The ethanol yield was calculated as follows:

$$\text{Theoretical maximum ethanol yield}[\%] = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Sugar (g) in reactor} \times 0.511} \times 100$$

Note. Sugar is interpreted as glucose in SSF or glucose plus xylose in SSCF.

Analytical methods

The solid samples were analyzed for sugar and Klason lignin following the procedures given in NREL Chemical Analysis and Testing Standard Procedures No. (NREL, 2004). Each sample was analyzed in duplicate. The moisture content was measured by an infrared moisture balance (Denver Instruments, IR-30). Sugars were determined by HPLC using a Bio-Rad Aminex HPX-87P column. For the SSF tests, HPX-87P and HPX-87H columns were used for measurement of sugar content and ethanol, respectively. An YSI 2300 Glucose/Lactate analyzer was used for rapid analysis of glucose during inoculums preparation. A refractive index detector was used in the HPLC.

SEM (scanning electron microscope)

Untreated and treated corn stover was prepared using a freeze dry system. The microscope pictures of the biomass samples were taken with a ZEISS DSM940 scanning electron microscope.

RESULTS AND DISCUSSION

Compositional changes and enzymatic digestibility

Fig. VI-1 summarizes the change of composition with soaking time. The major composition change was in the lignin. Approximately half of the lignin was removed within 4 days (Fig. VI-1-b). Delignification reached 55.8% after 10 days and 73.5% after 60 days. Fig. VI-1 also shows that most of the compositional changes occur within 10

days. Xylan dissolution was 10% after 4 days, 11.3% after 10 days, and 13% after 60 days. The glucan content was well preserved, showing no significant changes over the entire treatment period.

The enzymatic digestibilities of the SAA-treated corn stover for various soaking times and solid-to-liquid ratios are shown in Fig. VI-2. The data indicate that delignification and digestibility increase as the treatment time increases. However, the increase beyond 10 days was relatively insignificant. The 72-h digestibilities of the samples treated for 10–60 days were 92–97% (Fig. VI-2-a) with an enzyme loading of 60 FPU/g-glucan. With 15 FPU/g-glucan, the digestibilities were considerably lower, being in the range of 86–89%. The 10-day SAA-treated corn stover showed a digestibility of 86.3% with 15 FPU/g-glucan, whereas the 4-day sample showed 79% of digestibility. Increasing the soaking time beyond 10 days had no significant effect on the enzymatic digestibility (Fig. VI-2-a). The figure also shows that the initial hydrolysis rates of SAA-treated corn stover were substantially higher than for α-cellulose.

The enzymatic digestibilities as affected by solid-to-liquid ratios are summarized in Fig. VI-2-b. The enzymatic digestibility of the 1:4-sample is 86.1% with 15 FPU/g-glucan after 60 days soaking. Digestibility of this sample is equivalent to that of the 1:12-10 days treated sample. Data in this figure indicate that the solid-to-liquid ratio can be reduced to 1:4 with no significant adverse effects on the digestibility.

The enzymatic digestibility results for xylan in treated corn stover are shown in Fig. VI-3. The xylan digestibility was in the range of 84–85% with 60 FPU/g-glucan and 72–75% with 15 FPU/g-glucan for the samples treated for 10 days. As seen in this figure, no improvement in xylan digestibility was seen with increase of the soaking time beyond

10 days. The observed xylan digestibilities of 72–75% correspond to 86–89% for glucan digestibilities. The xylanase activity in Spezyme CP, while it is quite substantial, does not match the cellulase activity on glucan. Figure VI-4 shows the xylan hydrolysis profile of a representative SSA-treated corn stover (solid-to-liquid ratio=1:8, soaking time=10 or 60 days).

Tests with varying solid-to-liquid ratio indicate that lignin removal and enzymatic digestibility increased slightly when the L/S ratio was increased from 8 to 15 (Fig. VI-5). Delignification increased from 54 to 59%, and digestibility with 15 FPU/g-glucan increased from 85 to 87%. Xylan content decreased only by 1%. This indicates that the presence of excess ammonia does not increase digestibility, nor does it affect xylan removal significantly.

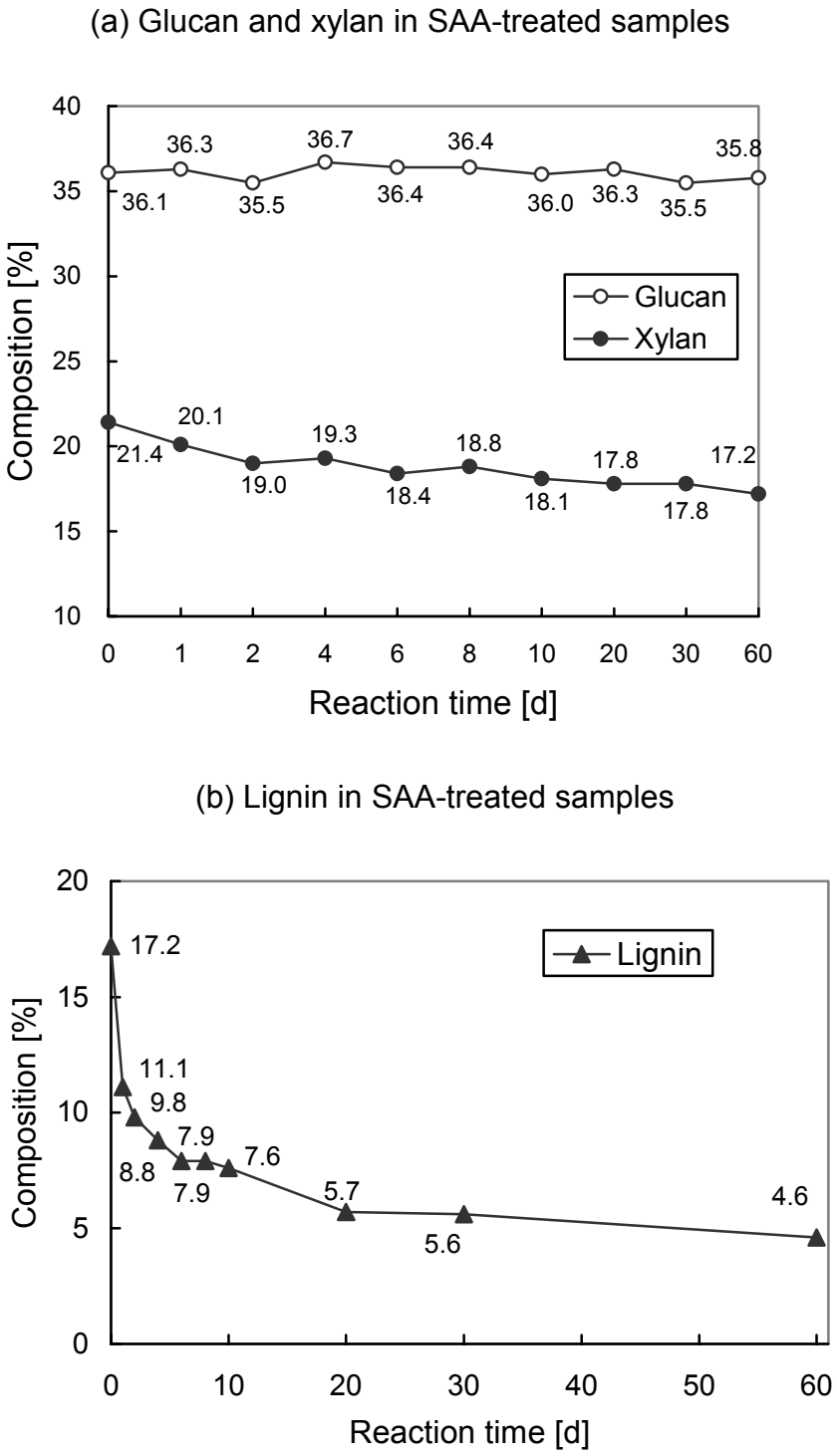


Fig. VI-1. Variation of solid composition with soaking time.

Note. 1–60 days; solid:liquid ratio=1:12, reaction temperature=23±1ºC (room temperature), 29.5 wt% ammonia.

All sugar and lignin content are based on the oven-dry untreated biomass.

