This is to certify that the
dissertation entitled

A NEW PARADIGM FOR CELLULOSIC ETHANOL PRODUCTION FROM AMMONIA FIBER EXPANSION (AFEX)-PRETREATED BIOMASS

presented by

Ming Woei Lau

has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Chemical Engineering

Bruce T. Dale

Major Professor's Signature

April 8, 2010

Date

A NEW PARADIGM FOR CELLULOSIC ETHANOL PRODUCTION FROM AMMONIA FIBER EXPANSION (AFEX)-PRETREATED BIOMASS

By

Ming Woei Lau

A DISSERTATION

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

DOCTOR OF PHILOSOPHY

CHEMICAL ENGINEERING

2010

ABSTRACT

A NEW PARADIGM FOR CELLULOSIC ETHANOL PRODUCTION FROM AMMONIA FIBER EXPANSION (AFEX)-PRETREATED CORN STOVER

By

Ming Woei Lau

Lignocellulosic ethanol can provide an environmentally-friendly alternative to petroleum-based fuels. By utilizing locally-produced feedstocks as carbon sources for transportation fuel, ethanol can substantially alter the energy profile of transportation fuels, reducing geopolitical implications and dependence on foreign energy supplies. Obtaining fermentable sugars in a cost-effective fashion is the central barrier to realizing the commercial potential of lignocellulosic ethanol. The costs associated with hydrolysate conditioning, nutrients, and enzymes, which were projected at 45% of the total processing cost, must be reduced to improve the overall economics.

Feedstock pretreatment has pervasive impacts on downstream processes, particularly on fermentation. On a comparable basis, Ammonia Fiber Expansion (AFEX)-pretreated biomass is significantly more fermentable than that from dilute acid

pretreatment. This study confirmed that fermentation of AFEX-pretreated corn stover using *Saccharomyces cerevisiae* eliminated the requirement for washing of pretreated biomass, detoxification and nutrient supplementation. Overall ethanol yield at 191 g/kg (64 gal/ton) corn stover was achieved at a final titer of 40 g/L. Fermentations were completed within 72 hr in a high-cell-density fermentation. Fermentations can be conducted with similar effectiveness with the recycled cells, for at least another three generations without the addition of fresh cells.

The proposed integrated cellulosic ethanol production utilizes AFEX-pretreated biomass as the exclusive source of carbon, nitrogen and nutrients for ethanol fermentation and cellulase production. The carbon (sugar) source is divided between ethanol and enzyme production at a weight ratio of 4:1. Maximum ethanol yield under this carbon partition scheme is 267 g EtOH/kg CS or 90 gal/ton. About 60% of the extractable nitrogen and nutrients from AFEX-pretreated corn stover is projected to be used for enzyme production. This in-house production would be self-sustained if 12% of the bioavailable nitrogen source was assimilated for saccharolytic enzyme production and to provide optimal set of activities for biomass deconstruction.

To my parents, Kai Karn Lau and Geok Lan Tey.
Their unconditional love and support made this work possible

ACKNOWLEDGEMENTS

I thank all those who counseled, encouraged, and supported me throughout this endeavor of my Ph.D. career. I would like to specially thank my mentor, Prof. Bruce E. Dale, for providing guidance and support in my academic and professional developments. I am grateful to many other faculty members who offer invaluable advice and resources to my project; they include my Ph.D. committee members, Prof. Lonnie O. Ingram from University of Florida and Prof. Lee R. Lynd from Dartmouth College. I also deeply appreciate all the members of Biomass Conversion Research Laboratory (BRCL) for encouragement and support. Among them, I particularly appreciative of Dr. Venkatesh Balan, Christa Gunawan and Ming Jie Jin from the fermentation group that worked with me on multiple projects. Thanks to Bryan D. Bals and Rebecca J. Garlock for providing editing and peer-review assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AFEX	Ammonia Fiber Expansion
ATCC	American Type Culture Collection
CBP	Consolidated Bioprocessing
CSL	Corn Steep Liquor
CS	Corn Stover
Conc	Concentration
Glc	Glucose
MSU	Michigan State University
NREL	National Renewable Energy Lab
OD	Optical Density
SLE	Solids Loading Equivalent
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and Co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
Xyl	Xylose
YEP	Yeast Extract and Peptone
YNB	Yeast Nitrogen Base

CHAPTER I BACKGROUND AND GENERAL INTRODUCTION

WHAT IS LIGNOCELLULOSIC BIOMASS?

