# INTEGRATED BIOLOGICAL PROCESSES FOR CONVERSION OF AFEX™ PRETREATED BIOMASS TO ETHANOL

By

Mingjie Jin

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

CHEMICAL ENGINEERING

2012

# ABSTRACT


INTEGRATED BIOLOGICAL PROCESSES FOR CONVERSION OF AFEX™ PRETREATED BIOMASS TO ETHANOL

By

Mingjie Jin

Ethanol production from lignocellulosic biomass has gained much momentum due to its benefits to energy security, reduction of green house gas emission, as well as both environmental and social sustainability. The technology for lignocellulosic ethanol production, however, is not yet fully commercialized. The major issues impeding the cellulosic ethanol production in the biochemical route include the high enzyme loadings needed, long enzymatic hydrolysis time, slow xylose fermentation and low ethanol productivity, which result in a high production cost.

Ammonia Fiber Expansion (AFEX™) is a leading alkaline pretreatment. It provides a biomass substrate with high enzymatic digestibility and high fermentation potential. Previous studies on ethanol production from AFEX™ pretreated biomass focused on a separate enzymatic hydrolysis and fermentation process (SHF). However, integrated biological processes, such as simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP), are strongly believed to have lower cost compared to SHF. This dissertation work studies the integrated biological processes performed on AFEX™ pretreated biomass and resolves the aforementioned issues for biochemical production of cellulosic ethanol.

The slow xylose fermentation issue in hydrolysate by *Saccharomyces cerevisiae* 424A (LNH-ST) was quantitatively studied. The xylose fermentation inhibition was not only from degradation products but also from ethanol and metabolites generated during glucose fermentation (Chapter II). Based on such understanding, a two-step SSCF process was developed, in which xylan was hydrolyzed and fermented ahead of glucan/glucose. As a result, xylose fermentation was greatly improved (Chapter III). Through the study of conventional SSCF on AFEX$^{TM}$ treated corn stover (CS), it was found that pre-hydrolysis to generate a certain amount of glucose was crucial for achieving a good result (Chapter IV). A high solids loading process can save much cost for ethanol production. However, ethanol yield decreased with increasing solids loading. From an economic point of view, 6% (w/w) glucan loading was the optimal solids loading during SSCF of AFEX$^{TM}$ CS (Chapter V). For improvement of productivity, a continuous SSCF process using multi-stage continuous stirred tank reactors (CSTRs) was developed based on the kinetic studies of the two reactions in SSCF (enzymatic hydrolysis and fermentation)(Chapter VI). Based on the fundamental understandings of the cellulosic ethanol production, a novel industrially-relevant integrated biological process was developed. This process shortened the biological processing time (including enzymatic hydrolysis and fermentation) from 11 days to around 2 days, reduced enzyme loading by more than 1/3 and enhanced ethanol productivity by 2-3 times (Chapter VII). Consolidated bioprocessing (CBP) eliminates the enzyme cost and is believed to be the ultimate low-cost industrial configuration for cellulosic ethanol production. CBP studies using *Clostridium phytofermentans* on AFEX$^{TM}$ CS at both low and high solids loadings showed promising results (Chapter VIII & IX).

*AFEX is a trademark of MBI, International of Lansing, Michigan.*

This work is dedicated to my parents, Jianlin Jin and Meiying Yu, without whose unconditional love and support this effort could not have been possible.

# ACKNOWLEDGMENTS

I would like to thank my distinguished committee members: Professors Bruce E. Dale (Chair), Venkatesh Balan, Gemma Reguera, Robert M. Worden and Robert P. Hausinger, for their support, encouragement and valuable suggestions. I would like to specially thank my advisor Dr. Dale and Dr. Balan for providing detailed guidance and support during my Ph.D. study period. I am grateful to my QB program advisor Dr. Reguera and Professor Lee R. Lynd from Dartmouth College for providing anaerobic fermentation training and resources.

I am indebted to all the Biomass Conversion Research Laboratory (BCRL) members for their encouragement, insightful comments and support. I would like to particularly thank Christa Gunawan for working with me on many projects and Bryan Bals for support on the techno-economic analyses. I would also like to thank Ming Woei Lau, Dahai Gao, Leonardo da Costa Sousa, Rebecca Garlock, James Humpula, Pete Donald, Mary Ann Vigil, Nirmal Uppugundla, Nick Posawatz and Derek Marshall for their support.

My thanks go to all the people ever helped and encouraged me. Thank the Great Lakes Bioenergy Research Center (GLBRC). I thank Xiongjun Shao (Dartmouth College) and Jenna Young for their help on the anaerobic fermentation training.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# ABBREVIATIONS

| | |
|---|---|
| AFEX | Ammonia Fiber Expansion |
| BCRL | Biomass Conversion Research Laboratory |
| CBP | Consolidated bioprocessing |
| CFU | Colony forming unit |
| CS | Corn Stover |
| CSL | Corn steep liquor |
| CSTR | Continuous stirred tank reactor |
| EH | Enzymatic hydrolysis |
| EtOH | Ethanol |
| Glc | Glucose |
| LCC | Lignin-carbohydrates complex |
| MESP | Minimum ethanol selling price |
| SG | Switchgrass |
| SHF/SHcF | Separate hydrolysis and fermentation/co-fermentation |
| SSCF | Simultaneous saccharification and co-fermentation |
| SSF | Simultaneous saccharification and fermentation |
| WE | Water extract |
| XD | Xylitol dehydrogenase |

| | |
|---|---|
| XK | Xylulokinase |
| XR | Xylose reductase |
| Xyl | Xylose |
| YE | Yeast extract |
| YEP/YP | Yeast extract and peptone medium |

# CHAPTER I BACKGROUND AND GENERAL INTRODUCTION

## 1.1 Cellulosic ethanol production

### 1.1.1 Background

Development of renewable transportation fuels (biofuels) has gained much momentum due to the increasing energy demand, limited reserves of petroleum, required energy independence as well as environmental concerns (Farrell et al., 2006; Greene & Council, 2004; Hallac & Ragauskas, 2011; Wyman, 2008; Wyman, 2007). Currently, the US is consuming 20 million barrels of crude oil per day with over 70% going to transportation (Gray et al., 2006; Shaw et al., 2008) and the transportation area almost totally relies on petroleum (Wyman, 2007). The large-scale use of fossil fuels is recognized as a major factor resulting in the green house effect (Houghton et al., 2001). Since the use of fossil fuels is not sustainable, fossil fuels utilization as the main world's energy is greatly threatened (Yu et al., 2003). In such an atmosphere, substitutes to fossil fuels for transportation are badly needed. Bioethanol ($CH_3$-$CH_2$-OH), which is renewable and produces little green house gas when consumed (Hansen et al., 2005), is thought to be a promising liquid biofuel to replace a large amount of petroleum in transportation.

Fuel ethanol production in the US keeps increasing from year to year (Fig. 1). Today, over 13 billion gallons of ethanol are produced per year by the US (www. http://www.eia.gov/) with corn as the major substrate. However, corn ethanol is not sufficient to make a big impact on the transportation fuel use (Yang & Wyman, 2008). Besides, fuel production using corn, which is a food, is controversial. The US government's goal is to replace 30% of the gasoline consumption by biofuels by 2030 (Perlack et al., 2005), which requires around 60 billion gallons ethanol. Lignocellulosic biomass, such as corn stover (CS), swithgrass (SG), agricultural and forestry residues, which amounts to 1.3 billion tons (corresponding to about 80 billion gallons ethanol)

per year produced by the US (Perlack et al., 2005), can offer sufficient ethanol volume to achieve the government's goal and make a big impact on the petroleum use in the transportation area. Corn ethanol production is a mature technology with glucose from enzymatic liquefaction and saccharification of starch fermented to ethanol by *Saccharomyces* yeasts (Gray et al., 2006). Cellulosic ethanol technology is more complicated and not yet fully commercialized.

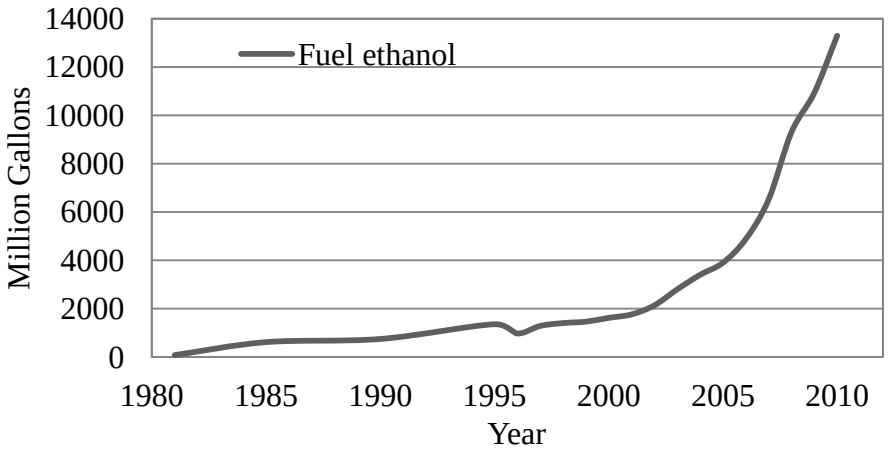



Figure 1 Fuel ethanol production in the U.S.

### 1.1.2 Cellulosic ethanol production

Cellulosic ethanol refers to the ethanol produced from lignocellulosic biomass. Lignocellulosic biomass typically contains 36-61% of cellulose and 13-39% of hemicelluloses (Olsson & Hahn-Hägerdal, 1996), which are the sugars for ethanol production. These sugars are buried in a complex carbohydrate-lignin network (Chundawat et al., 2011a), which is highly recalcitrant to enzymes hydrolysis and thus renders pretreatment necessary for the biochemical route of cellulosic ethanol production (Yang & Wyman, 2008). Biochemical cellulosic ethanol production process is composed of 7 steps (Fig. 2):

- Lignocellulosic biomass (feedstock) production and harvest.
- Pretreatment to disrupt the structure of plant cell wall making substrates more digestible

to enzymes (Yang & Wyman, 2008).

- Enzyme production. To hydrolyze cellulose to glucose, at least three types of cellulases are needed: endoglucanase (1,4-*β*-D-glucan glucanohydrolase; EC 3.2.1.4), exoglucanase (1,4-*β*-D-glucan cellobiohydrolase; EC 3.2.1.91) and *β*-glucosidase (cellobiase or *β*-D-glucoside glucohydrolase; EC 3.2.1.21) (Kabel et al., 2006). Xylanases are required for hydrolyzing hemicelluloses (Zhong et al., 2009).
- Enzymatic Hydrolysis (EH) of pretreated feedstock to fermentable sugars.
- Fermentation of hexose to ethanol. The most widely used genus for ethanol production is *Saccharomyces* yeasts. The reaction can be expressed as (Hamelinck et al., 2005):

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

- Fermentation of pentose to produce ethanol.

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5\ CO_2$$

The theoretical yield is 0.51 g ethanol / g sugar (Hamelinck et al., 2005).

- Ethanol Recovery.

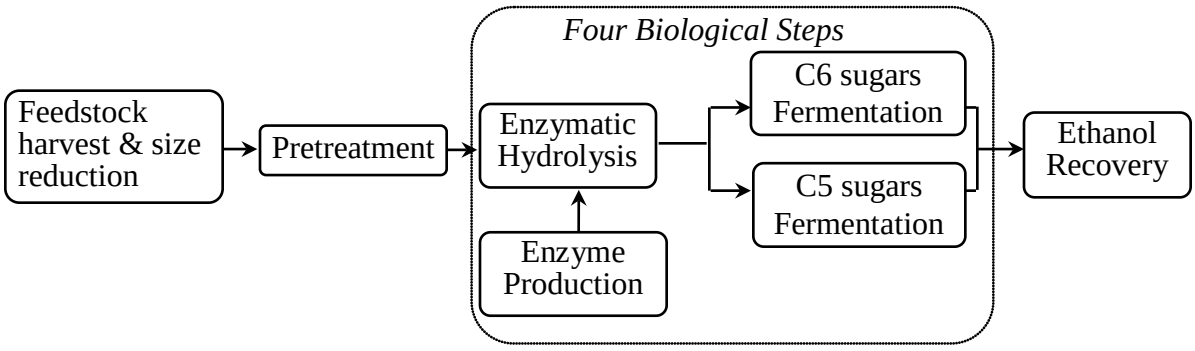



Figure 2 Biochemical process for cellulosic ethanol production

### 1.1.3 Biomass pretreatment

Linocellulosic biomass is composed of three major polymers: cellulose, hemicelluloses and lignin. Cellulose is linear and comprises *β*-D-glucopyranose moieties linked by *β*-(1,4)

glycosidic bonds (Somerville et al., 2004). Cellulose chains bundled together form microfibrils which are further packed to create cellulose fibers (Chundawat et al., 2011a). Hemicelluloses are heterogeneous polymers consisting of various sugars including xylose, arabinose, galactose, glucose and mannose (Saha, 2003). Hemicelluloses act like a "coat" to cellulose (Agbor et al., 2011). Lignin glues the components of lignocellulosic biomass together by forming lignin-carbohydrates complex (LCC) linkages and thus makes the lignocellulosic biomass recalcitrant to microbial/enzymatic attack. Three major mono-lignols for forming lignin are *p*-coumaryl alcohol (H lignin), coniferyl alcohol (G lignin) and sinapyl alcohol (S lignin) (Vanholme et al., 2010). Due to these plant cell wall properties, the major factors affecting enzymatic hydrolysis of lignocellulosic biomass include high crystallinity of cellulose, low accessible surface area for enzymes, high lignin content and high degree of polymerization. The role of pretreatment is to modify the properties of the lignocellulosic biomass for high digestibility by enzymes (e.g. decrystallize cellulose, increase accessible surface area, remove lignin, de-polymerization, alter lignin structure and remove hemicelluloses).

Many pretreatment technologies have been invented, such as physical pretreatment comminution and biological pretreatment using white-rot fungi (Agbor et al., 2011). However, the most efficient and cost-effective ones belong to physicochemical pretreatment such as dilute acid, steam explosion, liquid hot water, lime, ammonia recycle percolation (ARP), soaking aqueous ammonia (SAA) and ammonia fiber expansion (AFEX$^{TM}$) (Poth et al., 2011). Depending on the choice of pretreatment the composition and properties of the substrate may change and thereby affect the digestibility and fermentability during the downstream processes (Yang & Wyman, 2008). During physicochemical pretreatment, it is almost impossible to avoid the formation of degradation products, which are inhibitory to enzymatic hydrolysis and

fermentation. The degradation products could derive from glucose such as 5-hydroxymethyl-2-furaldehyde (HMF), or from xylose such as furfural, or from lignin such as phenolics (Almeida et al., 2007). Degradation compounds are also formed by cleaving LCC linkages (e.g. acetic acid) (Almeida et al., 2007). Ideal pretreatment technology should not only provide a biomass substrate with high enzyme digestibility and high fermentability but also have low capital and operating costs. Eggeman et al. (Eggeman & Elander, 2005) techno-economically compared the existing leading pretreatments and concluded that dilute acid and AFEX™ are the two most cost-effective pretreatments with a potential minimum ethanol selling price (MESP) of $1.35 and $1.41 per gallon, respectively.

### 1.1.4 Ammonia fiber expansion (AFEX™) pretreatment

AFEX™ is an alkaline thermo-chemical pretreatment using ammonia as the catalyst (Balan et al., 2010; Morales-Rodriguez et al., 2011). As shown in Fig. 3, untreated biomass with certain moisture content and ammonia are fed into the preheated AFEX™ reactor, in which

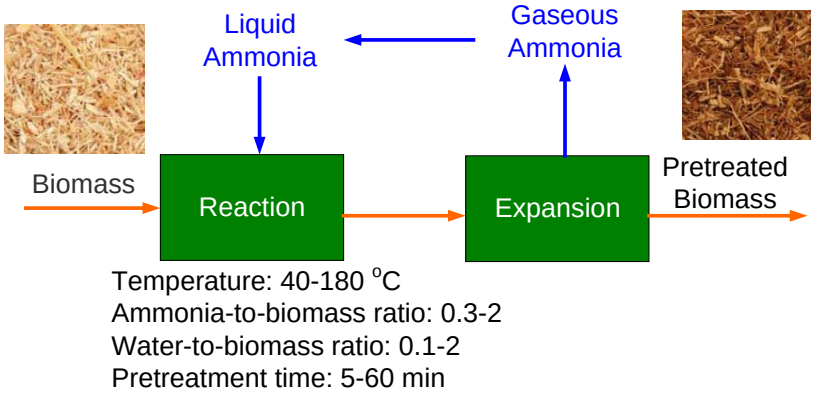



Figure 3 AFEX™ pretreatment.
This figure is in color. *For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.*

pretreatment reactions occur under certain temperature and pressure conditions. After 5-60 min, the pressure is released and ammonia is recycled. The pretreated biomass is typically darker than the untreated biomass.

AFEX$^{TM}$ pretreatment is a “dry to dry” process with no liquid stream. Two major reactions occur during AFEX$^{TM}$ are ammonolysis and hydrolysis, which cleave linkages in carbohydrate-lignin network. Some ammonia soluble lignin is extracted and relocated on the surface of the biomass (Chundawat et al., 2011c). Pores in the biomass are formed. Lignocellulosic ultra and macro structures are altered (Chundawat et al., 2011c). After AFEX$^{TM}$ pretreatment, cellulose is decrystallized, accessible surface area is increased, and hemicellulose is partially depolymerized. Cellulose and hemicellulose conversions for most AFEX$^{TM}$ treated biomass during enzymatic hydrolysis achieved greater than 90% (Yang & Wyman, 2008). Degradation products formed during AFEX$^{TM}$ pretreatment showed mild inhibitory effect on yeast fermentation (Lau & Dale, 2009). The level of organic acids like degradation products in AFEX$^{TM}$ pretreated corn stover is much lower than in diluted acid pretreated corn stover (Chundawat et al., 2010), probably due to the ammonolysis reaction during AFEX$^{TM}$ converting the compounds into amide rather than acid forms. As a result, the acetamide to acetic acid ratio in AFEX$^{TM}$ pretreated corn stover is around 5:1 (Chundawat et al., 2010). Moreover, AFEX$^{TM}$ preserves the nutrients naturally in plant biomass for yeast fermentation (Lau et al., 2012).

### 1.1.5 Process configurations based on four biological steps

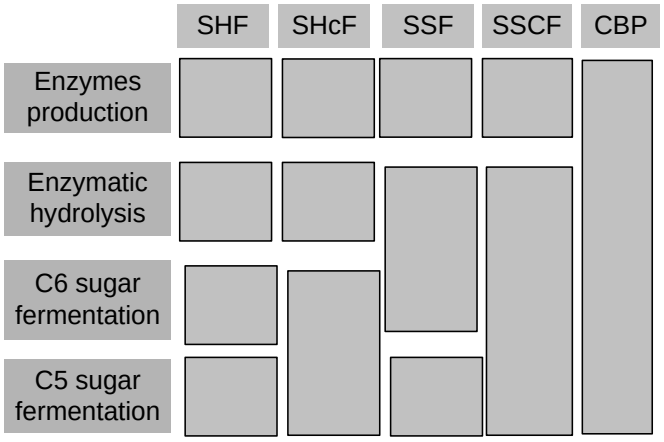



Figure 4 Process configurations (adaptated from (Lynd, 1996a)).
SHF: separate hydrolysis and fermentation; SHcF: separate hydrolysis and co-fermentation; SSF: simultaneous saccharification and fermentation; SSCF: simultaneous saccharification and co-fermentation; CBP: consolidated bioprocessing.

Biological events (Fig. 2) involved in the processing of harvested plant biomass for ethanol production include saccharolytic enzyme production, enzymatic hydrolysis, hexose fermentation and pentose fermentation (Lynd, 1996). Depending on how the four biological events are integrated, the process configuration can be separate hydrolysis and fermentation/co-fermentation (SHF/SHcF), simultaneous saccharification and (co)fermentation (SSF/SSCF), or consolidated bioprocessing (CBP) (Fig. 4) (Lynd, 1996). In the configuration of SHF/SHcF, the four biological events are conducted separately (co-fermentation performs hexose and pentose fermentation together). In the configuration of SSF/SSCF, enzymatic hydrolysis and fermentation are conducted in the same bioreactor. CBP carries out the four biological events together in a single bioreactor. This dissertation work involves SHcF, SSCF and CBP. But I would like to use term SHF to refer to SHcF since SHcF belongs to the category of SHF.

#### 1.1.5.1 Co-fermentation of glucose and xylose

Xylose in the form of xylan/hemicelluloses is the second most abundant sugar in the lignocellulosic biomass (Girio et al., 2010). From an economic-viability point of view, it is critical to convert xylan/xylose as well as cellulose/glucose to ethanol (Weber et al., 2010). During the past decades, numerous microorganisms, such as *Escherichia coli* (Olsson et al., 1995), *Zymomonas mobilis* (Zhang et al., 1995), and *Schefferosomyces* (*Pichia*) *stipitis* (Jeffries et al., 2007) have been genetically modified to accomplish this goal. *E.coli* can natively consume xylose as well as arabinose (Weber et al., 2010). Genetically modified strain *E.coli* KO11 possesses pyruvate decarboxylase and alcohol dehydrogenase genes (PET operon) from *Z. mobilis*, which makes it a homoethanolic fermentation strain (Ingram et al., 1999). *Z. mobilis* AX101 with the seven xylose and arabinose metabolism genes integrated into the genome was also able to ferment pentose to ethanol with high ethanol yield (Mohagheghi et al., 2002). *S. stipitis* is also a native xylose consumer. Dissolved oxygen control is the key for ethanol production using *S. stipitis* (Skoog & Hahn-Hagerdal, 1990).

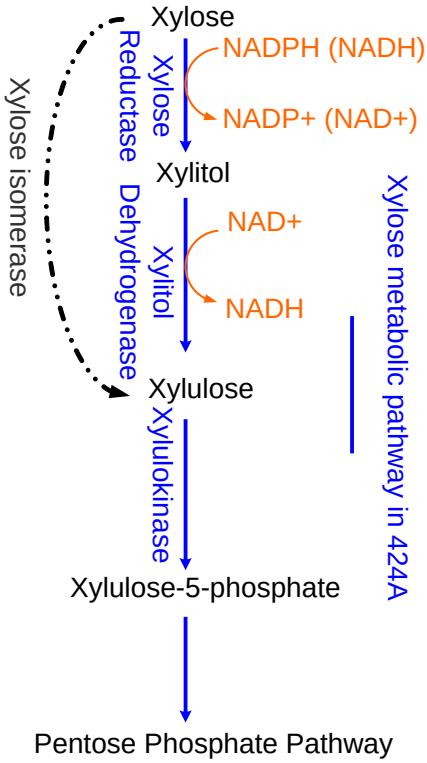



Figure 5 Xylose metabolic pathway in *S. cerevisiae* 424A (LNH-ST).
The figure is in color.

*Saccharomyces cerevisiae*, the oldest ethanol producer used by humans has also been engineered to ferment xylose (Olofsson et al., 2008b; Sedlak & Ho, 2004). For instance, *S. cerevisiae* 424A (LNH-ST) with its chromosome integrated with multiple copies of xylose reductase (XR) and xylitol dehydrogenase (XD) genes from *S. stipitis* and endogenous xylulokinase gene (XK) was able to ferment xylose with high rates (Sedlak & Ho, 2004). However, due to the fact that

NADPH is the preferable cofactor for XR and $NAD^+$ is the sole cofactor for XD (Fig. 5), the redox of those two reactions is imbalanced, which results in xylitol production (Ha et al., 2011). Mutation of XR and screen of the XR mutants preferring NADH is a way to solve this problem but this approach reduced the xylose fermentation rate (Bengtsson et al., 2009; Watanabe et al., 2007). Ha et al. applied both wild XR and XR mutants, which turned out to be a better way to reduce xylitol production and at the same time keep high xylose consumption rate (Ha et al., 2011). Strains with the xylose isomerase gene instead of XR and XD genes could also bypass the redox imbalance problem (Kuyper et al., 2005; Madhavan et al., 2009).

After all those endeavors, the xylose fermentation rate, however, is still much lower compared to glucose fermentation. One reason for slow xylose consumption is that *S. cerevisiae* does not have a specialized xylose transport system. Xylose relies on the hexose transporters to be transported into the cells (Bertilsson et al., 2008). However, the affinities of hexose transporters are much lower for xylose when compared to glucose. As a result, xylose fermentation begins only after glucose is nearly depleted in the fermentation broth. It is interesting to note that the expression of hexose transporters is regulated by the concentration of glucose (Bertilsson et al., 2008; Diderich et al., 1999). In order to create the optimum conditions for xylose uptake, the glucose concentration must be maintained at a low but not zero level (Bertilsson et al., 2008). Recently, Ha et al. constructed a recombinant xylose-fermenting *S. cerevisiae* with a cellodextrin transporter and intracellular *β*-glucosidase (Ha et al., 2011). The strain is able to co-ferment cellobiose and xylose and thus bypasses the problem of co-fermenting glucose and xylose since the glucan in the lignocellulosic biomass can be degraded to just cellobiose.

Lau et.al (Lau et al., 2010) compared ethanologens *E. coli* KO11, *S. cerevisiae* 424A(LNH-ST) and *Z. mobilis* AX101 in AFEX$^{TM}$ pretreated corn stover hydrolysate fermentation and concluded that *S. cerevisiae* 424A(LNH-ST) is the best among the tested strains in terms of xylose fermentation, tolerance to biomass degradation products and ethanol yield.

**1.1.5.2 Separate enzymatic hydrolysis and (co)fermentation (SHF/SHcF)**

Currently, the most efficient cellulase enzymes are produced from *Trichoderma reesei*. During past a few years, the cost of cellulase enzymes has been reduced by more than tenfold (Olofsson et al., 2008a). The current cellulase enzymes cost estimated by National Renewable Energy Laboratory (NREL) is about $4.24/kg protein (Humbird et al., 2011). Nevertheless, the enzyme expense is still one of the major outlays for cellulosic ethanol production, accounting for 15.7% of the total cost based on the near future technology (Humbird et al., 2011). Enzyme companies such as Novozymes and Genencor are leading the production of cellulase enzymes.

The optimal conditions using *Trichoderma* cellulases for enzymatic hydrolysis are 45-50 °C, pH 4.8 and high agitation rate (250 rpm in shake flask (Lau & Dale, 2009)) (Taherzadeh & Karimi, 2007). On the other hand, the optimal conditions for ethanol fermentations using current ethanologens (*S. cerevisiae, S. stipitis, Z. mobilis* and *E. coli*) are around 30-37 °C, pH 5.5-7.0, and low agitation rate (around 150 rpm in shake flask (Lau & Dale, 2009)) (Lau et al., 2010; Taherzadeh & Karimi, 2007). The major advantage of SHF/SHcF compared to SSF/SSCF is that enzymatic hydrolysis and fermentation can be carried out at their own optimal conditions (Taherzadeh & Karimi, 2007). However, enzymes during hydrolysis is easily inhibited by its end-products (sugars), especially during high solids loading enzymatic hydrolysis (Kristensen et al., 2009; Philippidis & Smith, 1995), which demands somewhat longer hydrolysis time and high

enzyme loading to achieve high sugar conversions. Another problem of this process is the high risk of contamination during enzymatic hydrolysis due to the long reaction time and high sugar concentrations (Taherzadeh & Karimi, 2007). Enzymatic hydrolysis is the limiting step for SHF, which determines the overall ethanol yield (Lau & Dale, 2009).

#### 1.1.5.3 Simultaneous saccharification and (co)fermentation (SSF/SSCF)

With enzymatic hydrolysis and sugar fermentation combined, SSF/SSCF removes sugar inhibition on enzymatic hydrolysis thus increases the hydrolysis sugar yield and reduces contamination risk. Moreover, SSF/SSCF reduces the overall reaction time and reactor volume (Kristensen et al., 2009). However, the optimal conditions for hydrolysis and fermentation are different. SSF/SSCF sacrifices the optimal conditions for both enzymatic hydrolysis and fermentation. Typically, for *Trichoderma reesei* cellulases – *Saccharomyces* SSF system the temperature is kept at 37°C as a compromise (Dien et al., 2003b). In addition, SSF/SSCF introduces a new inhibitor (ethanol) for enzymatic hydrolysis. But the inhibitory effect from ethanol is much lower compared to cellobiose or glucose (Taherzadeh & Karimi, 2007). SSF has been widely investigated on the steam and diluted acid pretreated biomass while SSCF studies were limited (Olofsson et al., 2008a).

#### 1.1.5.4 Consolidated bioprocessing (CBP)

Both SHF/SHcF and SSF/SSCF require expensive enzymes production in a separate bioreactor, which is a major cost barrier for commercialization of cellulosic ethanol (Banerjee et al., 2010). CBP which carries out the four biological events together in a single bioreactor reduces the enzyme production cost and at the same time possesses all the advantages of SSCF. A strong case has been made that CBP is the ultimate low-cost industrial configuration to produce cellulosic ethanol (Lynd et al., 2005; Sendich et al., 2008). However, currently there's

no perfect microbe or microbe consortium capable of degrading lignocelluloses, utilizing all the carbohydrates and at the same time producing ethanol at a high yield as CBP requires (Lynd et al., 2005).

Intensive research has been carried out focusing on two strategies to develop CBP microbes (Lynd et al., 2005). The first one is genetic engineering of an excellent ethanol producer for production of cellulases and hemicellulases. So far, many kinds of endoglucanases, exoglucanases, $\beta$-glucosidase, $\beta$-xylanases and $\beta$-mannanases, of which most are fungal origin, have been expressed in *S. cerevisiae* (Crous et al., 1995; Den Haan et al., 2007; Ilmen et al., 2011; Reinikainen et al., 1992; Romanos et al., 1991; Vanarsdell et al., 1987). With high initial cell density, direct fermentation of amorphous cellulose resulting in a yield of 0.45 g ethanol per g substrate corresponding to 88.5% of the theoretical yield was achieved by co-expression of three types of cellulase in *S. cerevisiae* (Fujita et al., 2004). The other strategy is genetic modification of a cellulolytic microbe, which can efficiently degrade lignocellulosic materials, to make it also capable of producing ethanol at a high yield. Among cellulolytic microbes, the thermophilic anaerobic bacterium *Clostridium thermocellum* received the most attention (Demain et al., 2005). *C. thermocellum* can degrade cellulose efficiently through a cellulase complex system called "*cellulosome*" whose specific activity is much higher compared to *Trichoderma* cellulases (Carere et al., 2008). *C. thermocellum* can also produce ethanol by utilizing sugars from cellulose degradation but with production of a large amount of by-products: acetate and lactate (Desvaux, 2006). Xylanases are included in the enzymes secreted by *C. thermocellum* which can break down hemicelluloses (Morag et al., 1990). However, *C. thermocellum* cannot utilize pentose. So co-culture of *C. thermocellum* with other thermophilic microbes which can consume pentose is a good option and received considerable investigation

(Demain et al., 2005). Ethanol yield reached as high as 1.8 mol per mol of anhydroglucose on MN300 cellulose by co-culturing *C.thermocellum* and *Clostridium thermohydrosulfuricum* (Ng et al., 1981). *Thermoanaerobacterium saccharolyticum* which can ferment xylan and almost all the soluble sugars from biomass with ethanol, acetate and lactate as the end metabolites is also considered as a suitable species to co-culture with *C. thermocellum* (Lynd et al., 2005). Shaw and co-workers have successfully developed an ethanologic strain *T. saccharolyticum* ALK2 by knockout of the genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase) (Shaw et al., 2008). Similar targeted gene knockouts have also been done on *C. thermocellum* to eliminate side product synthesis and improve the ethanol yield (Argyros et al., 2011). By co-culture of the engineered *C. thermocellum* with *T. saccharolyticum* ALK2 on 92 g/L Avicel (pure cellulose), an ethanol titer of 38 g/L was achieved, which is the highest ethanol titer achieved by thermophilic CBP microbes to date (Argyros et al., 2011). Nevertheless, the performance of this co-culture system on real biomass is still unknown. The low tolerance of the two microbes to biomass degradation products as well as ethanol could be an issue.

The advantages of anaerobic thermophilic microbes over yeast as CBP strains are: 1) they ferment almost all the sugars in lignocellulosic materials; 2) no air supply is needed since enzyme production is also anaerobic as well as ethanol fermentation, which reduces costs; 3) High fermentation temperature accelerates enzymatic hydrolysis, prevents contamination, facilitates ethanol recovery, and cuts cooling cost. 4) Low cell yield makes most of the sugars convert to ethanol. 5) Much higher enzyme specific activities require less quantity of enzymes and in turn less nutrients and sugars. 6) Utilizing hexose and pentose at the same time results in

higher pentose consumption rate. 7) Oligosaccharides can also be used, leading to a higher ethanol yield (Zhang & Lynd, 2005).

The mesophilic anaerobic strain *Clostridium phytofermentans* whose genome encodes the highest number of enzymes for degradation of lignocellulosic material among sequenced clostridial genomes (Weber et al., 2010), is also a promising CBP microbe. It secretes individual enzymes (non-complexed) like fungi and can degrade both cellulose and hemicelluloses to fermentable sugars. In addition, *C. phytofermentans* can consume almost all the sugars present in lignocellulosic biomass and produce ethanol and acetate as the major products (Warnick et al., 2002; Weber et al., 2010). It is named Q-Microbe by Qteros Company. The major disadvantage compared to *C. thermocellum* is that the enzymes cannot function at their optimal/high temperature because of the low thermo-tolerance of the microbe.

The summary of the three biological processes is listed in Table 1. CBP is the most promising process but currently no perfect CBP microbe has been reported in the literature. SHF/SHcF and SSF/SSCF are the current feasible industrial processes. So far, there is no systematic comparison study of the three processes on any pretreated biomass.

Table 1 Comparison of SHF/SHcF, SSF/SSCF and CBP

| | SHF/SHcF | SSF/SSCF | CBP[1] | CBP[2] | CBP[3] |
|---|---|---|---|---|---|
| Sugar inhibition during enzymatic hydrolysis | High | Low | Low | Low | Low |
| Enzyme loading | High | Medium-High | None | None | None |
| Contamination risk | High | Low | Low | Low | Lowest |
| Overall processing time | Long | Medium | N/A | N/A | Short |
| No. of reactors | 4-3 | 2-3 | 1 | 1 | 1 |
| Overall cost | High | Medium | Low | Low | Low |

Footnote: CBP[1] use engineered *S. cerevisiae*; CBP[2] use mesophilic anaerobic microbes such as *C. phytofermentans*; CBP[3] use thermophilic anaerobic microbes such as *C. thermocellum* and *T. saccharolyticum*

## 1.2 Ethanol production from AFEX™ pretreated biomass

Before this dissertation work, the studies of cellulosic ethanol production from AFEX™ pretreated biomass were almost all based on the SHF configuration. The critical properties of one pretreated biomass for cellulosic ethanol production include enzyme digestibility and fermentability. AFEX™ pretreated biomass is both high digestible and high fermentable, which make it a suitable substrate for cellulosic ethanol production.

### 1.2.1 High digestibility and high fermentability of AFEX™ pretreated biomass

High sugar conversions have been achieved on various AFEX™ pretreated feedstocks at high solids loading (6% (w/w) glucan loading) using commercial enzymes, which showed the high digestibility of AFEX™ pretreated biomass (Bals et al., 2010; Krishnan et al., 2010; Lau & Dale, 2009). For instance, enzymatic hydrolysis of AFEX™ treated corn stover at 6% (w/w) glucan loading reached glucan and xylan conversions as high as 86.7% and 85.0%, respectively (Fig. 6). However, 19% of the output sugars were oligomers, which cannot be fermented to ethanol by *S. cerevisiae*. Moreover, it took 96 h to reach such high sugar conversions, which is too long for an industrial process.

Fermentability of a pretreated biomass is largely determined by two factors: 1) toxicity of degradation products; 2) nutrients level. AFEX™ pretreatment generates low-toxicity degradation products and preserves nutrients naturally in the plant biomass. The residual ammonia (unrecovered) in the pretreated biomass also serves as nutrients (nitrogen source) during fermentation. Hence, AFEX™ pretreated biomass is well known having high

fermentability. The degradation products and nutrients profiles in the AFEX$^{TM}$ pretreated corn stover have been quantified, which are shown in Table 2 and Table 3, respectively. AFEX$^{TM}$ pretreated corn stover contains much lower concentrations of toxic compounds (such as acetic acid) compared to diluted acid pretreatment but has higher concentrations of low-toxic amides, which is the result of ammonolysis reaction during AFEX$^{TM}$ and is the major reason for low toxicity. AFEX$^{TM}$ pretreated corn stover hydrolysate has a total amino acid concentration and ammonia concentration of as high as 1231±44 mg/L and 750±50 mg/L, respectively, with excess trace elements for yeast fermentation, which renders nutrients supplementation unnecessary during fermentation (Lau et al., 2012; Lau & Dale, 2009). Due to its high fermentability, almost all the tested xylose-fermenting ethanologens such as *S. cerevisiae* 424A(LNH-ST), *E. coli* KO11, *Z. mobilis* AX101 and *P. stipitis* FPL-061 grew well in AFEX$^{TM}$ pretreated biomass hydrolysate without detoxification and nutrients supplementation (Lau et al., 2010; Li et al., 2010; Shao et al., 2010).

Lau & Dale (Lau & Dale, 2009) conducted a mass balance study based on AFEX$^{TM}$ pretreatment using commercial enzymes for enzymatic hydrolysis and *S. cerevisiae* 424A(LNH-ST) for fermentation (Fig. 6). It was found that little sugar was lost during pretreatment (high sugar recovery), which guaranteed potential high ethanol yield. Both sugar yield during enzymatic hydrolysis and ethanol yield during fermentation were high. Without washing, detoxification and nutrients supplementation, 191.5 g ethanol was produced from 1 kg untreated corn stover with the final ethanol concentration of 40 g/L. The ethanol metabolic yield during hydrolysate fermentation was 92.9%, which was higher than that (83.6%) for fermentation in

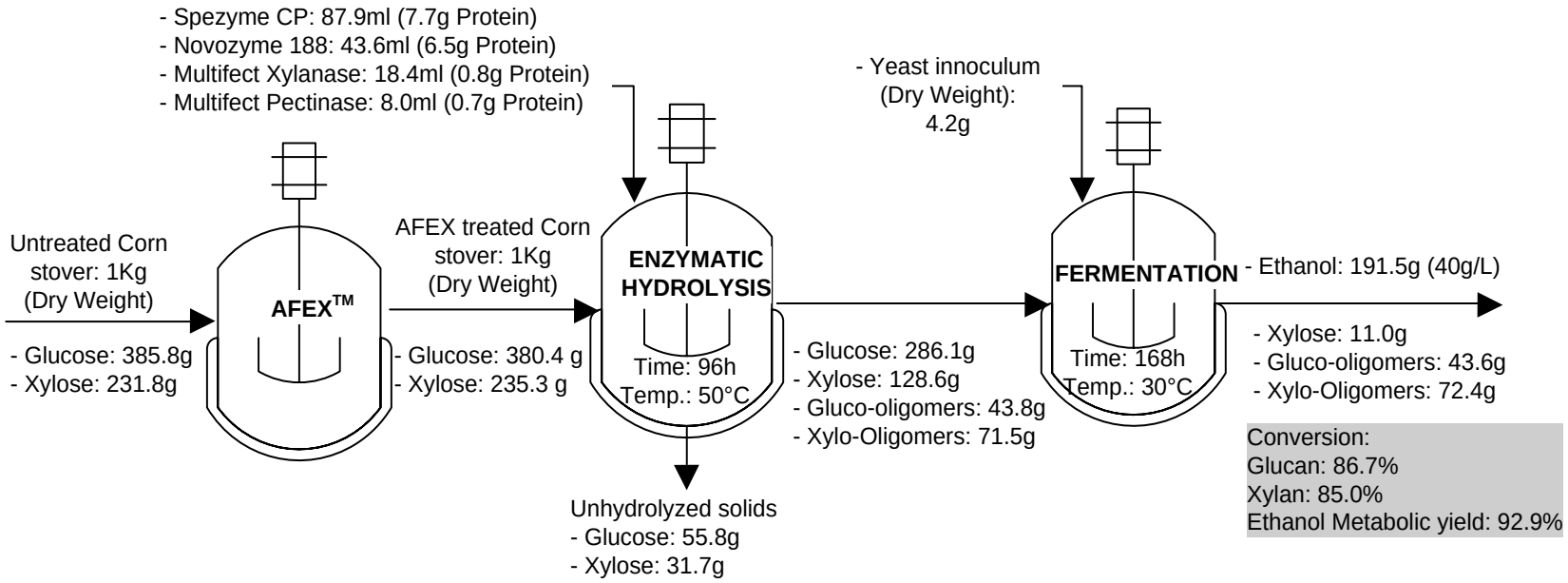



Figure 6 Mass balance of SHF process based on AFEX$^{TM}$ pretreatment (adapted from (Lau & Dale, 2009)).
Enzymatic hydrolysis was carried out at 6%(w/w) glucan loading using commercial enzymes. Glucose and xylose co-fermentation was conducted using *S. cerevisiae* 424A(LNH-ST).

YEP medium (yeast extract (10 g/L) and peptone (20 g/L) medium). Degradation products were attributed to this increment (Lau & Dale, 2009). Similar high ethanol metabolic yield using *S. cerevisiae* 424A(LNH-ST) was also found on other AFEX$^{TM}$ pretreated biomass (Table 4). This was probably due to the fact that some ATP being used for resistance of degradation products such as weak organic acids rather than for cell growth (Bellissimi et al., 2009), which in turn reduced carbon flux into cell production and hence increased carbon flux into ethanol production.

Table 2 Major degradation products (μg analyte/g substrate) in AFEX™-CS
Adapted from (Chundawat et al., 2010).

| | Untreated corn stover | AFEX™ treated corn stover | Dilute-acid treated corn stover |
|---|---|---|---|
| Acetic acid | 1610 | 4610 | 34770 |
| Levulinic acid | 171 | 24 | 3649 |
| Furfural/HMF | 72 | 645 | 23640 |
| Acetamide/phenolic amides | - | 39801 | - |
| Pyrazine/imidazole derivatives | - | 945 | - |
| Syringaldehyde | 3 | 11 | 149 |
| Phenolic acids | 196 | 1183 | 3151 |
| Total soluble sugars | 35,927 | 81,930 | 318,300 |

### 1.2.2 Problems of ethanol production from AFEX™ pretreated biomass

To make the cellulosic ethanol production process cost-effective, it is essential for fermentation technology to meet the following criteria: ethanol titer > 40 g/L, ethanol metabolic yield > 90% and productivity > 1.0 $g \cdot L^{-1} \cdot h^{-1}$ (Dien et al., 2003a). While requirements for ethanol titer and ethanol metabolic yield have been achieved on some AFEX™ pretreated biomass, the ethanol productivity was far away from 1.0 $g \cdot L^{-1} \cdot h^{-1}$ (Table 4).

The major cause for low ethanol productivity is slow xylose fermentation. Typically, in 6% (w/w) glucan loading AFEX™ hydrolysate, glucose is completely consumed in 18-24 h. However, it requires more than 96 h to consume around 28 g/L xylose. As a result, the ethanol productivity can be as high as 4.8 $g \cdot L^{-1} \cdot h^{-1}$ during the glucose fermentation period (Table 4), but the overall average productivity is low. Xylose fermentation in corn steep liquor (CSL) or YEP, however, is much faster compared to hydrolysate fermentation (Lau et al., 2010). The reduced xylose consumption rate in hydrolysate fermentation was attributed to both cell growth inhibition and xylose metabolism inhibition by degradation products (Lau & Dale, 2009). Xylose

fermentation in AFEX$^{TM}$ hydrolysates is also affected by nutrient levels in the feedstocks. For instance, the nutrient level in sugar cane leaf is higher compared to sugar cane bagasse. As a

Table 3 Nutrients in 6% glucan loading AFEX$^{TM}$ treated corn stover hydrolysate. Adapted from (Lau et al., 2012).

| **Amino acids (mg/L)** | **Free** | **Total** | **Trace elements** | |
|---|---|---|---|---|
| Asp | 8.4±1.7 | 75.9±1.7 | Mg (mg/L) | 168.42±3.24 |
| Glu | 0.0±2.4 | 133.8±2.4 | Ca (mg/L) | 242.87±7.72 |
| Ser | 16.8±3.8 | 104.2±3.8 | Mn (mg/L) | 2.32±0.53 |
| Gly | 5.2±5.8 | 127.2±5.8 | Co (µg/L) | 11.3±3.8 |
| His | 4.5±2.3 | 34.3±2.3 | Ni (µg/L) | 13.5±5.3 |
| Thr | 17.6±4.6 | 98.9±4.6 | Cu (µg/L) | 116.2±9.3 |
| Arg | 17.1±3.2 | 55.0±3.2 | Zn (µg/L) | 505.7±51.3 |
| Ala | 11.6±2.9 | 110.2±2.9 | Mo(µg/L) | 15.9±0.6 |
| Pro | 30.4±2.3 | 108.7±2.3 | Fe (µg/L) | 296.4±74.5 |
| Tyr | 30.0±2.5 | 28.6±2.5 | **Vitamins (µM)** | |
| Val | 9.9±2.2 | 68.8±2.2 | Pantothenic Acid | 1.50±0.12 |
| Met | 2.6±1.9 | 19.4±1.9 | Pyridoxine | 1.26±0.18 |
| Ile | 7.6±2.2 | 55.4±2.2 | Nicotinic Acid | 10.87±1.38 |
| Leu | 0.0±3.8 | 93.6±3.8 | Biotin | ~0.05 |
| Lys | 18.4±1.3 | 25.7±1.3 | Thiamine | ~0.66 |
| Phe | 15.7±3.8 | 91.6±3.8 | | |
| Total AA | 195.8±28.3 | 1231±43.8 | | |
| **$NH^{4+}$ (mg/L)** | 750±50 | | | |

Table 4 Fermentation performance (SHF) on AFEX$^{TM}$ treated biomass

| AFEX$^{TM}$ pretreated biomass | Max. EtOH produc. ($g \cdot L^{-1} \cdot h^{-1}$) | Average EtOH produc. ($g \cdot L^{-1} \cdot h^{-1}$) | EtOH metabolic yield | EtOH titer (g/L) | Xyl. cons. (%) | Final xyl. conc. (g/L) | Ref. |
|---|---|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* 424A(LNH-ST) | | | | | | | |
| Corn Stover | 1.6 | 0.24 | 92.9% | 40.0 | 82.2 | 5.0 | (Lau & Dale, 2009) |
| Cane leaf | 2.4 | 0.51 | 91.6% | 36.4 | 87.0 | 3.8 | (Krishnan et al., 2010) |
| Sugarcane Bagasse | 3.0 | 0.28 | 91.6% | 33.7 | 62.7 | 12.3 | (Krishnan et al., 2010) |
| Rice straw | 1.7 | 0.26 | 95.3% | 37.0 | 69.2 | 7.4 | (Zhong et al., 2009) |
| Poplar | 4.8 | 0.74 | 93.0% | 35.5 | 78.7 | 3.5 | (Lu et al., 2009) |
| Forage sorghum | N/A | 0.32 | 86.2% | 30.9 | 57.0 | 13.0 | (Li et al., 2010) |
| Corn silage | 1.1 | 0.39 | 93.2% | 28.4 | 72.0 | 2.1 | (Shao et al., 2010) |
| Whole corn plant | 1.2 | 0.41 | 89.2% | 29.8 | 79.5 | 1.6 | (Shao et al., 2010) |
| *P. stipitis* FPL-061 | | | | | | | |
| Rice straw | 0.8 | 0.25 | 71.7% | 29.7 | 92.4 | 1.8 | (Zhong et al., 2009) |
| *P. stipitis* DX-26 | | | | | | | |
| Rice straw | 0.52 | 0.23 | 67.7% | 27.6 | 88.4 | 2.8 | (Zhong et al., 2009) |
| *Z. mobilis* AX101 | | | | | | | |
| Corn Stover | 0.42 | 0.22 | 96.5% | 32.0 | 31.0 | 20.0 | (Lau et al., 2010) |
| *E. Coli* KO11 | | | | | | | |
| Corn Stover | 0.46 | 0.22 | N/A | 31.0 | 10.3 | 26.0 | (Lau et al., 2010) |

result, *S. cerevisiae* 424A(LNH-ST) grew to an $OD_{600}$ of 14 in AFEX$^{TM}$ pretreated sugar cane leaf hydrolysate, while it grew to an $OD_{600}$ of 9 in bagasse hydrolysate, which in turn affected xylose consumption (87% in 72 h vs. 62.7% in 120 h; table 4) (Krishnan et al., 2010).

Moreover, if calculation is based on enzymatic hydrolysis time plus fermentation time (process ethanol productivity), the ethanol productivity value for the current SHF process is much lower since at least 96 h is required for enzymatic hydrolysis reaction. Besides, high enzyme loading is required during high solids loading enzymatic hydrolysis, which is a barrier for commercialization of cellulosic ethanol due to the high cost of cellulase enzymes.

### 1.3 Research objectives

The integrated biological processes (SSCF&CBP) are strongly believed to have lower cost compared to the SHF process. But so far, they have not been studied on AFEX$^{TM}$ pretreated biomass. Moreover, the current SHF processing of AFEX$^{TM}$ biomass has the following problems: 1) relatively long enzymatic hydrolysis time; 2) high enzyme loading; 3) slow xylose fermentation and low ethanol productivity. This dissertation work's goal is to: 1) study the integrated biological processes on AFEX$^{TM}$ pretreated biomass with the results compared to SHF process; 2) solve the aforementioned problems in the current process.

# CHAPTER II QUANTITATIVELY UNDERSTANDING REDUCED XYLOSE FERMENTATION PERFORMANCE IN AFEX™ TREATED CORN STOVER HYDROLYSATE


## Abstract

Reduced xylose fermentation performance has been an issue during fermentation of AFEX™ hydrolysate using *Saccharomyces cerevisiae* 424A (LNH-ST) or *Escherichia coli* KO11. To better understand why fermentation performance is reduced, the effects of compounds present in the fermentation broth on xylose consumption were quantitatively studied. The compounds include biomass degradation products, ethanol and fermentation metabolites. The xylose consumption capability of *E. coli* KO11 was almost totally inhibited by the presence of both degradation products and ethanol. On the other hand, for *S. cerevisiae* 424A, 89% reduction of xylose consumption rate was found during hydrolysate fermentation. Degradation products, ethanol and fermentation metabolites were responsible for 32%, 24% and 33% of such reduction, respectively. Those results suggest that to further improve the xylose fermentation in hydrolysate, strains should be selected not only for degradation products tolerance but also for ethanol and fermentation metabolites tolerance.

## 2.1 Introduction

*Saccharomyces cerevisiae* 424A (LNH-ST) is a genetically engineered xylose-fermenting strain. Its chromosome is integrated with multiple copies of xylose reductase (XR) and xylitol dehydrogenase (XD) genes from *Schefferosomyces stipitis* and an endogenous xylulokinase gene (XR) (Ho et al., 1999). *Escherichia coli* KO11 possesses pyruvate decarboxylase and alcohol dehydrogenase genes (PET operon) from *Zymomonas mobilis*, which makes it a homoethanolic fermentation strain (Ingram et al., 1999). Both strains were able to consume xylose quickly in corn steep liquor or YEP (yeast extract and peptone) medium (Lau et al., 2010). Nevertheless, in 6% (w/w) glucan loading AFEX$^{TM}$ treated corn stover hydrolysate, the xylose consumption was slow for both of the strains.

It is well known that glucose is always fermented first during co-fermentation of glucose and xylose by *S. cerevisiae* or *E. coli* (Weber et al., 2010). *S. cerevisiae* does not have specialized xylose transporters (Bertilsson et al., 2008). It depends on hexose transporters to transport xylose. Due to the much lower affinity of hexose transporters for xylose, glucose is always transported and hence fermented first (Bertilsson et al., 2008). Xylose consumption in *E. coli* is typically repressed due to carbon catabolite repression in the presence of glucose which reduces the expression of both xylose transporters and key enzymes for xylose metabolism (Görke & Stülke, 2008; Ren et al., 2009). Therefore, xylose consumption does not start until glucose is nearly depleted. Thus, during fermentation of high solids loading AFEX$^{TM}$ hydrolysate there are always fermentation metabolites and 20-40 g/L ethanol (mostly generated from glucose fermentation) as well as degradation products present in fermentation broth when xylose is being consumed (Lau et al., 2010). Degradation products generated from pretreatment

were considered to cause poor xylose fermentation (Lau & Dale, 2009; Lau et al., 2010). However, the effects of ethanol and fermentation metabolites on xylose consumption have not been quantified, especially in combination with degradation products.

The present study investigates and quantifies the effects of degradation products, ethanol, fermentation metabolites and nutrients, on xylose consumption in AFEX$^{TM}$ corn stover hydrolysate using *S. cerevisiae* 424A or *E. coli* KO11.

## 2.2 Materials and Methods

### 2.2.1. AFEX$^{TM}$ treated corn stover

AFEX$^{TM}$ pretreated corn stover (AFEX$^{TM}$-CS) was supplied by the Biomass Conversion Research Laboratory (BCRL), Michigan State University, and contained glucan and xylan contents of 34.1% and 20.4%, respectively. The AFEX$^{TM}$ pretreatment procedure has been described in Chapter I. Pretreatment conditions included: ammonia to biomass loading 1.0 g/g dry biomass, water loading 0.6 g/g dry biomass, temperature 140 $^{o}$C and residence time 15 min. Unless otherwise stated, AFEX$^{TM}$ treated corn stover was used for enzymatic hydrolysis and fermentation experiments with no washing, conditioning, nutrient supplementation or detoxification.

### 2.2.2 AFEX$^{TM}$-CS hydrolysate preparation

AFEX$^{TM}$-CS hydrolysate was prepared by using a commercial enzyme mixture including Spezyme CP (Genencor Inc, USA) 22.4 mg protein/g glucan (15 FPU/g glucan), Novozyme 188 (Sigma-Aldrich, USA) 38.4 mg protein/g glucan (64 *p*NPGU/g glucan), Multifect xylanase 2.6 mg protein/ g glucan and Multifect pectinase (Genencor Inc, USA) 4.7 mg protein/ g glucan.

Enzymatic hydrolysis was carried out at 6% (w/w) glucan loading (corresponding to 18% (w/w) solid loading) in a 2.0 L baffled flask with 500 g total mixture at pH 4.8, 50 $^{o}$C, and 250 rpm. 50 mg/L chloramphenicol (Cm) was used to avoid microbial contamination. After 96 h hydrolysis, hydrolysate was centrifuged at 14,000 rpm for 30 min. Supernatant was sterile filtrated through a 0.22 μm filter and kept in a sterile bottle for fermentation use.

### 2.2.3 AFEX$^{TM}$-CS water extract preparation

Water extract of AFEX$^{TM}$-CS was prepared by spraying 1L of preheated distilled water (around 50 $^{o}$C) on 240 g of AFEX$^{TM}$-CS and then pressing the wetted biomass to a moisture content of 55 ± 3%. The water extract obtained was sprayed on the biomass again and then the biomass was pressed again. The extraction process was finished by 3 times of spraying and pressing. The final water extract obtained was equivalent to a concentration of 8% (w/w) glucan loading. Sugars contained in the water extract included 0.8 g/L glucose, 1.5 g/L xylose, 2.9 g/L oligomeric glucose and 8.8 g/L oligomeric xylose. The water extract (concentration: 8% (w/w) glucan loading) was then sterile filtrated and diluted to different concentrations (2%, 4% and 6% (w/w) glucan loading) for this study. The water extract was used as a surrogate to represent soluble degradation products in the AFEX$^{TM}$-CS hydrolysate. Xylose and ethanol were supplemented in the water extract for xylose conversion experiments.

### 2.2.4 Microorganisms and seed culture preparation

The genetically engineered xylose-fermenting strain *S. cerevisiae* 424A(LNH-ST) (Ho et al., 1999) was obtained from Purdue University. *E. coli* KO11 was purchased from American Type Culture Collection (ATCC) with a designated number of 55124. To facilitate comparisons between the two strains, seed cultures of both strains were prepared on YEP medium (10 g/L

yeast extract and 20 g/L tryptone) with 50 g/L glucose in a 250 ml Erlenmeyer flask with working volume 100 ml. MOPS (pH 7.0) at a concentration of 0.05 M was used for the seed culture of *E. coli* KO11. Frozen glycerol stocks were used for inoculation. The cultures of *S. cerevisiae* 424A and *E. coli* KO11were grown at 30 $^{O}$C and 37 $^{O}$C, respectively and 150 rpm under micro-aerophilic conditions for 24 h.

**2.2.5 Fermentations in YEP medium or hydrolysates**

Fermentations on YEP medium (yeast extract 5 g/L; tryptone 10 g/L) or hydrolysate were conducted at 150 rpm in 125 ml Erlenmeyer flasks with a working volume of 50 ml. 0.05 M phosphate buffer and 0.05 M MOPS buffer were used for *S. cerevisiae* 424A and *E. coli* KO11 fermentations to maintain pH at 5.5 and 7.0, respectively. During *E. coli* KO11 fermentations, pH was adjusted twice per day to maintain the set point. Temperatures for *S. cerevisiae* 424A and *E. coli* KO11 fermentations were 30 $^{O}$C and 37 $^{O}$C, respectively (Lau et al., 2010). To maintain largely anaerobic conditions and release carbon dioxide, rubber stoppers with a needle piercing them were used to cap the flasks. *S. cerevisiae* 424A and *E. coli* KO11 fermentations were initiated with an $OD_{600}$ of 2.0 and 0.5, respectively.

**2.2.6 Xylose conversion experiments using non-growing cells**

After 24 or 48 h fermentations on YEP medium or hydrolysate, 10 ml fermentation broth was harvested and centrifuged at 4400 rpm for 10 min. Supernatant was discarded. Cell pellets were washed using 0.9% NaCl solution. The cell pellets obtained were re-suspended in various solutions with a total volume of 10 ml. The various solutions included water extract at different concentrations and water extract with different concentrations of ethanol (Table 5). Either 0.05 M phosphate buffer (pH 5.5, for *S. cerevisiae* 424A) or 0.05 M MOPS buffer (pH 7.0, for *E. coli*

KO11) was used for pH control. The 10 ml cell and other component mixtures were transferred to a 15 ml screw-capped vial and incubated at 150 rpm and 30 $^{o}$C (for *S. cerevisiae* 424A) or 37$^{o}$C (for *E. coli* KO11) for 3 hours. Samples were taken for xylose concentration analysis by HPLC both before and after the incubation. Specific xylose consumption rate was calculated by dividing the amount of consumed xylose by the length of reaction time (3 h) and by the density of the cells.

Experiments were conducted in triplicates and the averages and standard deviations were given in the figures. Minitab15 Statistical Software (2006 Minitab Inc, Pennsylvania, USA) was used to perform *t*-test for the determination of statistical significance. All the values of specific xylose consumption rate reduction and cell biomass production reduction shown in the figures 8,

Table 5 Xylose conversion experiment design for investigating the effects of water extract, ethanol, cell culture medium and fermentation metabolites on xylose consumption [a]

| Parameter | Effect of water extract | Effect of ethanol | Effect of culture medium | Effect of ferm. metabolites |
|---|---|---|---|---|
| Water extract conc. [b] | 0, 2%, 4%, 6% | 6% | 6% | 0 |
| Ethanol conc. (g/L) | 0 | 0, 20,30,40 | 30 | 35 |
| Cells from | Hyl.Ferm. | Hyl.Ferm. | Hyl.Ferm. & YEP Ferm. | YEP Ferm. |
| CHT for *S. cerevisiae* [c] | 24 h | 24 h | 24 h & 48 h | 24 h |
| CHT for *E. coli* | 48 h | 48 h | 24 h & 48 h | N/A |
| Relevant data | Fig. 8 | Fig. 9 | Fig. 10 | Fig. 10 |

[a] The experiments were conducted using non-growing cells in a 15 ml screw-capped vial with 10 ml solution. The solution with xylose 30 g/L contained water extract and ethanol at different concentrations as shown in the table. Either 0.05 M phosphate buffer (pH 5.5, for *S. cerevisiae* 424A) or 0.05M MOPS buffer (pH 7.0, for *E. coli* KO11) was used for pH control. The 15 ml vial was incubated at 150 rpm and 30 $^{o}$C (for *S. cerevisiae* 424A) or 37$^{o}$C (for *E. coli* KO11) for 3 h.

[b] Water extract concentrations were expressed as glucan loading equivalent.

[c] Cell harvest time (CHT) from hydrolysate fermentation or YEP fermentation for xylose conversion experiments.

9, 10 &12 are statistically significant ($p$<0.05).

### 2.2.7 HPLC analyses

Concentrations of glucose, xylose and ethanol were determined using the Shimadzu HPLC system equipped with a refractive index detector and a BioRad Aminex HPX-87H column. The mobile phase was 5 mM aqueous sulfuric acid solution flowing at a rate of 0.6 mL/min. The column temperature was maintained at 60 $^{o}$C.

### 2.2.8 Measurement of viable cells density

Viable cell density was measured in colony forming unit (CFU) per ml. Samples were diluted and 20 µL of each diluted sample was plated on YPD agar medium (5 g/L yeast extract, 10 g/L tryptone, 25 g/L glucose, 20 g/L agar and 50 mg/L chloramphenicol). Dilution rate was varied to make sure the number of colony on a single plate was between 20 to 100. During dilution, the sample solutions were vigorously vortexed to prevent cell clumping or adhering to the solid biomass. Single colonies were counted after the plate was incubated for 24 h at 30 $^{o}$C and viable cell density was calculated accordingly.