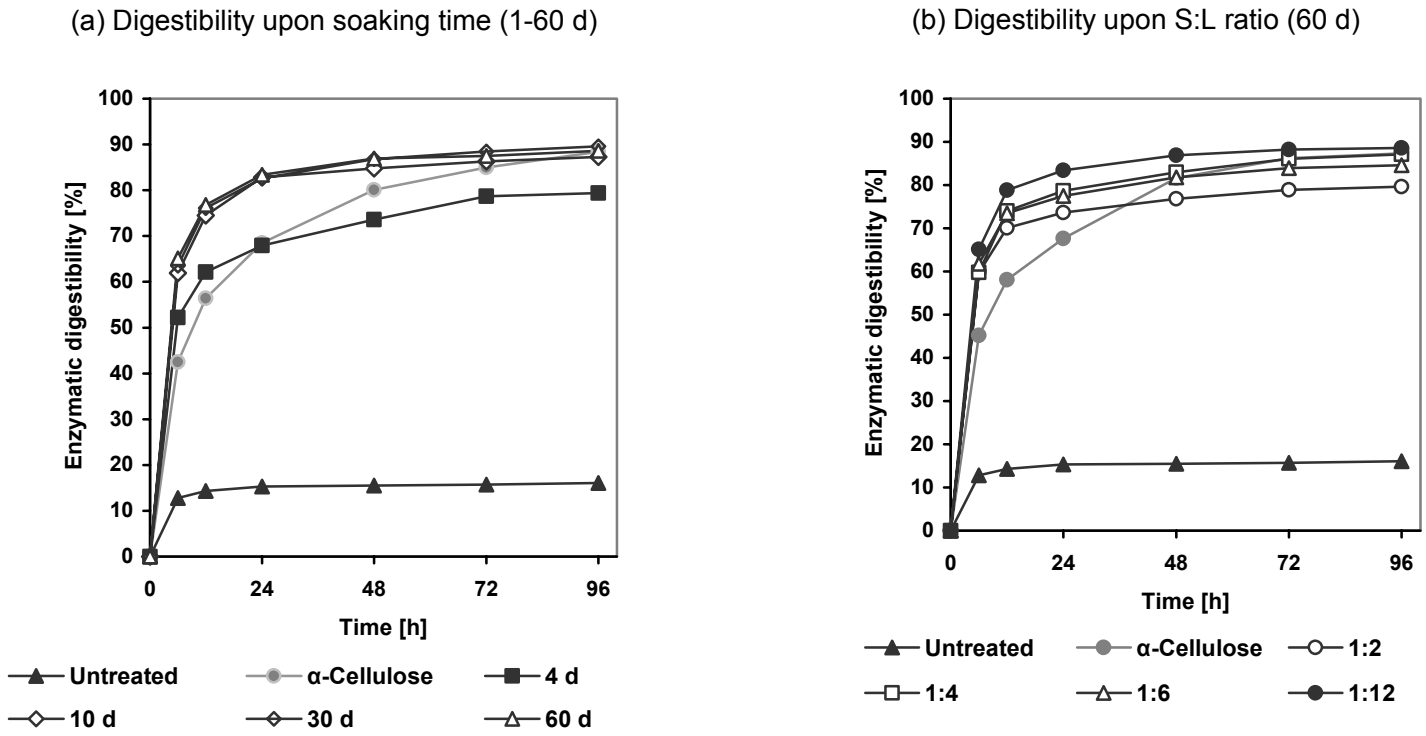


Fig. VI-2. Effect of soaking time and solid-to-liquid ratio on digestibility

Note. (a) solid:liquid ratio=1:12, 1–60 days treatment; (b) Solid:liquid ratio=1:2–1:12, 60 days of soaking, (a) & (b) reaction temperature=23±1ºC (room temperature); 29.5 wt.% ammonia; solid-to-liquid ratio is based on wt. Enzymatic digestibility; 15 FPU/g-glucan, digestibility at 50ºC and 150 rpm.

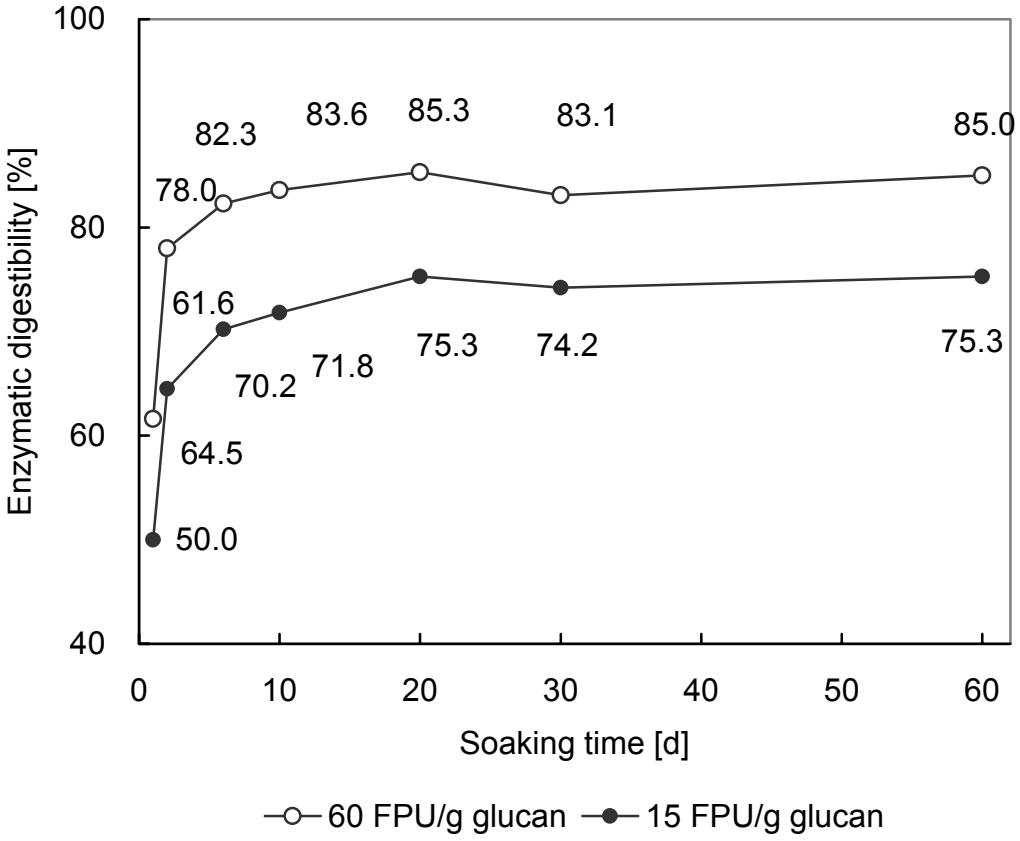


Fig. VI-3. Effect of soaking time on digestibility of xylan.

Note. Reaction temperature=23±1ºC (room temperature); 29.5 wt.% ammonia; solid-to-liquid ratio is 1:12 (based on wt.). enzymatic digestibility; 15 or 60 FPU/g-glucan, digestibility at 50ºC and 150 rpm.

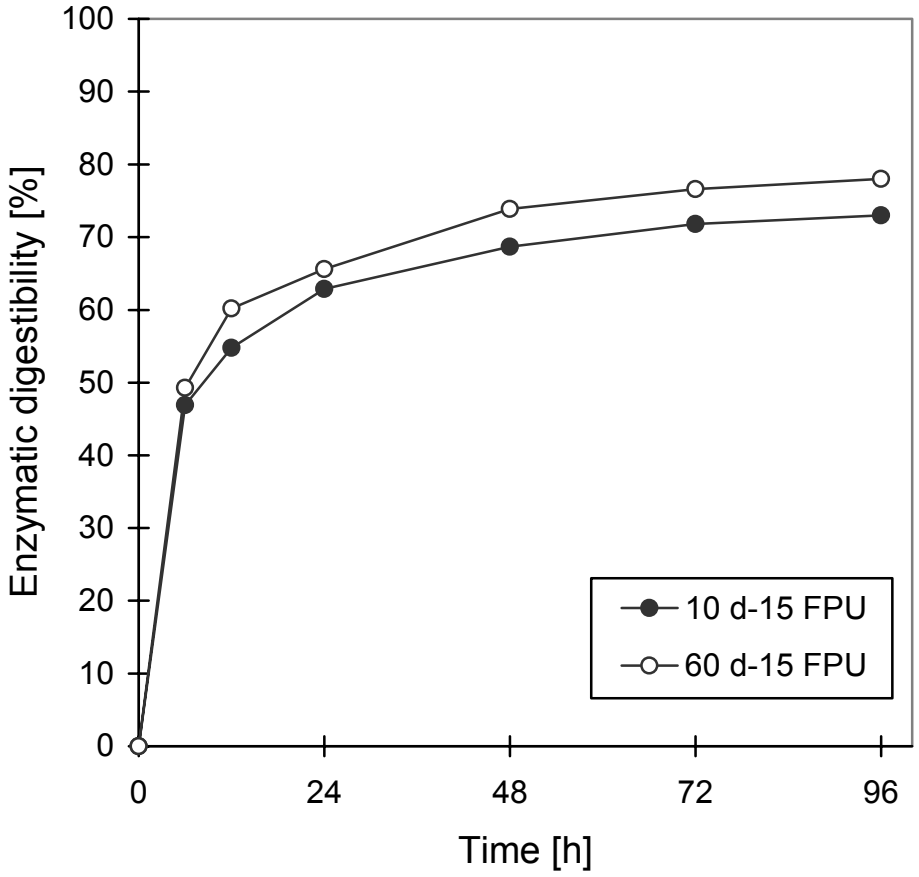


Fig. VI-4. Xylan digestibility of a representative SAA-treated sample

Note. Solid:liquid ratio=1:8, 10 or 60 days treatment; reaction temperature=23±1ºC (room temperature; 29.5 wt.% ammonia; solid-to-liquid ratio is based on wt. Enzymatic digestibility; 15 FPU/g-glucan, digestibility at 50ºC and 150 rpm.