Lignocellulosic biomass, a mix of complex organic polymers, consists of cellulose, hemicellulose, lignin, and other extractive (phenolics and proteins) and non-extractive components. Cellulose is a linear polymer of β-1,4-D-glucopyranose units with a highly ordered and crystallized structure [1]. Hemicellulose, a branched carbohydrate, consists of several types of sugars including xylose, glucose, arabinose, galactose and mannose. Xylose is the predominant sugar in hemicellulose for most herbaceous biomass. Lignin is a highly cross-linked phenylpropylene polymer which is covalently bonded to hemicellulose [2]. It provides structural integrity to plant materials and protects plant carbohydrates from hydrolytic activity. Typical composition of lignocellulosic biomass is as listed in Table 1.

Due to the abundance and biorenewability of lignocellulosic materials, the ability to utilize these materials as the carbon sources would dramatically reduce the cost for raw materials and the carbon footprints of the chemicals produced.

Table 1 Typical biomass constituents in the energy crops

	Cellulose	Xylan	Lignin	Protein	Reference
Corn Stover	37.5	22.4	17.6	3.0	[3]
Switchgrass	32.2	22.3	17.3	3.0	[4]
Reed Canarygrass	26.5	16.3	14.8	4.5	[4]
Bagasse	44	22	22	2	[5]
Wheat Straw	31	20	25	-	[6]
Poplar	47.4	16.3	31.9	-	[7]

Unit: Percentage of the total dry biomass

WHY CORN STOVER

Corn stover, an agricultural residual from the existing farming operation, can be harvested at 80-100 million dry tonnes per year in a sustainable fashion [8]. Corn stover is also the most abundant existing agricultural residue in the United States and its production is concentrated in the Midwestern region [8]. Effective conversion of this lignocellulosic feedstock to ethanol could double the total ethanol production from the corn industry. Due to the similarity of herbaceous biomass in term of composition, understanding on how to process corn stover will also be applicable to other important energy crops such as switchgrass.

WHY LIGNOCELLULOSIC ETHANOL?

Ethanol, in the search for a sustainable source for transportation fuel, has emerged as the leading alternative to replace petroleum. Currently, ethanol is produced from edible crops such as corn and sugarcane. However, apart from competing with food supply, these feedstocks may not have the potential to substantially alter the energy profile in

the transportation sector due to the scale of production required [9]. Lignocellulosic feedstocks, the non-edible part of plant materials, are abundant carbohydrate sources and available at a competitive cost [10]. Thus, the capability to utilize plant materials for ethanol production could substantially reduce global dependence on fossil fuels.

LIGNOCELLULOSIC BIOMASS PROCESSING

Feedstock pretreatment – overcoming the recalcitrance of plant materials

Lignocellulosic biomass is inherently recalcitrant primarily due to the protection of cellulose by lignin, high crystallinity of cellulose, low accessible surface area and cellulose sheathing by hemicellulose [11, 12]. Thermochemical pretreatments are commonly used to increase susceptibility of plant carbohydrate for enzymatic hydrolysis [2]. Several features have been identified to be important for feedstock pretreatment, including the following:

- Effective in creating reactive fiber
- High hemicellulose recovery
- Low levels of inhibitor production
- Low requirement for particle size reduction
- Low cost for construction materials

Various chemicals such as sulfuric acid, sulfur dioxide, ammonia, sodium hydroxide and lime have been investigated as the potential catalyst for pretreatment. The effects commonly found in thermochemical pretreatments include increase in accessible surface area, decrystallization of cellulose, removal of hemicellulose and lignin removal

[13]. Among them, dilute acid pretreatment (using sulfuric acid as catalyst) and ammonia fiber expansion (AFEX) are two important feedstock pretreatment methods due to their ability to generate highly reactive biomass [14].

Dilute acid pretreatment has been extensively investigated for the past two decades. Acid hydrolyzes hemicellulose and other extractives in the plant materials, leaving cellulose in the remaining water-insoluble solids. Although effective at breaking down hemicellulose, acid-catalyzed reactions on these complex materials have significant drawbacks; approximately 10-30% of the hemicellulose is degraded to non-sugar byproducts [15]. These degradation products from sugars and lignin are oftentimes inhibitory toward saccharolytic enzymes and fermentation [16-19]. The primary degradation products found in the hydrolysate are furfural, 4-hydroxymethylfurfural, and acetic acid [17]. Detoxification through calcium hydroxide treatment is incorporated with cellulosic ethanol production using dilute acid as the feedstock pretreatment platform [20, 21].

Ammonia fiber expansion (AFEX) uses concentrated ammonia at temperatures from 90 to 150°C and explosive decompression to disrupt the lignocellulosic structure. AFEX is a dry-to-dry process due to low water use and the volatility of anhydrous ammonia at normal conditions [22]. This facilitates ethanol production at higher concentration, reducing water use and energy input during distillation. Furthermore, AFEX-pretreated feedstocks are highly fermentable presumably due to reduced production of inhibitory compounds and preservation of nutrient content during the pretreatment [2].