## 2.3 Results and discussion

### 2.3.1 Slow xylose fermentation in AFEX$^{TM}$-CS hydrolysate

AFEX$^{TM}$-CS hydrolysate fermentations and the fermentations in YEP medium with similar concentrations of glucose and xylose by *S. cerevisiae* 424A and *E. coli* KO11 are shown in Figure 7. Both strains fermented xylose rapidly in YEP medium with approximate 30 g/L of xylose completely consumed in 48 and 96 h, respectively (Fig. 7b&d). The xylose fermentation performance in hydrolysate, however, was reduced significantly (Fig. 7a&c). *S. cerevisiae* 424A and *E. coli* KO11 consumed around 21 and 5 g/L xylose in 168 h, respectively, thereby reducing

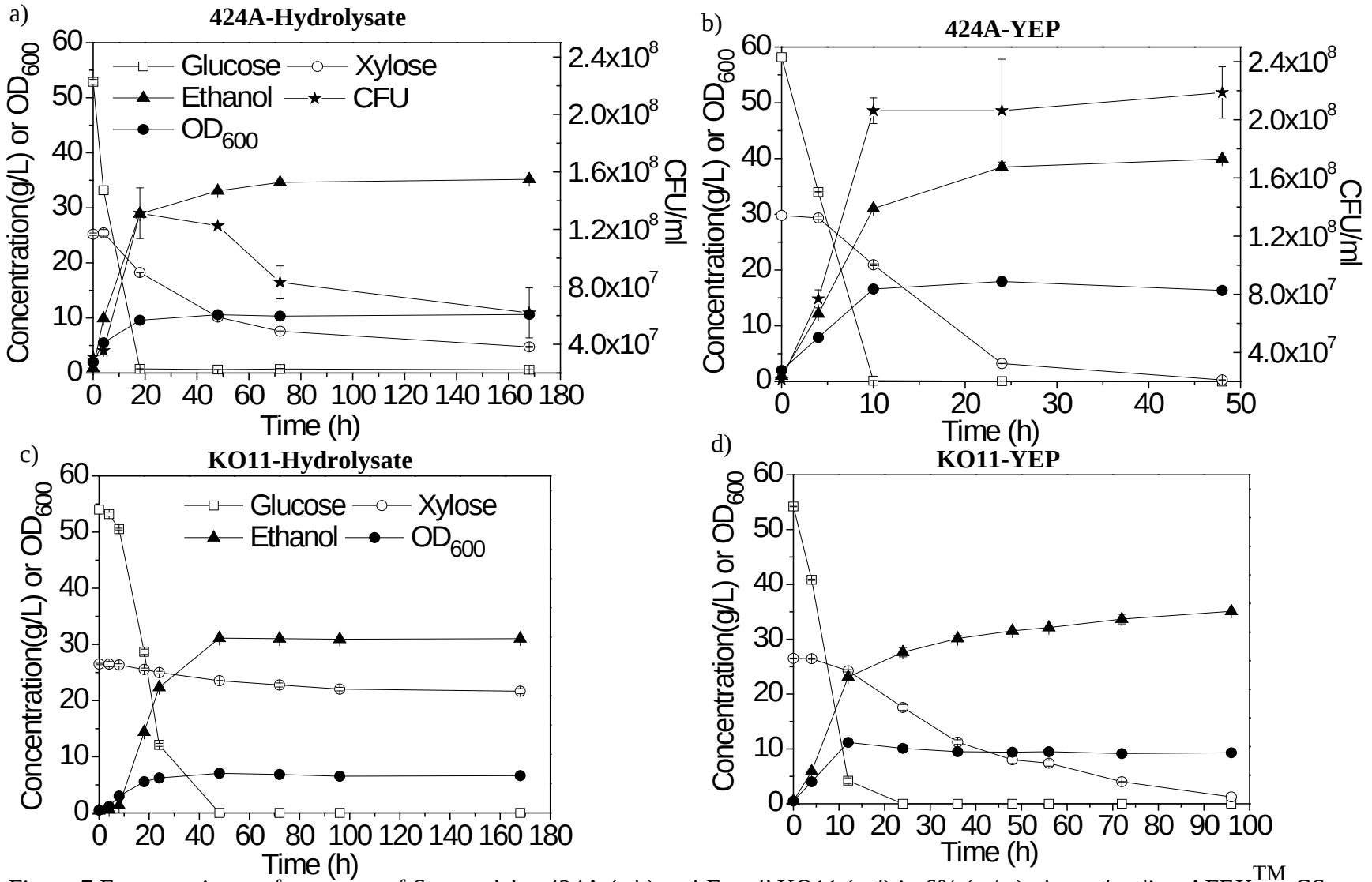



Figure 7 Fermentation performances of *S. cerevisiae* 424A (a,b) and *E. coli* KO11 (c,d) in 6% (w/w) glucan loading AFEX$^{TM}$-CS hydrolysate (a,c) and YEP medium (b,d).

0.05 M phosphate buffer and 0.05 M MOPS buffer were used for *S. cerevisiae* 424A and *E. coli* KO11 fermentations to maintain pH at 5.5 and 7.0, respectively. YEP medium contained 5 g/L yeast extract and 10 g/L tryptone.

both ethanol yield and productivity. To understand the reduced xylose fermentation performances in hydrolysate, we investigated the effects of possible inhibitors such as degradation products, ethanol and the growth medium on specific xylose consumption rate. The inhibitory effect of degradation products on cell growth was also quantified since the xylose consumption rate was determined by both specific xylose consumption rate and cell density. (Xylose consumption rate equals to cell density multiply by specific xylose consumption rate.)

**2.3.2 Effect of degradation products on specific xylose consumption rate**

Degradation products (Table 2; profile reported by Chundawat et al (Chundawat et al., 2010)) were represented using the AFEX$^{TM}$-CS water extract prepared as described above. The AFEX$^{TM}$-CS water extract should have a very similar degradation products profile compared to AFEX$^{TM}$-CS hydrolysate. The effect of water extracts at different concentrations was investigated by performing xylose conversion experiments using non-growing cells from hydrolysate fermentations. The non-growing cells for *S. cerevisiae* 424A and *E.coli* KO11 were harvested at 24 h and 48 h, respectively. At this time, glucose was depleted and cell growths had reached the stationary phase (Fig. 7a&c). During the xylose conversion experiment, no significant cell growth was observed.

With no inhibitors in the solution, the specific xylose consumption rates for *S. cerevisiae* 424A and *E. coli* KO11 reached as high as 0.408 and 0.435 g·h$^{-1}$·g cell$^{-1}$, respectively (Fig. 8). However, in real hydrolysate fermentations these values were reduced to 0.056 and 0.017 g·h$^{-1}$·g cell$^{-1}$, respectively. About 86% and 96% reductions on specific xylose consumption rate were,

therefore, observed for *S. cerevisiae* 424A and *E. coli* KO11 fermentations in 6% (w/w) glucan loading hdyrolysates, respectively.

Water extract showed no significant impact on the specific xylose consumption rate of *S. cerevisiae* 424A until the concentration reached as high as 6% glucan loading equivalent, at which point a 13% reduction in specific xylose consumption rate was observed (Fig. 8a). The robustness of *S. cerevisiae* 424A was also highly rated by Lau et al. (2010). The specific xylose consumption rate of *E. coli* KO11, however, was severely affected by water extract. Up to 76% reduction was observed in water extract at the concentration of 6% (w/w) glucan loading equivalent (Fig. 8b).

### 2.3.3 Effect of ethanol on specific xylose consumption rate

During fermentation of 6% (w/w) glucan loading hydrolysate, there is always 20-40 g/L ethanol (mostly produced from glucose) present in fermentation broth during the xylose consumption period (Fig. 7). Therefore, the effect of 0, 20, 30, and 40 g/L ethanol on specific xylose consumption rate was investigated in 6% (w/w) glucan loading equivalent water extract by applying xylose conversion experiments. With more than 20 g/L ethanol in water extract, the specific xylose consumption rate of *S. cerevisiae* 424A was reduced from 0.355 to around 0.230 $g \cdot h^{-1} \cdot g\ cell^{-1}$ (Fig. 9a). This is a 31% reduction when compared to the control (without any inhibitor). No significant difference of specific xylose consumption rate was found under these conditions for different concentrations of ethanol in the range of 20-40 g/L.

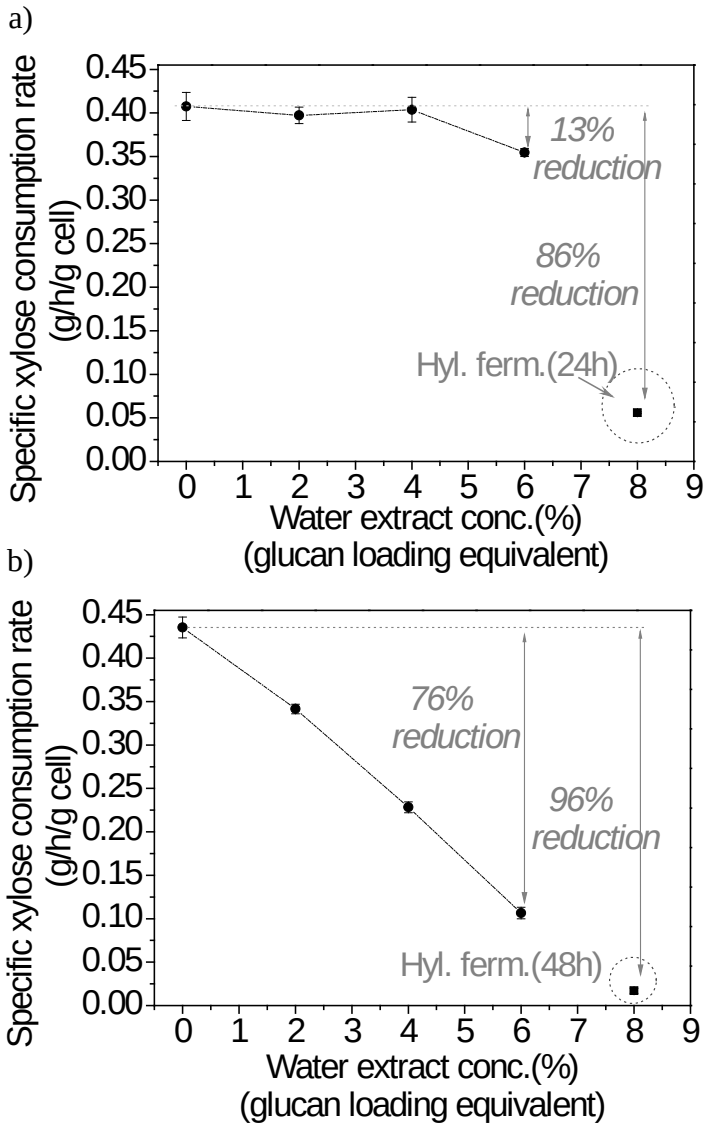



Figure 8 Effect of water extract of AFEX$^{TM}$ pretreated corn stover on specific xylose consumption rates of *S. cerevisiae* 424A (a) and *E. coli* KO11 (b).

Xylose conversion experiments were conducted in a 15 ml screw-capped vial and incubated at 150 rpm and 30 $^{o}$C (for *S. cerevisiae* 424A) or 37 $^{o}$C (for *E. coli* KO11) for 3 h. Non-growing cells were harvested from hydrolysate fermentations at 24 h (for *S. cerevisiae* 424A) or 48 h (for *E. coli* KO11). The solutions used for these experiments contained different concentrations of water extract and buffer for pH control.

For *S. cerevisiae* 424A, the specific xylose consumption rate of the cells (from hydrolysate fermentation) in xylose conversion solution (containing 6% glucan loading equivalent water extract, 30 g/L ethanol and 30 g/L xylose) is higher than the value in a real

hydrolysate fermentation. Most of the components and their concentrations in the solution and in the fermentation broth were similar. Two major factors could be responsible for this observation. One is the additional degradation products, such as acetate that might be released during enzymatic hydrolysis. But *S. cerevisiae* 424A had high degradation products tolerance (Fig.8a), which renders this explanation less likely. The other factor was the inhibitory metabolites

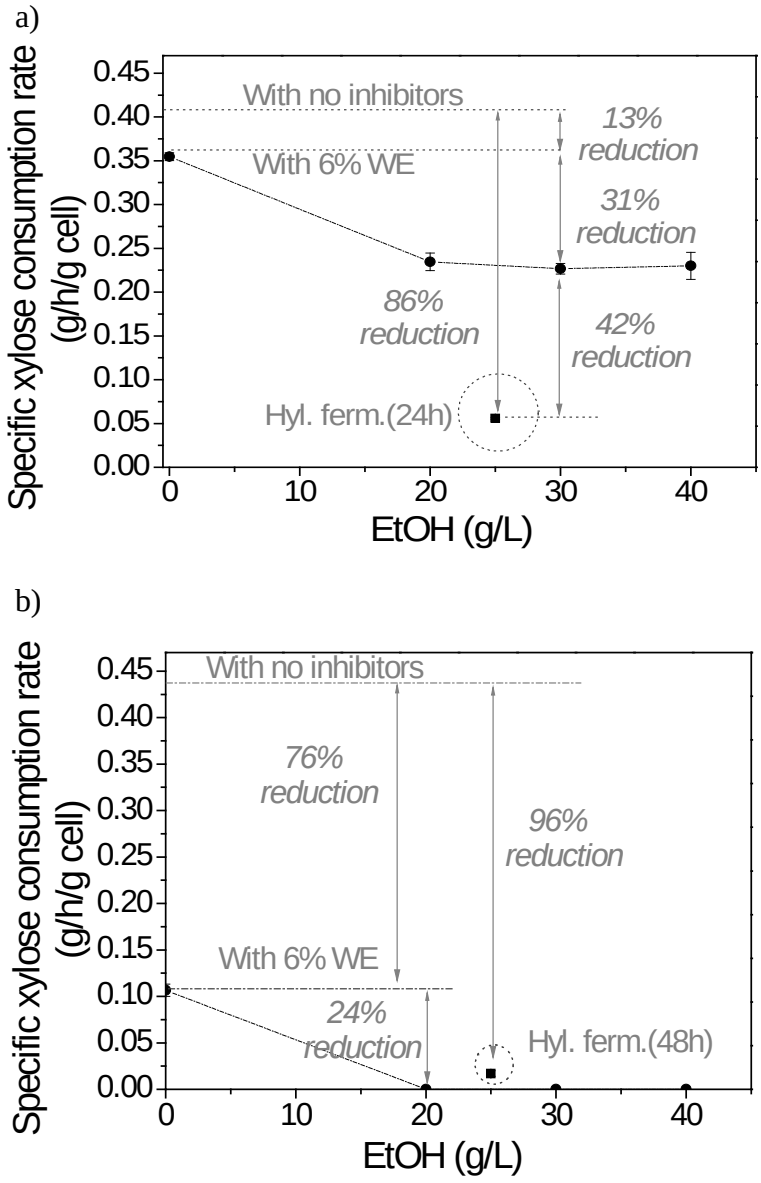



Figure 9 Effect of ethanol on specific xylose consumption rates of *S. cerevisiae* 424A (a) and *E. coli* KO11 (b).
Results were obtained from xylose conversion experiments in 6% (w/w) glucan loading water extract (WE). Non-growing cells harvested from hydrolysate fermentation were used.

produced by yeast fermentation while cells were in a dynamic process of growing and dying. In order to explore the second possibility, we harvested the 24 h non-growing yeast cells cultured in YEP medium and tested the specific xylose consumption rate of these cells in the solution containing 35 g/L ethanol, 30 g/L xylose and 0.05 M phosphate buffer (pH 5.5). This solution is similar to the YEP fermentation broth at 24h except that there were no fermentation metabolites in this solution. The specific xylose consumption rate was around two times higher than the one in the YEP fermentation, which indicates that the fermentation metabolites reduced the specific xylose consumption rate in YEP medium by about half (Fig. 10a).

The xylose consumption of *E. coli* KO11 was totally inhibited by ethanol concentrations greater than 20 g/L ethanol in 6% (w/w) glucan loading equivalent water extract (Fig. 9b). However, during real hydrolysate fermentations, *E. coli* KO11 at 48 h (ethanol: 31.1 g/L) still consumed xylose at a rate of 0.017 $g\cdot h^{-1}\cdot g\ cell^{-1}$. Such differences might be caused by the activity loss during cell sample preparation including centrifuge, washing, and cell re-suspension.

**2.3.4 Effect of culture medium on specific xylose consumption rate**

The xylose consumption ability of cells cultured in YEP medium and in hydrolysate was compared by performing xylose conversion experiment in the solution containing 6% glucan loading equivalent water extract, 30 g/L ethanol and 30 g/L xylose (Fig. 10). For both *S. cerevisiae* 424A and *E. coli* KO11, cells from YEP medium showed higher specific xylose consumption rates compared to the cells from hydrolysate. Particular nutrient(s) in YEP medium probably helped induce the expression of sugar transporters or enzymes involved in xylose metabolism, which in turn enhanced the xylose-fermenting ability or improved tolerance to various inhibitors. We also observed that the yeast cells harvested at 48 h showed slightly higher xylose consumption rates compared to the cells harvested at 24 h.

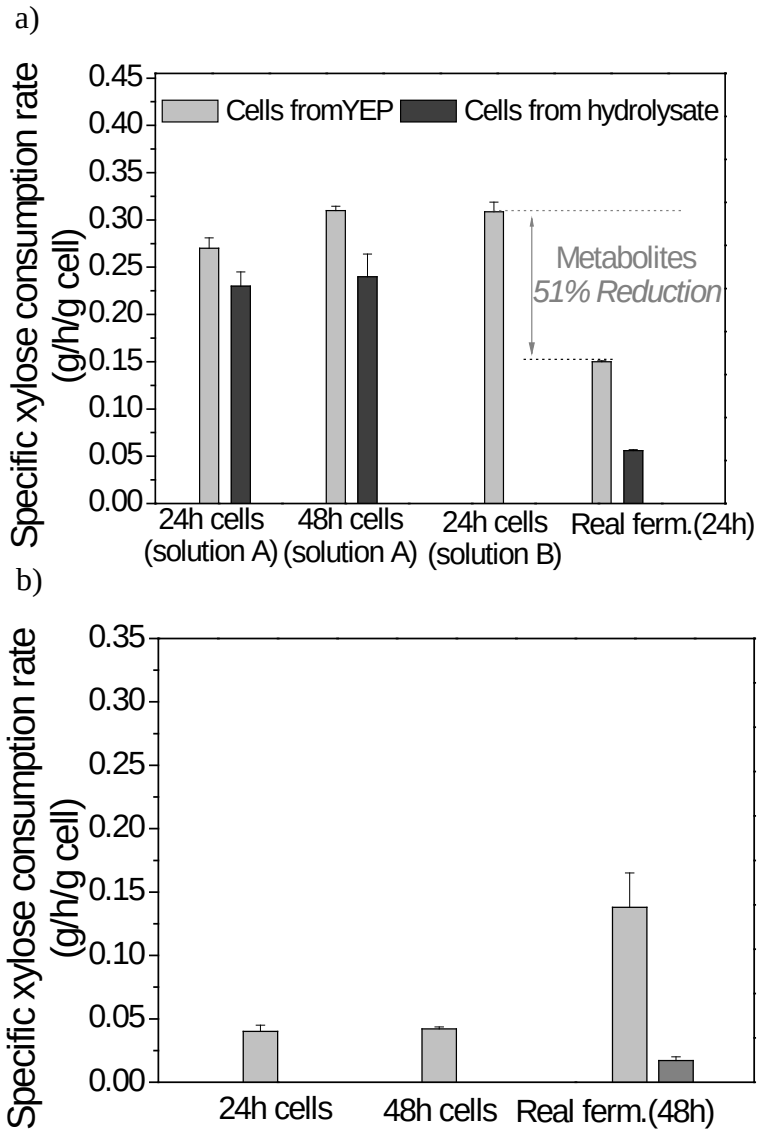



Figure 10 Effect of culture medium (YEP and hydrolysate) and culture time (24h and 48h) on specific xylose consumption rates of *S. cerevisiae* 424A (a) and *E. coli* KO11 (b).
Results were obtained from xylose conversion experiments in solution A: 6% glucan loading equivalent water extract, 30 g/L ethanol, 30 g/L xylose and 0.05M phosphate buffer (pH 5.5); and solution B: 35 g/L ethanol, 30 g/L xylose and 0.05M phosphate buffer (pH 5.5) for *S. cerevisiae* 424A and in solution A' (6% glucan loading equivalent water extract, 30 g/L ethanol, 30 g/L xylose and 0.05M MOPS buffer (pH 7.0) for *E. coli* KO11.

### 2.3.5 Effects of degradation products on cell growth and nutrients addition on xylose consumption

Since it is very difficult to separate degradation products from nutrients, and both are present in AFEX$^{TM}$-CS, the effect of degradation products on cell growth was investigated by comparing maximum cell density in YEP medium (plus pure sugars) to the maximum cell density in the hydrolysate with supplementation of YEP. Both glucose and xylose concentrations were the same. Under such conditions, nutrients in both media were in excess and were assumed not to affect performance. A 22% reduction of yeast cell biomass production and 11% reduction of *E. coli* cell biomass production were observed in hydrolysate supplemented with YEP when compared to the fermentation in YEP medium supplemented with sugars (Fig. 11). Such mild inhibitory effects of degradation products on cell growth was probably due to the AFEX$^{TM}$ pretreatment forming much less acids compared to dilute acid pretreatment (Chundawat et al., 2010). The ammonolysis reaction occurring during AFEX$^{TM}$ pretreatment converted a large portion of compounds into amides instead of acids. For instance, the ratio of acetamide over acetic acid in AFEX$^{TM}$ pretreated corn stover was 5.4 and the acetic acid concentration in AFEX$^{TM}$ pretreated corn stover was 7.5 times lower compared to the diluted acid pretreated sample (Chundawat et al., 2010).

Surprisingly, the 24 h specific xylose consumption rate of *S. cerevisiae* 424A in hydrolysate with YEP was very close to that without YEP. YEP can act as an electron acceptor like furfural to alleviate the imbalance of redox caused by xylose fermentation in anaerobic conditions (Van Zyl et al., 1989; Wahlbom & Hahn-Hägerdal, 2002). Besides, as mentioned above, enzyme systems involved in xylose metabolism might also be enhanced in the presence of

YEP. However, apparently, YEP did not help to increase the specific xylose consumption rate in the hydrolysate, although it enhanced yeast cell density and overall xylose consumption when compared to the hydrolysate without YEP (Fig. 11a). For *E.coli*, the xylose consumption rate in hydrolysate with YEP was very close to that for hydrolysate without YEP although the maximal cell density was higher (Fig. 11b). This was probably due to the extremely low specific xylose consumption rate of *E. coli* in the presence of both degradation products and ethanol as shown above.

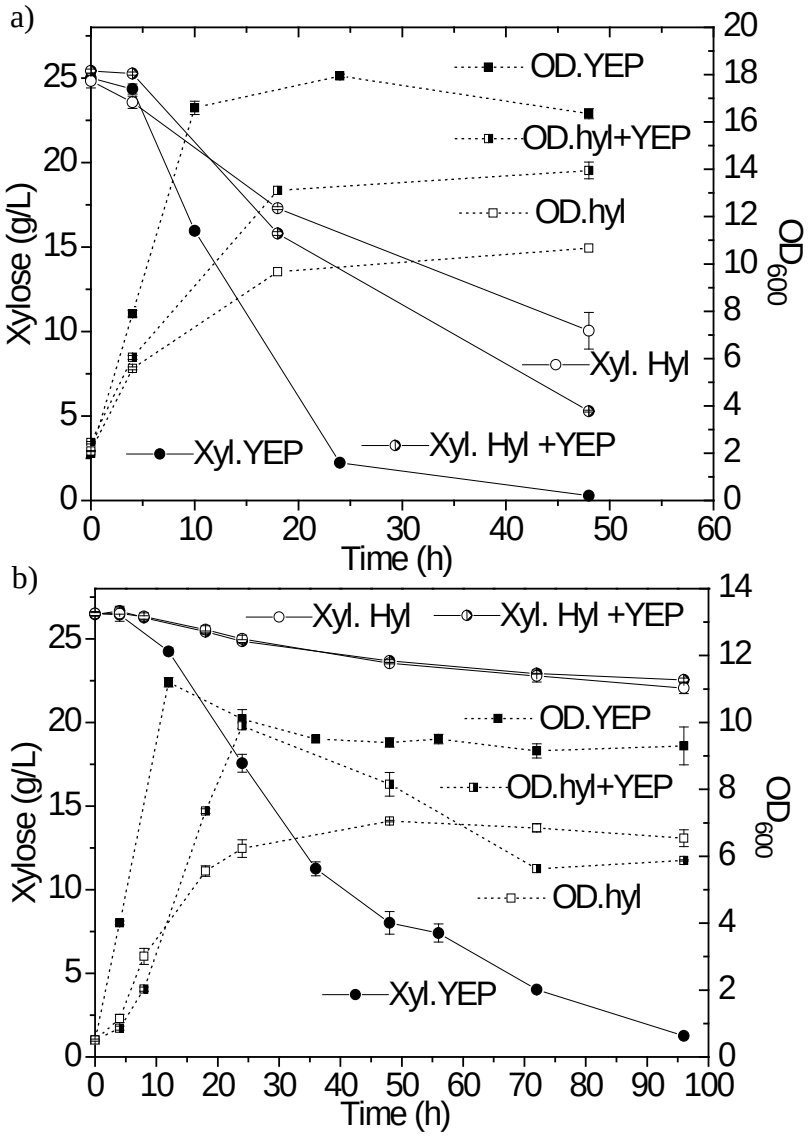



Figure11 Cell growth and xylose consumption of *S. cerevisiae* 424A (a) and *E. coli* KO11(b) during fermentations in YEP medium, AFEX$^{TM}$-CS hydrolysate and hydrolysate with YEP. The initial glucose and xylose concentrations were approximately 55 and 25 g/L, respectively.

### 2.3.6 Understanding the xylose consumption problem during hydrolysate fermentation by *S. cerevisiae* 424A

The overall extent of xylose consumption is determined by both cell density and the specific xylose consumption rate. During hydrolysate fermentation by *S. cerevisiae* 424A, we typically observed cell growth only during glucose fermentation. Cells were in a stationary or dying phase during xylose fermentation (Fig. 7a). Shortage of ATP could be one major cause for the inability of *S. cerevisiae* 424A to grow on xylose anaerobically (Kuyper et al., 2004). Due to the inhibitory effect of degradation products on cell growth, 22% less cell biomass was produced during glucose fermentation, which resulted in 22% fewer cells for xylose consumption/fermentation (Fig.11a&Fig.12). After glucose fermentation, the composition of broth changed with the release of ethanol and fermentation metabolites, which caused 31% and 42% reduction of specific xylose consumption rate. Degradation products were responsible for a 13% reduction in the specific xylose consumption rate. Therefore, compared to the ideal conditions without any inhibitors present (maximal cell growth and maximal specific xylose

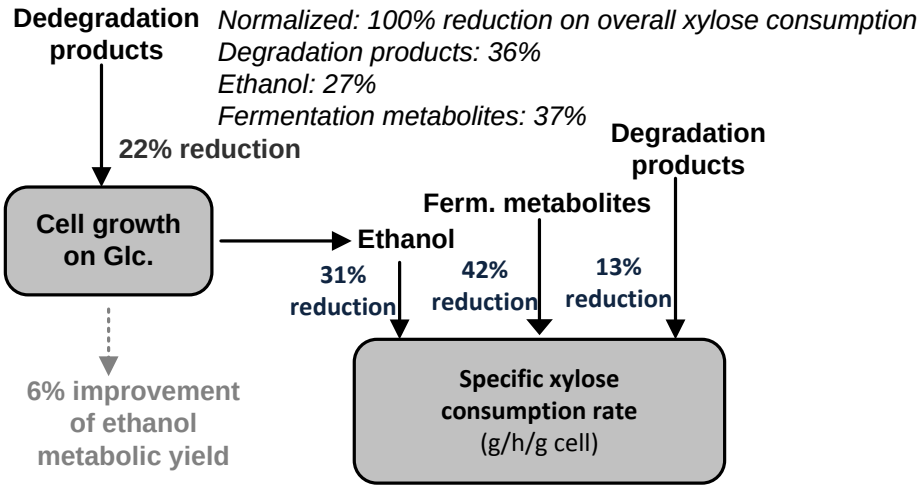



Figure 12 Understanding the xylose fermentation problem during AFEX$^{TM}$-CS hydrolysate fermentation by *S. cerevisiae* 424A.

consumption rate), the overall xylose consumption rate ($g \cdot L^{-1} \cdot h^{-1}$) was reduced by 89% during hydrolysate fermentation, among which degradation products, ethanol and fermentation metabolites accounted for 32%, 24%, and 33%, respectively.

Under ideal conditions without any inhibition, *S. cerevisiae* 424A is able to grow to a dry cell weight of 6.5 g/L in hydrolysate and reaches a maximum specific xylose consumption rate of 0.408 $g \cdot h^{-1} \cdot g\ cell^{-1}$. Under such conditions, xylose in AFEX$^{TM}$-CS hydrolysate (typically 28-30 g/L) can be completely consumed in 10.6-11.3 hours with a xylose consumption rate of 2.65 $g \cdot h^{-1} \cdot L^{-1}$. In real hydrolysate fermentations, however, the dry cell weight and 24 h specific xylose consumption rate decreased to 5.1 g/L and 0.056 $g \cdot h^{-1} \cdot g\ cell^{-1}$, respectively. Based on those values, the 28 g/L xylose in hydrolysate can be theoretically consumed in 98 h. Nevertheless, the xylose consumption rate was continually decreasing during fermentation due to the decreasing viable cell density and xylose concentration (Fig. 7a). Therefore, it took 150 h (excluding the glucose fermentation time of 18 h) to reduce xylose concentration to around 5 g/L. It was noteworthy that there was a short but higher specific xylose consumption rate period during late glucose fermentation at around 10-18 h. This was due to the fact that a low concentration of glucose could help induce sugar transporters that have higher affinity for xylose (Bertilsson et al., 2008). The specific xylose consumption rate reached about 0.15 $g \cdot h^{-1} \cdot g\ cell^{-1}$ at that time.

With YEP supplementation, cell density was enhanced to 6.7 g/L in hydrolysate (Fig. 11a) and 28 g/L xylose was reduced to around 5 g/L in 30 h (excluding glucose fermentation time 18 h; Fig. 11a). Nutrients affected cell growth and hence affected xylose fermentation. Different feedstocks have different levels of nutrients and hence exhibit different xylose fermentation

performance. For instance, in 6% (w/w) glucan loading of AFEX$^{TM}$ treated sugar cane leaf hydrolysate, the *S. cerevisiae* 424A cell density reached 6.9 g/L (dry weight basis) and xylose consumption reached 87.0% in a 72 h fermentation. However, cell density in AFEX$^{TM}$ sugar cane bagasse hydrolysate was 4.4 g/L and xylose consumption of 62.7% was achieved in 120 h (Krishnan et al., 2010). Fermentation of mixed biomass hydrolysates containing more nutrients could be a potential way to enhance xylose fermentation.

**2.4 Conclusions**

*S. cerevisiae* 424A and *E. coli* KO11 both have the potential to finish AFEX$^{TM}$-CS hydrolysate xylose fermentation in 24h. However, due to its poor tolerance to degradation products and ethanol, *E. coli* KO11 lost most of its ability to consume xylose during hydrolysate fermentation. The xylose consumption rate of *S. cerevisiae* 424A in hydrolysate was reduced to a great extent (89%) due to the presence of not only degradation products but also ethanol and fermentation metabolites. Degradation products reduced yeast cell biomass production by 22% and the specific xylose consumption rate by 13%. Ethanol and fermentation metabolites reduced the specific xylose consumption rate by 31% and 42%, respectively.

# CHAPTER III SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTATION OF AFEX™ PRETREATED SWITCHGRASS


## Abstract

It is well known that simultaneous saccharification and co-fermentation (SSCF) reduces cellulosic ethanol production cost compared to separate hydrolysis and fermentation (SHF). However, the traditional SSCF process of converting Ammonia Fiber Expansion (AFEX™) pretreated switchgrass to ethanol using commercial enzymes and *Saccharomyces cerevisiae* 424A(LNH-ST) gave reduced ethanol yield due to lower xylose consumption. To overcome this problem a two-step SSCF process was developed, in which xylan was hydrolyzed and fermented first followed by the hydrolysis and fermentation of glucan. Important parameters such as temperature, cellulases loading during xylan hydrolysis and fermentation, initial $OD_{600}$ for inoculation of *S. cerevisiae* 424A(LNH-ST), and pH were studied for best performance. Compared with traditional SSCF, the two-step SSCF showed higher xylose consumption and higher ethanol yield. The sugar conversion was also enhanced from 70% by enzymatic hydrolysis to 82% by two-step SSCF. One important finding is that the residue from enzymatic hydrolysis of switchgrass plays a significant role in reducing xylose consumption and ethanol metabolic yield during SSCF.

## 3.1 Introduction

Switchgrass (*Panicum virgatum*), a C4 perennial grass, has been identified as one of the most promising biomass feedstocks to produce cellulosic ethanol (Walsh et al., 2003). It has been estimated that 171 million tons of switchgrass (SG) can be economically produced in the US (Walsh et al., 2003) and that 94% life cycle greenhouse gas emission reductions are possible using ethanol produced from SG compared to gasoline (Schmer et al., 2008).

Simultaneous saccharification and co-fermentation (SSCF) carries out enzymatic hydrolysis, xylose and glucose fermentation in a single bioreactor. It has several advantages such as: lower cost, shorter process time, reduced contamination risk and less inhibitory effects during enzymatic hydrolysis compared to separate hydrolysis and fermentation (SHF) (Taherzadeh & Karimi, 2007). By applying both AFEX$^{TM}$ and SSCF on corn stover, the minimum ethanol selling price (MESP) was estimated to be as low as $1.03/gallon based on the future technology (Sendich et al., 2008). In the model it was assumed that both monomeric glucose and xylose from AFEX$^{TM}$ treated biomass were completely fermented to ethanol. However, relatively low xylose consumption (< 40%) was found when SSCF was performed on steam-pretreated biomass at 9% water insoluble solids (WIS) by using xylose-fermenting strain *S. cerevisiae* TMB3400 (Olofsson et al., 2008b). By applying pre-fermentation of xylose, the xylose utilization was enhanced to 77% in fed-batch SSF at 10% WIS loading (Bertilsson et al., 2009).

Previous results in Chapter II have shown that *S. cerevisiae* 424A (LNH-ST) consumed xylose slowly compared to glucose, especially in hydrolysates. Degradation products, ethanol and fermentation metabolites are the major inhibitors for xylose consumption. To alleviate ethanol and fermentation metabolite inhibition, performing xylose fermentation ahead of glucose

fermentation might help. In the present study, a two-step SSCF process on AFEX$^{TM}$-treated switchgrass (using commercial enzymes and *S. cerevisiae* 424A) was developed. Two-step SSCF gives higher ethanol yield with improved xylose consumption compared to the traditional SSCF process on AFEX$^{TM}$-SG. Process improvements include hydrolyzing AFEX$^{TM}$-SG with hemicellulases (xylanase and pectinase) first to release xylose which was then fermented by *S. cerevisiae* 424A, followed by adding cellulases to hydrolyze glucan to glucose and continue the fermentation. Several process parameters for the two-step SSCF were optimized and results were compared with both traditional SSCF and SHF process.

## 3.2 Materials and Methods

### 3.2.1 Switchgrass

The species SG used in this study was Cave-in-Rock grown at Michigan State University (East Lansing, MI, USA) and harvested in mid October (Bals et al., 2010). The glucan, xylan and lignin contents were estimated using the standard method published by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2004) and have been reported in our previous publication (Bals et al., 2010) as 33.6%, 25.3%, and 16.7%, respectively.

### 3.2.2 AFEX$^{TM}$ Pretreatment

AFEX$^{TM}$ pretreatment conditions used for this study include: ammonia to biomass loading 2.0 g/g dry biomass, water loading 0.5 g/g dry biomass, temperature 140 $^{o}$C and residence time 30 minutes.

### 3.2.3. Enzymatic Hydrolysis (EH)

A commercial enzyme mixture was used for enzymatic hydrolysis. The mixture was composed of Spezyme CP (Genencor Inc, USA; protein 88 mg/mL) 89.3 ml/kg SG, Novozyme

188 (Sigma-Aldrich, USA; protein 150 mg/ml) 89.9 ml/kg SG, Multifect xylanase (protein 35 mg/mL) 31.5 ml/kg SG and Multifect pectinase (Genencor Inc, USA; protein 90 mg/mL) 22.2 ml/kg SG. The protein concentrations were estimated by Dairyone (Ithaka, NY) using Kjeldahl's method. These enzymes activities have been reported by Dien et al. (Dien et al., 2008). The glucan loading (Zhong et al., 2009) used in this study was 6% by weight. The reaction was carried out in a 2.0 L baffled flask with 500 g total mixture at pH 4.8 (50 mM phosphate buffer), 50 $^{o}$C, and 250 rpm for 96 h. A final concentration of 50 mg/L chloramphenicol (Cm) was used to avoid microbial contamination. Biomass and enzymes were fed in two batches at a 3 h time interval. Hydrolyzate was harvested by centrifuging at 14,000 rpm for 30 min and sterile filtering the supernatant through a 0.22 μm filter.

### 3.2.4 Microorganism and seed culture preparation

Xylose-fermenting *S. cerevisiae* 424A(LNH-ST) (Ho et al., 1999) obtained from Prof. Nancy W. Y. Ho, Purdue University was used for this study. Seed culture was prepared on YEP medium (5 g/L yeast extract, 10 g/L peptone and 50 g/L glucose) by inoculating a frozen glycerol stock into a 250 ml flask with a working volume of 100 ml. The initial OD of the seed culture was approximately at 0.1. The strain was grown at 30 $^{o}$C and 150 rpm under microaerophilic conditions (Zhong et al., 2009) for 20 h. The final optical density at 600 nm ($OD_{600}$) and glucose concentration of the seed culture were around 10 and below 1 g/L, respectively.

### 3.2.5 Fermentation of hydrolyzate

Fermentations of hydrolyzate were carried out in 250 ml Erlenmeyer flasks with a working volume of 100 ml at pH 5.5, 30 $^{o}$C and 150 rpm. The flasks were capped with rubber stoppers to

maintain microaerophilic conditions. Yeast cell pellets used for inoculation were obtained by centrifuging the seed culture at 4000 rpm for 8 min. The initial OD for fermentation of hydrolyzate was 2.0. Glucose, xylose and ethanol concentrations were analyzed by HPLC with a Biorad Aminex HPX-87H column described previously in Chapter II section 2.2.7.

Ethanol metabolic yield was calculated based on the theoretical ethanol yield from available glucose and xylose, which is 0.51 g ethanol/g sugar.

### 3.2.6 SSCF of AFEX$^{TM}$-SG

SSCF experiments were conducted in 250 ml baffled flasks with 100 g total mixture at 180 rpm in a shaking incubator (Innova, New Brunswick, NJ). Enzyme loading and glucan loading were the same as for enzymatic hydrolysis. For traditional SSCF (Fig.13A), all the enzymes were added at the beginning during pre-hydrolysis at pH 4.8, 50 $^{o}$C and 250 rpm for 8 h. Then pH, temperature, and agitation rate were adjusted to 5.2, 30 $^{o}$C, and 180 rpm, respectively. Yeast cells were inoculated at an initial OD of 4 to perform SSCF for another 144 h. For two-step SSCF (Fig.13B), xylanase and pectinase were added at first to perform pre-hydrolysis for 8 h. Then yeast cells and a small percentage of Spezyme CP and Novozyme 188 were added to the hydrolyzate to carry out SSCF (step 1) for 60 h. During step 1, most of the xylan was hydrolyzed to xylose and then fermented by yeast cells. Little glucan was hydrolyzed and fermented during this step. After 60 h, the rest of Spezyme CP and Novozyme 188 enzymes were fed to release glucose from glucan, which was subsequently fermented to ethanol by the yeast (SSCF step 2).

Unless otherwise stated, fermentations were performed with no washing of pretreated biomass, detoxification or nutrient supplementation.

### 3.2.7 Preparation of washed AFEX$^{TM}$-treated SG

AFEX$^{TM}$-treated SG wash experiments were conducted using 50 ml distilled water per g dry AFEX$^{TM}$ treated SG. The pretreated biomass was washed 5 times to make sure all the soluble degradation products were removed. The washed biomass was used the same day.

### 3.2.8 Mass balance

Mass balance calculation was based on the analyses of ethanol, monomeric, oligomeric, and polymeric sugars before and after each process (enzymatic hydrolysis and fermentation for SHF; fermentation for SSCF) as reported earlier (Lau & Dale, 2009).

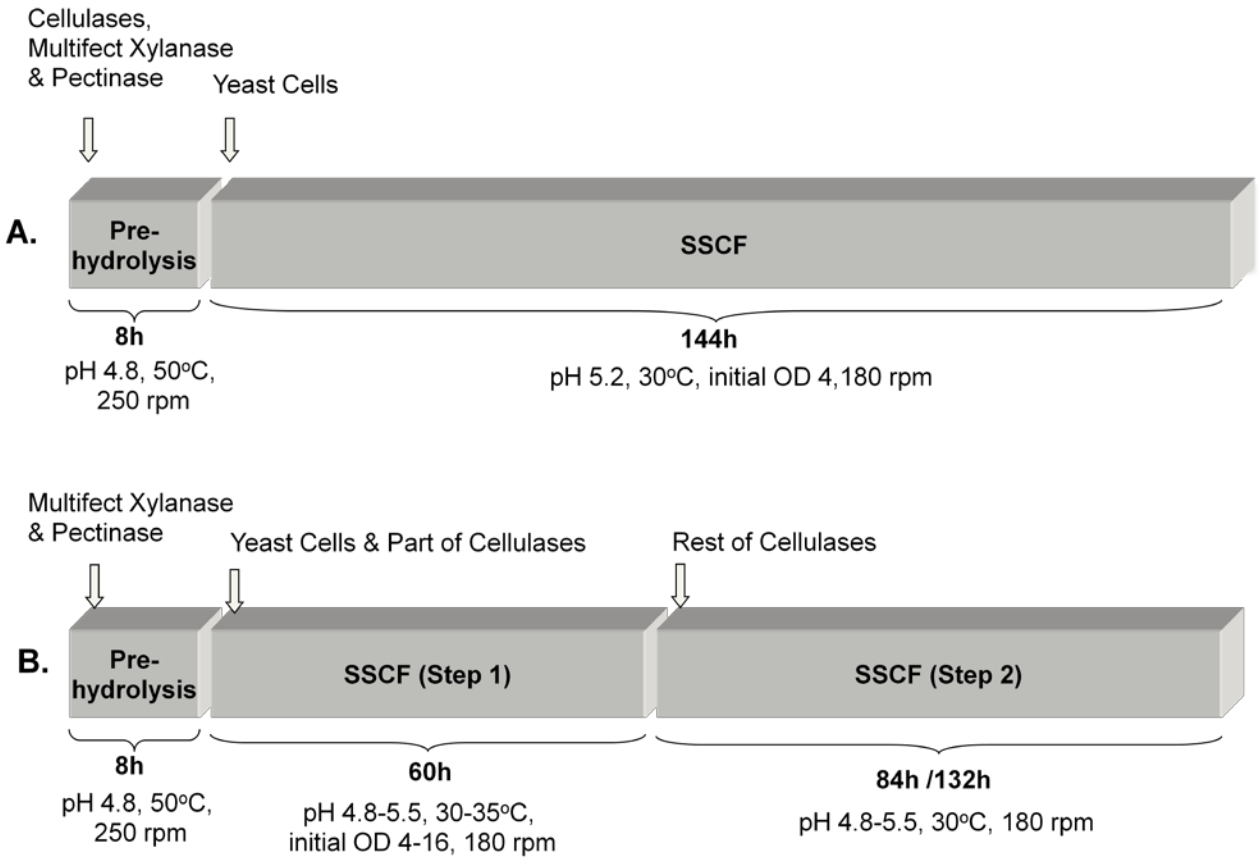



Figure 13 Schematic diagram of traditional SSCF (A) and two-step SSCF (B).

Table 6 Summary of parameters for the experiments examining the effects of the fraction of total cellulases loaded and temperature during step 1, pH and initial OD on two-step SSCF performance

| Parameter | Effect of percentage of total cellulases loading | Effect of temperature | Effect of initial $OD_{600}$ | Effect of pH |
|---|---|---|---|---|
| Percentage of total cellulases loading | 0%,2%,4%,6%,8% | 4% | 4% | 4% |
| Temperature (°C) | 30 | 30, 33, 35 | 35 | 35 |
| pH | 5.2 | 5.2 | 5.2 | 4.8, 5.2, 5.5 |
| Initial $OD_{600}$ | 4 | 4 | 1, 4, 10, 16 | 4 |
| Relevant figure | Fig. 15A1, A2 | Fig. 15B1,BA2 | Fig. 15C1, C2 | Fig. 15D1, D2 |

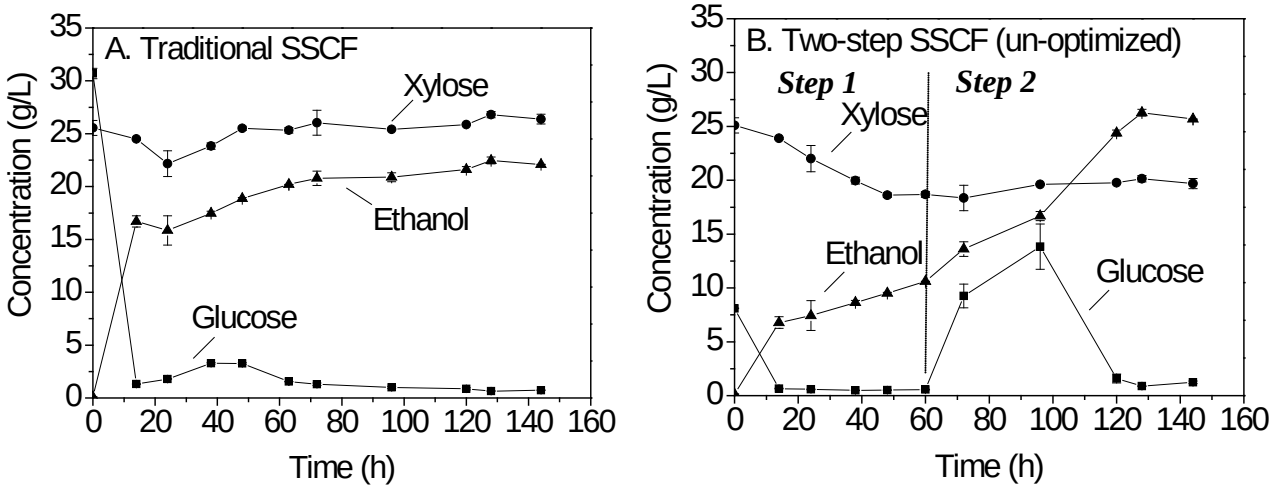



Figure 14 Traditional SSCF (A) vs. two-step SSCF (B).

Both experiments were carried out at 6% glucan loading, initial OD 4, pH 5.2, 30 $^{o}$C, and 180 rpm. For this experiment of two-step SSCF, no cellulases were added during step 1.

### 3.3 Results and discussion

#### 3.3.1 Traditional SSCF vs. two-step SSCF (un-optimized)

Traditional and two-step SSCF experiments were conducted at identical conditions at 6.0% (w/w) glucan loading (equivalent to 17.9% (w/w) total solids loading) of AFEX$^{TM}$-treated SG.

In this two-step SSCF experiment, no cellulases were added during step 1. Results are compared side-by-side in Fig. 14. For the traditional SSCF process, after 8 h pre-hydrolysis using hemicellulase and cellulase, glucose and xylose concentrations reached 30.7 and 25.5 g/L, respectively. After inoculation of the yeast, glucose concentration was quickly reduced to 1.3 g/L in 14 h and then maintained at a low level (< 4 g/L) during the whole fermentation time. However, xylose concentration remained at nearly the same level (about 25 g/L) during the entire process. A relatively low final ethanol concentration (22 g/L) was achieved after 144 h fermentation. For the two-step SSCF process (un-optimized), glucose and xylose concentrations were 8.1 and 25.1 g/L, respectively, after 8 h pre-hydrolysis. During step 1 of SSCF (0-60 h), glucose and xylose concentrations were reduced to 0.6 and 18.7 g/L, respectively with 10.6 g/L ethanol produced. In the step 2 (after cellulase addition after 60 h), xylose concentration remained the same at about 19 g/L and glucose concentration increased to 15 g/L in 36 h. However, glucose concentration was reduced to 0.9 g/L at 128 h and ethanol concentration reached the maximum of 26.3 g/L. These results clearly demonstrate that the two-step SSCF process results in higher ethanol yield by enhanced xylose consumption and is superior to the traditional SSCF process.

### 3.3.2 Optimization of two-step SSCF conditions

Conditions optimized during the two-step SSCF process include: the fraction of the total cellulases loaded and temperature during step 1 of SSCF, initial OD, and pH (Table 6). Different cellulases fractions loaded during step 1 resulted in different xylose consumption rates (Fig. 15A1) (assuming that xylan hydrolysis was not affected by feeding cellulases at low levels). Among the tested values, 4% of total cellulases led to the fastest xylose consumption, the lowest final xylose concentration (18.5 g/L), and highest ethanol yield (27.3 g/L, Fig. 15A2). Regarding

temperature optimization, xylose was consumed much faster at both 33 and 35 $^{o}$C than at 30 $^{o}$C (Fig. 15B1) even without considering the higher xylose release rate at higher temperature (Taherzadeh & Karimi, 2007). The final ethanol and xylose concentrations were higher and lower, respectively when step 1 of SSCF was conducted at either 33 or 35 $^{o}$C (Fig. 15B2). Higher initial OD resulted in higher xylose consumption rates, lower final xylose concentration (6.27 g/L at OD 16), and higher ethanol yield (36.1 g/L at OD 16, Fig. 15C1, C2). Judging from final xylose and ethanol concentrations, pH 5.5 was the optimum for two-step SSCF among the tested values (Fig. 15 D2). Glucose concentrations (data are not shown) in all tested conditions were below 2 g/L after 192 h. Considering the above analysis and the cost of cellulosic ethanol production, the following conditions were chosen as the optimal ones to conduct two-step SSCF: 4% of the total cellulases loading and 35 $^{o}$C during step 1, initial OD 4, and pH 5.5. Under the optimum conditions, 32.1 g/L final ethanol concentration was achieved with a final xylose concentration of 11.2 g/L (Fig. 16).

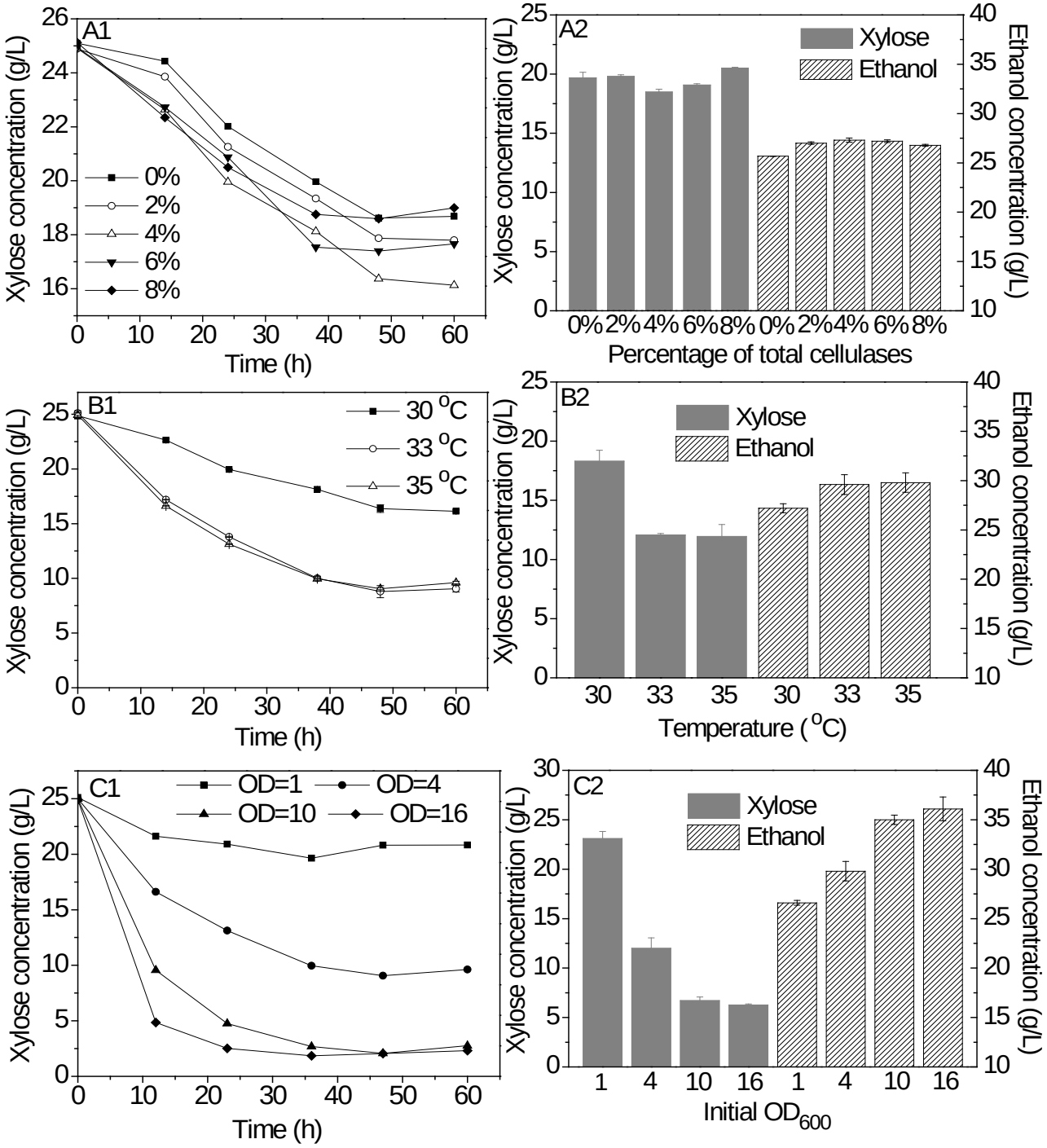
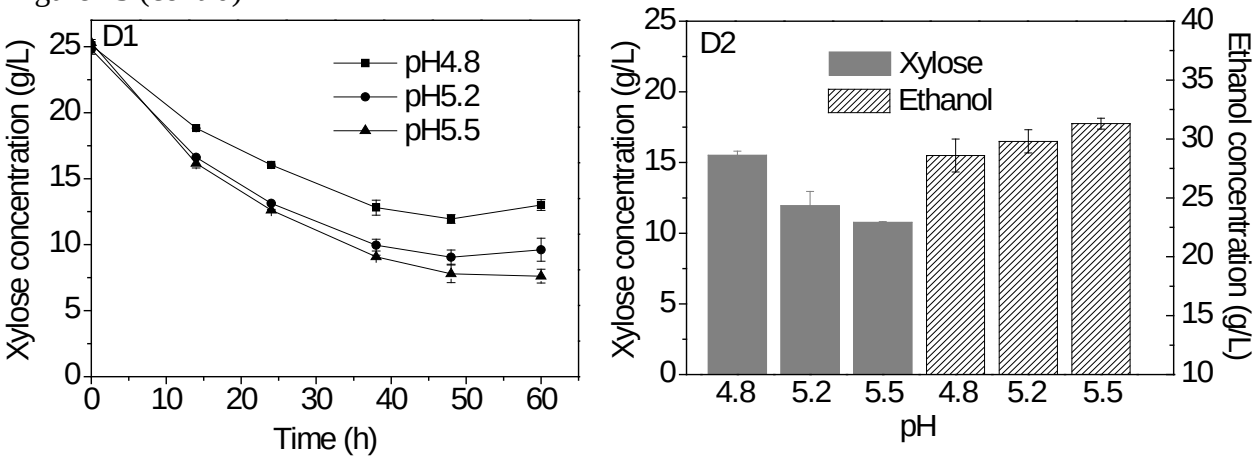



Figure 15 Effects of various factors on xylose fermentation and final ethanol concentration during two-step SSCF.
Fraction of total amount of cellulases fed during step 1 (A1), Initial OD (B1), Temperature during step 1 (C1), pH (D1) and the corresponding SSCF results after 192 h (A2, B2, C2, and D2).

Figure 15 (cont'd)




Poor xylose fermentation is a key bottleneck in traditional SSCF processes for cellulosic ethanol production. This is probably due to the large amount of glucose released during the process, which inhibited xylose uptake by hexose transporters (Bertilsson et al., 2008).

In addition, xylose consumption was also inhibited by ethanol and fermentation metabolites released during glucose fermentation (Chapter II). By performing the two-step SSCF under optimal conditions, xylose fermentation was greatly improved. This process avoided large amount of ethanol and fermentation metabolites inhibition on xylose fermentation. Glucose concentration was kept at a low but not zero level during step 1 of SSCF by supplementing with a low concentration of cellulases, which could not only help induce the sugar transporters expression used for xylose uptake (Bertilsson et al., 2008), but also help overcome the inhibition from degradation products (Bellissimi et al., 2009). At the beginning of fermentation, there was a low oxygen concentration in the broth. This was also important to xylose fermentation since oxygen could help to alleviate the redox imbalance caused by xylose metabolism (Ruohonen et al., 2006). Presence of nutrients at the beginning of the two-step SSCF process could be another beneficial factor which probably stimulated the yeast growth and hence stimulated the xylose consumption.

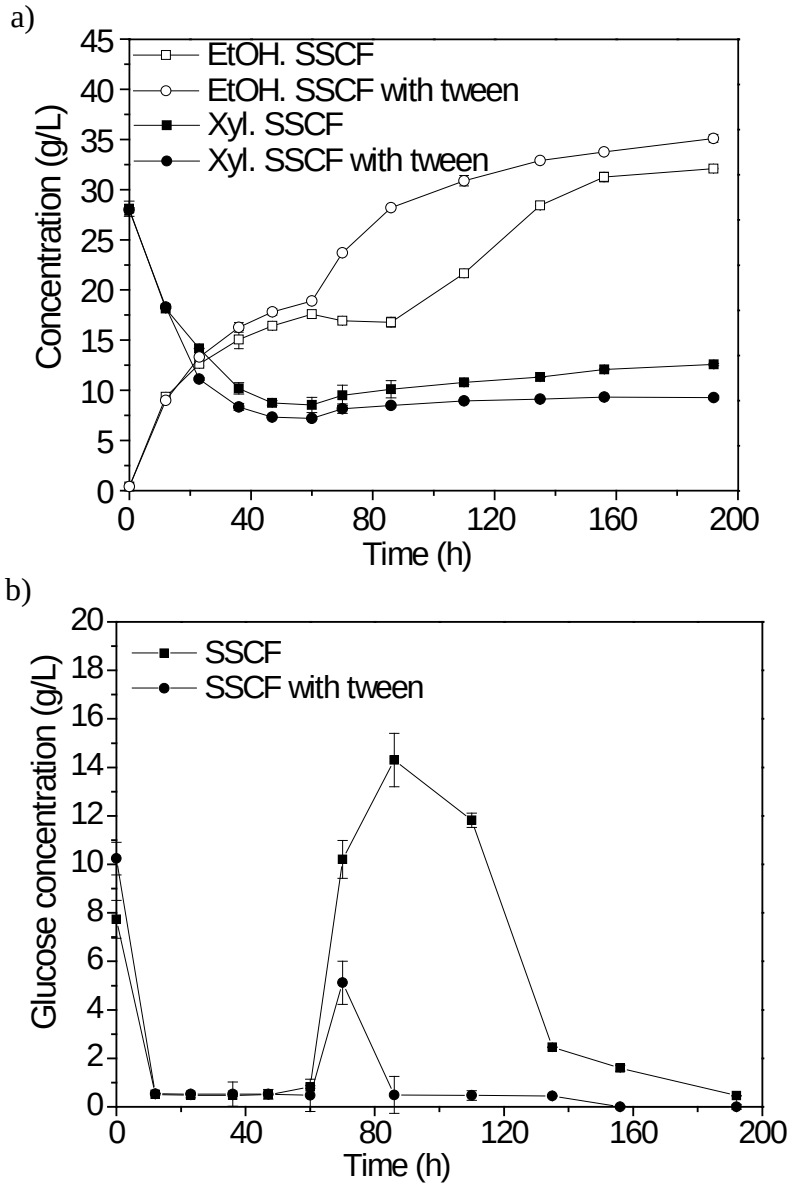



Figure 16 Effect of adding 2% (w/w) Tween 80 on xylose consumption, ethanol production (a), and glucose fermentation (b) during two-step SSCF.

The experiments were conducted at optimized conditions: initial OD 4, pH 5.5, 35 $^{o}$C and 4% of total cellulases loading during step 1 of SSCF.

In all tested SSCF experiments, little xylose was consumed after 48 h even with high xylose concentration and low glucose concentration in the broth. This was also found by the SSCF studies with TMB3400 (Bertilsson et al., 2009; Olofsson et al., 2008b) and was mostly

attributed to the decreased cell viability (Rudolf et al., 2005) and the inactivation of transport systems.

### 3.3.3 Effects of EH residue and washed AFEX$^{TM}$-SG on hydrolyzate fermentation

To investigate the effects of solids content during SSCF on xylose fermentation and ethanol metabolic yield, EH residue and washed AFEX$^{TM}$-SG biomass were added to the hydrolyzate derived from 6% glucan loading enzymatic hydrolysis (Fig. 17). Both glucose and xylose were fermented well in the absence of solid residues (Fig. 17A). Glucose (45.5 g/L) was consumed completely in 18 h and xylose was reduced from 31.8 g/L to 1.9 g/L in 96 h. The final ethanol concentration reached 34.6 g/L with an ethanol metabolic yield of 89.7%. With the solid EH residue (75 g/L, equivalent to the same concentration in final SSCF broth), however, only 11.8 g/L xylose was consumed during 144 h fermentation (Fig. 17B). The ethanol metabolic yield was reduced to 82% (Fig. 17D). The xylose consumption rate and ethanol metabolic yield were also lowered by adding 29 g/L (equivalent to 1% glucan loading AFEX$^{TM}$-SG) or 114 g/L (equivalent to 4% glucan loading AFEX$^{TM}$-SG) washed AFEX$^{TM}$-SG (Fig. 17C, D). These results clearly suggest that the presence of solid biomass (especially the EH residue) during SSCF affected both xylose consumption and ethanol metabolic yield. The mechanisms by which EH residue affects the xylose metabolism are unclear at present.

