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Bioethanol production from lignocellulosic feedstock using aqueous ammonia pretreatment and simultaneous saccharification and fermentation (SSF): process development and optimization

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Bioethanol production from lignocellulosic feedstock using aqueous ammonia pretreatment and simultaneous saccharification and fermentation (SSF): process development and optimization

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THESIS ABSTRACT

An integrated bioconversion process, which incorporated soaking in aqueous ammonia (SAA) pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF), was investigated. The TPSSF process consists of pentose conversion using recombinant *Escherichia coli* KO11 in the first phase (0-48h) and hexose conversion with *Saccharomyces cerevisiae* D_5A in the second phase (48-96h). With the xylan-rich SAA-pretreated corn stover as substrate, the TPSSF process resulted in 84% of theoretical maximum ethanol yield based on the total sugars (glucan+xylan) in untreated corn stover.

Cascade two-phase simultaneous saccharification and fermentation (CTPSSF) was also studied to improve overall efficiency of enzymes. SAA-pretreated corn stover was subjected to two-phase SSF performed in series. The enzymes in liquid fraction were recycled from each stage for the use in the subsequent fermentation stage by separating the solid residues from fermentation broth. The results showed that approximately 60% of theoretical maximum ethanol yield based on the total sugars in untreated corn stover was achieved, while enzyme loadings were significantly reduced (up to 50%).

A low-liquid pretreatment method of corn stover with aqueous ammonia was evaluated for the purpose of ethanol production. The effects of a variety of factors, i.e., ammonia loading, solid-to-liquid ratio and reaction time, on the composition and enzyme digestibility of corn stover were thoroughly investigated. Optimal low-liquid ammonia pretreatment conditions (50 wt. % of ammonia loading, 1:5 of solid-to-liquid ratio, 30˚C, 4 weeks) resulted in up to 55% delignification and 86.5% glucan digestibility with 15

FPU/g-glucan of cellulase and 30 CBU/g-glucan of β-glucosidase. With the corn stover treated under the conditions of 50 wt. % ammonia loading, 1:2 solid-to-liquid ratio, 30˚C and 4 weeks as the substrate, an ethanol yield of 73% of theoretical maximum was obtained on the basis of the total carbohydrates (glucan+xylan) content in untreated corn stover. The results demonstrated that the pretreatment of corn stover could be achieved with reduced ammonia loading and water consumption compared to those for soaking in aqueous ammonia (SAA) pretreatment. By applying the mild reaction conditions and low ammonia and moisture dosage, the low-liquid aqueous ammonia pretreatment reduces the pretreatment severity and liquid throughput, and has the potential of making the ethanol production process more cost-effective.

CHAPTER 1. GENERAL INTRODUCTION

Objectives

The overall objective of this study was to develop an efficient biological conversion process of lignocellulosic biomass for ethanol production and to optimize the various operating parameters to improve the process efficiency.

Specifically, this study was focused on efficient conversion of both hexoses and pentoses derived from lignocellulosic biomass, efficient utilization of enzymes in the bioconversion process, and effective pretreatment with low energy and chemical input.

Thesis organization

This thesis contains a general introduction, three chapters of descriptive research procedures and results, one chapter of overall conclusions and future work, as well as cited references and acknowledgements.

The body of this thesis is divided into four chapters. Chapter 1 is a general introduction that includes the objectives and organization of this thesis, a literature review and the list of references. Chapter 2 entitled “Bioethanol production from corn stover using aqueous ammonia pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF)” is a research article modified from a paper that has been published in Bioresource Technology (2010, 101(15), 5910-5916). In this work, a novel integrated bioconversion process using soaking in aqueous ammonia (SAA) pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF) is introduced and this process is demonstrated to be efficient for the utilization of both hexoses and pentoses in corn stover for

ethanol production. The third chapter entitled “Bioconversion of corn stover using cascade two-phase simultaneous saccharification and fermentation (CTPSSF)” is a report of the trial tests of a novel bioconversion scheme in which the efficiency of enzyme recycling and ethanol production via the CSSF process was evaluated. This report is modified from a manuscript prepared for the submission as a journal article. The fourth chapter is a research paper in which a low-liquid pretreatment method of corn stover using aqueous ammonia is proposed. In this work, the various factors that might influence the pretreatment effectiveness were evaluated and the potential of the pretreated corn stover as the substrate for ethanol fermentation was also investigated via simultaneous saccharification and cofermentation (SSCF) tests. This chapter is modified from a manuscript to be submitted as a journal article.

Author’s role: the author of this thesis has made a direct and substantial contribution to the work reported in this thesis. The author participated in conceiving and designing the study with major professor. The author was the main person who performed the lab procedures as well as the collection, analysis and interpretation of experimental data as described in this thesis. The author was also responsible for writing the manuscripts based on the research approaches and the results obtained.

Literature Review

1. Background

Global depletion of fossil fuels, rising fuel prices, environmental concerns, and pressures for oil independence are creating a strong market for biofuels (USDA ERS, 2009). Biofuels have the potential to be domestically and globally available for energy security, with most being carbon neutral (introducing no additional carbon to the global carbon cycle) or potentially carbon negative (if coupled with carbon sequestration) and supportable within the

current agricultural infrastructure (DOE, 2005a). Presently, one of the most promising alternatives for petro-fuels is bioethanol. Ethanol is a simple alkyl alcohol that can be used as a transport fuel in spark ignition engines. It has high octane levels and can be either blended into petrol or used in unmodified vehicles, or run as 100 percent ethanol in a converted engine (Rudkin, 2002).

Ethanol can be produced from a variety of feedstocks, including sugar substances, such as sugarcane juice and molasses, as well as starch-based materials such as wheat and corn (Jones et al., 1994), where the corn starch based ethanol production is dominant in the US bioethanol industry (Korves, 2008). However, this technology may not be practical in the long run due to the fact that wide planting of corn for ethanol production will compete for the finite arable land and thus threaten the national food security (Giampietro et al., 1997). Lignocellulosic biomass has been suggested as the most promising alternative for the traditional starch feedstock. Lignocellulosic feedstocks have the best well-to-wheel assessment, considering its abundance, low cost and high polysaccharides (cellulose and hemicellulose) content (Fujii et al., 2009). Intensive research and developments in the last decades on lignocellulosic materiasls will most likely make them important feedstock for ethanol production in the future (Taherzadeh et al., 2007).

The currently available approach for converting lignocellulosic feedstock into ethanol applies a complex and expensive multi-step process that combines thermochemical and biological methods in large, centralized processing plants (DOE, 2005b). Biomass conversion involves three basic steps: (1) Pretreatment of raw feedstock to increase the accessibility of enzymes to the polysaccharides (cellulose and hemicellulose); (2) enzymatic hydrolysis to break down the lignocellulose constituents (polysaccharides) into a mixture of

fermentable sugars; and (3) microbial fermentation, mediated by bacteria or yeast, to convert these sugars to ethanol. Making the transformation of lignocellulose to ethanol more economical and practical will require the development of molecular redesign of numerous enzymes, biochemical pathways, and full cellular systems (DOE, 2005b).

Ethanol production from lignocellulosic biomass using enzymatic hydrolysis and fermentation can be improved by: (1) development of effective pretreatment technologies that do not require expensive chemicals and/or high pressure equipment; (2) maintaining a high density of cells within the reactor to convert sugars to ethanol quickly; (3) integrating enzymatic hydrolysis of cellulose and hemicellulose with fermentation to keep sugar levels low, improving enzymatic conversion rates by minimizing the product (sugar) inhibition; (4) converting both the cellulose (glucose) and hemicellulose (xylose) to ethanol to increase the overall ethanol yield; (5) co-producing crude cellulase enzyme and/or recycle the enzymes so as to reduce enzyme costs; (6) incorporation of low temperature separation of ethanol from the reactor broth so as to keep fermentation reaction rates high, and allow recycle of enzymes w/o thermal destruction (Dale and Moelhman, 2005). Fig. 1.1 shows the coordinated actions for improvement of biomass to ethanol that are being taken (Chandel et al., 2007).

2. Lignocellulosic feedstock

Lignocellulosic materials are renewable resources that can be directly or indirectly used for the production of biomolecules and commodity chemicals (Ramos and Fontana, 2004). Lignocellulose mainly consists of three major components, i.e., cellulose, hemicelluloses, and lignin. The cell wall polysaccharides of lignocellulosic biomass are composed of crystalline cellulose fibrils surrounded by a matrix of non-crystalline hemicelluloses, which are a group of heteropolysaccharides that bind with pectin to cellulose,

forming a net-work of cross-linked fibers (Ramos, 2003). The chemical structure of the plant cell wall is shown in Fig. 1.2.

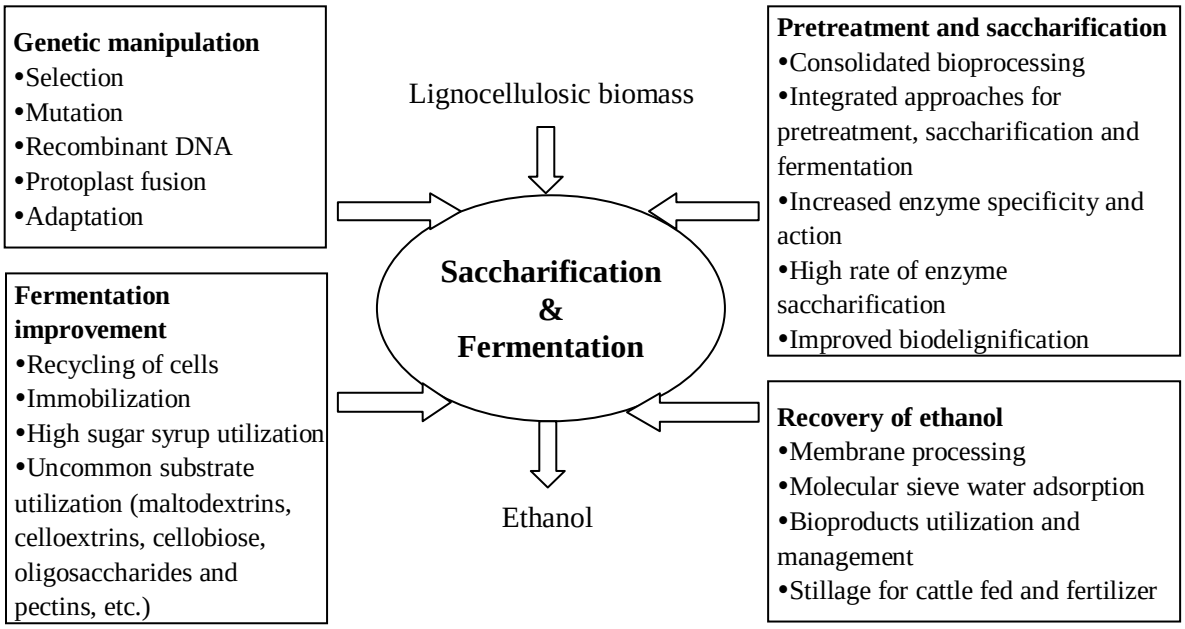

Fig. 1.1. Coordinated action for improvement of biomass to ethanol.

Cellulose is a linear polymer chain which is formed by joining the anhydroglucose units into glucan chains. These anhydroglucose units are linked together by β-(1, 4)-glycosidic bonds. Due to this linkage, cellobiose is established as the repeat unit for cellulose chains (Demirbas, 2009). The degree of polymerization (DP) of native cellulose is in the range of 7,000-15,000 (Bodîrlău et al., 2007).

$$DP = \frac{\text{Molecular weight of cellulose}}{\text{Molecular weight of one glucose unit}}$$

By forming intra-molecular and inter-molecular hydrogen bonds between -OH groups within the same cellulose chain and the surrounding cellulose chains, the chains tend to arrange in parallel and form a crystalline supermolecular structure. Then, bundles of linear

cellulose chains (in the longitudinal direction) form a microfibril which is oriented in the cell wall structure (Ibrahim, 1998).

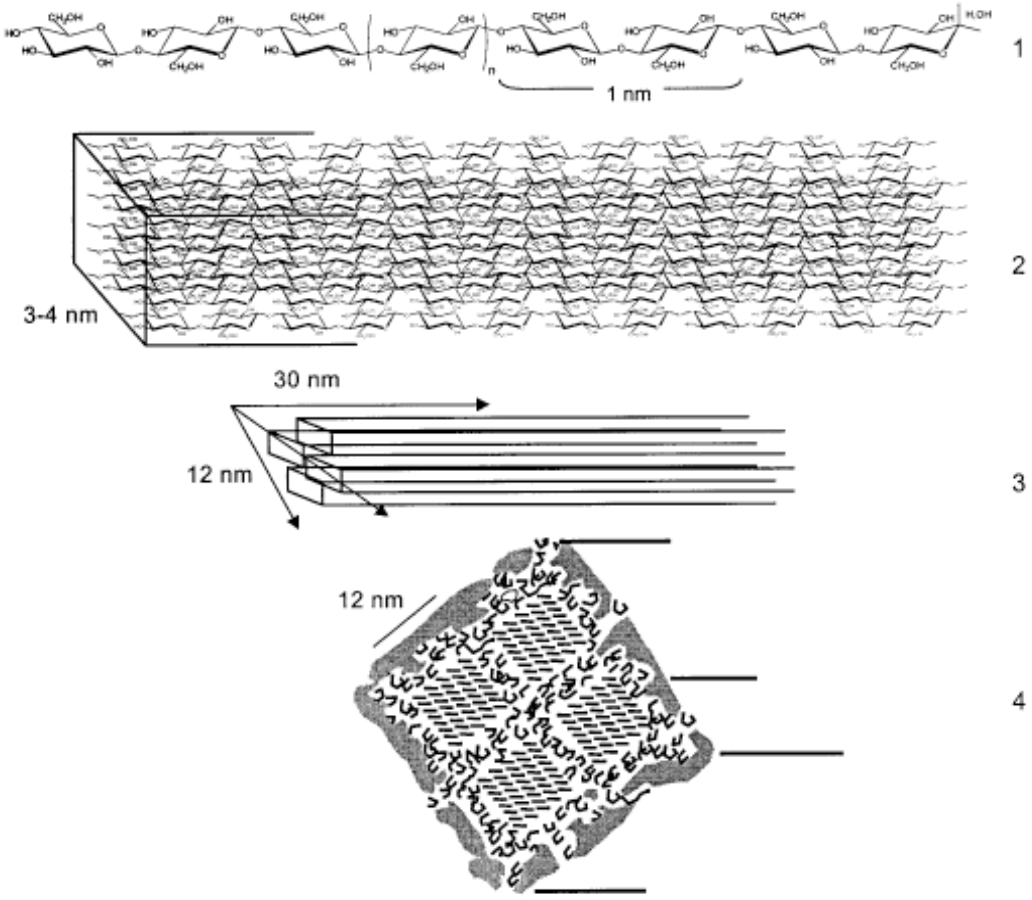

Fig. 1.2. Chemical association in the plant cell wall: (1) the cellulose backbone, with an indication the length of its basic structural unit, cellobiose; (2) frame work of cellulose chains in the elementary fibril; (3) cellulose crystalline; (4) microfibril cross section, showing stands of cellulose molecules embedded in a matrix of hemicellulose and proto lignin (Ramos, 2003).

Unlike cellulose, hemicelluloses consist of different monosaccharide units. The polymer chains of hemicelluloses are short-branched and amorphous. Owing to the amorphous morphology, hemicelluloses are partially soluble in water (Demirbas, 2008). The backbone of hemicellulose chains can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars) (Speight, 2008). These

polysaccharides are formed by a wide variety of building blocks including pentoses (e.g., xylose, and arabinose), hexoses (e.g., glucose, mannose and galactose) and uronic acids (e.g., 4-O-methyl-glucuronic and galacturonic acids). Generally, these polysaccharides fall into four classes: (a) unbranched chains such as (1-4)-linked xylans or mannans; (b) helical chain such as (1-3)-linked xylans; (c) branched chains such as (1-4)-linked galactoglucomannans; and (d) pectin substances such as polyrhamnogalacturonans (Ramos and Fontana, 2004). Some hemicelluloses, particularly heteroxylans, also show a considerable degree of acetylation (Ramos and Fontana, 2004). The most important sugar of the hemicelluloses component is xylose (Speight, 2008). Hemicelluloses are able to bind to cellulose by multiple hydrogen bonds and to bind to lignin by covalent bonds.

Lignin is a complex hydrophobic network of phenylpropanoid units that is thought to result from the oxidative polymerization of one or more of three types of hydroxycinnamyl alcohol precursors (Higuchi, 1985). Lignin has been recognized not only to give mechanical strength or rigidity to a plant (Chabannes et al., 2001), but also to prevent the invasion by pathogens and pests (Sarkanen and Ludwig, 1971). Moreover, lignin serves as a disposal mechanism for metabolic waste (McCrady, 1991). Lignin is described as a random, three-dimensional network of phenylpropane units (Sjöström, 1993). The precursors of these phenylpropane units are coniferyl, sinapyl, and p-coumaryl alcohols, which are transformed into lignin by a complex dehydrogenative polymerization process (FAO, 2002). These three aromatic monomers in lignin are referred to as p-hydroxyphenyl, guaiacyl and syringyl residues, respectively (Boerjan et al., 2003). Depending upon the number and type of functional groups on the aromatic rings and propane side chains, the solubility of lignin is highly variable (FAO, 2002). Lignin in plant cell walls is physically and chemically

associated with wall polysaccharides and proteins. The association between lignin and polysaccharides includes glycosidic linkages, ether cross-linkages, ester cross-linkages and cinnamic acid bridges (Sánchez, 2002).

Except for the three major components above, lignocellulosic biomass also contains extractives, which refer to the organic substances which have low molecular weight and are soluble in neutral solvents. Resins, fats, waxes, fatty acids and alcohols, phenolics, phytosterols, salts, minerals, and other compounds are categorized as extractives. Moreover, the residue remaining after ignition (dry oxidation at $575 \pm 25^{\circ}C$) of lignocellulosic biomass is ash, which is composed of minerals such as silicon, aluminum, calcium, magnesium, potassium, and sodium (Lee et al., 2007).

3. Pretreatment

Lignocellulosic biomass, as the prospective source of fermentable sugars for ethanol bio-production, has great potential use in industry. However, lignocellulosic feedstocks are not easily broken down into their composite sugar molecules. As a result, an effective pretreatment is required to liberate the polysaccharides from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step (Mosier et al., 2005).

Ammonia as a pretreatment reagent has many advantages for an effective delignification as well as swelling of biomass. Meanwhile, ammonia pretreatment does not significantly produce the inhibitors, e.g., furfural and hydroxymethyl furfural (HMF), which are by far considered as the most toxic inhibitors present in lignocellulosic hydrolysate (Tian et al., 2009) for the downstream biological processes. The presence of inhibitors complicates the ethanol production and increases the cost of production due to required detoxification steps.

The soaking in aqueous ammonia (SAA) at low temperature retains the hemicellulose in the solids by minimizing the interaction with hemicellulose during treatment, which was reported as a feasible approach to increase the ethanol yield based on total sugars and simplify the bioconversion scheme (Kim and Lee, 2005b; Kim and Lee, 2007). Retained xylan can usually be hydrolyzed to fermentable pentoses by most commercial cellulase and xylanase mixtures (Kim and Lee, 2007) . The fact that 100% of glucan and over 85% of xylan remained in the solids after SAA pretreatment provides the opportunity for increasing the ethanol yield based on totals sugars, i.e., hexose and pentose, and simplifying the bioconversion scheme.

4. Bioconversion strategies and process integration

4.1. Separate enzymatic hydrolysis and fermentation (SHF)

The terminology "separate hydrolysis and fermentation (SHF)" refers to a process in which enzymatic hydrolysis (saccharification) of polysaccharides and the microbial fermentation are performed sequentially in separate units (Fig. 1.3(a)). The major advantage of this method is that the enzymatic hydrolysis and fermentation can be carried out at their own optimum conditions: 45-50˚C for enzymatic hydrolysis with cellulase and β-glucosidase, and 30-37˚C for ethanol fermentation with fermentative microorganisms (Olsson et al., 2006; Saha et al., 2005).

However, the drawbacks of SHF process include: (1) the inhibition of the released sugars, mainly cellobiose and glucose, on cellulase activity. At a cellobiose concentration as low as 6 g/l, the activity of cellulase is reduced by 60%. Although glucose decreases the cellulase activity as well, the inhibitory effect of glucose is lower than that of cellobiose. On the other hand, glucose is a strong inhibitor for β-glucosidase. At a level of 3 g/l of glucose,

the activity of β -glucosidase is reduced by 75% (Philippidis et al., 1993; Philippidis and Smith, 1995); (2) high probability of contamination since separate vessels are used for hydrolysis and fermentation. The hydrolysis process is long and the released sugars as carbon source provide the opportunity of contamination with naturally-occurring microbes. A possible source of contamination could be the enzyme preparation. In practice, it is difficult to sterilize enzymes in large scale, since it must be filter-sterilized owing to the denaturation of enzymes in an autoclave (Taherzadeh and Karimi, 2007). In addition, it is not feasible to add antibiotics in hydrolysis reactor because antibiotics may affect the growth and fermentation of microorganisms in the subsequent fermentation step.

4.2. Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) integrates enzymatic hydrolysis and microbial fermentation into one step: the sugars released from enzymatic hydrolysis are immediately consumed by the fermentative microorganism for ethanol production (Fig. 1.3(b)). By SSF, enzymatic hydrolysis rates can be maximized by reducing the product (sugar) inhibition. SSF gives higher reported ethanol yields from cellulose than SHF and requires lower amounts of enzyme (Eklund and Zacchi, 1995; Sun and Cheng, 2002). Another advantage of SSF is that the risk of contamination is reduced due to the low sugar concentration and ethanol accumulation in the fermentation system. Furthermore, the number of vessels required for SSF is reduced in comparison to SHF, resulting in lower capital cost of the process (Taherzadeh and Karimi, 2007).

SSF has the drawback that the enzymatic hydrolysis and fermentation have to be performed under the comprised conditions, particularly with respect to pH and temperature. The optimum temperature and pH of enzyme activity and fermentation are always different.

Hydrolysis is usually the rate-limiting step in SSF (Philippidis and Smith, 1995), and the optimal temperature of enzyme reaction is typically higher than that of fermentation. Therefore, several thermotolerant bacteria and yeasts, e.g., *Candida acidothermophilum* and *Kluyveromyces marxianus* have been proposed for SSF in order to raise the temperature close to the optimum temperature of enzyme reactions (Ballesteros et al., 2004; Golias et al., 2002). Inhibition of ethanol accumulated during the SSF process on enzymes and microorganisms may also be a disadvantage of SSF. It was reported that the enzyme activities are reduced by 25% when ethanol concentration is 30 g/l (Wyman and Abelson, 1996).