Bio-mediated processes – cellulase production, enzymatic hydrolysis and fermentation

Cellulose utilization and ethanol fermentation are the two important processes in the bio-mediated conversion for cellulosic ethanol production. Cellulose utilization involves the breakdown of the carbohydrates to fermenting sugars and the sugars are converted to ethanol through microbial catalysts during fermentation.

These processes are envisioned to be conducted in one of three configurations i.e. (i) separate hydrolysis and fermentation (SHF), (ii) simultaneous saccharification and (co)-fermentation (SSF/SSCF) and (C) consolidating bioprocessing (CBP) [2]. These configurations are described in Figure 1.

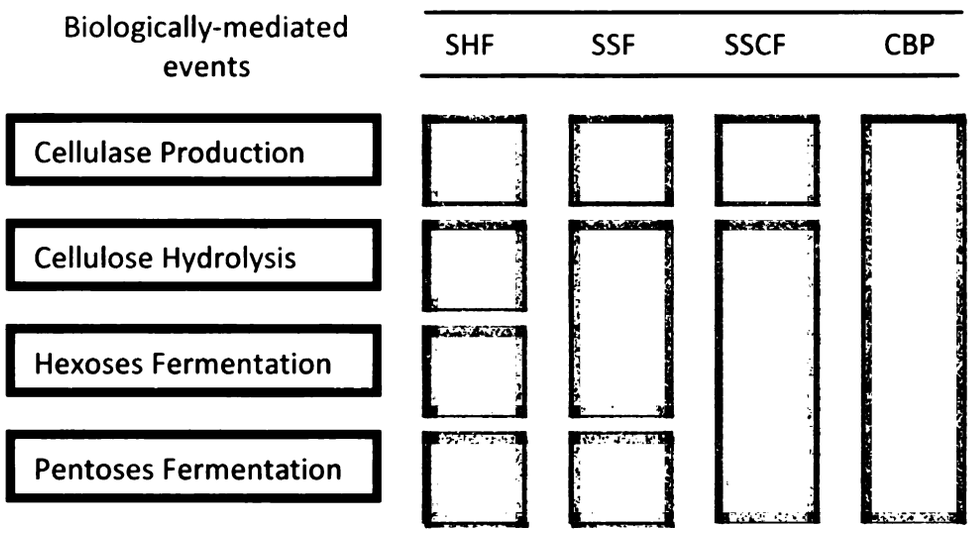


Figure 1 Schematic illustration of biological-mediated events in various biomass processing strategies

Note: Modified from [2]. SHF: Separate hydrolysis and fermentation, SSF: Simultaneous saccharification and fermentation, SSCF: Simultaneous saccharification and cofermentation, CBP: Consolidated bioprocessing.

SHF allows hydrolysis and fermentation to be conducted in their respective optimal conditions. The optimal pH and temperature for celluloytic enzymes fall in the ranges of pH4-5 and 50-60°C, respectively. However, conventional ethanologenic strains are mesophiles which ferment between 25 and 35°C. The significant challenge of SHF compared to SSF is overcoming sugar inhibition[23]. Cellulases and hemicellulases are reported to be inhibited by the simpler forms of sugars (end-product inhibition) which reduce fermentable sugar yield [24]. The effect of sugar inhibition is particularly noticeable when the solid loading is greater than 10%.

Both SHF and SSCF require addition of exogenous cellulase. Due to the complex nature of lignocellulosic biomass, a wide spectrum of enzymatic activity includes endoglucanase, exoglucanase, β-glucosidase, endoxylanase, exoxylanase, β-xylosidase and α-arabinofuranosidase are required for biomass deconstruction[25]. The cost of these enzymes remains the primary challenge to the overall economics of cellulosic ethanol production. Hence, development of strains that are able to effectively saccharify plant carbohydrates and convert fermentable sugars to ethanol in the configuration of consolidated bioprocessing (CBP) has been pursued. Mature technology of CBP is projected to reduce the cost of biological processing by 78% compared to SSCF [26].

Ethanologenic strains for cellulosic ethanol production

The ethanol fermentation using lignocellulosic biomass must achieve high overall yield, titer and productivity. Relative to corn ethanol industry, co-fermentation of hexoses and pentoses in a plant hydrolysate is essential in cellulosic ethanol production.