In an attempt to alleviate these effects, 2% (w/w) of Tween 80 was added during the two-step SSCF process (Fig. 16). With Tween 80 addition, glucose concentration was enhanced to 10.2 g/L after pre-hydrolysis. Adding surfactants is known to improve sugar yield during enzymatic hydrolysis (Alkasrawi et al., 2003; Kim & Chun, 2004; Kim et al., 2006; Tu et al.,

2009). This improvement is attributed to reduction of unproductive binding to the lignin (Eriksson et al., 2002a). The final sugar conversion, however, was slightly decreased when

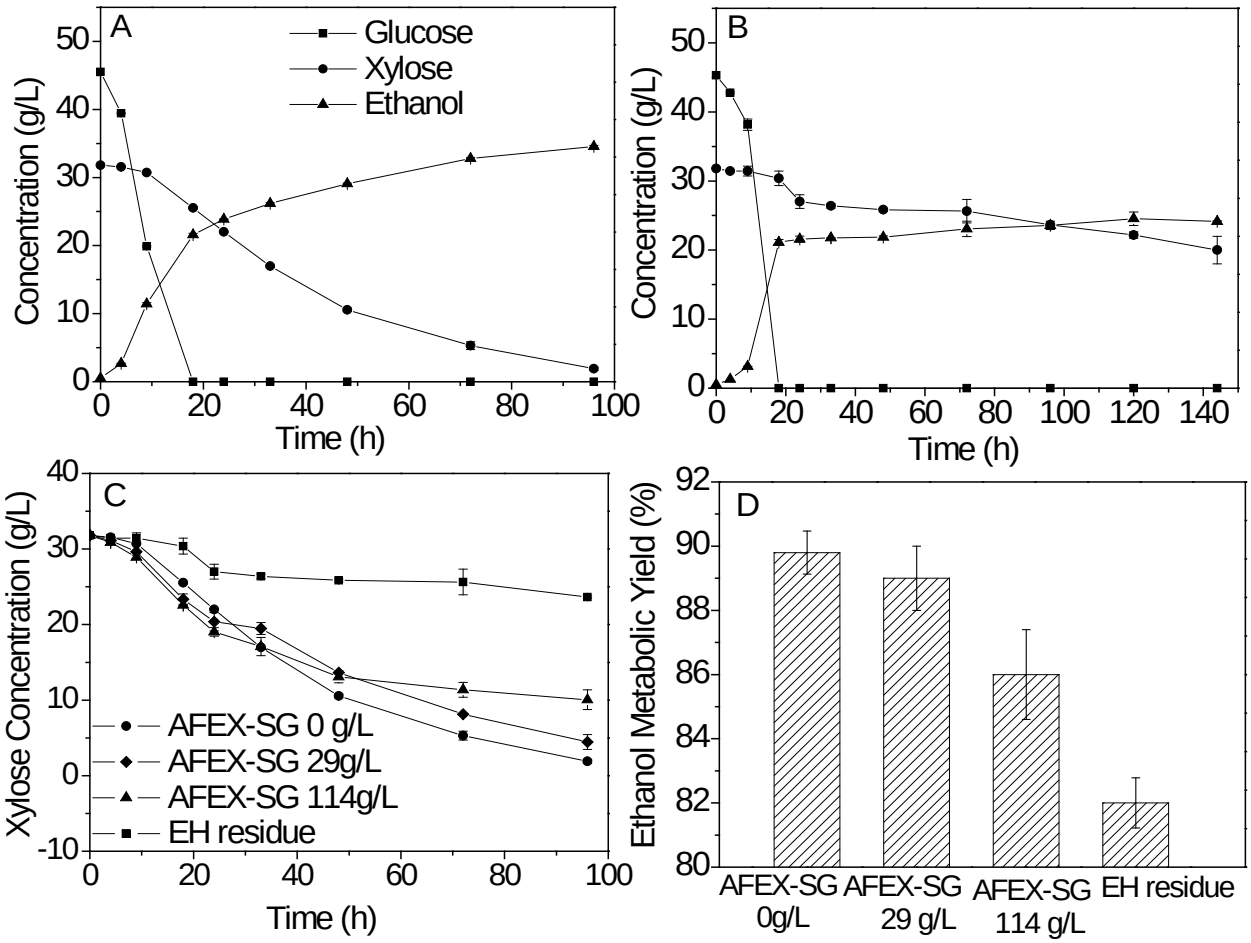



Figure 17 Effects of EH residue and washed AFEX$^{TM}$-SG on fermentation performance in 6% glucan loading AFEX$^{TM}$-SG hydrolyzate.
A, fermentation of hydrolyzate without solids; B, fermentation of hydrolyzate with EH residue; C, effects of washed AFEX$^{TM}$-SG and EH residue on xylose fermentation in hydrolyzate; D, effects of washed AFEX$^{TM}$-SG and EH residue on ethanol metabolic yield. Fermentations carried out at initial OD 2, pH 5.5, 30 $^{o}$C and 150 rpm.

compared with the two-step SSCF without Tween 80 (Fig. 18B1, B2). This was probably because of the higher ethanol concentration present in the fermentation broth due to the addition of Tween 80. Ethanol is an important factor limiting enzymatic hydrolysis during SSCF

(Taherzadeh & Karimi, 2007). The maximum glucose accumulation after feeding cellulases at 60 h was reduced from 14.3 to 5.1 g/L (Fig. 16b), which indicated that cellular activity was improved. The xylose consumption rate and ethanol metabolic yield were also enhanced with the addition of Tween 80 (Fig. 16a, Fig. 18B2). Similar behavior was also found during SSF by the addition of Tween 20 (Alkasrawi et al., 2003). Since Tween 80 did not show any influence during solid-free fermentation (Tu et al., 2009), it probably reduced the binding of yeast cells to the solid biomass during SSCF, which inhibited the cell viability.

### 3.3.4 Mass balance comparison between SHF and two-step SSCF

To demonstrate the current status and bottlenecks of the technology, mass balances were done for both SHF and two-step SSCF of AFEX$^{TM}$-SG (with and without 2% Tween 80 addition) (Fig. 18, Table 7). The SHF process yielded 178.4 g ethanol from 1 kg AFEX$^{TM}$-SG with glucan and xylan conversions of 66.5% and 74.7%, respectively (Fig. 18A). Ethanol metabolic yield was 89.7%. SHF of Cave-in-Rock switchgrass (cut in October) showed low sugar conversion during enzymatic hydrolysis, which was also found in an earlier report (Bals et al., 2010). However, ethanol metabolic yield was high during fermentation of the solid-free hydrolyzate. Enzymatic hydrolysis has been identified as the bottleneck for SHF (Zhong et al., 2009). Two-step SSCF with initial OD 4 enhanced glucan and xylan conversions to 80.3% and 84.3%, respectively (Fig. 18B1). These enhancements were probably due to the removal of enzymatic hydrolysis products (sugars) by the yeast as soon as they were produced, thus preventing sugar inhibition (Xiao et al., 2004). The glucan conversion enhancement was also due to the hydrolysis and fermentation of xylan before cellulases were added, which reduced the inhibition of xylan and xylose oligomers to cellulases (Kumar & Wyman, 2009b). However, the ethanol metabolic yield was reduced to 72.7% and the final ethanol yield was 165.3 g per kg

AFEX$^{TM}$-SG. About 55.0 g monomeric xylose remained unutilized in the fermentation broth. Based on the total sugars in the AFEX$^{TM}$-SG, 76.8% of glucose and 51.0% of xylose were consumed by yeast; 3.5% of glucose and 33.2% of xylose remained in the fermentation broth; 19.7% of glucose and 15.7% of xylose were unhydrolyzed and left in the residue. By adding 2% of Tween 80, ethanol metabolic yield was improved to 77.7% (Fig. 18B2) and the final ethanol yield was enhanced to 183.5 g per kg AFEX$^{TM}$-SG. However, 40.7 g monomeric xylose was still un-utilized.

Xylose consumption and the final ethanol yield (but not the ethanol metabolic yield) of two-step SSCF were improved by increasing the initial OD (Table 7). However, this may not be a cost-efficient approach unless the yeast cells can be reused. Though Tween 80 addition improved both xylose consumption and ethanol metabolic yield, this is probably too expensive for cellulosic ethanol production. A cheap surfactant with the same effect of Tween 80 would be an important advance. About 191 g ethanol per kg of washed AFEX$^{TM}$-SG (all the degradation products removed) was achieved by SSCF when supplementing YEP (Table 7). Overall, 91% xylose consumption and 77.2% ethanol metabolic yield were realized. However, sugar conversion was reduced to 77% probably because of the high ethanol concentration which is known to inhibit enzymes (Taherzadeh & Karimi, 2007). Washing and nutrient supplementation will also increase the production cost of cellulosic ethanol from AFEX$^{TM}$ treated biomass.

### 3.3.5 The limiting factors during SSCF

Reduced xylose consumption and ethanol metabolic yield are two key issues for SSCF, which affect the overall ethanol yield and titer. The presence of solid AFEX$^{TM}$ treated biomass,

especially the EH residue (mostly insoluble lignin) was partly responsible for reduced performance versus solid-free fermentation. Another factor which may affect xylose consumption and ethanol metabolic yield during SSCF is the interaction between solid biomass and degradation products produced during AFEX. Degradation products caused lower yeast cell production and thereby increased ethanol metabolic yield but decreased xylose consumption during hydrolyzate fermentation (Chapter II). Based on this assumption, cell production should be increased when SSCF is conducted on washed AFEX$^{TM}$-SG (degradation products removed) with supplementation of YEP, which in turn increase xylose consumption and decrease ethanol metabolic yield. Though the results showed enhanced xylose consumption, the ethanol metabolic yield was also increased (Table 7). High cell density (Table 7; SSCF with initial OD 10) only benefited xylose consumption with the presence of degradation products. Therefore, it seems that the removal of degradation products helped increase the ethanol metabolic yield. This implies the interaction between solid biomass and degradation products also played a role in the reduction of ethanol metabolic yield since degradation products alone do not reduce ethanol metabolic yield.

**3.4 Conclusions**

The two-step strategy was an efficient way to improve xylose consumption and ethanol yield during SSCF. The optimized conditions for two-step SSCF were found to be: 4% of total amount of cellulases loading and 35 $^{o}$C during step 1, initial OD 4, and pH 5.5. Compared to the traditional SSCF (at 6% glucan loading), the two-step SSCF reduced the final xylose concentration from 26.4 g/L to 11.2 g/L and increased the final ethanol concentration from 22.0 g/L to 32.1g/L. 80.3% of glucan conversion and 84.3% of xylan conversion were achieved with ethanol metabolic yield 72.7%. Enzymatic hydrolysis residue played a significant role in the low xylose consumption and ethanol metabolic yield.

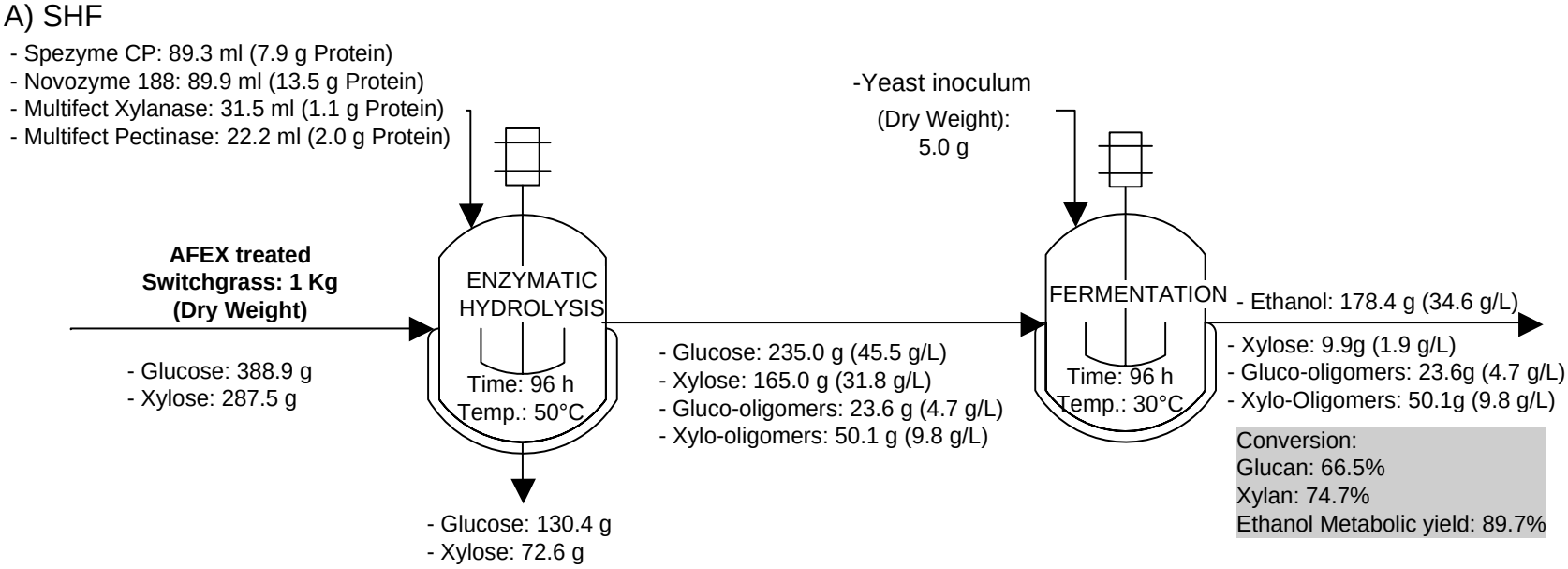



Figure 18 Mass balance analysis for SHF (A), two-step SSCF (B1), and two-step SSCF with 2% Tween 80 (B2).
For SHF, enzymatic hydrolysis was carried out at 6% glucan loading, pH 4.8, 50 $^{o}$C, 250 rpm for 96 h. Fermentation of hydrolyzate was conducted at initial OD 2, pH 5.5, 30 $^{o}$C, 150 rpm for 96 h. For two-step SSCF, experiments were performed at 35 $^{o}$C and 4% of total cellulases loading during step 1 of SSCF, initial OD 4, pH 5.5, and 180 rpm.

Figure 18 (cont'd)

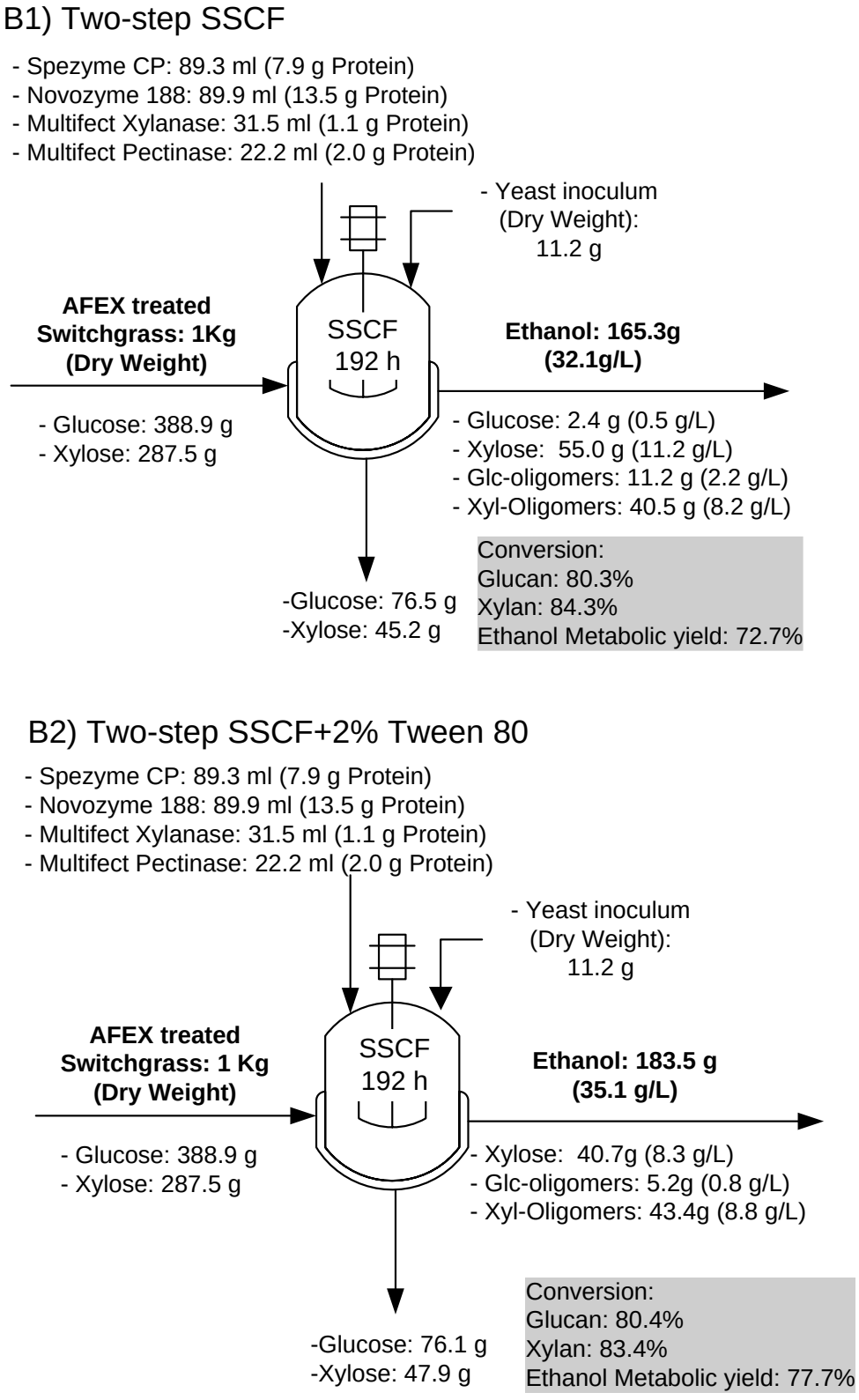


Table 7 Summary of SHF and two-step SSCF experiments on 6% glucan loading AFEX™-SG[a]

| | Sugar conversion[b] (%) | Xylose consumption[c] (%) | Final ethanol concentration (g/L) | Ethanol metabolic yield[d] (%) | Ethanol yield (g/kg AFEX SG) |
|---|---|---|---|---|---|
| SHF | 70 | 94 | 34.6 | 89.7 | 178 |
| SSCF-OD 4 | 82 | 73 | 32.1 | 72.7 | 165 |
| SSCF-OD10 | 85 | 84 | 35.0 | 71.7 | 180 |
| SSCF-tween[e] | 82 | 79 | 35.1 | 77.7 | 184 |
| SSCF-washed SG[f] | 77 | 91 | 36.4 | 77.2 | 191 |

a, For SHF, enzymatic hydrolysis was carried out at 6% glucan loading, pH 4.8, 50 $^{o}$C, 250 rpm for 96 h. Fermentation of hydrolyzate was conducted at initial OD 2, pH 5.5, 30 $^{o}$C, 150 rpm for 96 h. For two-step SSCF, experiments were performed at 35 $^{o}$C and 4% of total cellulases loading during step 1 of SSCF, pH 5.5, and 180 rpm.
b, Related to both oligmeric and monomeric sugars (glucose and xylose)
c, Based on available monomeric xylose
d, Based on the theoretical yield
e, two-step SSCF with initial OD 4 and 2% (w/w) Tween 80
f, two-step SSCF of washed AFEX-SG with initial OD 4 and supplementation of YEP.

# CHAPTER IV SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTATION OF AFEX™ PRETREATED CORN STOVER


## Abstract

Xylose consumption by *Saccharomyces cerevisiae* 424A(LNH-ST) during simultaneous saccharification and co-fermentation (SSCF) of AFEX™ pretreated switchgrass was inhibited by unhydrolyzed solids. Such inhibitory effects were not found in unhydrolyzed solids from AFEX™ pretreated corn stover (AFEX™-CS). However, the xylose consumption was still unsatisfactory during 6 h pre-hydrolysis SSCF. By extending the pre-hydrolysis time to 24 h or longer, the xylose consumption was improved significantly. In order to better understand the reasons for such improvement, the hydrolysate slurries after 6 h pre-hydrolysis and 24 h pre-hydrolysis were studied and compared. We found that the glucose concentration after pre-hydrolysis was the critical factor that determined cell viability and hence xylose consumption during SSCF. Low temperature (30$^{o}$C) and ethanol inhibition were shown to be the factors limiting hydrolysis rate and hence productivity during SSCF.

## 4.1 Introduction

Corn stover is an agricultural residue abundantly available in the US, which makes it a suitable feedstock for biofuel production (Perlack et al., 2005). Regarding biofuel production from corn stover, much effort has focused on environmental impacts (Mann et al., 2002), energy analysis (Luo et al., 2009), and technology development (Lau & Dale, 2009). Lack of commercial conversion technologies is currently a bottleneck.

Due to the merits of high digestibility and high fermentability of AFEX$^{TM}$ pretreated biomass, a yield of 191.5 g ethanol per kg corn stover was realized without detoxification or nutrient supplementation after AFEX$^{TM}$ pretreatment (Lau & Dale, 2009). However, these experiments were done by separate hydrolysis and fermentation (SHF) which has issues of higher capital cost, longer processing time, higher contamination risk and sugar inhibition for enzymatic hydrolysis compared to simultaneous saccharification and co-fermentation (SSCF) (Taherzadeh & Karimi, 2007). While SSCF is generally superior to SHF, there are still several concerns about this process such as the optimum temperature discrepancies between saccharolytic enzymes and fermentation microbes as well as ethanol inhibition on enzymes (Taherzadeh & Karimi, 2007). *S. cerevisiae* 424A(LNH-ST) works best at 30$^{o}$C (Lau & Dale, 2009) while, the *Trichoderma* enzymes have an optimum temperature of around 50$^{o}$C (Taherzadeh & Karimi, 2007). Therefore, the SSCF of AFEX$^{TM}$ pretreated switchgrass (AFEX$^{TM}$-SG) was performed at 30-35$^{o}$C (Chapter III). Nevertheless, the sugar conversions during SSCF were still higher compared to SHF. That study also identified the inhibitory effect of unhydrolyzed biomass solids on xylose fermentation, which caused poor xylose consumption.

By applying two-step SSCF, which mainly hydrolyzes xylan and ferments xylose prior to glucose, xylose consumption was greatly improved. Xylose fermentation before glucose has the advantages of alleviating redox imbalance and promoting xylose consumption due to the presence of oxygen and many nutrients at the beginning as well as avoiding ethanol inhibition on xylose consumption. The key for the two-step SSCF is to keep the glucose release rate at a low level during the xylose fermentation period so that it does not affect xylose uptake by *S. cerevisiae* (Bertilsson et al., 2008).

In order to improve our understanding of the SSCF process, the performance of two-step SSCF and traditional SSCF were further compared on AFEX$^{TM}$ pretreated corn stover (AFEX$^{TM}$-CS). The effect of unhydrolyzed AFEX$^{TM}$-CS solids and the causes of the unsatisfactory xylose consumption during SSCF of AFEX$^{TM}$-CS were studied. The effect of ethanol and temperature on enzymatic hydrolysis was also investigated.

## 4.2 Materials and Methods

### 4.2.1 AFEX$^{TM}$ treated corn stover

Please see Chapter II section 2.2.1.

### 4.2.2 Microorganism and seed culture preparation

Seed cultures of *S. cerevisiae* 424A(LNH-ST) were prepared on YEP medium (10 g/L yeast extract and 20 g/L tryptone) with 75 g/L glucose and 25 g/L xylose in a 250 ml Erlenmeyer flask with a working volume of 100 ml. Frozen glycerol stock was used for inoculation and the initial seed culture $OD_{600}$ was 0.1. The culture was incubated at 30 $^{o}$C and 150 rpm under micro-aerophilic conditions for 24 h.

### 4.2.3 Separate hydrolysis and fermentation (SHF) of AFEX$^{TM}$-CS

Enzymatic hydrolysis (EH) of AFEX$^{TM}$-CS was performed in a 250 ml baffled flask with 100 g total mixture. The EH procedure is shown in Chapter II section 2.2.2. After 96 h hydrolysis, the hydrolysate was harvested by centrifuge at 14,000 rpm for 30 min to remove unhydrolyzed solids and was then sterile filtered through a 0.22 μm filter. The filtered hydrolysate was stored at 4 $^{o}$C in a sterile bottle for fermentation use. For study of the unhydrolyzed solids effect on fermentation, the hydrolysate was used as is without centrifuge and filtration (unfiltered hydrolysate).

Fermentation of hydrolysate procedure is the same as described in Chapter III section 3.2.5.

### 4.2.4 Simultaneous Saccharification and Co-fermentation

SSCF was conducted using the same enzymes and biomass loading as used for the SHF experiments. SSCF was also conducted in a 250 ml baffled flask with a total mixture weight of 100 g. For the traditional SSCF process, all the enzymes were added at the beginning and pre-hydrolyzed the biomass at 50 $^{o}$C, pH 4.8 and 250 rpm. After 6 h pre-hydrolysis, the temperature, pH and agitation rate were changed to 30 $^{o}$C, 5.5 and 180 rpm, respectively, and yeast cells were inoculated at an $OD_{600}$ of 2.0 to initiate the SSCF. For two-step SSCF, xylanase, pectinase and 25% of the Spezyme CP enzymes were added at the beginning for 6 h pre-hydrolysis. The other 75% of the Spezyme CP and all of Novozyme 188 enzymes were added 48 h after yeast inoculation. Glucose, xylose and ethanol concentrations were analyzed using HPLC with a Biorad Aminex HPX-87H column as described previously in Chapter II section 2.2.7.

### 4.2.5 Preparation of washed AFEX$^{TM}$-CS

The procedure used is the same as described in Chapter III section 3.2.7.

### 4.2.6 Mass balance

Please see Chapter III section 3.2.8.

### 4.2.7 Measurement of viable cell density

Please see Chapter II section 2.2.8.

## 4.3 Results and Discussion

### 4.3.1 Traditional SSCF process vs. Two-step SSCF process

The two-step SSCF process with xylose fermentation ahead of glucose fermentation resulted in better xylose consumption and higher ethanol yields compared to traditional SSCF when processing AFEX$^{TM}$-SG. Here the performance of traditional SSCF and two-step SSCF on AFEX$^{TM}$-CS was compared (Fig. 19). For the traditional process, xylose in the fermentation broth remained at a nearly constant concentration (around 11 g/L) during the whole process time.

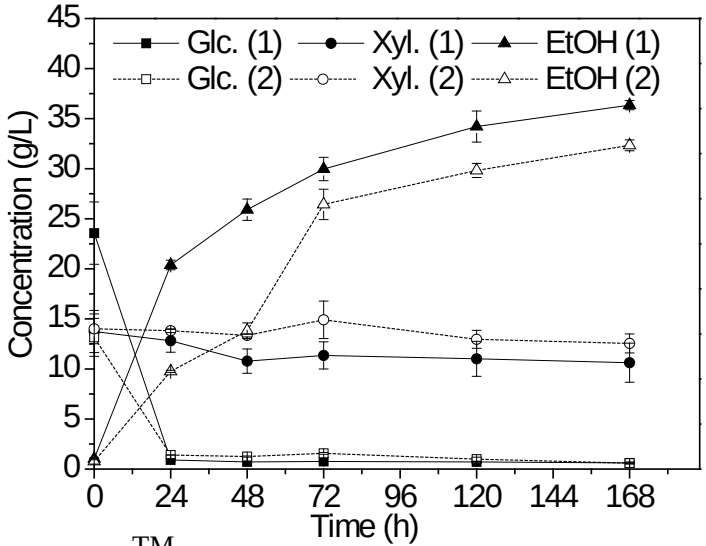



Figure 19 SSCF of AFEX$^{TM}$-CS by applying two processes.
Process 1 (Traditional SSCF): 6h pre-hydrolysis using all of the enzymes followed by inoculation of yeast; Process 2 (two-step SSCF): 6 h pre-hydrolysis using xylanase, pectinase and 1/4 Spezyme CP followed by inoculation of yeast, the rest of Spezyme CP and Novozyme 188 was added at 48 h.

This indicates that the xylose consumption rate by the yeast was the same as the xylose release rate by the enzymes. At the end of the process, a concentration of 10.6 g/L xylose was left in the broth unutilized. The two-step SSCF process did not improve xylose fermentation. About 12.5 g/L xylose remained in the broth at the end of the process. Ethanol produced during the two processes was 36.3 and 32.3 g/L, respectively.

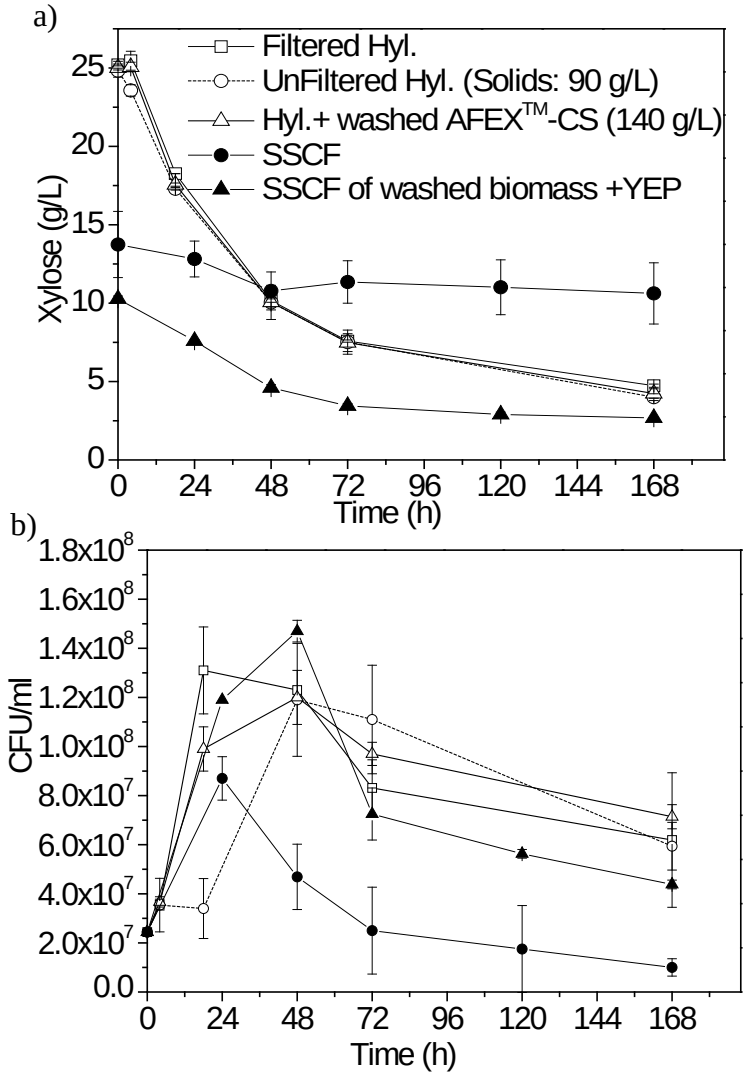



Figure 20 Comparison of xylose fermentation (a) and cell viability (b) during SHF and traditional SSCF.
SHF fermentations were carried out in filtered hydrolysate (without solids), unfiltered hydrolysate (with unhydrolyzed solids), and filtered hydrolysate with 140 g/L washed AFEX™-CS. 6 h pre-hydrolysis SSCF was performed on unwashed AFEX™-CS without nutrients supplementation and washed AFEX™-CS with supplementation of YEP (tryptone: 10 g/L & yeast extract 5 g/L).

Different biomass properties/compositions might have caused different results for two-step SSCF on AFEX$^{TM}$-SG and AFEX$^{TM}$-CS. AFEX$^{TM}$-SG contains 25.3% xylan and was easily solubilized by using only xylanase and pectinase (released around 25 g/L xylose after pre-hydrolysis). On the other hand, AFEX$^{TM}$-CS contains 20.4% xylan and was found to be very difficult to solubilize using only xylanase and pectinase even with a 10% higher dosage. Because of this solubilization issue, there was almost no free water and little xylose available for fermentation after pre-hydrolysis. Therefore, we tried adding part of Spezyme CP enzyme, which has low β-glucosidase activity, to help solubilize the biomass during the pre-hydrolysis. Several loadings were tried. The one reported here with 25% of the total Spezyme CP enzymes added at the start gave the best xylose consumption results. We found it difficult to balance solubilization of the biomass with minimization of the glucose release so as not to influence xylose fermentation. Apparently, 25% of the total Spezyme CP enzymes gave a too high glucose release rate and negatively affected xylose fermentation. The effect of cellulases loading on xylose consumption was reported previously in Chapter III. There are two potential ways to apply two-step SSCF on AFEX$^{TM}$-CS and give good results: (i) by increasing the amount of xylanase and pectinase use for pre-hydrolysis; (ii) by using pure endo-glucanase to help solubilize biomass. However, both methods will increase the cost of the process since enzymes are still expensive for cellulosic ethanol production (Humbird et al., 2011). Since the two-step SSCF method did not help improve the xylose fermentation on AFEX$^{TM}$-CS, we subsequently chose to work on traditional SSCF to better understand the poor xylose fermentation issue.

### 4.3.2 Effect of unhydrolyzed solids on xylose fermentation

It was found that the inhibitory effect of unhydrolyzed solids was the major cause for poor xylose fermentation during SSCF of AFEX$^{TM}$-SG. Therefore, we investigated the unhydrolyzed solids of AFEX$^{TM}$-CS and AFEX$^{TM}$-CS biomass itself on xylose fermentation in hydrolysate the same way as we did for AFEX$^{TM}$-SG (Fig. 20). The hydrolysate containing 52.3 g/L glucose and 25.5 g/L xylose was obtained by 96 h enzymatic hydrolysis. Surprisingly, no inhibitory effect of those solids on xylose fermentation (Fig. 20a) and cell viability (Fig. 20b) was observed except for a longer lag phase for the unfiltered hydrolysate fermentation (with unhydrolyzed solids 90 g/L). Xylose was consumed to levels below 5 g/L in 168 h, which was a typical rate for AFEX$^{TM}$-CS SHF fermentation using *S. cerevisiae* 424A(LNH-ST) (Lau & Dale, 2009). Comparing to SHF fermentations, SSCF showed lower cell viability (Fig. 20b) and poor xylose fermentation (Fig. 20a). It appears that the xylose fermentation was strongly correlated with cell viability. To test this hypothesis, we performed SSCF on washed AFEX$^{TM}$-CS with supplementation of YEP. Using this approach, we found the cell viability was enhanced to a similar level as in SHF (Fig. 20b) and the xylose consumption was also improved to approximately the SHF level with the final xylose concentration around 3 g/L (Fig. 20a). The correlation between cell viability and xylose fermentation was also observed on AFEX$^{TM}$-SG. The unhydrolyzed solids of AFEX$^{TM}$-SG severely reduced cell viability (data not shown), which was likely the cause for poor xylose fermentation in the presence of AFEX$^{TM}$-SG solids.

Since the poor xylose fermentation during SSCF of AFEX$^{TM}$-CS was probably not due to the solids inhibition and xylose consumption during fermentation of unfiltered hydrolysate was good, which could be considered as an SSCF process with 96h pre-hydrolysis period, we decided it would be worthwhile to investigate the effect of pre-hydrolysis time.

### 4.3.3 Effect of pre-hydrolysis time on SSCF performance

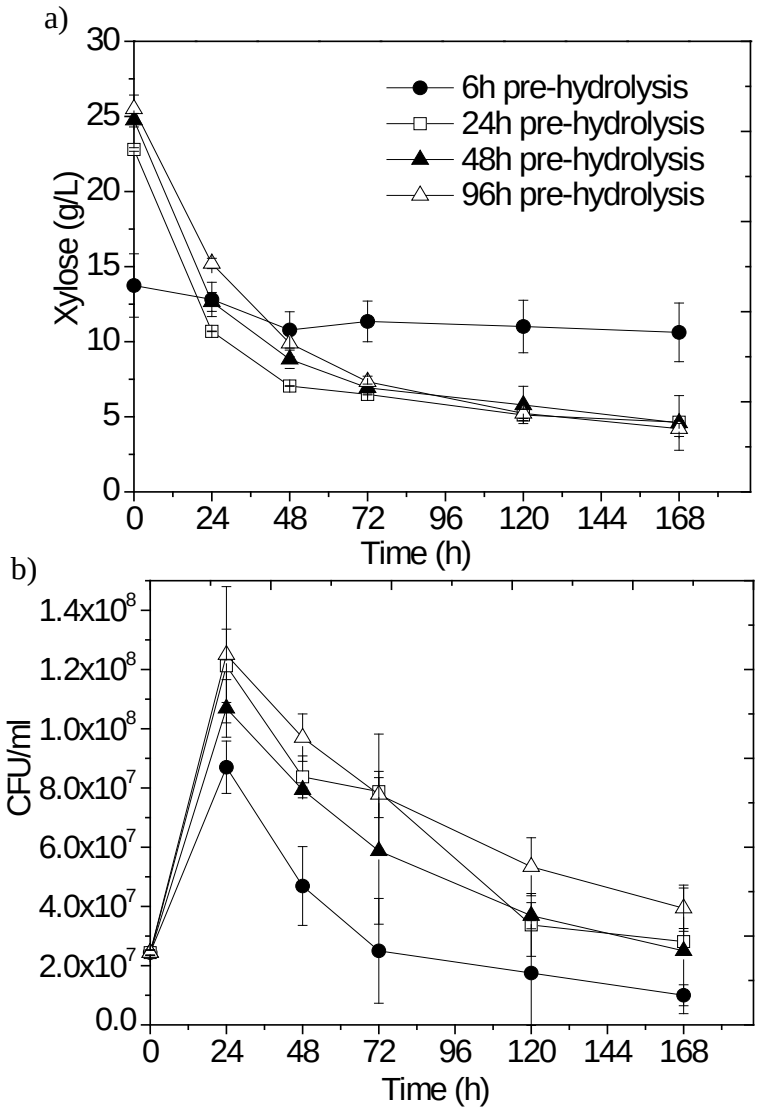



Figure 21 Effect of pre-hydrolysis time on xylose fermentation (a) and cell viability (b) during traditional SSCF.
The glucose concentrations after 6, 24, 48, and 96 h pre-hydrolysis were 23.5, 46.0, 50.2 and 52.3 g/L, respectively.

Four pre-hydrolysis times were tested (6, 24, 48, and 96 h) during SSCF (Fig. 21). As discussed above, the xylose consumption during 6 h pre-hydrolysis SSCF was not as good as during SHF experiments. However, as the pre-hydrolysis time was extended to 24, 48 or 96 h, the xylose consumption was improved and achieved the same level as SHF (Fig. 21a). The yeast cell viabilities were also enhanced (Fig. 21b), which is a possible reason for improved xylose consumption. The correlation between cell viability and xylose consumption was further validated by these results.

The initial purpose of pre-hydrolysis was to solubilize the biomass quickly at a high temperature so that a liquid, more homogenous environment could be formed for yeast fermentation. Though 6 h pre-hydrolysis solubilized the biomass well, the xylose fermentation during SSCF was poor. Hence, in addition to solubilizing the biomass, it is important to create a better environment in which to initiate yeast cell growth and thereby xylose consumption. Pre-hydrolysis time directly influences the concentration and composition of unhydrolyzed solids as well as the composition of liquid hydrolysate. To understand why a longer pre-hydrolysis time is superior, we studied the effects of both the solids portion of the medium and the liquid portion on xylose fermentation and yeast cell viability.

### 4.3.4 Effect of solids and liquids from different pre-hydrolysis on xylose fermentation and yeast cell viability

After pre-hydrolysis for different time periods, the unhydrolyzed solids were harvested by centrifugation and washed using water and 1M sodium chloride to remove both degradation products and enzymes adsorbed on the solids. Removal of enzymes avoided further sugar release and changes in solids composition. The compositions of solids obtained were determined using the NREL protocol (Sluiter et al., 2004) and are shown in Table 8. The effects of such solids on

xylose fermentation and yeast cell growth were investigated in hydrolysate produced in 96 h (Fig. 22). The solids concentrations were the same as after pre-hydrolysis during SSCF and ranged from 90 to 135 g/L (Table 8). No significant differences of xylose consumption (Fig. 22a) and cell viability (Fig. 22b) were observed between fermentations with 6 h pre-hydrolyzed solids and fermentations with longer time pre-hydrolyzed solids. All the xylose consumption rates and cell viabilities were as good as achieved during SHF. Therefore, the solids portion of the media is probably not the factor causing xylose consumption difference among the SSCFs using different pre-hydrolysis time.

Table 8 Concentration and composition of pre-hydrolyzed solids [a]

| Pre-hydrolysis time (h) | Solids conc. (g/L)[b] | Solids composition [c] | | | | |
|---|---|---|---|---|---|---|
| | | Glucan (%) | Xylan(%) | Arabinan (%) | Acid insoluble lignin (%) | Ash(%) |
| 0 | 195 | 34.1 | 20.4 | 4.2 | 11.0 | 6.1 |
| 6 | 135±5.0 | 27.0±0.1 | 15.5±0.4 | 1.9±0.0 | 27.9±0.6 | 7.2±0.3 |
| 24 | 117±2.4 | 22.7±1.3 | 13.9±0.6 | 1.8±0.0 | 33.4±1.4 | 8.5±0.1 |
| 48 | 102±6.8 | 21.9±1.2 | 13.0±0.6 | 1.8±0.0 | 34.4±1.5 | 8.8±0.4 |
| 96 | 90±3.3 | 20.0±1.2 | 12.3±0.9 | 1.8±0.0 | 37.0±1.9 | 8.7±0.2 |

[a] Pre-hydrolysis was carried out at 50 $^{o}$C, 250 rpm, pH 4.8

[b] Unhydrolyzed solids, dry weight basis

[c] Composition analysis was performed on unhydrolyzed solids using the NREL protocol

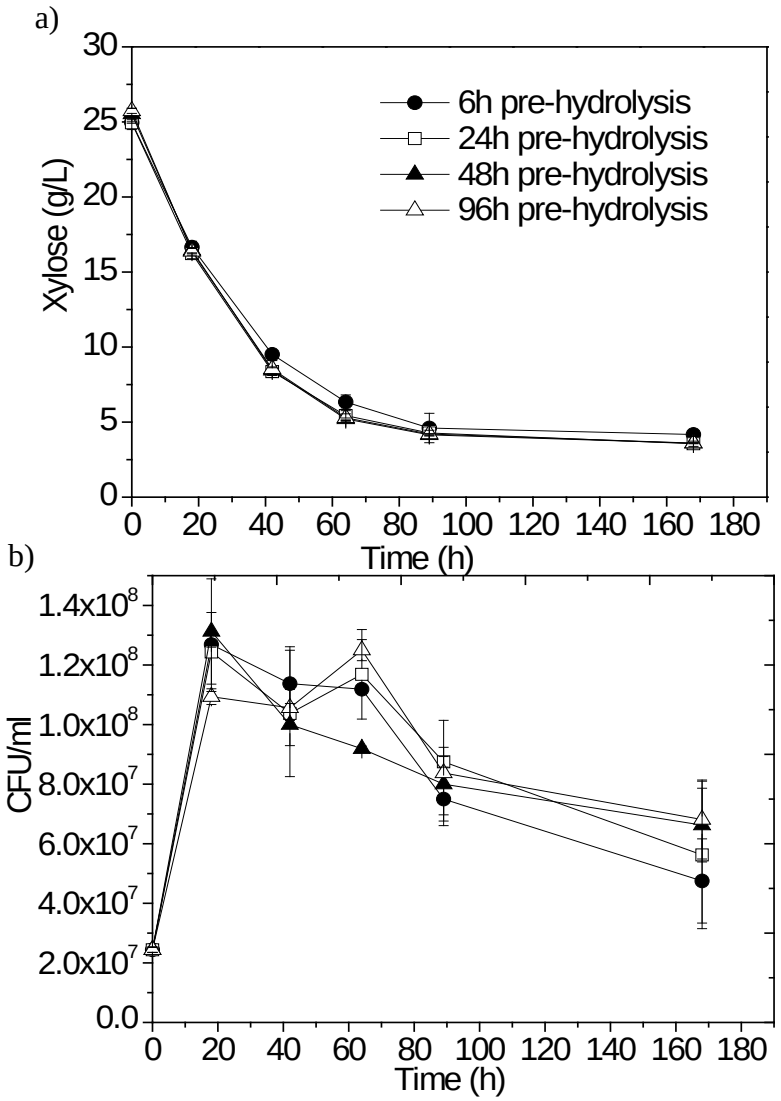



Figure 22 Effect of pre-hydrolyzed solids on xylose fermentation (a) and cell viability (b). Solids were harvested after hydrolyzing at different time and washed using water, 1M NaCl, and water for 3 times. The washed solids were then washed again using 96h hydrolysate and added into 96 h hydrolysate for fermentation study. The glucose concentration was 50 g/L. The solids concentrations were the same as SSCF. Solids concentration and solids compositions were shown in Table 8.

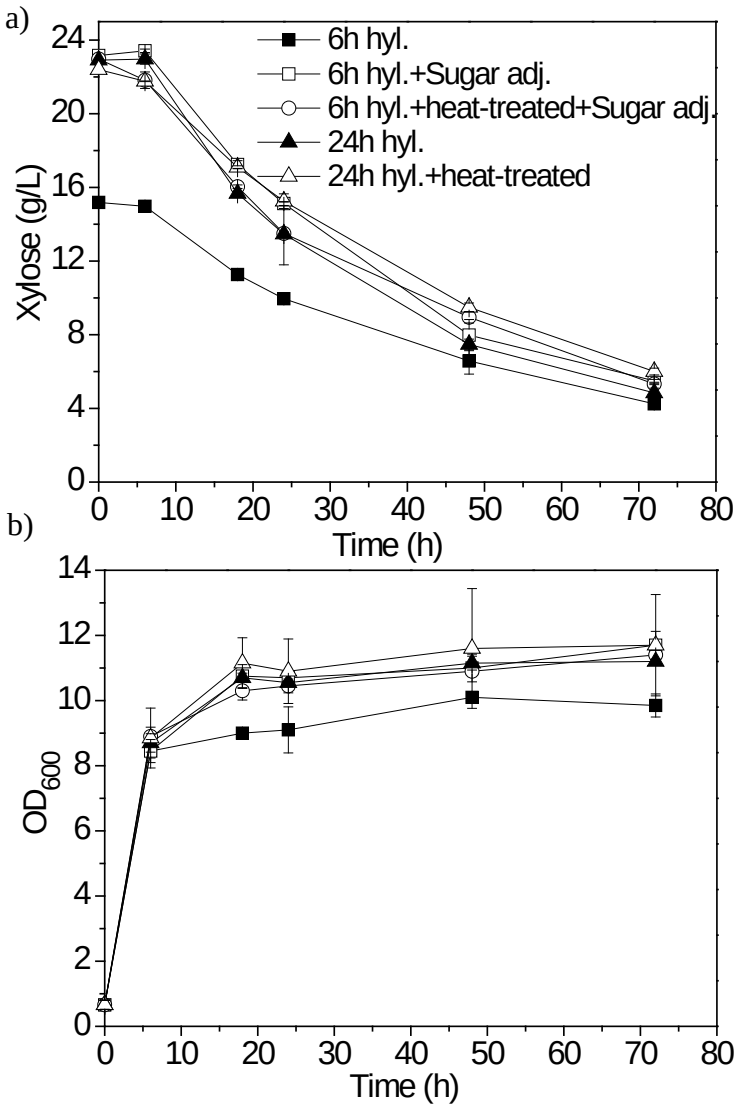



Figure 23 Xylose consumption (a) and cell growth (b) during fermentations of hydrolysate from 6 h pre-hydrolysis and 24 h pre-hydrolysis.
Liquid hydrolysate was harvested after 6 h or 24 h hydrolysis by centrifuge and sterile filtration. Fermentations were performed on both heat-treated and untreated hydrolysate. To fair comparison, glucose and xylose in 6h hydrolysate was adjusted to the same concentrations as 24 h hydrolysate. The one with no sugar adjustment was also investigated. Oligomeric glucose and xylose in 6h hydrolysate, 6 h heat-treated hydrolysate, 24 h hydrolysate, and 24 h heat-treated hydrolysate were 4.0 and 17.1 g/L, 1.7 and 14.8 g/L, 3.6 and 15.2 g/L, and 5.3 and 16.2 g/L, respectively. Heat treatment was conducted at 105 $^{o}$C for 10 min.

The liquid portion after 6 h pre-hydrolysis (6 h hydrolysate) and 24 h pre-hydrolysis (24 h hydrolysate) were also obtained by centrifugation. Fermentations were performed under 5

different sets of conditions to investigate the effect of the liquid portion on xylose fermentation and yeast cell growth (Fig. 23): (i) 6 h hydrolysate, (ii) 6 h hydrolysate with glucose and xylose adjusted to the same concentrations as 24 h hydrolysate, (iii) heat-treated 6h hydrolysate with glucose and xylose adjusted, (iv) 24 h hydrolysate and (v) heat-treated 24 h hydrolsyate. Heat treatment was used to denature the enzymes in the hydrolysate. No significant differences were observed for xylose consumption (Fig. 23a) and cell growth (Fig. 23b) between the 6 h hydrolysate fermentations and 24 h hydrolysate fermentations with the same sugar concentrations (no matter if heat-treated or not). The 6h hydrolysate fermentation (without adjusting sugar concentrations) showed lower cell density and hence slower xylose consumption but still consumed xylose to a similar low level as did the 24 h hydrolysate fermentation (Fig. 23).

In summary, the solids portion and the liquid portion prepared under different pre-hydrolysis regimes showed no significant effect on xylose fermentation and cell viability. The improved cell viability and better xylose consumption for longer pre-hydrolysis SSCFs compared to 6h pre-hydrolysis SSCF are likely due to the higher glucose concentrations achieved after pre-hydrolysis. The glucose concentrations after 6, 24, 48, and 96h pre-hydrolysis were 23.5, 46.0, 50.2 and 52.3 g/L, respectively. Assuming the yeast cell biomass yield per g glucose was fixed, higher glucose concentrations would produce more yeast cells. During SSCF, glucose was still being released, but the release rate was probably too slow to satisfy yeast cell growth demand, especially at a low temperature (30 $^{o}$C) (Fig. 27a). Besides, *S. cerevisiae* 424A(LNH-ST) can hardly grow on xylose anaerobically might due to the deficiency of ATP during xylose metabolism (Kuyper et al., 2004). Therefore, the cell growth was only seen in the first 24 h (Fig. 21b), which was mostly the glucose fermentation period (Fig. 19). After 24 h, the cell viabilities began to decrease (Fig. 21b). It seems that the first 24 h cell growth determines

the overall cell viability and hence xylose consumption and that the glucose concentration after pre-hydrolysis determines the first 24 h cell growth. To test this hypothesis, we conducted the following investigation.

### 4.3.5 Glucose concentration after pre-hydrolysis is the key for xylose fermentation during SSCF

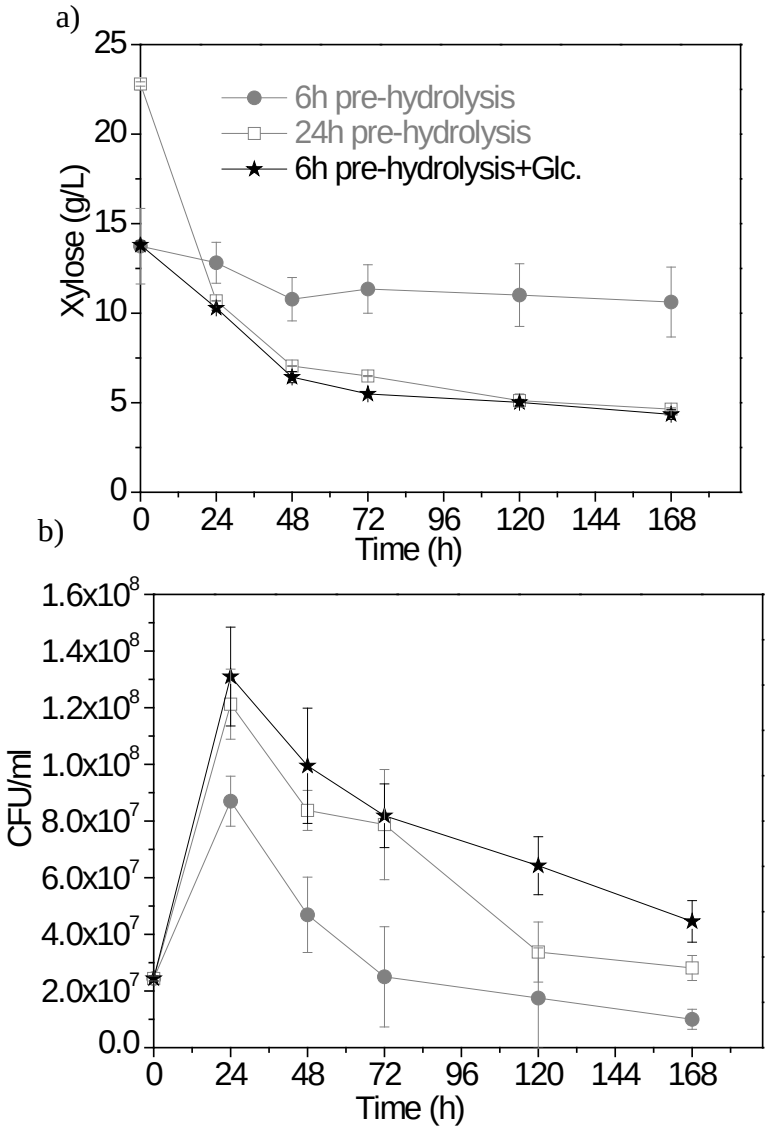



Figure 24 Effect of glucose supplementation on xylose consumption (a) and cell viability (b) during 6 h pre-hydrolysis SSCF.
Glucose was supplemented after 6 h pre-hydrolysis to the similar concentration as 24 h pre-hydrolysis (around 46.0 g/L).

We supplemented glucose after 6 h pre-hydrolysis to 46.0 g/L (the same concentration as 24 h pre-hydrolysis) and conducted the SSCF study. The cell viability was enhanced to a similar level as 24 h pre-hydrolysis SSCF (Fig. 24b) and the xylose consumption was also improved to a similar level (Fig. 24a). This observation supports the hypothesis that the low glucose concentration after 6 h pre-hydrolysis caused low viable cell density and thereby poor xylose consumption during SSCF.

To further test this hypothesis, we substituted the Spezyme CP enzymes with Accellerase enzymes which had higher activities and performed 6h pre-hydrolysis SSCF (Fig. 25). Glucose concentration reached approximately 32.3 g/L after 6 h pre-hydrolysis, higher than that achieved using Spezyme CP enzymes (23.5 g/L). Once again, the cell viability and xylose consumption were better than those for Spezyme CP. As a result, after 168 h, 39.9 g/L ethanol was produced with 3.2 g/L xylose left in the broth.

### 4.3.6 Mass balance comparison of different process

For a better understanding of the system, mass balance studies were performed on the processes of SHF, 24 h pre-hydrolysis SSCF using Spezyme CP enzymes and 6 h pre-hydrolysis

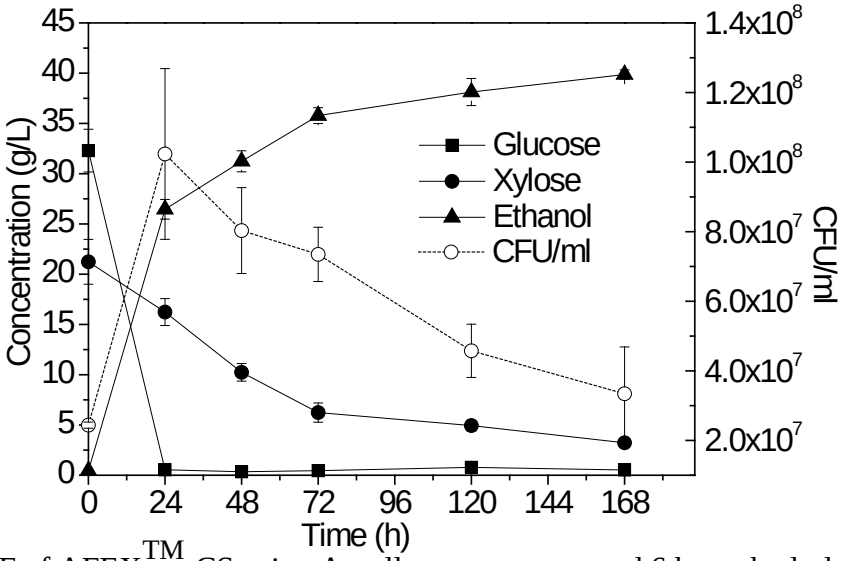



Figure 25 SSCF of AFEX$^{TM}$-CS using Accellerase enzymes and 6 h pre-hydrolysis. Accellerase 1500, Accellerase XY, and Multifect pectinase with the protein loadings of 24, 6, and 6 mg/g glucan, respectively were used for this experiment.

SSCF using Accellerase enzymes. The sugar conversions and ethanol yield of SHF process (Fig. 26a) were lower than those observed in a previous study (Lau & Dale, 2009), likely due to enzyme activity loss during storage in a refrigerator for almost 3 years. Compared to SHF, 24 h pre-hydrolysis SSCF increased sugar conversions and hence increased ethanol yield from 168.0 to 188.9 g ethanol per kg AFEX$^{TM}$-CS (Fig. 26b). 6 h pre-hydrolysis SSCF using Accellerase enzymes gave a higher ethanol yield (193.2 g ethanol per kg AFEX$^{TM}$-CS) (Fig. 26c). Oligomeric sugar loss, especially oligomeric xylose loss, was an issue for all the processes. Compared to SHF, SSCFs resulted in lower oligomeric glucose content in the fermentation broth. However, the oligomeric xylose content was found to be similar in both processes.

Another feature of these processes is low ethanol productivity. The process ethanol productivities for SHF, 24 h pre-hydrolysis SSCF using Spezyme CP enzymes and 6 h pre-hydrolysis SSCF using Accellerase enzymes were 0.13, 0.20, and 0.23 $g \cdot L^{-1} \cdot h^{-1}$, respectively. SSCF shortened the overall process time by combining enzymatic hydrolysis and fermentation and hence had a higher productivity than SHF. However, the low processing temperature ($30^{o}C$) and the presence of ethanol limited the hydrolysis rates (Fig. 27) during SSCF and hence limited the further improvement of productivity. Thermo-tolerant strains and ethanol-tolerant enzymes are required to fully exploit the merits of the SSCF process. It is interesting to note that with the increase of ethanol concentration the hydrolysis rates dropped most rapidly for hydrolyses conducted at $50^{o}C$, and the optimum temperature for enzymatic hydrolysis in the presence of ethanol was found to be $45^{o}C$ (Fig. 27).

a)

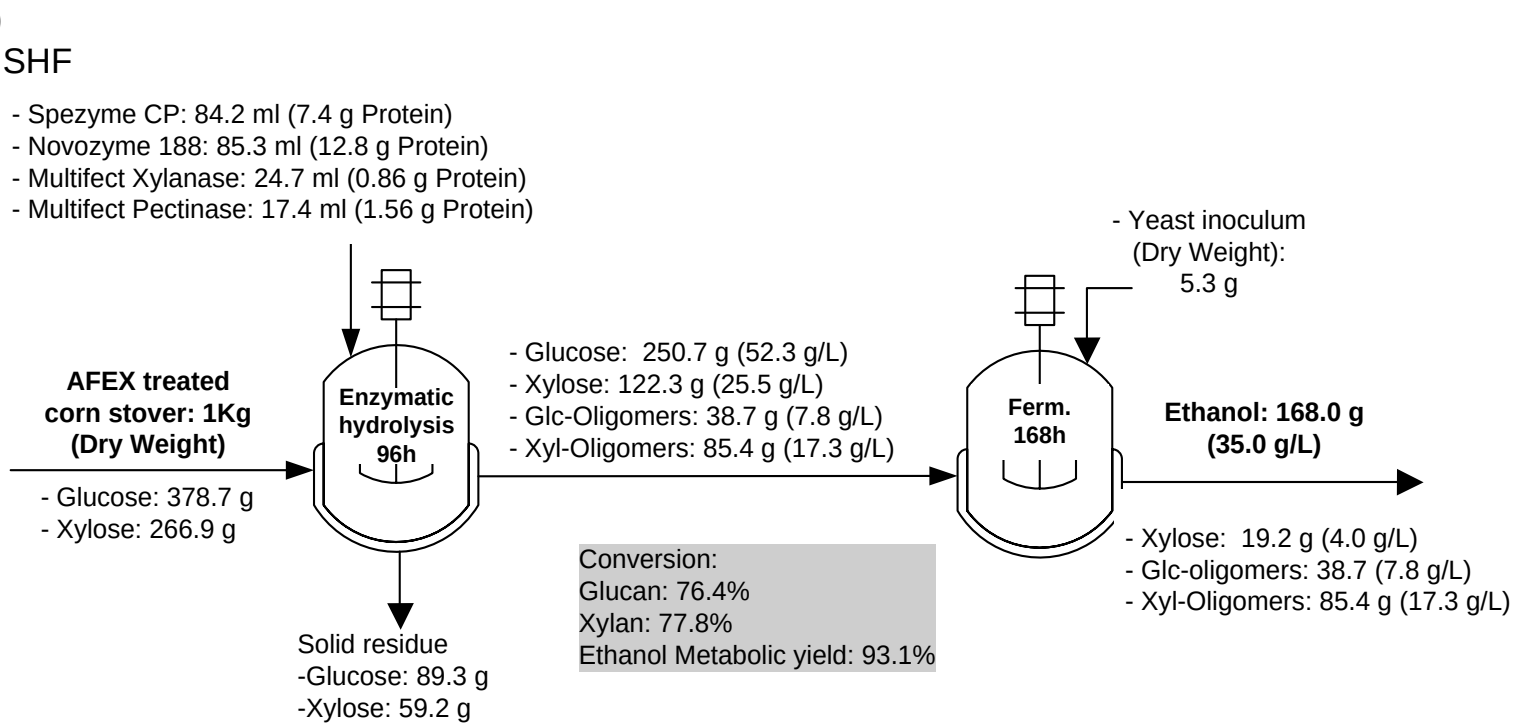


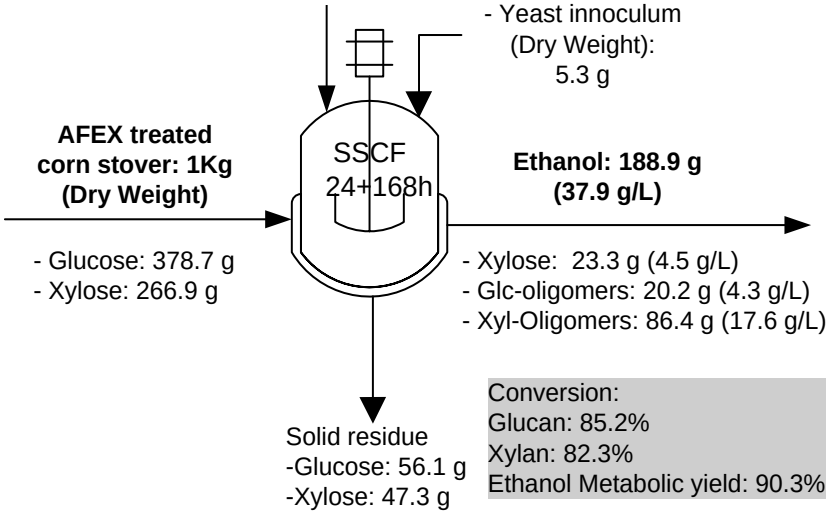

Figure 26 Mass balance for the SHF (a), SSCF with 24 h pre-hydrolysis (b), and SSCF with 6 h pre-hydrolysis using Accellerase enzymes (c) on AFEX$^{TM}$-CS.