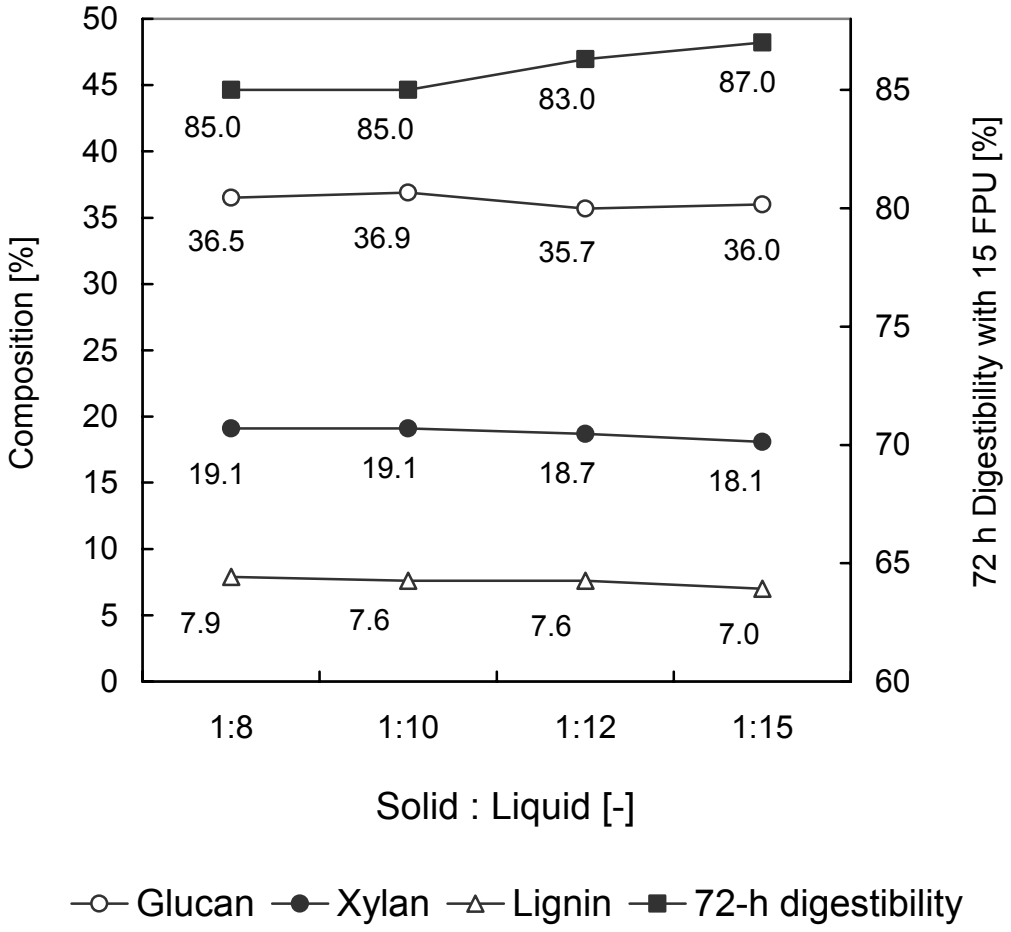


Fig. VI-5. Effect of solid-to-liquid ratio on solid composition and digestibility

Note. Reaction time= 10 days; reaction temperature=23±1ºC (room temperature); 29.5 wt.% ammonia; solid-to-liquid ratio is based on wt. All sugar and lignin content are based on the oven-dry untreated biomass. Enzymatic digestibility; 15 FPU/g-glucan, digestibility at 72 h.

Simultaneous saccharification and fermentation/co-fermentation of SAA-treated corn stover

Simultaneous saccharification and fermentation (SSF) of SAA-treated corn stover and α-cellulose was performed using *Saccharomyces cerevisiae* ATCC® 200062 (NREL-D_5A). Samples treated for 10 days with a 1:8 solid:liquid ratio were used. The ethanol and glucose data for SAA-treated corn stover and α-cellulose are presented in Fig. VI-6. The SAA-treated corn stover contained 49% glucan; therefore a solid concentration based on 3% w/v glucan loading became approximately 6% w/v in the bioreactor. The theoretical maximum ethanol yield (100%) thus corresponded to 17.0 g/L with 3% w/v glucan loading. At the 24 h point, the ethanol yield of the treated sample reached 73% (12.4 g/L) of the theoretical maximum. At this point, the ethanol yield from the treated corn stover was substantially higher than that of α-cellulose (56%, 11.0 g/L).

Table VI-1 summarizes the compositional changes after pretreatment by the SAA treatment and the ammonia treatment at high temperature and high pressure (ARP). The major difference in the compositions is for lignin and xylan. In order to verify the possibility of interaction between released lignin/sugars and ethanol during the reaction, the enzymatic digestibility tests were repeated with the addition of 5 % v/v of ethanol to the two differently treated samples (see the data in Table VI-1). However, the digestibilities and ethanol concentrations were unaffected in this test.

According to the digestibility test, 72-h digestibility (85%) of SAA-treated corn stover was slightly lower than that of the ARP treated corn stover (90%). However, the xylan content of 18.7% in the SAA-treated corn stover was much higher than the 9.9% of the ARP-treated corn stover. This translates to 85% xylan retention for the SSA treatment

compared to only 48% for ARP. The digestibility test using α-cellulose was performed to verify the effect of xylose on the enzyme activity (Fig. VI-7). One reactor contained 3% of glucan as a control while the other reactor contained 3% glucan plus 3% of xylose. In Fig. VI-7, the 72 h-digestibility decreased by 12% after supplementation with 3% of xylose. This result indicates that xylose has a direct inhibitory effect on the glucan hydrolysis by cellulase enzymes, a finding that agrees with previous studies (Nigam and Prabhu, 1991; Todorovic and Grujic, 1987; Xiao et al., 2004).

We speculate that the low ethanol yield observed from the SSF of SAA-treated sample was due to cellulase inhibition by xylose. Xylose accumulation during the SSF is shown in Fig. VI-9-a.

Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover and α-cellulose was performed using the recombinant *E.coli* ATCC® 55124 (KO11). The main advantage of SSCF is that microorganisms can utilize hexose and pentose in a single reactor. Fig. VI-8 presents the ethanol and sugar concentrations in the SSCF. SAA-treated corn stover contains about 49% of glucan and 24% of xylan based on weight, which corresponds to 50 g/L of the total carbon source in 3% w/v glucan loading fermentation. Maximum ethanol concentration was obtained in approximately 96 h (19.8 g/L), which corresponds to 77% of the maximum theoretical yield. This was substantially higher than the 16.3 g/L for α-cellulose and the 12.4 g/L of the SSF test. At the 24 h point, the ethanol concentration of the SAA-treated corn stover reached 16.8 g/L, again higher than the 11.0 g/L observed for α-cellulose. The SSCF results for the SAA-treated corn stover were deemed satisfactory in view of the fact that corn stover is a complex and very heterogeneous feedstock.

Xylose and glucose concentration profiles of SSF and SSCF are presented in Fig. VI-9. In the early phase of SSF and SSCF (Fig. VI-9-a and Fig. VI-9-b), where the cells are in the growth phase, glucose accumulation is noticeable (Fig. VI-9). After 24 h, however, glucose was undetectable in either the SSF or the SSCF tests, indicating that the process proceeds under glucose-limited conditions. The SSF/SSCF process is therefore controlled by the hydrolysis reaction rather than microbial action. The advantage of the SSF/SSCF process, namely that it eliminates the glucose inhibition on cellulase enzyme, is therefore reaffirmed in this work. It is interesting to note the contrasting xylose profiles showing accumulation of xylose in the SSF and consumptions of xylose in the SSCF (Fig. VI-9), reflecting the difference between the two different microorganisms. When fermenting a treated corn stover by SSCF, the microorganism used glucose preferentially, followed by xylose (Fig. VI-8 and Fig. VI-9-b) when they exist in appreciable quantities. This agrees with a previous study that the recombinant *E.coli* does not metabolize until the glucose is completely consumed (Dien et al., 1998). However, the sugar profiles in Fig IV-9 indicate that when the SSCF proceeds under sugar-limited conditions, where the glucose and xylose concentrations are extremely low, the recombinant *E.coli* consumes both sugars concurrently.

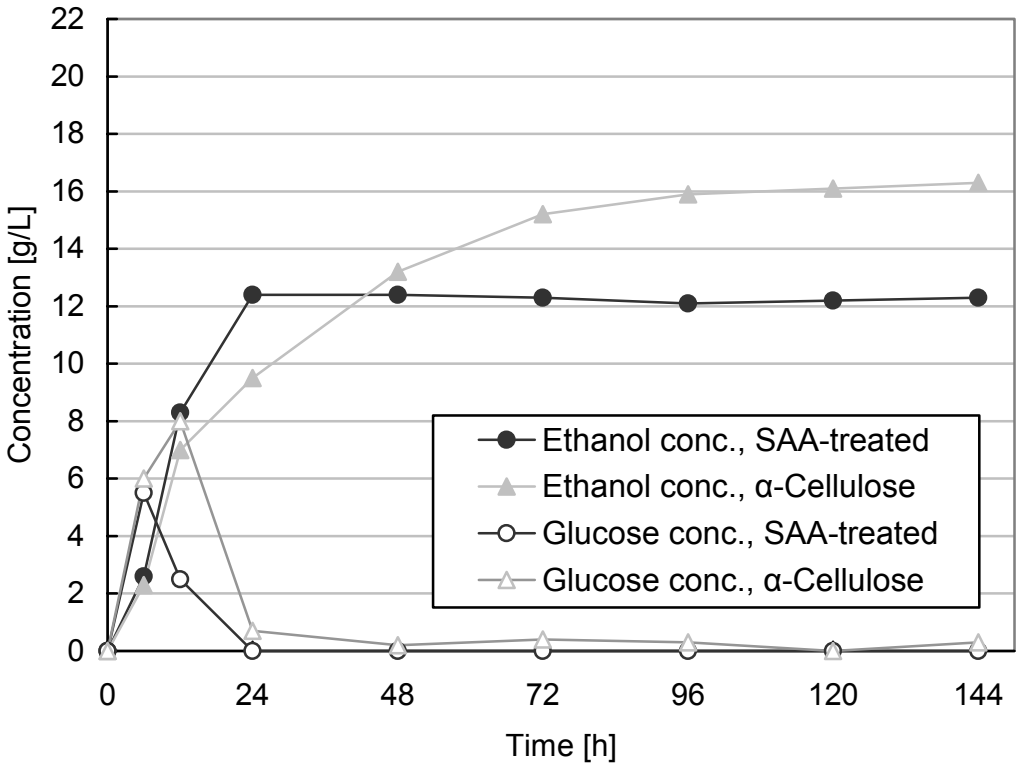


Fig. VI-6. Simultaneous saccharification and fermentation (SSF) of SAA-treated corn stover by D_5A yeast.