Lignocellulosic hydrolysate that contains degradation compounds from pretreatment exposes the fermenting strain to potential inhibitory chemicals that can interfere with cellular activities. Hence, an ethanologenic strain that is relevant for commercial production must have the following features [27]:

- Ethanol yield higher than 90% based on total plant carbohydrate
- Final ethanol concentration greater than 40 g/L
- Overall productivity greater than 1.0 g/L/hr, respectively
- Robust growth in lignocellulosic hydrolysate
- Simple nutrient requirement for growth

Besides traditional brewer's yeast (*Saccharomyces cerevisiae*), there are several known ethanologenic strains that have advantageous features as cellulosic ethanol producers. *Zymomonas mobilis* produces ethanol from hexoses at higher metabolic yield, concentration (>100 g/L) and specific rate than *S. cerevisiae* due to its ability to utilize glucose anaerobically through the Enter-Doudoroff pathway [27]. *Escherichia coli* K12 utilizes both hexoses and pentoses to produce ethanol. However, the native abilities of the ethanologens are limited particularly regarding overall ethanol yield. Two general metabolic engineering strategies were exploited for strain development, (i) expand fermentable sugar of ethanologens to include pentose sugars and (ii) improve metabolic ethanol yield by minimizing byproduct formation. The summary of the genetic engineering approach of three promising ethanologens is presented in Table 2.

Table 2 Summary for the features and genetic engineering approaches for selected cellulosic ethanol producer

	***E. coli* KO11**	***Z. mobilis* AX101**	***S. cerevisiae* 424A(LNH-ST)**
Advantageous Features (of Native Strain)	Ability to utilize hexoses and pentoses	Ability to produce ethanol at near theoretical maximum	-- Ability to produce ethanol from hexose sugars at near theoretical maximum -- The value of yeast cells as coproduct of fermentation
Disadvantageous Features (of Native Strain)	-- Produce ethanol at 25% of the maximum yield -- Relative low ethanol tolerance	-- Inability to utilize pentose sugar such as xylose and arabinose	-- Inability to utilize pentose sugar such as xylose and arabinose
Metabolic Engineering Approach	-- Genes encoding for pyruvate decarboxylase and alcohol dehydrogenase II from *Zymomonas mobilis* was heterologously expressed in *E. coli* K12 -- The succinic acid pathway was disrupted through gene knockout	-- Genes encoding for xylose isomerase, xylulokinase, arabinose isomerase, ribulokinase, ribulase, 5-phosphate 4 epimerase, transaldolase and transketolase from *E. coli* were cloned and expressed in *Zymomonas mobilis*	-- Gene cloning multiple copies of xylose reductase, xylose dehydrogenase and xylulokinase into the yeast's chromosome
Improvements	Ethanol is produced at near theoretical maximum, 4 fold increase from the native capability	Both xylose and arabinose can be utilized for ethanol production	Xylose can be utilized for ethanol production at 90% of the theoretical maximum
Reference	[28]	[29]	[30-32]

Conventional approach in cellulosic ethanol production

Biomass processing and conversion technologies centered on dilute acid pretreatment involve (i) cellulose solids and hemicellulose hydrolysate separation; (ii) detoxification unit of the hemicelluloses hydrolysate and (iii) nutrient supplementation to improve general fermentability of the acid-pretreated materials as shown in Figure 2[33].