Figure 26 (cont'd)

b)

SSCF (24h pre-hydrolysis)

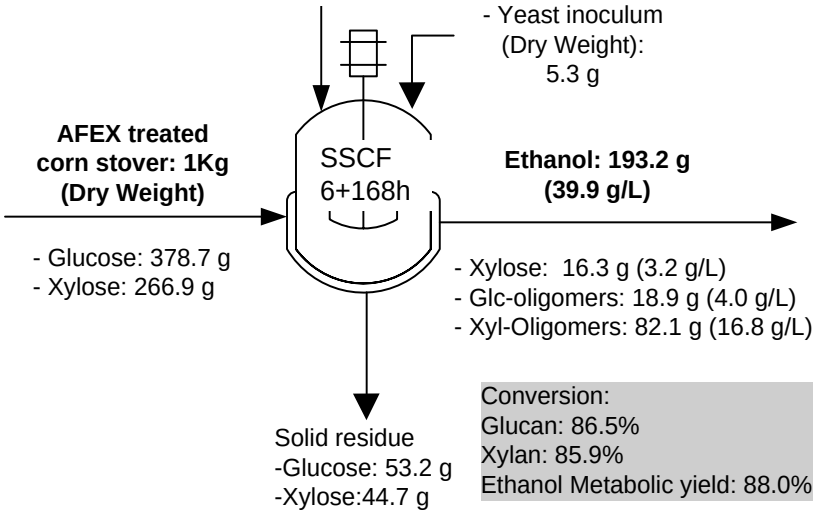
- Spezyme CP: 84.2 ml (7.4 g Protein)
- Novozyme 188: 85.3 ml (12.8 g Protein)
- Multifect Xylanase: 24.7 ml (0.86 g Protein)
- Multifect Pectinase: 17.4 ml (1.56 g Protein)




c)

SSCF (6h pre-hydrolysis)

- Accellerase 1500: 122.1 ml (8.18 g Protein)
- Accellerase XY: 70.6 ml (2.05 g Protein)
- Multifect Pectinase: 23.0 ml (2.05 g Protein)



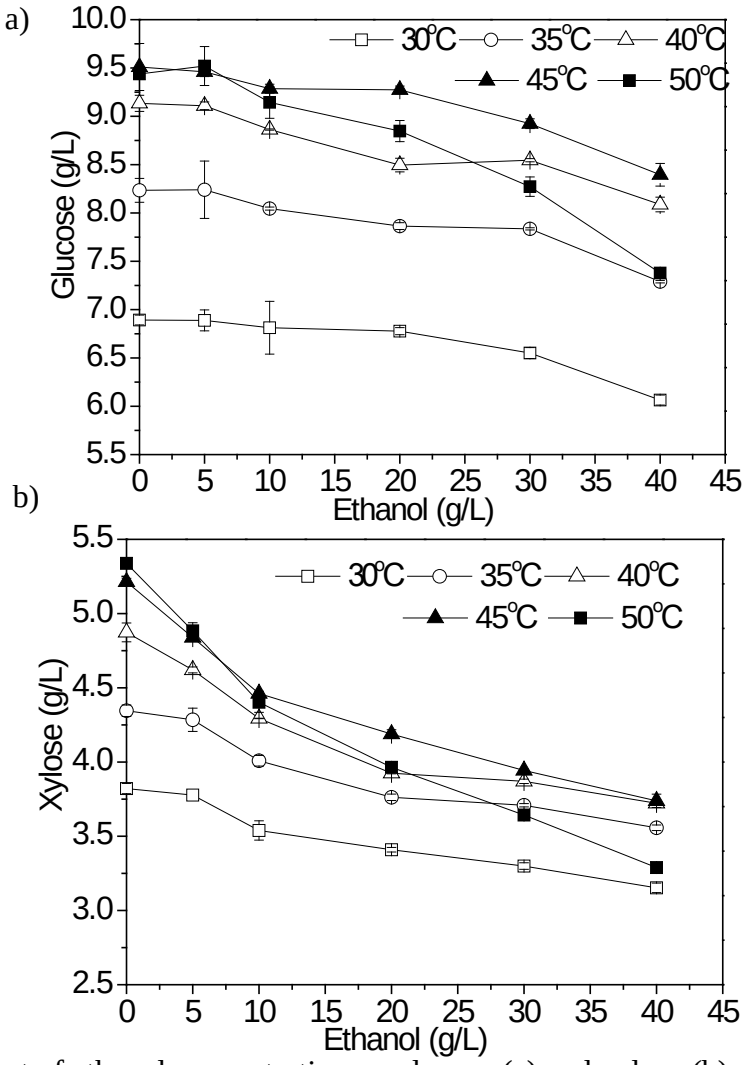



Figure 27 Effect of ethanol concentration on glucose (a) and xylose (b) release during enzymatic hydrolysis at different temperature.
Enzymatic hydrolysis was carried out at pH 4.8, 200 rpm and 1% glucan loading for 24 h. Spezyme CP, Novozyme 188, Mutifect Xylanase, and Mutlfict Pectinase loadings were 22.4, 38.4, 2.6 and 4.7 mg/g glucan, respectively.

**4.4 Conclusions**

In *S. cerevisiae* 424A(LNH-ST) fermentations, xylose consumption has a strong correlation with yeast cell viability. Glucose concentration after pre-hydrolysis determines the cell viability and hence xylose fermentation during SSCF of AFEX$^{TM}$-CS. Unhydrolyzed AFEX$^{TM}$-CS solids has no inhibitory effect on cell growth and xylose fermentation. We found that 45$^{o}$C is the ideal temperature for SSCF.

# CHAPTER V EFFECT OF SOLIDS LOADING ON SSCF


## Abstract

A high solid loading is desired in an industrial relevant process. However, the sugar conversions typically decrease with increasing solids loading during enzymatic hydrolysis and SSF. So far, no one has investigated solids loading effect on SSCF process, especially SSCF on AFEX™ pretreated biomass. In the present study, the solids loading effect during SSCF of AFEX™ pretreated corn stover was investigated. Techno-economic analyses were done to show the solids loading effect on the economics of cellulosic ethanol production. A decreased sugar conversion with increasing solids loading was also observed during SSCF. The increased degradation products and xylose accumulation with increasing solids loading were proven not to be the major factors causing such phenomenon but they did affect the overall economics of cellulosic ethanol production. 6% (w/w) glucan loading was shown as the optimal solids loading for SSCF on AFEX™ corn stover.

## 5.1 Introduction

Processing lignocellulosic biomass at high solids loading is desired in a biorefinery. A high solids loading process can potentially generate a high concentration of sugars and in turn a high titer of products (such as ethanol), which facilitates products recovery (lower distillation cost for ethanol production). In addition, high solids loading typically means less reactor volume required, less energy for heating and cooling as well as less waste water (Kristensen et al., 2009). Thus, both capital cost and operating cost can be reduced when a high solids loading process is applied. However, sugar conversion during enzymatic hydrolysis normally linearly decreases when solids loading increases (Cara et al., 2007; Hodge et al., 2008; Kristensen et al., 2009). Numerous factors could be responsible for such phenomenon including increased degradation products inhibition (Panagiotou & Olsson, 2007), increased lignin concentration (Pan, 2008), enzyme non-productive adsorption to lignin (Kristensen et al., 2009), decreased water concentration (Kristensen et al., 2009), mass transfer limitations (Hodge et al., 2008), and increased end-product inhibition (Kristensen et al., 2009). Kristensen et al. (Kristensen et al., 2009) concluded that the major cause for decreased sugar conversion at high solids loading was decreased enzymes adsorption to biomass, which was inhibited by increased end-products (sugars) concentration. SSF/SSCF removes the enzymatic hydrolysis products (sugars) and thus reduces the sugar inhibition on enzymes. However, studies showed that sugar conversion still decreases with increasing solids loading during SSF (Jørgensen et al., 2007; Mohagheghi et al., 1992) but not as much as for SHF (Jørgensen et al., 2007). The inhibition from increased ethanol concentration might be a major reason. However, the accumulated xylose during SSF could be another factor limiting enzyme performances during SSF of lignocellulosic biomass since xylose was not consumed by the SSF microbe. To the author's knowledge, the effect of solids loading

on SSCF performance has not been studied. SSCF removes not only glucose but also xylose and thereby might be able to reduce the effect of high solids loading.

In the present study, the goal is to study the effect of high solids loading on enzymatic hydrolysis and ethanol yield during SSCF of AFEX$^{TM}$-CS and to figure out which solids loading is the most economic one based on current technologies.

## 5.2 Materials and Methods

### 5.2.1 AFEX$^{TM}$ pretreated corn stover

Untreated corn stover (GLBRC corn stover) with glucan content and xylan content of 36.2% and 22.5%, respectively, was AFEX$^{TM}$ pretreated in a 5 gallon reactor in Michigan Biotechnology Institute (MBI). AFEX$^{TM}$ pretreatment conditions were: ammonia to biomass loading 1.0 g/g dry biomass, water loading 0.6 g/g dry biomass, temperature 100 $^{o}$C and residence time 15 minutes.

### 5.2.2 Enzymatic hydrolysis

Enzymatic hydrolysis was carried out at 5%, 7% and 9% (w/w) glucan loading in a 250 ml baffled flask with a biomass-enzyme-water mixture of 100 g. Enzymes applied included Accellerase 1500, Accellerase XY and Multifect pectinase (Genencor Inc., USA) in protein loadings of 20, 5 and 5 mg/g glucan, respectively. Hydrolysis was conducted in an incubator at pH 4.8, temperature 50 $^{o}$C and 250 rpm for 7 days. After 7 days hydrolysis, the glucose concentrations for 5%, 7% and 9% glucan loading were 48.7, 64.1 and 74.5 g/L, respectively. The xylose concentrations were 25.7, 35.2, and 41.5 g/L, respectively.

### 5.2.3 Microorganism and seed culture preparation

*S. cerevisiae* 424A(LNH-ST) was used for fermentation of glucose and xylose in this study. Seed culture preparation has been described in Chapter IV section 4.2.2.

### 5.2.4 Fermentation of enzymatic hydrolysate (SHF)

The fermentation procedure has been described in Chapter III section 3.2.5. Fermentations were conducted on both sugar concentration unadjusted hydrolysates and sugar concentration adjusted hydrolysates. The sugar concentration adjusted hydrolysates were prepared by adding glucose and xylose to the hydrolysates to reach the same concentrations of glucose and xylose (74.5 and 43.0 g/L, respectively) in different hydrolysates.

### 5.2.5 Simultaneous saccharification and co-fermentation (SSCF)

SSCF experiments were performed in the same baffled flask and the same enzyme loading as enzymatic hydrolysis. Solids loading investigated included 4%, 5%, 6%, 7% and 9% (w/w) glucan loading. The 6 h pre-hydrolysis SSCF procedure described in the Chapter IV section 4.2.4 was used for this study. SSCF experiments were conducted on unwashed AFEX$^{TM}$-CS, unwashed AFEX$^{TM}$-CS with supplementation of YEP (yeast extract 5 g/L and tryptone 10 g/L), as well as washed AFEX$^{TM}$-CS with supplementation of YEP. The washed AFEX$^{TM}$-CS was prepared as described in Chapter III section 3.2.7.

Monomeric sugar conversions during SSCF were calculated based on the produced ethanol and remaining sugars in the fermentation broth with the assumption of 90% ethanol metabolic yield for fermentation.

### 5.2.6 Techno-economic analyses

Techno-economic analyses were performed based on a modified NREL 2011 model (Humbird et al., 2011). The modified model was developed by a colleague Dr. Bryan Bals. The model strips out biomass handling and pretreatment, and calculates capital, raw material, and energy costs as well as total ethanol and electricity revenue based on inputs regarding biomass composition, enzymatic hydrolysis and fermentation performance. The cellulase enzymes production area was also removed in the model and replaced with a fixed cost of enzyme (on a $/kg protein basis). In the modified model, AFEX$^{TM}$ pretreatment is assumed to be carried out in the local biomass processing depots (LBPDs). The costs of biomass pre-processing including pretreatment are assumed to be $50/tonne untreated biomass (Bals & Dale, 2012) with an ammonia use of 22 g/kg biomass. The calculations for all the costs and revenues are based on the NREL model and adjusted proportionally for differences in the composition, enzymatic hydrolysis and fermentation inputs. Different areas of the biorefinery were adjusted based on different inputs. For example, the costs of purifying the ethanol via molecular sieving were adjusted based on the difference in ethanol produced via NREL's assumptions and the user's assumptions, whereas the costs associated with fermentation were based on the differences in solid loading and total residence time. The cost adjustments were performed linearly for energy and raw material (for example, a 5% increase in ethanol production relative to NREL resulted in a 5% increase in energy use in the molecular sieve), whereas the cost of capital was adjusted based on the average sizing factor of equipment in that area (generally around 0.6). For example, a 5% increase in ethanol production would increase the capital cost of the molecular sieving by 1.05^0.6= 3% increase. All financial assumptions (lifetime of the plant, interest rate on loan, taxes, depreciation method, proportion of funding by equity, working capital, etc), all design

assumptions (equipment used, power requirements of equipment, design of wastewater treatment and boiler/turbogenerator, etc), and all assumptions around the efficiency of the generator are identical to the NREL model.

### 5.3 Results and Discussion

#### 5.3.1 SSCF performances at different solids loading

4%, 5%, 6%, 7% and 9% glucan loading SSCF were performed using unwashed AFEX$^{TM}$-CS. The results were compared in Fig. 28. Time 0 was set for the beginning of SSCF after 6 h pre-hydrolysis. Ethanol concentration increased with increasing solids loading but not proportionally. Glucose conversion to ethanol by fermentation was all rapid even at 9% glucan loading.

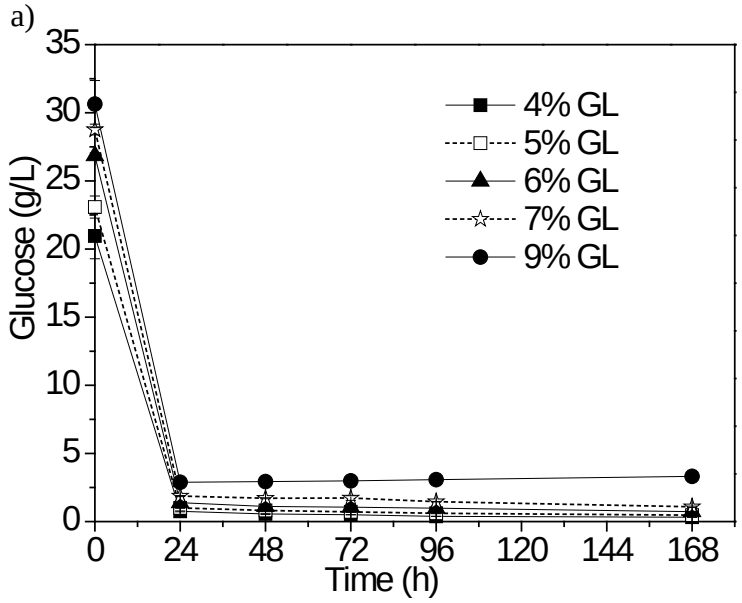
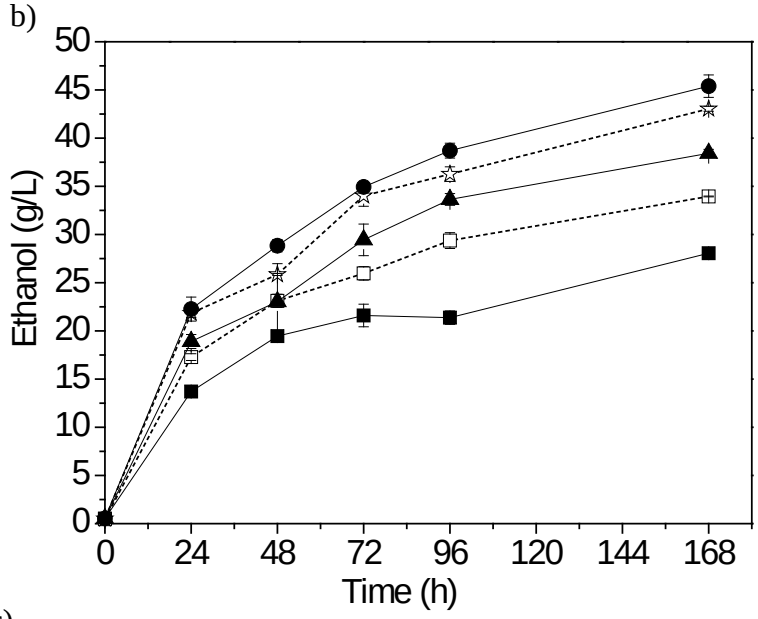


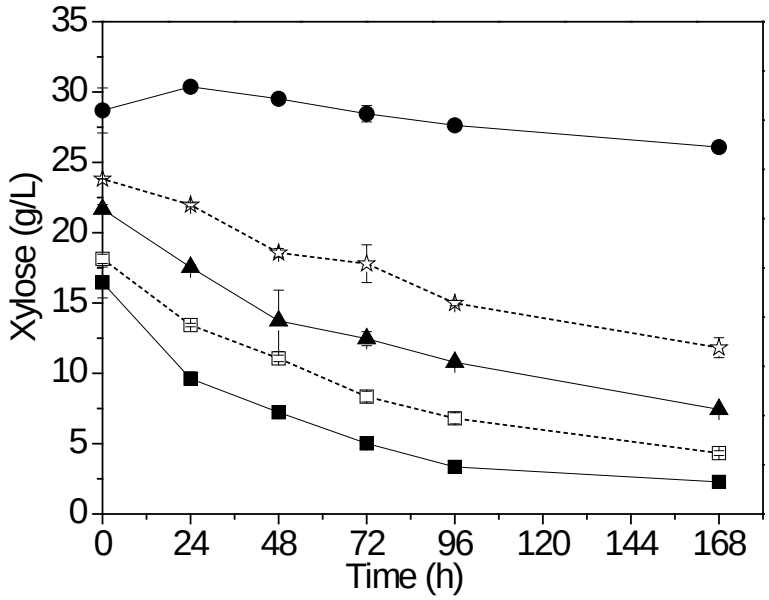

Figure 28 Glucose (a), xylose (b) and ethanol (c) profiles during SSCF of unwashed AFEX$^{TM}$-CS at different glucan loading (GL).

Figure 28 (cont'd)

b)




c)



However, the xylose consumption was reduced with increasing solids loading (Fig. 28b). At 9% glucan loading, there was 26.1 g/L xylose remaining in the fermentation broth at the end of fermentation, which was a huge sugar waste for a biorefinery. The reduced xylose fermentation performance with increasing solids loading is probably a synergistic effect of degradation products, ethanol and fermentation metabolites as already shown in Chapter II. Solids concentration might be another factor decreased the overall xylose consumption. Higher solids loading correspond to higher solids concentration during SSCF, which influence the viscosity and shear stress (Pimenova & Hanley, 2004). However, it is illustrated in Chapter VII Fig. 51 that solids concentration (even up to 200 g/L) has little effect on *S. cerevisiae* 424A fermentation performance including xylose consumption. To further prove the small effect of solids during SSCF, fermentations of solids-free hydrolysate were conducted.

### 5.3.2 Fermentation performances of solids-free hydrolysate at different solids loading

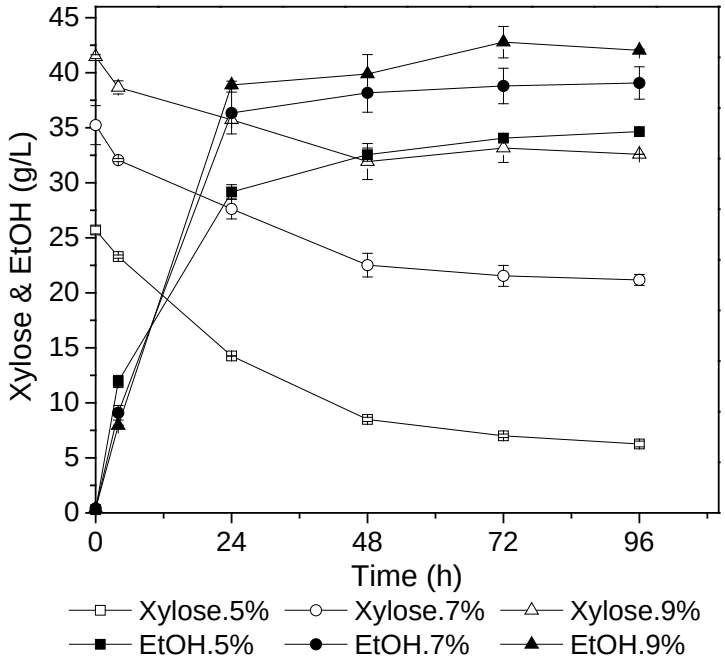



Figure 29 Xylose and ethanol profiles during fermentations of 5% glucan loading AFEX$^{TM}$-CS hydrolysate, 7% glucan loading AFEX$^{TM}$-CS hydrolysate and 9% glucan loading AFEX$^{TM}$-CS.

This was an SHF experiment. Enzymatic hydrolyses were conducted at 5%, 7% and 9% glucan loading for 7 days. The obtained hydrolysates with solids removed by centrifugation were used for fermentation. Initial glucose concentrations for 5%, 7% and 9% glucan loading hydrolysates were 48.7, 64.1 and 74.5 g/L, respectively. These glucose were all fermented to ethanol in 24 h. The xylose consumption trend is similar to the one in SSCF (Fig. 29): the higher solids loading the poorer xylose consumption, which proved that the solids during SSCF were not the main factor limiting xylose fermentation. At the end, around 34.7, 39.1 and 42.0 g/L ethanol, respectively, were produced from 5%, 7% and 9% glucan loading hydrolysate.

To illustrate the effect of increased degradation products concentration on xylose fermentation performance, sugars in 5%, 7% and 9% glucan loading hydrolysate were all adjusted to the same levels before fermentation was carried out (Fig. 30). High solids loading means more degradation products as well as more nutrients since AFEX$^{TM}$-CS contains both ingredients. In this fermentation at the same sugar concentrations, cell density (OD) reached the same level for all three glucan loading hydrolysates (Fig. 30). The benefits of more nutrients on cell growth at higher solids loading were probably offset by the inhibition of more degradation products. After 96 h fermentation, around 18.2, 12.8 and 8.0 g/L xylose were consumed in the 5%, 7% and 9% glucan loading hydrolysates, respectively. The increased concentration of degradation products substantially increased the inhibition on xylose fermentation probably through the synergistic effect with ethanol and fermentation metabolites.

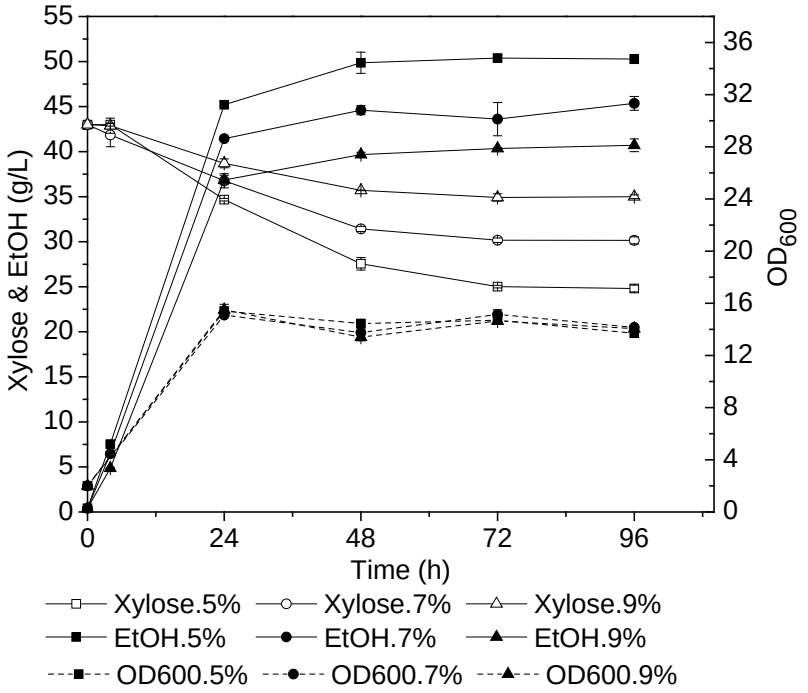



Figure 30 Xylose, ethanol and $OD_{600}$ profiles during fermentations of 5% glucan loading AFEX$^{TM}$-CS hydrolysate, 7% glucan loading AFEX$^{TM}$-CS hydrolysate and 9% glucan loading AFEX$^{TM}$-CS, with glucose and xylose concentrations all adjusted to 74.5 and 43.0 g/L, respectively.

### 5.3.3 SSCF performances with YEP supplementation

One goal of this study is to see if the sugar conversions decrease with increasing solids loading during SSCF with both glucose and xylose removed by fermentation. However, with the fact that xylose removal was not satisfactory at high solids loadings, the SSCF results on unwashed AFEX$^{TM}$-CS might not strong enough to see the effect of xylose removal. Therefore, two other strategies were applied. One approach used SSCF of unwashed AFEX$^{TM}$-CS with supplementation of YEP. Supplementation of YEP could help enhance yeast cell growth and thereby improve xylose consumption as shown in Chapter II Fig. 11. The other method involved SSCF of washed AFEX$^{TM}$-CS with supplementation of YEP. The removal of degradation

products could definitely help improve xylose consumption. Experimental results are shown in Fig. 31.

With the supplementation of YEP, xylose consumption was substantially improved during SSCF on unwashed AFEX$^{TM}$-CS (Fig. 31a), especially at solids loadings higher than 6% glucan

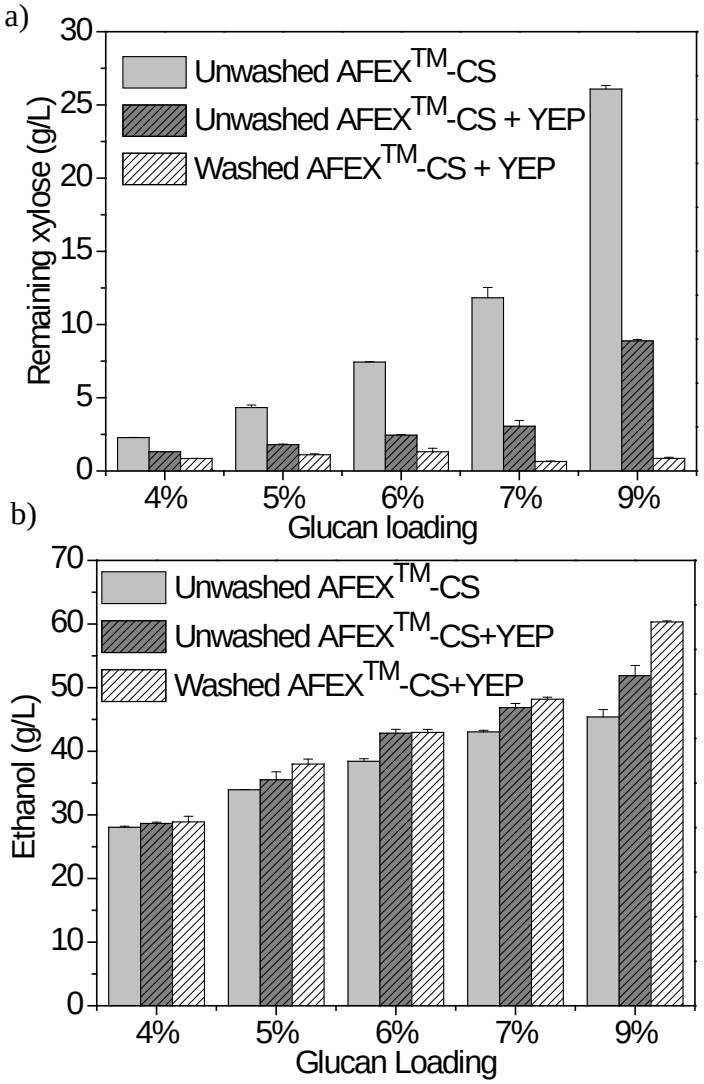



Figure 31 Comparison of final xylose and ethanol concentrations during SSCF of unwashed AFEX$^{TM}$-CS, unwashed AFEX$^{TM}$-CS with supplementation of YEP and washed AFEX$^{TM}$-CS with supplementation of YEP at different glucan loading.

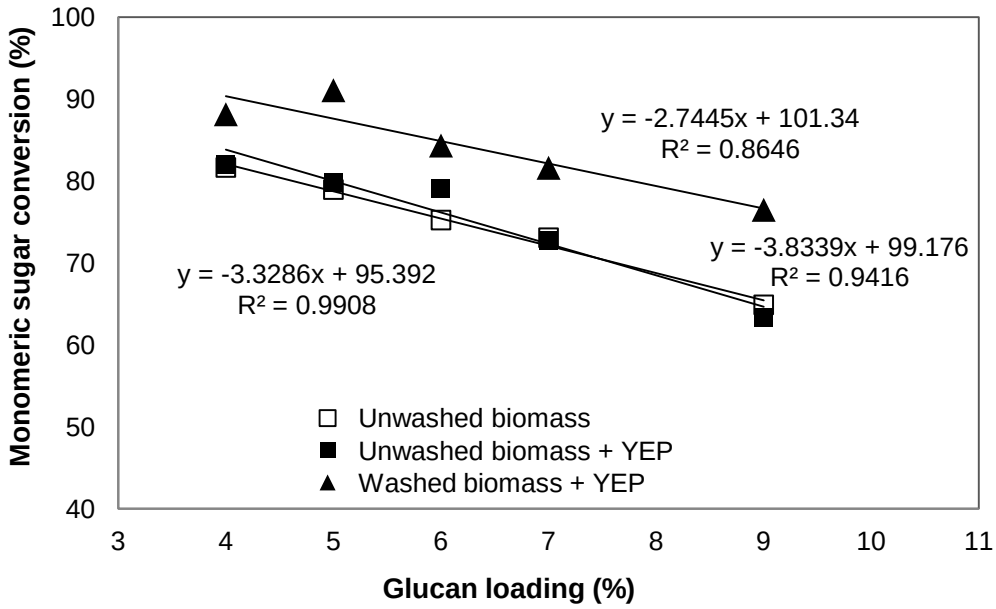
loading. For instance, the final (168 h) xylose concentration at 9% glucan loading SSCF was reduced from 26.1 g/L without YEP to 8.9 g/L with YEP. Due to the improvement of xylose

fermentation, the ethanol yield was enhanced (Fig. 31b). For 9% glucan loading SSCF, ethanol titer was enhanced from 45.4 g/L without YEP to 51.9 g/L with YEP. The xylose removal effect was even better when SSCF was performed on washed AFEX$^{TM}$-CS with supplementation of YEP. In this situation, the final xylose concentrations for different solids loadings were all reduced to around 1 g/L (Fig. 31a). As a result, an ethanol titer as high as 60.3 g/L was achieved at 9% glucan loading (Fig. 31b). The successful removal of both glucose and xylose during SSCF guaranteed the following analyses of the solids effect on sugar conversions and ethanol yield during SSCF.

**5.3.4 Effect of solids loading on monomeric sugar conversion and ethanol yield**

In the SSCF experiments, a decreased monomeric sugar conversion with increasing solids loading was still observed (Fig. 32a). The sugar conversion decreased with increasing glucan loading at a similar rate on unwashed AFEX$^{TM}$-CS and on unwashed AFEX$^{TM}$-CS with YEP (slope: -3.3 vs. -3.8). As already shown above, xylose accumulation in fermentation broth increased with increasing glucan loading during SSCF on unwashed biomass. Substantial reduction of xylose accumulation by supplementing YEP during SSCF for better xylose fermentation did not help on the increase of sugar conversion. This indicates that some determining factors might “mask” the effect of xylose inhibition on sugar conversion. With no degradation products and no accumulation of xylose, the sugar conversion was still decreasing with increase of glucan loading during SSCF of washed biomass. The slope for this decrease is -2.7 (Fig. 32a), which indicates that by eliminating the degradation products effect the decrease rate was slightly slowed down. Therefore, neither accumulated xylose or degradation products was not the determining factor causing the decrease of sugar conversion with increasing solids loading.

a)




b)

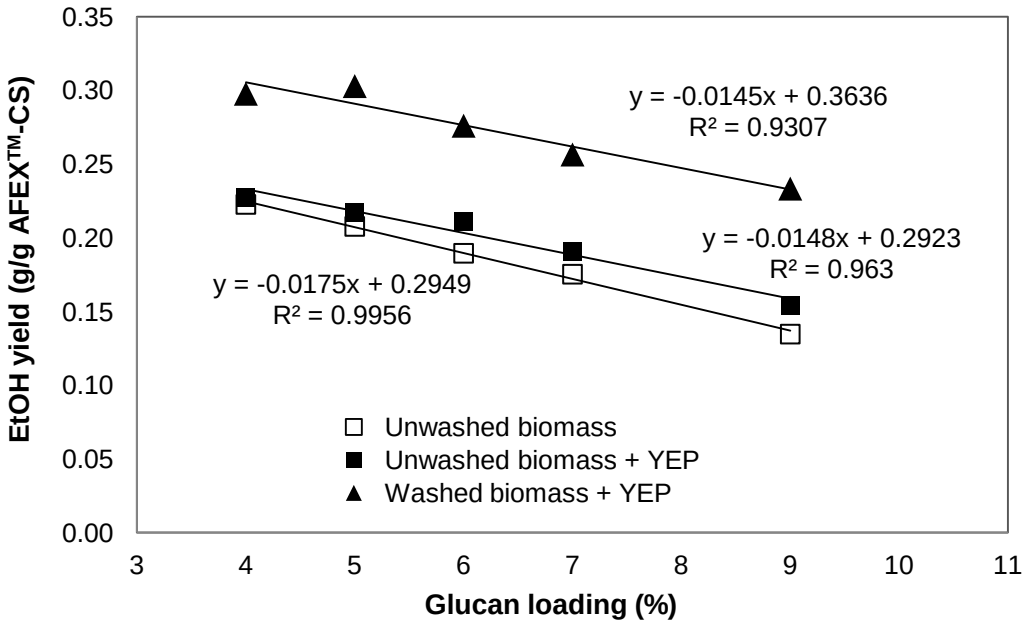



Figure 32 Effect of solids loading on monomeric sugar conversion (a) and ethanol yield (b). The monomeric sugar conversion was calculated by dividing released total glucose and xylose by total glucose and xylose in the initial biomass.

Since it has been concluded that end-products (sugars) are the major cause for the decreased sugar conversion at high solids loading during enzymatic hydrolysis (Kristensen et al., 2009) and the sugar conversion still decreased with increasing solids loading during SSCF, it is most likely that ethanol is the major cause of sugar conversion reduction during SSCF. The impact of ethanol on enzymatic hydrolysis has been shown in Chapter IV Fig. 27. Experiments similar to SSCF but without production of ethanol (e.g. perform SSCF at aerobic conditions) will be an interesting study to further test this. Oligomeric sugars, especially oligomeric xylose, increase in concentration with increasing solids loading and are another inhibitor for enzymatic hydrolysis (Qing et al., 2010). Studies in Chapter III (Fig. 18) and Chapter VI (Fig. 26) showed that SSCF did not reduce the production of oligomeric xylose much compared to SHF. Thus, oligomeric xylose is an unavoidable inhibitor in high solids loading processes based on current pretreatment and enzymes system.

### 5.3.5 Techno-economic analyses

For determining the optimal solids loading of SSCF for lignocellulosic ethanol production, techno-economic analyses were performed using a modified NREL model. Minimum ethanol selling price as a function of glucan loading is shown in Fig. 33. On unwashed AFEX$^{TM}$-CS, 5% glucan loading displayed the lowest MESP, which was around $2.18/gal. However, with the improvement of xylose fermentation by supplementation of YEP, the lowest MESP shifted to 6% glucan loading, which is around $2.02/gal. This indicates that xylose fermentation (besides sugar conversion) is also a determining factor in terms of production economics. A better xylose-fermenting strain or a better xylose-fermenting process can easily reduce the current MESP. On washed AFEX$^{TM}$-CS, 5%, 6%, 7% and 9% glucan loading showed almost the same lowest MESP, which is around $1.85/gal. Removal of degradation products reduced the rate of sugar

conversion decrease with solids loading and in turn made high solids loading process more profitable. However, the costs for washing and YEP addition, which are expensive, were not included. The techno-economic analyses on unwashed AFEX$^{TM}$-CS with supplementation of YEP and on washed AFEX$^{TM}$-CS were more about to show the benefits of xylose fermentation improvement and reduction of degradation products inhibitors through pretreatment improvement rather than showing the real economics for these two scenarios.

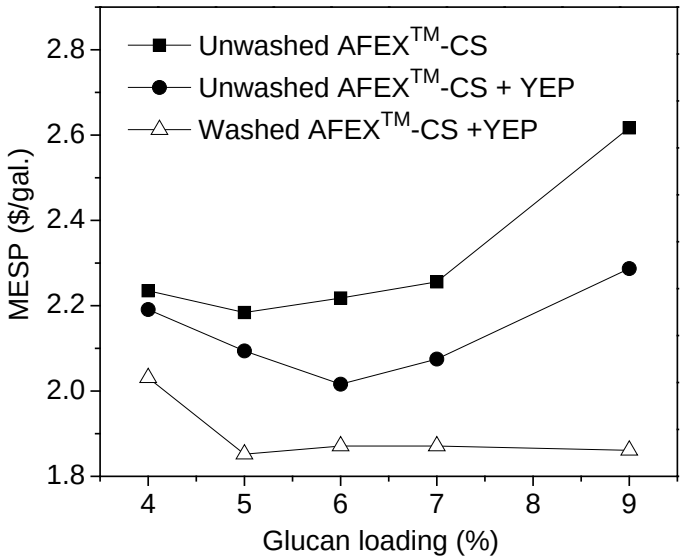



Figure 33 Effect of SSCF solids loading on minimum ethanol selling price (MESP). SSCF was performed on unwashed AFEX$^{TM}$-CS, unwashed AFEX$^{TM}$-CS with supplementation of YEP (yeast extract 5 g/L and tryptone 10 g/L), and washed AFEX$^{TM}$-CS with supplementation of YEP. Techno-economic analyses were conducted with the assumptions of ethanol metabolic yield 90%, no oligomeric sugars generation as well as no difference between glucan/glucose and xylan/xylose in terms of monomeric sugar conversion and conversion to ethanol. The costs for YEP addition and washing were not accounted.

This techno-economic model applied does not consider the threshold of ethanol concentration that makes distillation economic, which is 40 g/L (Dien et al., 2003a). When considering this factor, 6% (w/w) glucan loading should be the optimal solids loading for SSCF

on unwashed AFEX$^{TM}$-CS since 6% is the minimum solids loading that made 40 g/L ethanol titer possible (Fig. 31). In addition, the techno-economic analyses were done with the assumptions of no oligomeric sugars generated and no difference between glucan/glucose and xylan/xylose in terms of monomeric sugar conversion and conversion to ethanol, which made the calculated MESPs lower than real values. But this did not affect the economic comparison of different solids loadings.

# CHAPTER VI CONTINUOUS SSCF OF AFEX™ PRETREATED CORN STOVER


## Abstract

High productivity processes are critical for commercial production of cellulosic ethanol. One high productivity process - continuous hydrolysis and fermentation - has been applied in the sugarcane and corn ethanol industries. However, little research related to this process has been conducted on cellulosic ethanol production. In the present study, the kinetics of both batch SHF (separate hydrolysis and co-fermentation) and SSCF (simultaneous saccharification and co-fermentation) of AFEX™ (Ammonia Fiber Expansion) pretreated corn stover (AFEX™-CS) were studied and compared. Subsequently, a SSCF process was designed to evaluate continuous hydrolysis and fermentation performance on AFEX™-CS in a series of continuous stirred tank reactors (CSTRs). Based on similar sugar to ethanol conversions (around 80% glucose-to-ethanol conversion and 47% xylose-to-ethanol conversion), the overall process ethanol productivity for continuous SSCF was 2.3 and 1.8 fold higher than batch SHF and SSCF, respectively. Slow xylose fermentation and high concentrations of xylose oligomers were the major factors limiting further enhancement of productivity.

## 6.1 Introduction

Batch SHF (i.e. performing enzymatic hydrolysis and fermentation separately) has been widely applied for ethanol production from AFEX$^{TM}$ treated biomass using commercial enzymes and a xylose-fermenting yeast strain *Saccharomyces cerevisiae* 424A (LNH-ST) (Krishnan et al., 2010; Lau & Dale, 2009; Li et al., 2010; Shao et al., 2010; Zhong et al., 2009). The maximum volumetric ethanol productivities during the glucose fermentation period in 6% (w/w) glucan loading hydrolysates were all above 1.0 $g \cdot L^{-1} \cdot h^{-1}$. However, the average fermentation productivities were all below 1.0 $g \cdot L^{-1} \cdot h^{-1}$, which does not meet industrial production requirements (Dien et al., 2003a). If the ethanol productivity is calculated based on not only fermentation time but also on enzymatic hydrolysis time, the productivity values are even lower. SSCF, in which enzymatic hydrolysis and fermentation are performed in a single bioreactor, is a potential way to reduce the overall reaction time and hence enhance productivity (Taherzadeh & Karimi, 2007). However, the differences between optimum temperature and pH between enzymatic hydrolysis (EH) and yeast fermentation decrease this advantage of SSCF as shown in Chapter IV. Increasing solids loading and hence increasing ethanol titer is one way to enhance productivity. However, with increasing solid loading the sugar conversions and xylose fermentation efficiencies decrease as shown in Chapter V. The most economic solids loading based on current enzymes and microbes is 6% (w/w) glucan loading as discussed in Chapter V. Another way to improve ethanol productivity is by applying continuous hydrolysis and fermentation (Brethauer & Wyman, 2010).

The continuous stirred tank reactor (CSTR) system, especially with more than two reactors, provides a way to save the total reactor volume and hence enhance the volumetric productivity

(Brethauer & Wyman, 2010). With automation, CSTR reduces operations such as filling and cleaning of the reactors, as done during batch fermentation, and thus reduces the overall production cost. Moreover, the inocula required for CSTR seeding are negligible if the reactions proceed for a long time (autocatalytic system). Continuous hydrolysis and fermentation has been applied successfully in both industrial sugarcane ethanol production (Zanin et al., 2000) and corn ethanol production in China (Bai et al., 2008; Brethauer & Wyman, 2010). The CSTR system has also been studied on fermentations of pretreated biomass hydrolysate (such as enzymatic hydrolysate of diluted acid pretreated spruce wood) but not on AFEX$^{TM}$ hydrolysate (Brethauer & Wyman, 2010). In addition, to the author' knowledge, a continuous system has not yet been applied on SSCF of pretreated biomass.

In the present study, the ethanol productivity was enhanced by applying multi-stage CSTRs during fermentation of AFEX$^{TM}$-CS. Batch SHF and SSCF processes were studied and compared first. To compare SHF and SSCF processes, ethanol productivity was calculated based on enzymatic hydrolysis time plus fermentation time (process ethanol productivity). Since it gave higher ethanol productivity, further investigation on SSCF was performed in a continuous mode. Three flow rates for continuous process were investigated. Based on the mass balance, batch SHF, SSCF and continuous SSCF were comprehensively compared.

## 6.2 Materials and Methods

### 6.2.1 AFEX$^{TM}$ pretreated corn stover

The pretreated corn stover used in this study was the same as used in Chapter II (see section 2.2.1 for details). The AFEX$^{TM}$-CS (particle size 4 mm) was dried in the hood, until the

moisture content was below 10%, milled to pass a 0.5 mm sieve, and then used as is with no washing, detoxification or nutrient supplementation for enzymatic hydrolysis or fermentation.

### 6.2.2 Enzymatic Hydrolysis

Enzymatic hydrolysis (EH) of AFEX$^{TM}$-CS at 6% (w/w) glucan loading was conducted in a 500 ml fermentor (Biostat, Sartorius) with 400 g total mixture of biomass, enzymes and water. Enzymes used for the hydrolysis include Accellerase 1500, Accellerase XY, and Multifect pectinase (Genencor Inc, USA) with protein loadings of 24, 6, and 6 mg/g glucan, respectively. Temperature, pH and agitation speed were controlled at 50 $^{o}$C, 4.8, and 800 rpm, respectively. 50 mg/L chloramphenicol (Cm) was used during hydrolysis to avoid microbial contamination. Samples (5 ml) were taken during hydrolysis using a 10 ml syringe for sugar analysis. After 168 h hydrolysis, pH was adjusted to 5.5 and the hydrolysate was used for SHF fermentations at 220 rpm and 30 $^{o}$C without removing the unhydrolyzed solids.

### 6.2.3 Microorganisms and seed culture preparation

*S.cerevisiae* 424A was used for this study. For details, please see Chapter IV section 4.2.2.

### 6.2.4 Hydrolysate fermentation (SHF) and batch SSCF

Batch SHF/ SSCF fermentations were also carried out in 500 ml fermentors (Biostat, Sartorius) with 400 g total mixture. For SHF fermentation, hydrolysate (168 h hydrolysis) was inoculated with yeast cell pellets (obtained by centrifuging the seed culture) at an initial $OD_{600}$ of 2.0. The fermentation was carried out for 168 h. For batch SSCF, the same glucan and enzyme loadings which were employed in enzymatic hydrolysis were used. 24 h pre-hydrolysis of AFEX$^{TM}$-CS was performed first at 50 $^{o}$C, pH 4.8, and 800 rpm. Then temperature, pH, and agitation speed were changed to 30 $^{o}$C, 5.5 and 220 rpm, respectively, with yeast cell pellets

inoculated at an initial $OD_{600}$ of 2.0. After inoculation, fermentation was started and proceeded for another 168 h. Samples (5 ml) were taken during SSCF using a 10 ml syringe for sugar/ethanol analysis (mass balance). Triplicates of the experiments were performed. Averages and standard deviations of the experimental results are shown in the figures and table.

### 6.2.5 Continuous SSCF

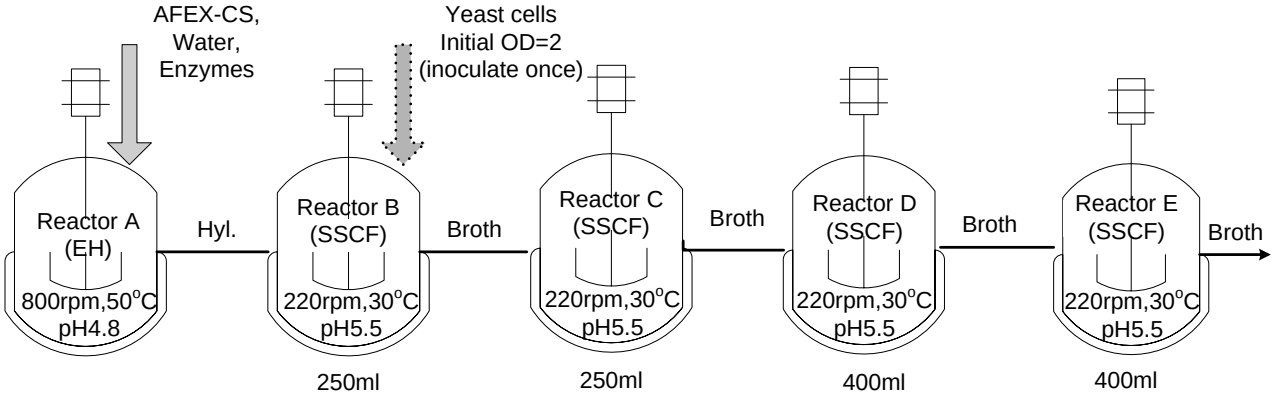



Figure 34 Continuous SSCF process diagram

Continuous SSCF was performed in a bio-reactor train (CSTR) as shown in Fig. 34. Five bioreactors (Biostat, Sartorius) were connected in series. Reactor A was used for enzymatic hydrolysis with temperature, pH, and agitation speed controlled at 50 $^{o}$C, 4.8 and 800 rpm, respectively. Reactors B, C, D, E were for SSCF with conditions controlled at 30 $^{o}$C, pH 5.5 and 220 rpm. AFEX$^{TM}$-CS, water and enzymes should keep flowing into Reactor A and the resulting hydrolysate was pumped to Reactor B. The fermentation broth from Reactor B was pumped to Reactor C, and then, D and E as shown in Fig. 34. Flow rates from one reactor to another were

the same. The volume of Reactor A ($V_A$) depends on the flow rate and pre-hydrolysis time (residence time). In this study, the pre-hydrolysis time was fixed at 24 h. Therefore, theoretically, $V_A$ is equal to flow rate multiplied by 24 (Table 9). However, due to equipment limitations in the laboratory, we were unable to continuously feed solid AFEX$^{TM}$-CS into Reactor A and did not have appropriate bioreactors to accommodate $V_A$. Instead, we performed enzymatic hydrolysis (400 g total) in another 500 ml bio-reactor (Biostat, Sartorius) for 24 h and then transferred the contents to Reactor A and made sure the reactor was never empty. Therefore, the pre-hydrolysis time for biomass in Reactor A varies from 24 h to 54 h depending on the flow rate. Between 24 h and 54 h during the enzymatic hydrolysis period, sugar concentrations were relatively constant, with glucose and xylose concentrations between 52-58 g/L and 31-35g/L, respectively (Fig. 35, Fig. 38a, & Fig. 38b). Thus pseudo-steady state operation was achieved with this setup. Our criterion for pseudo-steady state was that glucose and xylose concentrations in each SSCF reactor varied less than 2 g/L in 28 h. The working volumes for Reactor B, C, D, and E were 250, 250, 400, and 400 ml, respectively.

To initiate the continuous SSCF, 24 h pre-hydrolysis of AFEX$^{TM}$-CS was conducted in Reactor B followed by inoculation of yeast cells (initial OD=2) and fermenting the mixture for 24 h. At the end of this fermentation period, hydrolysate was continuously pumped from Reactor A to Reactor B and fermentation broth began to be continuously pumped from Reactor B to Reactor C. Once Reactor C was filled, the fermentation broth in Reactor C started being pumped to D. When all of the reactors reached the designated volume, the broth was continuously pumped out from Reactor E. Samples were taken from time to time to verify pseudo-steady state by measuring sugar/ethanol concentrations and for mass balance calculations.

### 6.2.6 Measurement of viable cells density

Please see Chapter II section 2.2.8.

### 6.2.7 Sugar, fermentation products analyses and mass balance

Glucose, xylose, ethanol, xylitol, glycerol and acetate concentrations were analyzed by HPLC using Biorad Aminex HPX-87H column as described previously Chapter II section 2.2.7. For mass balance details, please see Chapter III section 3.2.8.

## 6.3 Results and Discussion

### 6.3.1 Enzymatic hydrolysis comparison of SHF and SSCF

Two reactions existing in both SHF and SSCF processes are enzymatic hydrolysis and fermentation, which determine fermentable sugar yields and conversion of fermentable sugars to ethanol, respectively. Enzymatic hydrolysis was found to be the limiting step during SHF process affecting the final ethanol yield (Lau & Dale, 2009). The performance of this reaction in both processes was compared in Fig. 35. For the SHF process, enzymatic hydrolysis and fermentation were performed separately. Therefore, enzymatic hydrolysis was conducted at its optimal conditions (50 $^{o}$C, pH 4.8 and 800 rpm). For the SSCF process, the enzymatic hydrolysis reaction was carried out at the same optimal conditions during the first 24 h (pre-hydrolysis). But after 24 h, the conditions were changed to favor fermentation (30 $^{o}$C, pH 5.5 and 220 rpm).

Apparently, during the first 24 h the sugar release patterns were the same for SHF and for SSCF. Glucan and xylan were hydrolyzed quickly to monomeric/oligomeric glucose and xylose, respectively. Monomeric glucose and xylose concentrations reached 51.5 g/L and 32 g/L, respectively with 5.9 g/L oligomeric glucose and 14.4 g/L oligomeric xylose produced. The corresponding monomeric glucose and xylose, oligomeric glucose and xylose conversions were

64.5%, 57.0%, 7.4% and 25.6%, respectively. After 24 h, the hydrolysis rates in both processes were decreased with SHF showing larger hydrolysis rates compared to SSCF. For SSCF, we

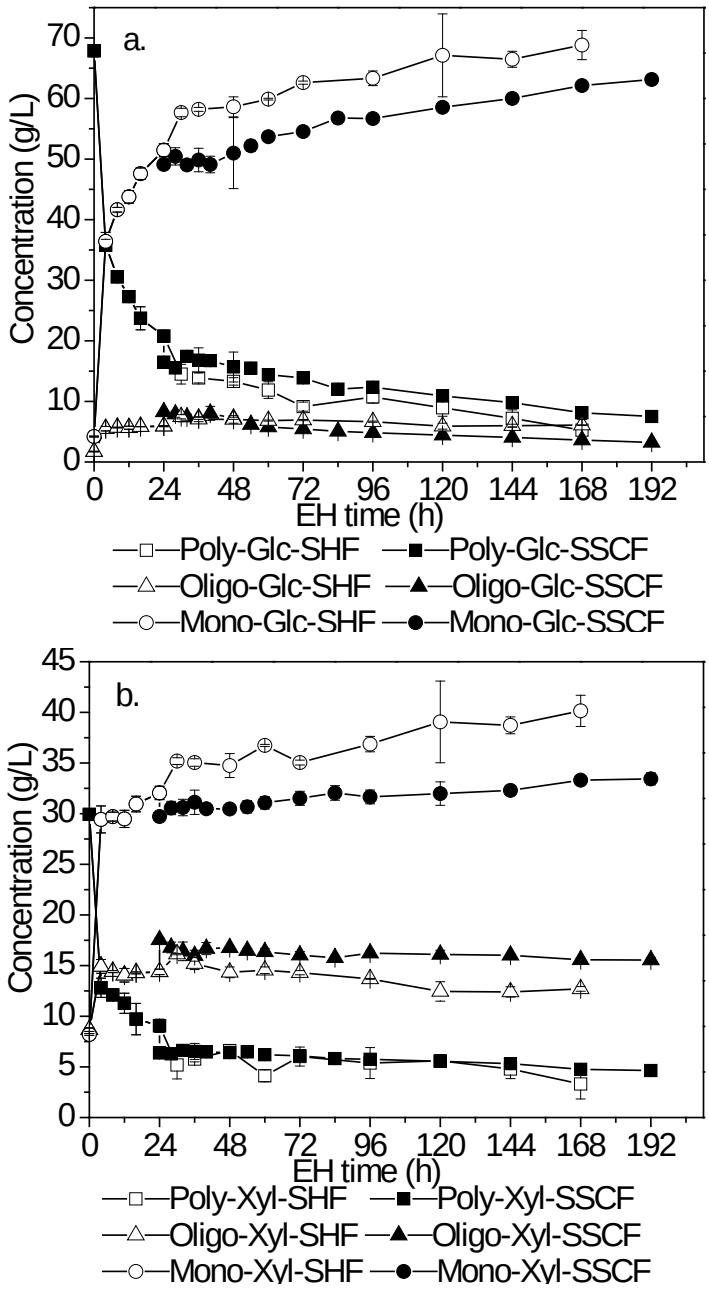



Figure 35 Time courses for polymeric (Poly-), oligomeric (Oligo-) and monomeric (Mono-) glucose (a) /xylose (b) during enzymatic hydrolysis of batch SSCF and SHF.
For SSCF, prehydrolysis was performed for the first 24 h at 800 rpm, pH 4.8 and 50 $^{o}$C followed by a change to 220 rpm, pH 5.5, and 30 $^{o}$C with yeast cells inoculated. Monomeric sugars released during SSCF were calculated based on total input sugars and remaining polymeric and oligomeric sugars.

observed about a 20 h lag phase after yeast inoculation (Fig. 36). During the lag phase, the enzymatic hydrolysis reaction seemed to stop with almost no additional sugars released (Fig. 35). The enzyme activities could have been fully inhibited by high concentrations of glucose and xylose at low temperature (30 $^{o}$C). After the lag phase, with glucose consumed by yeast fermentation, the monomeric glucose release continued to increase to 63.1 g/L at 192 h with the oligomeric glucose decreased to 3.2 g/L. Lower oligomeric glucose concentration was found during SSCF when compared to SHF. This could be a benefit of glucose removal by the yeast fermentation and thereby removing the end-product inhibition of $\beta$-glucosidase. The released monomeric xylose during SSCF after inoculation was increased slightly to 33.4 g/L (from 32 g/L) with oligomeric xylose also increased to 15.6 g/L. Slow xylose removal by yeast fermentation, and the presence of ethanol could be possible reasons why little xylose is released during SSCF at low temperature (30 $^{o}$C) (Chapter IV, Fig. 27). Overall, for a given EH time, SHF showed higher monomeric and total sugar conversions compared to SSCF. Thermo-tolerant strains, which can ferment at high temperatures, closer to the optimum temperature of EH, could improve EH performance during SSCF (Chapter IV, Fig. 27).

**6.3.2 Fermentation comparison between SHF and SSCF**

During the fermentation period, glucose was consumed first and rapidly (Fig. 36a) for both SHF and SSCF. Xylose fermentation was much slower. After 168 h fermentation, SHF consumed 64.7 g/L glucose and 31.2 g/L xylose with the remaining broth xylose concentration of 6.5 g/L. SSCF consumed 62.5 g/L glucose and 29.6 g/L xylose with the remaining xylose concentration of 3.8 g/L. The same ethanol concentration (38.8 g/L) was produced. The ethanol metabolic yields for SHF and SSCF were 78.0% and 81.3%, respectively (Fig. 40), which were

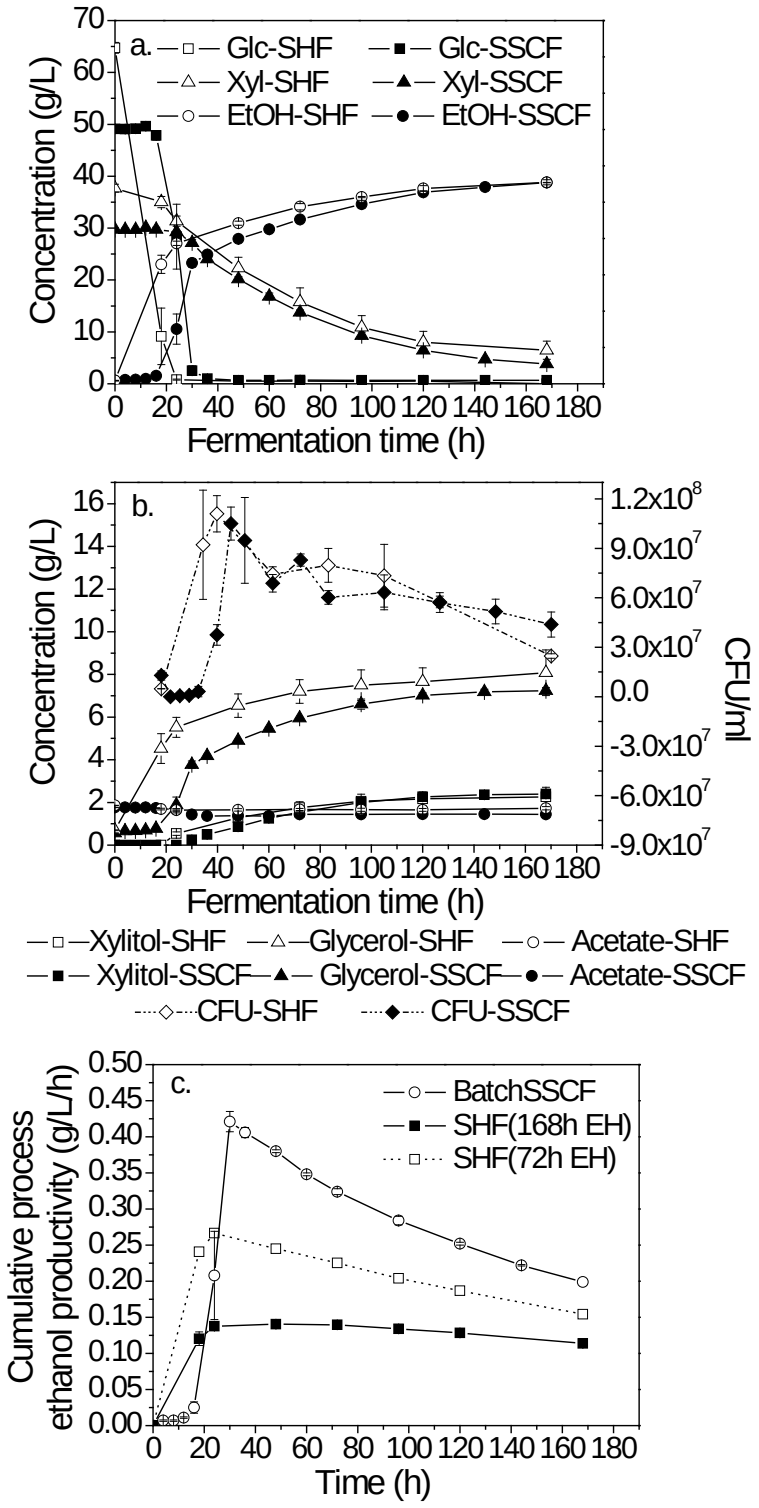



Figure 36 Fermentation comparison of batch SSCF and SHF.
Only the fermentation period is shown in this figure. The productivity of SHF (72h EH) was calculated based on the assumption that the fermentation kinetics were exactly the same as SHF with 168h EH.

lower than the previous study on SHF (Lau & Dale, 2009). The reduced ethanol metabolic yields in this study might be due to the presence of fine solids during fermentation in both SHF and SSCF processes. The byproducts produced during SHF and SSCF process were xylitol (1.1 and 2.4 g/L) and glycerol (8.1 and 7.2 g/L) respectively (Fig. 36b). Glycerol production could help reoxidize NADH produced during xylose fermentation in anaerobic conditions (Medina et al., 2010) and hence alleviate redox imbalance and facilitate xylose metabolism, but would also reduce ethanol metabolic yield. Acetate in the fermentation broth was mostly generated from plant biomass by pretreatment and EH. During yeast fermentation, acetate concentrations were slightly reduced (Fig. 36b).