4.3. Simultaneous saccharification and cofermentation (SSCF)

Basically, simultaneous saccharification and cofermentation (SSCF) has the same mechanism with SSF. Cofermentation refers to the fermentation of both hexoses (C_6) and pentoses (C_5) to ethanol. The hydrolyzed hemicellulose during pretreatment and the solid cellulose are not separated after pretreatment, allowing the conversion of cellulose and hemicellulose to be carried out in a single reactor via a single step (Teixeira et al., 2000).

Some conventional wild type fermentative microorganisms, e.g., *Saccharomyces cerevisiae*, are only capable of utilizing glucose as the carbon source for ethanol production, while some can consume both hexoses and pentoses, e.g., *Pichia stipitis*. However, the growth and fermentation of *Pichia stipitis* require expensive media, making the fermentation with *Pichia stipitis* less cost-effective (Salgadot et al., 2009). Therefore, one important requirement is an efficient microorganism capable of fermenting a wide range of substrates (pentoses and hexoses) as well as to tolerate stress conditions. There have been multiple efforts to develop these recombinant microorganisms to allow co-fermentation of both pentose and hexose sugars (Deanda et al., 1996; Dien et al., 1997; Eliasson et al., 2000;

Kötter and Ciriacy, 1993; Moniruzzaman et al., 1997; Zhang et al., 1995). Paticularly, three main microbial platforms, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Escherichia coli*, have emerged and their performance has been demonstrated in pilot studies (Zaldivar et al., 2004).

4.4. Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) (Fig. 1.3(c)) combines cellulase production, cellulose hydrolysis and fermentation in one step. CBP is distinguished from other less highly integrated configurations in that it does not involve a dedicated process step for cellulase production (Lynd et al., 2005). Progress in developing CBP-enabling microorganisms is being made through two strategies: engineering naturally occurring cellulolytic microorganisms to improve product-related properties, such as yield and titer, and engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase system enabling cellulose utilization (Lynd et al., 2005). Application of CBP entails no operating costs or capital investment for purchasing enzyme or its production (Hamelinck et al., 2005; Lynd et al., 2005).

4.5. Pentose fermentation

Hemicellulose is one of the major components of lignocellulosic biomass. The content of hemicellulose counts for approximately 25 wt. % in lignocellulosic biomass. The conversion of both cellulose and hemicellulose for production of fuel ethanol is being studied intensively with a view to develop a technically and economically viable bioprocess (Chandrakant and Bisaria, 1998). The ethanol yield in the process is an important parameter with regard to economy both because the cost of the raw material constitutes a major part of the total production cost and also because the processing costs are typically associated with

the amount of material passing through the process and not the amount of product made (Rocha et al., 2009). To achieve maximum ethanol yield, all monosaccharides have to be fermented. The pentose content in the raw material is of importance as pentoses are difficult to ferment to ethanol (Olsson and Hahn-Hägerdal, 1996). The strategies that have been put forward for the conversion of both hexose and pentoses to achieve high ethanol concentration, yield, and productivity mainly include: (1) separate pentose and hexose fermentation with glucose or xylose-fermenting microbes alone; (2) cofermentation of hexoses and pentoses via SSCF with microorganisms that are capable of metabolizing both hexoses and pentoses; (3) coupled isomerization and fermentation with xylose isomerase enzyme as well as microbes.

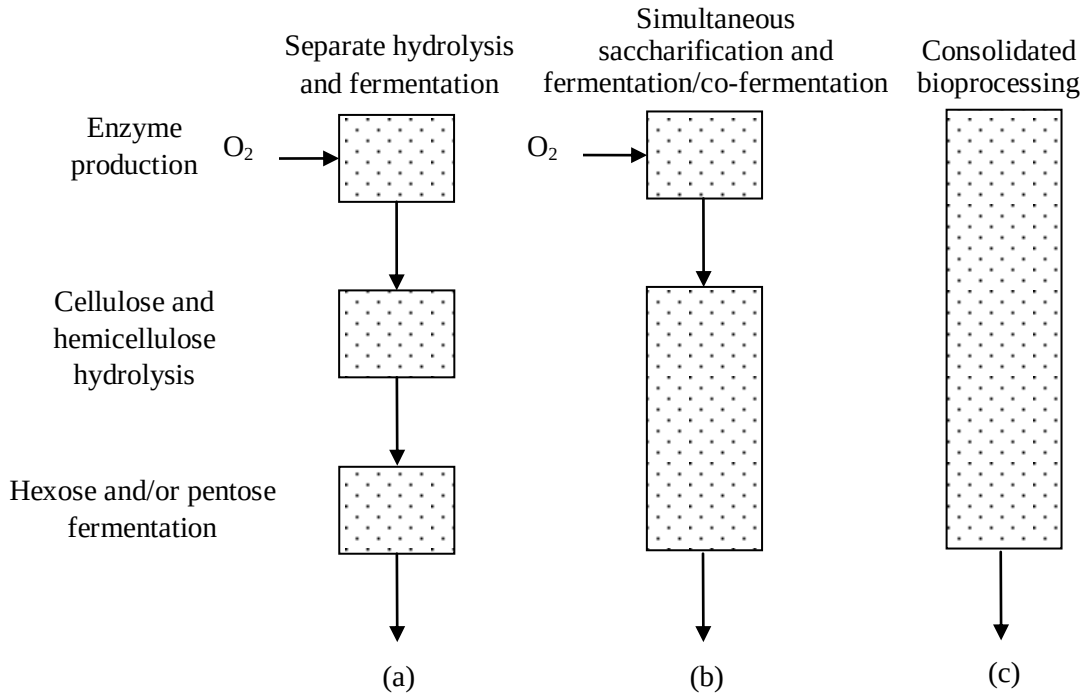

Fig. 1.3. A variety of processes that have show potential for lignocellulose conversion into ethanol*: (a) separate hydrolysis and fermentation (SHF); (b) simultaneous saccharification and fermentation/co-fermentation (SSF/SSCF); (c) consolidated bioprocessing (CBP).
*Source: USDA Agricultural Research Service

4.5.1. Separate pentose and hexose fermentation

In dilute acid and hot water pretreatment, the hemicellulose component and lignin is solubilized into the liquid fraction, while cellulose remains in the solids. Cellulose is then hydrolyzed with cellulase and β-glucosidase to glucose, which is then fermented to ethanol by yeast or some bacteria. The process can be carried out either with separate hydrolysis and fermentation (SHF) or simultaneous saccharification and fermentation (SSF). The liquid fraction which contains hemicellulose is subjected to the processes of lignin separation and conditioning before being fermented with pentose-fermenting microorganisms. The fermentation processes on hexose (from cellulose) and pentose (from hemicellulose) are performed in two separate vessels. The flow diagram of separate pentose and hexose fermentation is shown in Fig. 1.4.

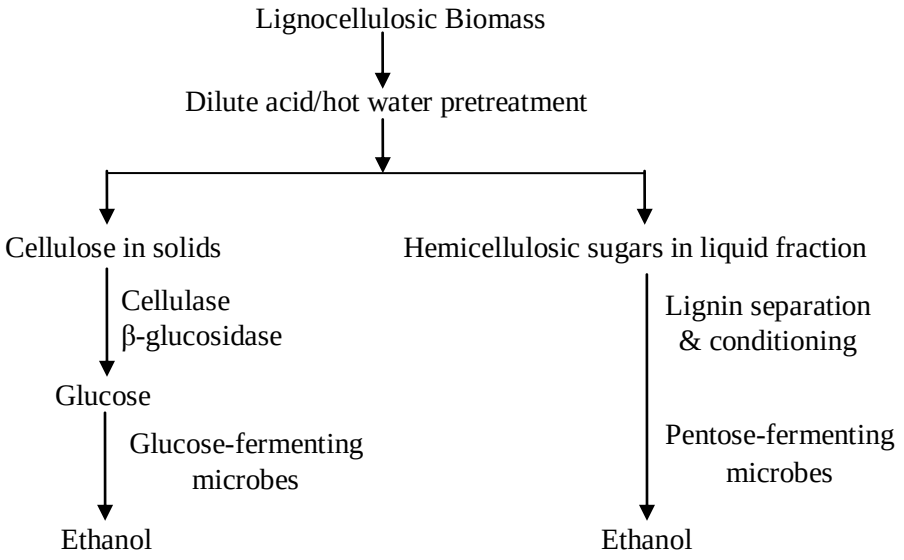

Fig. 1.4. Flow diagram of separate pentose and hexose fermentation

4.5.2. Simultaneous saccharification and co-fermentation (SSCF)

In alkaline pretreatment, nearly 100% of cellulose and most of hemicellulose remain in the solids. As described in section 1.3.3, in SSCF glucose and xylose are fermented to ethanol with the microorganism that is capable of converting both glucose and xylose. With

SSCF, hexose and pentose fermentation are carried out in the same vessel, as shown in Fig. 1.5. Recombinant *Escherichia coli* KO11 was considered to be the most promising ethanol producer from both glucose and xylose (Dumsday et al., 1999). However, hexoses are the preferred carbon source for recombinant *E. coli*; the problem of "glucose compression" or "xylose sparing", i.e., incomplete fermentation of xylose, has been found when hexoses dominate in the medium (Lindsay et al., 1995). Hexoses and pentoses can be utilized simultaneously be enthanologenic *E. coli*, but exercise mutual inhibition, with glucose exerting 15 times stronger inhibition on xylose fermentation than vice versa (Olsson and Hahn-Haegerdal, 1995).

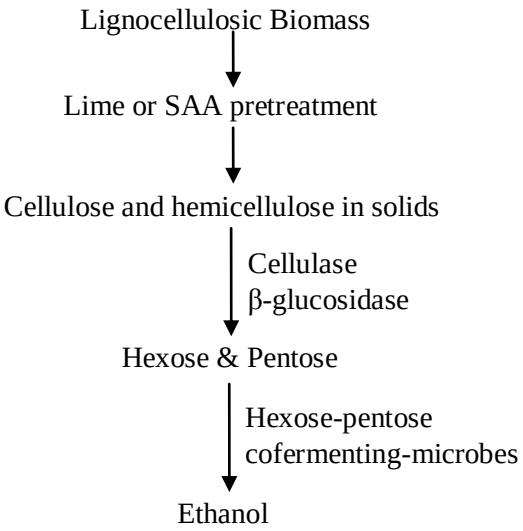

Fig. 1.5. Flow diagram of simultaneous saccharification and co-fermentation (SSCF)

4.5.3. Coupled isomerization and fermentation

It has been reported that yeasts are able to ferment xylulose to ethanol under anoxic conditions (Wang et al., 1980). Therefore, a coupled isomerization and fermentation process is proposed in which xylose is converted with exogenous, immobilized xylose isomerase (already commercially derived from bacteria) to an equilibrium mixture of xylose and

xylulose, which would be then be fermented to ethanol and the residual xylose recycled over the xylose isomerase (Fig. 1.6). The process would be continued until all xylose was consumed. Xylose isomerase could be incorporated directly into the fermentation vessel or the xylulose could be produced exogenously and separated from the xylose prior to fermentation (Jeffries, 1984).

Although sequential xylose isomerization and fermentation is technically feasible, it is impeded by several factors: the cost of the enzymatic isomerization, the formation of xylitol as a by-product, inhibition of xylose isomerase by xylitol, the use of separate optimal pile and temperatures for isomerization and fermentation, and the low rate of xylulose fermentation (Jeffries, 1984).

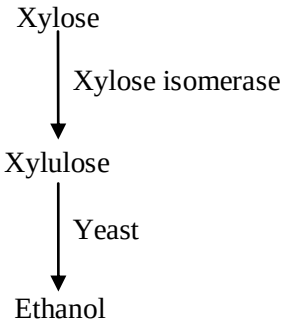

Fig. 1.6. Flow diagram of coupled isomerization and fermentation

5. Enzyme Recycling

Despite recent improvement in cellulase enzymes properties, the high cost associated with the hydrolysis step remains a major impediment to the commercialization of full-scale lignocellulose-to-ethanol bioconversion process (Tu et al., 2007). Strategies to reduce enzyme cost include increasing enzyme production efficiency, increasing enzyme specific activity and recycling cellulase enzymes to be used in subsequent hydrolysis (Cherry, 2003).

During hydrolysis, cellulase enzymes typically are present in the system in two different forms (Tu et al., 2007). Some cellulase enzymes remain free in the solution (free enzyme), while others are bound to the residual solids (both cellulose and lignin). An efficient enzyme recycling requires effective recovery of both free and adsorbed cellulase (Tu et al., 2007). Previous studies have demonstrated that free enzyme could be recovered potentially by membrane filtration (Tjerneld, 1994) and affinity re-adsorption with fresh substrates (Tu et al., 2007). However, there is a lack of effective strategy to recover bound enzyme after lignocellulose hydrolysis (Deshpande and Eriksson, 1984). This is primarily due to the non-productive binding between enzyme and residual lignin (Tu et al., 2007).

6. Overcoming the challenges to lignocellulosic ethanol

6.1. Challenges with current lignocellulosic ethanol production technologies

Presently, the major challenges in lignocellulose-to-ethanol processes include: (1) the currently available pretreatment technologies are chemical and energy intensive (Brown, 2006; Galbe et al.,. 2007; Wooley et al., 1999; Yang and Wyman, 2008); (2) the consumption and cost of enzymes associated with the hydrolysis step is high (Tu et al., 2009); (3) fermentation of pentoses is restricted on real substrates (Mohagheghi et al., 2002); (4) the concentration of ethanol in final broth is low, contributing to high energy demand in recovery process (Öhgren et al., 2006; Zacchi and Axelsson, 1989).

6.2. Efforts required to overcome the challenges from lignocellulose to ethanol

Future development of lignocellulose-to-ethanol processes should necessarily involve investigations on cost-effective pretreatment technologies, applications of biotechnology to industrial microorganisms and biocatalysts (enzymes) together with bioprocess engineering to integrate and optimize the production strategies.

6.2.1. Development of cost-effective pretreatment

Pretreatment has been viewed as one of the most expensive processing steps in cellulosic biomass-to-fermentable sugars conversion with costs as high as 30¢ per gallon ethanol produced (Mosier et al., 2005). There is great potential to improve the efficiency and lowering of the cost of pretreatment (Kohlman et al., 1995; Lee et al., 1994; Lynd et al., 1996; Mosier et al., 2003a, 2003b). The improvements in pretreatment technologies are based on better understanding of the chemistry of plant cell walls and the chemical reactions that occur during pretreatment (Eggeman and Elander, 2005). The future efforts on pretreatment research would be focused on the following areas: (1) developing pretreatment methods that are universally successful with multiple crops, sites, ages, and harvest times (Mosier, et al., 2005); (2) minimizing water and chemical consumption for pretreatment and post treatment (Mosier, et al., 2005; Zheng et al., 2009); (3) increasing total sugars yields (Wyman, 2006); (4) reducing the generation of inhibitors to eliminate hydrolysate conditioning (Hsu, 1996; Yang and Wyman, 2008); (5) minimizing energy requirements (Wyman, 2006).

6.2. 2. Improvement in enzymatic hydrolysis efficiency and economy

In addition to making more reactive solids through pretreatment, enzymes with better properties are needed to increase reaction rates and achieve high yields of fermentable sugars with much less enzymes, leading to cost reduction in enzymes (Wyman, 2007). Other representative strategies to reduce enzyme cost include increasing enzyme production efficiency and enzyme recycling (Cherry, 2003). Furthermore, the efficiency of enzymatic hydrolysis can also be enhanced by optimizing the composition of enzyme mixtures (cellulase mixtures for optimal synergy) and identifying the role of additional enzymes (hemicellulases, lignin modifying, etc.) (Viikari et al., 2006). Enzyme cocktails that can

effectively release the hemicellulose left in pretreated solids are also important for achieving the high yields needed for large-scale competitiveness (Wyman et al., 2005).

6.2.3. Increase of overall yield and final concentration of ethanol

The best opportunity for reducing the cost of cellulosic ethanol is, by far, through enhancing sugar yields from cellulose and hemicellulose (Öhgren et al., 2006). Advances in metabolic and genetic engineering have led to the development of microorganisms capable of efficiently converting biomass sugars into ethanol. Generally, such development relies on broadening the substrate range to include other biomass sugars such as arabinose or xylose in strains that cannot ferment sugars other than glucose (das Neves et al., 2007). On the other hand, the utilization of multiples sugars derived from lignocellulosic biomass can be achieved with appropriate fermentation strategy via single or multiple microorganisms. Further optimization of these strategies is necessary.

Increasing the ethanol concentration in the feed to the distillation reduces the production costs considerably (Wingren et al., 2003). To lower the cost of ethanol distillation of fermentation broths, a high initial substrate concentration is desirable. However, an increase in the substrate concentration typically reduces the ethanol yield owing to insufficient mass and heat transfer (Um and Hanley, 2008). Therefore, the high-solid fermentation process requires further development and optimization, or an alternative strategy should be necessarily introduced.

6.3. Research topics of this thesis

This work in particular aims at efficient conversion of corn stove-derived pentoses and hexoses for ethanol production via process development/integration and optimization, efficient utilization of enzymes in bioconversion process and increase of ethanol

concentration in the final broth, and effective pretreatment with reduced energy, water and chemical input.

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CHAPTER 2. BIOETHANOL PRODUCTION FROM CORN STOVER USING AQUEOUS AMMONIA PRETEATMENT AND TWO-PHASE SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (TPSSF)

Modified from a paper published in *Bioresource Technology*

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Abstract

An integrated bioconversion process was developed to convert corn-stover derived pentose and hexose to ethanol effectively. In this study, corn stover was pretreated by soaking in aqueous ammonia (SAA), which retained glucan (~100%) and xylan (>80%) in the solids. The pretreated carbohydrates-rich corn stover was converted to ethanol via two-phase simultaneous saccharification and fermentation (TPSSF). This single-reactor process employed sequential simultaneous saccharification and fermentation (SSF), i.e., pentose conversion using recombinant *Escherichia coli* KO11 in the first phase, followed by hexose conversion with *Saccharomyces cerevisiae* D_5A in the second phase. In the first phase, 88% of xylan digestibility was achieved through the synergistic action of xylanase and endo-glucanase with minimal glucan hydrolysis (10.5%). Overall, the TPSSF using 12-h SAA-treated corn stover resulted in the highest ethanol concentration (22.3 g/L), which was equivalent to 84% of the theoretical ethanol yield based on the total carbohydrates (glucan+xylan) in the untreated corn stover.

Key words: integrated bioconversion process, bioethanol, soaking in aqueous ammonia (SAA), pretreatment, KO11, D_5A yeast.

1. Introduction

Due to the foreseeable depletion of fossil fuels, ethanol, which can be obtained via the bioconversion of renewable feedstocks, is widely regarded as an efficient alternative for gasoline as transportation fuel. Currently, corn is the primary feedstock for commercial fuel ethanol production. The quantity of ethanol that can be produced from corn is limited (maximum 15-20 billion gallons) (NCGA, 2006) and therefore we need another feedstock like biomass that can be used to produce much more ethanol. It has been suggested that the most promising alternative for the traditional starch feedstock is lignocellulosic biomass considering its abundance, low cost, and high polysaccharides (cellulose and hemicellulose) contents (Fujii et al., 2009).

The production of bioethanol from lignocellulosic feedstock at commercial scale is much more difficult compared with the starch-based ethanol production, both technologically and economically, due to the heterogeneous nature and the rigid structure of lignocellulose, which is attributed to the inter-association of its three major components, cellulose, hemicellulose, and lignin. Lignocellulose contains five major sugar units of two categories; the hexoses include glucose, mannose, and galactose, and the pentoses include xylose and arabinose. Among them, xylose, the major unit of hemicellulose, is the second most abundant sugar in corn stover following glucose. Commercial bioconversion of lignocellulose to ethanol requires efficient fermentation of the sugar mixtures, which contain xylose as a major component (Hinman et al., 1989). If this cannot be achieved, the yield will be too low to make the lignocellulosic ethanol cost-competitive. Currently, co-fermentation of hexose and pentose with recombinant microorganisms is considered to be one of the most promising methods for lignocellulosic ethanol production. There have been multiple efforts to develop these recombinant microorganisms to allow co-fermentation of both pentose and hexose

sugars (Deanda et al., 1996; Dien et al., 1997; Eliasson et al., 2000; Kötter and Ciriacy, 1993; Moniruzzaman et al., 1997; Zhang et al., 1995). One of the major problems of these recombinant organisms is they ferment glucose preferentially and do not begin to metabolize xylose until low glucose concentrations have been reached. The result normally is incomplete conversion of xylose in mixed sugar solutions obtained from lignocellulosic biomass. To overcome this hurdle, we have developed a two-phase process in which xylose is preferentially released from a pretreated biomass and simultaneously fermented to ethanol first using an organism capable of highly efficient xylose metabolism, followed by release of glucose and its simultaneous conversion to ethanol by the yeast *Saccharomyces cerevisiae*, which has been known for high ethanol yields.

In this study, corn stover was pretreated by soaking in aqueous ammonia (SAA), which has been known for high retention of both glucan (~100%) and xylan in the solids (>80%) (Kim and Lee, 2005; Kim and Lee, 2007). High retention of these carbohydrate fractions will help to increase the overall ethanol yield and simplify the bioconversion process (Kim and Lee, 2005; Kim and Lee, 2007). The pretreated corn stover was subsequently used in a novel bioconversion process, which we termed two-phase simultaneous saccharification and fermentation (TPSSF). Fig. 2.1 shows the schematic diagram of TPSSF. This process is unique in that it uses a single fermentor for sequential simultaneous saccharification and fermentation (SSF) of xylan and glucan to produce ethanol at high yields. In the first phase, xylanase and endo-glucanase are used to produce xylose and small quantities of glucose, which subsequently are fermented to ethanol by the recombinant organism *Escherichia coli* KO11. In the second phase, cellulase and β-glucosidase are used to produce glucose, which then is fermented to ethanol by the yeast *Saccharomyces*

cerevisiae D_5A. *E coli* KO11 has been known to be highly effective in converting xylose to ethanol whereas *S. cerevisiae* can ferment glucose to ethanol at very high yields.