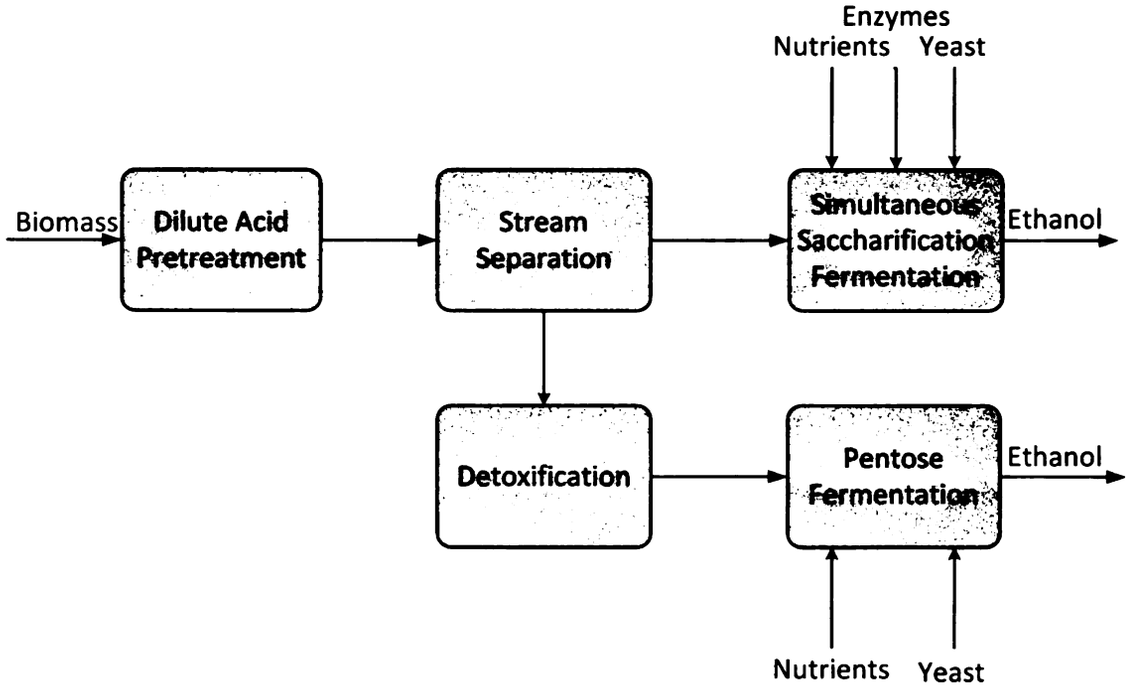
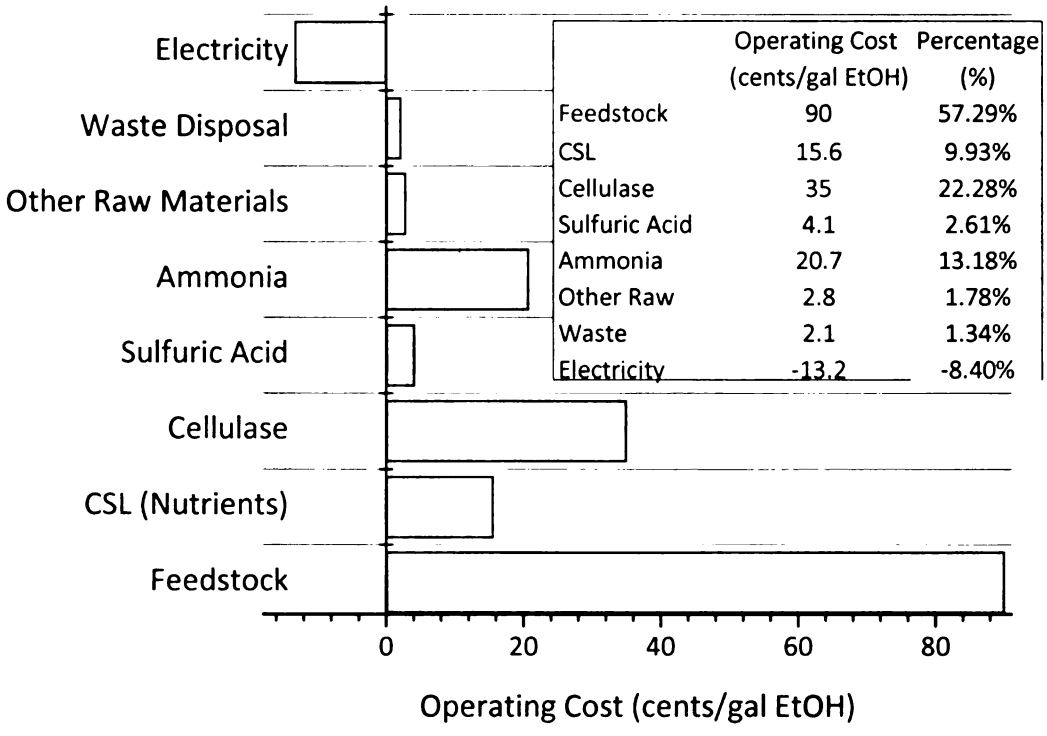


Figure 2 Conventional cellulosic ethanol production approach adopted by the National Renewable Energy Laboratory (NREL)

In an industrial setting, countercurrent washing has been proposed to separate the solids and liquid streams. This separation method introduces a greater water use [34]. The pretreatment must also be conducted at a greater solids loading to achieve a given final titer due to the introduction of the additional water wash stream.

Degradation products from acid pretreatment such as furfural, 4-hydroxymethyfurfural and acetic acid are shown to be inhibitory toward microorganisms. Removal of these inhibitors through physical, chemical and biological interactions has been extensively investigated [17, 35, 36]. Detoxification through calcium hydroxide (overliming) or activated carbon was reported to be effective [20, 37]. However, these detoxification methods often remove sugars and reduce the nutrient content of the plant hydrolysate. Detoxification, if applied, is estimated to comprise 20% of the total processing cost [38].