Overall, based on 168 h EH, SHF showed much lower process ethanol productivity compared to SSCF after the first 24 h of fermentation during which period SSCF was in the lag phase (Fig. 36c). To compare SHF and SSCF more fairly, we also calculated the process ethanol productivity of SHF based on 72 h EH with the assumption that the fermentation kinetics were exactly the same as the 168 h EH's. However, the process ethanol productivity of SHF was still lower compared to SSCF. Productivity comparison was based on the similar sugar-to-ethanol conversions. Glucose-to-ethanol conversion, xylose-to-ethanol conversion and ethanol concentration were 78.3%, 46.9% and 36.0 g/L, respectively for SHF (72 h EH + 96 h ferm.) and 80.3%, 48.2% and 36.9 g/L, respectively for SSCF (24 h prehydrolysis + 120 h SSCF), respectively. The process ethanol productivities for SHF and SSCF were 0.20 and 0.25 $g \cdot L^{-1} \cdot h^{-1}$, respectively. Although the EH during SHF yielded more sugars at a given time compared to SSCF, SSCF was more efficient in producing ethanol. Therefore, SSCF was chosen as the mode to conduct continuous fermentation for enhancing the overall productivity.

### 6.3.3 Fermentation kinetics of batch SSCF

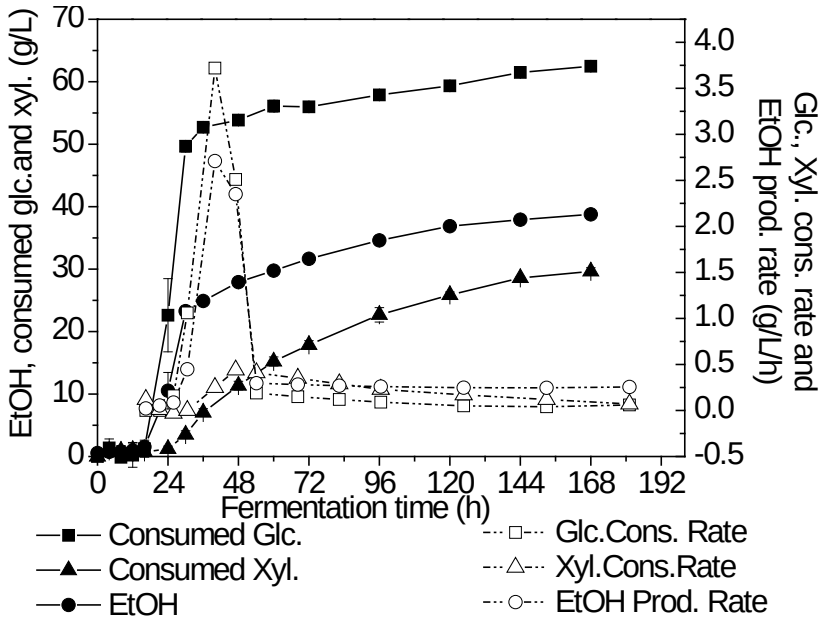



Figure 37 Consumed glucose/xylose, ethanol production, glucose/xylose consumption rate, and ethanol production rate during batch SSCF.
Those rates can be used to estimate dilution rate for continuous SSCF.

Glucose/xylose consumption, ethanol production and their respective rates are shown in Fig. 37. The maximum glucose consumption rate, xylose consumption rate and ethanol production rate were 3.72, 0.44 and 2.71 $g \cdot L^{-1} \cdot h^{-1}$, respectively. At such rates, the glucose, xylose and ethanol concentrations were 28.36, 27.17 and 10.54 g/L, respectively (Fig. 36a). Therefore, if the design of continuous SSCF aims to maximize glucose consumption rate, the dilution rate (D) should be approximately 0.13 $h^{-1}$ (the ratio of maximum glucose consumption rate over the corresponding glucose concentration(Govindaswamy & Vane, 2010)). If the maximum xylose consumption rate is desired, the dilution rate should be around 0.02 $h^{-1}$. To maximize the ethanol production rate, the dilution rate is 0.26 $h^{-1}$. To avoid yeast cell washout, the dilution rate should be controlled below the maximum specific cell growth rate 0.30 $h^{-1}$

(calculated from cell growth kinetics, Fig. 36b). Since the xylose consumption rate is much lower compared to the glucose consumption rate, a multi-stage continuous fermentation applying a larger working volume (reduced dilution rate and longer residence time) for xylose fermentation reactors could achieve both high glucose consumption rates and high xylose consumption rates (Govindaswamy & Vane, 2010). Due to the limitations of our laboratory reactors, we set the volumes of SSCF reactors (B, C, D, E) at 250, 250, 400 and 400 ml, respectively (Fig. 34).

### 6.3.4 Continuous SSCF

Continuous SSCF was conducted according to the diagram in Fig. 34. The flow rates tested were 12, 24, and 48 ml/h with the corresponding dilution rates shown in Table 9. At a flow rate of 12 ml/h when pseudo-steady state was reached, glucose consumption was almost complete in the first SSCF reactor Reactor B (Fig. 38a) with the glucose consumption and glucose-to-ethanol conversion at 56.5 g/L (Table 9) and 73.6% (Fig. 38e), respectively. The overall glucose consumption and glucose-to-ethanol conversion were 60.9 g/L and 79.2%, respectively. The cumulative xylose consumptions in Reactors B, C, D, and E were 11.3, 20.8, 25.0 and 27.5 g/L, respectively (Table 9) with the corresponding xylose-to-ethanol conversions of 20.9%, 38.4%, 46.2% and 50.8%, respectively (Fig. 38f). When the flow rate was raised to 24 or 48 ml/h, the residence time was reduced with less glucose/xylose consumed and converted to ethanol (Fig. 38a, b, c, e, f, Table 9). The overall glucose and xylose consumptions for a flow rate of 24 ml/h was 58.5 and 25.5 g/L, respectively with glucose and xylose-to-ethanol conversions of 77.0% and 47.5%, respectively. The overall glucose and xylose consumption for 48 ml/h were 55.3 and 17.7 g/L, respectively, with glucose and xylose-to-ethanol conversions of 75.6% and 33.3%, respectively. The final ethanol concentrations for the flow rates 12, 24, and 48

ml/h were 38.0, 36.5, and 33.8 g/L, respectively. Therefore, the overall trend was that sugar consumption and ethanol production decreased as the flow rate increased. However, higher final process ethanol productivity was observed at higher flow rates (Fig. 39a). The productivity values for the flow rates 12, 24, and 48 ml/h were 0.28, 0.46 and 0.65 $g \cdot L^{-1} \cdot h^{-1}$, respectively. This means higher flow rates converted sugars more efficiently to ethanol but the overall sugar conversions were reduced. For cost-efficient production of cellulosic ethanol, those two factors (sugar conversion and ethanol productivity) must be balanced to reach an optimum economic point.

Xylose fermentation rate was the major factor limiting ethanol productivity. For instance, at a flow rate 12 ml/h, it required 3 reactors (250 ml + 400 ml + 400 ml) to consume 27.5 g/L xylose while one reactor (250 ml) was sufficient to consume 56.5 g/L glucose (Fig 5a, b, Table 9). Slow xylose consumption is a major concern for lignocellulosic fermentation (Dien et al., 2003a; Hahn-Hagerdal et al., 2007). Recently, engineered *S. cerevisiae* which can simultaneous co-ferment cellobiose and xylose with the glucose repression of xylose fermentation minimized (Ha et al., 2011), and naturally xylose-fermenting thermophilic bacterium with knockout of organic acid genes (Shaw et al., 2008) showed interesting features in xylose fermentation. The presence of considerable concentrations of oligomeric sugars is another factor affecting overall sugar to ethanol conversion which in turn affects ethanol titer and ethanol productivity. For instance, at a flow rate of 24 ml/h, the overall xylan-to-xylose (monomers and oligomers) conversion reached a high of 86.9%. However, monomeric xylose conversion was merely 58.5% with 28.4% of oligomeric xylose conversion (16.5 g/L; Fig. 40, Table 9), which resulted in relatively low xylose-to-ethanol conversion (47.5%) since *S. cerevisiae* 424A was unable to consume oligomeric sugars.

Table 9 Byproduct profiles of continuous SSCF at different flow rates

| | Consumed glucose (g/L) | Consumed xylose (g/L) | Oligo-glc. (g/L) | Oligo-xyl. (g/L) | Xylitol (g/L) | Glycerol (g/L) | Acetate (g/L) |
|---|---|---|---|---|---|---|---|
| *12 ml/h, $V_A$=288 ml* | | | | | | | |
| Reactor A(D=0.04h$^{-1}$) | **0** | **0** | **6.19**±0.43 | **15.13**±1.13 | **0.00**±0.00 | **0.56**±0.02 | **1.86**±0.02 |
| Reactor B(D=0.05h$^{-1}$) | **56.54**±0.92 | **11.32**±0.33 | **5.50**±0.08 | **16.40**±0.29 | **0.38**±0.02 | **5.45**±0.11 | **1.47**±0.01 |
| Reactor C(D=0.05h$^{-1}$) | **56.69**±0.94 | **20.80**±0.77 | **4.89**±0.15 | **16.38**±0.23 | **0.68**±0.01 | **5.59**±0.03 | **1.40**±0.06 |
| Reactor D(D=0.03h$^{-1}$) | **57.52**±1.22 | **25.00**±1.31 | **4.56**±0.34 | **16.58**±0.37 | **1.04**±0.06 | **6.38**±0.28 | **1.35**±0.01 |
| Reactor E(D=0.03h$^{-1}$) | **60.58**±1.81 | **27.49**±1.41 | **4.18**±0.41 | **16.33**±0.42 | **1.10**±0.02 | **6.63**±0.17 | **1.40**±0.02 |
| *24 ml/h, $V_A$=576 ml* | | | | | | | |
| Reactor A(D=0.04h$^{-1}$) | **0** | **0** | **6.96**±0.44 | **16.14**±0.92 | 0.00±0.00 | 0.53±0.02 | 1.85±0.05 |
| Reactor B(D=0.10h$^{-1}$) | **46.42**±2.11 | **4.55**±0.84 | **6.51**±0.14 | **17.07**±0.12 | 0.00±0.00 | 3.63±0.19 | 1.55±0.05 |
| Reactor C(D=0.10h$^{-1}$) | **51.11**±0.56 | **10.12**±0.66 | **5.88**±0.10 | **17.56**±0.08 | 0.37±0.03 | 5.15±0.21 | 1.43±0.05 |
| Reactor D(D=0.06h$^{-1}$) | **54.09**±**2.23** | **19.75**±1.03 | **5.35**±0.06 | **16.99**±0.13 | 0.77±0.01 | 6.38±0.17 | 1.41±0.03 |
| Reactor E(D=0.06h$^{-1}$) | **58.47**±1.49 | **25.51**±0.94 | **4.87**±0.07 | **16.52**±0.05 | 0.93±0.01 | 6.97±0.21 | 1.44±0.03 |
| *48 ml/h, $V_A$=1152 ml* | | | | | | | |
| Reactor A (D=0.04h$^{-1}$) | **0** | **0** | **6.90**±0.25 | **15.96**±0.81 | **0.00**±0.00 | **0.52**±0.01 | **1.83**±0.04 |
| Reactor B (D=0.19h$^{-1}$) | **31.06**±2.46 | **2.02**±0.42 | **6.87**±0.09 | **16.49**±0.02 | **0.00**±0.00 | **2.07**±0.26 | **1.63**±0.08 |
| Reactor C (D=0.19h$^{-1}$) | **50.58**±0.20 | **4.99**±0.18 | **6.37**±0.04 | **17.03**±0.40 | **0.05**±0.11 | **4.00**±0.21 | **1.44**±0.07 |
| Reactor D (D=0.12h$^{-1}$) | **55.92**±0.18 | **13.65**±0.64 | **5.89**±0.02 | **17.15**±0.40 | **0.56**±0.03 | **5.45**±0.15 | **1.36**±0.04 |
| Reactor E (D=0.12h$^{-1}$) | **55.34**±0.47 | **17.67**±0.07 | **5.64**±0.04 | **17.05**±0.03 | **0.74**±0.01 | **5.96**±0.14 | **1.33**±0.02 |

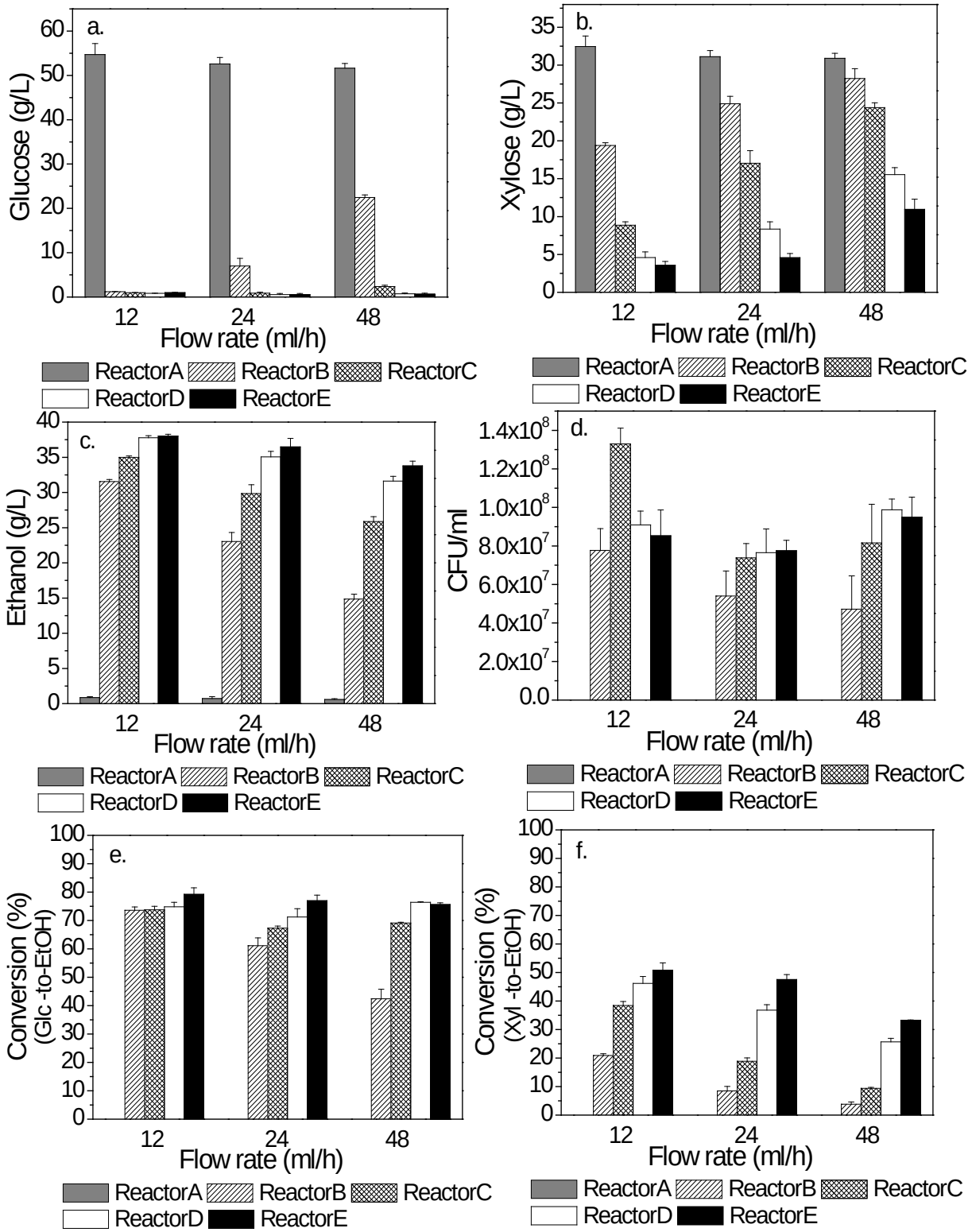



Figure 38 Comparison of three flow rates (12, 24, and 48 ml/h) on glucose (a) and xylose (b) consumption, ethanol production (c), viable cell density (d), glucose-to-ethanol (e) and xylose-to-ethanol (f) conversions during continuous SSCF.

The glucose-to-ethanol and xylose-to-ethanol conversions were calculated based on total glucose and total xylose in AFEX$^{TM}$-CS.

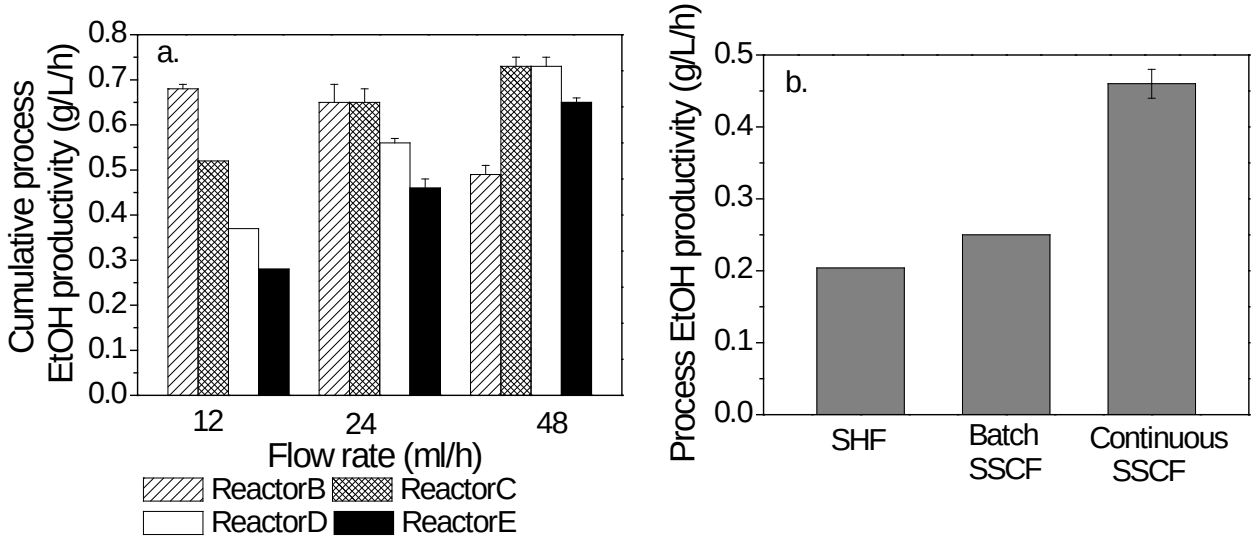



Figure 39 Process ethanol productivity comparisons of different flow rates during continuous SSCF (a), and productivity comparisons of SHF, batch SSCF and continuous SSCF (b). In (b), process ethanol productivities were compared based on similar glucose-to-ethanol and xylose-to-ethanol conversions. For SHF, the calculation was based on 72 h enzymatic hydrolysis and 96 h fermentation assuming the fermentation kinetics are the same as fermentation of 168 h hydrolysate. The glucose-to-ethanol conversion, xylose-to-ethanol conversion and ethanol concentration were 78.3%, 46.9% and 36.0 g/L, respectively. For batch SSCF, the calculation was based on 24 h prehydrolysis and 120 h SSCF. The glucose-to-ethanol conversion, xylose-to-ethanol conversion and ethanol concentration were 80.3%, 48.2% and 36.9 g/L, respectively. For continuous SSCF, the calculation was based on flow rate 24 ml/h. The glucose-to-ethanol conversion, xylose-to-ethanol conversion and ethanol concentration were 77.0%, 47.5% and 36.5 g/L, respectively.

### 6.3.5 Comparison of continuous SSCF with batch SHF and batch SSCF

Mass balances were completed for comprehensive comparison of those processes (Fig. 40). The highest sugar conversions were accomplished by SHF (168 h EH+168 h fermentation) followed by batch SSCF (24 h pre-hydrolysis + 168 h SSCF). The highest ethanol yield (195.1 g ethanol/kg AFEX$^{TM}$-CS) was achieved by batch SSCF (24 h prehydrolysis + 168 h SSCF) followed by SHF (168 h EH+168 h fermentation) 194.1 g ethanol/kg AFEX$^{TM}$-CS and

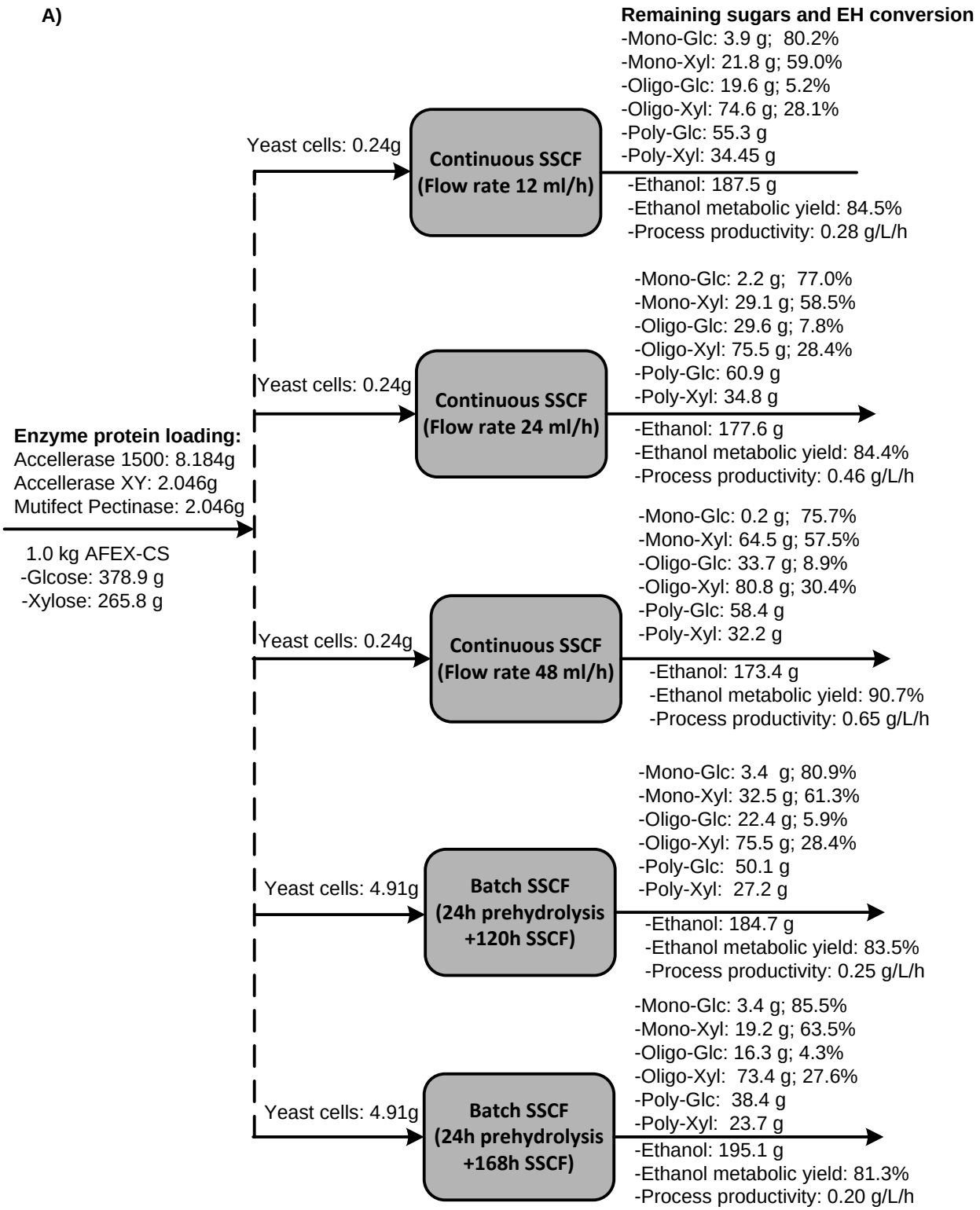
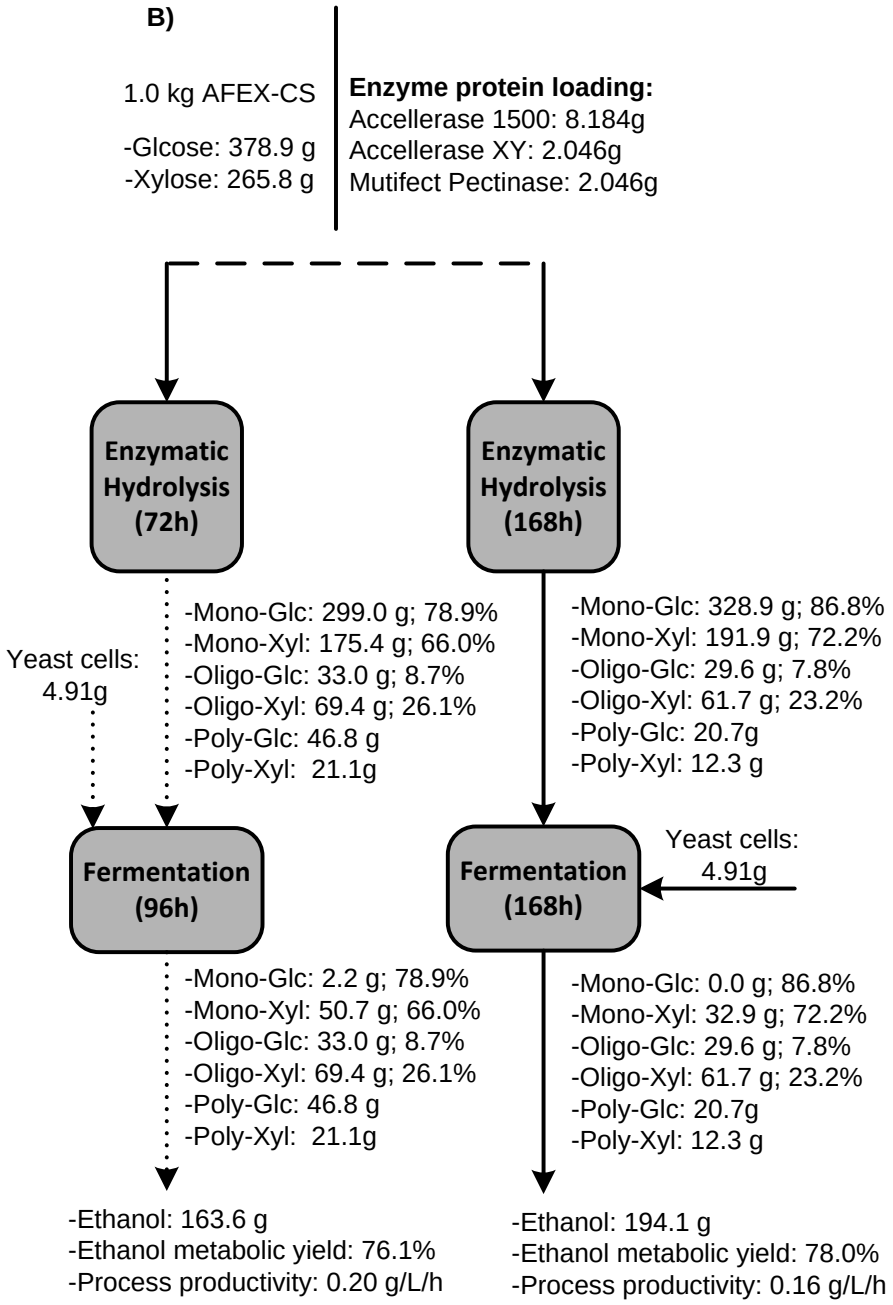



Figure 40 Mass balance of continuous SSCF, batch SSCF(A) and SHF(B).
For SHF (72 h EH+96h fermentation), the fermentation kinetics were assumed to be the same as those of 168 h hydrolysate fermentation.

Figure 40 (cont'd)




continuous SSCF (flow rate 12 ml/h) 187.5 g ethanol/kg AFEX$^{TM}$-CS. However, the corresponding process ethanol productivities were low at 0.20, 0.16, and 0.28 g·L$^{-1}$·h$^{-1}$,

respectively. The highest process ethanol productivities were found in continuous SSCF with flow rates of 48 and 24 ml/h, and were 0.65 and 0.46 $g \cdot L^{-1} \cdot h^{-1}$, respectively. To more fairly compare the three processes , the process ethanol productivity was compared based on similar sugar to ethanol conversions (glucose and xylose-to-ethanol conversions and ethanol concentration were 78.3%, 46.9% and 36.0 g/L, respectively for SHF (72 h EH +96 h ferm.); 80.3%, 48.2% and 36.9 g/L, respectively for batch SSCF (24 h pre-hydrolysis + 120 h SSCF); 77.0%, 47.5% and 36.5 g/L, respectively for continuous SSCF with 24 ml/h flow rate) in Fig. 39b. Under such a comparison, continuous SSCF also showed the highest process ethanol productivity 0.46 $g \cdot L^{-1} \cdot h^{-1}$, followed by batch SSCF 0.25 $g \cdot L^{-1} \cdot h^{-1}$ (Fig. 39b) and SHF 0.20 $g \cdot L^{-1} \cdot h^{-1}$. This means that to achieve the same sugar-to-ethanol conversion, continuous SSCF is a more efficient process. Ethanol productivity increased by around 2 times, therefore the capital cost for enzymatic hydrolysis and fermentation was reduced by around half. Besides, yeast inoculation for continuous SSCF is required only once and only in one bioreactor, which would also reduce the processing cost. For batch SSCF and SHF, however, yeast inoculation is needed for every batch. For example, the yeast cell inoculation required is 0.24 g per kg AFEX$^{TM}$-CS or even per ton AFEX$^{TM}$-CS based on continuous SSCF. However, for batch SSCF or SHF, 4.91 g yeast inoculum is required per 1 kg AFEX$^{TM}$-CS (Fig. 39).

### 6.3.6 Discussion on the continuous SSCF process design

The bioreactors configuration design in the continuous SSCF process was based on the CSTR design equation: $V = -\frac{F_{io}}{r_i} X_i$ (V is the reactor volume (L); $F_{io}$ is the mass flow in rate (g/h); i could be glucose or xylose; $r_i$ is the conversion rate (g/L/h); $X_i$ is the sugar to ethanol

conversion. $F_{io}$ equals the volumetric flow rate (L/h) multiplied by the initial sugar concentration. Levenspiel plots ($-F_{io}/r_i \sim X_i$) were used for reactor sizing (Fogler, 1999). The correlations between $-F_{io}/r_i$ and $X_i$ were calculated based on experimental data. When the volumetric flow rate is 12 ml/h, the plots are shown in Fig. 41. The shade areas are the designed reactor volumes. The designated glucose conversion for the first SSCF reactor (Reactor B) is around 68%, at which point the curve has a value close to minimum (Fig. 41a), corresponding to a reactor volume of 250 ml. After that point, a plug flow reactor (PFR) would be most efficient (PFR: $V = \int_0^X -\frac{F_{io}}{r_i} dX_i$). A series of CSTRs function like a plug flow reactor. Thus, three reactors (C, D, E) were used for achieving high volume efficiency. The obtained experiments results were similar to the design expectations. For instance, the Reactor B achieved 73.6% glucose-to ethanol conversion and Reactor E reached 79.2% (design expectation is 68% and 79%, respectively). The xylose-to-ethanol conversions for Reactor B and Reactor E were 20.9% and 50.8%, respectively (design expectation is around 16% and 49%, respectively).

An SSCF model, which could describe the reactions better, might be helpful in further optimization of the continuous SSCF process. However, even if the process is further optimized a large reaction volume is still required due to the slow xylose fermentation (Fig. 41b) and a high enzyme loading is needed, which make the economics of this process not that promising based on current enzymes and fermenting strain. However, this process is definitely superior to batch processes. Better enzymes and fermenting strains are needed for better reactions for this process.

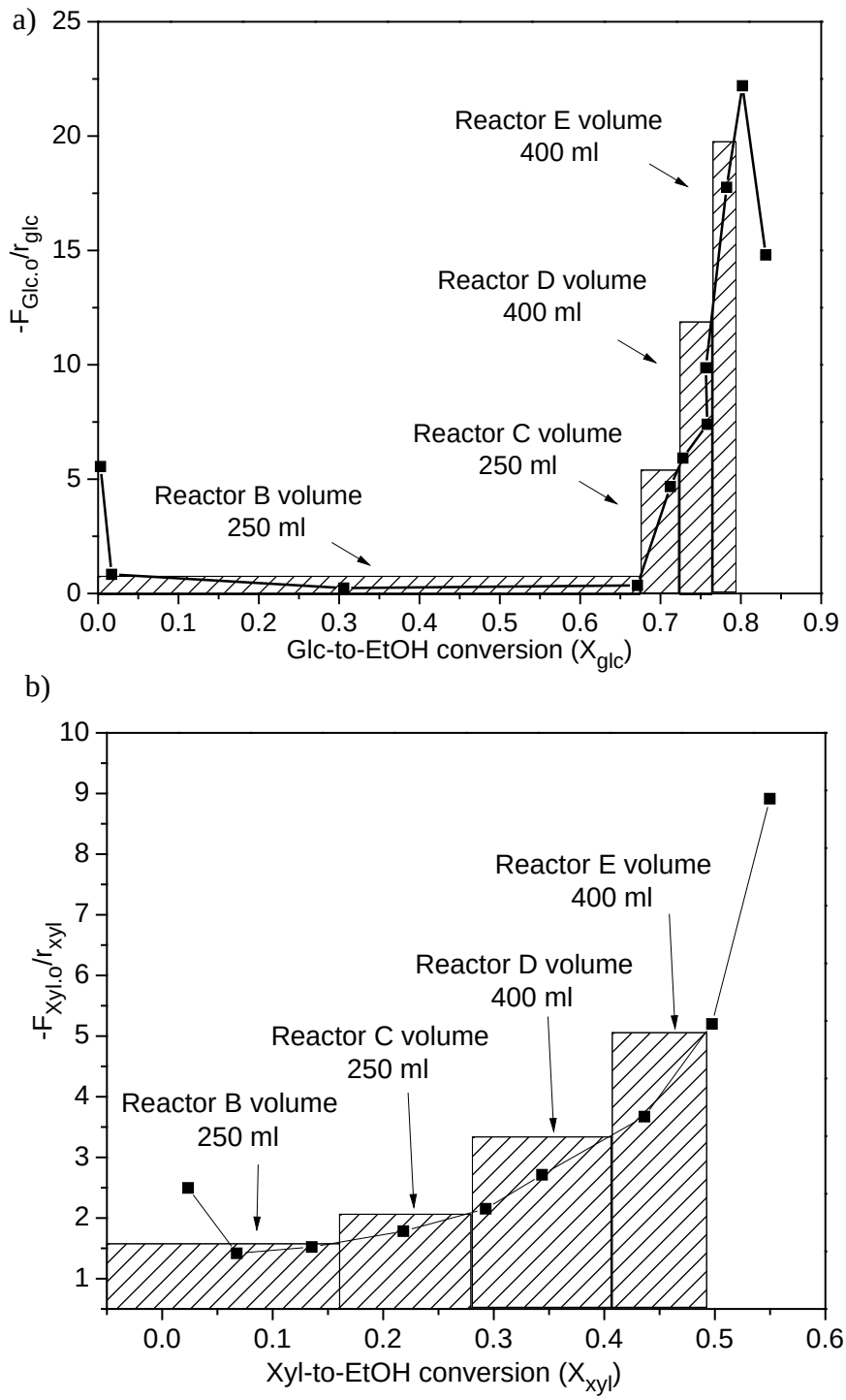



Figure 41 Levenspiel plots of $-F_{glc.o}/r_{glc}$ against $X_{glc}$ (a) and $-F_{xyl.o}/r_{xyl}$ against $X_{xyl}$ (b). *F* is the mass flow in rate (g/h); r is the sugar to ethanol conversion rate (g/L/h); X is the sugar to ethanol conversion.

## 6.4 Conclusions

Enzymatic hydrolysis performed better in the SHF process when compared to SSCF based on xylose-fermenting strain *S. cerevisiae* 424A(LNH-ST) and commercial enzymes (including Accellerase 1500, Accellerase XY and Multifect pectinase). However, SSCF was more efficient for ethanol production. Based on similar sugar to ethanol conversions (around 80% glucose-to-ethanol conversion and 47% xylose-to-ethanol conversion), the process ethanol productivity of continuous SSCF was 2.3 and 1.8 fold higher than that of SHF and batch SSCF, respectively. Increasing flow rates enhanced ethanol productivity, but decreased overall sugar conversions of continuous SSCF.

# CHAPTER VII NOVEL INTEGRATED BIOLOGICAL PROCESSES FEATURING HIGH ETHANOL PRODUCTIVITY, ENZYME RECYCLING AND YEAST CELLS REUSE


## Abstract

High enzyme loading requirements, slow xylose fermentation and low ethanol productivity are three of the major issues impeding commercial biochemical production of cellulosic ethanol. A novel integrated biological process was developed in this study to overcome these problems. Enzymatic hydrolysis was performed for only 24 h to avoid the slow rate period which begins at about that time. Unhydrolyzed recalcitrant solids with adsorbed enzymes were recycled to the subsequent cycles. By this approach, easily digestible biomass was processed first and recalcitrant biomass was given enough residence time to get hydrolyzed during subsequent processing steps. Fermentation was conducted using a high yeast inoculation level and was also completed in 24 h. The yeast cells were then recycled. With this novel processing approach, the enzyme loading was reduced from 36 to 22.3 and 25.8 mg protein per gram glucan, respectively, for separate hydrolysis and fermentation (SHF) and for simultaneous saccharification and co-fermentation (SSCF) on AFEX$^{TM}$ pretreated corn stover. The process ethanol productivity was enhanced by 2 to 3 fold due to both fast enzymatic hydrolysis and fast fermentation.

## 7.1 Introduction

Cellulosic ethanol production from lignocellulosic biomass has gained considerable momentum due to both environmental and social sustainability benefits (Farrell et al., 2006; Greene & Council, 2004; Wyman, 2008). However, the technology is not yet fully commercialized. There are major issues impeding the cellulosic ethanol production using the sugar platform, including high enzyme loadings needed, slow xylose fermentation and low ethanol productivity.

The enzyme loadings typically used for high solids loading enzymatic hydrolysis were around 30 to 36 mg protein per gram glucan (previous chapters). According to the National Renewable Energy Laboratory's (NREL, Golden Colorado) 2011 report, the enzyme cost accounts for 15.7% of the total cost even if the enzyme loading used is 20 mg per gram glucan (Humbird et al., 2011). The high enzyme loadings required for the process to obtain high sugar yields are largely due to the reduced rate of enzymatic hydrolysis with increased conversion/time (Yang et al., 2006) (Chapter VI, Fig. 35). Cellulose and hemicelluloses in plant cell wall exist in complex structures within a recalcitrant lignin matrix, and are difficult to hydrolyze (Chundawat et al., 2011a). Pretreatment disrupts such structures and improves the digestibility of lignocellulosic biomass (Yang & Wyman, 2008). After pretreatment, much of the heterogenous biomass is easily digestible while there is a considerable amount that is still recalcitrant (Kumar & Wyman, 2009a; Zhang et al., 1999; Zhou et al., 2010). Therefore, high rates are typically seen at the beginning of the enzymatic hydrolysis due to the easily digestible part being hydrolyzed before the recalcitrant part. Concerning the hydrolysis of the recalcitrant parts of biomass, there are several factors that likely further reduce the hydrolysis rates, including product inhibition

(Eriksson et al., 2002b), enzyme inactivation (Gusakov & Sinitsyn, 1992) and thermal instability of cellulases (Eriksson et al., 2002b). Reduced hydrolysis rates not only lead to high enzyme loadings but also result in long enzymatic hydrolysis times. Based on the current separate hydrolysis and fermentation (SHF) process, it takes 72~168 h to achieve high sugar conversions at high solids loading and another 96-168 h are required to ferment those sugars (mostly glucose and xylose) to ethanol since the xylose fermentation is slow. Thus overall biological processing times are well over 5 days, leading to higher capital and operating costs.

Xylose can be co-fermented with glucose to ethanol by genetically modified strains such as *Escherichia coli* KO11 (Olsson et al., 1995), *Zymomonas mobilis* AX101 (Zhang et al., 1995) and *Saccharomyces cerevisiae* 424A(LNH-ST) (Sedlak & Ho, 2004). Lau et al. compared the performance of those strains in AFEX$^{TM}$ (ammonia fiber expansion) pretreated hydrolysate and concluded that *S. cerevisiae* 424A(LNH-ST) was a good strain for AFEX$^{TM}$ hydrolysate fermentation (Lau et al., 2010). However, *S. cerevisiae* 424A(LNH-ST) fermented xylose slowly in AFEX$^{TM}$ hydrolysates. Slow xylose fermentation by this yeast is caused by redox imbalance (Ha et al., 2011), no specialized xylose transporter (Bertilsson et al., 2008), degradation products inhibition, ethanol inhibition as well as fermentation metabolites inhibition (Chapter II). Degradation products mostly inhibit the yeast cell growth during fermentation and hence reduce the overall xylose fermentation rate. Due to the slow xylose consumption, the fermentation ethanol productivity (ethanol produced per unit of time) was much lower than the industrial criterion of about 1.0 $g \cdot L^{-1} \cdot h^{-1}$ (Dien et al., 2003a). If the ethanol productivity is calculated based on enzymatic hydrolysis time plus fermentation time (process ethanol productivity), the productivity of current SHF process would be even lower due to the long enzymatic hydrolysis

time. By integrating enzymatic hydrolysis and fermentation, simultaneous saccharification and co-fermentation (SSCF), especially continuous SSCF, reduces the time required for enzymatic hydrolysis and hence improves productivity (Chapter III,V&VI). However, the high enzyme loading required is still a major concern.

Here a novel integrated biological process was invented (BCRL process; BCRL: Biomass Conversion Research Laboratory) to reduce the enzyme loading, enhance the xylose fermentation and increase the ethanol productivity. This process can be performed either in a SHF mode or in a SSCF mode (Fig. 42) and was tested on AFEX$^{TM}$ pretreated corn stover (AFEX$^{TM}$-CS) using commercial enzymes and *S.cerevisiae* 424A(LNH-ST). In this process, we hydrolyzed the easily digestible biomass first and avoided the slow-down period by performing enzymatic hydrolysis only for 24 h. The remaining unhydrolysed solids with some enzymes adsorbed (Azevedo et al., 2002; Gao et al., 2011; Tu et al., 2007) were recycled to the next cycle for further hydrolysis. By this approach, part of the enzymes was recycled and thereby the required enzyme loading was reduced. To achieve fast xylose fermentation, we used a high inoculum level ($OD_{600}$=20 instead of 0.5~2) and completed fermentation in 24 h. The high inoculum level fermentation bypassed the degradation products inhibition on yeast cell growth and hence improved xylose fermentation. The yeast cells were subsequently recycled. It took 24 h for enzymatic hydrolysis and another 24 h for fermentation to complete one cycle. Then the liquid part of the hydrolysis/fermentation mixture was harvested and subjected to distillation. The unhydrolyzed solids (recalcitrant part) were repeatedly transferred to the next hydrolysis/fermentation cycle to allow more hydrolysis time. After five cycles of

hydrolysis/fermentation, the accumulated recalcitrant residual solids were further hydrolyzed in the last step. Since some enzymes were recycled by this approach and part of the inhibitory

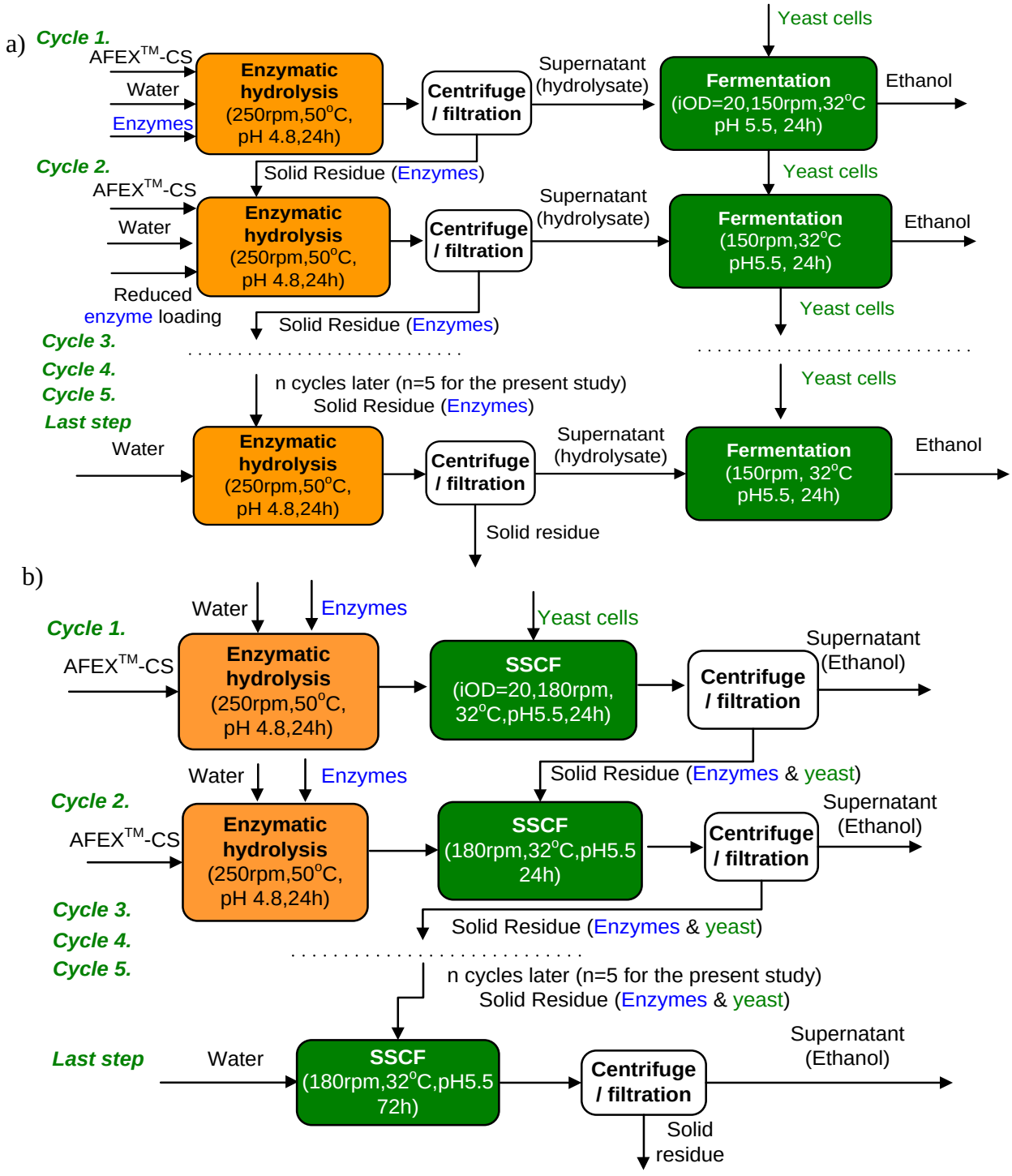



Figure 42 Flow chart of the Biomass Conversion Research Laboratory (BCRL) SHF process (a) and SSCF process (b).
The figure is in color.

degradation products were removed with liquid during the five cycles, the hydrolysis of the residual solids was both more rapid and more complete (higher sugar conversions). No enzymes were supplemented during the last step of enzymatic hydrolysis.

## 7.2 Materials and Methods

### 7.2.1 AFEX$^{TM}$ pretreated corn stover

Please see Chapter II section 2.2.1

### 7.2.2 Microorganisms and seed culture preparation

Please see Chapter IV section 4.2.2.

### 7.2.3 BCRL SHF and SSCF processes

The BCRL SHF process performed enzymatic hydrolysis for 24 h, followed by centrifugation at 5300 rpm for 20 min (Fig. 42a). The resulting unhydrolyzed solids were recycled to the next enzymatic hydrolysis tank and thereby the working volume increased from cycle to cycle (Fig. 43). The supernatant (hydrolysate) was used for ethanol fermentation at 150 rpm, 32 $^{o}$C and pH 5.5 with an initial $OD_{600}$ of 20 for 24 h. After fermentation, the yeast cells were collected by centrifugation at 4400 rpm for 8 min and used for the next fermentation inoculation. As shown in Fig. 42a, solid biomass residues and yeast cells were continually recycled to the next round of enzymatic hydrolysis and fermentation, respectively. For enzymatic hydrolysis in the last step, 5 ml phosphate buffer and 55 ml water were added without any new enzymes or fresh biomass. Enzymatic hydrolysis for cycles 1, 2, and 3 was conducted in a 250 ml baffled flask while cycles 4, 5 and the last step were performed in a 500 ml baffled flask.

The BCRL SSCF process was performed using enzymatic hydrolysis for 24 h followed by changing conditions to pH 5.5, 32 $^{o}$C, and 180 rpm, and inoculating yeast cells (initial OD = 20)

(Fig. 42b). After 24 h of SSCF, centrifugation was carried out at 5300 rpm for 20 min. The supernatant was ready for distillation. The solids were recycled and transferred to the next cycle SSCF tank and thereby the working volume increased from cycle to cycle (Fig. 43). After 5 cycles, 5 ml phosphate buffer and 55 ml water were added to the solids with no enzymes and fresh biomass supplemented for the last step. SSCF were conducted at 32 $^{o}$C, pH 5.5 and 180 rpm for 72 h.

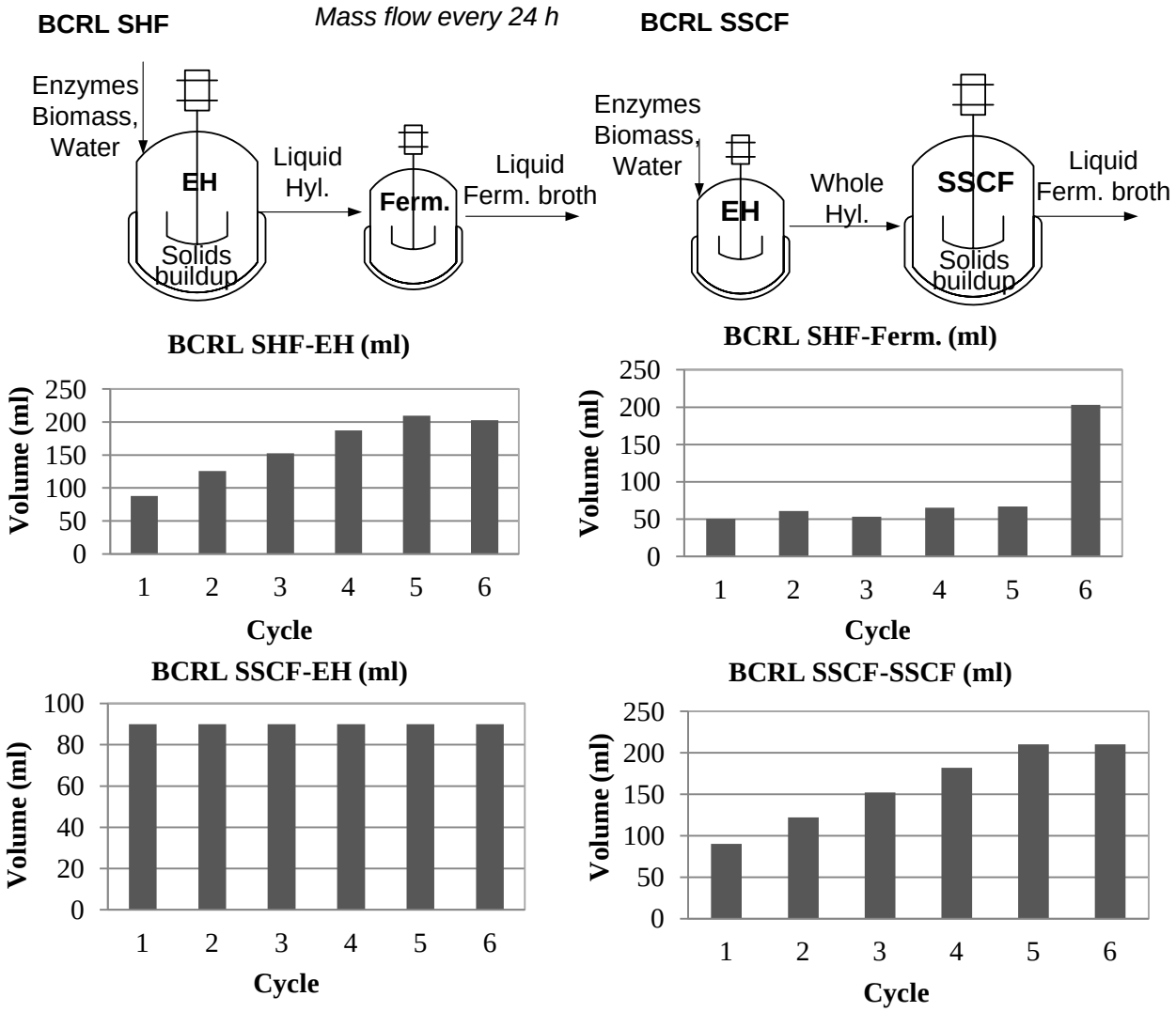



Figure 43 Tank setup and volume change for BCRL SHF and SSCF processes.
The last step is denoted as cycle 6. Basically, the volume increase happened in the enzymatic hydrolysis (EH) tank for the BCRL SHF process and in the SSCF tank for BCRL SSCF process due to the buildup of solid residues. The last step of fermentation for BCRL SHF process was carried out in the EH tank after 24 h hydrolysis without solid-liquid separation.

For enzymatic hydrolysis (EH) in both processes, the inputs for each cycle including fresh AFEX$^{TM}$-CS, water, phosphate buffer, and enzymes were 100 g in total. Glucan loadings for BCRL SHF and SSCF processes were 7.5% and 7% (w/w), respectively, which correspond to 22.0 g and 20.5 g AFEX$^{TM}$-CS biomass in 100 g total mixture, respectively. Enzymes used for the hydrolysis included Accellerase 1500, Accellerase XY, and Multifect pectinase (Genencor Inc, USA) with protein loadings of 30, 3, and 3 mg/g glucan, respectively, in the first cycle. The protein loadings varied during cycles 2~5. Enzymatic hydrolysis was performed at 50 $^{o}$C, pH 4.8, and 250 rpm. Fifty mg/L chloramphenicol (Cm) was used during hydrolysis to avoid microbial contamination.

### 7.2.4 Conventional SHF and SSCF processes

The conventional SHF process performed enzymatic hydrolysis at 50 $^{o}$C, pH 4.8 and 250 rpm for 96 h. The resulting hydrolysate was used for fermentation at 30 $^{o}$C, pH 5.5 and 150 rpm with an initial OD of 2.0. The conventional SSCF process was carried out by pre-hydrolyzing biomass at 50 $^{o}$C, pH 4.8 and 250 rpm for 6 h. The conditions were then changed to 30 $^{o}$C, pH 5.5 and 180 rpm with yeast cells inoculated at an initial OD of 2.0. The enzyme loading for the conventional processes was the same as for the first cycle of the BCRL processes. All the experiments were conducted in duplicate with the average and standard deviation shown in figures.

### 7.2.5 Measurement of viable cell density

Please see Chapter II section 2.2.8.

### 7.2.6 HPLC analyses and mass balance

For HPLC analyses, please see Chapter II section 2.2.7.

For mass balance details, please see Chapter III section 3.2.8.

## 7.3 Results and discussion

### 7.3.1 Effect of solids loading

The effect of solids loading (6%, 7% and 9% glucan loading) was investigated by performing 24 h enzymatic hydrolysis followed by 24 h hydrolysate fermentation or SSCF (Fig. 44a). One objective was to produce ethanol at an industrially relevant titer of 40 g/L (Dien et al., 2003a). The ethanol titer of 40 g/L was achieved by a 9% glucan loading for both SSCF and hydrolysate fermentation. However, the remaining xylose concentration in the fermentation broth was almost 2 times higher than 7% glucan loading, which means a greater loss of fermentable sugars if 9% glucan loading is applied since the broth will be removed and subject to distillation after 24 h fermentation (Fig. 42). Moreover, the cell viability during SSCF for 9% glucan loading was lower compared to the other solids loadings (Fig. 44b). This implies that the yeast cells were dying more rapidly at 9% glucan loading and thus fewer viable yeast cells could be recycled for the subsequent cycles. A glucan loading of 7% produced 38.6 g/L ethanol with 6.0 g/L xylose remaining in the broth after 24 h hydrolysis and 24 h SSCF. Similar studies using separate hydrolysis and fermentation (SHF) mode yielded 38.1 g/L ethanol with 4.8 g/L xylose remaining unutilized. By converting 4 g/L more xylose to ethanol, 40 g/L ethanol could be achieved. Therefore, the fermentation conditions were optimized.

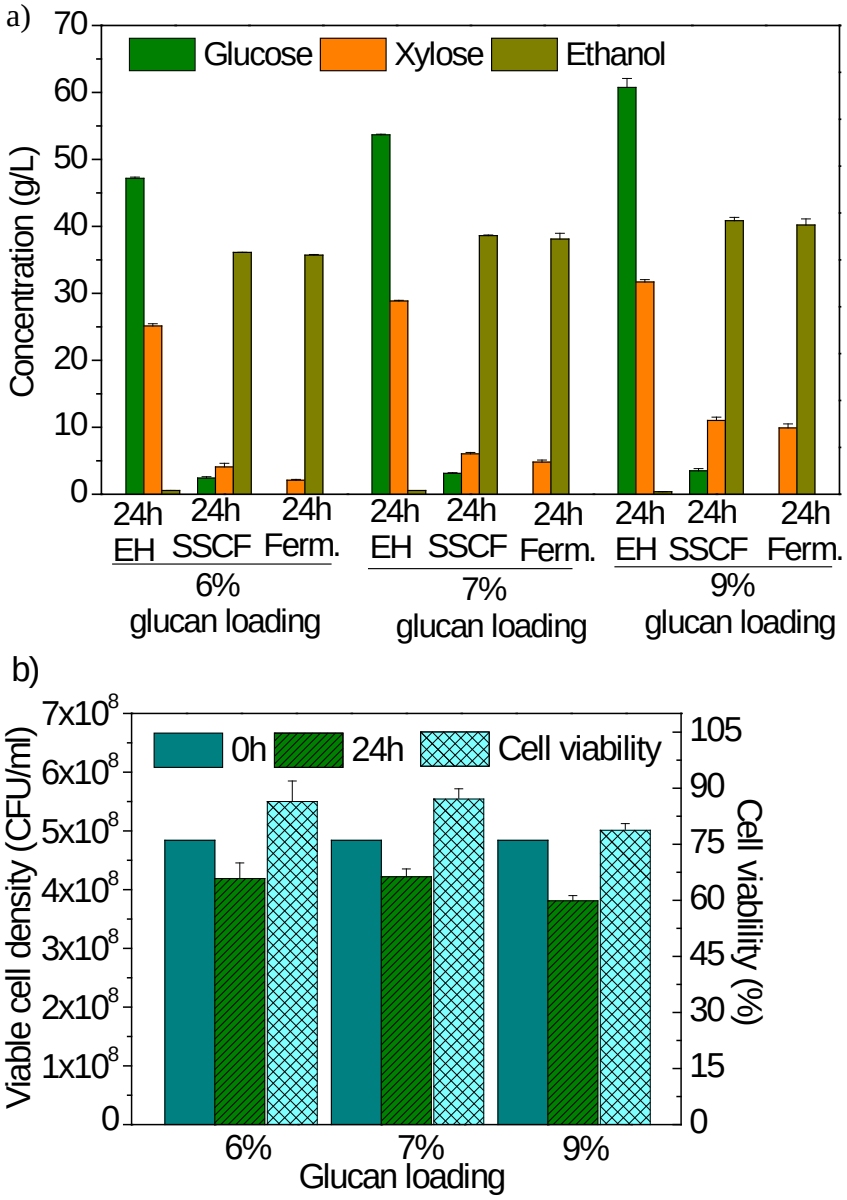



Figure 44 Effect of solids loading on 24 h enzymatic hydrolysis, the following 24 h SSCF or hydrolysate fermentation (a), viable cell density and cell viability after 24 h SSCF (b). Hydrolysate fermentation (fermentation of the liquid hydrolysate) and SSCF were conducted in 250 ml unbaffled and baffled flasks, respectively, at 30 oC, pH 5.5, $OD_{600}$=20, and 150 rpm. Viable cell density was measured in colony forming unit (CFU) per ml. The cell viability was calculated by using the viable cell density after 24 h SSCF dividing the initial viable cell density. The figure is in color.