Note. Microorganism: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A); substrate: 3% w/v glucan loading/ 100 mL reactor, 10 days SAA-treated corn stover (1:8 of S:L ratio, room temp., 29.5 wt.% NH_3); SSF: 15 FPU of Spezyme CP/g-glucan; 30 CBU of Novozyme 188/g-glucan; YP medium (1% of Yeast extract, 2% of Peptone); anaerobic condition; 38°C, 150 rpm.

Table VI-1. Compositions upon two different pretreatment methods

Compositions	Unit	Untreated corn stover	Low-liquid ARP	Soaking in aqueous ammonia
S.R.	[%]	100	57.50 ±0.61	75.90 ±2.26
Glucan	[%]	36.10	36.40 ±0.46	36.60 ±0.33
Xylan	[%]	21.40	9.90 ±0.35	18.70 ±0.67
Other Sugar	[%]	7.80	1.30 ±0.17	2.50 ±0.78
Acetyl group	[%]	2.20	0.30±0.03	0.35±0.01
Klason Lignin	[%]	17.20	5.08 ±0.04	7.51 ±0.39
Enzymatic digestibility	[%]	15.1	90.0	85.0

Note. 1. S.R.: solid remaining % based on untreated corn stover.
2. All data in table are based on original untreated corn stover.
3. Values are expressed as mean and standard deviation (n=3 for low-liquid ARP, n=5 for soaking in aqueous ammonia).
4. Pretreatment conditions: low-liquid ARP=170°C, 3.3 mL of 15 wt.% NH_3 liquid throughput per g of corn stover, 2.5 MPa; soaking in aqueous ammonia=10 days of reaction time, 23±1ºC (room temperature) of reaction temperature, 29.5 wt.% ammonia; solid-to-liquid ratio=1:10 (based on wt.)
5. Enzymatic digestibility test conditions: 15 FPU of cellulase/g-glucan, 0.05 M citrate buffer, 50°C, 150 rpm

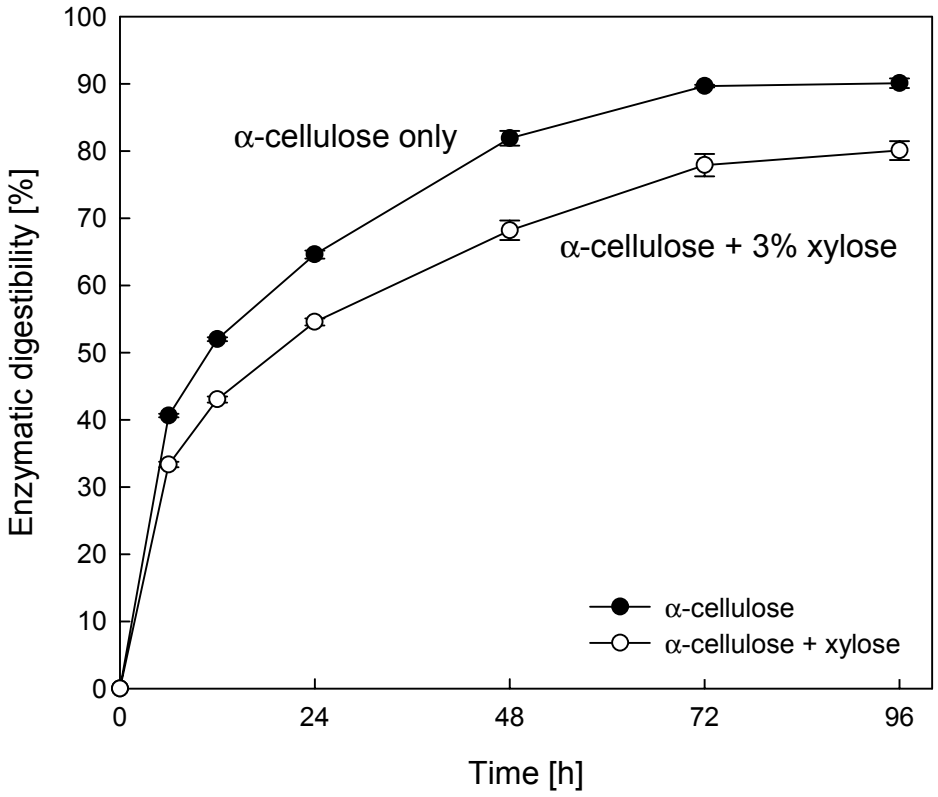


Fig. VI-7. Xylose inhibition on enzyme activity in the cellulose hydrolysis

Note. Substrate: α-cellulose (Sigma, Cat. No. C-8200, Lot No. 11K0246), enzymatic digestibility; 15 FPU/g-glucan, digestibility at 50ºC and 150 rpm.

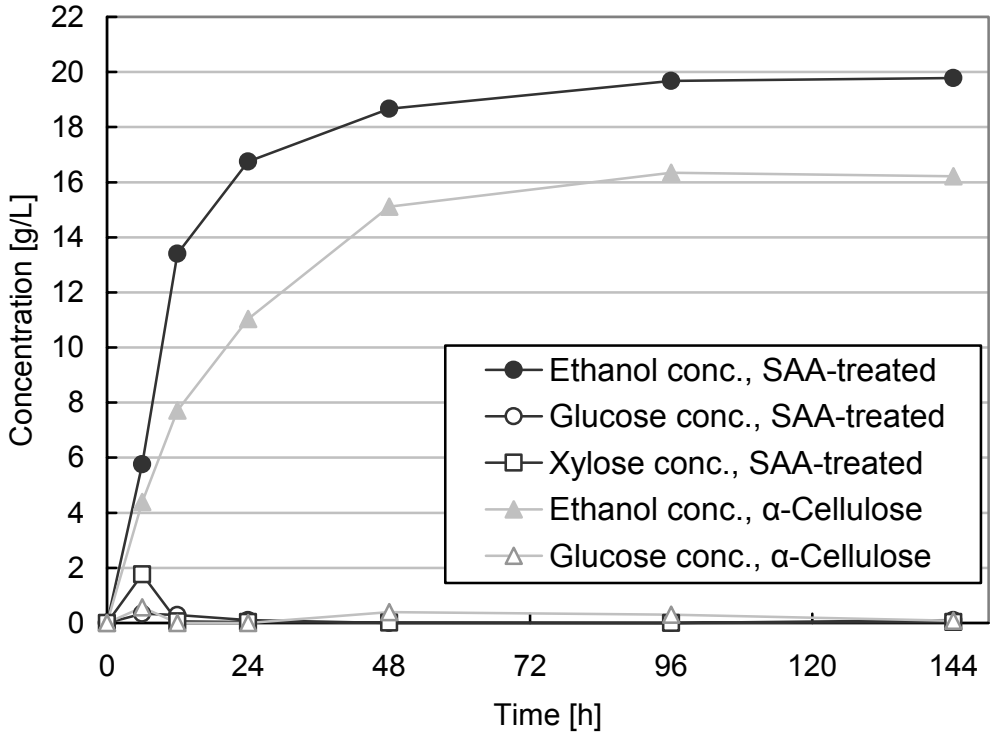
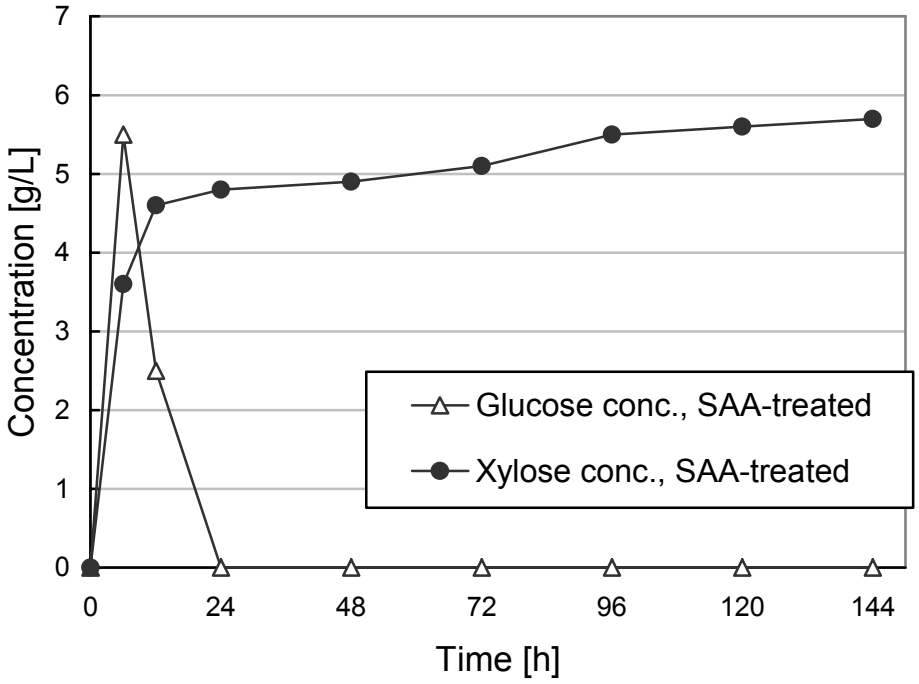


Fig. VI-8. Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover by recombinant *E.coli* (KO11)

Note. Microorganism: *Escherichia coli* ATCC® 55124; substrate: 3% w/v glucan loading/ 100 mL reactor, 10 days SAA-treated corn stover (1:8 of S:L ratio, room temp., 29.5 wt.% NH_3); SSF: 15 FPU of Spezyme CP/g-glucan; 30 CBU of Novozyme 188/g-glucan; LB medium (0.5% of Yeast extract, 1% of Tryptone); anaerobic condition; 38°C, 150 rpm.