Nutrient supplementation is generally regarded as the integral part of cellulosic ethanol production. Expensive complex nutrients such as yeast extract are not relevant as a supplement for ethanol production. Corn steep liquor (CSL), a co-product from corn ethanol industry, is regarded as a relatively economical source of nutrients for cellulosic ethanol fermentation [39]. The cost of commercial nutrients is expected to be at 5-8 cents/gal ethanol [39]. The cost of cellulase, detoxification through ammonium hydroxide, and nutrient supplementation using corn steep liquor consists of 45% of the total operating cost (Figure 3) from the economic projection by the National Renewable Energy Lab [40].



	Operating Cost (cents/gal EtOH)	Percentage (%)
Feedstock	90	57.29%
CSL	15.6	9.93%
Cellulase	35	22.28%
Sulfuric Acid	4.1	2.61%
Ammonia	20.7	13.18%
Other Raw	2.8	1.78%
Waste	2.1	1.34%
Electricity	-13.2	-8.40%

Figure 3 Operating cost distribution for the conventional cellulosic ethanol production projected by NREL

Note: Modified from[40]. Ethanol yield = 72.6 gal/ton; Assumed feedstock cost = USD65.3/dry ton.

RESEARCH SCOPE AND OBJECTIVES

My work addresses applied and fundamental understanding on overall lignocellulosic processing from an integrated perspective

- Comparison of feedstock pretreatments and ethanologenic microorganisms for lignocellulose bioprocessing
- Investigation of cellulosic ethanol production using AFEX as pretreatment and *S. cerevisiae* 424A(LNH-ST) as ethanologenic strain

- Comprehensive nutrient content analysis of plant materials hydrolysate

Ultimately, this research seeks to demonstrate a novel and straightforward paradigm for cellulosic ethanol production centered on AFEX pretreatment to reduce the overall processing cost.

CHAPTER II PRETREATMENT COMPARISON BETWEEN AFEX AND DILUTE ACID PRETREATMENT

INTRODUCTION

Feedstock pretreatment in cellulosic ethanol production plays an integral role in lignocellulosic biomass processing due to the inherent recalcitrance of plant material [1]. The selection of pretreatment method has far-reaching impacts on the overall process, including feedstock handling, biological conversions, and downstream processing [41].

Among pretreatments, dilute acid pretreatment and AFEX are regarded as promising candidate for large scale cellulosic biofuels production. High sugar recovery can be achieved by both pretreatments for corn stover from a previous comparative study[42]. However, a comprehensive comparison of pretreatments concerning their impacts on fermentation is needed. In this chapter, the impacts of these two pretreatments from a system-wide perspective are examined. I evaluate the interactions of dilute acid pretreatment and AFEX with enzyme requirements, hydrolysate fermentability and

lignin preservation. The microbial platform used for the pretreatment comparison involves *Saccharomyces cerevisiae* 424A(LNH-ST) and *Escherichia coli* KO11.

MATERIALS AND METHOD

Corn stover (CS)

Corn stover was supplied by the National Renewable Energy Laboratory (NREL, Golden, Colorado). It was milled and passed through a 4 mm screen. The moisture content was approximately 7% (total weight basis). The milled corn stover was kept at 4°C for long term storage. This corn stover contains 34.1% cellulose, 20.4% xylan, 3.3% arabinan and 2.3% protein on a dry weight basis.

Dilute acid pretreated corn stover from pilot-scale continuous (Sund) reactor at NREL

This dilute acid pretreatment has been carried out as described previously [15]. Pretreatment was conducted at 190°C. The solids and sulfuric acid loading of the pretreatment were reported as 30% (w/w) and 0.048 g/g dry corn stover, respectively. The whole slurry from the reactor was used in this study.

Pretreatment

Ammonia fiber expansion (AFEX)

The AFEX pretreatment was performed in a 2.0 L pressure vessel (Parr Instruments, Moline, IL). The reactor was equipped with thermocouples and a pressure sensor. AFEX on CS was conducted at 62.5% solids loading. The reactor was preheated to 100-110°C and prewetted corn stover (150 g dry CS + 90 g distilled water) was loaded into the vessel. The lid was bolted shut. Anhydrous ammonia (150 g) was preheated in a 500 mL stainless steel cylinder (Parker Instrumentation, Jacksonville, AL) until its pressure reached 4.48 MPa (650 psi). Heated ammonia was then transferred into the reactor to initiate the reaction. The initial and final temperatures of the pretreatment were 130±5°C and 110±5°C, respectively. The reactor pressure was released after 15 min through an exhaust value. AFEX-pretreated CS was then air-dried in a fume hood overnight.