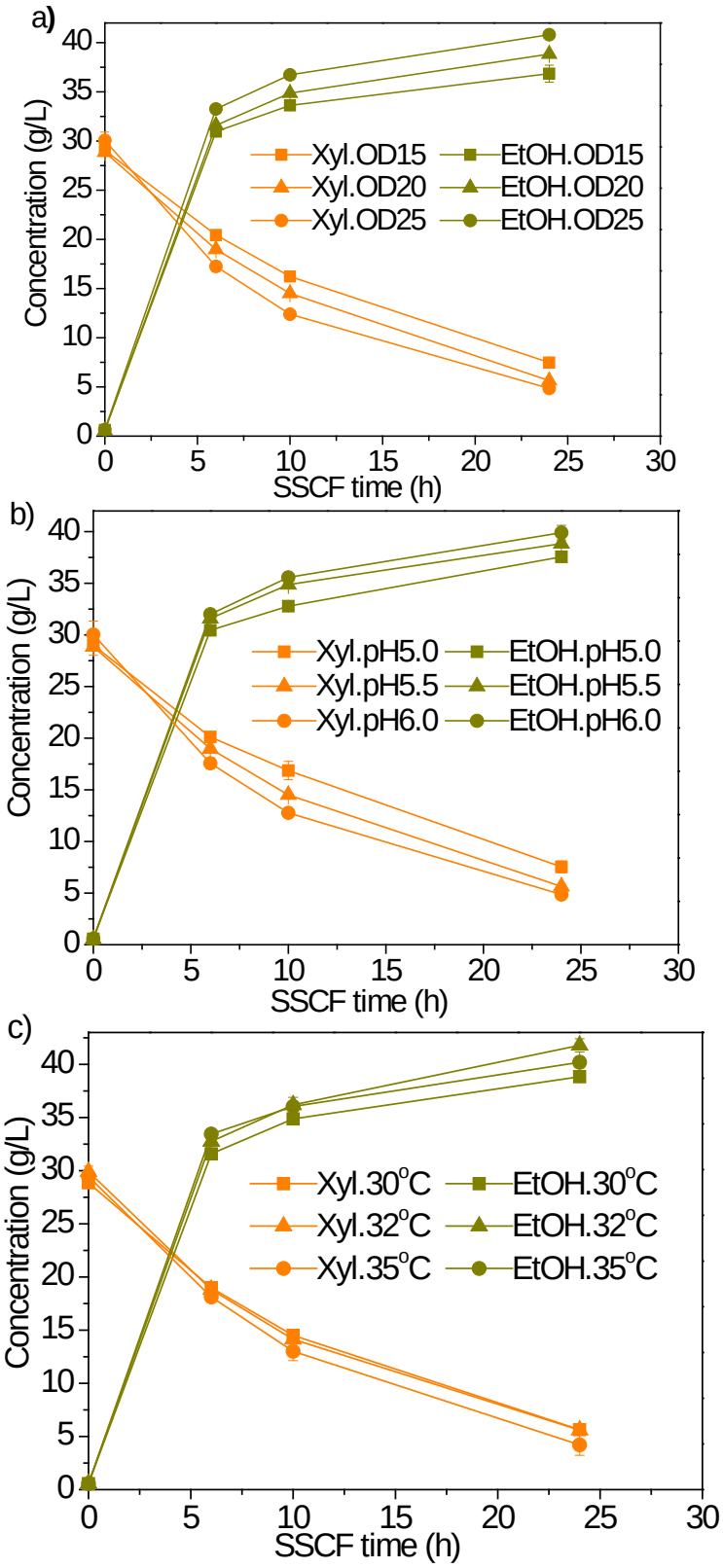
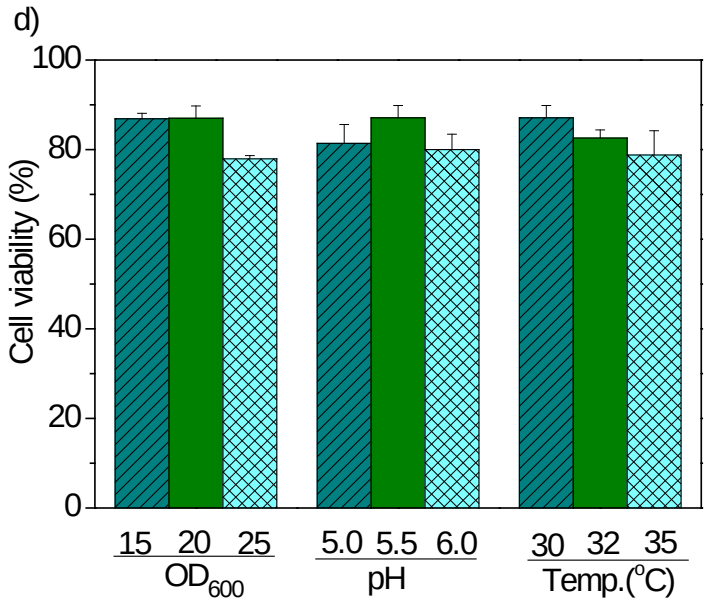



Figure 45 Effect of initial OD (a), pH (b) and temperature (c) on xylose consumption, ethanol production and cell viability (d) during the first cycle 24 h SSCF at 7% (w/w) glucan loading. The default conditions were OD=20, pH 5.5 and temperature 30 $^{o}$C. The figure is in color.

Figure 45 (cont'd)



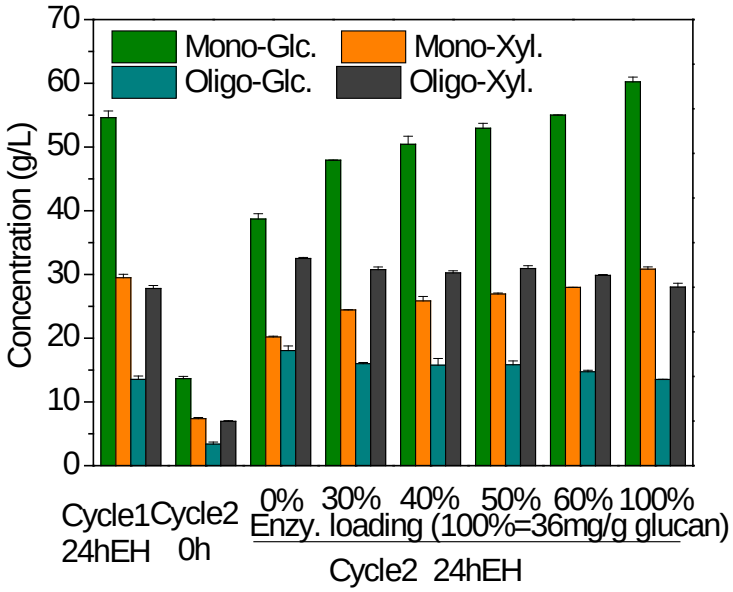




Figure 46 Optimization of enzyme loading for cycle 2 during the BCRL SHF process.
The enzyme loading for cycle 1 is 36 mg/g glucan (100%). Enzymes ratio is fixed at Accellerase 1500 (cellulases): Accellerase XY (xylanase): Mutifect pectinase=10:1:1. The figure is in color.

### 7.3.2 Optimization of fermentation conditions

The initial OD (yeast cell density), pH and temperature were optimized for the process of 24 h enzymatic hydrolysis followed by 24 h SSCF at 7% glucan loading. From the figure (Fig. 45a) it is clear that by increasing the OD more xylose was consumed and more ethanol was produced. However, the highest OD tested (OD=25) showed lower cell viability (Fig. 45d). The fact that this yeast strain cannot grow well on xylose anaerobically (probably due to redox imbalance (Kuyper et al., 2004)) and that the glucose release rate during SSCF might not support a high enough cell growth rate to balance the cell death rate could explain this result. pH 6.0 was the best for xylose fermentation (Fig. 45b). Nevertheless, the cell viability was not as good at this pH as it was at pH 5.5 (Fig. 45d) and it was also far removed from the optimum enzymatic hydrolysis pH 4.8, which would also affect the overall sugar conversion. A temperature of 32 $^{o}$C produced more than 40 g/L ethanol with reasonable cell viability. The higher ethanol yield might be due to higher sugar conversions at higher temperature. However, the ethanol metabolic yield is lower at 35 $^{o}$C as is the cell viability (Fig. 45d), which might explain the lower ethanol yield at 35 $^{o}$C (Fig. 45c). Therefore, an initial OD of 20, pH 5.5 and a temperature of 32 $^{o}$C were chosen as the optimum conditions for BCRL SSCF. The same conditions were used for the BCRL SHF process.

### 7.3.3 Optimization of enzyme loading for cycle 2 of BCRL SHF process

To make sure 40 g/L ethanol was produced, we applied a 7.5% glucan loading for the BCRL SHF process. Here, unhydrolyzed solids along with adsorbed enzymes were recycled for subsequent cycles of hydrolysis. By this approach we reduced the enzyme loadings for cycles 2~5. Experiments were carried out to optimize the enzyme loading for cycle 2 of BCRL SHF

process to obtain similar sugar concentrations as cycle 1 (Fig. 46). Without adding additional enzymes, 38.7 g/L glucose and 20.2 g/L xylose were obtained after 24 h enzymatic hydrolysis during cycle 2, which means the recycled enzymes from cycle 1 were functioning well. About 60% of the cycle 1's enzyme loading applied in cycle 2 yielded similar sugar concentrations as were obtained in cycle 1. Therefore, this enzyme loading (21.6 mg enzyme protein per glucan) was used for cycle 2, resulting in a 40% enzyme saving. Since the enzymes were being recycled and were likely accumulating, we used 50% of the cycle 1 enzyme loading (18 mg enzyme protein per glucan) for cycles 3 to 5.

### 7.3.4 BCRL SHF process results

By applying the enzyme loadings discussed above and a glucan loading of 7.5%, consistently high sugar concentrations were produced for cycles 1 to 5 (Fig. 47a). The last step produced lower sugar concentrations compared to other cycles due to no addition of fresh enzymes or fresh biomass. It is likely that 24 h was too short a time for the hydrolysis of the final residual solids, and that these solids were much more difficult to hydrolyze (Zhang et al., 1999). However, the sugar concentrations obtained were still reasonable. The removal of degradation products with liquid in previous cycles and lower sugar concentrations present at the beginning of the last step helped achieve reasonable sugar conversions from the most recalcitrant part of biomass. Ethanol produced in each cycle was always approximately 40 g/L and the OD was increasing from cycle to cycle (Fig. 47b), which guaranteed fast fermentation of xylose (Fig. 47c).The ethanol metabolic yield was maintained at around 90% (Fig. 47c).Though this is an SHF process, the enzymes (mostly $\beta$–glucosidase and $\beta$–xylosidase) present in the hydrolysate continued to function. Hence part of the oligmeric sugars was converted to monomeric sugars during the fermentation process and then fermented to ethanol (Fig. 47c).The overall glucan

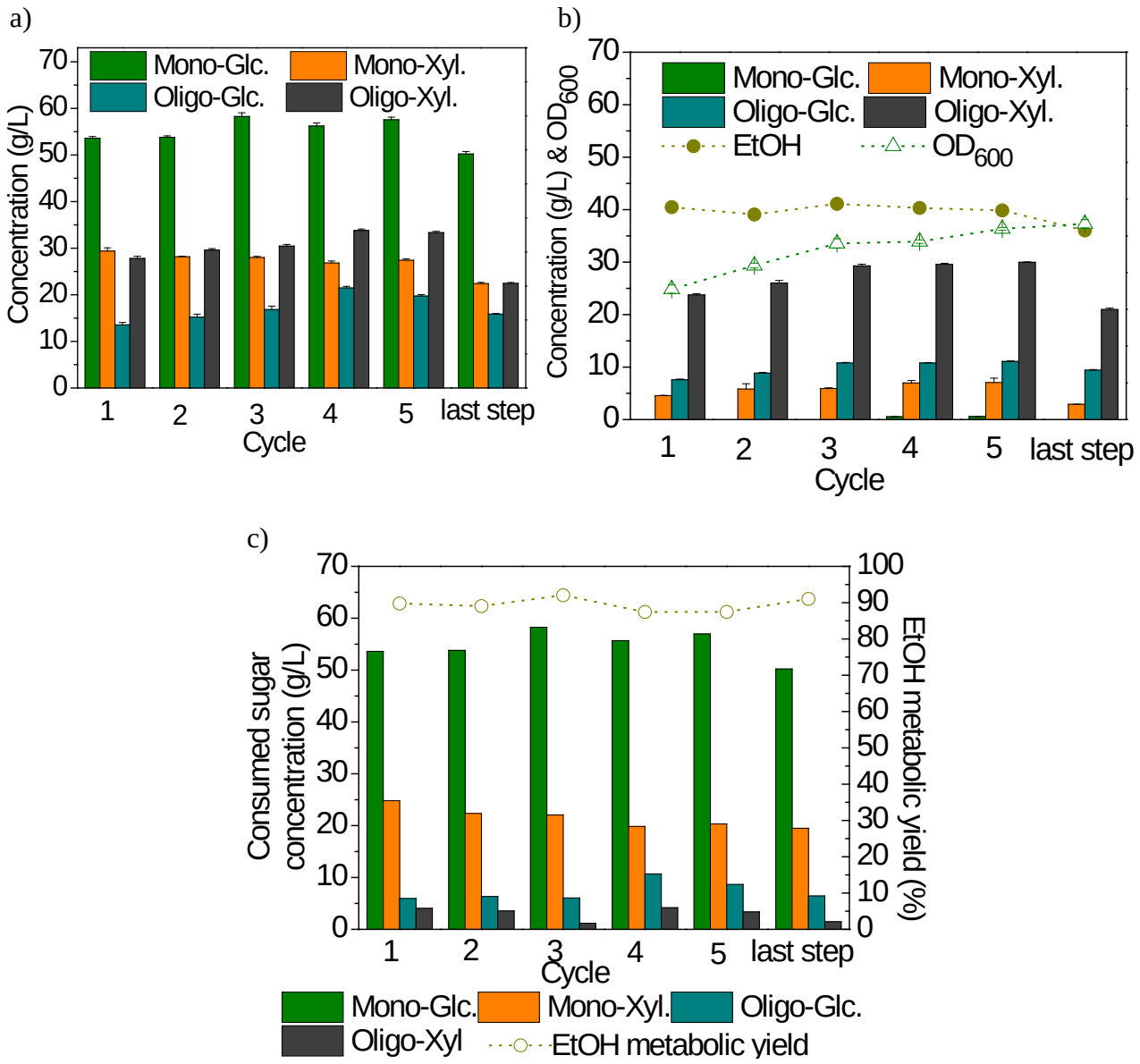
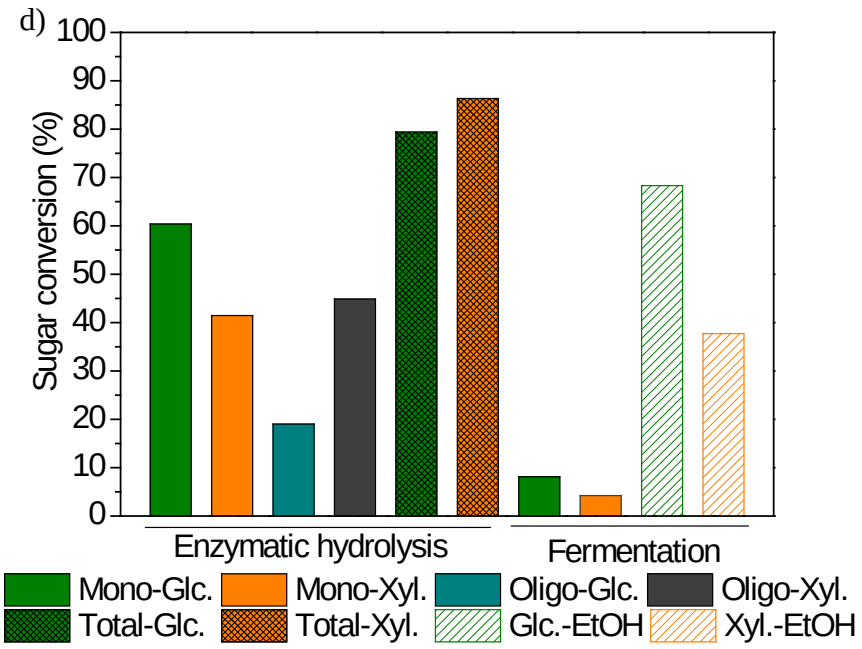



Figure 47 BCRL SHF process results.
a: Sugar concentrations after 24 h enzymatic hydrolysis; b: fermentation performance; c: sugar consumption and ethanol metabolic yield during fermentations; d: sugar conversions during enzymatic hydrolysis, oligomeric to monomeric sugar conversions during fermentation and overall sugar to ethanol conversions. The enzyme loading for cycle 1 is 36 mg/g glucan (100%). Enzyme loadings used for cycle 2, 3, 4, 5 were 60%, 50%, 50%, and 50% of the cycle 1 respectively. The average enzyme loading was 22.3 mg/ g glucan. All the conversions were calculated based on total input glucan and xylan as glucose or xylose equivalents. During fermentation, some oligomeric glucose/xylose was converted to monomeric glucose/xylose (Mono-Glc./Mono-Xyl. conversion). The Glc.-EtOH/Xyl.-EtOH conversion was calculated based on total fermented glucose/xylose. The figure is in color.

Figure 47 (cont'd)




conversion and xylan conversion were 79.4% and 86.3%, respectively for this process (Fig. 47d). However, the glucose to ethanol and xylose to ethanol conversions were only 68.3% and 37.7%, respectively. The major sugar loss was due to the oligomeric sugars (Fig. 49a), especially xylose oligomers (Fig. 47b), which cannot be consumed by yeast strains. Improved enzymes or engineered yeasts able to consume oligomers would help resolve this problem.

The enzymes used in this study were commercial enzyme cocktails rather than the optimum defined enzyme mixtures used for AFEX$^{TM}$-CS (Gao et al., 2010) to generate highest concentrations of monomeric sugars, especially xylose. (Our laboratory does not have the capacity to produce enough purified enzymes for work such as that reported here.) Large amounts of xylose oligmers always exist in AFEX$^{TM}$ hydrolysates at high solid loadings. In addition, we kept the enzyme loading ratio (cellulases: xylanases: pectinases) the same for each

cycle of the process. However, by analyzing the enzymes in the liquid hydrolysate for each cycle, we found that more $\beta$-glucosidase and xylanase were removed or denatured compared to cellulases (Fig. 50). This means that more xylanase and $\beta$-glucosidase should have been supplemented during late cycles and therefore the enzyme loading can probably be further reduced. Optimization of the enzyme cocktail for each cycle of this process needs to be performed in order to determine the overall potential for enzyme saving using the BCRL process.

### 7.3.5 BCRL SSCF process results

Since the BCRL SSCF process recycles solids and enzymes to the next cycle of the SSCF tank, the enzyme loading can be reduced during later cycles. However, as the strain cannot tolerate high temperatures and the cells were recycled along with the enzymes, the recycled enzymes must work at the fermentation temperature (32 $^{o}$C). Hence, the enzyme reduction achieved in the BCRL SSCF process is less than it is for the BCRL SHF process. We tested three enzyme loading profiles (Fig 6): *Enzyme loading a*: 36 mg/g glucan for each cycle; *Enzyme loading b*: 36.0 (100%), 32.4 (90%), 30.6 (85%), 28.8 (80%) and 27.0 (75%) mg/g glucan for cycles 1~5, respectively, with an average enzyme loading of 30.9 mg/g glucan; *Enzyme loading c*: 30.0 (100%), 27.0 (90%), 25.5 (85%), 24.0 (80%) and 22.5 (70%) mg/g glucan for cycles 1~5, respectively, with an average enzyme loading of 25.8 mg/g glucan. The ratio of enzymes cocktails applied (cellulases:xylanase:pectinase) was fixed at 10:1:1. The ethanol concentration for each cycle largely met the criterion of 40 g/L for all three cases (Fig. 48a). Glucose to ethanol conversions were around 75% to 80% (Fig. 48b). However, the xylose to ethanol conversions were quite low, around 40% to 50%. Most of the xylose was in oligomeric form and was not available for fermentation (Fig. 49).

One challenge in this process system is the viable cell density. Decreasing viable cell densities were observed from cycle to cycle (Fig. 48c), which caused a buildup of xylose (xylose loss as monomers) during later cycles (Fig. 48a & Fig. 49).The solid residue concentrations in cycles 1 to 6 of the BCRL SSCF process were around 88, 125, 153, 170, 185, and 185 g/L, respectively. One might suspect that high solids concentration could lead to loss of viable cells. However, the solids had no effect on hydrolysate fermentation (Fig. 51).

### 7.3.6 Comparisons of process ethanol productivity and ethanol yield of different process

The BCRL SHF and SSCF processes showed much higher process ethanol productivities compared to conventional SHF or SSCF processes (Fig. 52a) probably due to the time saved (Fig. 52c) by fast hydrolysis and fast fermentation. For the ethanol yield, 7% glucan loading was less effective than a 6% glucan loading using conventional processes (Fig. 52b). Decreased ethanol yield with increasing solids loading has been shown and discussed in Chapter V. The BCRL SHF process at 7.5% glucan loading showed similar ethanol yields compared to conventional processes at similar glucan loadings but with much lower enzyme loading (22.3 vs. 36 mg protein/ g glucan). The BCRL SSCF process showed higher or similar ethanol yields (depending on enzyme loading) at 7% glucan loading compared to conventional fermentation processes at 7% or 6% glucan loading.

Regarding enzyme reduction, it appears that the BCRL SHF process has greater potential than the BCRL SSCF process since the recycled enzymes can perform at optimal conditions, while the recycled enzymes for the BCRL SSCF process cannot perform to their potential due to the temperature limitations of the strain. If a thermo-tolerant strain is used, the full advantage of the BCRL SSCF process can perhaps be realized. The enzymes recycling depended on the enzyme adsorption to the residual solids. But high sugar concentrations in the fast SHF process

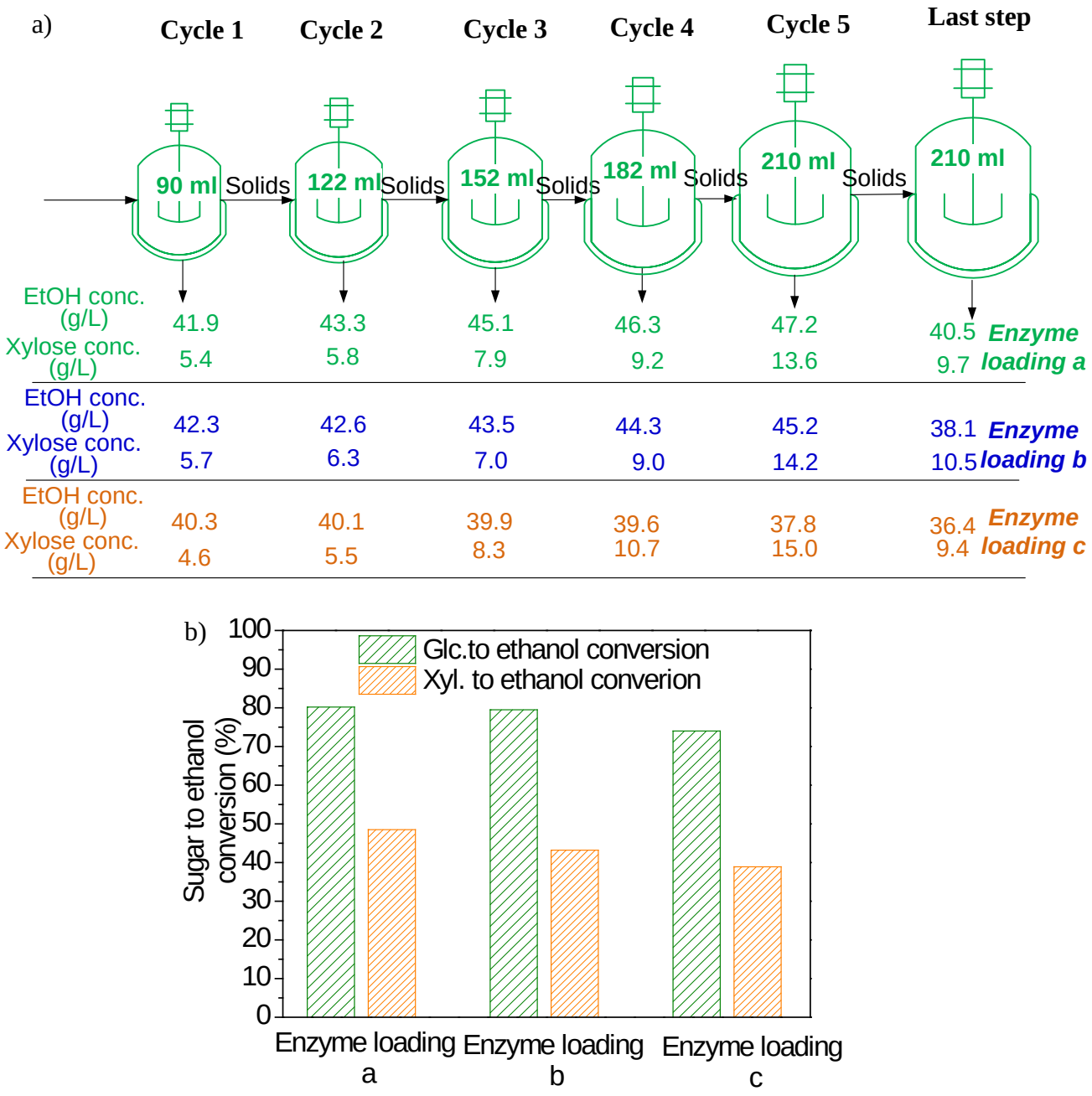
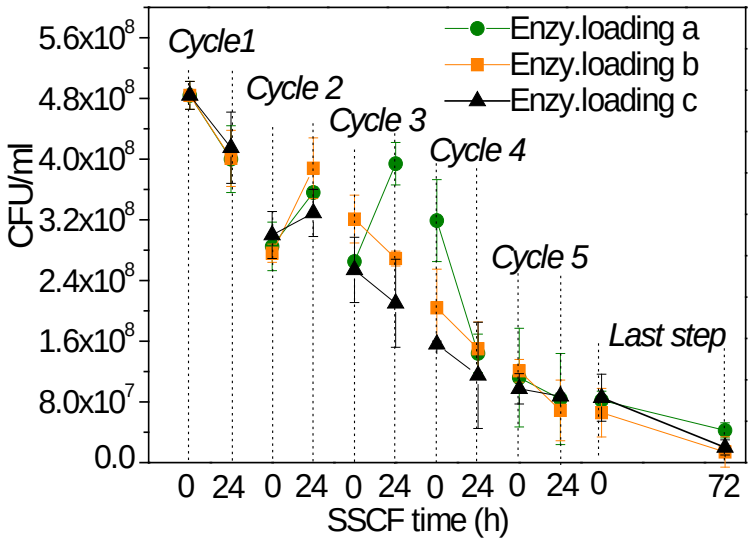



Figure 48 Effect of enzyme loading profile on ethanol production (a), sugar to ethanol conversions (b), and viable cell density (c) during BCRL SSCF process.
Enzyme loading a: 36 mg/g glucan for each cycle; Enzyme loading b: 36.0 (100%), 32.4 (90%), 30.6 (85%), 28.8 (80%) and 27.0 (75%) mg/g glucan for cycle 1~5, respectively with average enzyme loading 30.9 mg/g glucan; Enzyme loading c: 30.0 (100%), 27.0 (90%), 25.5 (85%), 24.0 (80%) and 22.5 (70%) mg/g glucan for cycle 1 to 5, respectively with average enzyme loading 25.8 mg/g glucan. The ratio for enzymes (cellulases:xylanase:pectinase) was fixed at 10:1:1. The figure is in color.

Figure 48 (cont'd)

c)




probably inhibited such adsorption (Kristensen et al., 2009) and hence affected enzyme recycling. The fast SSCF process removed most of the sugars by fermentation but produced ethanol whose effect on enzyme adsorption is unclear. Therefore, more study is needed in order to effectively recycle enzymes and specific enzyme activities using this approach. The BCRL SSCF process achieved higher ethanol yield compared to the BCRL SHF process probably because of the additional 24 h enzymatic hydrolysis during fermentation even though the hydrolysis was not performed at its optimal conditions.

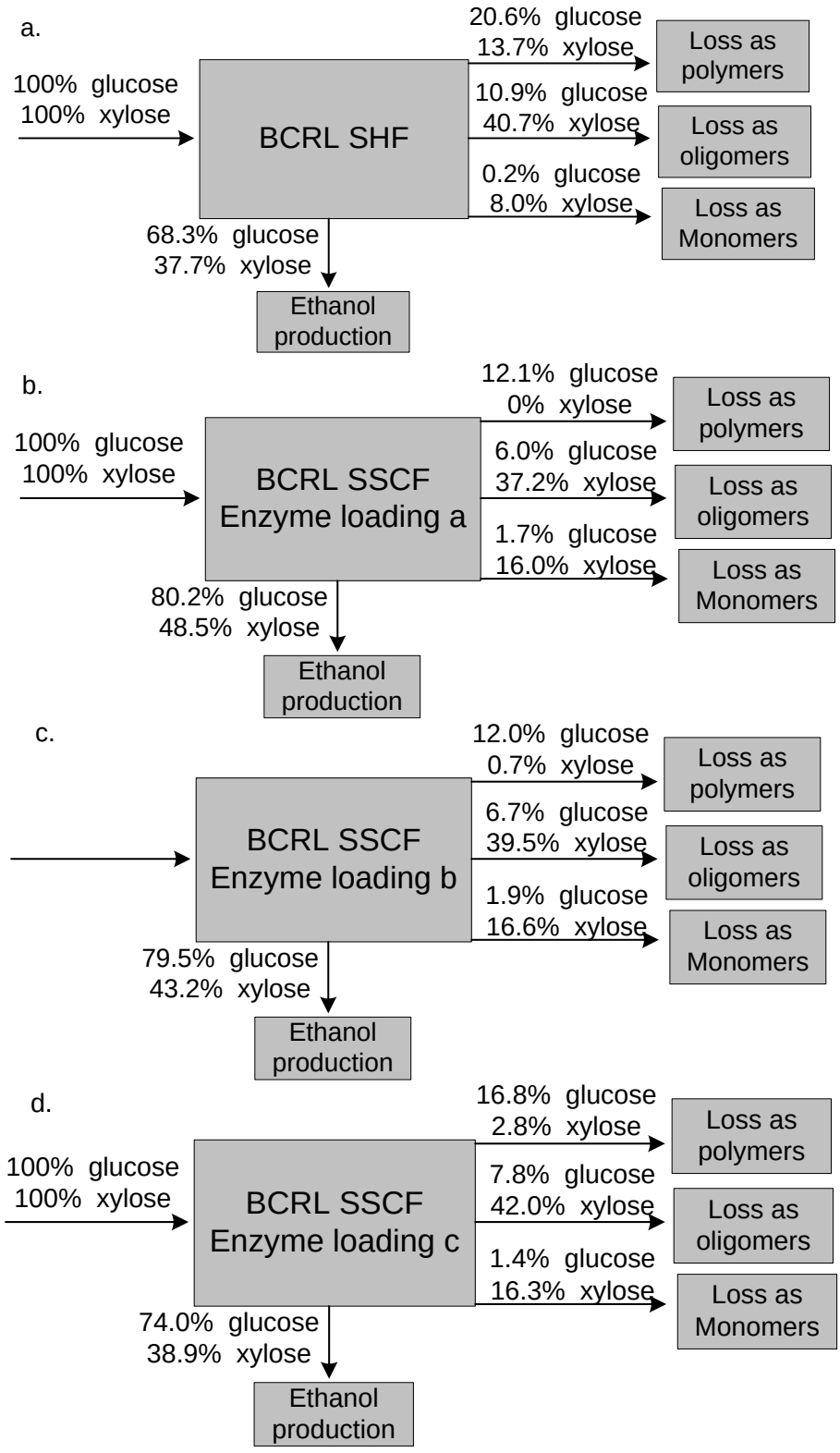
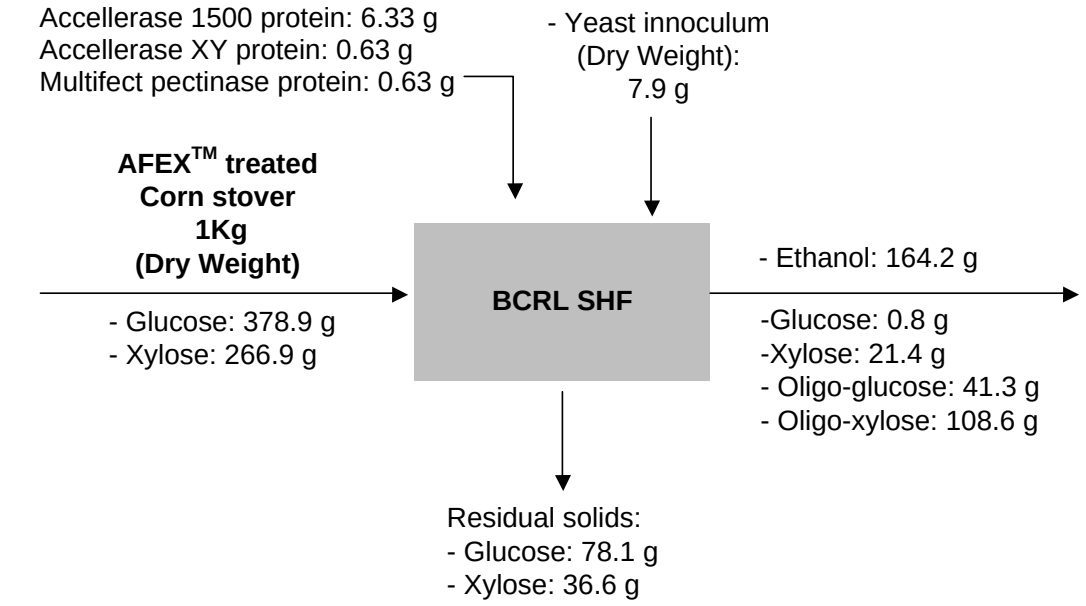


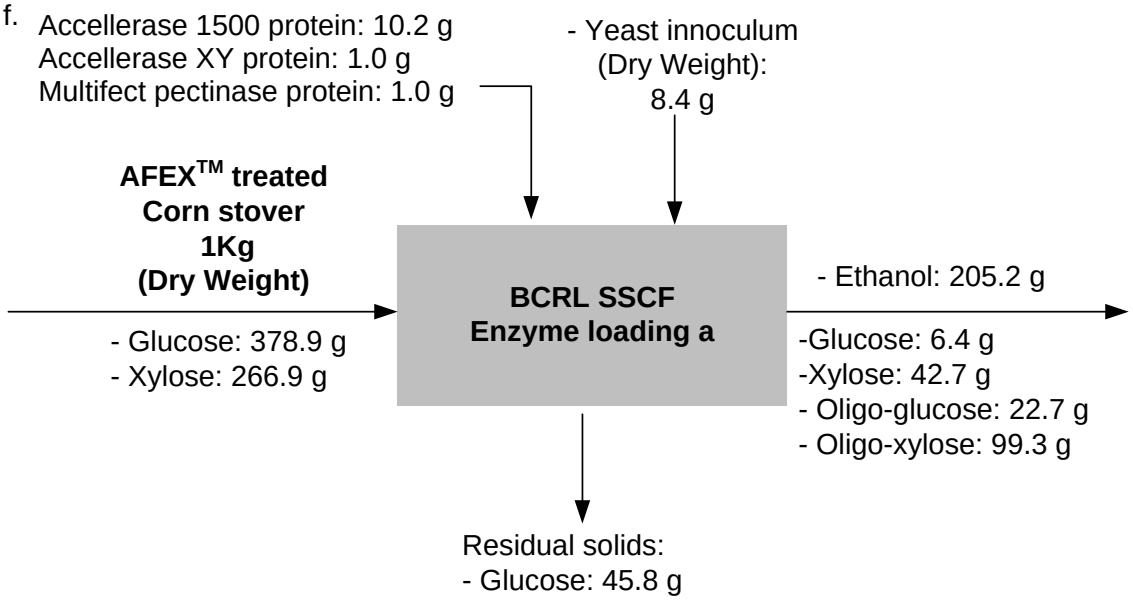

Figure 49 Mass balance of BCRL SHF and SSCF processes

Figure 49 (cont'd)

e.




f.



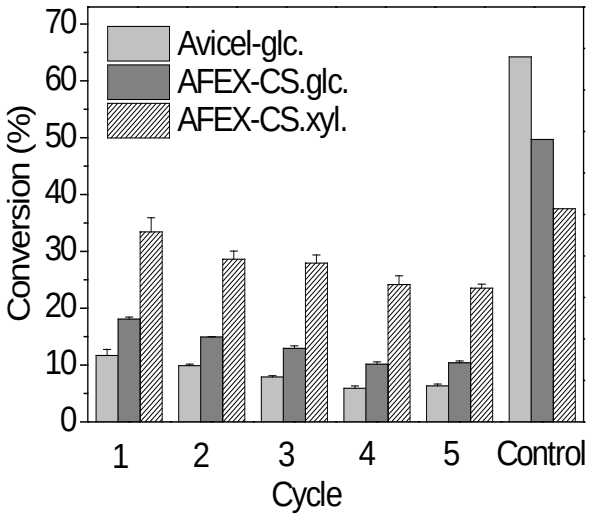



Figure 50 Enzyme activities of proteins in the removed hydrolysate after enzymatic hydrolysis during fast SHF process.

The assay was performed on Avicel and AFEX$^{TM}$-CS. Monomeric glucose/xylose conversion is shown in the figure. The protein concentrations in each cycle hydrolysate were 3.2, 2.7, 2.6, 3.2 and 2.8 mg/ml. The control assumed all the enzymes were removed in the hydrolysate after cycle 1 (i.e., no enzyme recycle with unhydrolyzed solids stream). The protein concentration for the control was 5.2 mg/ml. The enzyme activity assay was conducted in micro-plates with working volume 1.5ml/well, substrate loading 1 mg/well, protein loading 30 $\mu$g/well, reaction time 12 h.

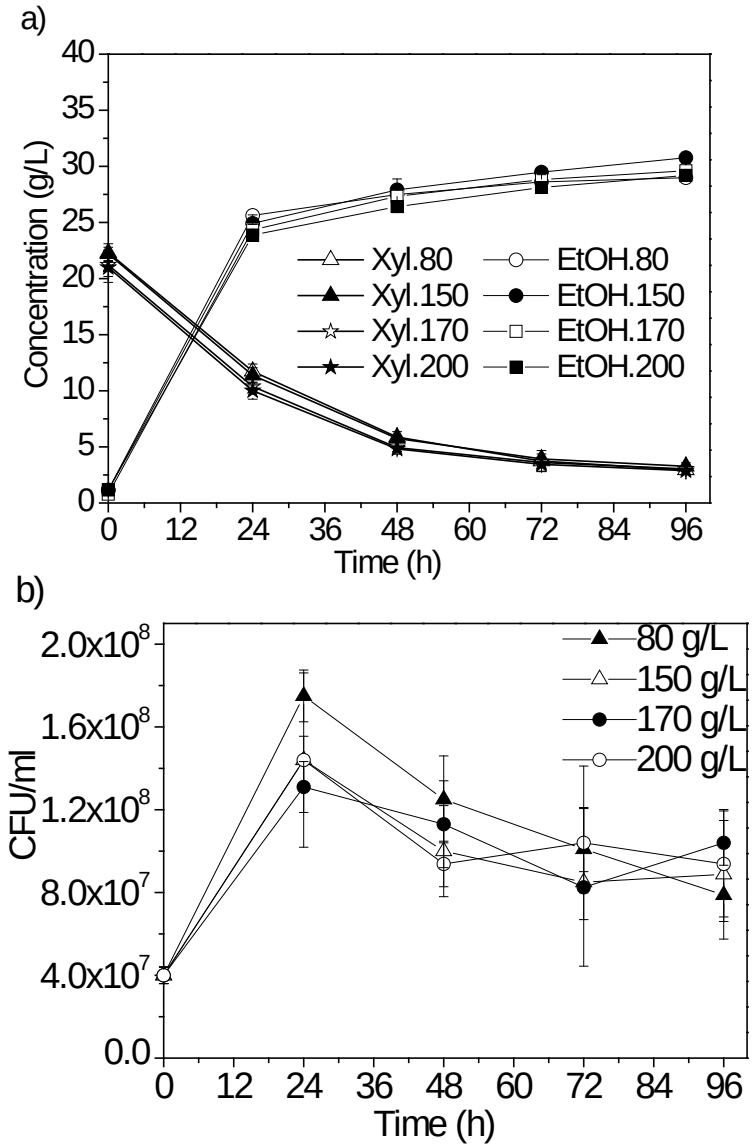



Figure 51 Effect of enzymatic hydrolysis residual solids concentration on fermentation in hydrolysate.

Solids concentrations investigated included 80, 150, 170, and 200 g/L. Fermentations were performed in 250 ml shake flasks with 100 ml working volume at 180 rpm, 32oC, pH 5.5 and initial OD 2.0. The initial glucose concentration was 43.8±1.4 g/L and was consumed completely in 24 h for all of the cases. The solids concentrations in cycle 1-6 of the BCRL SSCF process were around 88, 125, 153, 170, 185, and 185 g/L, respectively.

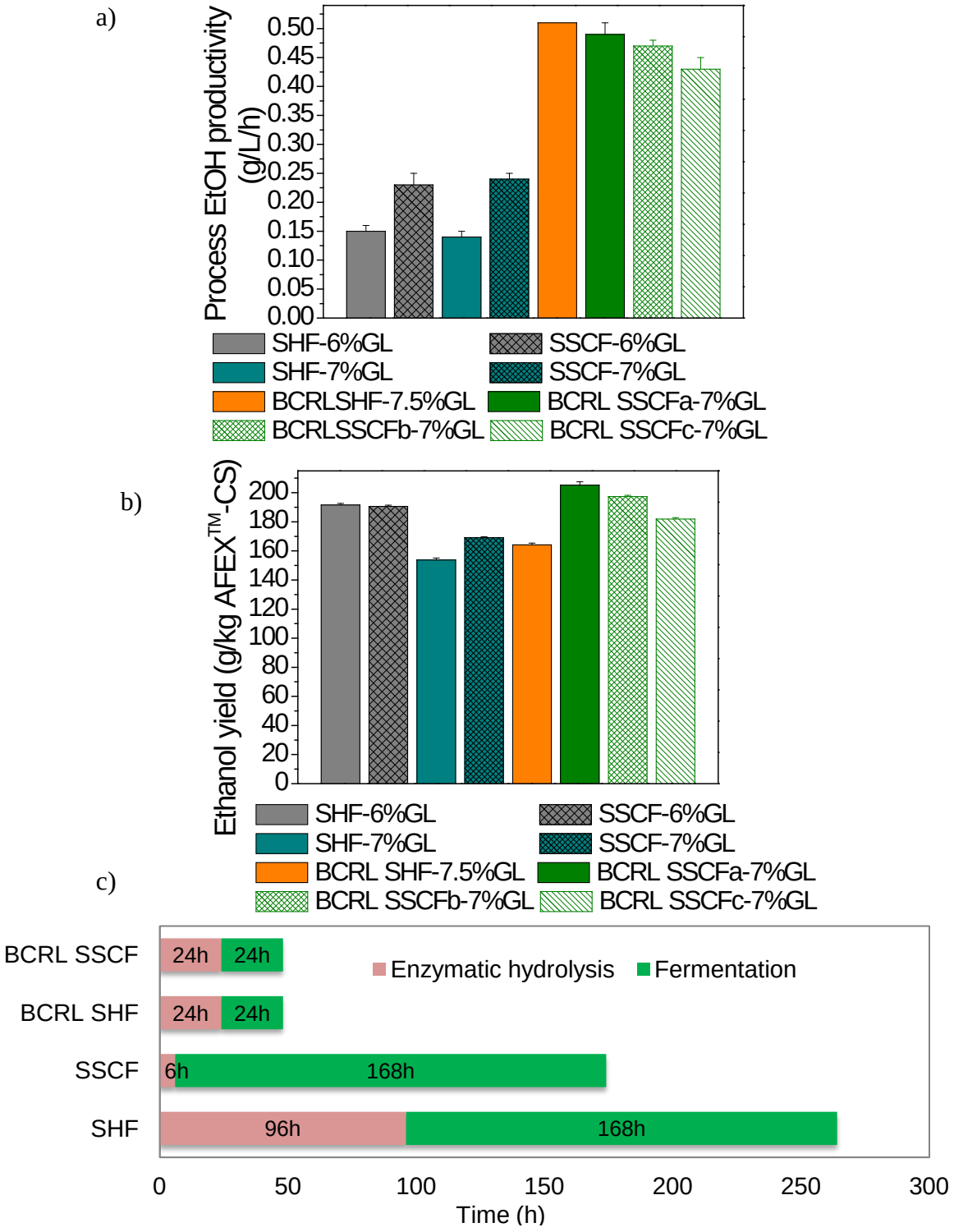



Figure 52 Comparisons of process ethanol productivity (a) and ethanol yield (b) of different processes.

SHF results were based on 96 h enzymatic hydrolysis and 168 h fermentation (c). SSCF results were based on 6 h pre-hydrolysis and 168 h fermentation. Enzyme loadings for these processes were 36, 36, 36, 36, 22.3, 36, 30.9, and 25.8 mg/g glucan, respectively. GL: glucan loading. The figure is in color.

High productivity achieved by BCRL processes could potentially reduce the reactor sizes and hence reduce both the capital costs and operating costs (Humbird et al., 2011). Based on a rough calculation using the NREL 2011 model (Humbird et al., 2011) and the conventional SHF process performance on AFEX$^{TM}$-CS, 12 saccharification tanks (250,000 gallon each) and 36 fermentors (1,000,000 gallon each) are required to process corn stover at a scale of 2,205 dry U.S. ton /day (2,000 metric tonne/day). However, when the BCRL SHF process is applied, the number of fermentors required is reduced to 12 with the number of saccharification tanks remaining the same, which means the total reaction volume is reduced by around 62%. The cost of centrifugation and filtration for the BCRL processes are almost the same as for the conventional process. Therefore, around 62% of the capital cost in saccharification tanks, fermentors and their accessories can be saved. Enzymes cost accounts for around 16% of the total ethanol production cost according to the NREL 2011 model, which is one of the major costs. The BCRL SHF process saves 38% of the enzymes compared to conventional process and hence reduces the cost of enzymes by around 38%.

### 7.4 Conclusions

This novel integrated biological process, the “BCRL Process” saves time by quickly fermenting xylose, processing the easily digestible biomass first to fully utilize the high enzymatic hydrolysis rate period, and hydrolyzing the more recalcitrant part of biomass with less inhibition by degradation products and sugars, and thereby enhances the process ethanol productivity. Biocatalyst resources were better utilized by recycling yeast cells and enzymes and thereby reduced the overall processing cost.

# CHAPTER VIII CBP OF AFEX™ PRETREATED CORN STOVER AT LOW SOLIDS LOADING


## Abstract

Consolidated bioprocessing (CBP) is believed to be a potentially cost-efficient and commercially-viable way to produce cellulosic biofuels. In this study, the performance of the CBP organism *Clostridium phytofermentans* (ATCC 700394) on AFEX™ treated corn stover (AFEX™-CS) was evaluated. Fermentation conditions including temperature, inoculation size, nutrients, and pH were investigated. At optimal conditions with 0.5% (w/w) glucan loading of AFEX™-CS, *C. phytofermentans* hydrolyzed 76% of glucan and 88.6% of xylan in 10 days. These values reached 87% and 102% of those obtained by simultaneous saccharification and co-fermentation (SSCF) using commercial enzymes and *S. cerevisiae* 424A. Ethanol titer for CBP was found to be 2.8 g/L which was 71.8% of that yielded by SSCF (3.9 g/L). Decomposition products from AFEX™-CS helped to increase ethanol yield somewhat during CBP. Particle size played a crucial role in the enhancement of sugar conversion by CBP.

## 8.1 Introduction

Both SHF and SSCF processes require expensive commercial enzymes production in a separate bioreactor, a major cost barrier for commercialization of cellulosic ethanol (Banerjee et al., 2010). Consolidated bioprocessing (CBP) (Lynd, 1996) which carries out enzyme production, enzymatic hydrolysis and fermentation in a single bioreactor reduces the enzyme production cost and at the same time possesses all the advantages of SSCF. A strong case has been made that CBP is the ultimate low-cost industrial configuration to produce cellulosic ethanol (Lynd et al., 2005; Sendich et al., 2008).

Currently, there is no perfect CBP microbe which can degrade lignocellulosic biomass efficiently and at the same time utilize all the sugars released from biomass to produce mostly ethanol. Researchers are focusing on either engineering an ethanologen (such as *Saccharomyces cerevisiae*) to be able to produce cellulases/ hemicellulases or engineering a lignocellulose degrader (such as *Clostridium thermocellum*) to be an efficient ethanol producer (Lynd et al., 2005). For *S. cerevisiae*, xylose fermentation will be another challenge and for *C. thermocellum,* which cannot consume xylose, co-culture with other bacteria (such as *C. thermosaccharolyticum*) (Demain et al., 2005) will be another concern. *Clostridium phytofermentans* (ATCC 700394), whose genome encodes the highest number of enzymes for degradation of lignocellulosic material among sequenced clostridial genomes (Weber et al., 2010), is a promising native anaerobic CBP microbe. It secretes individual enzymes (non-complexed) like fungi and can degrade both cellulose and hemicelluloses to fermentable sugars. In addition, *C. phytofermentans* can consume almost all the sugars present in lignocellulosic biomass and produce ethanol and acetate as the major products (Warnick et al., 2002; Weber et al., 2010).

To the author's knowledge, no detailed study has been reported in the literature on the performance of *C. phytofermentans* on pretreated biomass. In the present study, *C. phytofermentans* was used to conduct CBP on AFEX$^{TM}$ treated corn stover (AFEX$^{TM}$-CS) to produce ethanol. Conditions that might affect CBP performance (including temperature, inoculation size, nutrients, and pH) were investigated. Effects of biomass washing and biomass particle size were also studied.

## 8.2 Materials and Methods

### 8.2.1 AFEX$^{TM}$ pretreated corn stover

Please see Chapter II section 2.2.1.

### 8.2.2 Particle size reduction

Particle size reduction of AFEX treated corn stover (4 mm) was achieved by using a centrifugal mill (Model ZM 200, Retsch, Newtown, PA) fitted with various ring sieve attachments.

### 8.2.3 CBP microbe and seed culture preparation

*C. phytofermentans* ATCC 700394 was used for this CBP study. Seed culture was prepared in a 125 ml serum vial with $N_2$ atmosphere and 50 ml GS-2 cellobiose (5 g/L) medium (Cavedon et al., 1990) containing (g/L): $KH_2PO_4$ 1.5, $K_2HPO_4$ 2.9, urea 2.1, Cysteine-HCl 2.0, MOPS 1.0, NaCitrate$\cdot 2H_2O$ 3, yeast extract (YE) 6, $MgCl_2 \cdot 6H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 0.15, $FeSO_4 \cdot 7H_2O$ 0.00125, pH 7.0. The culture was incubated at 35 $^{o}$C and 200 rpm in a shaking incubator (Innova, New Brunswick, NJ).

### 8.2.4 CBP fermentation

CBP experiments were conducted in 125 ml serum vials (except for the experiments at varying pH) with $N_2$ atmosphere, 50 ml GS-2 medium (without cellobiose) and 0.5% (w/w) glucan loading AFEX$^{TM}$-CS. Experiments were conducted at 200 rpm. Fermentation experiments with varying pH was carried out in 500 ml fermentors (Biostat, Sartorius) with working volume 300 ml and agitation speed of 250 rpm. The fermentation medium was purged with $N_2$ before inoculation. CBP conditions including temperature, inoculation size, nutrients, and pH were studied according to Table 10. For nutrient investigation, 6 g/L YE in GS-2 medium were substituted by the nutrients indicated in Table 10. Particle size of AFEX$^{TM}$-CS used for these studies was 0.5 mm.

Glucose, cellobiose, xylose, ethanol, acetate, lactate and formate concentrations were analyzed using HPLC with a Biorad Aminex HPX-87H column described previously in Chapter II section 2.2.7. In all of the CBP fermentation samples tested, no glucose, cellobiose and xylose were detected.

### 8.2.5 Simultaneous Saccharification and Co-fermentation

SSCF was used as a control for this study and was conducted by using commercial enzymes and genetically modified xylose-fermenting yeast *S. cerevisiae* 424A(LNH-ST) (Ho et al., 1999). The commercial enzymes mixture was composed of Spezyme CP (Genencor Inc, USA) 22.4 mg protein/g glucan (15 FPU/g glucan), Novozyme 188 (Sigma-Aldrich, USA) 38.4 mg protein/g glucan (64 *p*NPGU/g glucan), Multifect xylanase 2.6 mg protein/ g glucan and Multifect pectinase (Genencor Inc, USA) 4.7 mg protein/ g glucan.

SSCF was conducted in the same 125 ml serum vials similar to CBP experiments at 30 $^{o}$C, 200 rpm with 50 ml medium containing 5 g/L yeast extract and 10 g/L tryptone. AFEX-CS glucan loading was 0.5% (w/w). The fermentation medium pH was controlled at 5.2 with 0.05 M phosphate buffer. The initial $OD_{600}$ for *S. cerevisiae* 424A(LNH-ST) was 0.5. SSCF experiments proceeded for 10 days.

### 8.2.6 Measurement of glucan and xylan conversions

Unutilized residual biomass samples resulting from CBP or SSCF were obtained by the following procedures: 1) centrifuge samples at 5300 rpm for 15 min in Falcon centrifuge tubes; 2) discard supernatant; 3) add water (same amount as supernatant) to wash the pellets; 4) centrifuge again; 5) discard supernatant; 6) dry the solid residue in vacuum oven at 90 $^{o}$C. The glucose and xylose contents in solid residual biomass were determined by quantitative saccharification (Lu et al., 2006; Zhang & Lynd, 2003). The glucan/xylan conversions were calculated based on the initial total glucose/xylose and residual total glucose/xylose.

### 8.2.7 Preparation of washed AFEX$^{TM}$-CS

Please see Chapter III section 3.2.7.

## 8.3 Results and discussion

### 8.3.1 Effect of temperature on CBP performance

Table 10 Summary of experimental parameters for the effects of temperature, inoculation size, nutrients, and pH on CBP of AFEX™-CS by *C.phytofermentans*.[a]

| Parameter | Effect of temperature | Effect of inoculation size | Effect of nutrients | Effect of pH |
|---|---|---|---|---|
| Temperature (°C) | 25, 30, 35, 40 | 35 | 35 | 35 |
| Inoculation size (v/v) | 10% | 5%,10%,20%,40% | 10% | 10% |
| Nutrients[b] | 6 g/L YE | 6 g/L YE | 6 g/L YE, 12 g/L YE, 0.3YP,0.4YP,0.5YP | 6 g/L YE |
| pH[c] | 7.0 | 7.0 | 7.0 | 6.5,7.0,7.5 |
| Relevant data | Fig. 53a,Table 11 | Fig. 53b,Table 11 | Fig. 53c,Table 11 | Fig. 53d, Table 12 |

[a] The experiments were conducted in 125 ml serum vials with working volume 50 ml and agitation at 200 rpm, except for the experiments investigating the effects of pH, which were carried out in 500 ml fermentors with working volume of 300 ml and agitation at 250 rpm.
[b] YE: yeast extract. YP medium: yeast extract 10 g/L and tryptone 20 g/L. The number beside the YP represents the fraction of YP concentration.
[c] Initial pH for serum vial experiments and controlled pH for fermentor experiments.

CBP experiments using AFEX™-CS at different temperatures including 25, 30, 35, 40 $^{o}$C were performed with initial pH 7.0 and inoculation size of 10% (v/v) (Table 10). Glucan and xylan conversion results after 7 and 10 days are shown in Fig. 53a. At higher temperatures (35 and 40 $^{o}$C), the two day fermentation results were very close with the 7$^{th}$ day's glucan conversions of 51% and 20%, xylan conversions of 82% and 77% and 10$^{th}$ day's glucan conversions of 52.5% and 21%, xylan conversions of 82.5% and 77.7%, respectively. However,

at lower temperatures (25 and 30 $^{o}$C), we observed a significant increase in glucan conversions from 7$^{th}$ day's 43% and 60% respectively to the 10$^{th}$ day's 62.4% and 71.6%, respectively.

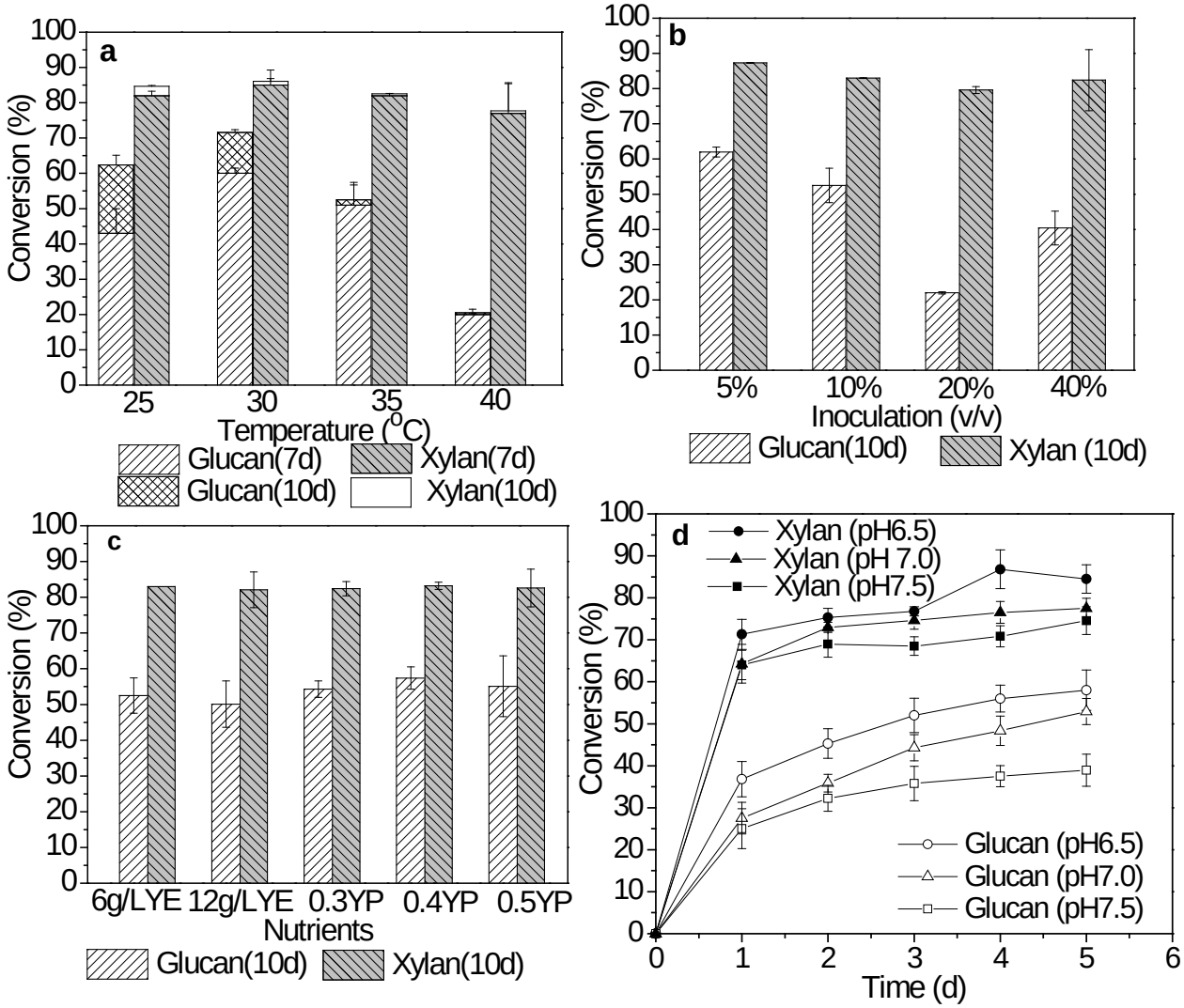



Figure 53 Effects of temperature (a), inoculation size (b), nutrient addition (c) and pH (d) on glucan and xylan conversion during CBP of AFEX$^{TM}$-CS by *C.phytofermentans*.

By microscopy we observed few viable cells in fermentation broth after 7 days at higher temperatures (35 or 40 $^{o}$C), while there were still many viable cells after 7 days at lower temperatures (25 or 30 $^{o}$C), especially 25 $^{o}$C. It seems the microbe can survive and function

longer at lower temperatures, which probably caused the 7 day and 10 day sugar conversion differences at 25 and 30 $^{o}$C. It is worthwhile to mention that the density of viable cells after 10 days fermentation at 25 $^{o}$C was similarly high when compared to the 7$^{th}$ days fermentation, which means the sugar conversions might be further enhanced by extending fermentation time at 25 $^{o}$C. At 30 $^{o}$C, however, most of the cells were dead after 10 days.

Table 11 Effects of temperature, inoculation size and nutrients on lactate, acetate, formate, and ethanol production during CBP of AFEX$^{TM}$-CS by *C.phytofermentans*.[a]

| Temperature /Inoculation /nutrients | Lactate (g/L) | Formate (g/L) | Acetate (g/L) | EtOH (g/L) | EtOH /Acetate |
|---|---|---|---|---|---|
| 25 $^{o}$C | 0.00±0.00 | 0.86±0.05 | 2.43±0.19 | 1.17±0.06 | 0.48 |
| 30 $^{o}$C | 0.00±0.00 | 0.89±0.01 | 2.58±0.03 | 1.39±0.11 | 0.54 |
| 35 $^{o}$C | 0.38±0.08 | 0.88±0.03 | 2.38±0.04 | 0.92±0.07 | 0.39 |
| 40 $^{o}$C | 0.16±0.12 | 1.14±0.01 | 2.33±0.60 | 0.51±0.13 | 0.22 |
| 5% | 0.14±0.06 | 0.77±0.17 | 2.23±0.25 | 0.96±0.01 | 0.43 |
| 10% | 0.38±0.08 | 0.88±0.03 | 2.38±0.04 | 0.92±0.07 | 0.39 |
| 20% | 0.63±0.00 | 0.91±0.06 | 2.33±0.07 | 0.48±0.05 | 0.21 |
| 40% | 0.53±0.03 | 0.81±0.13 | 2.39±0.11 | 0.73±0.09 | 0.31 |
| 6 g/LYE | 0.38±0.08 | 0.88±0.03 | 2.38±0.04 | 0.92±0.07 | 0.39 |
| 12 g/LYE | 0.42±0.09 | 0.79±0.05 | 2.31±0.07 | 0.87±0.09 | 0.38 |
| 0.3YP | 0.20±0.08 | 1.00±0.02 | 2.66±0.18 | 1.03±0.01 | 0.39 |
| 0.4YP | 0.22±0.02 | 1.05±0.09 | 2.67±0.06 | 1.30±0.02 | 0.49 |
| 0.5YP | 0.35±0.03 | 1.07±0.08 | 2.57±0.08 | 1.17±0.06 | 0.46 |

[a] The concentrations were 10$^{th}$ day results with inoculum concentrations subtracted.

In general, we found that xylan conversions were always higher than glucan conversions. At both the 7$^{th}$ and 10$^{th}$ day, a 30 $^{o}$C fermentation gave the highest glucan and xylan conversions and hence was chosen as the optimal temperature. Our results contradict reports by Warnick et. al., (2002) that the optimum temperature for *C. phytofermentans* was 35 to 37 $^{o}$C. The discrepancy might be caused by differences in carbon source as well as the criteria used. Warnick et al. used cellobiose as carbon source, which can be consumed directly and quickly by the strain. Moreover, the criterion for optimum temperature was a high growth rate. In the present study, however, AFEX$^{TM}$-CS was used as the carbon source, which must be hydrolyzed before the strain can utilize it. Also, the temperature was selected for better sugar conversion. Higher cell density with slower growth rate at relatively low temperature phenomena were also observed by Warnick et al., consistent with the results in this study.