(a) SSF using *Saccharomyces cerevisiae* ATCC® 200062 (D_5A)



(b) SSCF using Recombinant *Escherichia coli* ATCC® 55124

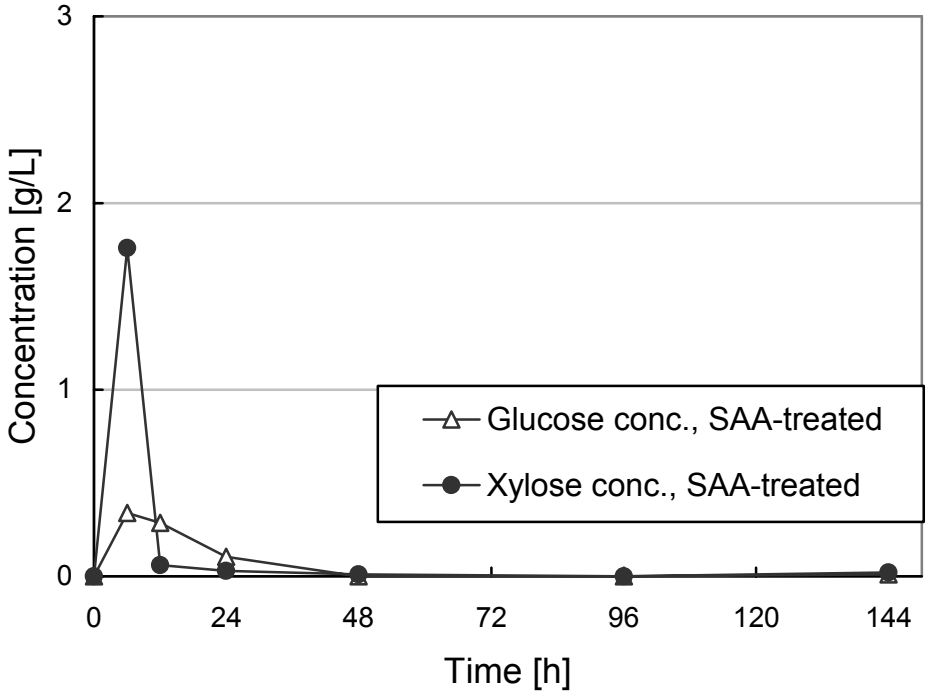
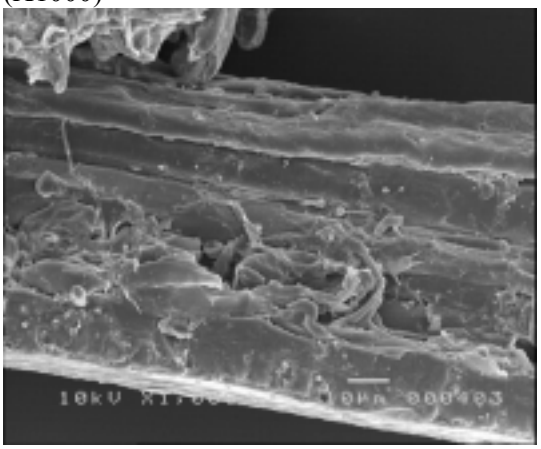


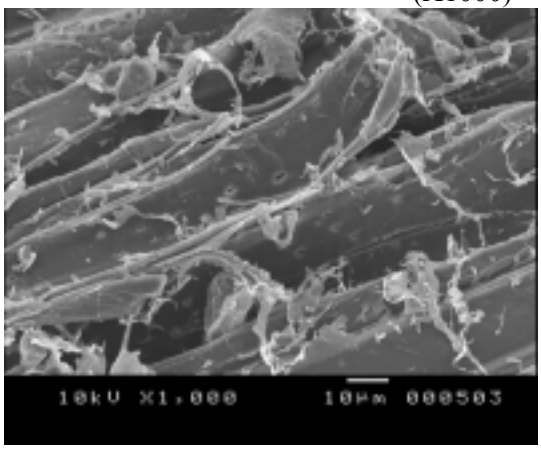
Fig. VI-9. Xylose accumulation and consumption in SSF/SSCF.
Note. See note in Fig. VI-6 for (a) and Fig. VI-8 for (b)

SEM (scanning electron microscope)

Physical changes were observed using SEM pictures of treated and untreated samples. Fig. VI-10 shows that SAA treatment altered the biomass structure significantly. The untreated sample shows rigid and highly ordered fibrils (Fig. VI-10-a), whereas the fibers of treated samples are seen to have been separated and peeled off from the initial connected structure and fully exposed (Fig. VI-10-b). Pinholes are also visible in the treated corn stover (Fig. VI-10-b), leading to speculation that the surface area and the porosity have also increased.

(X1000) (X1000)





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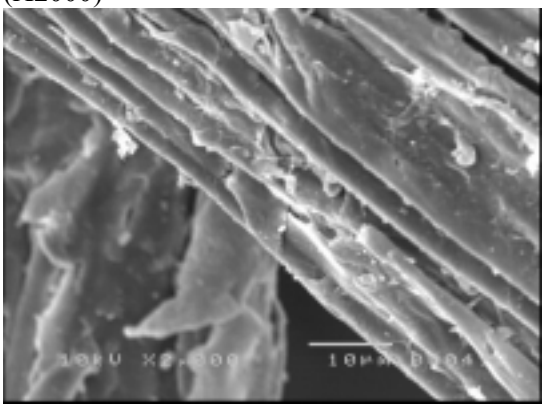

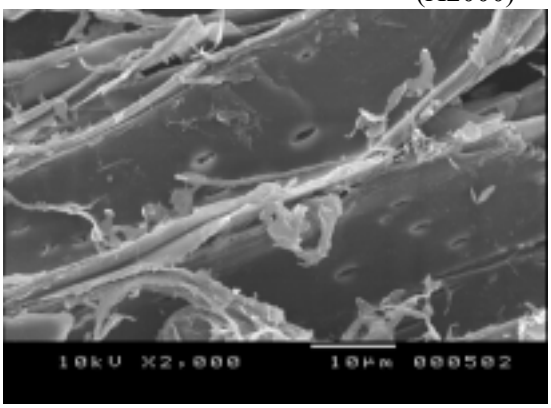

(a) Untreated corn stover (b) SAA-treated corn stover

Fig. VI-10. Scanning electron micrographs (SEM) of treated and untreated corn stover

Note. Reaction time= 10 days ; reaction temperature=23±1ºC (room temperature); 29.5 wt% ammonia; solid-to-liquid ratio is 1:8 (based on wt.).

CONCLUSIONS

SAA at room temperature is a pretreatment method that is technically feasible for corn stover. The process is simple, less capital intensive, and retains a higher fraction of xylan than the high temperature process (ARP). Consumption of ammonia is expected to be low due to reduced acetate generation.

The major effects of SAA at room temperature are swelling of the cellulose and delignification, both of which contribute to improved digestibility.

SAA treatment beyond 10 days has only a marginal effect on delignification and digestibility. However, it is beneficial in reducing the solid-to-liquid ratio.

The ethanol yield for the SAA treated corn stover in the SSF test using *S. cerevisiae* (D_5A) was 73% of the theoretical maximum. The unused xylose appears to inhibit the cellulase activity.

In the SSCF using recombinant *E. coli*, xylose was effectively utilized. The ethanol yield reached 77% of the theoretical maximum (116% based on glucose alone).

VI-II. SOAKING IN AQUEOUS AMMONIA AT MODERATE TEMPERATURE

ABSTRACT

Soaking in aqueous ammonia (SAA) at moderate temperature was investigated as a pretreatment method for corn stover. SAA reduces the reaction time to 12 h and the amount of liquid input to a 1:6 solid-to-liquid ratio. In the SSA process reported here, corn stover was soaked in 15–30 wt.% aqueous ammonia at 40–90°C for 6–24 h. The optimum treatment conditions of this process were 15 wt.% of NH_3, 60°C, 1:6 solid-to-liquid ratio, and 12 h of treatment time. This retained 85% of the xylan and removed 62% of the lignin. Under optimum treatment conditions, the enzymatic digestibility was enhanced from 17 to 85% for glucan with 15 FPU/g-glucan enzyme loading, and 78% of the xylan in the treated biomass was also hydrolyzed by cellulase enzyme. SSCF (3% w/v glucan loading) by recombinant *E.coli* (KO11) utilized a xylose very effectively, and its maximum ethanol concentration was 19.2 g/L after 96 h, which corresponds to 77% of the maximum theoretical yield based on glucan and xylan.