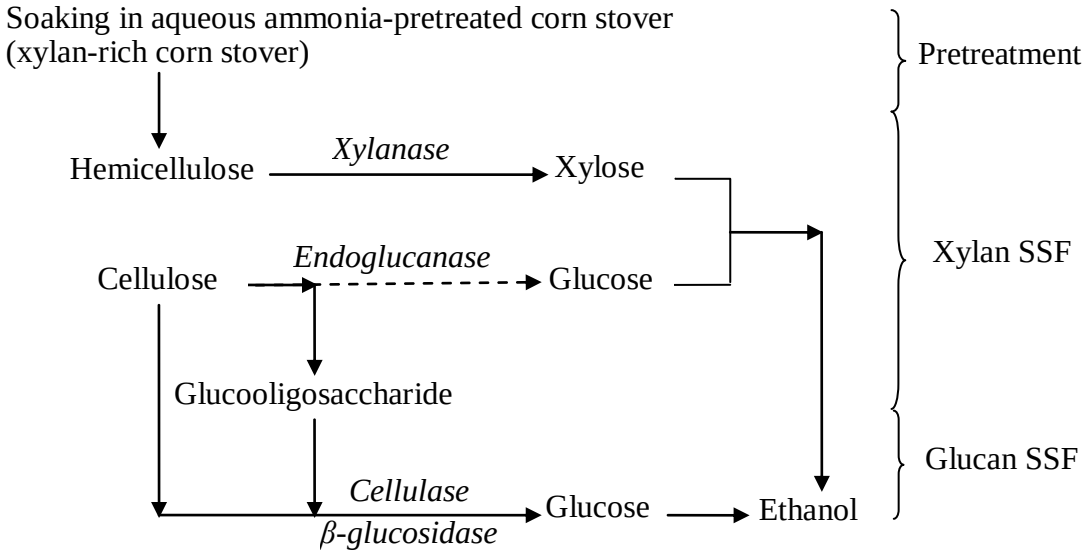


Fig. 2.1. Schematic diagram of Two-Phase Simultaneous Saccharification and Fermentation (TPSSF)

The primary objective of this study was to develop and optimize an efficient bioconversion process to convert pentose and hexose sugars derived from corn stover to ethanol, using multiple enzymes and two industrially viable microorganisms to achieve high ethanol concentration and yield. This study provided a solution to help overcome the hurdles that limit the efficiency of the biological production of fuel ethanol from agricultural residues. The effects of various enzymes on the digestibility of SAA-treated corn stover also were investigated.

2. Materials and Methods

2.1. Materials

2.1.1. Feedstock

Two batches of corn stover were used in this work. The first batch was corn stover locally harvested and the other was provided by National Renewable Energy Laboratory (NREL, Golden, CO). Both corn stover were ground and screened in our laboratory. The fractions between 10 and 35 mesh (0.5 mm and 2.0 mm) were collected and air dried at room temperature (~25˚C). The compositions of the local corn stover and NREL corn stover were determined by our lab and NREL, respectively, following the standard method developed by NREL (NREL, 2005a, 2005b, 2008a, 2008c). The compositions were glucan 38.2%, xylan 21.0%, arabinan 2.7%, galactan 2.1%, lignin 17.4%, extractives 13.3%, and ash 5.3%. The composition of the corn stover provided by NREL was given as glucan 34.2%, xylan 22.3%, arabinan 3.1%, galactan 1.6%, lignin 12.2%, sucrose 6.2%, acetate 3.9%, uronic acid 4.0%, and protein 1.6%.

2.1.2. Enzymes

Cellulase GC-220 (Lot. # 301-04232-162) and Multifect xylanase (Lot. # 301-04021-015) were generously provided by the Genencor, a Danisco Division (Rochester, NY); the average activities of these enzymes were 45 FPU (filter paper unit)/mL and 8000 GXU (Genencor xylanase unit)/ml, respectively. Endoglucanase was purchased from Sigma-Aldrich (St. Louis, MO) (Sigma Cat. # 068K1214) and the activity of enzyme 1, 000 units/g-solids (isolated endoglucanase activity). Novozyme 188, a β-glucosidase, (Sigma Cat. # C-6150, Lot. # 11K1088) also was purchased from Sigma-Aldrich and the activity was 750 CBU (cellobiase unit)/mL.

2.1.3. Microorganisms

Recombinant *E. coli* KO11 (ATCC® 55124) and *Saccharomyces cerevisiae* D_5A (ATCC® 200062) were obtained from the American Type Culture Collection (location). *E.*

coli KO11 was maintained on LB solid medium (Luria-Bertani Medium (Sigma, Cat. # L-3152); 5 g/L yeast extract, 10 g/L Tryptone, and 5 g/L NaCl; agar (Sigma Cat. # B0128234): 15 g/L), supplemented with 2 g/L dextrose (Fisher Cat. # D16) and 40 mg/L chloramphenicol (Sigma Cat. # C-0378). Dextrose and chloramphenicol were added after autoclaving. The culture was transferred monthly. *S. cerevisiae* D_5A was maintained on YPD solid medium, which contained 2% Bacto yeast extract (Lot. # 6215213), 1% Bacto peptone (Lot. # 6352003), 2% dextrose, and 1.5% agar. Transfer of the yeast culture also was performed monthly. To prepare the plates, the media were autoclaved at 121˚C for 15 min, allowed to cool to about 50˚C, poured onto the plates, and allowed to solidify. The plates were kept refrigerated at 4˚C.

2.2. Methods

2.2.1. Pretreatment

Corn stover was pretreated by soaking in aqueous ammonia (SAA) using 15% (w/w) ammonia solution (diluted from 29.54% ammonia hydroxide-certified ACS plus, Fisher Cat. # A669C) and a solid-to-liquid ratio of 1:11. The pretreatment was performed in screw-capped Pyrex solution bottles (250 ml) at 60˚C without agitation for 8h, 12h, and 24h. These conditions were chosen based on the results obtained previously for SAA pretreatment of corn stover (Kim and Lee, 2005; Kim and Lee, 2007). The pretreated corn stover was washed with deionized (DI) water using vacuum filter and fluted filter paper (Fisher Cat. # 09-790-14F) until the wash water had a neutral pH. The washed solids were collected for composition analysis and enzyme digestibility and fermentation tests.

2.2.2. Enzyme digestibility test

The enzyme digestibility tests of xylan in SAA-pretreated corn stover were performed in duplicates following the procedure of the NREL Laboratory Analytical Procedure (LAP) (NREL, 2008b). The tests were performed at 1% (w/v) initial glucan loading in 0.05 M citrate buffer (pH=4.8) supplemented with tetracycline at 40 mg/L and cyclohexamide at 30 mg/L using 250 ml screw-capped Erlenmeyer flasks. The flasks were incubated at 50˚C and 150 rpm in the incubator shaker (Excella E24, New Brunswick Scientific, Edison, NJ).

Xylan digestibility was determined at two xylanase loadings of 8000 GXU/g glucan and 1600 GXU/g glucan without and with endo-glucanase supplemented at 10 and 50 units/g glucan. Endoglucanase, substrate, and enzyme blanks were run in parallel as controls. The xylan digestibility (%) was calculated by ((total released xylose monomer at 72 h × 0.88)/total xylan in untreated corn stover) × 100). Similarly, the glucan digestibility was calculated by ((total released glucose x 0.9)/total glucan in treated corn stover) × 100) (where, 0.9 and 0.88 are the conversion factors of glucose-to-glucan and xylose-to-xylan, respectively).

2.2.3. Two-Phase Simultaneous Saccharification and Fermentation (TPSSF)

Inoculum preparation: The *E. coli* KO11 culture was first transferred to a new LBD agar plate without antibiotics, cultivating for 24 h at 37˚C. Single colonies were selected to inoculate 50 mL sterile LB supplemented with 20 g/L glucose in 250 mL flasks. The medium contained no antibiotics and was previously sterilized by autoclaving at 121˚C for 15 min. The inoculated flasks were incubated at 37˚C and 150 rpm in the incubator shaker for 10-14 hours (NREL, 2001). When the glucose concentration reached below 2 g/L, the cells were harvested by centrifugation (IEC MODEL HN-S Centrifuge) at 2,000 rpm (605 g-force) for 5 min. The supernatant was removed, and the cell pellets were re-suspended in 5 ml of DI

water. The cells harvested from two flasks were combined (10 ml) and used as the inoculum for the experimental flasks. Similar procedure was used to prepare the inoculum for *S. cerevisiae* D_5A except that sterile YP medium supplemented with 50 g/L glucose was used.

TPSSF experiments: All TPSSF experiments were performed using 100 mL LB medium in 250-mL flasks capped with rubber stoppers perforated with syringe needle for CO_2 venting and incubated at 37˚C and 150 rpm. The initial substrate loading was 3% w/v glucan (approximately 6% w/v pretreated biomass). Chloramphenicol also was added to the flasks at 40 mg/L. Initial pH of the first phase and the second phase SSFs were ~7.0 and ~5.0, respectively. The pH was not controlled in these experiments. TPSSF experiments were performed with *E. coli* KO11 alone (1^{st} phase only) and with *E. coli* KO11 plus *S. cerevisiae* D_5A (both 1^{st} and 2^{nd} phase). In all experiments xylanase was added at 8,000 GXU/g glucan and endo-glucanase added at 50 units/g glucan. The flasks were inoculated with the *E. coli* KO11 recovered from two inoculum flaks as described previously. In the flasks that used both *E. coli* KO11 and *S. cerevisiae* D_5A the yeast inocula obtained from two inoculum flasks as described previously also were added at 0 h and 48 h respectively. At 48 h, cellulase and β-glucosidase were added at 15 FPU/g glucan and 30 CBU/g glucan, respectively. All experiments were run for 120 h. Samples were taken at intervals for analysis.

Growth and fermentability of *S. cerevisiae* D_5A in LB medium: Since the *S. cerevisiae* D_5A culture was maintained on YP medium but the TPSSF experiments used the LB medium experiments also were performed to test the growth and fermentability of the yeast in the latter. In these experiments single colonies of *S. cerevisiae* D_5A from an YP agar plate were used to inoculate 100 mL of LB and YP, both supplemented with 30 g/L glucose,

in 250 mL flasks. The flasks were incubated at 37˚C and 150 rpm and the experiments were run for 48 h. Final samples were taken and analyzed for glucose and ethanol.

2.2.4. Analysis

The enzyme digestibility and ethanol fermentation samples were analyzed for individual sugars and ethanol by high performance liquid chromatography (HPLC) using refractive index (RI) detectors (Varian ProStar 355 and 356 RI detectors, Varian Inc, Palo Alto, CA) and Bio-Rad Aminex HPX-87P and HPX-87H columns (Hercules, CA), which were operated on two separate HPLC systems. HPLC analysis conditions were (1) HPX-87P columns: 85˚C of column temperature, 0.6 ml of DI water/min, and 60˚C of column temperature, (2) HPX-87H columns: 0.6 ml of 0.005 M H_2SO_4/min.

The treatment method for enzyme digestibility and fermentation samples was developed in our laboratory. The enzyme digestibility and fermentation samples were treated at 95-100˚C for 5 min and 85˚C for 45 min respectively in water bath to precipitate enzyme protein in the samples prior to HPLC analysis to prevent from clogging problems. Screw caped microcentrifuge tube with silicon O-ring (Fisher Cat. # 02-707-352) was used. The treated samples were centrifuged at 14,000 rpm for 15 min to remove to precipitated protein. The supernatant obtained after centrifuge was filtered with 0.2 μm sterile filters into HPLC vials and analyzed for the sugars using Aminex HPX-87P column and ethanol using Aminex HPX-87H column.

2.2.5. Ethanol yield calculation

The ethanol yields were calculated as follows:

$$\text{Ethanol yield } (Y_E)\ [\%] = \frac{\text{Ethanol produced [g] in reactor}}{\text{Initial sugars in reactor [g]} \times 0.511} \times 100\%$$

Note. Sugar is interpreted as glucose plus xylose in the SSF work.

2.2.6. Scanning Electron Microscopy (SEM)

A JEOL 5800LV Scanning Electron Microscope (JEOL Inc., Tokyo, Japan) was used to take images of the corn stover samples generated from each stage of the integrated bioconversion process. The samples were air-dried and mounted on aluminum sample stubs. The mounted samples were then placed in the chamber of the sputter coater and coated with a thin layer of gold before SEM examination.

3. Results and Discussion

3.1. Soaking in aqueous ammonia (SAA) pretreatment

The compositions of untreated and SAA-pretreated corn stover are summarized in Table 2.1. Fig. 2.2 summarizes the sugar retentions of SAA-pretreated corn stover. The 8 h, 12 h, and 24 h SAA pretreatment resulted in 99%, 98%, and 98% of glucan retention, respectively. In the case of xylan, the retention were 90%, 86%, and 80% of xylan, respectively. These results confirmed previous observation that SAA pretreatment is an efficient method for glucan and xylan retention. The SAA retains the hemicellulose in the solids by minimizing the interaction of ammonia with hemicellulose during treatment (Kim et al., 2008) at moderate temperatures and without agitation. The fact that virtually all of glucan and over 80% of xylan remained in the solids after SAA pretreatment provides the opportunity for increasing the ethanol yield from the available fermentable sugars and simplifies the bioconversion process (Kim and Lee 2005; Kim and Lee 2007). Moreover, the mild reaction conditions of SAA pretreatment prevents the formation of various toxic compounds, e.g., furfural and hydroxymethyl furfural (HMF) (Kim, et al., 2008; Kim and Lee, 2007), which are generated from the decomposition of sugars in most other

pretreatments involving harsh reaction conditions (Dunlop, 1948; Mills et al., 2009; Ulbricht et al., 1984).

Table 2.1 Composition of untreated and SAA-pretreated corn stover

Pretreatment conditions	Glucan [%]	Xylan [%]	Arabinan [%]	Galactan [%]	Lignin [%]
untreated	38.3	21.0	2.7	2.1	17.4
8h SAA-treated	37.9	18.9	2.3	1.7	9.6
12h SAA-treated	37.6	18.1	2.2	1.7	7.3
24h SAA-treated	37.5	16.8	2.2	1.3	6.8

Pretreatment conditions: 15 wt. % aqueous ammonia, 1:10 solid: liquid ratio, 60˚C for 8 h, 12 h, and 24 h.

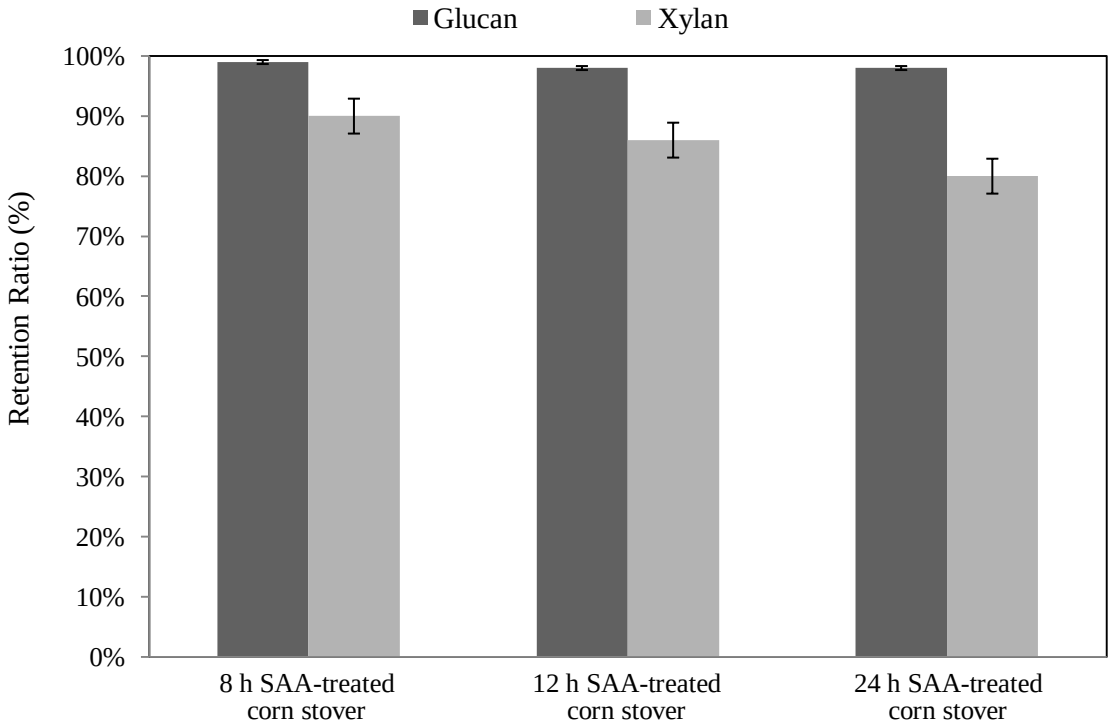


Fig. 2.2. Glucan and xylan retentions after SAA pretreatment. Pretreatment conditions: 15 wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C for 8 h, 12 h, and 24 h. (n=2)

3.2. Effects of xylanase and endoglucanase on hemicellulose hydrolysis

In order to investigate the effect of xylanase on hemicellulose hydrolysis, the enzyme digestibility tests of 24 h SAA-pretreated corn stover were performed using xylanase

supplemented with endoglucanase and the effects of endoglucanase on hemicellulose hydrolysis were evaluated (Fig. 2.3). The enzyme digestibility tests of 24 h SAA-pretreated corn stover with xylanase alone were performed in parallel. The hydrolysis of 24 h SAA-treated corn stover at enzyme levels of 8,000 and 16,000 GXU (1 and 2 ml) xylanase with no endoglucanase at initial solid loading of 1% glucan resulted in 3.8 g/L and 4.4 g/L of xylose respectively in saccharification reactor at 96 h, correspondingly with 0.25 g/L and 0.30 g/L of glucose (data with 16,000 GXU are not shown in Fig. 2.3(a)). When the xylanase dosage was doubled, xylose yield was only increased from 58.1% to 65.7%/54.5% to 63.1% based on the xylan content in pretreated/untreated corn stover. Considering the cost of enzymes, doubling the xylanase loading for the improvement of xylan digestibility probably would not be economically justifiable. In Fig. 2.3, the xylanase loading was fixed at 8,000 GXU/g-glucan, while the dosage of endoglucanase varied. As shown in Fig. 2.3(a), the endoglucanase loadings of 10 and 50 units (0.01 and 0.05 g) /g glucan resulted in 5.5 g/L and 5.8 g/L of xylose in the hydrolysate, respectively, compared to 3.8 g/L when endoglucanase was not added. Fig. 2.3(b) indicates that with the addition of 50 units/g glucan of endoglucanase, the xylan digestibility was increased from 58% to 88%/55% to 83% based on the xylan content in pretreated/untreated corn stover. Glucan hydrolysis was maintained at low levels (2-10%) in these cases.

Therefore, it was proven that the efficient hemicellulose hydrolysis with minimal cellulose hydrolysis is a feasible concept with the synergistic action of xylanase and endoglucanase. The effective enzymatic hydrolysis of hemicellulose in SAA-pretreated corn stover ensures the efficiency of xylan conversion in the first phase SSF. Moreover, it has been reported that the removal of xylan enhances cellulose digestion by reducing the xylan

coating and linkages to cellulose (Allen et al., 2001; Ishizawa et al., 2007). Therefore, the glucan SSF in the second phase conversion can be facilitated as well.

3.3. Two-Phase Simultaneous Saccharification and fermentation (TPSSF)

The effect of reaction time of SAA pretreatment on the two-phase SSF was studied so as to determine the most appropriate substrate for the two-phase SSF. The SAA-treated corn stover (15 wt. % aqueous ammonia solutions, 1:11 solid-to-liquid ratio, 60˚C, for 8, 12 and 24 h) was subject to the TPSSF with recombinant *E. coli* KO11. Fig. 2.4 indicates the ethanol concentrations resulted from the 8 h, 12 h, and 24 h SAA-pretreated corn stover. With other fermentation conditions being consistent, the 12 h and 24 h SAA-pretreated corn stover gave almost equal final ethanol concentrations (20.8 g/L and 20.5 g/L, respectively), while the 8 h SAA-pretreated corn stover gave lower ethanol concentration 16.6 g/L. Considering the pretreatment severity and fermentation results, the 12 h SAA-pretreated corn stover was determined to be the optimal substrate for TPSSF in terms of sufficient pretreatment effect and high ethanol fermentation yield. Fig. 2.5(a) presents the ethanol and sugar concentration profiles of the two-phase fermentation with 12 h SAA-treated corn stover as the substrate. With the initial substrate loading of 3% w/v glucan, after the second phase fermentation, the ethanol concentration reached 20.8 g/L, which was 78% theoretical yield based on total glucan plus xylan in the untreated corn stover.