Bench-scale dilute acid pretreatment

The dilute acid pretreatment was performed with a 1.0 L Parr reactor made of Hastelloy C (Parr Instruments, Moline, IL) equipped with a thermocouple (Extech Instruments, Waltham, MA) and a helical impeller (3.5 in.) on a two-piece shaft. The impeller was driven by a variable speed DC motor assembly (Parr Instruments, Moline, IL). Corn stover was presoaked in 1.0% w/v dilute sulfuric acid solution at 5.0% and 7.5% solids (w/w) overnight. Total weight of the pretreatment mixture was 800 g. The presoaked slurry was transferred into the reactor which was then sealed and fitted to the impeller driver motor. The impeller speed was set at 150 rpm. The reactor was heated rapidly to and maintained at 140±2°C in about 2 min and maintained at this temperature in a sand

bath for 40 min. At the end of the reaction time, the reactor was cooled to below 50°C. The diluted acid pretreated corn stover slurry was filtered through Whatman no. 1 filter paper. Details on the apparatus and experimental procedure are as described [43]

Fermentation on water extract of soluble compounds from pretreated CS

Water extract preparation

Four water extract of pretreated corn stover was prepared for fermentation studies, (i) AFEX-CS pretreated at 62.5% solids loading, (ii) dilute-acid-CS pretreated at 5.0% solids loading in the benchscale reactor, (iii) dilute-acid-CS pretreated at 7.5% solids loading in the benchscale reactor and (iv) dilute-acid-CS pretreated at 30.0% solids loading in continuous pilot reactor (Sund)

AFEX-pretreated corn stover was washed with distilled water at a ratio of 1 g dry CS to 5 mL of water to produce a water extract (20% solids loading equivalent). In each batch of washing, distilled water was preheated to 60-70°C and added to 100 g (dry weight equivalent) of AFEX-CS. The water content of the wetted AFEX-CS was reduced by using an in-house manufactured press. The washing was conducted in three cycles, i.e. water-extract from a previous cycle of washing was used for the next cycle of washing (Figure 4). In the final cycle of washing, the moisture content of the washed AFEX-CS was reduced to 77±3%. The AFEX-CS water extract was used for the fermentation.

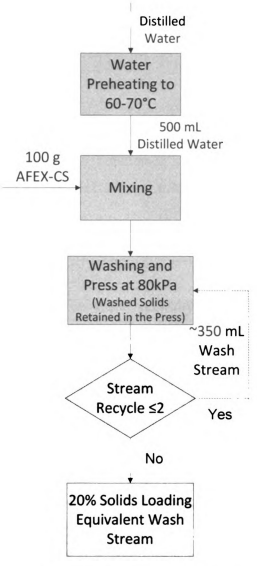


Figure 4 Schematic describing the preparation of AFEX-pretreated corn stover water extract

Hemicellulose hydrolysate from the dilute acid pretreatment stage was used as the water extract. Hemicellulose hydrolysates prepared from 5% and 7.5% solids loading during bench-scale dilute acid pretreatment were concentrated to 20% solids loading equivalent (1 g input CS in 5 mL liquid) through rotary evaporation under vacuum at

75°C.

achie

Where

respec

For CS

mL of

was mi

solids l

availabl

insolubl

benchsc

Seed cul

Seed cul

prepared

peptone

an initial

KO11, Soc

75°C. Xylose concentration was used as the indicator for the concentration factor achieved during the evaporation (Eq. 1).

$$(\text{Solids Loading})_f = \frac{[\text{Xyl}]_f}{[\text{Xyl}]_i} \times (\text{Solids Loading})_i \quad \text{---- Eq. 1}$$

Where [Xyl], f and i denote for concentration of xylose, final and initial condition, respectively.

For CS from the Sund reactor, distilled water was added so that the mixture contained 5 mL of liquid to 0.51 g of dry water-insoluble pretreated corn stover. The diluted slurry was mixed by rigorous shaking and centrifuged at 6000 ×g. The supernatant was as 20% solids loading equivalent. No mass balance around Sund pretreatment was made available, therefore it was assumed that percentage of input CS remaining as water-insoluble-solids after the pretreatment in the Sund reactor is the same as that of benchscale dilute acid pretreatment, i.e. 51%.

Seed culture preparation

Seed cultures of *E. coli* KO11, *S. cerevisiae* 424A(LNH-ST) and *Z. mobilis* AX101 were prepared in 100 mL of complex media YEP_GX (5 g/L bacto yeast extract + 10 g/L bacto peptone + 30 g/L glucose + 20 g/L xylose) by inoculating frozen (-80°C) culture stock at an initial cell density of 0.1 unit OD600nm. Culture temperature and period of *E. coli* KO11, *Saccharomyces cerevisiae* 424A(LNH-ST) and *Zymomonas mobilis* AX101 are 37°C,

18hr; 3

anaero

initiate

Fermen

Fermen

extracts

corn sto

Jose, CA

ammoni

xylose in

respectiv

solid loac

contamin

Each well

to aid stir

culture w

microplat

An openin

produced.

cerevisiae 4

18hr; 30°C, 18hr; 30°C, 42hr, respectively. The cultures were conducted under largely anaerobic conditions and mixed at 150 rpm agitation. The grown cells were used to initiate fermentations.