Moderate temperature and a simple process scheme (retaining hemicellulose in the solid) may contribute to low energy input and equipment costs.

INTRODUCTION

Soaking in aqueous ammonia (SAA) at room temperature was introduced as a pretreatment method in the first part of this chapter and was found to produce a remarkable improvement in the enzymatic digestibility (Kim and Lee, 2004). However, the process described in Chapter VI-I identified two main problems. The first is the high solid-to-liquid ratio, which results in increased operating costs. The second problem is that a very long treatment time (8–10 days) is required in order to achieve an appreciable pretreatment effect. This would induce a large capital cost. The main intent of the new process is to resolve these two main problems.

In this study, soaking in aqueous ammonia (SAA) at moderate temperature was investigated as a method for improving enzymatic saccharification of lignocellulosic biomass. The intent here was to reduce the reaction time to <1 day. In this process, the corn stover is pretreated by SAA at moderate temperatures (40–90°C) for treatment times of 4–24 h. The effects of different solid-to-liquid ratios and reaction times on the composition and digestibility were also investigated. The selectivity and activation energy of lignin were determined experimentally in order to describe the delignification effect in this process. Fermentation of pentose sugar xylose into ethanol is essential for an economically feasible production of fuel ethanol from biomass. The utilization of pentose sugar was therefore tested using recombinant *Escherichia coli* (KO11) in a simultaneous saccharification and co-fermentation (SSCF) process.

MATERIALS AND METHODS

This study used the same methods as those described in the first part of the of chapter in the experimental setup and operation, digestibility test, SSCF, and analytical methods.

Ground corn stover (9–35 mesh), which was also used previously, was treated with 29.5 wt.% of aqueous ammonia in screw-capped laboratory bottles at 40–90°C for 6–24 hours. Solid-to-liquid ratios ranging from 1:2 to 1:10 were applied. After the soaking, the solids were separated by filtering, washed with DI water, and subjected to enzymatic digestibility tests. Cellulase enzyme (Spezyme CP, Lot 301-00348-257, activity: 31.2 FPU/mL) supplemented with β-glucosidase (Novozyme 188, Lot 11K1088) was used for the digestibility test. Klason lignin, carbohydrate content, and digestibility were determined by NREL standard procedures (NREL, 2004).

For the simultaneous saccharification and co-fermentation (SSCF), recombinant *Escherichia coli* ATCC® 55124 (KO11) was employed for the co-fermentation test. LB medium (Sigma Cat. No. L-3152) was used for KO11; the medium contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloramphenicol.

RESULTS AND DISCUSSION

Compositional change and its effect on enzymatic digestibility

The effects of treatment conditions in SAA were investigated. The results are summarized in Table VI-2–3. In Table VI-2, two different ammonia concentrations were applied, and three different temperatures were tested at each concentration. As seen in Table VI-2, the major compositional changes are seen in the lignin. Delignification

increases in the range of 50–79% (Table VI-2 and Fig. VI-11), as temperature and ammonia concentration increase. However, the glucan content was well preserved during the treatment. Xylan loss was discernible but much lower than with other treatment methods using aqueous ammonia (Iyer et al., 1996; Kim et al., 2001; Kim et al., 2003). About 80% of the xylan was preserved in the solid in SAA treated corn stover, which can be directly fermented into ethanol by SSCF. Increasing the ammonia concentration from 15 wt.% to 30 wt.% did not improve the pretreatment effect significantly. Moreover, this pretreatment using 15 wt.% of ammonia at 60°C pressure is almost atmospheric pressure. Fifteen percent of ammonia, therefore, was chosen as optimum ammonia concentration.

Table VI-3 show that delignification and digestibility increase with the treatment time. However, increase beyond 12 hours of treatment was relatively insignificant.

The effect of solid-to-liquid ratio was tested at 60°C and is summarized in Table VI-4. Lignin removal and enzymatic digestibility increased when the solid-to-liquid ratio was increased from 2 to 10. Delignification increased from 38% to 67%, and digestibility with 15 FPU/g-glucan increased from 74% to 91%. Xylan removal increased significantly. It was also found that a 1:6 ratio of solids to liquids resulted in a satisfactory pretreatment effect. The enzymatic digestibilities of the representative SAA-treated corn stover are summarized in Fig. VI-12. The results were 85% and 78% for glucan and xylan, respectively, with 15 FPU/g-glucan.

Table VI-2
Effect of reaction temperature and ammonia concentrations on the compositions, enzymatic digestibility, and selectivity in SAA-treated corn stover [1]

NH3 Conc.	Temp	S.R.[2]	Lignin[3]	Deligni-fication	Solid		Enzymatic Digestibility[4]		Select-ivity[5]
					Glucan	Xylan	Glucan	Xylan	
[wt.%]	[°C]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[-]
Untreated	-	-	17.2	-	36.1	21.4	17.2	12.5	-
30	40	77.4	8.4	51.3	36.1	17.7	86.5	67.4	2.4
	60	69.6	5.1	70.5	35.9	17.5	90.3	82.2	2.9
	90	68.1	3.9	79.0	35.6	16.0	98.0	85.2	2.5
15	40	76.0	8.6	50.1	36.9	17.9	80.0	72.5	2.5
	60	71.4	5.6	67.2	36.1	17.2	90.1	79.8	2.8
	90	67.3	3.9	77.2	35.8	16.5	93.4	81.5	2.7

Note. 1. Data in the table are based on the oven dry untreated biomass. Pretreatment conditions: 24 h, solid-to-liquid ratio is 1:10 (based on wt.).
2. S.R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 15 FPU/g-glucan, pH 4.8, digestibility at 50ºC and 150 rpm.
5. Selectivity = m_{Lignin}/m_{Xylan} (where, m_{Lignin} and m_{Xylan} are the mass loss rate in lignin and xylan in the solid, respectively)

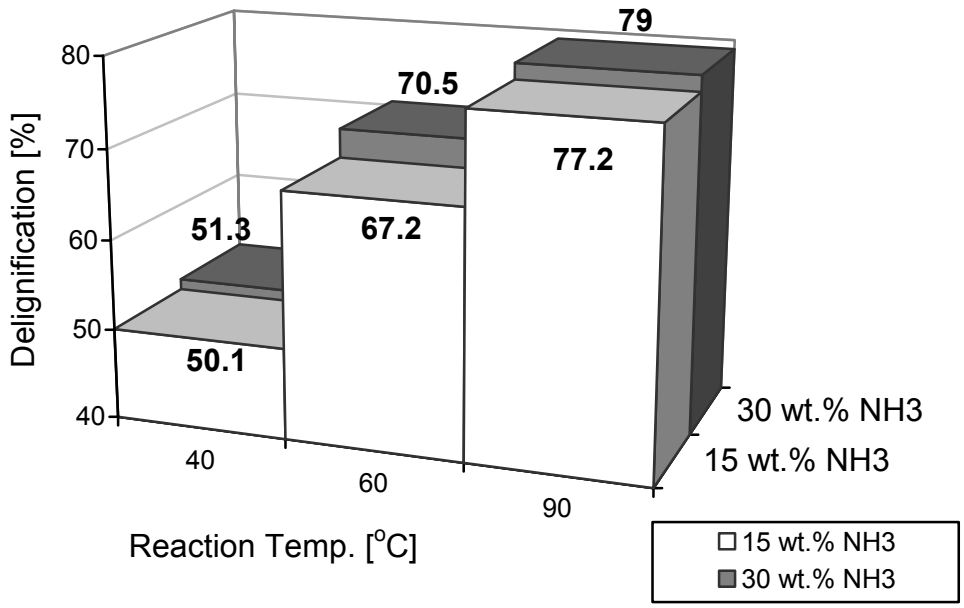

Fig. VI-11. Effect of reaction temperature and ammonia concentration

Note. 1. Data in the table are based on the oven dry untreated biomass. Pretreatment conditions: 15 or 30 wt.% of ammonia, 40–90°C reaction temperature, solid-to-liquid ratio is 1:10 (based on wt.), 24 h treatment time.
2. Klason lignin.

Table VI-3
Effect of reaction time on the compositions and enzymatic digestibility in SAA-treated corn stover [1]

Time	S.R.[2]	Lignin[3]	Deligni-fication	Solid		Enzymatic Digestibility[4]	
				Glucan	Xylan	Glucan	Xylan
[h]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	-	17.2	-	36.1	21.4	17.2	12.5
6	75.2	9.1	47.1	36.1	17.8	79.7	71.9
12	71.3	6.4	62.3	36.1	18.2	85.0	77.9
24	71.1	5.3	69.0	35.9	18.1	86.4	78.4

Note. 1. Data in the table are based on the oven dry untreated biomass. Pretreatment conditions: 15 wt.% of ammonia, 60°C reaction temperature, solid-to-liquid ratio is 1:6 (based on wt.).
2. S.R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 15 FPU/g-glucan, pH 4.8, digestibility at 50ºC and 150 rpm.