In the previous study, simultaneous saccharification and co-fermentation (SSCF) of SAA-treated corn stover using recombinant *E. coli* ATCC 55124 (KO11) was conducted and the final ethanol concentration 19.2 g/L (73% of theoretical maximum ethanol yield based on the total sugars in untreated corn stover) was reported (Kim and Lee, 2007). In that previous cofermentation of hexose and pentose study, *E. coli* KO11 was shown to consume the hexose

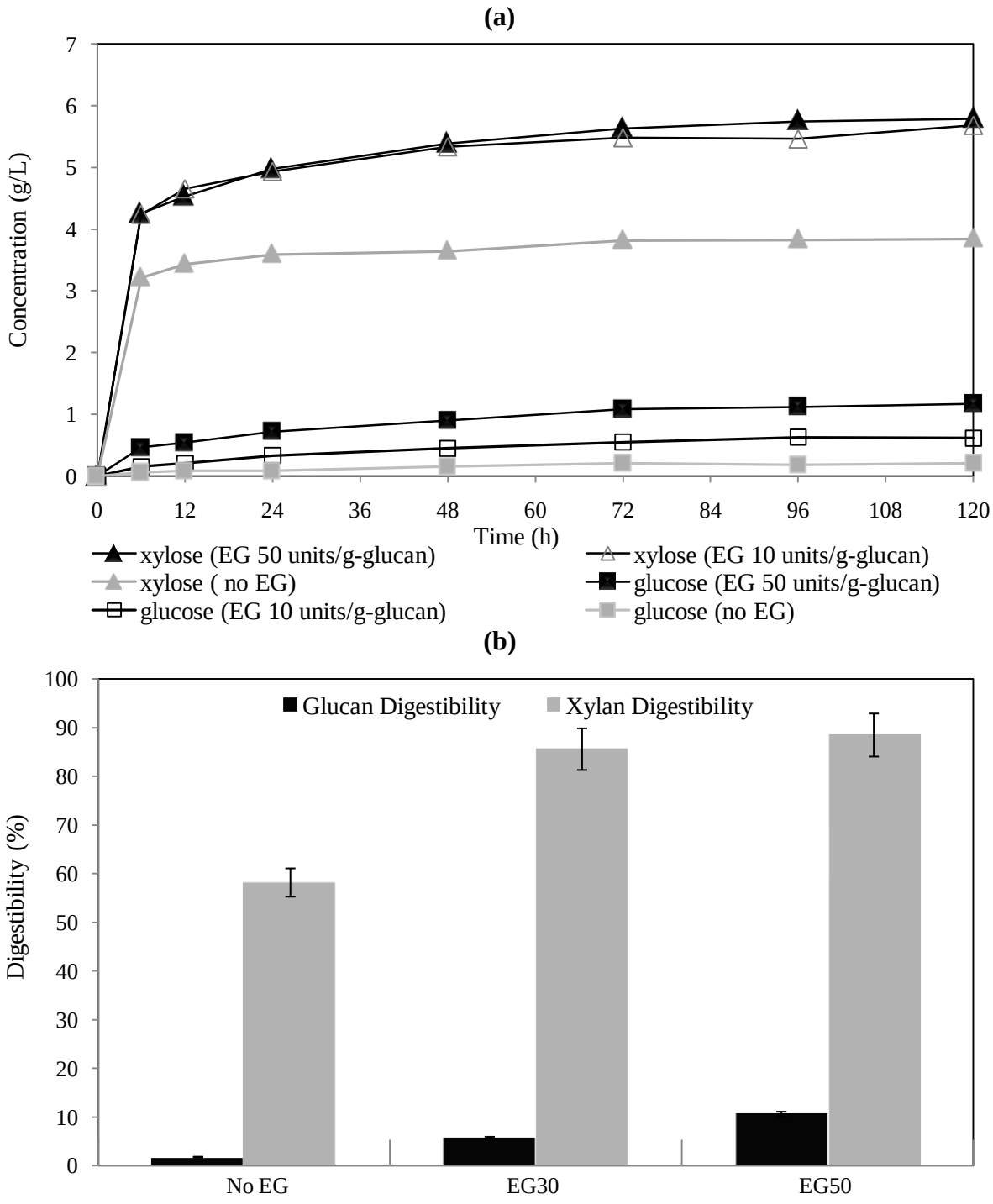


Fig. 2.3. Effects of xylanase and endoglucanase (EG) on xylan digestibility of SAA pretreated corn Stover. (a) Sugar concentrations of enzyme digestibility tests with various EG loadings; (b) 72-h digestibilities with various EG loadings. Pretreatment: 15wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C, 24 h. Enzymatic hydrolysis: 1% w/v glucan/100 mL working volume; endoglucanase 0, 10, 50 units/g-glucan; xylanase 8,000 GXU/g-glucan, 40˚C, pH 4.8 maintained by 0.05 M citrate buffer, 150 rpm. Note: XYN: Multifect Xylanase; EG: endo-glucanase. (n=2)

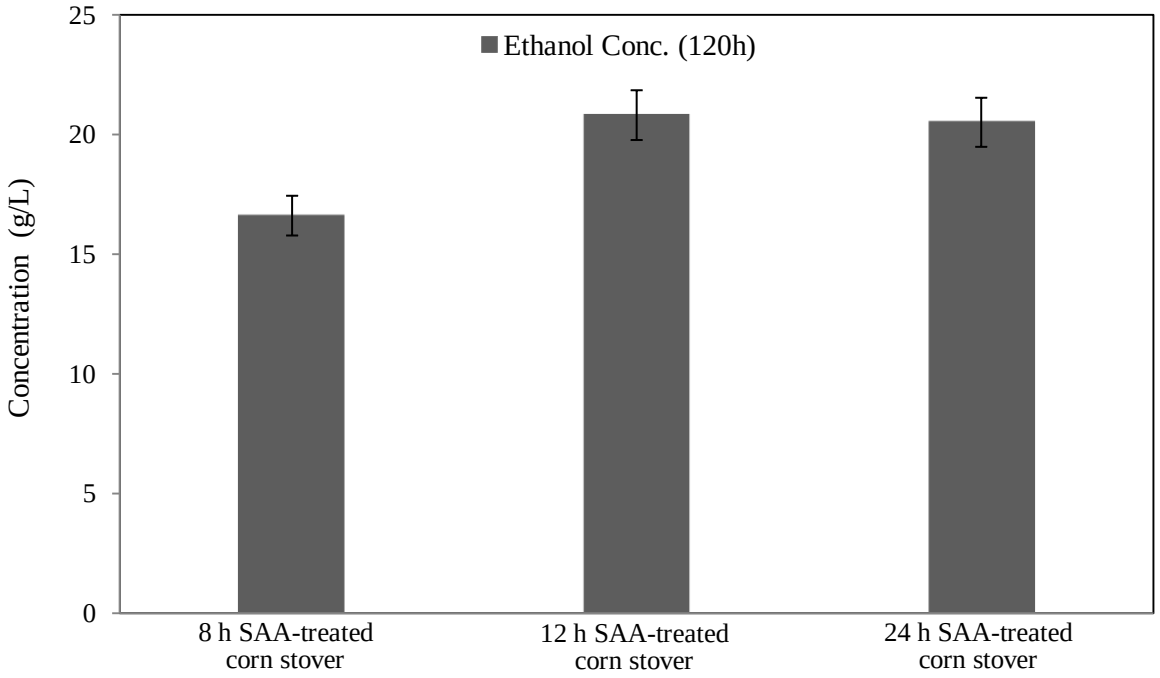


Fig. 2.4. Effects of SAA pretreatment reaction time on the Two-Phase Simultaneous Saccharification and Fermentation (TPSSF). Microorganism: *Escherichia coli* ATCC® 55124; substrate: 8 h, 12 h, 24 h SAA-treated corn stover, 3% w/v glucan/100 mL working volume; 1st phase: Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd phase: 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; LB medium, anaerobic condition; 37 ℃, 150 rpm. (n=2)

preferably to pentose. It is speculated that the higher ethanol yield obtained from the TPSSF of SAA-treated corn stover was resulted from the circumvention of glucose preference in co-fermentation, which was achieved by the sequential utilization of pentose and hexose.

3.4. TPSSF using *E. coli* KO11 and *S. cerevisiae* D_5A

In the two-phase SSF, LB medium was used for the growth of *E.coli* KO11 in the first phase and *S. cerevisiae* D_5A would take over the fermentation in the second phase. However, *S. cerevisiae* D_5A was maintained on YP medium supplemented with glucose. Therefore, it was necessary to test the growth and fermentability of *S. cerevisiae* D_5A on LBD medium. The ethanol production and glucose consumption of *S. cerevisiae* D_5A on YPD and LBD medium did not show significant difference (data are not shown), indicating that the growth

and fermentability of *S. cerevisiae* D_5A were not affected when it was transferred from YPD to LBD medium.

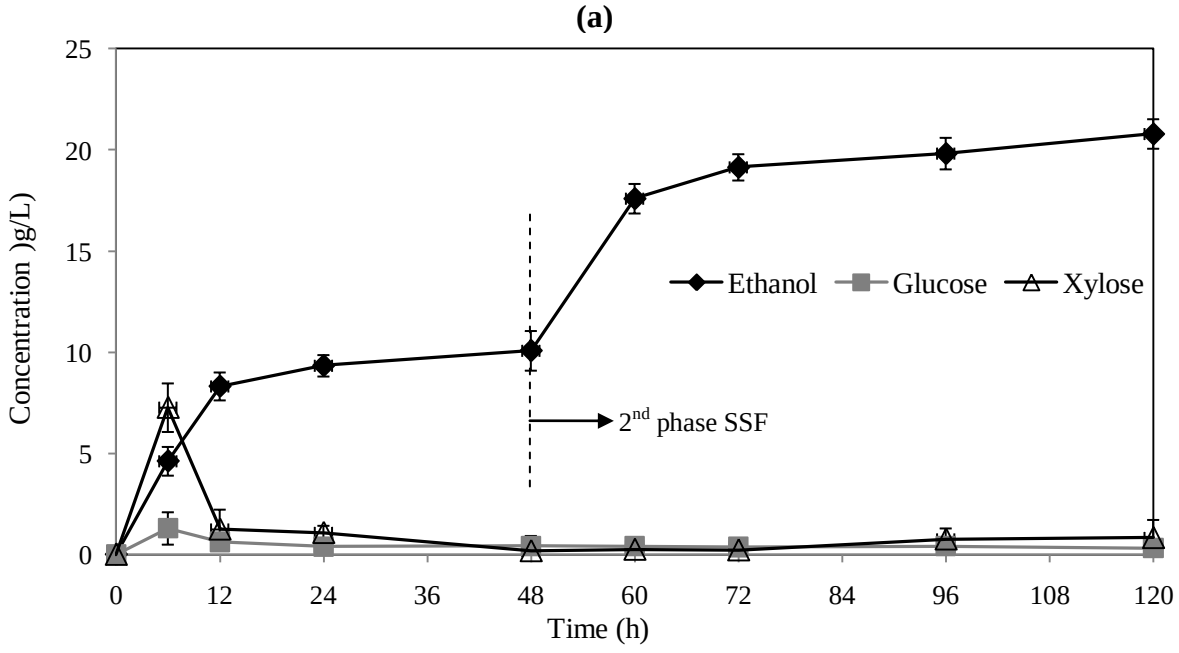
The results of the TPSSF with recombinant *E. coli* ATCC 55124 (KO11) for pentose conversion in the first phase and *S. cerevisiae* ATCC 20062 (D_5A) for the conversion of hexose in the second phase are shown in Fig. 2.5(b). The results show that in the TPSSF using both organisms 22.3 g/L ethanol was produced within 96 h of fermentation, which was equivalent to 84% of the theoretical maximum ethanol yield based on the total sugars (glucan+xylan) in untreated corn stover. The concentration profiles clearly show the sequential fermentation of xylose and glucose. In the early phase of TPSSF, xylose accumulated due to the lag phase of fermentation. As the microorganism propagated, the accumulated xylose was consumed. After 36 h, the ethanol concentration profile showed a plateau, indicating the completion of fermentation. At the end of the first-phase SSF (at 48 h), the ethanol concentration reached 11 g/L. In the second phase (48 h~120 h), there was no substantial increase in xylose concentration. The little increase in xylose concentration also indicated that most of the xylan in the pretreated solids had already been converted in the first phase SSF with xylanase, endoglucanase and *E.coli* KO11. During the first phase (pentose SSF) using *E. coli* KO11, pH started from 7.0 which was the optimum pH for the strain, and then decreased to pH ~5, which was near the optimum pH for *S. cerevisiae* D_5A strain in the hexose SSF. Therefore, another advantage of our bioconversion process is that additional pH adjustment is not required. After the second phase (hexose SSF), fermentation ended (at 96 h), the pH further decreased to 4.2.

The application of *S. cerevisiae* D_5A as the fermentative microorganism in the second phase hexose SSF shortens the fermentation using recombinant *E. coli* and ensures the high

efficient utilization of glucose for ethanol production. In addition, the excellent ethanol tolerance of *S. cerevisiae* D_5A (~12 wt. %) makes the fermentation not restricted by the ethanol tolerance of the recombinant *E. coli*. It is possible that ethanol concentration of 50-100 g/L could be achieved by way of TPSSF if the solid loading could be made sufficiently high to support that ethanol level, for example in a fed-bath fermentation. The overall mass balance of the integrated SAA pretreatment and TPSSF was presented in Fig. 2.6.

3.5. SEM

Changes in the structure of corn stover throughout the integrated two-phase bioconversion process were studied via scanning electron microscope (SEM) (Fig. 2.7). The image of the intact fibers of untreated corn stover shows that the structure of which is highly crystalline and rigid. After 12 SAA pretreatment, the crystalline structure of cellulose fibers is disrupted and the surface is modified in certain degree. In addition, the typical reticulate structure of xylan is observed indicating that xylan remains with the solids after pretreatment.



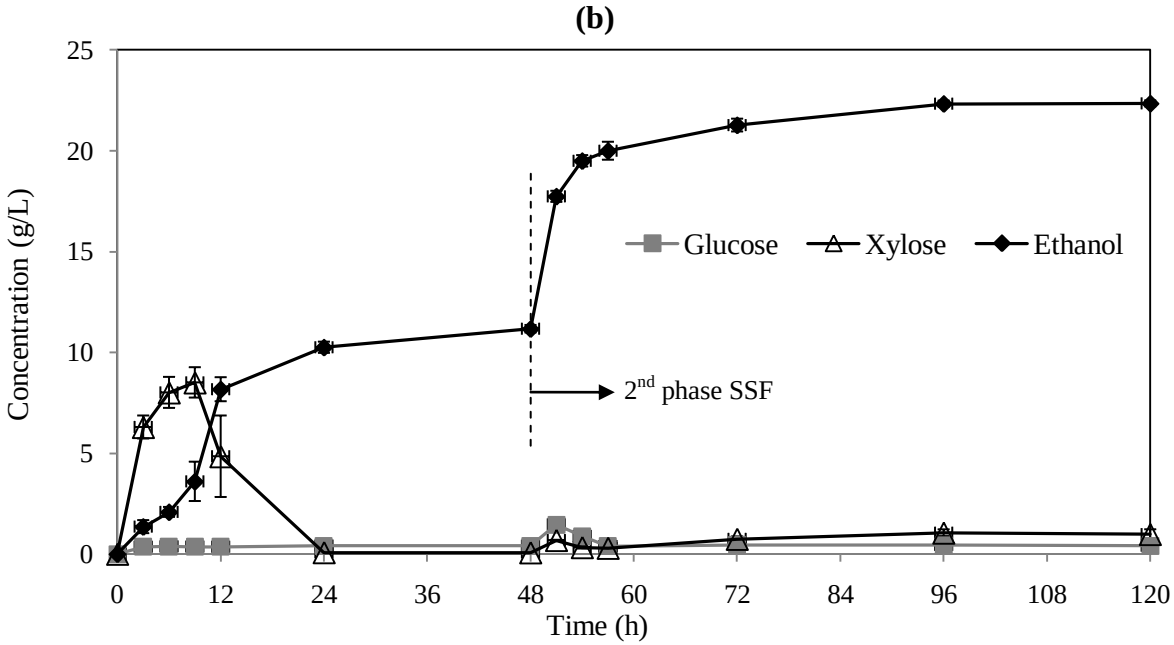


Fig. 2.5. Ethanol and sugar concentration profiles in two-phase simultaneous saccharification and fermentation (TPSSF)[1]. (a) TPSSF with KO11 using 12 h SAA-pretreated corn stover[2]; (b) TPSSF with KO11 and D_5A using 12 h SAA-pretreated corn stover[3]. 1. substrate: 12 h SAA-treated corn stover 3% w/v glucan/100 mL working volume; 1st phase: Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd phase: 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; LB medium, anaerobic condition; 37 ℃, 150 rpm. (n=2); 2. Microorganism: 1st and 2nd phase: *Escherichia coli* ATCC® 55124; 3. Microorganism: 1st phase: *Escherichia coli* ATCC® 55124; 2nd phase: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A).

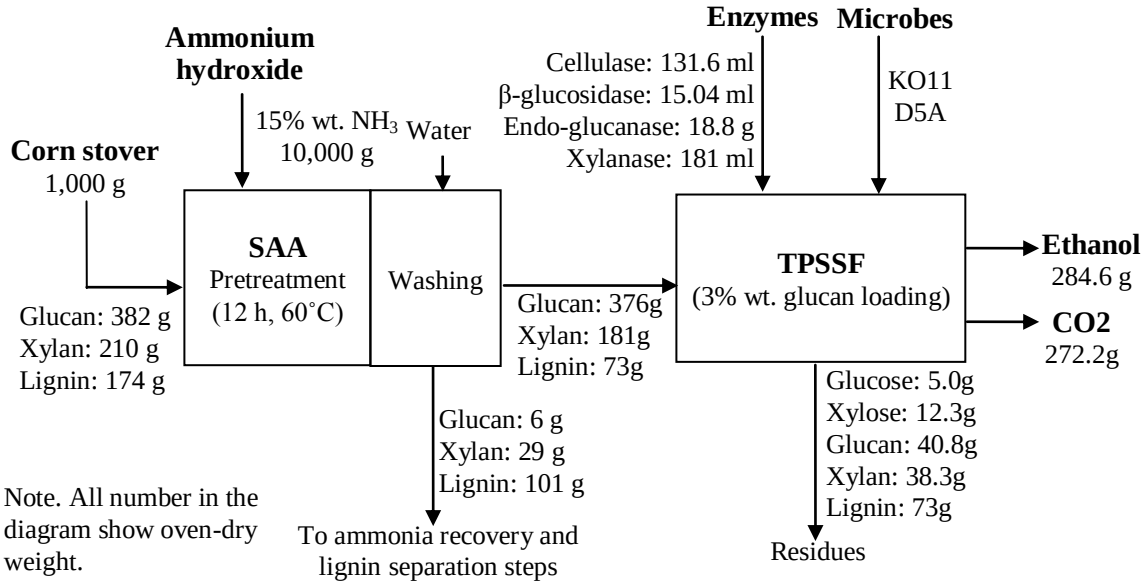


Fig. 2.6. Mass balance of the integrated bioconversion process using SAA pretreatment and Two-Phase Simultaneous Saccharification and Fermentation (TPSSF)

The image of the solid residues after the first phase SSF showed the further disrupted structure of corn stover and the breakages on the individual cellulose fibers which are resulted from the action of xylanase and endoglucanase. Finally, the surface of the corn stover residues after the second-phase SSF is shown to be strongly modified. It is speculated that these residues are mainly the most crystalline cellulose in the inner part of fiber bundles and lignin.

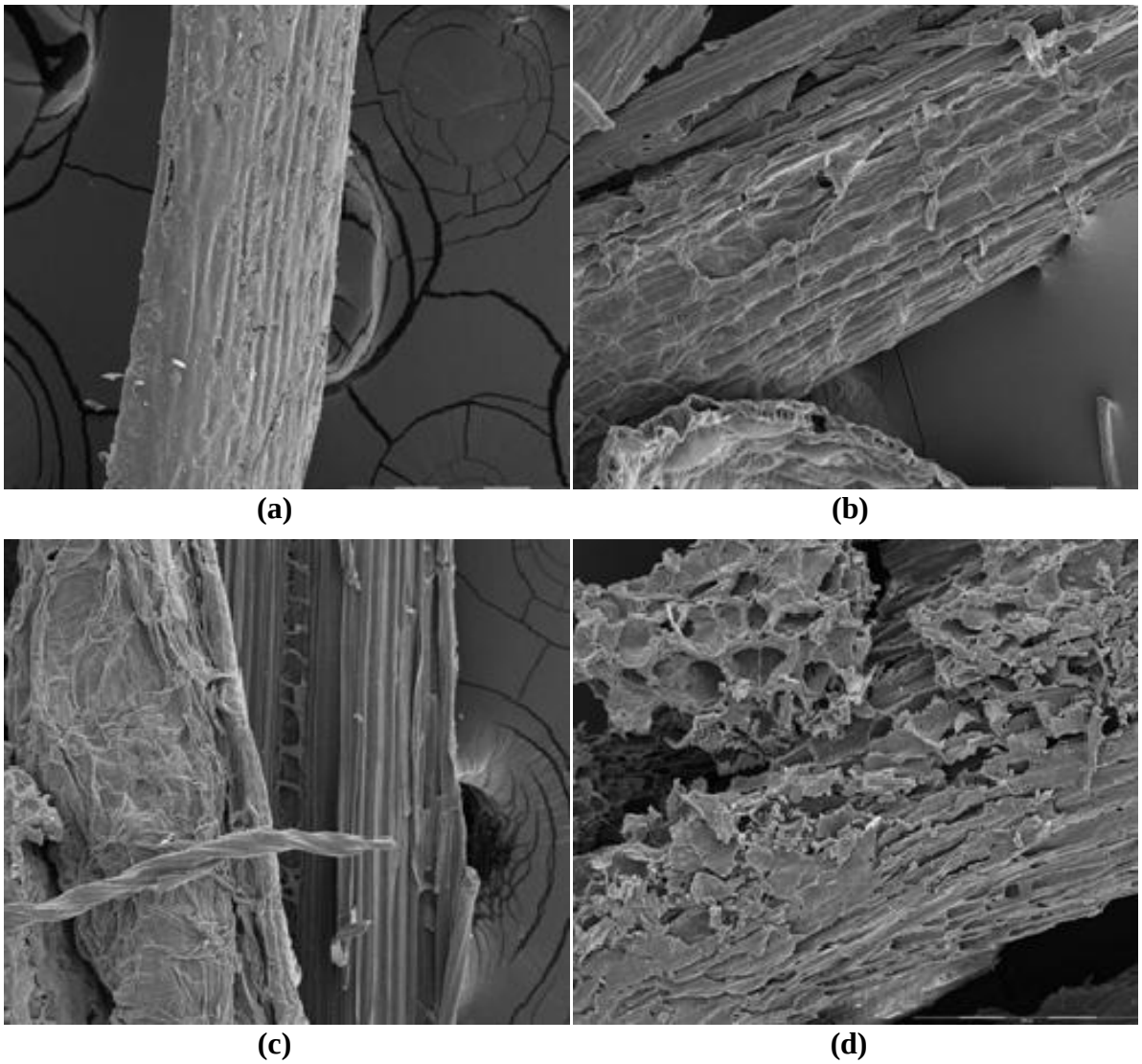


Fig. 2.7. Scanning electronic microscopy (SEM) of corn stover throughout the pretreatment and TPSSF. (a) Raw corn stover (×300); (b) 12h SAA-treated corn stover (×300); (c) Corn stover residue after 1st phase SSF (×300); (d) Corn stover residue after 2nd phase SSF (×300).

4. Conclusion

The integrated bioconversion process using SAA pretreatment and TPSSF can solve the problems with the conventional processes involving pentose utilization from three aspects: (1) pentose and hexose fermentation can be integrated into a single reactor using xylan-rich pretreated-corn stover; (2) the inhibition of glucose on xylose fermentation, which is always the case in conventional SSCF, can be circumvented by converting xylan prior to glucan; (3) the application of *S. cerevisiae* in the second phase ensures the stable and efficient utilization of glucose and overcomes the relatively low ethanol yield of genetically engineered strains.

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CHAPTER 3. BIOCONVERSION OF CORN STOVER DERIVED PENTOSE AND HEXOSE TO ETHANOL USING CASCADE SIMULTANEOUS SACCHARIFICATION AND FERMENTATION (CSSF)

Modified from a paper prepared for submission to *Bioresource Technology*

Xuan Li and Tae Hyun Kim

Abstract

In the previous study, two-phase simultaneous saccharification and fermentation (TPSSF) was developed. In this study, cascade type of TPSSF was studied to convert corn stover derived pentose and hexose to ethanol with minimal enzyme input. This bioconversion method, which we termed cascade simultaneous saccharification and fermentation (CSSF), produced up to 60% of the theoretical maximum ethanol yield based on the total sugars in untreated corn stover, while enzyme loadings were reduced by 50% (v/v). In detail, each step of CSSF utilizes two sequential simultaneous saccharification fermentation (SSF) phases operating on pentose and hexose, i.e., pentose conversion using xylanase, endo-glucanase, and recombinant *Escherichia coli* (KO11) with minimal glucose conversion in the first phase SSF and hexose conversion in the second phase SSF using cellulase, β-glucosidase, and the well-proven strain *Saccharomyces cerevisiae* (D_5A). In this cascade scheme, multiple stages of $1^{st}/2^{nd}$ phase SSF were performed in series; enzymes are recycled from the fermentation broth of the last stage for the use of the next stage.

Key words: cascade fermentation, enzyme recycling, bioethanol, soaking in aqueous ammonia (SAA), simultaneous saccharification and fermentation (SSF)

1. Introduction

Alternative energy resources have acquired great attention in recent years due to environmental, economic and geopolitical concerns over sustainability and a dwindling supply of fossil fuels in present days. Bioconversion of lignocellulosic feedstock to produce fuel-grade ethanol presents an attractive opportunity for the production of renewable, environmentally friendly biofuels (Farrell et al., 2006).