We also observed that ethanol production was affected by temperature (Table 11). Lower temperatures (25 and 30$^{o}$C) resulted in higher ethanol/acetate ratios. The 10$^{th}$ day's highest ethanol/acetate ratio (0.54) was found at 30$^{o}$C.

Table 12 Effect of pH on lactate, acetate, formate, and ethanol production during CBP of AFEX-CS by *C. phyfermentans.*[a]

| | Lactate (g/L) | Acetate (g/L) | Formate (g/L) | Ethanol (g/L) | Ethanol /Acetate |
|---|---|---|---|---|---|
| pH 6.5 | 0.18±0.07 | 2.71±0.08 | 1.03±0.09 | 1.02±0.11 | 0.38 |
| pH 7.0 | 0.10±0.03 | 2.77±0.10 | 1.05±0.05 | 0.97±0.09 | 0.35 |
| pH 7.5 | 0.16±0.06 | 2.74±0.12 | 1.17±0.10 | 0.71±0.02 | 0.26 |

[a] The concentrations were 5$^{th}$ day results with inoculum concentrations subtracted.

### 8.3.2 Effect of inoculation size on CBP performance

Inoculation introduced not only microbial cells, cellulases and hemicellulases produced during seed cultivation but also other metabolites (such as acetate and lactate) which could inhibit CBP performance. The larger inoculums, the more microbial cells, enzymes as well as inhibitory metabolites are introduced into CBP systems. Microbial cells and enzymes should certainly help initiate the CBP process, but some metabolites do not. The optimal inoculation size is the one best balancing these advantages and disadvantages. It was found that 5% (v/v) inoculation size led to highest glucan and xylan conversions among the tested values followed by 10% (Fig. 53b). 20% (v/v) inoculation size gave the lowest sugar conversions. 40% (v/v) inoculation size yielded higher sugar conversions than 20% probably due to the additional enzymes introduced along with the inoculums from seed culture.

It is well known that CBP microbes tend to produce much less enzymes when grown on non-cellulosic substrates such as cellobiose (Demain et al., 2005; Lynd et al., 2002). Therefore, the relatively poor CBP performances caused by large inoculation sizes might also be due to an inadequate ratio between enzymes and cells in the seed culture (grown on cellobiose) which was not high enough to give good results at large inoculums levels. If so, the enzymes in the seed culture were insufficient to hydrolyze biomass rapidly enough to meet the cells' need for sugars at the beginning of CBP. As a result, more cells might have died during the early stages of CBP fermentation when bigger inoculums were used, thereby releasing inhibitory compounds to affect both enzymatic hydrolysis and cell growth and hence diminish CBP performance.

The ethanol production results (Table 11) showed the same trend as sugar conversions. A 5% (v/v) inoculation size resulted in the highest ethanol production (0.96 g/L) as well as the highest ethanol/acetate ratio (0.43).

### 8.3.3 Effect of nutrients and pH on CBP performance

During CBP, both enzyme production and cell growth require nitrogen sources. Apparently, *C. phytofermentans* prefers amino acids or peptides (Warnick et al., 2002), which are abundant in either yeast extract or tryptone. Increasing the concentration of yeast extract did not further increase either sugar conversion or ethanol production (Fig. 53c and Table 11). When 6 g/L yeast extract in GS-2 medium was replaced by different fractions of YP medium (yeast extract 10 g/L and tryptone 20 g/L), enhancements in both sugar conversions and ethanol production were observed. Addition of 0.4YP resulted in the highest sugar conversions and highest ethanol/acetate ratio, which were 57.4% of glucan conversion, 83.2% of xylan conversion and 0.49 of ethanol/acetate ratio, respectively. Compared to the control (6 g/L YE), glucan conversion was increased by 4.9% while ethanol/acetate ratio enhancement was 25.6% of the control. A higher YP fraction (0.5) resulted in reduced glucan conversion and ethanol/acetate ratio when compared to 0.4YP. This might be due to the higher initial salt concentration caused by the higher YP fraction since there are salts in yeast extract and tryptone. As CBP proceeds, more salts (acetate, lactate and formate) accumulated in the fermentation broth, perhaps inhibiting cell growth (Lynd et al., 2001) and thereby CBP performance.

Studies of pH effects in fermentors were only able to proceed for 5 days without nitrogen purging. After 5 days, the fermentation stopped producing gas (mostly $CO_2$ and $H_2$ (Warnick et al., 2002)) and the pressure of the fermentors became negative, perhaps due to more rapid cell death at 35 $^{o}$C compared to low temperatures (section 8.3.1). Among the tested pH values, the lower pH resulted in higher ethanol/acetate ratios (Table 12). Low pH stimulating ethanol production was also found in a mixed culture fermentation (Zhao et al., 2009). Based on the

sugar conversions (Fig. 53d) and ethanol/acetate ratio, pH 6.5 was found to be the optimum among the tested values.

At optimal conditions (30 $^{o}$C, 5% (v/v) inoculation size, and 0.4YP), 76% glucan conversion and 88.6% xylan conversion with 2.8 g/L ethanol (ethanol/acetate ratio 1.08) were

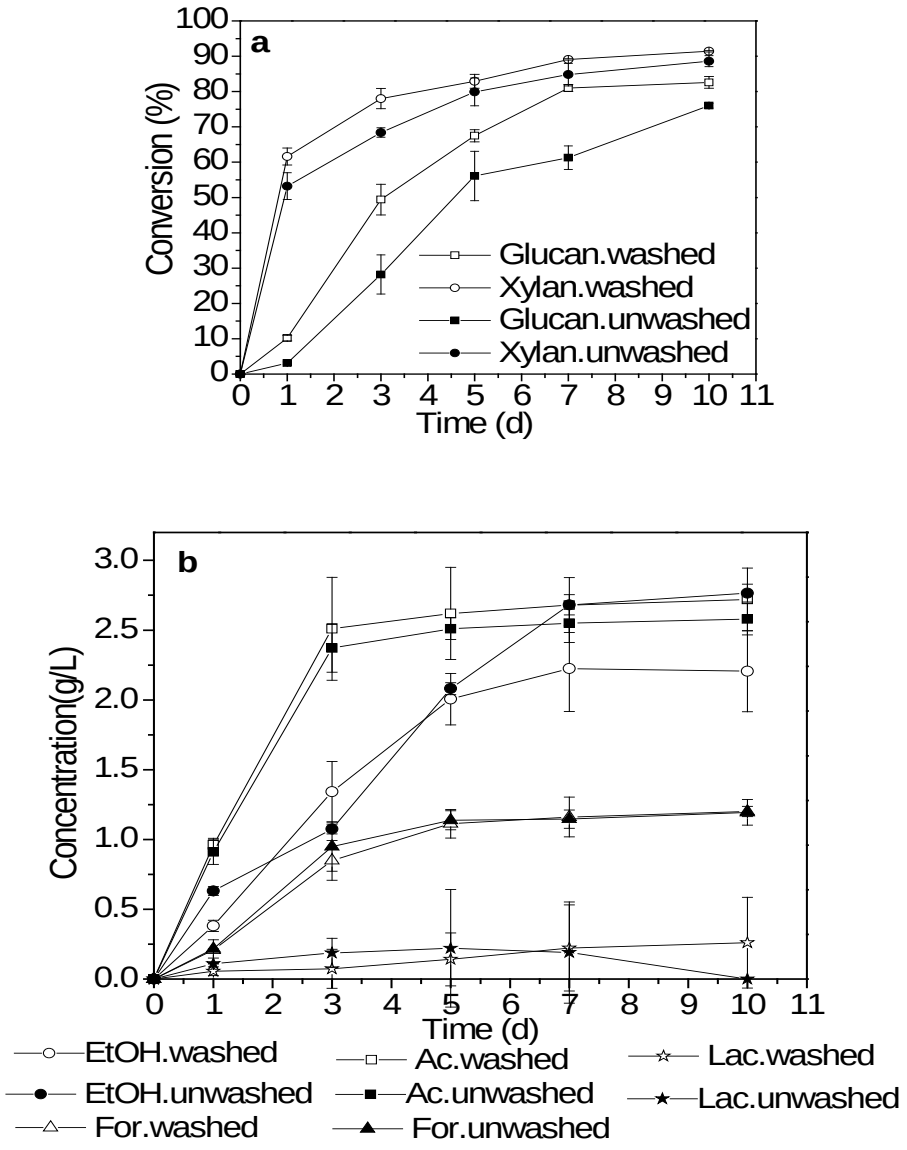



Figure 54 Comparison of sugar conversions (a) and product generation (b) during CBP on washed AFEX$^{TM}$-CS and un-washed AFEX$^{TM}$-CS.
Abbreviations: EtOH: ethanol; Ac: acetate; Lac: lactate; For: formate.

obtained in serum vials (initial pH 7.0, final pH 6.1±0.2) after 10 days' CBP fermentation by *C.phytofermentans* (Fig. 54). During degradation of AFEX$^{TM}$-CS there was around 0.2 g/L acetate released from the biomass itself. If that amount were subtracted from the observed total acetate, the ethanol/acetate ratio produced by fermentation would be increased to 1.17.

### 8.3.4 Comparison of CBP on washed AFEX$^{TM}$-CS and unwashed AFEX$^{TM}$-CS

CBP experiments on washed and unwashed AFEX-CS were performed at optimal conditions in serum vials (initial pH 7.0). The results are compared side by side in Fig. 54. Higher sugar conversions were observed on washed AFEX$^{TM}$-CS with final glucan and xylan conversions of 82.6% and 91.4%, respectively. Apparently, removal of decomposition products present in AFEX$^{TM}$ treated corn stover (Chundawat et al., 2010) by water washing helped improve the CBP performance. Although sugar conversions were improved by washing, the ethanol yield and ethanol/acetate ratio were reduced from 2.8 g/L and 1.08 to 2.2 g/L and 0.81, respectively. Enhancement of ethanol metabolic yield by decomposition products was also observed during yeast fermentation of AFEX$^{TM}$-treated biomass (Lau & Dale, 2009).

### 8.3.5 Effect of particle size on CBP performance

Particle size is one of the factors limiting enzymatic hydrolysis (Lynd et al., 2002). Reduction of particle size increases the surface area accessible to enzymes and thereby increases enzymatic hydrolysis rate (Chundawat et al., 2007; Yeh et al., 2010). Similar results were also observed in this CBP study (Fig. 55). As the particle size was reduced, the glucan and xylan conversion rates increased. Among the tested values (4 mm, 0.5 mm, and 0.08 mm), the smallest particle size (0.08 mm) gave the highest glucan and xylan conversions (83.1% and 89.4%, respectively) after 10 days' CBP fermentation. For a particle size of 4 mm, glucan was

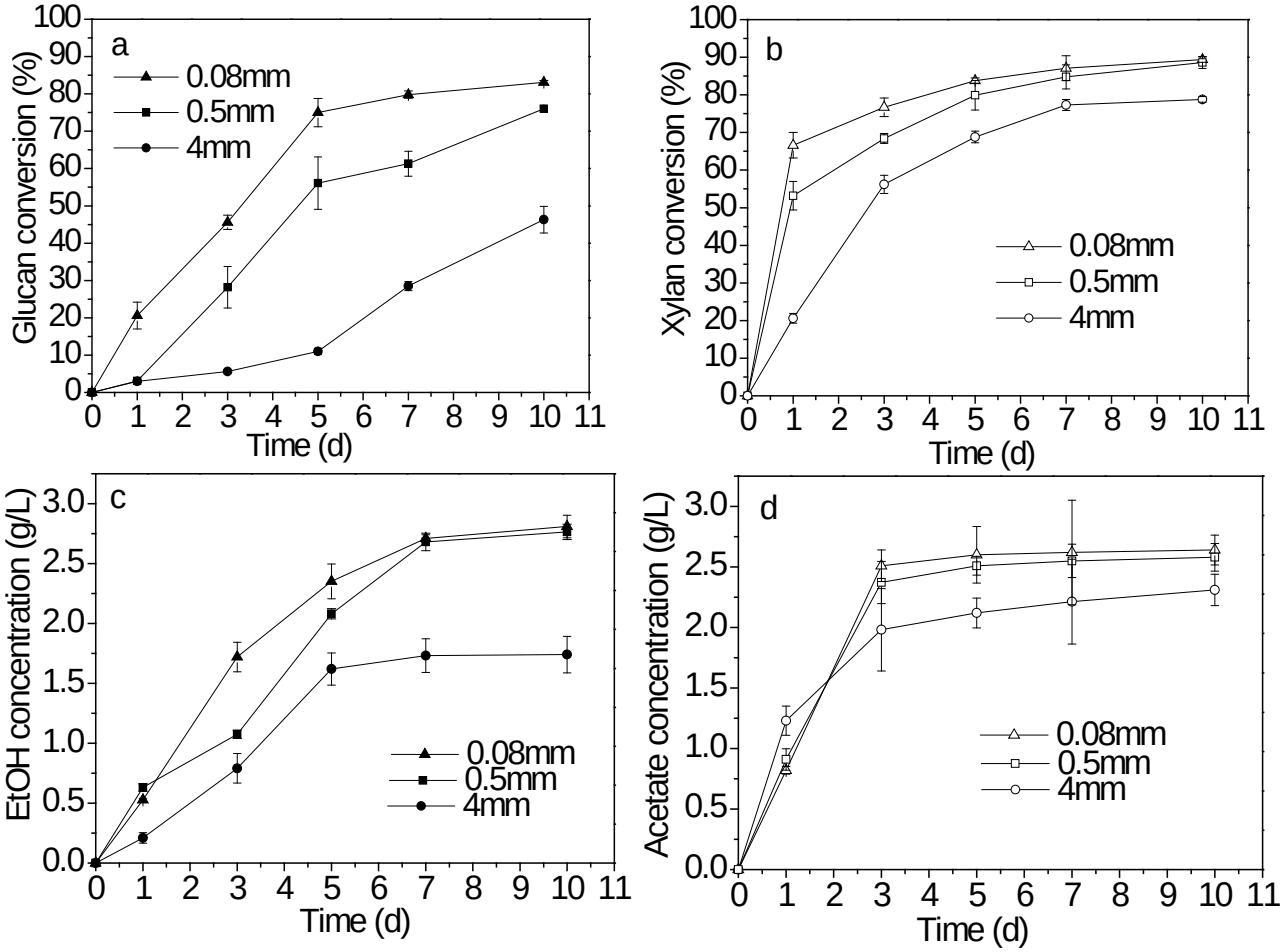



Figure 55 Effect of particle size of AFEX$^{TM}$-CS on sugar conversion and product generation during CBP by *C. phytofermentans.*

hydrolyzed very slowly during the first 5 days while xylan hydrolysis was still relatively rapid. After 10 days, the glucan and xylan conversions reached 46.3% and 78.8%, respectively. A particle size of 0.5 mm showed slower conversion rates compared to 0.08 mm but similar xylan conversion rates and somewhat lower glucan conversions with nearly the same final ethanol and acetate concentrations (around 2.8 g/L ethanol and 2.6 g/L acetate).

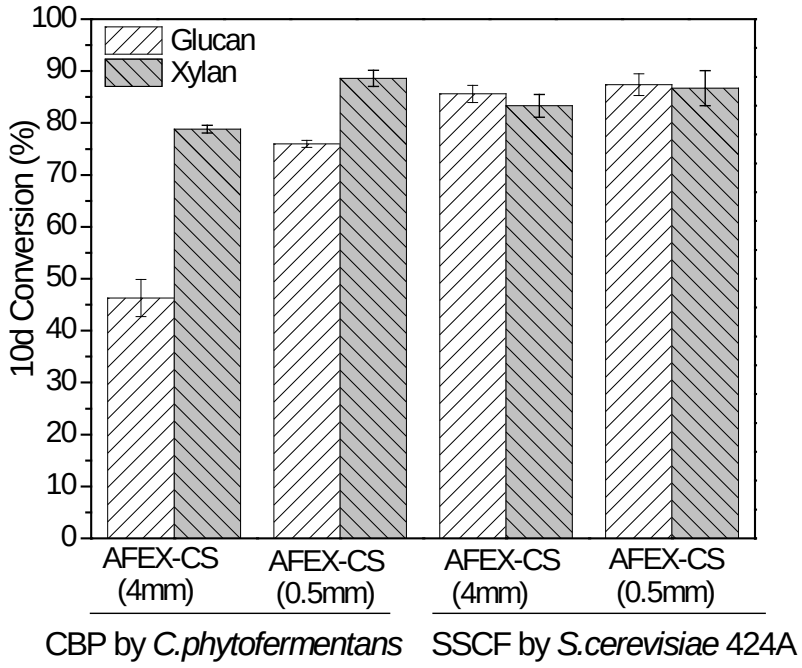



Figure 56 Comparison of sugar conversions achieved by CBP using *C. phytofermentans* and SSCF using *S. cerevisiae* 424A on AFEX$^{TM}$-CS.

### 8.3.6 Comparison between CBP by *C. phytofermentans* and SSCF by *S. cerevisiae* 424A

A commercial enzyme mixture (containing 15 FPU cellulases) and xylose-fermenting yeast *S. cerevisiae* 424A were used to perform SSCF at similar conditions (30 $^{o}$C and 200 rpm) as for CBP. After 10 days' SSCF, 85.6% glucan conversion and 83.3% xylan conversion were achieved for a particle size of 4 mm (Fig. 56). Slightly higher conversions (87.4% glucan conversion and 86.7% xylan conversion) were obtained for the particle size 0.5 mm. Compared to the SSCF process, CBP using *C. phytofermentans* reached 54% and 87% of the glucan and xylan conversions achieved by SSCF at particle size 4 mm. However, at a particle size of 0.5 mm, CBP was able to reach 87% and 102% of the glucan and xylan conversions achieved by SSCF. This indicates that CBP has a more stringent requirement for effective sugar release profiles than SSCF does, probably due to the fact that CBP needs sugars to produce enzymes in addition to

sugars for microbial fermentation. Small particle sizes led to faster sugar release rate which facilitated simultaneous microbial growth and enzyme production, thereby enhancing the sugar conversions. Although at particle size 0.5 mm CBP sugar conversions were close to those of SSCF's it is noteworthy that SSCF reached almost the same conversions in 5 days' reaction, twice as fast as CBP.

After 10 days, at a particle size of 0.5 mm, CBP yielded 2.8 g/L ethanol, which is 71.8% of that yielded by SSCF (3.9 g/L). Potentially, *C. phytofermentans*is is able to produce more ethanol than *S. cerevisiae* from corn stover since it can also ferment galactan/galactose, arabinan/arabinose, and mannan/mannose (Warnick et al., 2002) present in corn stover. The lower ethanol concentration obtained in this study is due to byproduct formation, especially acetate production. Based on the results obtained in this study at low solid loading, 174.1 g ethanol and 161.6 g acetate are generated from 1 kg AFEX$^{TM}$-CS through CBP using *C. phytofermentans* (Fig. 57). The ethanol yield for SSCF using *S. cerevisiae* 424A is 242.4 g per kg AFEX$^{TM}$-CS. The yields, especially for CBP will be significantly reduced if the experiments are conducted at high solid loading due to inhibition by solids and products.

**8.4 Conclusion**

The optimal conditions for CBP of AFEX-CS by *C. phytofermentans* ATCC 700394 were found to be: 30 $^{o}$C, 5% (v/v) inoculation size and 0.4YP addition. At these conditions in serum vials with an initial pH 7.0, 76% of glucan conversion and 88.6% of xylan conversion were achieved in 10 days with an ethanol yield of 2.8 g/L. Decomposition products removal by washing improved sugar conversions slightly but also decreased ethanol yield. Higher sugar yields were observed at smaller particle sizes during CBP.

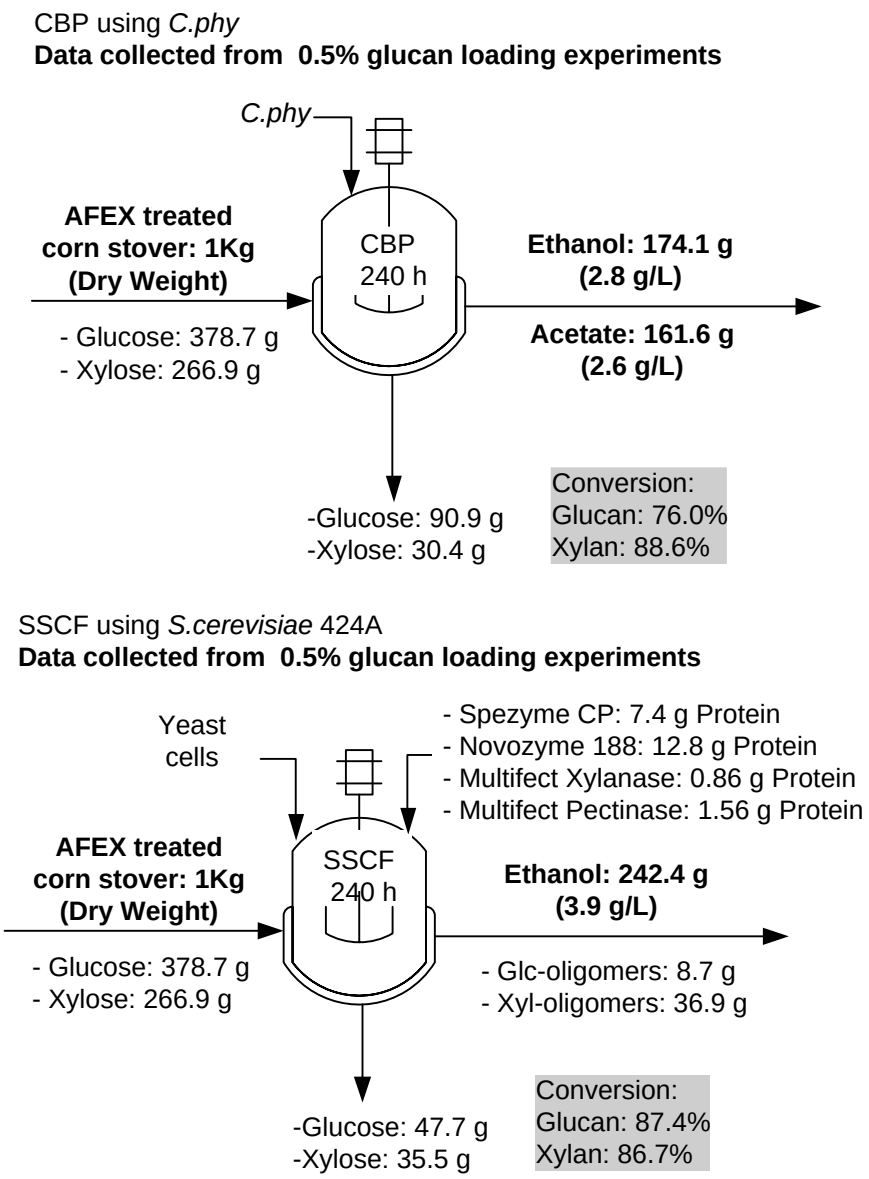



Figure 57 Mass balance comparison between CBP by *C. phytofermentans* and SSCF by *S. cerevisiae* 424A on AFEX$^{TM}$-CS

# CHAPTER IX CBP OF AFEX™ PRETREATED CORN STOVER AT HIGH SOLIDS LOADING


## Abstract

Consolidated bioprocessing (CBP) using *Clostridium phytofermentans* (ATCC 700394) on AFEX™ treated corn stover (AFEX™-CS) at low solids loading showed promising results (Chapter VIII). However, an industrially relevant process requires high solids loading. Therefore, high solids loading CBP performance on AFEX™-CS was studied. The factors potentially affecting the performance including solids loading, CBP products acetate and ethanol, and degradation products resulting from pretreatment were investigated. At 4% (w/w) glucan loading, *C. phytofermentans* performed well on AFEX™-CS with no nutrients supplementation and reached similar sugar conversions as a fermentation with nutrients supplementation. A glucan conversion of 48.9% and a xylan conversion of 77.9% were achieved after 264 h with 7.0 g/L ethanol and 8.8 g/L acetate produced. Relatively high concentrations of acetate produced at high solids loading was found to be the major factor limiting the CBP performance. Degradation products in AFEX™-CS helped enhance ethanol production.

## 9.1 Introduction

CBP produces enzymes during the fermentation process and eliminates the requirement of external enzymes addition. *Clostridium thermocellum* and *Clostridium phytofermentans* are two of the promising CBP microbes. *C. thermocellum* is a thermophilic anaerobic strain and produces cellulosomes to degrade cellulose (Alexande.Jk, 1968; Lynd et al., 2005; Lynd et al., 2002; McBee, 1954). This organism solubilized AFEX$^{TM}$ pretreated corn stover (AFEX$^{TM}$-CS) of particle size 0.5 mm with sugar conversions higher than 80% in 4 days at low solids loading (Shao et al., 2011). However, *C. thermocellum* cannot ferment xylose in the lignocellulosic biomass and produced around 0.9 g/L ethanol in that study (data unpublished). *C. phytofermentans* is a mesophilic anaerobic strain with its genome encoding the highest number of cellulases and hemicellulases among the sequenced clostridial genomes (Weber et al., 2010). It can ferment almost all of the carbohydrates in lignocellulosic biomass (Warnick et al., 2002). *C. phytofermentans* also reached around 80% sugar conversion at low solids loading but the fermentation required 10 days. However, it produced more ethanol (2.8 g/L) than *C. thermocellum* did and the ethanol yield of 174.1 g ethanol per kg corn stover is promising. Nevertheless, the study was conducted at a low solids loading (0.5% glucan loading). Higher solids loading is preferred for industrial process due to the benefits of lower capital cost, higher ethanol titers and hence low distillation costs, and less waste water (Kristensen et al., 2009). Industrially relevant solids loading (high solids loading) should have the potential to give enough fermentable sugars for ethanol production at a titer of $> 40$ g/L (Dien et al., 2003a), which corresponds to at least 4% (w/w) glucan loading for corn stover. To the author's knowledge there is very limited information on high solids loading CBP reported in the literature. Here, the high solids loading CBP performance of *C. phytofermentans* on AFEX$^{TM}$-CS was studied. The

possibility of performing the CBP without nutrients supplementation and the factors potentially affecting the CBP performance at high solid loadings were investigated.

## 9.2 Materials and Methods

### 9.2.1 AFEX$^{TM}$ pretreated Corn Stover

Please see Chapter II section 2.2.1.

### 9.2.2 Particle size reduction

Please see Chapter VIII section 8.2.2.

### 9.2.3 Preparation of washed AFEX$^{TM}$-CS

Please see Chapter III section 3.2.7.

### 9.2.4 CBP microbe seed cultures preparation

*C. phytofermentans* ATCC 700394 was used for this study. For seed cultures preparation please see Chapter VIII section 8.2.3.

### 9.2.5 Water extract of AFEX$^{TM}$-CS preparation

Please see Chapter II section 2.2.3.

### 9.2.6 CBP fermentation

CBP experiments at 2%, 3% and 4% (w/w) glucan loading (corresponding to 5.9%, 8.8% and 11.7% (w/w) total solids loading, respectively) were carried out in a 500 ml fermentor (Biostat, Sartorius) with a working volume of 300 ml, agitation speed 400 rpm, pH 6.7, and temperature of 30 $^{o}$C. The fermentation medium was purged with $N_2$ before inoculation. The inoculation size was 10% (v/v). GS-2 medium (without cellobiose) and washed AFEX$^{TM}$-CS were used for those experiments except the experiment without nutrient supplementation. CBP of 4% (w/w) glucan loading AFEX$^{TM}$-CS without nutrients supplementation was started with 4%

glucan loading equivalent water extract as the medium and 2% (w/w) glucan loading equivalent washed AFEX$^{TM}$-CS. Another 2% glucan loading equivalent washed AFEX$^{TM}$-CS was fed after 96 h. The particle size of AFEX$^{TM}$-CS used was 0.5 mm except for the experiment investigating different solids loadings, in which a particle size of 4.0 mm was used.

Experiments investigating the effects of AFEX$^{TM}$-CS water extract, acetate, and ethanol on CBP performance and sugar fermentations were conducted in 125 ml serum vials with $N_2$ atmosphere, 50 ml GS-2 medium (without cellobiose). For CBP, 0.5% (w/w) glucan loading AFEX$^{TM}$-CS with particle size 0.5 mm was used. For fermentation of pure sugars: 5 g/L cellobiose or 10 g/L glucose or 10 g/L xylose was used. Experiments were conducted at 200 rpm and 30 $^{o}$C. Glucose, cellobiose, xylose, ethanol, acetate, and lactate concentrations were analyzed by HPLC with a Biorad Aminex HPX-87H column as described previously in Chapter II section 2.2.7.

### 9.2.7 Measurement of glucan and xylan conversions

Please see Chapter VIII section 8.2.6.

## 9.3 Results and discussion

### 9.3.1 CBP at 4% glucan loading with and without nutrients supplementation

AFEX$^{TM}$ pretreatment is a dry to dry process which conserves nutrients in the plant biomass and hence there is no nutrients supplementation required for yeast fermentation of AFEX$^{TM}$ treated biomass (Lau & Dale, 2009; Zhong et al., 2009). Here the CBP of AFEX$^{TM}$-CS without nutrients supplementation was tried. One experiment with the nutrients supplementation (GS-2 medium) was used as a control. To avoid high salts concentration that

potentially inhibits anaerobic microbe fermentation (Lynd et al., 2001), and that could be caused by the buildup of salts from both GS-2 medium and AFEX$^{TM}$-CS, we used washed biomass (0.5 mm) with soluble compounds removed.

The CBP sugar conversions and products profiles with and without nutrients supplementation showed a similar trend (Fig. 58). The sugar conversion rates for the nutrients supplementation experiment were higher with the final conversions also slightly higher than the experiment with no nutrients supplementation (Fig. 58a). The relatively slower sugar conversions for the no nutrients supplementation condition could be caused by the inhibition from the degradation products generated during pretreatment. After 264 h CBP, the runs with and without nutrients supplementation reached glucan and xylan conversions of 50.9% and 83.1%, and 48.9% and 77.9%, respectively. For both cases, around 7.0 g/L ethanol and 9.0 g/L acetate (subtracting 1.6 g/L acetate originally present in AFEX$^{TM}$-CS) was produced after 264 h CBP fermentation (Fig. 58b).

At 4% glucan loading, AFEX$^{TM}$-CS provided sufficient nutrients for the CBP fermentation by *C. phytofermentans*. However, the glucan conversions for both cases were low (around 50%). Sugar conversions did not increase even when the fermentation time was extended (data not shown). To better understand the CBP performance at high solids loading, we subsequently investigated several factors that could potentially affect CBP performance on AFEX$^{TM}$-CS.

### 9.3.2 Effect of solids loading on CBP performance

It is well known that solids loading plays an important role in the enzymatic hydrolysis of lignocellulosic biomass (Kristensen et al., 2009). Here we investigated different glucan loadings

(2%, 3% and 4%) on the CBP performances. To eliminate the effects of degradation products, washed biomass with particle size 4 mm was used. The sugar conversions showed similar trends

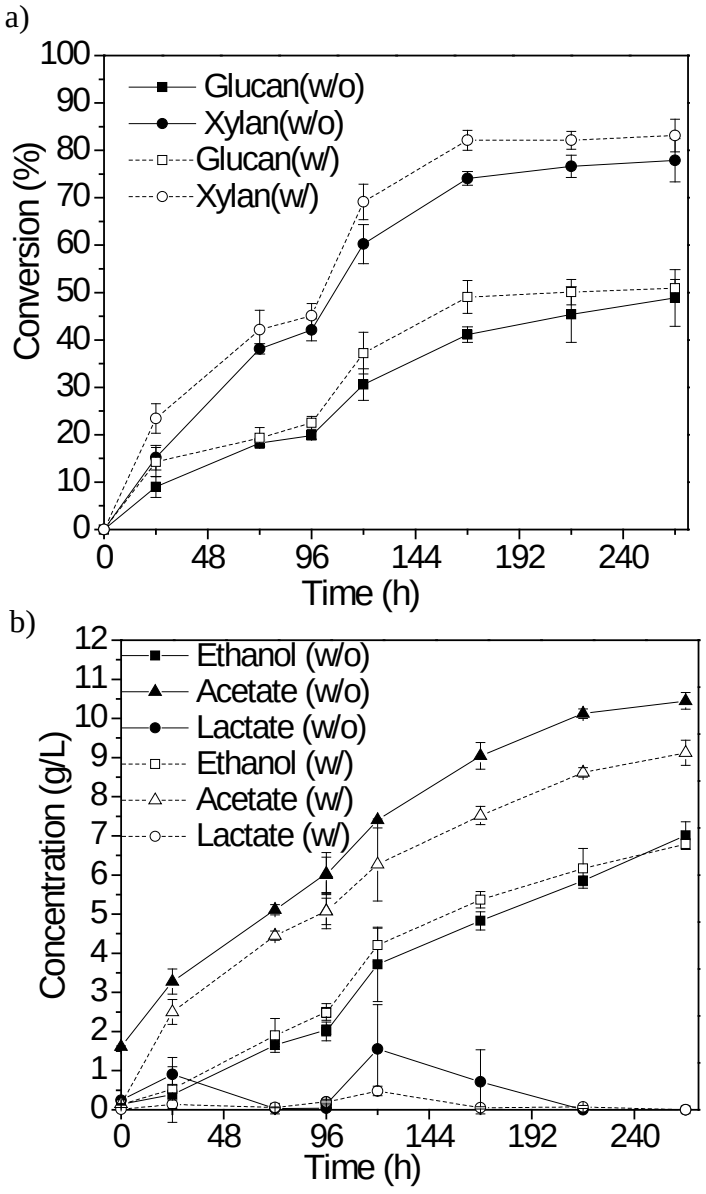



Figure 58 CBP of 4% glucan loading AFEX$^{TM}$-CS with (w/) and without (w/o) nutrients supplementation.
The experiments were started with half of the total biomass. The other half was fed at 96 h. 4% glucan loading equivalent water extract was used as medium for no nutrients supplementation experiment. GS-2 medium was used for nutrients supplementation experiment. Washed biomass with particle size 0.5 mm was used for those experiments. pH was controlled at 6.7.

as observed for enzymatic hydrolysis (conversion decreased with increase of solids loading) (Fig. 59a). For enzymatic hydrolysis, high sugar (hydrolysis products) concentration limited the final sugar conversion at high solids loading (Kristensen et al., 2009). However, for CBP we did not observe sugar accumulation during the process. The potential inhibitors of CBP could be the accumulated fermentation products such as acetate and ethanol (Fig. 59b), which might inhibit sugar fermentation, cell growth, enzyme production, and enzymatic hydrolysis. With the addition of 7.5 FPU/glucan cellulases (Spezyme CP, the optimal pH and temperature are around 4.8 and 50 $^{o}$C, respectively) at the beginning of the fermentation, the glucan and xylan conversions of 3% glucan loading increased from 44.7% and 75.4% to 73.1% and 82.9%, respectively, and the ethanol and acetate concentrations also increased from 6.2 and 7.6 to 11.2 and 10.9 g/L, respectively. These data indicate that the inhibition probably acted more on the enzyme production process than on sugar fermentation.

### 9.3.3 Effects of acetate and ethanol on CBP performance and sugar consumption

The experiments investigating the effects of acetate and ethanol on CBP performance were conducted at 0.5% glucan loading in a serum vial using unwashed AFEX$^{TM}$-CS with particle size 0.5 mm. Different concentrations of acetate and ethanol were investigated (Fig. 60). Sugar conversions decreased with increased ethanol or acetate concentration and the reduction of glucan conversion was more pronounced than that of xylan conversion. Compared to ethanol, acetate showed more severe inhibitory effects on sugar conversions. With the addition of acetate to 10 g/L, the glucan and xylan conversions dropped from 76.0% and 88.6% to 31.1% and 73.5%, respectively. A reduction this large was not observed until the ethanol concentration was increased to 20 g/L, at which point the glucan and xylan conversions dropped to 37.7% and 76.8%, respectively. During high solids loading CBP fermentation, we typically see more and

earlier acetate production when compared to ethanol (Fig. 58b). This fact probably played a major role in the inhibition of sugar conversion during CBP process. The inhibitory effects of ethanol on glucose and xylose consumptions were similar (Fig. 61a&c). Ethanol concentrations lower than 10 g/L showed mild inhibition while 20 g/L severely inhibited sugar consumption. At

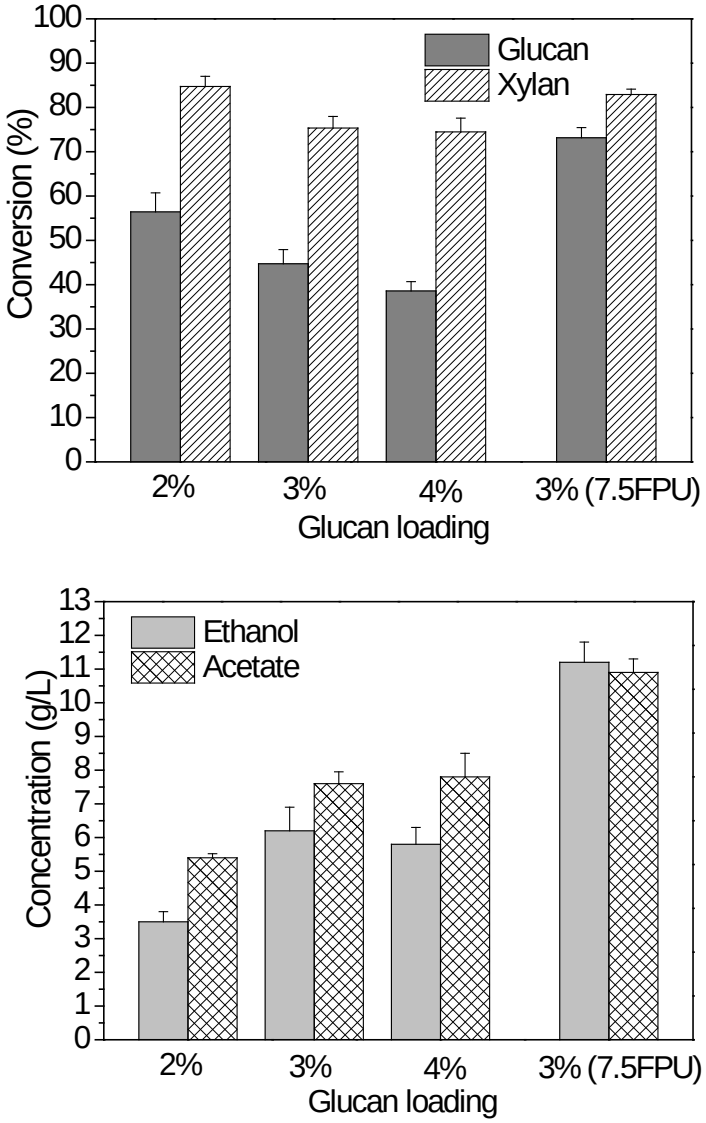



Figure 59 Effect of solids loading on sugar conversions (a) and products production (b) during CBP.
3% glucan loading CBP performance was also tested with addition of 7.5 FPU cellulase (Spezyme CP) per glucan. Particle size of the washed biomass used was 4 mm. pH was controlled at 6.7. Experiments were performed for 264 h.

the tested levels (2, 5, and 10 g/L), acetate did not show severe inhibition on glucose consumption. However, acetate with the concentrations higher than 5 g/L showed severe inhibition of xylose consumption. The inhibitory effects of 20 g/L ethanol and 10 g/L acetate were similar on xylose consumption (Fig. 61c&d).

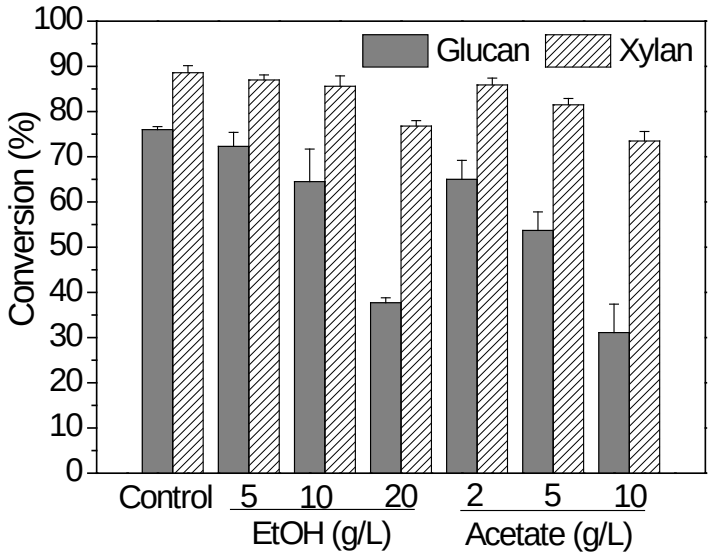



Figure 60 Effect of ethanol and acetate on sugar conversions of CBP.
The experiment was conducted at 0.5% glucan loading using unwashed AFEX$^{TM}$-CS with particle size 0.5 mm for 240 h. Ethanol and acetate at different concentrations were added at the beginning. There was no addition of ethanol or acetate for the control.

In summary, it is likely that relatively high concentrations of acetate produced during high solids loading CBP inhibited both enzyme production, especially production of cellulases, and xylose metabolism which in turn further affected enzymes production, and hence reduced sugar conversions (Fig. 58).

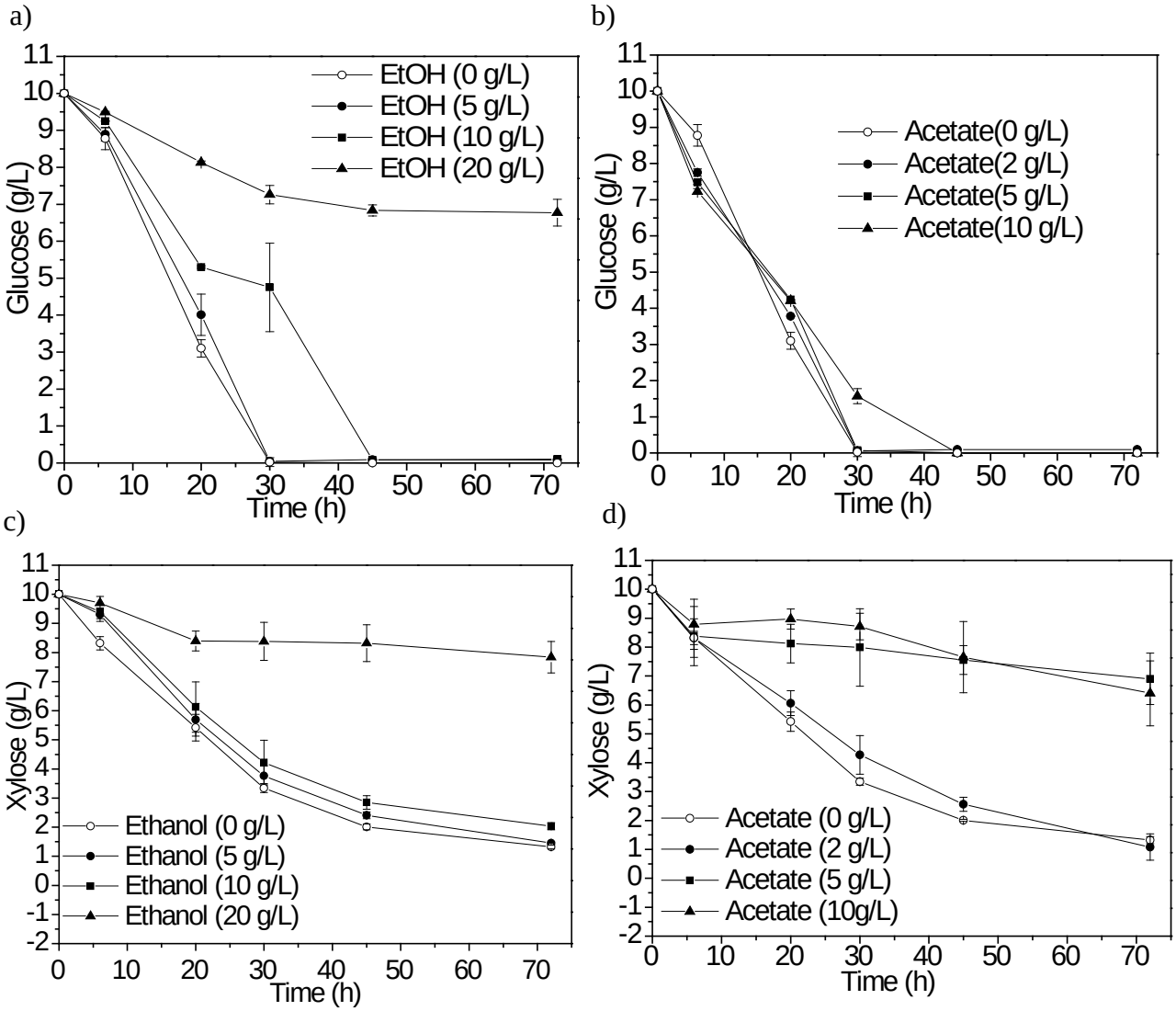



Figure 61 Effect of ethanol and acetate on glucose and xylose consumption.
Experiments were conducted using GS-2 medium and glucose or xylose. Ethanol and acetate were added at the beginning.

### 9.3.4 Effect of degradation products on fermentation

It has been shown that without nutrients supplementation, *C. phytofermentans* performed well on AFEX$^{TM}$-CS. AFEX$^{TM}$-CS contains not only nutrients but also degradation products resulting from pretreatment which could potentially affect sugar conversions and fermentation performance during CBP. However, the effect of degradation products on sugar conversions does

not seem significant (Fig. 58). Therefore, we only investigated the effect of degradation products on fermentation performance. The water extract of AFEX$^{TM}$-CS was used to represent the degradation products and cellobiose was used as the carbon source (Fig. 62 & Table 13). At high concentration (6% glucan loading), degradation products showed inhibition on the sugar consumption and cell growth (Fig. 62a&b). However, at lower concentrations (0.5%, 2% and 4% glucan loading), the inhibitory effect was not substantial (Fig. 62). With low water extract concentrations, the ethanol metabolic yield and ethanol to acetate ratio were enhanced (Table 13). For example, with the addition of 2% glucan loading water extract, the ethanol metabolic yield and ethanol to acetate ratio increased from 48.5% and 1.76 to 67.2% and 2.77, respectively. We also observed higher ethanol metabolic yield with the addition of 4% glucan loading water extract.

In Chapter VIII, it has been shown that more ethanol was produced during CBP of unwashed AFEX$^{TM}$-CS compared to washed material at 0.5% glucan loading. However, CBP at 4% glucan loading without nutrients supplementation produced similar amounts of ethanol as washed biomass with nutrients supplementation (Fig. 58). During CBP, there are two key types of reactions taking place inside the cell and which determine the flow of carbon flux. One is cell growth and enzymes production, which require ATP. Acetate production can provide more ATP than ethanol production (Zhang & Lynd, 2005). The other reaction is fermentation producing ethanol and acetate. Apparently, with 4% water extract, more ethanol can be produced during fermentation since water extract can enhance ethanol metabolic yield. However, with these amounts of degradation products, more enzyme production might be required to reach similar sugar conversions as without degradation products since degradation products inhibit enzymatic hydrolysis (Chundawat et al., 2010). Therefore, it seems likely that more sugars were used for

acetate production to generate more ATP for enzyme production, which offset the higher ethanol metabolic yield of fermentation with the presence of degradation products.

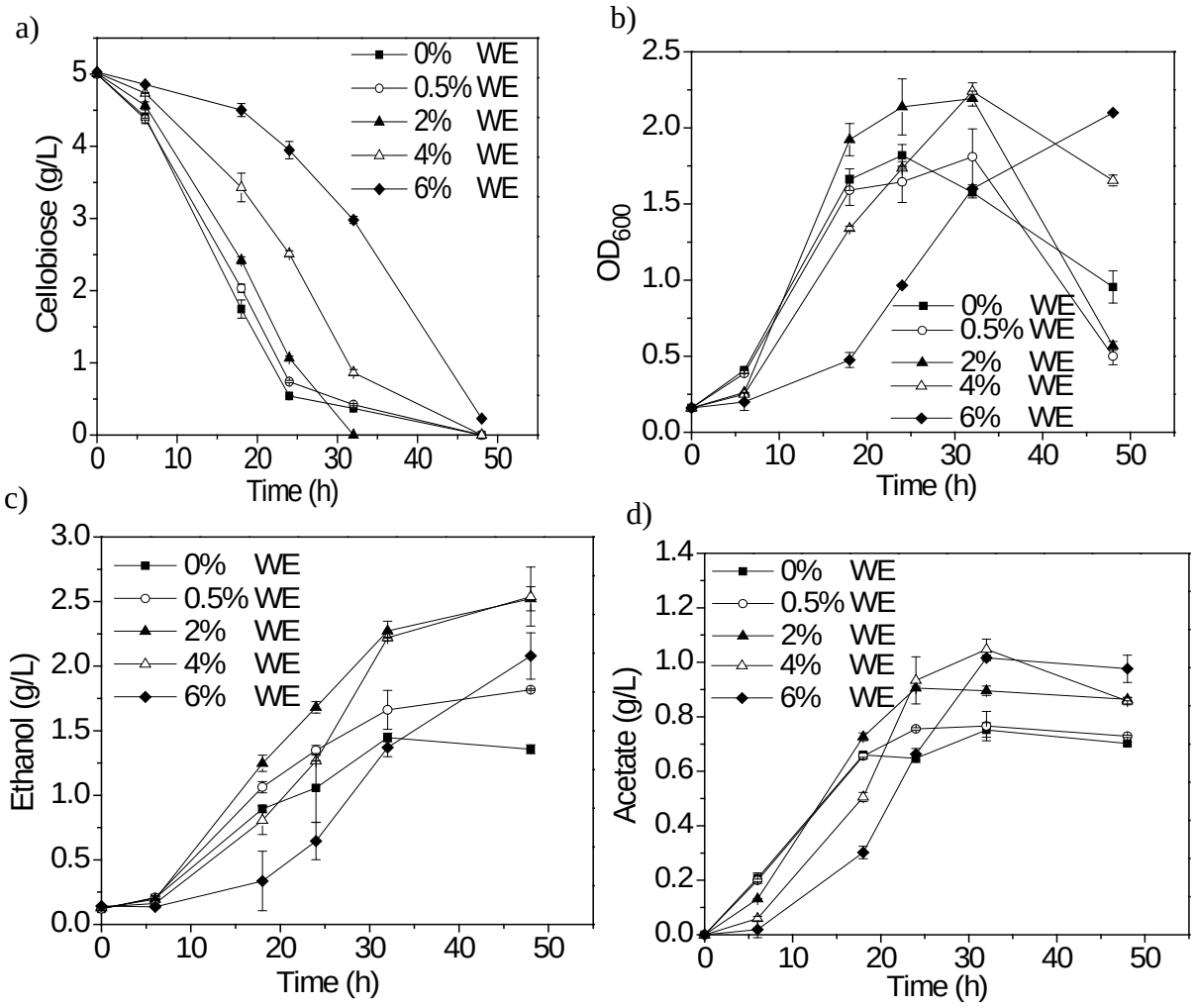



Figure 62 Effect of AFEX$^{TM}$-CS water extract (WE) on cellobiose consumption (a), cell growth (b), ethanol (c) and acetate (d) production during cellobiose fermentation by *C.phytofermentans*. The experiments were conducted in 125 ml serum vials with working volume 50 ml at 30 ºC, 200 rpm with inoculation size 10% (v/v). Water extract concentrations were expressed as glucan loading equivalent.

## 9.4 Conclusions

Production of cellulosic ethanol from AFEX$^{TM}$ pretreated biomass through CBP is promising. However, the strain has to be improved for less acetic acid production, higher ethanol tolerance as well as fast hydrolysis of the biomass.

Table 13 Summary of AFEX$^{TM}$-CS water extract (WE) effect on cellobiose fermentation.

| Water extract conc. (glucan loading equivalent) | Glc. (g/L) | Cellobiose (g/L) | Xylose (g/L) | Oligo-Glc[a] (g/L) | Oligo-Xyl[a] (g/L) | EtOH (g/L) | Acetate (g/L) | EtOH Metabolic yield[b] | Acetate Metabolic yield[b] | EtOH/ Acetate |
|---|---|---|---|---|---|---|---|---|---|---|
| 0% (t=0 h) | 0.00 | 5.00 | 0.00 | 0.00 | 0.00 | 0.12 | 0.08 | | | |
| 0% (t=48 h) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.36 | 0.78 | 48.5% | 18.9% | 1.76 |
| 0.5% (t=0 h) | 0.19 | 5.00 | 0.12 | 0.18 | 0.55 | 0.12 | 0.25 | | | |
| 0.5% (t=48 h) | 0.10 | 0.00 | 0.00 | 0.15 | 0.32 | 1.82 | 0.98 | 55.2% | 18.2% | 2.33 |
| 2% (t=0 h) | 0.75 | 5.01 | 0.49 | 0.72 | 2.18 | 0.13 | 0.77 | | | |
| 2% (t=48 h) | 0.36 | 0.00 | 0.00 | 0.71 | 1.66 | 2.52 | 1.64 | 67.2% | 18.6% | 2.77 |
| 4% (t=0 h) | 1.51 | 5.02 | 0.97 | 1.45 | 4.37 | 0.14 | 1.47 | | | |
| 4% (t=48 h) | 0.64 | 0.00 | 0.00 | 1.43 | 4.35 | 2.54 | 2.32 | 63.2% | 17.3% | 2.80 |
| 6% (t=0 h) | 2.26 | 5.03 | 1.46 | 2.17 | 6.55 | 0.14 | 2.16 | | | |
| 6% (t=48 h) | 0.00 | 0.23 | 0.00 | 1.13 | 5.51 | 2.08 | 3.14 | 34.1% | 13.1% | 1.98 |

[a]Oligomeric sugars were from water extract.

[b] Ethanol and acetate metabolic yield were calculated based on consumed sugars. The theoretical yield for ethanol and acetate were 0.51 and 0.667 g per g consumed glucose or xylose.

# CHAPTER X PERSPECTIVES

This dissertation work studied integrated biological processes (SSCF and CBP) on AFEX$^{TM}$ pretreated biomass for cellulosic ethanol production, investigated fundamental issues in these processes and based on the gained understandings developed several processes for a better performance. The developed processes include two-step SSCF (Chapter III), continuous hydrolysis and fermentation process (continuous SSCF; Chapter VI), and BCRL integrated biological processes (Chapter VII). The fundamental issues studied include slow xylose fermentation (Chapter II), factors affecting SSCF (such as SG solids inhibition, CS solids effect, effects of ethanol and temperature on enzymatic hydrolysis, effect of pre-hydrolysis time and effect of solids loading), factors affecting CBP performance (such as fermentation conditions, biomass particle size, degradation products, fermentation products and solids loading). In particular, the BCRL integrated biological processes achieved rapid hydrolysis and rapid fermentation, shortened the cellulosic ethanol production time from 11 days to around 2 days, reduced enzyme loading by more than 1/3 and enhanced productivity by 2~3 times. The cellulosic ethanol production problems mentioned in Chapter I, such as long enzymatic hydrolysis time, high enzyme loading, slow xylose fermentation and low ethanol productivity, were largely resolved.

## 10.1 Techno-economic analyses of various processes

Techno-economic analyses based on the modified NREL model described in Chapter V were performed here for overall comparison of different processes. Results are shown in Fig. 63. The lowest minimum ethanol selling price (MESP) $2.24/gallon goes to the BCRL SSCF process with the enzyme loading profile *b*, following by BCRL SSCFs (enzyme loading profile *a* & *c*),

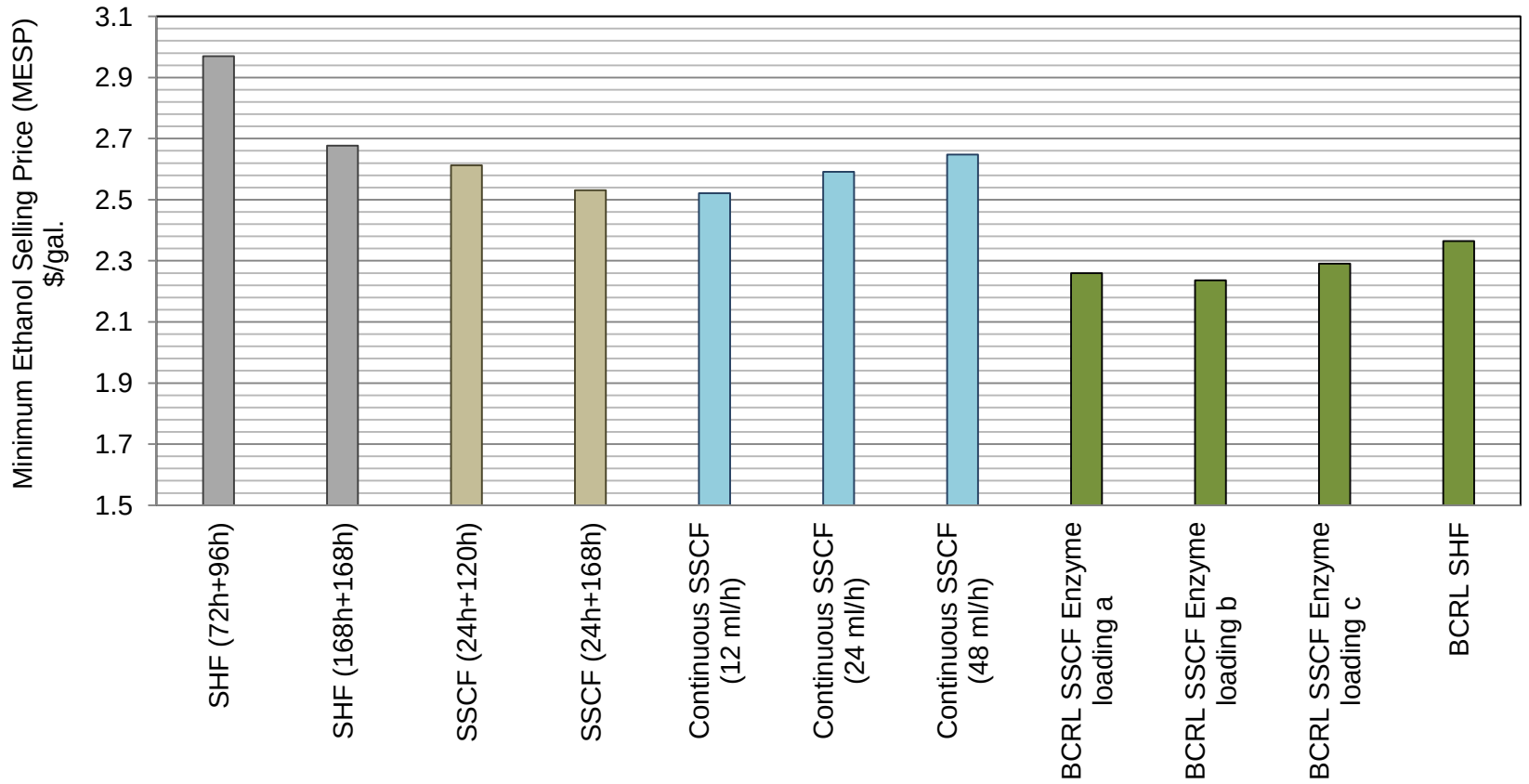



Figure 63 Techno-economic analyses of various processes.
The experimental data for SHF(72 h EH + 96 h Ferm.), SHF (168 h EH+168h Ferm.), SSCF(24 h EH +120 h Ferm.), SSCF(24 h EH + 168 h Ferm.), and continuous SSCF (flow rates: 12 ml/h, 24 ml/h and 48 ml/h) were from Chapter VI. The experimental data for BCRL process were from Chapter VII.

BCRL SHF process ($2.37/gallon) and continuous SSCF (flow rate 12 ml/h) $2.52/gallon. The process with the highest MESP $2.97/gallon is the SHF process with enzymatic hydrolysis 72 h and fermentation 96 h. The batch SSCF (24 h pre-hydrolysis + 168 h fermentation) has almost the same MESP as the continuous SSCF with flow rate 12 ml/h. This model did not consider the savings of the continuous process in terms of automation which reduces operations such as filling and cleaning of the reactors. In addition, the plant lifetime of this model is set for 30 years instead of 20 years in the 2002 NREL model. This change makes ethanol yield the most important factor and makes capital cost not as important as in the 2002 model. Therefore, this dissertation work's effort on reduction of capital cost by improving ethanol productivity might be underestimated.

Based on the techno-economic analyses, it seems ethanol yield is now the factor that limits further reduction of production costs. For instance, among the continuous SSCF processes, the lowest flow rate (12 ml/h) has the highest ethanol yield and thereby the lowest MESP although the productivity is lower than for the other two flow rates. The BCRL SHF process has the highest ethanol productivity and the lowest enzyme loading while it has higher MESP compared to BCRL SSCF probably due to the lower ethanol yield.

## 10.2 Perspectives on the BCRL integrated biological processes improvement

### 10.2.1 Ethanol yield enhancement

The glucose-to-ethanol conversion in the BCRL SSCF process can reach around 80% with around 12~17% lost as polymers and 6~8% lost as oligomers (Fig. 49). It would be difficult to convert additional polymeric glucose to ethanol since the conversion is already high and the remaining unhydrolyzed cellulose is of high recalcitrance. Conversion of oligomeric glucose to ethanol could increase around 10 g ethanol production from 1 kg corn stover. The xylose-to-

ethanol conversion in BCRL SSCF process was around 40%~50%, which is very low, with about 37%~ 42% lost as oligomeric sugars (Fig. 49). The conversion of oligomeric xylose to ethanol is crucial! It could not only add around 50 g ethanol per kg corn stover to the total yield making the production of around 260 g ethanol per kg corn stover possible but also could remove the inhibitory effect of oligomeric xylose on enzymatic hydrolysis of cellulose and oligomeric glucose. If the xylose-to-ethanol conversion reaches 80%, the MESP for BCRL SSCF process could be as low as $1.81/ gallon.

The current xylases are probably easily inhibited by high sugar concentrations or high ethanol concentrations (Fig. 27). Thus, SSCF did not help reduce the oligomeric xylose production (Fig. 26, 35 & 48). Ethanol tolerant or high sugar concentration tolerant xylanases are desired for reducing oligomeric xylose in hydrolysate. In addition, a structure study of the remaining oligomeric xylose would be also important for finding the right xylanase to degrade them. Obtaining a microbial strain that can consume oligomeric xylose and produce ethanol would be another approach.