Table VI-4
Effect of solid-to-liquid ratio on the compositions and enzymatic digestibility in SAA-treated corn stover [1]

Solid-to-Liquid	S.R.[2]	Lignin[3]	Deligni-fication	Solid		Enzymatic Digestibility[4]	
				Glucan	Xylan	Glucan	Xylan
[-]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
Untreated	-	17.2	-	36.1	21.4	17.2	12.5
1:2	79.0	10.7	37.7	36.1	18.1	74.0	66.2
1:4	74.4	8.2	52.7	36.1	17.4	81.3	73.6
1:6	71.3	6.4	62.3	36.1	18.2	85.0	77.9
1:8	71.6	6.1	64.9	35.3	18.4	91.2	80.6
1:10	71.4	5.6	67.2	35.8	17.2	90.1	79.8

Note. 1. Data in the table are based on the oven dry untreated biomass. Pretreatment conditions: 15 wt.% of ammonia, 60°C reaction temperature, solid-to-liquid ratio is 1:2–1:10 (based on wt.).•
2. S.R. stands for solid remaining after reaction.
3. Klason lignin.
4. Digestibility at 72 h, enzymatic hydrolysis conditions: 15 FPU/g-glucan, pH 4.8, digestibility at 50ºC and 150 rpm.

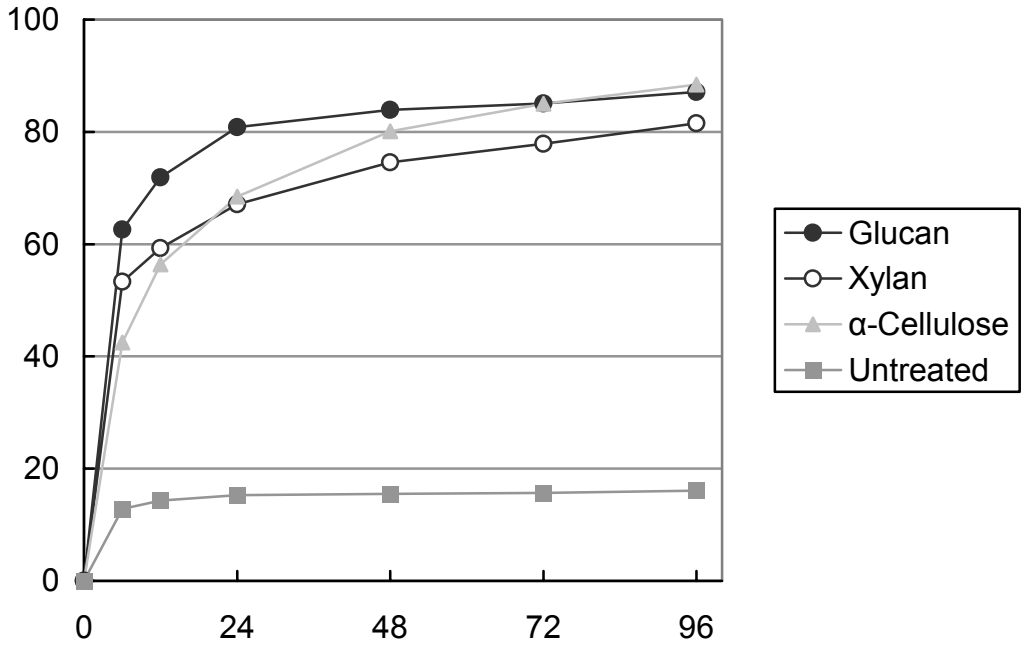

Fig. VI-12. Comparison of enzymatic hydrolysis between untreated and SAA-treated samples

Note. 15 FPU of cellulase/g-glucan, 30 CBU of β-glucosidase/g-glucan 50°C, 150 rpm
Pretreatment conditions: 15 wt.% of ammonia, 60°C of reaction temperature, 12 h reaction time, and 1:6 solid-to-liquid ratio.

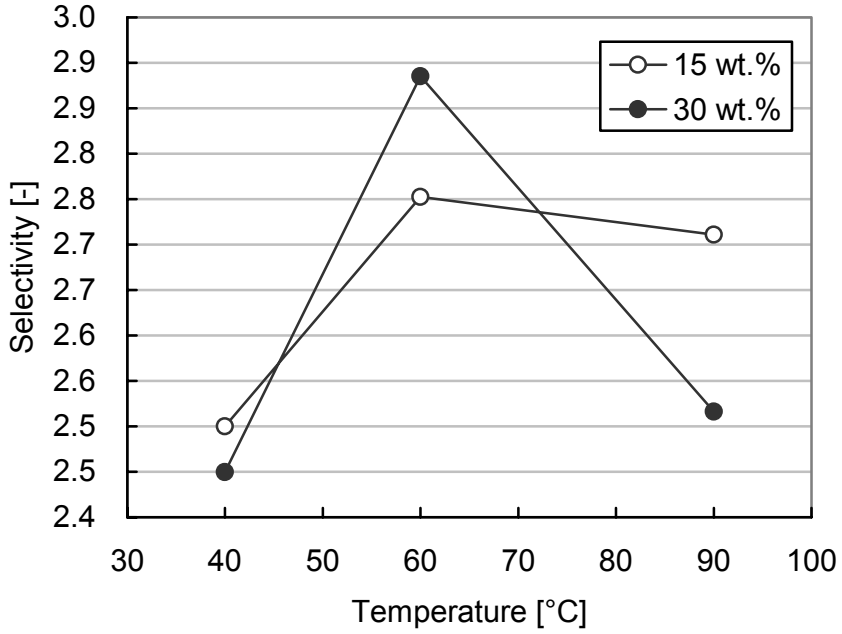

Fig. VI-13. Selectivity plot of the SAA-treated corn stover upon various temperature and ammonia concentrations

Note. 1. Data in the figure are based on the oven dry untreated biomass; Pretreatment conditions: 24 h, solid-to-liquid ratio is 1:10 (based on wt.), 15 or 30 wt.% of ammonia, 40–90°C reaction temperature.

2. Selectivity = m_{Lignin} /m_{Xylan} (where, m_{Lignin} and m_{Xylan} are the mass loss rate of lignin and xylan in the solid, respectively)

Selectivity and activation energy of lignin

Selectivity of lignin over xylan was calculated as below:

$$Selectivity = \frac{m_{Lignin}}{m_{Xylan}}$$

where, m_{Lignin} and m_{Xylan} are the mass loss rate of lignin and xylan in the solid, respectively.

The selectivity, namely the lignin removal rate over the xylan loss rate, results are presented in Fig. VI-13, which indicates that the selectivities for the 60°C SAA treatment are higher than those of other treatment conditions with 15 and 30 wt.% ammonia concentrations. The optimum operating conditions of SAA as a moderate temperature process, on the basis of selectivity and digestibility, are: 15 wt.% ammonia, 60°C, 12 h, and a 1:6 solid-to-liquid ratio.

In order to describe the temperature dependency of the rate of mass, the activation energies were estimated based on SAA treatment conditions, assuming a simple first order reaction following the Arrhenius equation.

$$\dot{m} = -\frac{dm}{dt} = A \cdot \exp\left(-\frac{E}{RT}\right) \cdot m$$

where, $\dot{m}$ is the mass loss rate, E is the activation energy, R is the universal gas constant and T is the temperature in Kelvin.

The activation energy of lignin in the SAA reaction was 19 kJ/mol, which was

markedly lower than the 38 kJ/mol for the hot-water treatment and other reported values, such as 46 and 88 kJ/mol in sulfuric acid, 55–80 kJ/mol for Klason lignin, and 122 kJ/mol for milled-wood enzyme lignin (Beall, 1969; Tang, 1967; Ramiah, 1970; Parker and LeVin, 1989). The low activation energy in this process explains the high delignification of SAA-treated corn stover even at moderate temperatures because of its low temperature dependency.

Simultaneous saccharification and co-fermentation (SSCF)

Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover and α-cellulose was performed with recombinant *E.coli* ATCC® 55124 (KO11). Fig. VI-14 presents ethanol and sugar concentrations in fermentation over an extended period. Maximum ethanol concentrations were reached after approximately 96 h (19.2 g/L), with 77% of the maximum theoretical yield based on glucan and xylan. This was a substantially higher concentration than the 16.3 g/L obtained for α-cellulose.

Xylose and glucose concentration profiles of SSCF are presented in Fig. VI-15. After 24 h, glucose was undetectable in the SSCF, indicating that SSCF utilizes hexose and pentose in a single reactor very effectively.

Ethanol production process schemes using a conventional method and a SAA process are presented in Fig. IV-16. The high ethanol yield of SAA-treated corn stover was due to the contribution of retained hemicellulose to the ethanol conversion. The basic idea in a "biomass-to-ethanol conversion process using SAA pretreatment" is that only the treated solid is utilized for SSCF. This contrast with conventional pretreatments, such as acidic, alkali, and neutral treatments, which usually generate hydrolysate containing a

mixture of sugars and lignin. It is well-known that soluble lignin and other toxic materials in the hydrolysate inhibit the enzyme reaction and bioconversion step. In order to utilize the soluble hemicellulose in the hydrolysate, the conventional pretreatment methods require conditioning steps to remove the soluble lignin and toxic components. The theoretical process scheme of SAA-SSCF is much simpler than those of the conventional processes. Leaving hemicellulose in the solids, therefore, is a desirable pretreatment strategy.