In lignocellulosic biomass, cellulose, hemicellulose and lignin form structures termed microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall (Rubin, 2008). Deriving fermentable sugars from lignocellulose requires both physical and chemical disruption. Enzymatic methods of saccharification are the most common (Mosier et al., 2005). Despite recent improvement in cellulase enzymes properties, the high cost associated with the hydrolysis step remains a major impediment to the commercialization of the full-scale lignocellulose-to-ethanol bioconversion process (Tu et al., 2009). Strategies to reduce enzyme cost include increasing enzyme production efficiency, increasing enzyme specific activity and recycling cellulase enzymes to be used in subsequent hydrolysis (Cherry, 2003). The enzymatic hydrolysis of lignocellulose could become a more economical process if the enzyme could be recovered from the reaction mixture in active form and reused several times (Woodward, 1989). Economic analysis shows that the cost savings gained by enzyme recycling are sensitive to enzyme pricing and load. At the demonstrated recovery of 60% and current loading of 15 Filter paper units (FPU) of cellulase/g of glucan, enzyme recycling is expected to generate a cost savings of approximately 15%. If recovery efficiency can be improved to 70%, the savings will increase over 25%, and at 90% recovery the savings will be 50% (Steele et al., 2005). Therefore,

enzyme recycling technology is an important aspect to develop a commercial process for bioconversion of lignocellulosic feedstock to ethanol.

Another factor that significantly influences the production cost of ethanol from lignocellulosic biomass is the concentration of ethanol in the fermentation broth prior to distillation (Öhgren et al., 2006). The ethanol concentration in the feed has a major effect on the energy demand, especially at concentrations below 4 wt. % (Zacchi and Axelsson, 1989). Increasing the ethanol concentration in the feed to the distillation reduces the production costs considerably (Wingren et al., 2003). To lower the cost of ethanol distillation of fermentation broths, a high initial substrate concentration is desired. However, an increase in the substrate concentration typically reduces the ethanol yield due to insufficient mass and heat transfer (Um and Hanley, 2008).

The cascade simultaneous saccharification and fermentation (CSSF) is devised on the basis of two-phase simultaneous saccharification and fermentation (TPSSF). As described in Chapter 3, TPSSF, this single-reactor process applied sequential simultaneous saccharification and fermentation (SSF), i.e., pentose conversion using recombinant *Escherichia coli* KO11 in the first phase, followed by hexose conversion with *Saccharomyces cerevisiae* D5A in the second phase. The major problem associated with TPSSF is high enzyme input for complete hydrolysis of cellulose and hemicellulose.

The CSSF was initially intended to: (1) to reduce the overall enzyme loading and (2) to increase the ethanol concentration in final fermentation broth. In CSSF, to reduce the enzyme loading, enzymes that have functioned are recycled by centrifugation and reutilized in the subsequent stage of fermentation. Moreover, a stage-wise strategy of ethanol accumulation is applied in CSSF to achieve high ethanol concentration with moderate initial

substrate concentration in the fermentation system to avoid the insufficient mass and heat transfer typically associate with high solid fermentation.

In detail, the CSSF scheme comprises sequential glucan and/or xylan SSF interconnected as a cascade. Fig. 3.1 is the most typical and simplest cascade scheme investigated in this study. As shown in the schematic, soaking in aqueous ammonia (SAA)-pretreated biomass is introduced into the 1st stage SSF, hydrolyzed with xylanase and endoglucanase, and fermented with KO11; after the 1st stage fermentation, the liquid fraction containing the recovered free enzymes and solid residues are separated with the liquid fraction going to the 2nd stage SSF and the solid residues being collected for further utilization; in the 2nd stage SSF, new biomass and the liquid fraction from the 1st stage are mixed with the addition of new inoculum of KO11; cellulase, β-glucosidase, and D_5A inoculum are fed into the whole broth following the 2nd stage SSF to start the 3rd stage SSF (glucan SSF); the liquid fraction of the 3rd stage SSF is recycled and mixed with the solid residues generated from the 1st stage SSF to start the 4th stage conversion.

2. Materials and Methods

2.1. Materials

2.1.1. Feedstock

Locally harvested corn stover was ground and screened. The fractions between 10 and 35 mesh (0.5 mm and 2.0 mm) were collected and air dried at room temperature (~25˚C). The composition of corn stover was determined following the standard method developed by NREL (NREL, 2005a, 2005b, 2008) to be: glucan 38.2%, xylan 21.0%, arabinan 2.7%, galactan 2.1%, lignin 17.4%, extractives 13.3%, and ash 5.3%.

2.1.2. Enzymes

Cellulase GC-220 (Lot. # 301-04232-162) and Multifect xylanase (Lot. # 301-04021-015) were generously provided by the Genencor, a Danisco Division (Rochester, NY); the average activities of these enzymes were 45 FPU (filter paper unit)/mL and 8000 GXU (Genencor xylanase unit)/ml, respectively. Endoglucanase was purchased from Sigma-Aldrich (St. Louis, MO) (Sigma Cat. # 068K1214) and the activity of enzyme 1, 000 units/g-solids (isolated endoglucanase activity). Novozyme 188, a β-glucosidase, (Sigma Cat. # C-6150, Lot. # 11K1088) also was purchased from Sigma-Aldrich and the activity was 750 CBU (cellobiase unit)/mL.

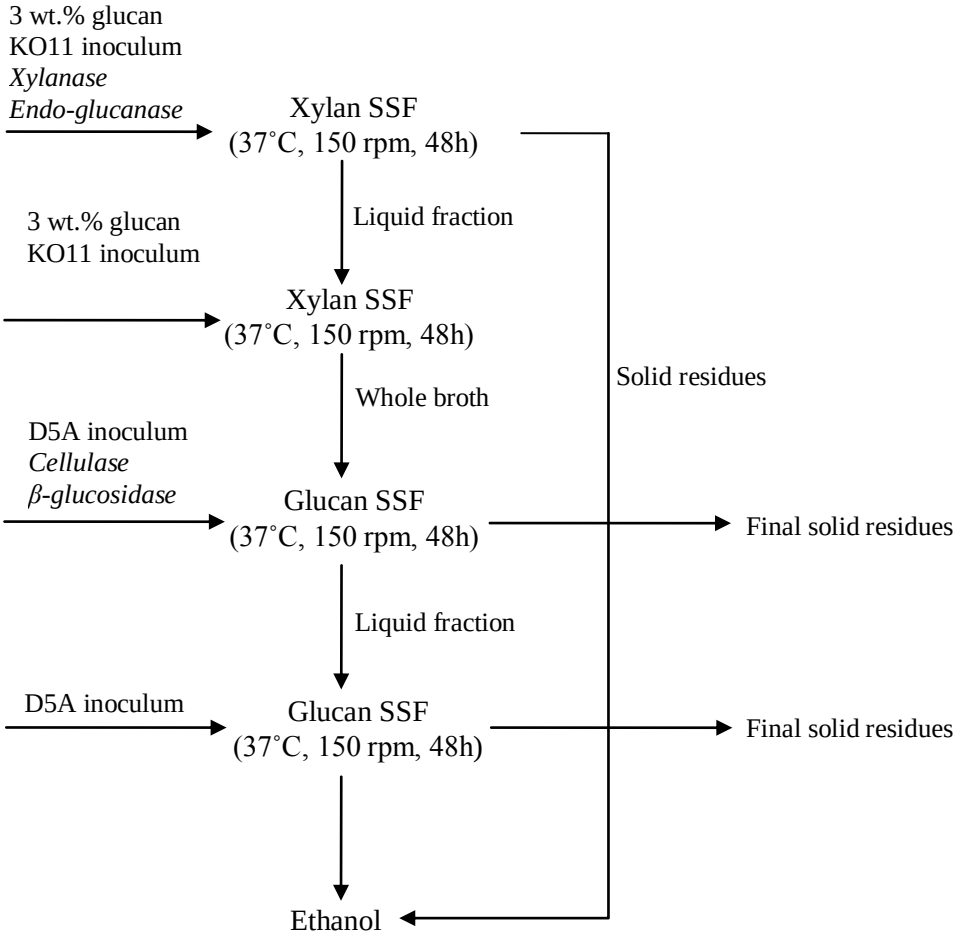

Fig. 3.1. Schematic diagram of the simple cascade simultaneous saccharification and fermentation (CSSF)

2.1.3. Microorganism

Recombinant *E. coli* KO11 (ATCC® 55124) and *Saccharomyces cerevisiae* D_5A (ATCC® 200062) were obtained from the American Type Culture Collection (location). *E. coli* KO11 was maintained on LB solid medium (Luria-Bertani Medium (Sigma, Cat. # L-3152); 5 g/L yeast extract, 10 g/L Tryptone, and 5 g/L NaCl; agar (Sigma Cat. # B0128234): 15 g/L), supplemented with 2 g/L dextrose (Fisher Cat. # D16) and 40 mg/L chloramphenicol (Sigma Cat. # C-0378). Dextrose and chloramphenicol were added after autoclaving. The culture was transferred monthly. *S. cerevisiae* D_5A was maintained on YPD solid medium, which contained 2% Bacto yeast extract (Lot. # 6215213), 1% Bacto peptone (Lot. # 6352003), 2% dextrose, and 1.5% agar. Transfer of the yeast culture also was performed monthly. To prepare the plates, the media were autoclaved at 121˚C for 15 min, allowed to cool to about 50˚C, poured onto the plates, and allowed to solidify. The plates were kept refrigerated at 4˚C.

2.2. Methods

2.2.1. Pretreatment

Corn stover was pretreated by soaking in aqueous ammonia (SAA) using 15% (w/w) ammonia solution (diluted from 29.54% ammonia hydroxide-certified ACS plus, Fisher Cat. # A669C) and a solid-to-liquid ratio of 1:11. The pretreatment was performed in screw-capped Pyrex solution bottles (250 ml) at 60˚C without agitation for 12h. These conditions were chosen based on the results obtained previously for SAA pretreatment of corn stover (Kim and Lee, 2005; Kim and Lee, 2007). The pretreated corn stover was washed with deionized (DI) water using vacuum filter and fluted filter paper (Fisher Cat. #

09-790-14F) until the wash water had a neutral pH. The washed solids were collected for composition analysis and fermentation tests.

2.2.2. Enzyme recycling

The fermentation broth was centrifuged (IEC MODEL HN-S Centrifuge) at 2,000 rpm (605 g-force) for 5 min. The supernatant (liquid fraction) containing the recovered enzymes was separated and collected from the solid residues. The solid residues were also kept in refrigerator at 4^{o}C for further utilization.

2.2.3. Cascade fermentation

Inoculum preparation: The *E. coli* KO11 culture was first transferred to a new LBD agar plate without antibiotics, cultivating for 24 h at 37˚C. Single colonies were selected to inoculate 50 mL sterile LB supplemented with 20 g/L glucose in 250-mL flasks. The medium contained no antibiotics and was previously sterilized by autoclaving at 121˚C for 15 min. The inoculated flasks were incubated at 37˚C and 150 rpm in the incubator shaker for 10-14 hours (NREL, 2001). When the glucose concentration reached below 2 g/L, the cells were harvested by centrifugation (IEC MODEL HN-S Centrifuge) at 2,000 rpm (605 g-force) for 5 min. The supernatant was removed, and the cell pellets were re-suspended in 5 ml of DI water. The cells harvested from two flasks were combined (10 ml) and used as the inoculum for the experimental flasks. Similar procedure was used to prepare the inoculum for *S. cerevisiae* D_5A except that sterile YP medium supplemented with 50 g/L glucose was used.

CSSF experiments: All CSSF experiments were performed in 250-mL flasks capped with rubber stoppers perforated with syringe needle for CO_2 venting and incubated at 37˚C and 150 rpm. The initial substrate loading was 3% w/v glucan (approximately 6% w/v pretreated biomass). The concentration of LB medium was 25g/L. The working volume of

the 1st stage SSF was 100 mL. The working volume of each following stage was measured with graduated cylinder. The pH was not controlled in these experiments. In the 1st stage (xylan SSF), xylanase was added at 8,000 GXU/g glucan and endo-glucanase was added at 50 units/g glucan. The flasks were inoculated with the *E. coli* (KO11) recovered from two inoculum flasks as described previously. After 48 h, the liquid fraction containing free enzymes and ethanol was separated from the solids which contain unconverted substrate and cell mass. The liquid fraction was mixed with new substrate and new KO11 inoculum to start the 2nd stage fermentation (xylan SSF). No new enzymes were added in the 2nd stage fermentation. The solids were collected for the 4th stage fermentation later. The 2nd stage fermentation also lasted for 48 h, and then the D_5A inoculum, cellulase and β-glucosidase were added to initiate the 3rd stage fermentation (glucan SSF). After 48h of the 3rd stage fermentation, the liquid fraction was recycled and mixed together with the solid residues from the 1st stage fermentation and D_5A inoculum to start the last (4th) stage fermentation.

2.2.5. Analysis

The ethanol concentration in fermentation samples was analyzed by high performance liquid chromatography (HPLC) with refractive index (RI) detector (Varian ProStar 356, Varian Inc, Palo Alto, CA) and Bio-Rad Aminex HPX-87H column (Hercules, CA).

The fermentation samples were treated at 85˚C for 45 min in water bath to precipitate enzyme protein in the samples prior to HPLC analysis to prevent from clogging of column at HPLC analysis temperature (60˚C in Bio-Rad Aminex HPX-87H column). Screw caped microcentrifuge tube with silicon O-ring (Fisher Cat. # 02-707-352) was used. The treated samples were centrifuged at 14,000 rpm for 15 min to remove to precipitated protein. The supernatant obtained after centrifuge was filtered with 0.2 μm sterile filters into HPLC vials.

2.2.6. Ethanol yield calculation

The ethanol yields were calculated as follows:

Ethanol yield (Y_E) [%]

$$= \frac{\text{Ethanol concentration [g/L] in reactor} \times \text{working volume}}{\text{Initial sugars in reactor [g]} \times 0.511} \times 100\%$$

Note. Sugar is interpreted as glucose plus xylose in the SSF work.

*The final totally working volume is not necessarily 100mL.

3. Results and Discussion

3.1. Overall ethanol production efficiency of CSSF

The ethanol concentrations after each stage of fermentation are summarized in Table 3.1. The ethanol profile of CSSF is also shown in Fig. 3.2. With the cascade fermentation scheme, on average 27.2 g/L of ethanol concentration was finally obtained, in other word, the overall yield of ethanol was 58% of theoretical maximum based on the total carbohydrates (glucan+xylan) in untreated corn stover.. The final total volume of fermentation broth was 113 mL. Meanwhile, the enzyme loading was reduced by 50%. The accumulation of ethanol after each stage of fermentation indicates that the enzymes were partially recycled by simple centrifugation and solid-liquid separation, and that ethanol accumulated in a stage-wise mode.

However, compared with the two-phase simultaneous saccharification and fermentation (TPSSF) the overall ethanol yield decreased from 84% to 58% based on the total carbohydrates (glucan+xylan) in untreated corn stover. The prime reason for these results probably lies in the low recovery yield of enzymes from fermentation broth. It has been reported that during hydrolysis cellulase enzymes typically are present in the system in

two different forms, free enzyme and bound enzyme (to both cellulose and lignin in residual substrate), and thus an efficient enzyme recycling requires effective recovery of both free and adsorbed cellulase (Tu et al., 2007). However, via single centrifugation, only free enzyme can be partially recovered from the fermentation broth, rendering the low enzyme activity in the next stage fermentation.

Table 3.1 Ethanol concentrations accumulated after each stage of fermentation

No.	Ethanol concentration [g/L]			
	1st stage Xylan SSF	2nd stage Xylan SSF	3rd stage Glucan SSF	4th stage Glucan SFF
1	10.4	16.8	26.2	30.5
2	9.1	10.1	19.7	24.3
3	9.3	12.1	22.9	26.8
Ave.	9.6	13.0	22.9	27.2
Std. dev.	0.7	3.4	3.3	3.1

Pretreatment: 15wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C, 12 h; CSSF: 1st stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, enzymes recycled from 1st stage; 3rd stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 2nd stage/100 mL working volume, 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; 4th stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 1st stage/100 mL working volume, enzymes recycled from 3rd stage; LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.

3.2. Effects of recycled enzymes on CSSF ethanol yield

The effects of recycled enzymes is evaluated in terms of the ethanol amount (g) produced in each stage. The initial working volume of CSSF was 100 mL. However, the working volume did not remain constant throughout all the four stages of CSSF process. Two factors contributed to the variation in working volume: (1) liquefaction of solid substrate increased the volume of liquid fraction; (2) the high-moisture solid cake generated after the solid-liquid separation by centrifugation removed some liquid, reducing the volume of liquid fraction. The increment in liquid volume caused by the liquefaction and the moisture

removed with the solid residues were not always equal, resulting in net change in the volume of liquid fraction before and after SSF. When the liquid fraction was used for the next stage fermentation, the total working volume (liquid fraction + solids) was also changed.

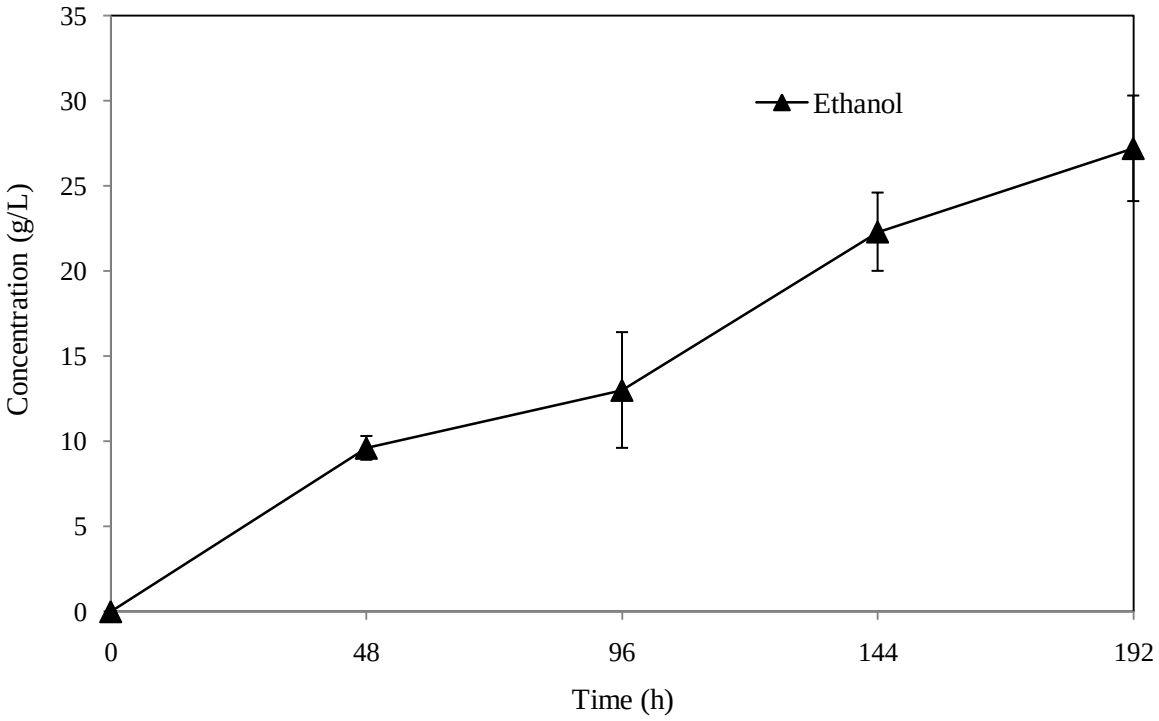

Fig. 3.2. Ethanol profile of cascade simultaneous saccharification and fermentation (CSSF). Pretreatment: 15wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C, 12 h; CSSF: 1st stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, enzymes recycled from 1st stage; 3rd stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 2nd stage/100 mL working volume, 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; 4th stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 1st stage/100 mL working volume, enzymes recycled from 3rd stage; LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.

The total working volumes at the end of each stage of SSF were summarized in Table 3.2(a). The ethanol produced in each stage of CSSF was calculated and presented in Table 3.2(b). The 1st stage fermentation is typical xylan SSF, which results in stable ethanol production with an average of 0.96 g. In the 2nd stage fermentation, the recovered xylanase

and endoglucanase were used for the hydrolysis of xylan. An average of 0.31g of ethanol was obtained with a standard deviation of 0.30 g. The 3rd stage fermentation is typical glucan SSF, which also gave stable ethanol production (1.06 g). In the 4th stage of fermentation, with recovered cellulase and β-glucosidase 0.74 g ethanol was generated. These data show that the ethanol produced in the 2nd and 4th stage are respectively 32.4% and 69.6% of those in the 1st and 3rd stage.

The decline in ethanol production of the 2nd and 4th stage SSF can be explained from the following aspects: (1) enzymes were only partially recovered; (2) accumulation of ethanol inhibited both enzyme activities (Bezerra and Dias, 2005; Chen and Jin, 2006; Waugh and Purchase, 1987; Wu and Lee, 1997; Wyman and Abelson, 1996) and the fermentability of microbes. For the 2nd stage SSF, the fermentation with *E.coli* KO11 was also impeded by low pH (~4), and yet the optimal pH for *E. coli* KO11 is 6-7 (Moniruzzaman et al., 1998). Additionally, the data in Table 3.2(b) shows that the ethanol produced in the 3rd stage fermentation (1.06 g) is slightly lower than that produced in the glucan SSF of a TPSSF with D_5A (1.11 g) (Li et al., 2010). It is possibly because that the higher ethanol concentration at the beginning of the 3rd stage fermentation (13.0 g/L), compared with that of the glucan SSF of a TPSSF (11.2 g/L), led to more significant inhibitory effects on cellulase activity (Wu and Lee, 1997) and fermentability of microorganisms. It has been reported that the glucan digestibility exhibited a linear decreasing pattern with ethanol concentration and that for a typical SSF temperature of 38˚C, the cellulase lost 9%, 36%, and 64% of its original activity at ethanol concentrations of 9 g/L, 35 g/L, and 60 g/L respectively (Wu and Lee, 1997).

Table 3.2
(a) Working volume at the end of each stage of fermentation

No.	Working volume [mL]			
	1st stage Xylan SSF	2nd stage Xylan SSF	3rd stage Glucan SSF	4th stage Glucan SFF
1	100	100	104	114
2	100	94	98	112
3	100	98	102	112
Ave.	100	97.3	101.3	112.7
Std. dev.	0	3.1	3.1	1.2

(b) Ethanol produced in each stage of fermentation

No.	Ethanol produced [g]*			
	1st stage Xylan SSF	2nd stage Xylan SSF	3rd stage Glucan SSF	4th stage Glucan SFF
1	1.04	0.64	1.04	0.75
2	0.91	0.04	0.98	0.79
3	0.93	0.26	1.15	0.67
Ave.	0.96	0.31	1.06	0.74
Std. dev.	0.07	0.30	0.09	0.06

Pretreatment: 15wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C, 12 h; CSSF: 1st stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, enzymes recycled from 1st stage; 3rd stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 2nd stage/100 mL working volume, 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; 4th stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 1st stage/100 mL working volume, enzymes recycled from 3rd stage; LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.
*Ethanol produced in the n-th stage SSF: ethanol (n) = volume (n) × ethanol concentration (n) – volume (n-1) × ethanol concentration (n-1).