### 10.2.2 Thermo-tolerant xylose-fermenting ethanologen

In Chapter IV Fig. 27, it has been shown that the optimal temperature for SSCF is 45 $^{o}$C and the enzymatic hydrolysis rates are much lower when the temperature is 30-32 $^{o}$C. Using the current nonthermo-tolerant strain *S. cerevisiae* 424A for the BCRL SSCF process, the recycled enzymes have to work at a temperature around 30 $^{o}$C, in which temperature the enzymes cannot function to their best. A thermo-tolerant ethanologen could make the reduction of enzyme loading for BCRL SSCF process at least as much as BCRL SHF achieved if the SSCF

temperature can be 45 $^{o}$C. In addition, the ethanol yield or productivity could be further enhanced due to the higher sugar yield at 45 $^{o}$C.

### 10.2.3 Improvement of ethanol yield for the BCRL SHF process

The reducing cell viability in the BCRL SSCF process is an issue. The BCRL SHF process does not have this problem. Yeast cells were growing well and were staying at a high level (OD>20) during the entire process. In addition, based on the current microbial strain, the BCRL SHF has higher productivity and higher enzyme loading reduction potential compared to the BCRL SSCF process. The only disadvantage of the BCRL SHF process is the lower ethanol yield. Further improvement of enzymatic hydrolysis for higher sugar yield by optimization of the enzymes combination/cocktail will probably make the BCRL SHF process reach the same ethanol yield as the BCRL SSCF.

### 10.2.4 Improvement of enzyme adsorption

Enzyme adsorption to biomass is strongly affected by high sugar concentrations. The effect of ethanol on enzyme adsorption is not clear. Enzyme adsorption is strongly correlated to the hydrolysis performance. In the BCRL integrated processes, enzyme recycling is dependent on the adsorption to biomass. The improvement of the adsorption capability of cellulases and hemicellulases to biomass would not only help improve hydrolysis rate and hence reduce enzymes loading but also help on the enzymes recycling during the BCRL processes.

### 10.2.5 Alleviation of solids accumulation

Solids accumulation in the BCRL integrated biological processes is an issue, which limited the cycles that can be performed. Removal of lignin during pretreatment is a way to alleviate this problem. Another approach is to reorganize the configuration. For instance, in the BCRL SHF process, the solids content in the enzymatic hydrolysis tank (Fig. 43) can be

maintained to a constant level by directing part of the residual solids to another enzymatic hydrolysis tank as shown in Fig. 64. This way, the process can be performed with unlimited cycles and hence probably can further reduce the enzyme loading and enhance the productivity. The "another enzymatic hydrolysis" tank would replace the "last step" in the previous configuration. The solids content level in the major enzymatic hydrolysis tank needs to be investigated.

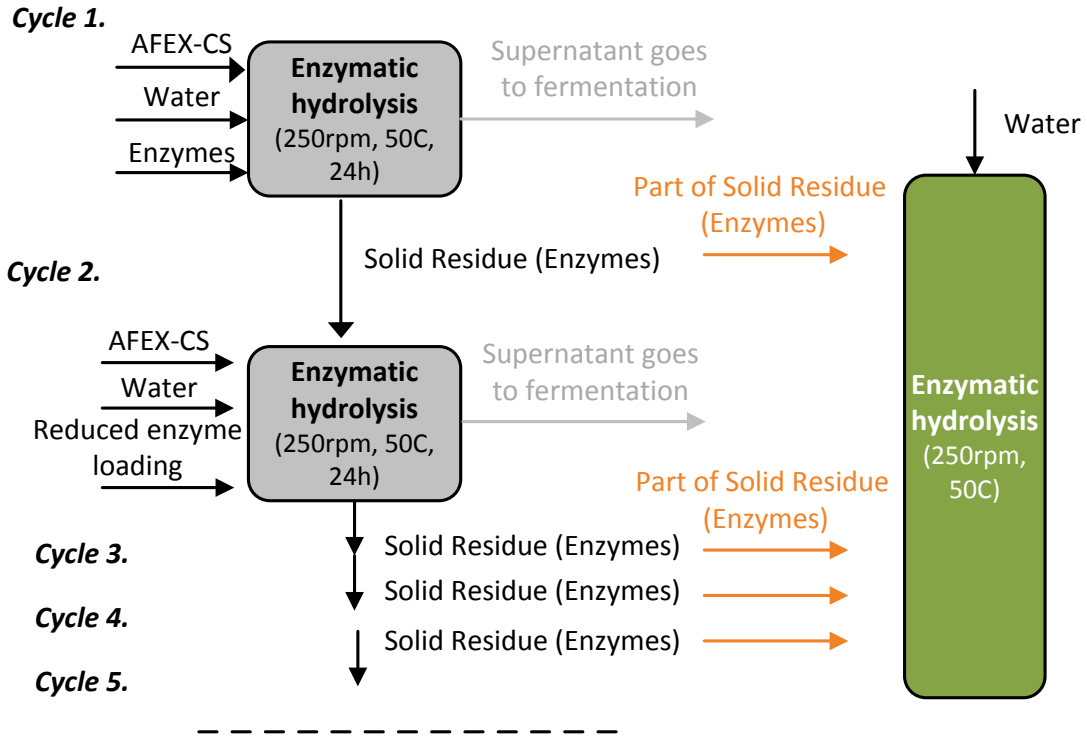



Figure 64 Rearranged BCRL SHF process for solving accumulated solids problem. The figure is in color.

### 10.3 Perspectives on the solids inhibitory effect on fermentation

This dissertation work found that the enzymatic hydrolysis residue of switchgrass was highly inhibitory to xylose fermentation by *S. cerevisiae* 424A while the residue of corn stover was not. SSCF and CBP processes both have solids together with the microbial cells in the

fermentation broth. Therefore, understanding why some feedstock solids have inhibitory effect on fermentation while others do not is important. Various feedstocks including sugarcane bagasse, miscanthus, poplar and rice straw have been further investigated (results not shown in this dissertation). None of those solids showed inhibitory effect. The switchgrass harvested in different years (2008, 2009, and 2010) were also studied. Only the one harvested in 2008 has inhibitory effect on both yeast cell growth and xylose fermentation. Currently, the various solids samples are under characterization in the BioEnergy Science Center (BESC) using various techniques such as $^{13}$C NMR and $^{31}$P NMR. The characteristics of the solids samples might not correlate with fermentation inhibition directly. However, they might be able to give some clues, with what more experiments need to be done to justify hypotheses.

**10.4 Perspectives on the CBP process**

CBP has much potential in the cellulosic ethanol production. In this dissertation work, the performances of CBP using *C. phytofermentans* have been investigated at both low and high solids loadings. AFEX$^{TM}$ pretreated biomass has been proven to be a good substrate for CBP. The current major bottlenecks for this CBP process on AFEX$^{TM}$ pretreated biomass include slow glucan degradation, production of acetic acid, and low tolerances to acetate and ethanol. Genetic engineering of this strain for high expression of cellulases and less production of acetic acid is a possible way to make this CBP process economical for cellulosic ethanol production. Pretreatment improvement is another approach to achieve faster hydrolysis of biomass through CBP. For instance, our lab is currently developing an Extractive AFEX$^{TM}$ (E-AFEX$^{TM}$) process for converting cellulose I to cellulose III and at the same time remove part of the lignin in the biomass. Cellulose III has around 5 times higher enzyme digestibility compared to cellulose I

(Chundawat et al., 2011b) which is the typical cellulose type in regular AFEX$^{TM}$ treated biomass. Removal of lignin could also reduce the recalcitrance of the pretreated biomass.

# APPENDIX

# APPENDIX MODIFIED NREL 2011 TECHNO-ECONOMIC MODEL PARAMETERS

The modified NREL model is developed by Dr. Bryan Bals (BCRL, Michigan State University) based on NREL's 2011 model (please see Chapter V section 5.2.6). BCRL SSCF process with enzyme loading b is used here as an example.

## ***Model inputs***

| | | |
|---|---|---|
| | Biomass Composition | |
| Fraction of untreated | 1000 | g/kg untreated |
| Glucan | 341 | g/kg input |
| Xylan | 234 | g/kg input |
| Arabinan | 30 | g/kg input |
| Klason Lignon | 150 | g/kg input |
| Ash | 50 | g/kg input |
| Water Extractives | 80 | g/kg input |
| Other | 165 | g/kg input |
| | Enzymatic Hydrolysis | |
| Solid loading | 20.5 | % solids input |
| Cellulase Loading | 8.78 | mg/g Biomass |
| Hemicellulase Loading | 1.756 | mg/g Biomass |
| High viscosity residence time | 28.8 | hours |
| Low viscosity residence time | 0 | hours |
| Cellulase Cost | 3600 | $/Mg |
| Hemicellulase Cost | 4500 | $/Mg |
| Glucan to oligomer conversion | 6.7 | % glucan |
| Glucan to monomer conversion | 81.4 | % glucan |
| Xylan to oligomer conversion | 39.5 | % xylan |
| Xylan to monomer conversion | 59.8 | % xylan |
| Arabinan to oligomer conversion | 0 | % arabinan |
| Arabinan to monomer conversion | 0 | % arabinan |
| | Fermentation | |
| Residence Time | 28.8 | hours |
| Glucose Conversion | 97.7 | % |
| O-Glucose Conversion | 0 | % |
| Glucose Metabolic Yield | 0.47 | g ethanol/g sugar |
| Xylose Conversion | 72.2 | % |

| | | |
|---|---|---|
| O-Xylose Conversion | 0 | % |
| Xylose Metabolic Yield | 0.47 | g ethanol/g sugar |
| Arabinose Conversion | 0 | % |
| O-Arabinose Conversion | 0 | % |
| Arabinose Metabolic Yield | 0.45 | g ethanol/g sugar |
| Inoculum size | 10 | % of hydrolysate volume |
| Others | | |
| Biomass Input Price | 40 | $/Mg |
| Pre-processing Costs | 50 | $/Mg Untreated |
| Biorefinery Size | 2000 | Mg/day |
| Electricity selling price | 5.72 | cents/kW*h |
| Lignin selling price | 0 | $/Mg |
| Internal Rate of Return | 10 | % |
| Ethanol selling price | 2.5 | $/gal |
| Pretreatment Costs | | |
| Pretreatment installed cost | 0 | Million $ |
| Thermal Energy Use | 0 | GJ/Mg biomass |
| Electricity Use | 0 | kWh/Mg biomass |
| Ammonia Use | 22 | g/kg biomass |
| Ammonia Price | 0 | $/Mg |
| Other variable costs | 0 | $/Mg |

## ***Model outputs***

| | MESP | IRR |
|---|---|---|
| MESP | 2.254 | 2.500 |
| IRR | 0.10 | 1.03 |
| NPV | 0.0000 | 0.0000 |

| | | |
|---|---|---|
| Ethanol yield (kg/Mg) | 195.6 | 195.6 |
| Ethanol yield (gal/ton) | 59.41 | 59.41 |
| Ethanol volume (Mmgal/yr) | 45.89 | 45.89 |
| Ethanol titer (w/w)% | 4.63% | 4.63% |
| Net electricity (kWh/Mg) | 359.6 | 359.6 |
| Economic consideration | | |
| Fixed Capital Investment (MM$) | $275.77 | $275.77 |
| TCI/Annual gallon | $6.349 | $6.349 |
| Ethanol Selling Price | $2.25 | $2.50 |
| IRR | 10.0% | 102.9% |
| Feedstock Prod Cost ($/gal) | $0.611 | $0.611 |
| Feedstock Del Cost ($/gal) | $1.374 | $1.374 |
| Enzyme Cost ($/gal) | $0.603 | $0.603 |
| Fixed Costs ($/gal) | $0.148 | $0.148 |

| | | |
|---|---|---|
| Other Op Costs ($/gal) | $0.072 | $0.072 |

## ***Mass balance***

| | | |
|---|---|---|
| Initial Water | 3871.429 | g/kg solids |
| Mglucose | 308.4156 | g/kg solids |
| Oglucose | 23.48164 | g/kg solids |
| Mxylose | 159.0136 | g/kg solids |
| Oxylose | 95.58102 | g/kg solids |
| Marabinose | 0 | g/kg solids |
| Oarabinose | 0 | g/kg solids |
| Pglucose | 40.579 | g/kg solids |
| Pxylose | 1.638 | g/kg solids |
| Parabinose | 30 | g/kg solids |
| Solids | 271.717 | g/kg solids |
| Water in solids | 271.717 | g/kg solids |
| Water reacted | 53.70885 | g/kg solids |
| Water in hydrolysate | 3546.003 | g/kg solids |
| Water added | 487.1429 | |
| Water in fermentation | 4033.146 | |
| Ethanol | 195.581 | g/kg solids |
| Ethanol conc | 0.046251 | w/w |
| Ethanol yield | 0.247885 | L/kg dry BM |
| | 65.48441 | gal/Mg dry BM |
| | 59.40646 | gal/ton dry BM |
| REFERENCE | 79 | |
| FRACTION | 0.751981 | |

## ***Capital Cost***

| | | | | |
|---|---|---|---|---|
| Total water use in hydrolysate | 3546.0 | Lignin present | 271.717 | |
| Baseline reference | 4093 | Reference lignin | 273 | |
| Fraction of baseline | 0.8663 | Fraction of baseline | 0.99530 | |
| Size fraction | 1 | Lignin energy | 1187.3 | cal/g entering bm |
| Total fermentation volume | 4228.7 | Reference energy | 1167.6 | |
| Reference fermentation | 4479.55 | Fraction of baseline | 1.0169 | |
| Fraction | 0.9440 | | | |

| | | | | | WITH BOILER |
|---|---|---|---|---|---|
| Process Area 2007 dollors | Purchased | Installed | | | |
| Area 100: Feedstock handling | 14,200,000 | 24,200,000 | | 0 | 0 |
| Area 200: Pretreatment | 19,900,000 | 29,900,000 | | 0 | 0 |
| Area 200: Conditioning | 1,500,000 | 3,000,000 | | 0 | 0 |
| Area 300: Enzymatic hydrolysis and fermentation | 18,500,000 | 31,200,000 | | 19,758,956 | 19,758,956 |
| Area 400: Enzyme production | 10,700,000 | 18,300,000 | | 0 | 0 |
| Area 500: Recovery | 11,100,000 | 22,300,000 | 104,700,000 | 20,590,828 | 20,590,828 |
| Area 600: Wastewater | 49,300,000 | 49,400,000 | | 47,721,280 | 47,721,280 |
| Area 700: Storage | 2,800,000 | 5,000,000 | | 4,585,139 | 4,585,139 |
| Area 800: Boiler | 36,500,000 | 66,000,000 | | 65,918,232 | 65,918,232 |
| Area 900: Utilities | 4,000,000 | 6,900,000 | | 6,719,605 | 6,719,605 |
| Totals (excl. Area 100) | 154,500,000 | 232,100,000 | 232,000,000 | 165,294,040 | 165,294,040 |
| Warehouse 4.0% of ISBL | | 4,200,000 | 0 | 1,613,991 | 1,613,991 |
| Site development 9.0% of ISBL | | 9,400,000 | 0 | 3,631,481 | 3,631,481 |
| Additional piping 4.5% of ISBL | | 4,700,000 | 0 | 1,815,740 | 1,815,740 |
| Total Direct Costs (TDC) | | 250,400,000 | 232,000,000 | 172,355,252 | 172,355,252 |
| Prorateable expenses 10.0% of TDC | | 25,000,000 | | 17,235,525 | 17,235,525 |
| Field expenses 10.0% of TDC | | 25,000,000 | | 17,235,525 | 17,235,525 |
| Home office & construction fee 20.0% of TDC | | 50,100,000 | | 34,471,050 | 34,471,050 |
| Project contingency 10.0% of TDC | | 25,000,000 | | 17,235,525 | 17,235,525 |
| Other costs (start-up, permits, etc.) 10.0% of TDC | | 25,000,000 | | 17,235,525 | 17,235,525 |
| Total Indirect Costs | | 150,200,000 | | 103,413,151 | 103,413,151 |
| Fixed Capital Investment (FCI) | | 400,600,000 | | 275,768,403 | 275,768,403 |
| Land | | 1,800,000 | | 1,800,000 | 1,800,000 |
| Working capital 5.0% of FCI | | 20,000,000 | | 13,788,420 | 13,788,420 |
| Total Capital Investment (TCI) | | 422,500,000 | | 291,356,823 | 291,356,823 |
| Lang Factor (FCI/purchased equip cost)b 3.1 | | | | | |
| TCI per annual gallon | 6.92/gal | | | | 6 |

## ***Energy Balance***

| Ref | Modified | | | Difference | |
|---|---|---|---|---|---|
| 1167.6 | 1187.368 | cal/g entering biomass | lignin | | |
| 1008 | 1008 | cal/g entering biomass | other | | |
| 1740.48 | 1756.294 | cal/g steam | | | |
| | | | | | |
| 10.30804 | 0 | kWh/Mg | Area 1 | | |
| 68.16027 | 0 | kW/Mg | Area 2 | | |
| 31.63213 | 20.25089 | kW/Mg | Area 3 | | |
| 64.08026 | 0 | kW/Mg | Area 4 | | |
| 25.4881 | 25.4881 | kW/Mg | Area 5 | | |
| 88.41635 | 83.46566 | kW/Mg | Area 6 | | |
| 0.132001 | 0.099262 | kW/Mg | Area 7 | | |
| 16.41607 | 16.41607 | kW/Mg | Area 8 | | |
| 37.69215 | 37.69215 | kW/Mg | Area 9 | | |
| | | | | | |
| 342.3254 | 183.4121 | | | 158.913244 | |
| | | | | | |
| Heat requirement | | | | | |
| 1.959 | 1.784815 | GJ/Mg | | | |
| 0.1557 | 0 | | | | |
| | | | | | |
| 2.1147 | 1.784815 | | 0.329885 | 40.3196355 | kW*hr/Mg |
| 258.4654 | 218.1458 | | | | |
| | | | | | kW*hr/Mg |
| | | | | 199.232879 | saved |

## ***Economics***

| | | | | | |
|---|---|---|---|---|---|
| **FIXED COSTS** | | | | | |
| Salaries | | 1.9184 | | 1.7836 | MM$/yr |
| Benefits | | 1.72656 | | 1.60524 | MM$/yr |
| Maintenance | | 1.2104935 | | 1.210494 | MM$/yr |
| Insurance | | 1.9303788 | | 1.192095 | MM$/yr |
| | | 6.7858323 | | 5.791428 | |
| **Raw Material Costs** | | | | | |
| Ammonia | | 0 | | 0 | MM$/yr |
| Feedstock | | 63.075001 | 28.03333 | 63.075 | MM$/yr |
| Ash disposal | 1.5319 | 1.5247006 | | 0 | MM$/yr |
| A900 Items | | 0.38055 | | 0.38055 | MM$/yr |
| A800 Items | | 0.01034 | | 0 | MM$/yr |
| A300 Items | 2.154 | 1.0166955 | | 1.016696 | MM$/yr |
| A600 Item | 6.821508 | 0.3891044 | | 0.389104 | MM$/yr |
| Enzymes | 39.51 | 27.689928 | | 27.68993 | MM$/yr |
| | | 94.086319 | | 92.55128 | |

| | | | |
|---|---|---|---|
| **Other Revenue** | | | |
| Lignin | | 0 | |
| Electricity | 14.417414 | | |
| Ethanol production | 45.89366 | 45.89366 | MM gal/yr |

| ***Raw materials*** | | kg/hr | LB/HR | $/USton | $/hr | MM$ /yr | Cents /gal |
|---|---|---|---|---|---|---|---|
| | Feedstock | 104,167 | 229,688 | 46.8 | 5,374.69 | 45.2 | 74.07 |
| A200 | Sulfuric | 1,981 | 4,367 | 81.39 | 177.73 | 1.49 | 2.45 |
| | Ammonia | 1,051 | 2,317 | 406.96 | 471.48 | 3.97 | 6.5 |
| A300 | CSL | 1,158 | 2,554 | 51.55 | 65.84 | 0.55 | 0.91 |
| | Diammonium phosphate | 142 | 313 | 895.32 | 140.33 | 1.18 | 1.94 |
| | Sorbitol | 44 | 98 | 1,021.93 | 49.96 | 0.42 | 0.69 |
| A400 | Glucose | 2,418 | 5,332 | 526.52 | 1,403.60 | 11.8 | 19.34 |
| | CSL | 164 | 363 | 51.55 | 9.35 | 0.08 | 0.13 |
| | Ammonia | 115 | 254 | 406.96 | 51.59 | 0.43 | 0.71 |
| | Host nutrients | 67 | 149 | 745.3 | 55.34 | 0.47 | 0.76 |
| | Sulfur dioxide | 16 | 36 | 275.7 | 4.99 | 0.04 | 0.07 |
| A600 | Caustic | 2,252 | 4,966 | 135.65 | 336.83 | 2.83 | 4.64 |
| A800 | Boiler | <1 | 1 | 4,532.17 | 1.23 | 0.01 | 0.02 |
| | FGD lime | 895 | 1,973 | 180.87 | 178.42 | 1.5 | 2.46 |
| A900 | Cooling Tower chemicals | 2 | 5 | 2,716.10 | 7.14 | 0.06 | 0.1 |
| | Makeup water | 147,140 | 324,443 | 0.23 | 38.11 | 0.32 | 0.53 |
| Subtotal | | | | | 8,328.49 | 70.36 | 115.3 |
| Waste | disposal | | | | | | |
| A800 | Ash Disposal | 5,725 | 12,623 | 28.86 | 182.15 | 1.53 | 2.51 |
| Subtotal | | | | | 182.15 | 1.53 | 2.51 |
| By-products | and | credits | | | | | |
| | Grid Electricity | 12,797 | kW | $0.0572/kWh | 731.57 | 6.15 | 10.08 |
| | Area 100 Elec | 859 | kW | $0.0572/kWh | 49.13 | 0.41 | 0.68 |
| Subtotal | | | | | 780.71 | 6.57 | 10.76 |
| Total | variable | operating | costs | | 7,779.08 | 65.33 | 107.76 |

| ***Rate of return*** | Ethanol price | 2.25391 | | Equity | 0.4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IRR | 0.1 | | Interest | 0.14903 | | | | | | | |
| | Tax | 0.35 | | | 0.08 | | | | | | | |
| | TCI | 275.768 | | | | | | | | | | |
| Year | | -2 | -1 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Ethanol sales | | | | | 90.51 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 |
| Electricity sales | | | | | 12.62 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 |
| Fixed costs | | | | | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 |
| Operating costs | | | | | 85.12 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 |
| Depreciation | | | | | 0.14 | 0.24 | 0.17 | 0.12 | 0.09 | 0.09 | 0.09 | 0.04 |
| Depreciation | | | | | 0.04 | 0.07 | 0.07 | 0.06 | 0.06 | 0.05 | 0.05 | 0.05 |
| Real Depreciation | | | | | 2.47 | 4.76 | 4.40 | 4.07 | 3.77 | 3.48 | 3.22 | 2.98 |
| Real Depreciation | | | | | 29.99 | 51.39 | 36.70 | 26.21 | 18.74 | 18.72 | 18.74 | 9.36 |
| PWFv | | 8.82 | 66.18 | 35.30 | | | | | | | | |
| Other capital | | 1.80 | | 13.79 | | | | | | | | |
| Loan Payment | | 1.06 | 9.00 | 13.24 | 24.66 | 24.66 | 24.66 | 24.66 | 24.66 | 24.66 | 24.66 | 24.66 |
| Actual Interest | | | | | 13.24 | 12.32 | 11.34 | 10.27 | 9.12 | 7.88 | 6.53 | 5.08 |
| | | | | | 11.42 | 12.34 | 13.32 | 14.39 | 15.54 | 16.78 | 18.12 | 19.57 |
| | | | | | 154.04 | 141.70 | 128.38 | 113.99 | 98.45 | 81.67 | 63.55 | 43.97 |
| PWFc | | 1.21 | 1.10 | 1.00 | 0.91 | 0.83 | 0.75 | 0.68 | 0.62 | 0.56 | 0.51 | 0.47 |
| Net Revenue | | | | | -34.48 | -51.49 | -35.45 | -23.57 | -14.64 | -13.09 | -11.51 | -0.44 |
| Losses Forward | | | | | | -34.48 | -85.97 | -121.42 | -144.99 | -159.63 | -172.72 | -184.23 |
| Taxable Income | | | | | -34.48 | -85.97 | -121.42 | -144.99 | -159.63 | -172.72 | -184.23 | -184.67 |
| Income Tax | | | | | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Annual Cash Income | | | | | -13.44 | -7.67 | -7.67 | -7.67 | -7.67 | -7.67 | -7.67 | -7.67 |
| | | | | | -12.22 | -6.34 | -5.76 | -5.24 | -4.76 | -4.33 | -3.94 | -3.58 |
| -159.165 | | 14.14 | 82.70 | 62.32 | **0.00** | | | | | | | |

| Year | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol sales | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 |
| Electricity sales | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 |
| Fixed costs | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 |
| Operating costs | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 |
| Depreciation | | | | | | | | | | | | |
| Depreciation | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 |
| Real Depreciation | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 | 2.94 |
| Real Depreciation | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| PWFv | | | | | | | | | | | | |
| Other capital | | | | | | | | | | | | |
| Loan Payment | 24.66 | 24.66 | | | | | | | | | | |
| Actual Interest | 3.52 | 1.83 | | | | | | | | | | |
| | 21.14 | 22.83 | | | | | | | | | | |
| | 22.83 | 0.00 | | | | | | | | | | |
| PWFc | 0.42 | 0.39 | 0.35 | 0.32 | 0.29 | 0.26 | 0.24 | 0.22 | 0.20 | 0.18 | 0.16 | 0.15 |
| Net Revenue | 10.53 | 12.22 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 | 14.04 |
| | - | - | - | - | - | - | - | | | | | |
| Losses Forward | 184.67 | 174.14 | 161.92 | 147.88 | 133.83 | 119.79 | 105.75 | -91.70 | -77.66 | -63.61 | -49.57 | -35.52 |
| | - | - | - | - | - | - | | | | | | |
| Taxable Income | 174.14 | 161.92 | 147.88 | 133.83 | 119.79 | 105.75 | -91.70 | -77.66 | -63.61 | -49.57 | -35.52 | -21.48 |
| | | | | | | | | | | | | |
| Income Tax | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Annual Cash | | | | | | | | | | | | |
| Income | -7.67 | -7.67 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 |
| | -3.25 | -2.96 | 5.95 | 5.41 | 4.92 | 4.47 | 4.07 | 3.70 | 3.36 | 3.05 | 2.78 | 2.52 |

| Year | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol sales | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 | 103.44 |
| Electricity sales | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 | 14.42 |
| Fixed costs | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 | 6.79 |
| Operating costs | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 | 94.09 |
| Depreciation | | | | | | | | | | |
| Depreciation | 0.02 | | | | | | | | | |
| Real Depreciation | 1.47 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Real Depreciation | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| PWFv | | | | | | | | | | |
| Other capital | | | | | | | | | | 0.00 |
| Loan Payment | | | | | | | | | | |
| Actual Interest | | | | | | | | | | |
| | | | | | | | | | | |
| PWFc | 0.14 | 0.12 | 0.11 | 0.10 | 0.09 | 0.08 | 0.08 | 0.07 | 0.06 | 0.15 |
| Net Revenue | 15.51 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 |
| Losses Forward | -21.48 | -5.96 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Taxable Income | -5.96 | 11.02 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 | 16.99 |
| | | | | | | | | | | |
| Income Tax | 0.00 | 3.86 | 5.94 | 5.94 | 5.94 | 5.94 | 5.94 | 5.94 | 5.94 | 5.94 |
| Annual Cash Income | 16.99 | 13.13 | 11.04 | 11.04 | 11.04 | 11.04 | 11.04 | 11.04 | 11.04 | 11.04 |
| | 2.30 | 1.61 | 1.23 | 1.12 | 1.02 | 0.93 | 0.84 | 0.77 | 0.70 | 1.64 |

## ***CAPEX***

| | | | | |
|---|---|---|---|---|
| Dependent on solid loading and hyd/fermentation time | | | | |
| A-300 | Ethanol Fermentor Agitator | 1 | $951,337 | |
| A-306 | Beer Surge Tank Agitator | 0.5 | $106,105 | |
| F-300 | Ethanol Fermentor | 1 | $15,293,882 | |
| | | 0.9968 | $16,351,324 | 0.9968 |
| Dependent on viscous hydrolysate residence time and solid loading | | | | |
| T-310 | Saccharification Tank | 0.7 | $8,006,772 | |
| | | 0.7 | $8,006,772 | 0.7000 |
| Dependent on fermentation OD | | | | |
| A-301 | Seed Hold Tank Agitator | 0.5 | $49,305 | 24652.5000 |
| A-304 | 4th Seed Vessel agitator | 0.5 | $39,262 | 19631.0000 |
| A-305 | 5th Seed vessel agitator | 0.5 | $64,933 | 32466.5000 |
| F-301 | 1st Seed fermentor | 0.7 | $136,630 | 95641.0000 |
| F-302 | 2nd Seed fermentor | 0.7 | $211,288 | 147901.6000 |
| F-303 | 3rd Seed fermentor | 0.7 | $285,582 | 199907.4000 |
| F-304 | 4th Seed fermentor | 0.7 | $708,721 | 496104.7000 |
| F-305 | 5th Seed fermentor | 0.7 | $2,375,827 | 1663078.9000 |
| P-301 | Seed Hold transfer pump | 0.8 | $18,795 | 15036.0000 |
| P-302 | Seed Transfer pump | 0.8 | $55,698 | 44558.4000 |
| T-301 | Seed Hold tank | 0.7 | $825,471 | 577829.7000 |
| | | | $4,771,512 | 0.6951 |
| Depends on solid loading only | | | | |
| H-300 | Fermentation Cooler | 1 | $192,524 | 192524.0000 |
| H-301 | Hydrolyzate Cooler | 0.7 | $214,500 | 150150.0000 |
| H-310 | Fermentor Batch cooler | 0.7 | $0 | 0.0000 |
| P-300 | Fermentation Recirc pump | 0.8 | $109,288 | 87430.4000 |
| P-306 | Beer Transfer pump | 0.8 | $58,165 | 46532.0000 |
| P-310 | Saccharification Tansfer pump | 0.8 | $113,746 | 90996.8000 |
| T-306 | Beer Storage tank | 0.7 | $1,199,170 | 839419.0000 |
| A-308 | Enzyme-Hydrolysate Mixer | 0.5 | $201,519 | 100759.5000 |
| | | | $2,088,912 | 0.7218 |

# REFERENCES

# REFERENCES


Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, **29**(6), 675-685.

Alexande.Jk. 1968. Purification and specificity of cellobiose phosphorylase from *Clostridium thermocellum*. *Journal of Biological Chemistry*, **243**(11), 2899-2904.

Alkasrawi, M., Eriksson, T., Borjesson, J., Wingren, A., Galbe, M., Tjerneld, F., Zacchi, G. 2003. The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol. *Enzyme and Microbial Technology*, **33**(1), 71-78.

Almeida, J.R.M., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G., Gorwa-Grauslund, M.F. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemical Technology & Biotechnology*, **82**(4), 340-349.

Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., Foden, J.M., Miller, B.B., Lynd, L.R., Hogsett, D.A., Caiazza, N.C. 2011. High Ethanol Titers from Cellulose by Using Metabolically Engineered Thermophilic, Anaerobic Microbes. *Applied and Environmental Microbiology*, **77**(23), 8288-8294.

Azevedo, H., Bishop, D., Cavaco-Paulo, A. 2002. Possibilities for recycling cellulases after use in cotton processing part II: Separation of cellulases from reaction products and released dyestuffs by ultrafiltration. *Applied Biochemistry and Biotechnology*, **101**(1), 77-91.

Bai, F.W., Anderson, W.A., Moo-Young, M. 2008. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances*, **26**(1), 89-105.

Balan, V., Bals, B., Chundawat, S.P.S., Marshall, D., Dale, B.E. 2010. Lignocellulosic Biomass Pretreatment Using AFEX. in: *Biofuels*, Vol. 581, pp. 61-77.

Bals, B., Rogers, C., Jin, M., Balan, V., Dale, B. 2010. Evaluation of ammonia fibre expansion (AFEX) pretreatment for enzymatic hydrolysis of switchgrass harvested in different seasons and locations. *Biotechnology for Biofuels*, **3**, 1.

Bals, B.D., Dale, B.E. 2012. Developing a model for assessing biomass processing technologies within a local biomass processing depot. *Bioresource Technology*, **106**(0), 161-169.

Banerjee, G., Scott-Craig, J.S., Walton, J.D. 2010. Improving Enzymes for Biomass Conversion: A Basic Research Perspective. *Bioenergy Research*, **3**(1), 82-92.

Bellissimi, E., van Dijken, J.P., Pronk, J.T., van Maris, A.J.A. 2009. Effects of acetic acid on the kinetics of xylose fermentation by an engineered, xylose-isomerase-based Saccharomyces cerevisiae strain. *Fems Yeast Research*, **9**(3), 358-364.

Bengtsson, O., Hahn-Hägerdal, B., Gorwa-Grauslund, M.F. 2009. Xylose reductase from Pichia stipitis with altered coenzyme preference improves ethanolic xylose fermentation by recombinant Saccharomyces cerevisiae. *Biotechnology for Biofuels*, **2**(1), 1-10.

Bertilsson, M., Andersson, J., Liden, G. 2008. Modeling simultaneous glucose and xylose uptake in *Saccharomyces cerevisiae* from kinetics and gene expression of sugar transporters. *Bioprocess and Biosystems Engineering*, **31**(4), 369-377.

Bertilsson, M., Olofsson, K., Liden, G. 2009. Prefermentation improves xylose utilization in simultaneous saccharification and co-fermentation of pretreated spruce. *Biotechnology for Biofuels*, **2**(1), 8.

Brethauer, S., Wyman, C.E. 2010. Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. *Bioresource Technology*, **101**(13), 4862-4874.

Cara, C., Moya, M., Ballesteros, I., Negro, M.J., González, A., Ruiz, E. 2007. Influence of solid loading on enzymatic hydrolysis of steam exploded or liquid hot water pretreated olive tree biomass. *Process Biochemistry*, **42**(6), 1003-1009.

Carere, C.R., Sparling, R., Cicek, N., Levin, D.B. 2008. Third generation biofuels via direct cellulose fermentation. *International Journal of Molecular Sciences*, **9**(7), 1342-1360.

Cavedon, K., Leschine, S., Canale-Parola, E. 1990. Cellulase system of a free-living, mesophilic clostridium (strain C7). *Journal of bacteriology*, **172**(8), 4222.

Chundawat, S., Beckham, G., Himmel, M., Dale, B. 2011a. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annu Rev Chem Biomol Eng*, **2**, 121-145.

Chundawat, S.P.S., Bellesia, G., Uppugundla, N., Sousa, L.D., Gao, D.H., Cheh, A.M., Agarwal, U.P., Bianchetti, C.M., Phillips, G.N., Langan, P., Balan, V., Gnanakaran, S., Dale, B.E. 2011b. Restructuring the Crystalline Cellulose Hydrogen Bond Network Enhances Its Depolymerization Rate. *Journal of the American Chemical Society*, **133**(29), 11163-11174.

Chundawat, S.P.S., Donohoe, B.S., Sousa, L.D., Elder, T., Agarwal, U.P., Lu, F.C., Ralph, J., Himmel, M.E., Balan, V., Dale, B.E. 2011c. Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. *Energy & Environmental Science*, **4**(3), 973-984.

Chundawat, S.P.S., Venkatesh, B., Dale, B.E. 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. *Biotechnology and Bioengineering*, **96**(2), 219-231.

Chundawat, S.P.S., Vismeh, R., Sharma, L.N., Humpula, J.F., Sousa, L.D., Chambliss, C.K., Jones, A.D., Balan, V., Dale, B.E. 2010. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresource Technology*, **101**(21), 8429-8438.

Crous, J.M., Pretorius, I.S., Vanzyl, W.H. 1995. Cloning and Expression of an Aspergillus-Kawachii Endo-1,4-Beta-Xylanase Gene in Saccharomyces-Cerevisiae. *Current Genetics*, **28**(5), 467-473.

Demain, A.L., Newcomb, M., Wu, J.H.D. 2005. Cellulase, clostridia, and ethanol. *Microbiology and Molecular Biology Reviews*, **69**(1), 124-154.

Den Haan, R., McBride, J.E., La Grange, D.C., Lynd, L.R., Van Zyl, W.H. 2007. Functional expression of cellobiohydrolases in *Saccharomyces cerevisiae* towards one-step conversion of cellulose to ethanol. *Enzyme and Microbial Technology*, **40**(5), 1291-1299.

Desvaux, M. 2006. Unravelling carbon metabolism in anaerobic cellulolytic bacteria. *Biotechnology Progress*, **22**(5), 1229-1238.

Diderich, J.A., Schepper, M., van Hoek, P., Luttik, M.A.H., van Dijken, J.P., Pronk, J.T., Klaassen, P., Boelens, H.F.M., de Mattos, R.J.T., van Dam, K., Kruckeberg, A.L. 1999. Glucose uptake kinetics and transcription of HXT genes chemostat cultures of *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, **274**(22), 15350-15359.

Dien, B., Cotta, M., Jeffries, T. 2003a. Bacteria engineered for fuel ethanol production: current status. *Applied Microbiology and Biotechnology*, **63**(3), 258-266.

Dien, B.S., Cotta, M.A., Jeffries, T.W. 2003b. Bacteria engineered for fuel ethanol production: current status. *Applied Microbiology and Biotechnology*, **63**(3), 258-266.

Dien, B.S., Ximenes, E.A., O'Bryan, P.J., Moniruzzaman, M., Li, X.L., Balan, V., Dale, B., Cotta, M.A. 2008. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol. *Bioresource Technology*, **99**(12), 5216-5225.

Eggeman, T., Elander, R.T. 2005. Process and economic analysis of pretreatment technologies. *Bioresource Technology*, **96**(18), 2019-2025.

Eriksson, T., Borjesson, J., Tjerneld, F. 2002a. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. *Enzyme and Microbial Technology*, **31**(3), 353-364.

Eriksson, T., Karlsson, J., Tjerneld, F. 2002b. A model explaining declining rate in hydrolysis of lignocellulose substrates with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. *Applied Biochemistry and Biotechnology*, **101**(1), 41-60.

Farrell, A., Plevin, R., Turner, B., Jones, A., O'hare, M., Kammen, D. 2006. Ethanol can contribute to energy and environmental goals. *Science*, **311**(5760), 506.

Fogler, H.S. 1999. *Elements of chemical reaction engineering*. Prentice-Hall International London.

Fujita, Y., Ito, J., Ueda, M., Fukuda, H., Kondo, A. 2004. Synergistic saccharification, and direct fermentation to ethanol, of amorphous cellulose by use of an engineered yeast strain

codisplaying three types of cellulolytic enzyme. *Applied and Environmental Microbiology*, **70**(2), 1207-1212.

Gao, D., Chundawat, S.P.S., Krishnan, C., Balan, V., Dale, B.E. 2010. Mixture optimization of six core glycosyl hydrolases for maximizing saccharification of ammonia fiber expansion (AFEX) pretreated corn stover. *Bioresource Technology*, **101**(8), 2770-2781.

Gao, D.H., Chundawat, S.P.S., Uppugundla, N., Balan, V., Dale, B.E. 2011. Binding Characteristics of Trichoderma reesei Cellulases on Untreated, Ammonia Fiber Expansion (AFEX), and Dilute-Acid Pretreated Lignocellulosic Biomass. *Biotechnology and Bioengineering*, **108**(8), 1788-1800.

Girio, F.M., Fonseca, C., Carvalheiro, F., Duarte, L.C., Marques, S., Bogel-Lukasik, R. 2010. Hemicelluloses for fuel ethanol: A review. *Bioresource Technology*, **101**(13), 4775-4800.

Görke, B., Stülke, J. 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nature Reviews Microbiology*, **6**(8), 613-624.

Govindaswamy, S., Vane, L.M. 2010. Multi-stage continuous culture fermentation of glucose-xylose mixtures to fuel ethanol using genetically engineered Saccharomyces cerevisiae 424A. *Bioresource Technology*, **101**(4), 1277-1284.

Gray, K.A., Zhao, L.S., Emptage, M. 2006. Bioethanol. *Current Opinion in Chemical Biology*, **10**(2), 141-146.

Greene, N., Council, N.R.D. 2004. *Growing energy: How biofuels can help end America's oil dependence*. Natural Resources Defense Council New York, NY.

Gusakov, A.V., Sinitsyn, A.P. 1992. A theoretical-analysis of cellulase product inhibition-effect of cellulase binding constant, enzyme substrate ratio, and beta-glucosidase activity on the inhibition pattern. *Biotechnology and Bioengineering*, **40**(6), 663-671.

Ha, S.J., Galazka, J.M., Rin Kim, S., Choi, J.H., Yang, X., Seo, J.H., Louise Glass, N., Cate, J.H.D., Jin, Y.S. 2011. Engineered *Saccharomyces cerevisiae* capable of simultaneous cellobiose and xylose fermentation. *Proceedings of the National Academy of Sciences*, **108**(2), 504.

Hahn-Hagerdal, B., Karhumaa, K., Fonseca, C., Spencer-Martins, I., Gorwa-Grauslund, M.F. 2007. Towards industrial pentose-fermenting yeast strains. *Applied Microbiology and Biotechnology*, **74**(5), 937-953.

Hallac, B.B., Ragauskas, A.J. 2011. Analyzing cellulose degree of polymerization and its relevancy to cellulosic ethanol. *Biofuels Bioproducts & Biorefining-Biofpr*, **5**(2), 215-225.

Hamelinck, C.N., Hooijdonk, G.v., Faaij, A.P.C. 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. *Biomass and Bioenergy*, **28**(4), 384-410.

Hansen, A.C., Zhang, Q., Lyne, P.W.L. 2005. Ethanol-diesel fuel blends - a review. *Bioresource Technology*, **96**(3), 277-285.

Ho, N., Chen, Z., Brainard, A., Sedlak, M. 1999. Successful Design and Development of Genetically Engineered *Saccharomyces* Yeasts for Effective Cofermentation of Glucose and Xylose from Cellulosic Biomass to Fuel Ethanol. in: *Recent Progress in Bioconversion of Lignocellulosics*, pp. 163-192.

Hodge, D.B., Karim, M.N., Schell, D.J., McMillan, J.D. 2008. Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresource Technology*, **99**(18), 8940-8948.

Houghton, J.T., Ding, Y., Griggs, D.J., Noguer, M., Van der Linden, P.J., Dai, X., Maskell, K., Johnson, C.A. 2001. Climate Change 2001: the scientific basis. Contribution of Working Group I to the Third Assessment Report of the Intergovernmental Panel on Climate Change (IPCC). . *Cambridge University Press, UK*.

Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A. 2011. Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol. *NREL report (NREL/TP-5700-47764)*.

Ilmen, M., den Haan, R., Brevnova, E., McBride, J., Wiswall, E., Froehlich, A., Koivula, A., Voutilainen, S.P., Siika-Aho, M., la Grange, D.C., Thorngren, N., Ahlgren, S., Mellon, M., Deleault, K., Rajgarhia, V., van Zyl, W.H., Penttila, M. 2011. High level secretion of cellobiohydrolases by Saccharomyces cerevisiae. *Biotechnology for Biofuels*, **4**.

Ingram, L.O., Aldrich, H.C., Borges, A.C.C., Causey, T.B., Martinez, A., Morales, F., Saleh, A., Underwood, S.A., Yomano, L.P., York, S.W., Zaldivar, J., Zhou, S.D. 1999. Enteric bacterial catalysts for fuel ethanol production. *Biotechnology Progress*, **15**(5), 855-866.

Jeffries, T., Grigoriev, I., Grimwood, J., Laplaza, J., Aerts, A., Salamov, A., Schmutz, J., Lindquist, E., Dehal, P., Shapiro, H. 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast Pichia stipitis. *Nature Biotechnology*, **25**(3), 319-326.

Jørgensen, H., Vibe‐Pedersen, J., Larsen, J., Felby, C. 2007. Liquefaction of lignocellulose at high‐solids concentrations. *Biotechnology and Bioengineering*, **96**(5), 862-870.

Kabel, M.A., van der Maarel, M., Klip, G., Voragen, A.G.J., Schols, H.A. 2006. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant materials. *Biotechnology and Bioengineering*, **93**(1), 56-63.

Kim, S.B., Chun, J.W. 2004. Enhancement of enzymatic digestibility of recycled newspaper by addition of surfactant in ammonia-hydrogen peroxide pretreatment. *Applied Biochemistry and Biotechnology*, **113**, 1023-1031.

Kim, S.B., Kim, H.J., Kim, C.J. 2006. Enhancement of the enzymatic digestibility of waste newspaper using tween. *Applied Biochemistry and Biotechnology*, **130**(1-3), 486-495.

Krishnan, C., Sousa, L.D., Jin, M.J., Chang, L.P., Dale, B.E., Balan, V. 2010. Alkali-Based AFEX Pretreatment for the Conversion of Sugarcane Bagasse and Cane Leaf residues to Ethanol. *Biotechnology and Bioengineering*, **107**(3), 441-450.

Kristensen, J.B., Felby, C., Jorgensen, H. 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. *Biotechnology for Biofuels*, **2**, 11.

Kumar, R., Wyman, C.E. 2009a. Does change in accessibility with conversion depend on both the substrate and pretreatment technology? *Bioresource Technology*, **100**(18), 4193-4202.

Kumar, R., Wyman, C.E. 2009b. Effect of xylanase supplementation of cellulase on digestion of corn stover solids prepared by leading pretreatment technologies. *Bioresource Technology*, **100**(18), 4203-4213.

Kuyper, M., Hartog, M.M.P., Toirkens, M.J., Almering, M.J.H., Winkler, A.A., van Dijken, J.P., Pronk, J.T. 2005. Metabolic engineering of a xylose-isomerase-expressing Saccharomyces cerevisiae strain for rapid anaerobic xylose fermentation. *Fems Yeast Research*, **5**(4-5), 399-409.

Kuyper, M., Winkler, A.A., Dijken, J.P., Pronk, J.T. 2004. Minimal metabolic engineering of Saccharomyces cerevisiae for efficient anaerobic xylose fermentation: a proof of principle. *Fems Yeast Research*, **4**(6), 655-664.

Lau, M.W., Bals, B.D., Chundawat, S.P.S., Jin, M., Gunawan, C., Balan, V., Jones, A.D., E, D.B. 2012. An integrated paradigm for cellulosic biorefineries: utilization of lignocellulosic biomass as self-sufficient feedstocks for fuel, food precursors and saccharolytic enzyme production. *Energy & Environmental Science*, **DOI: 10.1039/C2EE03596K.**

Lau, M.W., Dale, B.E. 2009. Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST). *Proceedings of the National Academy of Sciences of the United States of America*, **106**(5), 1368-1373.

Lau, M.W., Gunawan, C., Balan, V., Dale, B.E. 2010. Comparing the fermentation performance of Escherichia coli KO11, Saccharomyces cerevisiae 424A(LNH-ST) and Zymomonas mobilis AX101 for cellulosic ethanol production. *Biotechnology for Biofuels*, **3**, 11.

Li, B.Z., Balan, V., Yuan, Y.J., Dale, B.E. 2010. Process optimization to convert forage and sweet sorghum bagasse to ethanol based on ammonia fiber expansion (AFEX) pretreatment. *Bioresource Technology*, **101**(4), 1285-1292.

Lu, Y.L., Warner, R., Sedlak, M., Ho, N., Mosier, N.S. 2009. Comparison of Glucose/Xylose Cofermentation of Poplar Hydrolysates Processed by Different Pretreatment Technologies. *Biotechnology Progress*, **25**(2), 349-356.

Lu, Y.P., Zhang, Y.H.P., Lynd, L.R. 2006. Enzyme-microbe synergy during cellulose hydrolysis by Clostridium thermocellum. *Proceedings of the National Academy of Sciences of the United States of America*, **103**(44), 16165-16169.

Luo, L., van der Voet, E., Huppes, G. 2009. An energy analysis of ethanol from cellulosic feedstock-Corn stover. *Renewable & Sustainable Energy Reviews*, **13**(8), 2003-2011.

Lynd, L.R. 1996. Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy. *Annual review of energy and the environment*, **21**(1), 403-465.

Lynd, L.R., Baskaran, S., Casten, S. 2001. Salt accumulation resulting from base added for pH control, and not ethanol, limits growth of Thermoanaerobacterium thermosaccharolyticum HG-8 at elevated feed xylose concentrations in continuous culture. *Biotechnology Progress*, **17**(1), 118-125.

Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Current Opinion in Biotechnology*, **16**(5), 577-583.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, **66**(3), 506-577.

Madhavan, A., Tamalampudi, S., Ushida, K., Kanai, D., Katahira, S., Srivastava, A., Fukuda, H., Bisaria, V.S., Kondo, A. 2009. Xylose isomerase from polycentric fungus Orpinomyces: gene sequencing, cloning, and expression in Saccharomyces cerevisiae for bioconversion of xylose to ethanol. *Applied Microbiology and Biotechnology*, **82**(6), 1067-1078.

Mann, L., Tolbert, V., Cushman, J. 2002. Potential environmental effects of corn (Zea mays L.) stover removal with emphasis on soil organic matter and erosion. *Agriculture Ecosystems & Environment*, **89**(3), 149-166.

McBee, R.H. 1954. The characteristics of clostridium thermocellum. *Journal of Bacteriology*, **67**(4), 505-506.

Medina, V.G., Almering, M.J.H., van Maris, A.J.A., Pronk, J.T. 2010. Elimination of Glycerol Production in Anaerobic Cultures of a Saccharomyces cerevisiae Strain Engineered To Use Acetic Acid as an Electron Acceptor. *Applied and Environmental Microbiology*, **76**(1), 190-195.

Mohagheghi, A., Evans, K., Chou, Y.C., Zhang, M. 2002. Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of Zymomonas mobilis AX101. *Applied Biochemistry and Biotechnology*, **98**(1), 885-898.

Mohagheghi, A., Tucker, M., Grohmann, K., Wyman, C. 1992. High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. *Applied Biochemistry and Biotechnology*, **33**(2), 67-81.

Morag, E., Bayer, E.A., Lamed, R. 1990. Relationship of cellulosomal and noncellulosomal xylanases of Clostridium thermocellum to cellulose-degrading enzymes, Vol. 172, pp. 6098-6105.

Morales-Rodriguez, R., Gernaey, K.V., Meyer, A.S., Sin, G. 2011. A Mathematical Model for Simultaneous Saccharification and Co-fermentation (SSCF) of C6 and C5 Sugars. *Chinese Journal of Chemical Engineering*, **19**(2), 185-191.

Ng, T.K., Benbassat, A., Zeikus, J.G. 1981. Ethanol-Production by Thermophilic Bacteria - Fermentation of Cellulosic Substrates by Cocultures of Clostridium-Thermocellum and Clostridium-Thermohydrosulfuricum. *Applied and Environmental Microbiology*, **41**(6), 1337-1343.

Olofsson, K., Bertilsson, M., Liden, G. 2008a. A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnology for Biofuels*, **1**.

Olofsson, K., Rudolf, A., Liden, G. 2008b. Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of Saccharomyces cerevisiae. *Journal of Biotechnology*, **134**(1-2), 112-120.

Olsson, L., Hahn-Hägerdal, B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme and Microbial Technology*, **18**(5), 312-331.

Olsson, L., Hahn Hägerdal, B., Zacchi, G. 1995. Kinetics of ethanol production by recombinant Escherichia coli KO11. *Biotechnology and Bioengineering*, **45**(4), 356-365.

Pan, X. 2008. Role of functional groups in lignin inhibition of enzymatic hydrolysis of cellulose to glucose. *Journal of Biobased Materials and Bioenergy*, **2**(1), 25-32.

Panagiotou, G., Olsson, L. 2007. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. *Biotechnology and Bioengineering*, **96**(2), 250-258.

Perlack, R.D., Wright, L.L., Turhollow, A.F., Graham, R.L., Stokes, B.J., Erbach, D.C. 2005. Biomass as feedstock for a bioenergy and bioproducts industry: the technical feasibility of a billion-ton annual supply. *http://www.osti.gov/bridge*.

Philippidis, G.P., Smith, T.K. 1995. Limiting Factors in the Simultaneous Saccharification and Fermentation Process for Conversion of Cellulosic Biomass to Fuel Ethanol. pp. 117-124.

Pimenova, N.V., Hanley, T.R. 2004. Effect of corn stover concentration on rheological characteristics. *Applied Biochemistry and Biotechnology*, **114**(1), 347-360.

Poth, S., Monzon, M., Tippkotter, N., Ulber, R. 2011. Lignocellulosic biorefinery: process integration of hydrolysis and fermentation (SSF process). *Holzforschung*, **65**(5), 633-637.

Qing, Q., Yang, B., Wyman, C.E. 2010. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. *Bioresource Technology*, **101**(24), 9624-9630.

Reinikainen, T., Ruohonen, L., Nevanen, T., Laaksonen, L., Kraulis, P., Jones, T.A., Knowles, J.K.C., Teeri, T.T. 1992. Investigation of the Function of Mutated Cellulose-Binding

Domains of Trichoderma-Reesei Cellobiohydrolase-I. *Proteins-Structure Function and Genetics*, **14**(4), 475-482.

Ren, C., Chen, T., Zhang, J., Liang, L., Lin, Z. 2009. An evolved xylose transporter from Zymomonas mobilis enhances sugar transport in Escherichia coli. *Microbial Cell Factories*, **8**, 66-66.

Romanos, M.A., Makoff, A.J., Fairweather, N.F., Beesley, K.M., Slater, D.E., Rayment, F.B., Payne, M.M., Clare, J.J. 1991. Expression of Tetanus Toxin Fragment-C in Yeast - Gene Synthesis Is Required to Eliminate Fortuitous Polyadenylation Sites in at-Rich DNA. *Nucleic Acids Research*, **19**(7), 1461-1467.

Rudolf, A., Alkasrawi, M., Zacchi, G., Liden, G. 2005. A comparison between batch and fed-batch simultaneous saccharification and fermentation of steam pretreated spruce. *Enzyme and Microbial Technology*, **37**(2), 195-204.

Ruohonen, L., Aristidou, A., Frey, A.D., Penttila, M., Kallio, P.T. 2006. Expression of Vitreoscilla hemoglobin improves the metabolism of xylose in recombinant yeast Saccharomyces cerevisiae under low oxygen conditions. *Enzyme and Microbial Technology*, **39**(1), 6-14.

Saha, B.C. 2003. Hemicellulose bioconversion. *Journal of industrial microbiology & biotechnology*, **30**(5), 279-291.

Schmer, M.R., Vogel, K.P., Mitchell, R.B., Perrin, R.K. 2008. Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(2), 464-469.

Sedlak, M., Ho, N.W.Y. 2004. Production of ethanol from cellulosic biomass hydrolysates using genetically engineered Saccharomyces yeast capable of cofermenting glucose and xylose. pp. 403-416.

Sendich, E., Laser, M., Kim, S., Alizadeh, H., Laureano-Perez, L., Dale, B., Lynd, L. 2008. Recent process improvements for the ammonia fiber expansion (AFEX) process and resulting reductions in minimum ethanol selling price. *Bioresource Technology*, **99**(17), 8429-8435.

Shao, Q., Chundawat, S.P.S., Krishnan, C., Bals, B., Sousa, L.D., Thelen, K.D., Dale, B.E., Balan, V. 2010. Enzymatic digestibility and ethanol fermentability of AFEX-treated starch-rich lignocellulosics such as corn silage and whole corn plant. *Biotechnology for Biofuels*, **3**.

Shao, X., Jin, M., Guseva, A., Liu, C., Balan, V., Hogsett, D., Dale, B.E., Lynd, L. 2011. Conversion for Avicel and AFEX pretreated corn stover by Clostridium thermocellum and simultaneous saccharification and fermentation: Insights into microbial conversion of pretreated cellulosic biomass. *Bioresource Technology*, **102**(17), 8040-8045.

Shaw, A.J., Podkaminer, K.K., Desai, S.G., Bardsley, J.S., Rogers, S.R., Thorne, P.G., Hogsett, D.A., Lynd, L.R. 2008. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proceedings of the National Academy of Sciences of the United States of America*, **105**(37), 13769-13774.

Skoog, K., Hahn-Hagerdal, B. 1990. Effect of oxygenation on xylose fermentation by Pichia stipitis. *Applied and Environmental Microbiology*, **56**(11), 3389.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2004. Determination of structural carbohydrates and lignin in biomass. *NREL, Golden, CO*.

Somerville, C., Bauer, S., Brininstool, G., Facette, M., Hamann, T., Milne, J., Osborne, E., Paredez, A., Persson, S., Raab, T. 2004. Toward a systems approach to understanding plant cell walls. *Science's STKE*, **306**(5705), 2206.

Taherzadeh, M.J., Karimi, K. 2007. Enzyme-Based Hydrolysis Processes for Ethanol from Lignocellulosic Materials: a Review. *Bioresources*, **2**(4), 707-738.

Tu, M.B., Chandra, R.P., Saddler, J.N. 2007. Recycling cellulases during the hydrolysis of steam exploded and ethanol pretreated lodgepole pine. *Biotechnology progress*, **23**(5), 1130-1137.

Tu, M.B., Zhang, X., Paice, M., McFarlane, P., Saddler, J.N. 2009. Effect of Surfactants on Separate Hydrolysis Fermentation and Simultaneous Saccharification Fermentation of Pretreated Lodgepole Pine. *Biotechnology progress*, **25**(4), 1122-1129.

Van Zyl, C., Prior, B.A., Kilian, S.G., Kock, J.L.F. 1989. D-xylose utilization by Saccharomyces cerevisiae. *Microbiology*, **135**(11), 2791.

Vanarsdell, J.N., Kwok, S., Schweickart, V.L., Ladner, M.B., Gelfand, D.H., Innis, M.A. 1987. Cloning, Characterization, and Expression in *Saccharomyces-Cerevisiae* of Endoglucanase-I from Trichoderma-Reesei. *Bio-Technology*, **5**(1), 60-64.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W. 2010. Lignin biosynthesis and structure. *Plant physiology*, **153**(3), 895-905.

Wahlbom, C.F., Hahn-Hägerdal, B. 2002. Furfural, 5-hydroxymethyl furfural, and acetoin act as external electron acceptors during anaerobic fermentation of xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, **78**(2), 172-178.

Walsh, M.E., Ugarte, D.G.D., Shapouri, H., Slinsky, S.P. 2003. Bioenergy crop production in the United States - Potential quantities, land use changes, and economic impacts on the agricultural sector. *Environmental & Resource Economics*, **24**(4), 313-333.

Warnick, T.A., Methe, B.A., Leschine, S.B. 2002. *Clostridium phytofermentans* sp nov., a cellulolytic mesophile from forest soil. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 1155-1160.

Watanabe, S., Abu Saleh, A., Pack, S.P., Annaluru, N., Kodaki, T., Makino, K. 2007. Ethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing protein-engineered NADH-preferring xylose reductase from Pichia stipitis. *Microbiology*, **153**(9), 3044.

Weber, C., Farwick, A., Benisch, F., Brat, D., Dietz, H., Subtil, T., Boles, E. 2010. Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. *Applied Microbiology and Biotechnology*, **87**(4), 1303-1315.

Wyman, C.E. 2008. Cellulosic ethanol: A unique sustainable liquid transportation fuel. *Mrs Bulletin*, **33**(4), 381-383.

Wyman, C.E. 2007. What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology*, **25**(4), 153-157.

Xiao, Z.Z., Zhang, X., Gregg, D.J., Saddler, J.N. 2004. Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates. *Applied Biochemistry and Biotechnology*, **113**, 1115-1126.

Yang, B., Willies, D.M., Wyman, C.E. 2006. Changes in the enzymatic hydrolysis rate of avicel cellulose with conversion. *Biotechnology and Bioengineering*, **94**(6), 1122-1128.

Yang, B., Wyman, C.E. 2008. Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts and Biorefining*, **2**(1), 26-40.

Yeh, A.I., Huang, Y.C., Chen, S.H. 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. *Carbohydrate Polymers*, **79**(1), 192-199.

Yu, J., Corripio, A.B., Harrison, D.P., Copeland, R.J. 2003. Analysis of the sorbent energy transfer system (SETS) for power generation and CO2 capture. *Advances in Environmental Research*, **7**(2), 335-345.

Zanin, G.M., Santana, C.C., Bon, E.P.S., Giordano, R.C.L., de Moraes, F.F., Andrietta, S.R., Neto, C.C.D.C., Macedo, I.C., Lahr Fo, D., Ramos, L.P. 2000. Brazilian bioethanol program. *Applied Biochemistry and Biotechnology*, **84**(1), 1147-1161.

Zhang, M., Eddy, C., Deanda, K., Finkelstein, M., Picataggio, S. 1995. Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis*. *Science*, **267**(5195), 240.

Zhang, S., Wolfgang, D.E., Wilson, D.B. 1999. Substrate heterogeneity causes the nonlinear kinetics of insoluble cellulose hydrolysis. *Biotechnology and Bioengineering*, **66**(1), 35-41.

Zhang, Y.H., Lynd, L.R. 2003. Quantification of cell and cellulase mass concentrations during anaerobic cellulose fermentation: Development of an enzyme-linked immunosorbent assay-based method with application to *Clostridium thermocellum* batch cultures. *Analytical Chemistry*, **75**(2), 219-227.

Zhang, Y.H.P., Lynd, L.R. 2005. Cellulose utilization by *Clostridium thermocellum*: Bioenergetics and hydrolysis product assimilation. *Proceedings of the National Academy of Sciences of the United States of America*, **102**(20), 7321-7325.

Zhao, C.X., O-Thong, S., Karakashev, D., Angelidaki, I., Lu, W.J., Wang, H.T. 2009. High yield simultaneous hydrogen and ethanol production under extreme-thermophilic (70 degrees C) mixed culture environment. *International Journal of Hydrogen Energy*, **34**(14), 5657-5665.

Zhong, C., Lau, M.W., Balan, V., Dale, B.E., Yuan, Y.J. 2009. Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. *Applied Microbiology and Biotechnology*, **84**(4), 667-676.

Zhou, W., Xu, Y., Schuttler, H.B. 2010. Cellulose Hydrolysis in Evolving Substrate Morphologies III: Time-Scale Analysis. *Biotechnology and Bioengineering*, **107**(2), 224-234.