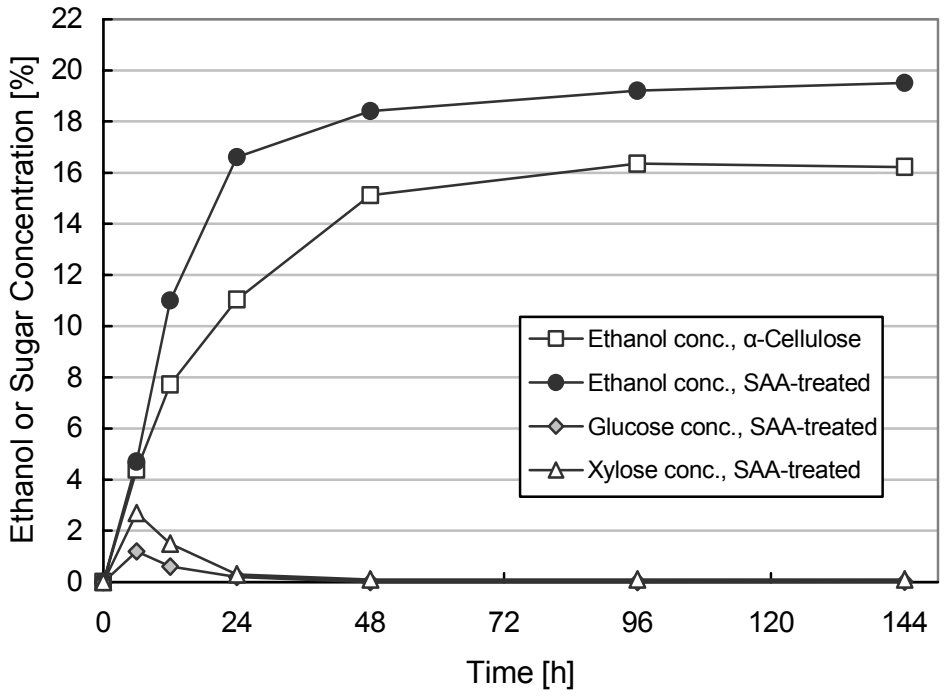

Fig. VI-14. Simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover by recombinant *E.coli* (KO11)

Note. Microorganism: *Escherichia coli* ATCC® 55124; substrate: 3% w/v glucan loading/ 100 mL working volume, 10 days SAA-treated corn stover (1:8 of S:L ratio, room temp., 29.5 wt.% NH_3); SSF: 15 FPU of Spezyme CP/g-glucan; 30 CBU of Novozyme 188/g-glucan; LB medium (0.5% of Yeast extract, 1% of Tryptone); anaerobic condition; 38°C, 150 rpm.

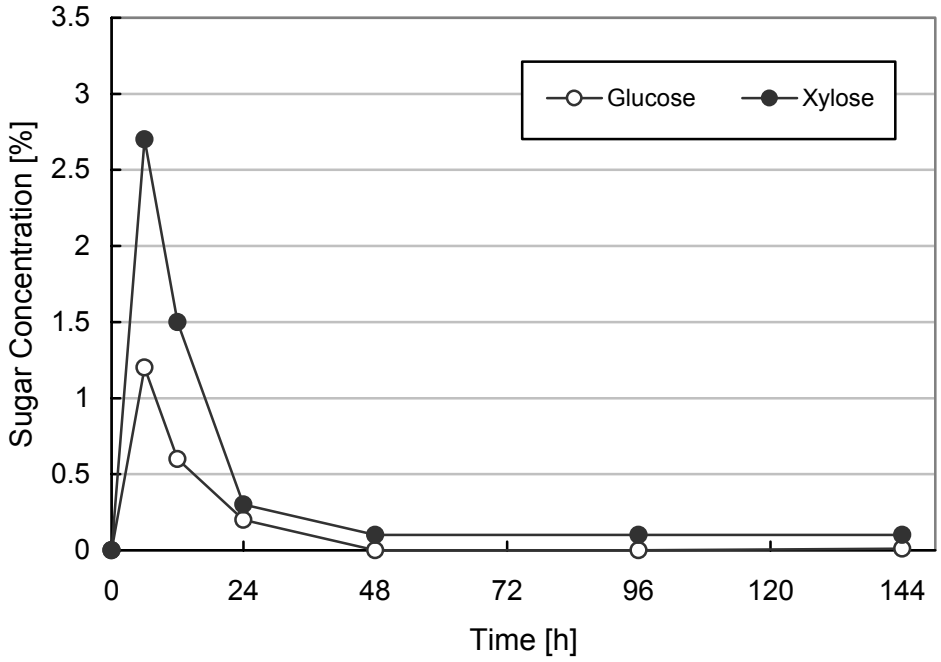

Fig. VI-15. Sugar accumulation and consumption in SSF/SSCF

Note. Microorganism: *Escherichia coli* ATCC® 55124; substrate: 3% w/v glucan loading/ 100 mL reactor, 10 days SAA-treated corn stover (1:8 of S:L ratio, room temp., 29.5 wt.% NH_3); SSF: 15 FPU of Spezyme CP/g-glucan; 30 CBU of Novozyme 188/g-glucan; LB medium (0.5% of Yeast extract, 1% of Tryptone); anaerobic condition; 38°C, 150 rpm.

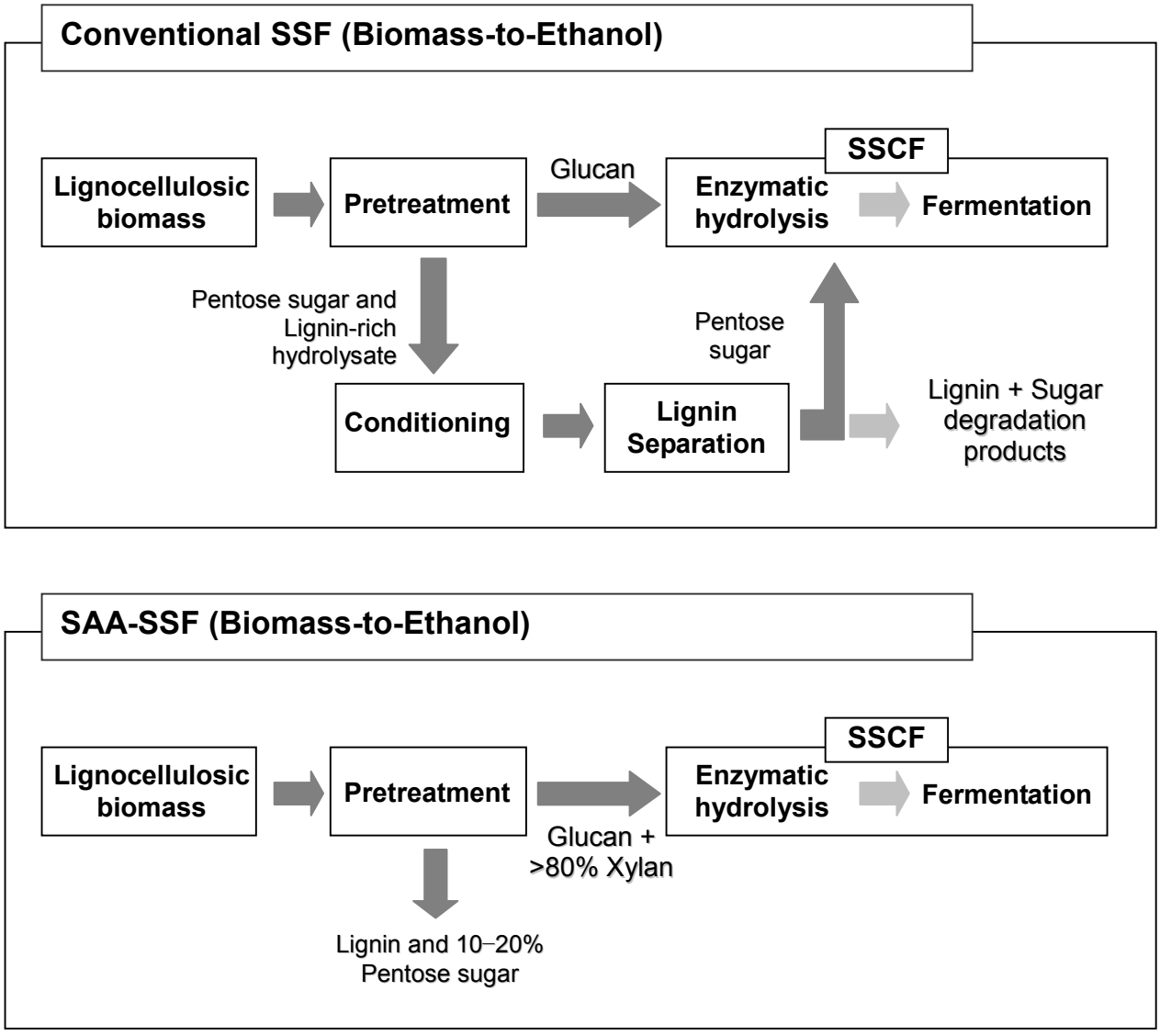

Fig. VI- 16. Comparison of the ethanol production process schemes for conventional pretreatment and the SSA process

CONCLUSION

Soaking in aqueous ammonia at moderate temperatures appears to be a feasible pretreatment method for corn stover. Its moderate treatment conditions make the process very simple and the moderate temperature reduces the treatment time compared with the old SAA process.

The optimum operating conditions for the new SAA at moderate temperature process are 15 wt.% ammonia, 60°C, 12 h, and a 1:6 of solid-to-liquid ratio. The main advantages of this method are that the process scheme is a simple and economical pretreatment method because most of the xylan is retained after treatment and the energy input of the process is low. SSCF test results proved that recombinant *E.coli* converted the glucose and xylose into ethanol very effectively.

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