Furthermore, the complex operation procedures between stages (e.g., solid-liquid separation, mixing of liquid fraction and solid substrate) might cause some loss of ethanol since these procedures increased the time of exposing the fermentation broth to open air. Ethanol loss might also occur in the disposal of substrate residues after the 3rd stage SSF because some ethanol could be removed with the high-moisture solids. However, this loss could be minimized by adopting efficient solid-liquid separation and dehydration equipment.

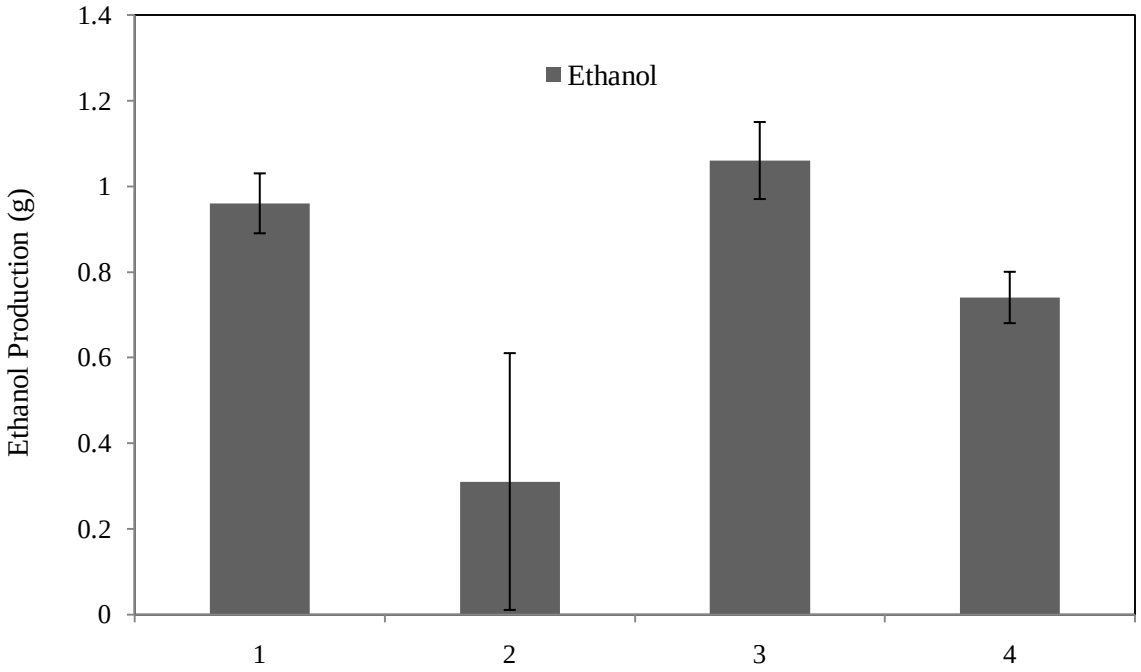

Fig. 3.3. Ethanol production in each stage of SSF. Pretreatment: 15wt. % aqueous ammonia, 1:11 solid: liquid ratio, 60˚C, 12 h; CSSF: 1st stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, Multifect Xylanase 8,000 GXU/g-glucan, endo-glucanase 50 units/g-glucan; 2nd stage: *Escherichia coli* ATCC® 55124, 3% w/v glucan/100 mL working volume of SAA-treated corn stover, enzymes recycled from 1st stage; 3rd stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 2nd stage/100 mL working volume, 15 FPU of GC 220/g-glucan; 30 CBU of Novozyme 188/g-glucan; 4th stage: *Saccharomyces cerevisiae* ATCC® 200062 (D_5A), solid residues from 1st stage/100 mL working volume, enzymes recycled from 3rd stage; LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.

3.4. Research suggestions

The cascade simultaneous saccharification and fermentation (CSSF) is designed to reduce the overall enzyme load in bioconversion process and to increase the final ethanol concentration in fermentation broth. The results of CSSF tests indicate that this process needs to be further developed and optimized. Considering the preliminary results obtained and the problems hypothesized in this work, the future efforts can be made from the following aspects: (1) to introduce or develop effective enzyme recycling technology; (2) to optimize the bioreactor configuration, making the operation simple for the multi-stage cascade

fermentation. The improvement in the efficiency of enzyme recycling can shorten the fermentation period, enhance ethanol yield and productivity. In order to realize simple and precise operation and to minimize the ethanol loss during the operation, further development of bioreactor configuration is also necessary.

Nevertheless, the CSSF is innovative and constructive in integrating enzyme recycling and simultaneous saccharification and fermentation, and increasing the final ethanol concentration by way of stage-wise accumulation, which serves as an alternative to direct high solid fermentation in which insufficient mass and heat transfer always constitutes a major difficulty for efficient bioconversion.

4. Conclusion

The cascade simultaneous saccharification and fermentation (CSSF) which applies sequential xylan SSF and glucan SSF interconnected as a cascade results in the accumulation of ethanol in a stage-wise mode, indicating that the enzymes were partially recycled by simple centrifugation and solid-liquid separation. The final ethanol concentration obtained via CSSF was increased compared with TPSSF process. However, the CSSF exhibits deficiencies in decreased ethanol yield, low productivity, extended fermentation period and high process complexity. The prime reason for these results lies in the low efficiency of enzyme recovery via single centrifugation, rendering the low enzyme loading in the subsequent stage of fermentation. Therefore, this scheme needs to be further developed and optimized.

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CHAPTER 4. LOW-LIQUID PRETREATMENT OF CORN STOVER WITH AQUEOUS AMMONIA

Modified from a paper to be submitted to *Bioresource Technology*

Xuan Li and Tae Hyun Kim

Abstract

A low-liquid pretreatment method of corn stover by aqueous ammonia was studied for the purpose of ethanol production. Corn stover was treated at 0.5-50.0 wt. % of ammonia loadings, 1: 0.2-5.0 (w/w) of solid-to-liquid ratio and 30˚C over an extended period of 4 and 12 weeks without mechanical agitation in closed plastic containers. The factors of interest include ammonia loading, solid-to-liquid ratio, and reaction time. The effects of these factors on the composition and enzyme digestibility of corn stover were thoroughly investigated. Optimal low-liquid ammonia pretreatment conditions (50 wt. % of ammonia loading, 1:5 of solid-to-liquid ratio, 30˚C, 4 weeks) resulted in up to 55% delignification and 86.5% glucan digestibility with 15 FPU/g-glucan of cellulase and 30 CBU/g-glucan of β-glucosidase. With the corn stover treated under the conditions of 50 wt. % ammonia loading, 1:2 solid-to-liquid ratio, 30˚C and 4 weeks as the substrate, an ethanol yield of 73% of theoretical maximum was obtained on the basis of the total carbohydrates (glucan+xylan) content in untreated corn stover. The low-liquid aqueous ammonia pretreatment reduced the pretreatment severity and had potential of reducing the energy cost, making overall bioconversion process more cost-effective.

Key words: aqueous ammonia, low-liquid, pretreatment, corn stover, composition, enzyme digestibility, SSCF

1. Introduction

The current and projected shortage of fossil fuels availability has led to the ever-increasing interest in the conversion of renewable lignocellulosic resources to liquid and gaseous fuels (O'Brien et al., 2004; Quintero et al., 2008; Reddy et al., 2007; Yu and Tao, 2008). Ethanol is the most widely used liquid biofuel for a sustainable and secure alternative to petroleum transportation fuels (Demirbas, 2005). Lignocellulosic biomass, as the prospective source of fermentable sugars for ethanol bio-production, has great potential in industry use (Mosier et al., 2005). However, bioconversion of lignocellulosic biomass to ethanol is significantly hindered by the structural and chemical complexity of biomass, which makes these materials a challenge to be used as feedstock for cellulosic ethanol production (Zheng et al., 2009). Pretreatments that constitute the means to disrupt the lignin-carbohydrate complex and crystalline structure of lignocellulosic biomass so as to render it accessible for a subsequent hydrolysis step have been therefore extensively studied for fuel ethanol production (Hsu., 1996; Mosier et al., 2005; Zerbe and Baker, 1987; Zheng et al., 2009).

Pretreatment has been identified as the unit operation, of second most costly (only to feedstock) in enzyme-based conversion of corn stover to ethanol (NREL, 2002; Wooley et al., 1999). The cost centers are steam, chemicals, and corrosion-resistant pretreatment reactors (Keller et al., 2003). Solvent loading in pretreatment, whether the solvent is water or some other chemical, is an important parameter since it affects the overall plant energy balance and capital costs for fermentation and downstream recovery equipment. The total process steam usage is proportional to the solvent concentration in the pretreatment reactor effluent (Eggeman and Elander, 2005). Pretreatment processes with high water and/or chemical usage

require high energy consumption, and this will adversely affect the production costs of final products. For instance, the pretreatment technologies based on flowing liquid through solid biomass have been shown to produce higher hemicellulose sugar recoveries, greater lignin removal, less inhibitors in the hydrolysate liquid, and highly digestible cellulose compared to conventional systems (Laser et al., 2001; Torget et al., 1996; van Walsum et al., 1996). However, the high water consumption required for such flow systems results in excessive energy consumption for pretreatment and product recovery (Yang et al., 2004); Pretreatment of lignocellulosic biomass with aqueous ammonia has been reported for the purpose of ethanol production (Kim et al., 2003; Kim and Lee, 2005a, 2005b, 2006, 2007). Among them, the soaking in aqueous ammonia (SAA) is a batch process applied under low-severity condition (Kim et al., 2009). The optimized conditions of SAA were 1:6-10 solid-to-liquid ratio (dry biomass (g): weight of 15 wt. % aqueous ammonia), 60-80˚C, 8-24h (Kim and Lee, 2005b, 2007). However, the problems associated with this method are the high water and chemical consumption.

The primary intent of this work was to seek an economically viable pretreatment process with aqueous ammonia that can further reduce the energy input and liquid throughput. The low-liquid treatment with aqueous ammonia is proposed as an effective pretreatment of lignocellulosic biomass with minimized water and chemical consumption.

This low-liquid ammonia treatment method originated from the ammoniation of forages in conventional agricultural application. Ammoniation improves forage nutritive value by increasing the crude protein content, intake and digestibility by ruminants, and by allowing storage of higher moisture roughage by inhibiting mold development (Lalman et al., 2008). Ammonia functions in the form of ammonium hydroxide. Owing to the hydroxide

group (OH-), ammonium hydroxide serves to solubilize hemicellulose by breaking chemical bonds holding lignin and hemicellulose together; and partially break down the structure of cellulose by disrupting hydrogen bonds. This reaction causes swelling of the fiber and allows cellulase better access to the fiber for digestion (Church, 1988). Ammoniation of forage by means of anhydrous and aqueous ammonia, urea and other ammonia releasing compounds have also been widely studied as reviewed by Berger et al. (1994), and Sundstøl and Coxworth (1984). Ammoniation is relatively simple and easy to accomplish. The process involves sealing forage in an air-tight, enclosed container and adding ammonia releasing compounds under ambient temperature for an extended period (Lalman et al., 2008; Sánchez, 2002).

Ammonia as a pretreatment reagent has many attractive features for fuel ethanol production from lignocellulosic biomass: (1) effective delignification of lignocellulosic biomass (Kim et al., 2008; Kim and Lee, 2005b; Streeter and Horn, 1982); (2) strong swelling effect (Broido and Weinstein, 1970; Holtzapple et al., 1992; Mosier et al., 2005; Taherzadeh and Karimi, 2008); (3) less significant influence on the carbohydrate contents (Kim et al., 2009); (4) nearly zero generation of toxic components that create an inhibitory environment in which microorganisms cannot sustain their viability required for efficient bioconversion (Kim and Lee, 2005b); (5) preservative effect against the development of bacteria and moulds in lignocellulosic biomass (Grotheer, 1986). Ammonia based pretreatment is a very effective method especially for substrates that have low lignin contents such as agricultural residues and herbaceous feedstock (Kim et al., 2009).

In this study, the low-liquid pretreatment of corn stover using aqueous ammonia was investigated for the purpose of fuel ethanol production. There are a number of factors that

influence the effectiveness of ammonia pretreatment, i.e., ammonia loading, moisture content, temperature and treatment time (Sánchez, 2002; Schneider and Flachowsky, 1990; Teymouri et al., 2005). The factors determining the effectiveness of pretreatment were thoroughly evaluated via the compositional change and enzyme digestibility of pretreated corn stover. Simultaneous saccharification and co-fermentation of pretreated corn stover was also performed so as to demonstrate the appropriateness of low-liquid aqueous ammonia treated corn stover as the substrate for microbial ethanol fermentation.

2. Materials and Methods

2.1. Materials

2.1.1. Feedstock

Locally harvested corn stover was ground and screened. The fractions between 10 and 35 mesh (0.5 mm and 2.0 mm) were collected and air dried at room temperature (~25˚C). The composition of corn stover was determined following the Laboratory Analytical Procedure (LAP) developed by NREL (NREL, 2005a, 2005b, 2008a) to be: glucan 38.2%, xylan 21.0%, arabinan 2.7%, galactan 2.1%, lignin 17.4%, extractives 13.3%, and ash 5.3%. Prior to pretreatment, the moisture content of the air dry corn stover was analyzed. Knowing the moisture level in the corn stover is essential for determining the amount of NH_3 to be added.

2.1.2. Enzymes

Cellulase GC-220 (Lot. # 301-04232-162) was generously provided by Genencor, a Danisco Division (Rochester, NY); the average activities of these enzymes provided by the manufacturer were 45 FPU (filter paper unit)/mL. Novozyme 188, a β-glucosidase, (Sigma

Cat. # C-6150) was purchased from Sigma-Aldrich and the activity was measured to be 750 CBU (cellobiase unit)/mL.

2.1.3. Microorganism

Recombinant *Escherichia coli* ATCC® 55124 (KO11) was employed for the simultaneous saccharification and cofermentation (SSCF) tests. *E. coli* KO11 was maintained on LB (Luria-Bertani Medium, Sigma, Cat. # L-3152) solid medium which consists of 5 g/L yeast extract, 10 g/L tryptone, and 5 g/L NaCl, supplemented with 15 g/L agar (Sigma Cat. # B0128234), 2 g/L dextrose (Fisher Cat. # D16) and 40 mg/L chloramphenicol (Sigma Cat. # C-0378). Dextrose and chloramphenicol were added after autoclaving. The culture was transferred monthly. To prepare the plates, the media were autoclaved at 121˚C for 15 min, allowed to cool to about 50˚C, poured onto the plates, and allowed to solidify. The plates were kept refrigerated at 4˚C.

2.2. Methods

2.2.1. Pretreatment

The pretreatment was performed in 60ml HDPE wide-mouth round bottles (Fisher Scientific Cat. # 02-912-031) with 5 g (oven dry weight) of corn stover at 0.5-50.0 wt. % of ammonia loading, 1: 0.2-5.0 (w/w) of solid-to-liquid ratio, and 30˚C for respectively 4 and 12 weeks.

The ammonia loading and solid-to-liquid ratio are defined as follows:

(1) Ammonia loading [wt. %]) = ammonia [g]/dry biomass [g] ×100%;

(2) Solid-to-liquid ratio [w/w] = dry biomass [g]: (ammonia [g] + moisture [g]).

The source of ammonia was 29.54% ammonium hydroxide (Fisher Scientific Lot. # 091276).

Water and aqueous ammonia were injected with syringe (20cc Lspk50, Fisher Scientific Cat. # 14-840-30) and needle. Injection of solution was done at multiple heights. The bottles were shaken periodically (approximately once per day) for better distribution of ammonia and moisture. The cap and neck of the bottle were tightly sealed with aluminum foil to prevent the leakage of ammonia and water vapor. The temperature was maintained by incubator (Fisher Scientific Cat. # 11-690-537D).

The ammoniated corn stover was washed with deionized water (DI) via vacuum filtration continuously until the wash water had a neutral pH. The fluted filter paper (medium pore) used for filtration was purchased from Fisher Scientific (Cat. # 09-790-14F). The washed solids were collected for composition analysis, enzyme digestibility tests and SSCF tests.

2.2.2. Enzyme digestibility test

The enzyme digestibility tests of glucan in pretreated corn stover were performed in duplicates following the procedure of the NREL LAP (NREL, 2008b). The tests were performed at 1% (w/v) initial glucan loading in 0.05 M citrate buffer (pH=4.8) supplemented with tetracycline at 40 mg/L and cyclohexamide at 30 mg/L using 250 ml screw-capped Erlenmeyer flasks. The flasks were incubated at 50˚C and 150 rpm in the incubator shaker (Excella E24, New Brunswick Scientific, Edison, NJ).

Glucan digestibility was determined at cellulase loading of 15 FPU/g of glucan with β-glucosidase supplemented at 30 CBU/g of glucan. Substrate and enzyme blanks were run in parallel as controls.

Glucan digestibility [%] = total released glucose×0.9/total glucan in treated corn stover ×100%.

0.9 is the conversion factors of glucose to equivalent glucan.

2.2.3. Simultaneous saccharification and cofermentation (SSCF) test

Single colonies of *E. coli* KO11 on LB solid medium were selected to inoculate 50 mL sterile LB medium supplemented with 20 g/L glucose in 250-mL flasks. The inoculated flasks were incubated at 37˚C and 150 rpm in the incubator shaker (Excella E24, New Brunswick Scientific, Edison, NJ) for 10-14 hours (NREL, 2001). When the glucose concentration dropped below 2 g/L, the cells were harvested by centrifugation (IEC MODEL HN-S Centrifuge) at 2,000 rpm (605 g-force) for 5 min. The supernatant was removed, and the cell pellets were re-suspended in 5 ml of DI water. The cells harvested from two flasks were combined (10 ml) and used as the inoculum for the experimental flasks.

SSCF tests were performed using 100 mL LB medium in 250-mL flasks capped with rubber stoppers perforated with syringe needle for CO_2 venting and incubated at 37˚C and 150 rpm in the incubator shaker (Excella E24, New Brunswick Scientific, Edison, NJ). The initial substrate loading was 3% w/v glucan (approximately 6% w/v pretreated biomass). Chloramphenicol also was added to the flasks at 40 mg/L. The SSCF runs were performed without buffer, starting at pH 7.0. The loading of cellulase enzyme (GC-220) was 15 FPU/g-glucan, and that of β-glucosidase (Novozyme 188) was 30 CBU/g-glucan.

The ethanol yields were calculated as follows:

$$\text{Ethanol yield } (Y_E)\ [\%] = \frac{\text{Ethanol produced [g] in reactor}}{\text{Initial sugars in reactor [g]} \times 0.511} \times 100\%$$

Note. Sugar is interpreted as glucose plus xylose in the SSF work.

2.2.4. Analysis

The enzyme digestibility and ethanol fermentation samples were analyzed for individual sugars and ethanol by high performance liquid chromatography (HPLC) using

refractive index (RI) detectors (Varian ProStar 355 and 356 RI detectors, Varian Inc, Palo Alto, CA) and Bio-Rad Aminex HPX-87P and HPX-87H columns (Hercules, CA), which were operated on two separate HPLC systems. HPLC analysis conditions were (1) HPX-87P columns: 85˚C of column temperature, 0.6 ml of DI water/min, and 60˚C of column temperature, (2) HPX-87H columns: 0.6 ml of 0.005 M H_2SO_4/min. The enzyme digestibility/fermentation samples were treated at 95˚C/85˚C for 5 min/45 min in water bath to precipitate enzyme protein in the samples prior to HPLC analysis to prevent from clogging of columns at HPLC analysis temperatures (85˚C in sugar column and 60˚C in organic column). Screw capped microcentrifuge tube with silicon O-ring (Fisher Cat. # 02-707-352) was used. The treated samples were centrifuged at 14,000 rpm for 15 min to remove the precipitated protein. The supernatant obtained after centrifuge was filtered with 0.2 μm sterile filters into HPLC vials and analyzed for sugars and ethanol.

3. Results and Discussion

3.1. Effects of various ammonia loadings on biomass composition and enzyme digestibility

The pretreatment effects were evaluated from the aspects of compositional change and enzyme digestibility of corn stover. The pretreatment conditions were 0.5-50 wt. % of ammonia loading, 1:0.2-5 of solid-to-liquid ratio, 4 weeks, and 30˚C. The effects of various ammonia loadings on compositional changes of biomass were shown in Fig. 4.1. The glucan content remained at the same level with various ammonia loadings (Fig. 4.1(a)). Compared with the untreated corn stover, averagely over 95% of glucan were preserved over the entire range of ammonia loading and various solid-to-liquid ratios. For each solid-to-liquid ratio, the xylan contents of pretreated corn stover resulted from the various ammonia loadings presented similar slightly descending trends (Fig. 4.2(b)). The major compositional change

was in lignin. For each solid-to-liquid ratio, the lignin contents of pretreated corn stover resulted from the various ammonia loading also decreased (Fig. 4.2(b)), e.g., at 1:2 of solid-to-liquid ratio, delignification reached 1.4%, 3.4%, 13.1%, 28.8%, 51.4%, and 55.4% respectively with the 0.5 wt. %, 2 wt. %, 5 wt. %, 10 wt. %, 30 wt. %, and 50 wt. % of ammonia loadings.

The enzyme digestibilities of the pretreated corn stover for various ammonia loading and solid-to-liquid ratios were conducted using 15 FPU of cellulase/g-glucan of cellulase and 30 CBU of β-glucosidase/g-glucan of β-glucosidase with initial solid loading of 1% glucan and the results are summarized in Fig. 4.2. The enzyme hydrolysis of ammonia-treated corn stover under the conditions of 50 wt. % ammonia loading, 1:5 solid-to-liquid ratio, 30˚C and 4 weeks resulted in the highest enzyme digestibility, 86.5% of glucan digestibility based on the glucan and xylan content respectively in untreated corn stover. Moreover, the data indicate that for each solid-to-liquid ratio, glucan digestibility increased as the ammonia loading increased (Fig. 4.2(c)), and the trends of enzyme digestibility increases for all the solid-to-liquid ratios are approximately parallel. For the solid-to-liquid ratio 1:2/1:5, as the ammonia loadings increased from 0.5 wt. % to 50 wt. %, the glucan digestibility were enhanced significantly from 20.7%/22.5% to 82.9%/86.5%. Therefore, tests with varying ammonia loadings indicated that delignification and enzymatic digestibility were significantly improved as the ammonia loadings were increased.

Lignin has been considered as the primary inhibitors in the enzyme hydrolysis; Disruption of lignin-hemicellulose linkages makes the hemicellulose more digestible. Ammonia treatment disrupts chemical linkages between lignin and hemicellulose. Cellulose digestibility also increases since lignified hemicellulose encases cellulose (Church, 1988;

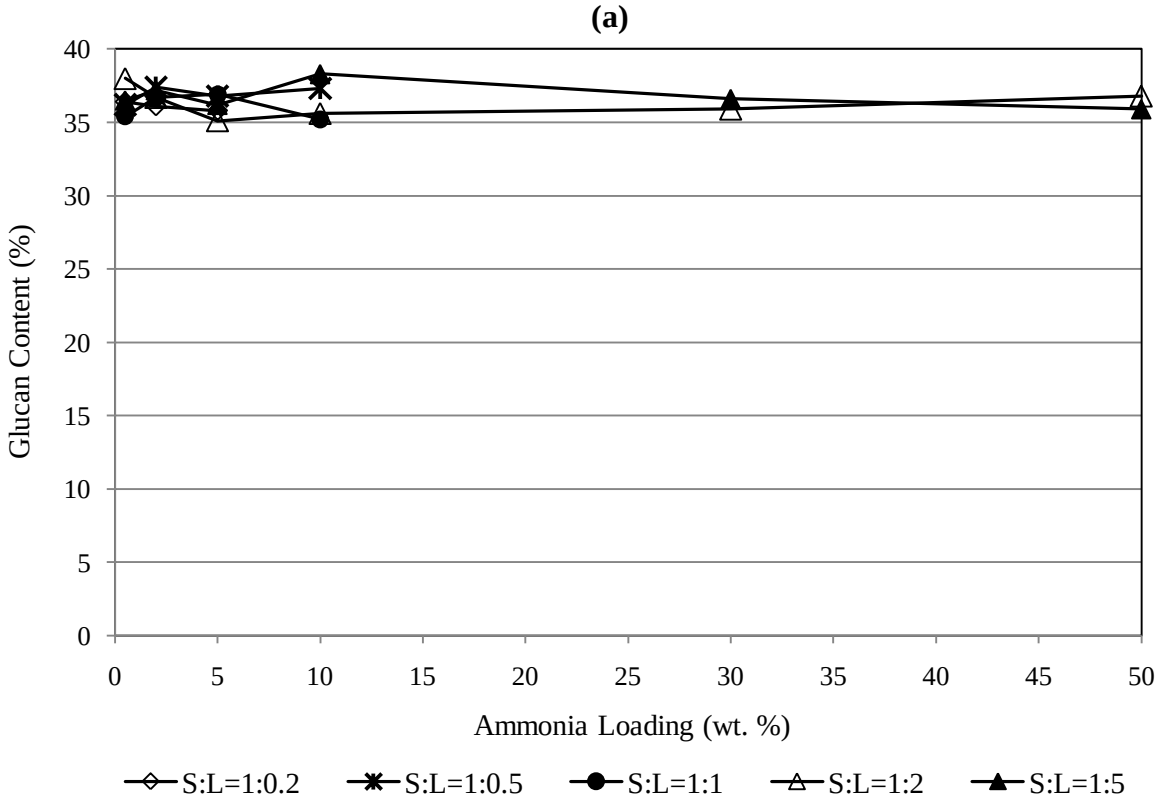
Lalman et al., 2008). Ammonia treatment changes the physical characteristics of lignocellulosic biomass making them more pliable and increases their uptake of water (hydration). Hydration rate has an important role in digestibility; the faster a biomass particle is hydrated, the faster it is digested (Weiss and Underwood, 1995). Therefore, it is probable that the increase in ammonia loading contributes to more efficient delignification and higher enzyme digestibility can be explained.

3.2. Effects of various solid-to-liquid ratios on biomass composition and enzyme digestibility

Moisture content of biomass is another important factor determining the effectiveness of treatment. In this study, solid-to-liquid ratio was applied as the index of moisture. Solid-to-liquid ratio was defined as the ratio of dry biomass weight to the weight sum of ammonia and moisture (water). The pretreatment was carried out under the conditions of 0.5-50 wt. % of ammonia loading, 1:0.2-5 of solid-to-liquid ratio, 4 weeks, and 30˚C. The compositional changes of pretreated biomass were plotted upon various solid-to-liquid ratios as shown in Figure 3. There was no significant deviation of glucan over the entire range of solid-to-liquid ratio for each ammonia loading (Fig. 4.3(a)). Similarly, the xylan retention remained at the same level over the range of solid-to-liquid ratio (Fig. 4.2(b)). The trend lines of lignin content (Fig. 4.3(c)) and enzyme digestibilities (Fig. 4.4) of pretreated corn stover for all the ammonia loadings are approximately parallel to each other and also to the horizontal axis, indicating that neither lignin content nor enzyme digestibility changes significantly as the solid-to-liquid ratio increases. Furthermore, the parallel trend lines indicate that there is no correlation between ammonia loading and solid-to-liquid ratio and thus the effects of ammonia loading and solid-to-liquid ratio can be evaluated independently. Therefore, paired t-tests were performed to confirm the observation that solid-to-liquid ratio

did not contribute to considerable changes in the composition and enzyme digestibility of corn stover. As shown in Table 1, the *p*-values (>0.05) demonstrated that there was no evidence of statistical significance between the data of different soli-to-liquid ratios. Therefore, with the same ammonia loading, the solid-to-liquid ratio, in other words, moisture did not significantly affect the pretreatment effects.

Since the pretreatment reagent used in this study is 29.54% ammonia hydroxide, for the ammonia loading 30 and 50 wt. %, solid-to-liquid ratio over 1:2 cannot be achieved. However, it is possible that in the case of high ammonia loading (e.g., 30-50 wt. %) and high solid-to-liquid ratio (e.g., >1:2) the limiting factor is moisture. This is the limitation of this test. The high ammonia loading and solid-to-liquid ratio cases can be tested by using anhydrous ammonia instead of aqueous ammonia as the source of ammonia.



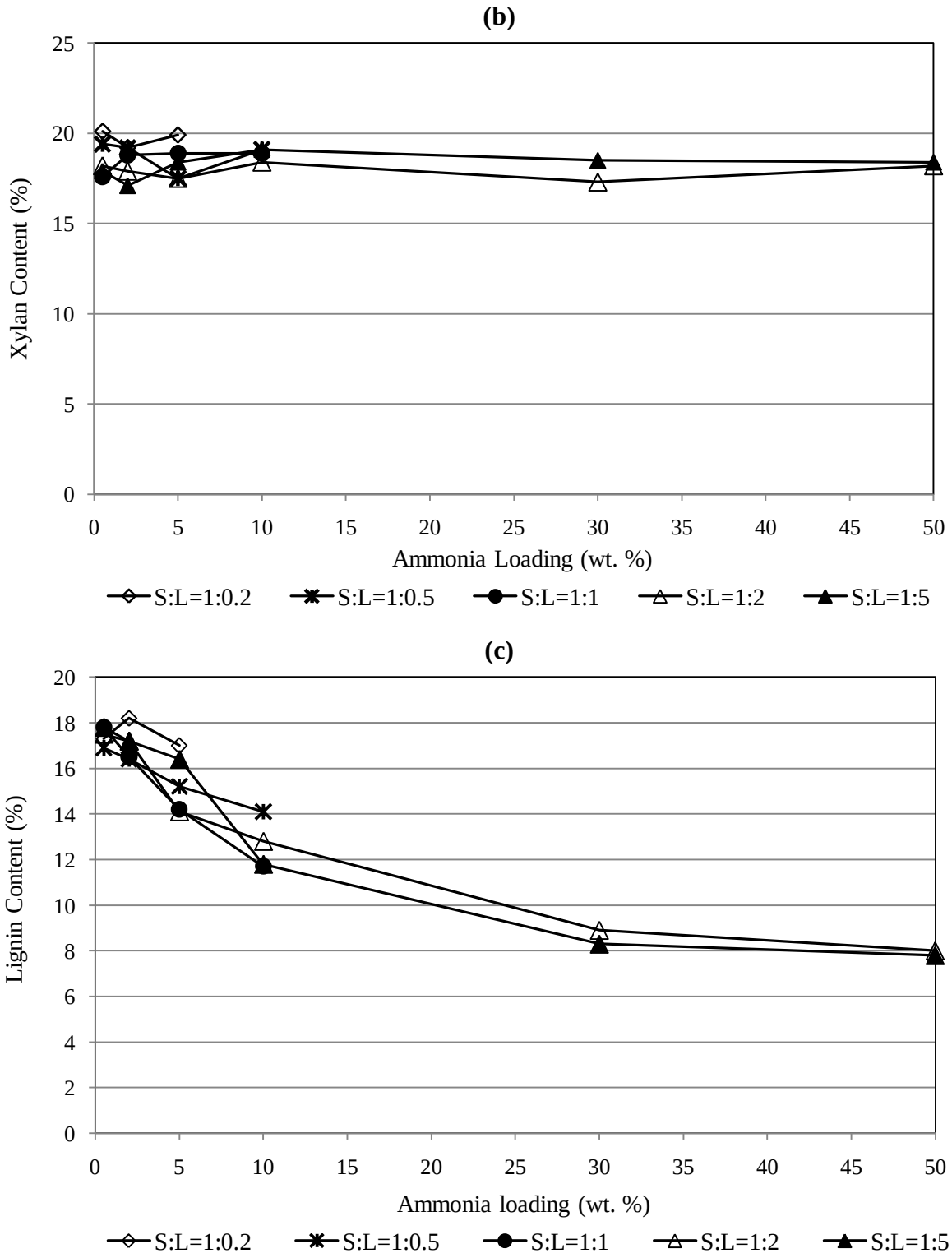


Fig. 4.1. Effects of ammonia loading on the composition of corn stover. (a) Effects of ammonia loading on glucan content; (b) effects of ammonia loading on xylan content; (c) effects of ammonia loading on lignin content. Pretreatment: 0.5, 2, 5, 10, 30, and 50 wt.% of ammonia loading, 1:0.2, 0.5, 1, 2, and 5 of solid-to-liquid ratio, 30˚C, for 4 weeks.

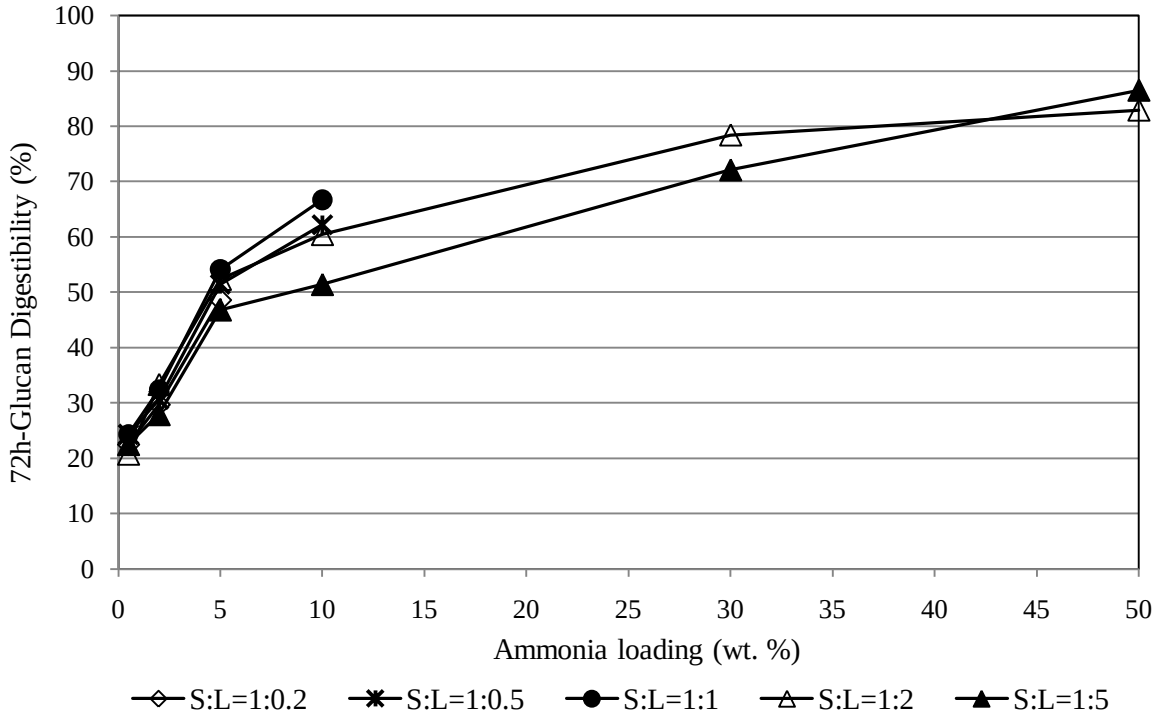
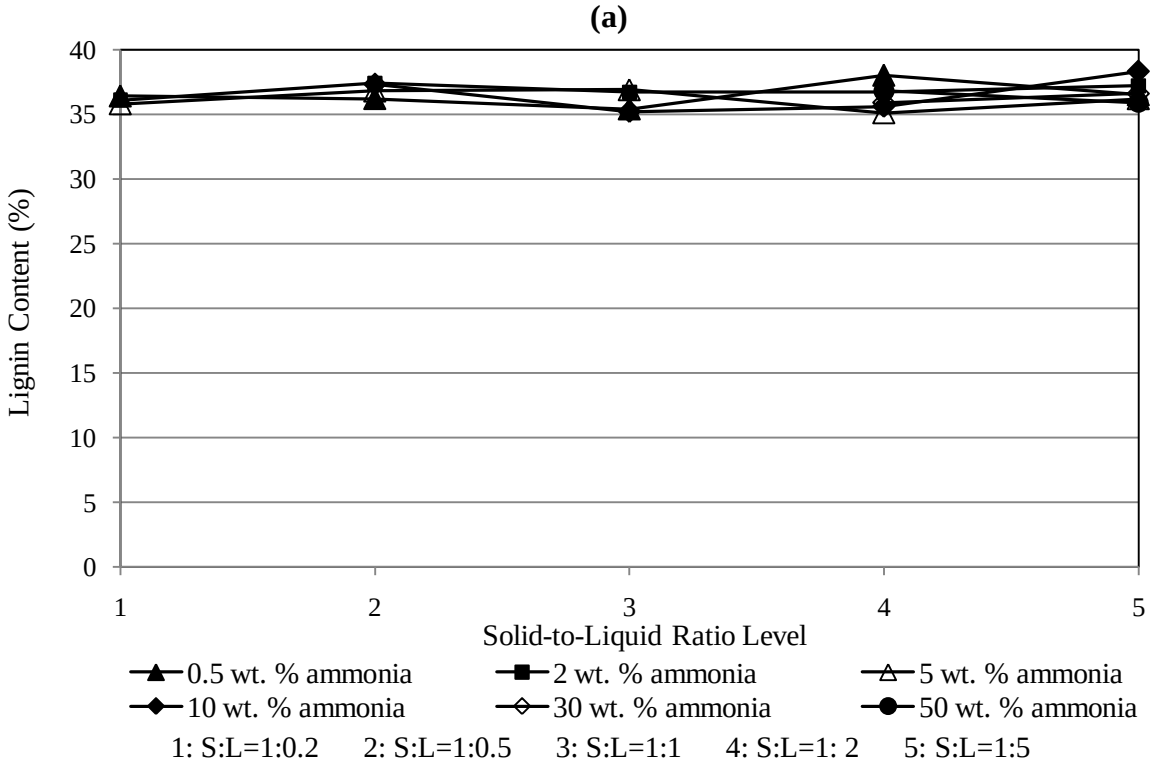


Fig. 4.2. Effects of ammonia loading on glucan digestibility. Pretreatment: 0.5, 2, 5, 10, 30, and 50 wt. % of ammonia loading, 1:0.2, 0.5, 1, 2, and 5 of solid-to-liquid ratio, 30˚C, for 4 weeks; Enzyme digestibility test: 1% w/v glucan/100 mL working volume, 15 FPU of GC220/g-glucan, 30 CBU of Novozyme 188/ g-glucan, 50˚C, pH 4.8 maintained by 0.05 M citrate buffer, 150 rpm.



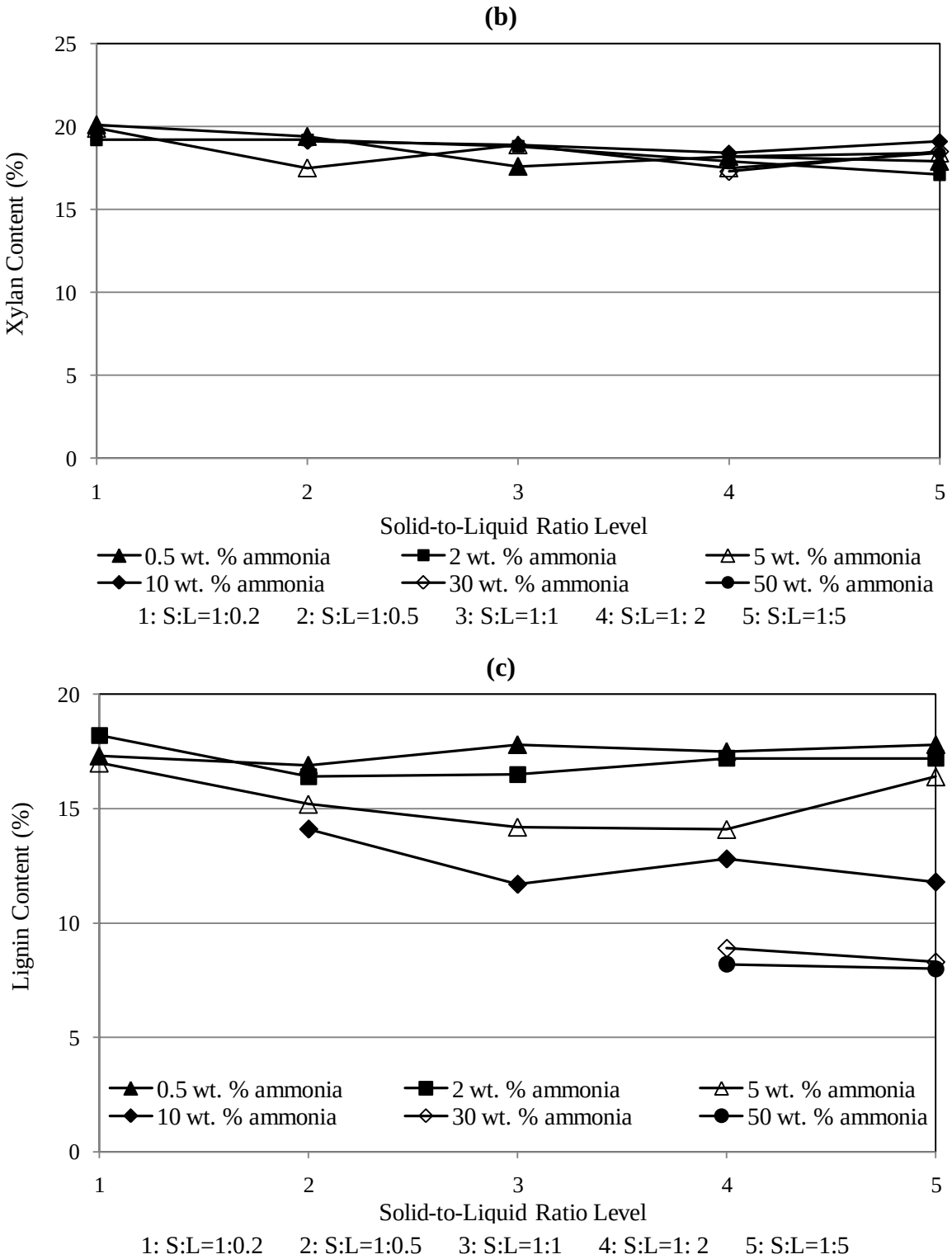


Fig. 4.3. Effects of solid-to-liquid ratio on the composition of corn stover. (a) Effects of solid-to-liquid ratio on glucan content; (b) effects of solid-to-liquid ratio on xylan content; (c) effects of solid-to-liquid ratio on lignin content**.** Pretreatment: 0.5, 2, 5, 10, 30, and 50 wt. % of ammonia loading, 1:0.2, 0.5, 1, 2, and 5 of solid-to-liquid ratio, 30˚C, for 4 weeks.

3.3. Effects of treatment time on biomass composition and enzyme digestibility

Treatment time is one of the factors affecting pretreatment effectiveness. Two different treatment times were tested, i.e., corn stover was pretreated for 4 and 12 weeks at 30˚C with 1: 0.2-5 solid-to-liquid ratio and various ammonia loadings (0.5-50 wt. % of ammonia). The lignin content and enzyme digestibilities of pretreated corn stover are summarized in Table 4.2. Paired t-test was carried out and the *p*-value was calculated to be 0.5857 and 0.0545 (>0.05), respectively for lignin contents and glucan digestibilities, indicating no and suggestive but inconclusive evidence for that extending reaction time over 4 weeks leads to significant changes in either composition or enzyme digestibility of pretreated corn stover over the entire range of ammonia loading and solid-to-liquid ratio applied in this study.

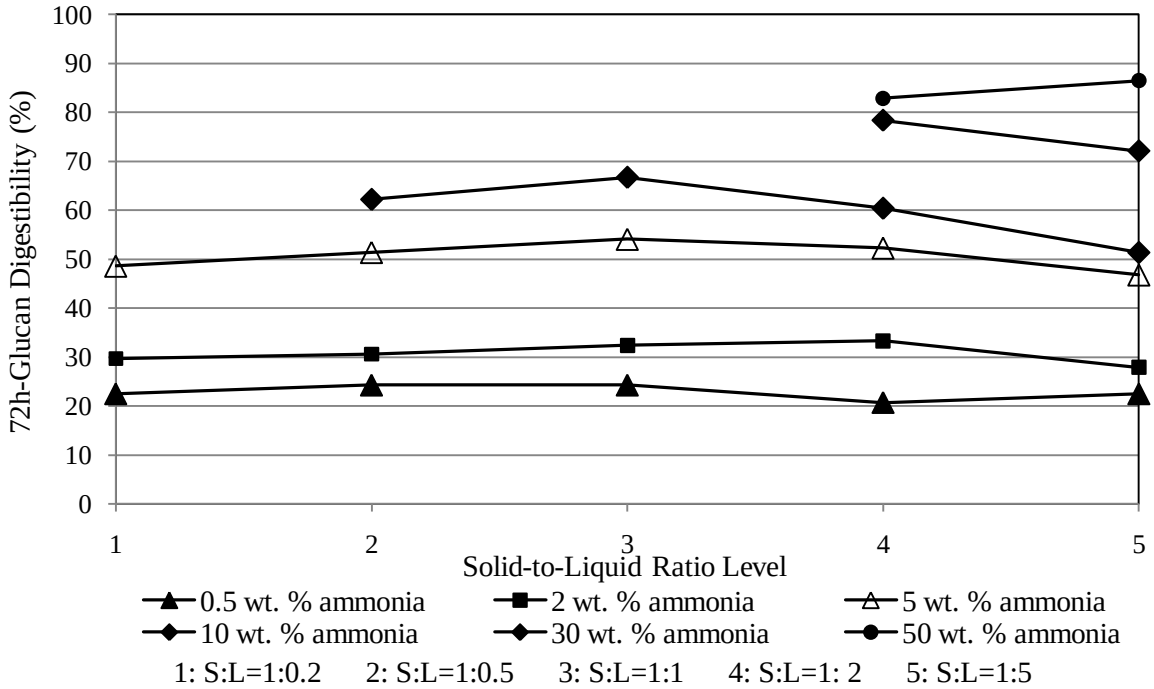


Fig. 4.4. Effects of solid-to-liquid ratio on glucan digestibility. Pretreatment: 0.5, 2, 5, 10, 30, and 50 wt. % of ammonia loading, 1:0.2, 0.5, 1, 2, and 5 of solid-to-liquid ratio, 30˚C, for 4 weeks; Enzyme digestibility test: 1% w/v glucan/100 mL working volume, 15 FPU of GC220/g-glucan, 30 CBU of Novozyme 188/ g-glucan, 50˚C, pH 4.8 maintained by 0.05 M citrate buffer, 150 rpm.

Table 4.1 Statistical difference in lignin contents and glucan digestibility resulted from the different solid-to-liquid ratio

(a) Statistical difference in lignin contents resulted from the different solid-to-liquid ratios

Solid-to-liquid ratio		*p*-value	95% CI	Degree of evidence against the null hypothesis
1:0.2	1:0.5	0.1038	-0.675 to 3.341	No
1:0.5	1:1	0.4632	-1.676 to 2.876	No
1:1	1:2	0.3672	-1.401 to 0.701	No
1:2	1:5	0.7887	-1.346 to 1.079	No
1:0.2	1:5	0.4995	-1.563 to 2.296	No

(b) Statistical difference in glucan digestibility resulted from the different solid-to-liquid ratios

Solid-to-liquid ratio		*p*-value	95% CI	Degree of evidence against the null hypothesis
1:0.2	1:0.5	0.0791	-4.194 to 0.528	Suggestive but inconclusive
1:0.5	1:1	0.0957	-5.231 to 0.731	Suggestive but inconclusive
1:1	1:2	0.1727	-2.121 to 7.521	No
1:2	1:5	0.1491	-1.764 to 8.697	No
1:0.2	1:5	0.1835	-1.382 to 3.782	No

Pretreatment: 0.5, 2, 5, 10, 30, and 50 wt. % of ammonia loading, 1:0.2, 0.5, 1, 2, and 5 of solid-to-liquid ratio, 30˚C, for 4 weeks; Enzyme digestibility test: 1% w/v glucan/100 mL working volume, 15 FPU of GC220/g-glucan, 30 CBU of Novozyme 188/ g-glucan, 50˚C, pH 4.8 maintained by 0.05 M citrate buffer, 150 rpm. The probability level 0.05 (p-value = 0.05) was used to test the significance.

3.4. Simultaneous saccharification of co-fermentation (SSCF) of pretreated corn stover

It was reported by Kim and Lee (2007) that retention of hemicellulose fraction with solids after pretreatment is a desirable feature. It is well known that commercial cellulase enzymes exhibit considerable xylanase activity as well as cellulase activity. Therefore, the ammonia-based pretreatment is well suited for simultaneous saccharification and co-fermentation (SSCF) because the treated biomass retains cellulose as well as hemicellulose (Kim et al., 2008). Simultaneous saccharification and co-fermentation (SSCF) of ammonia pretreated corn stover and was performed using recombinant *Escherichia coli* strain ATCC 55124® (KO11) at 15 FPU/g-glucan of cellulase and 30 CBU/g-glucan. The ethanol yields resulted from the corn stover that was pretreated with aqueous ammonia under various conditions are summarized in Table 4.3. Thirty percent of ammonia loading is

required so as to achieve more than 70% ethanol yield. The theoretical maximum ethanol yield (100%) on the basis of total carbohydrates (glucan+xylan) in untreated corn stover corresponds to 26.3 g/L with 3% w/v glucan loading. The highest ethanol yield achieved in the SSCF of low-liquid aqueous ammonia pretreated corn stover was 73% (of the theoretical maximum); the equivalent ethanol concentration was 19.2 g/L at 96 h. The yield of ethanol on the basis of glucan content alone was 113% of theoretical maximum, indicating that the xylan fraction was utilized in the SSCF by KO11. The glucose, xylose and ethanol profiles are presented in Fig. 4.5.

Table 4.2 Comparison of lignin contents and enzyme digestibilities of 4-week and 12-week pretreated corn stover

No.	Conditions		Lignin [%]		Glucan digestibility [%]	
	NH_3 (wt %)	Solid: liquid	4-week	12-week	4-week	12-week
1	0.5	1:0.2	17.2	17.5	22.5	21.9
2	0.5	1:0.5	16.9	17.2	24.3	24.6
3	0.5	1:1	17.8	17.0	24.3	25.8
4	0.5	1:2	17.5	16.9	20.7	22.0
5	0.5	1:5	17.8	17.8	22.5	22.5
6	2	1:0.2	18.2	17.3	29.7	30.8
7	2	1:0.5	16.4	17.2	30.6	30.5
8	2	1:1	16.5	18.8	32.4	32.6
9	2	1:2	17.2	17.0	33.3	33.2
10	2	1:5	17.2	16.0	27.9	28.5
11	5	1:0.3	17.0	17.3	48.6	49.0
12	5	1:0.5	15.2	16.5	51.4	51.4
13	5	1:1	14.2	16.6	54.1	53.9
14	5	1:2	14.1	15.8	52.3	52.1
15	5	1:5	16.4	14.9	46.8	47.4
16	10	1:0.5	14.4	14.1	62.2	62.3
17	10	1:1	14.1	14.0	66.7	66.8
18	10	1:2	11.7	12.3	60.4	60.6
19	10	1:5	12.8	11.8	51.4	52.4
20	30	1:2	8.9	9.7	78.4	78.4
21	30	1:5	8.3	6.3	72.1	78.0
22	50	1:2	8.0	6.8	82.9	82.8
23	50	1:5	7.8	4.8	86.5	86.1
Paired t-test *p*-value			0.5857		0.0545	

These results confirmed that the ammonia-based pretreatment is well suited for simultaneous saccharification and co-fermentation (SSCF) since virtually all of glucan and over 80% of xylan remained in the solids after ammonia pretreatment, providing the opportunity for increasing the ethanol yield from the available fermentable sugars and simplifies the bioconversion process (Kim and Lee 2005b; Kim and Lee 2007).

3.5. Low-liquid aqueous ammonia pretreatment vs. soaking in aqueous ammonia (SAA) pretreatment

The low-liquid aqueous ammonia pretreatment may reduce the energy cost, ammonia loading and water consumption compared with soaking in aqueous ammonia (SAA) pretreatment, of which the optimized conditions are 1:6-10 solid-to-liquid ratio (dry biomass (g): weight of 15 wt. % aqueous ammonia), 60-80˚C, 8-24 h (Kim and Lee, 2005b; Kim and Lee, 2007). For 100g of dry biomass, with SAA pretreatment, 90g of ammonia (if 1:6 solid-to-liquid ratio is applied) and 510g of water are required, whereas with low-liquid pretreatment, 50g of ammonia (if ammonia loading is 50 wt. %) and 150g of water (if solid-to-liquid ratio 1:2 is applied) are required. The ammonia loading was reduced by 44%. The water consumption for the pretreatment process was reduced by 71%. Meanwhile, the energy cost can be reduced as well by using lower temperature (30˚C instead of 60-80˚C). Although longer reaction time is required in low-liquid ammonia pretreatment, this drawback can be overcome by coupling with year-round storage and by utilizing continuous pretreatment system composed of sufficient number of vessels. In this proposed continuous pretreatment scheme, raw biomass is loaded by batch into individual vessels at certain time interval (defined by the downstream biological processes); once a batch of pretreatment completes, the pretreated feedstock is discharged from the vessel, and the vessel is loaded

with new raw feedstock; therefore batches of pretreated biomass can be obtained in succession and the pretreatment is not restricted by the long treatment period. Fig. 4.6 represents the overall mass balance of low-liquid pretreatment of corn stover using aqueous ammonia under the treatment conditions of 50 wt. % ammonia loading, 1:2 solid-to-liquid ratio, 30˚C, and 4 weeks, and the simultaneous saccharification and cofermentation (SSCF) of pretreated corn stover with recombinant *E. coli* (KO11).

Table 4.3
Ethanol yields resulted from the corn stover that was pretreated with aqueous ammonia under various conditions

Conditions				Ethanol Yield [%]
NH_3 (wt. %)	Solid: liquid	Temperature (˚C)	Time (Week)	
2	1:0.2	30	4	6
5	1:0.5	30	4	45
10	1:1	30	4	59
30	1:2	30	4	71
50	1:2	30	4	73

SSCF: *Escherichia coli* ATCC® 55124, 3% w/v glucan/ 100 mL working volume of low-liquid aqueous ammonia treated corn stover, 15 FPU of GC220/g-glucan, 30 CBU of Novozyme 188/g-glucan; LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.

4. Conclusion

Low-liquid treatment with aqueous ammonia is an effective pretreatment method for corn stover for the purpose of bioethanol production considering its delignification and carbohydrates retention effects, which contribute to high enzyme digestibility and overall ethanol yield in SSCF.

Within the range of interest in this study, the effect of ammonia loading was demonstrated to be significant on delignification and enzyme digestibility of corn stover, while increasing solid-to-liquid ratio and extending reaction time over 4 weeks do not exhibit significant effects on the delignification and enzyme digestibility of corn stover.

It is anticipated that this method can reduce the energy cost substantially, compared to conventional methods such as soaking in aqueous ammonia (SAA) pretreatment because this method requires low liquid throughput and moderate treatment conditions. Therefore, low-liquid aqueous ammonia treatment has potential to be an effective way for the pretreatment of corn stover with the advantage of being simple and more cost-competitive.

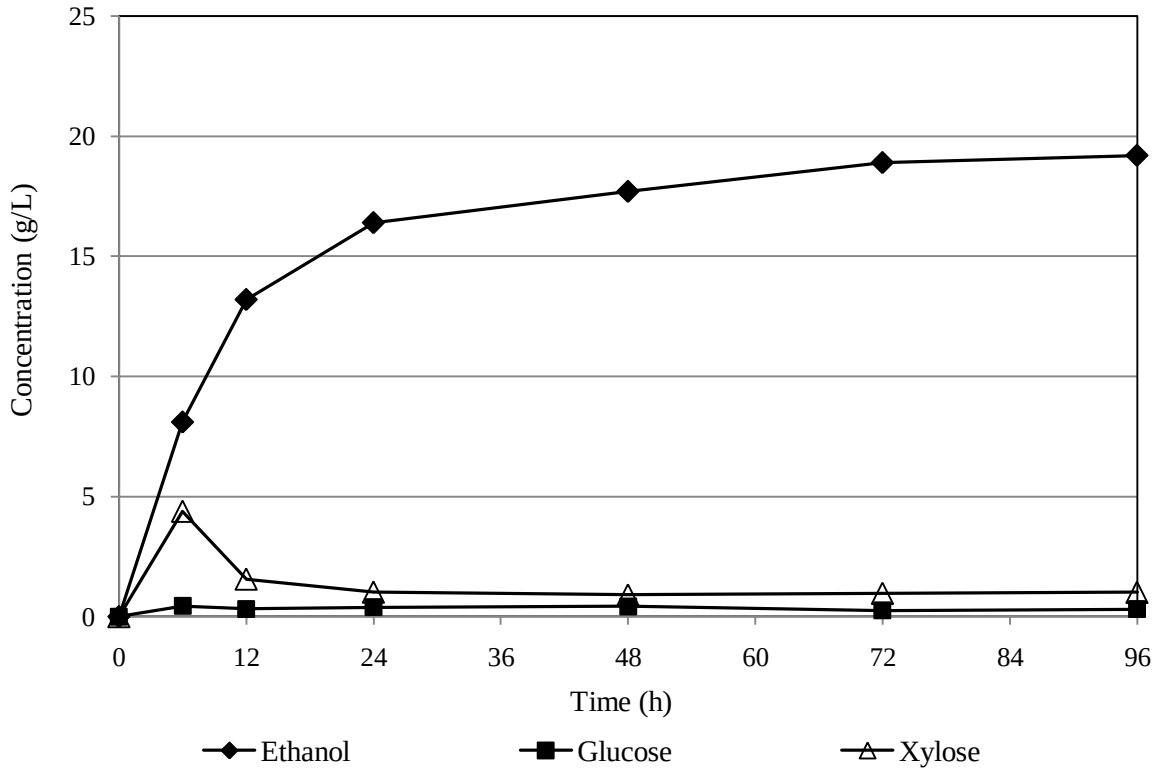


Fig. 4.5. Trajectory of simultaneous saccharification and co-fermentation (SSCF) of low-liquid aqueous ammonia treated corn stover by recombinant *E. coli* (KO11). Pretreatment: 30 wt. % of ammonia loading, 1: 2 of solid-to-liquid ratio, 70˚C, 4 weeks; SSCF: *Escherichia coli* ATCC® 55124, 3% w/v glucan/ 100 mL working volume of low-liquid aqueous ammonia treated corn stover, 15 FPU of GC220/g-glucan, 30 CBU of Novozyme 188/g-glucan, LB medium (0.5% of Yeast extract, 1% of Tryptone), 40mg/L of Chloramphenicol, anaerobic condition, 37˚C, 150 rpm.

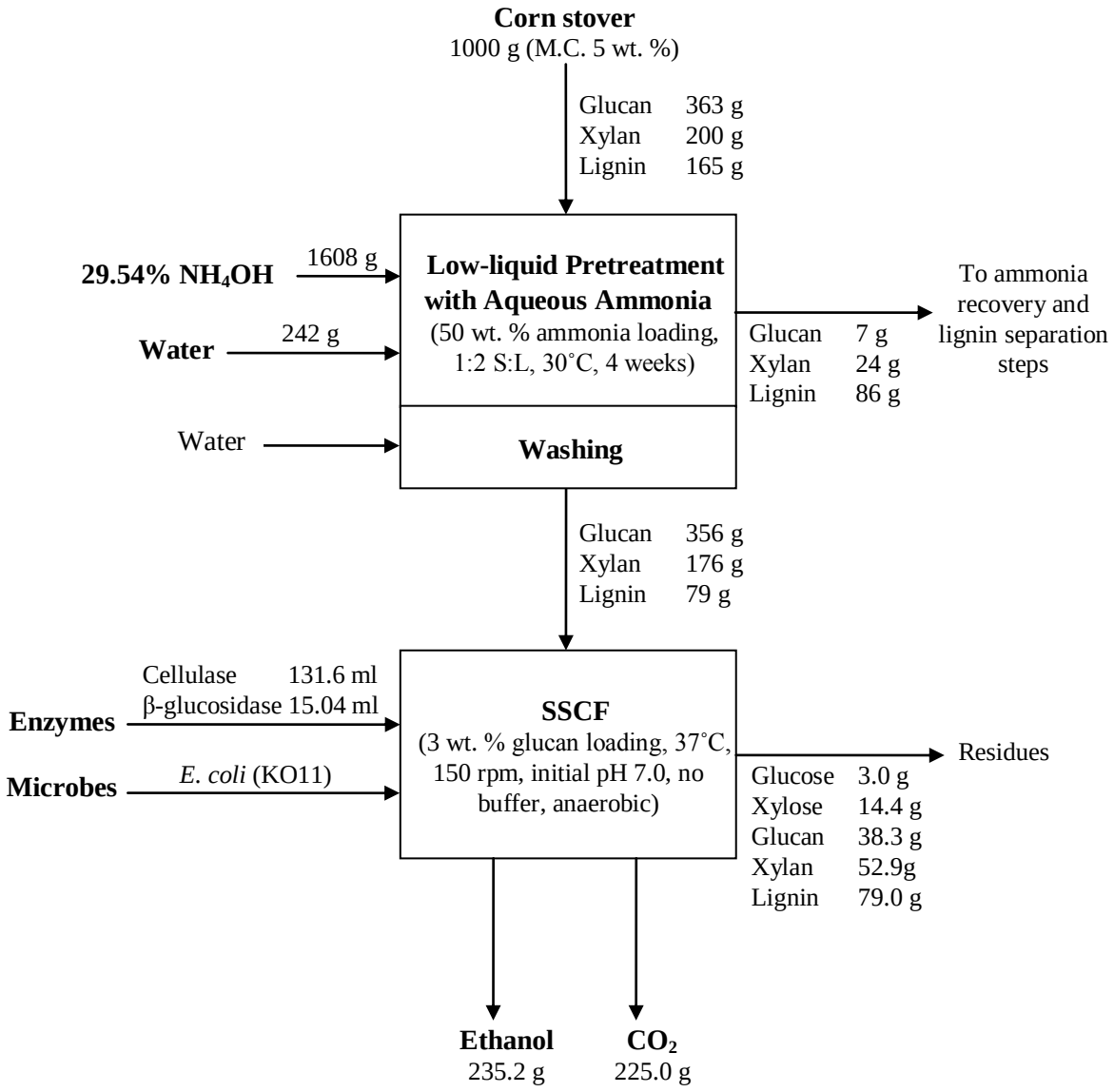


Fig. 4.6. Overall mass balance of low-liquid pretreatment of corn stover using aqueous ammonia and simultaneous saccharification and cofermentation (SSCF)

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CHAPTER 5. GENERAL CONCLUSIONS AND FUTURE WORK

1. Conclusions

This thesis represents a summary of the research project "bioethanol production from lignocellulosic feedstock using aqueous ammonia pretreatment and simultaneous saccharification and fermentation (SSF)". The bioconversion of lignocellulose to produce ethanol consists of three main steps, i.e., pretreatment, enzymatic saccharification and microbial fermentation, the latter two of which can be integrated to be the SSF process. In particular, this work focuses on the process development and optimization of chemical pretreatment and biological conversion of lignocellulosic biomass to ethanol.

In chapter two, a process integrating soaking in aqueous ammonia (SAA) pretreatment and two-phase simultaneous saccharification and fermentation (TPSSF) was developed. The TPSSF process consists of pentose conversion using xylanase (supplemented with endoglucanase) and recombinant *Escherichia coli* KO11 in the first phase and hexose conversion with cellulase, β-glucosidase and *Saccharomyces cerevisiae* D_5A in the second phase. With xylan-rich SAA-pretreated corn stover as substrate, the TPSSF simplified the bioconversion process by integrating pentose and hexose transformation into a single reactor and eventually realized the efficient utilization of both pentoses and hexoses derived from corn stover for ethanol production. The utilization of xylan was improved because the inhibition of glucose on xylose fermentation, which was always the case in conventional simultaneous saccharification and cofermentation (SSCF) of a mixture of glucose and xylose, was circumvented by converting xylan prior to glucan, contributing to the overall ethanol

yield based on the total carbohydrates in untreated feedstock. Moreover, in the second phase SSF by adopting *S. cerevisiae* D5A, a robust yeast strain capable of producing ethanol via anaerobic fermentation from glucose with high ethanol yield and excellent ethanol tolerance, stable and efficient utilization of glucose was guaranteed and the fermentation was no longer restricted by the relatively low ethanol tolerance of genetically engineered pentose-fermenting strains used in the first phase.

Chapter three documents the trial tests of cascade simultaneous saccharification and fermentation (CSSF), a process derived from the two-phase simultaneous saccharification and fermentation (TPSSF). The CSSF which applies sequential xylan SSF and glucan SSF interconnected as a cascade resulted in the accumulation of ethanol in a stage-wise mode, indicating that the enzymes were partially recycled by simple centrifugation and solid-liquid separation. The final ethanol concentration obtained via CSSF was increased compared with TPSSF process and the overall enzyme loading was reduced by half. However, the CSSF exhibits deficiencies in declined ethanol yield, low productivity, extended fermentation period and high process complexity. The prime reason for these results lies in the low recovery yield of enzymes via single centrifugation, rendering the low enzyme loading in the subsequent stage of fermentation. Considering the preliminary results obtained and the problems hypothesized in this work, the future efforts can be made from the following aspects: (1) to introduce or develop effective enzyme recycling technology; (2) to optimize the bioreactor configuration, making the operation simple and precise for the multi-stage cascade fermentation. Nevertheless, the CSSF is innovative and constructive in integrating enzyme recycling and SSF and increasing the final ethanol concentration by way of stage-wise accumulation, which serves as an alternative to direct high solid fermentation in

which insufficient mass and heat transfer always constitutes a major difficulty for efficient bioconversion.

In chapter four, a low-liquid pretreatment method of corn stover using aqueous ammonia was investigated and the reaction parameters that might influence the pretreatment effects were thoroughly evaluated. The research work demonstrates that the low-liquid pretreatment of corn stover with aqueous ammonia is effective for the purpose of ethanol bio-production taking into account its significant delignification and carbohydrates retention effects, which contribute to high enzyme digestibility and overall ethanol yield in simultaneous saccharification and cofermentation (SSCF). Within the range of interest in this study, the effect of ammonia loading was demonstrated to be significant on delignification and enzyme digestibility of corn stover, while increasing solid-to-liquid ratio and extending reaction time over 4 weeks do not exhibit significant effects on the delignification and enzyme digestibility of corn stover. This method reduces the ammonia loading, water consumption, and reaction severity in pretreatment process, and thus has the potential of reducing the energy input and liquid throughput compared with soaking in aqueous ammonia (SAA) pretreatment, offering the opportunity of making the lignocellulose-to-ethanol bioconversion process more cost-competitive.

2. Future work

2.1. High solid fermentation

Two factors influence the production cost of ethanol from biomass most significantly: the effective conversion of all the sugars to ethanol, and the concentration of ethanol in the fermentation broth prior to distillation (Öhgren et al., 2006). The ethanol concentration in the feed has a major effect on the energy demand, especially at concentrations below 4 wt. %

(Zacchi and Axelsson, 1989). Increasing the ethanol concentration in the feed to the distillation reduces the production costs considerably (Wingren et al., 2003). To lower the cost of ethanol distillation of fermentation broth, a high initial substrate concentration is desirable. However, an increase in the substrate concentration typically reduces the ethanol yield because of insufficient mass and heat transfer (Um and Hanley, 2008).

The high solid fermentation can be achieved via appropriate bioreactor configuration or fermentation scheme. Mohagheghi et al. (1992) used sulfuric acid pretreated wheat straw as the substrate and designed a simple rotary fermentor instead of the conventional impeller mixers used in laboratory fermentors to conduct the high solid SSF. The results indicate that the cellulose in pretreated wheat straw can be efficiently fermented into ethanol for up to a 15% cellulose concentration (24.4% straw concentration). Um and Hanley (2008) also reported that the enzymatic digestibility of Solka Floc with a baffled Rushton impeller was significantly higher than that obtained with a baffled marine impeller. With the corn stover pretreated by alkaline and acidic wet oxidation (WO) at 15% dry matter loading, 120 h SSF gave the highest ethanol concentration of 52 g/L (6 vol. %), which exceeds the technical and economical limit of the industrial-scale alcohol distillation. A further increase of substrate concentration reduced the ethanol yield significant as a result of insufficient mass transfer (Varga et al., 2004). High solid fermentation can also be achieved by adopting proper scheme, e.g., fed-batch fermentation, in which the substrate is not loaded all at once at the beginning of fermentation, allowing the liquefaction of solid substrate and therefore facilitating the mass and heat transfer. The optimization of fed-batch fermentation can be achieved by adjusting the feed rate of substrate.

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