Fermentation procedure--microplate fermentation

Fermentations of *E. coli* KO11, *S. cerevisiae* 424A(LNH-ST) and *Z. mobilis* AX101 in water extracts of 7.5% and 15.0% solids-loading-equivalent of the three types of pretreated corn stover were conducted in 24-well cell culture microplates (BD Falcon #353047, San Jose, CA). The media were supplemented with water extract, yeast base nitrogen with ammonium sulfate (YNB, MP Biomedicals, Lot Number: 4027512-119914), glucose and xylose in appropriate buffer (50mM) at final concentrations of 16.7 g/L, 9 g/L and 35 g/L, respectively. Distilled water was added to dilute the water extracts to 7.5% and 15.0% solid loading equivalent. Chloramphenicol (50 mg/L) was added to reduce risk of contamination.

Each well contained 2.0 mL media and was added with a glass bead (6 mm in diameter) to aid stirring. Seed cultures were prepared as described above and the microplate cell culture was initiated at OD(600nm) of 0.5. The microplate was sealed and fixed on the microplate clamp system (Applikon Inc, Springfield, IL) in an incubator shaker (150 rpm). An opening (about 1mm diameter) was made on the seal to vent carbon dioxide produced. Initial pH for *E. coli* KO11 was at 7.0 and at 5.5 for *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST). Incubation temperature was the same as seed culture

cond

424A

spect

HPLC

in the

Ferme

Ferme

extract

extract

10 g/L g

was adc

conduct

was pH-

designat

Enzymat

Enzymat

To prepa

washed w

water. For

water was

conditions. The fermentations were conducted for a designated period (KO11, 424A(LNH-ST): 24hr; AX101: 48hr). Cell density was measured using a spectrophotometer at 600nm. Sugars and fermentation products were analyzed using a HPLC system with a Biorad Aminex HPX-87H colume as described [3]. Error bars shown in the results are standard deviations of triplicates.

Fermentation procedure--shake flask fermentation

Fermentations of KO11 and 424A(LNH-ST) were further conducted in shake flask. Water extract from AFEX and dilute acid pretreatment were supplemented with 1 g/L yeast extract and 2 g/L peptone with appropriate buffer. Sugar levels were adjusted to about 10 g/L glucose and 50 g/L xylose. Final solids loading equivalent was at 7.5%. Inoculum was added to achieve an initial cell density of 0.1 OD600nm. Fermentation was conducted at the strains' respective optimal pH and temperature. KO11 fermentation was pH-adjusted every 24hr using 6M KOH. Fermentation samples were taken at designated period throughout the 120hr culture.

Enzymatic hydrolysis

Enzymatic hydrolysis of water-insoluble solids of the pretreated CS

To prepare water-insoluble materials, pretreated CS from both pretreatments was washed with distilled water at a ratio 1 dry g (input CS to pretreatment) to 50 mL of water. For bench-scale dilute acid pretreated CS, the designated amount of distilled water was poured into a filter system with Whatman filter paper (No 4) under vaccum.

The solid

pretreate

for 24 hr

After eac

system. T

content c

The wate

mixtures

cellulase

Novozym

Multifect

spectrum

and Mult

188 was

conducte

oligomer

triplicate

The solids remaining on filter paper were dried under vaccum at 60°C. For AFEX-pretreated CS, the washing was achieved in two stages; (i) Incubation at 250rpm, 50°C for 24 hr at 5% solids loading equivalent and (ii) two cycles of centrifugation at 6000 x g. After each cycle of centrifugation, the supernatant was decanted through the filter system. The total weight of water-insoluble solids was measured and the carbohydrate content of the solids was analyzed using NREL protocol LAP-002.

The water-insoluble materials were enzymatically-hydrolyzed using either (i) cellulase mixtures and (ii) cellulase + hemicellulase mixtures at pH 4.8, 50°C for 144 hr. The cellulase mixture consisted of Spezyme CP [86.7 mL/kg CS; 15 FPU/g cellulose] and Novozyme 188 [87.5 mL/kg CS; 64 *p*NPGU/g cellulose]. The hemicellulase mixture was Multifect Xylanase [12.7 mL/kg CS] and Multifect Pectinase [12.7 mL/kg CS]. The spectrum of activities for the commercial enzymes was as reported [44]. The Spezyme and Multifect enzymes were obtained from Genencor Inc. (Palo Alto, CA) and Novozyme 188 was purchased from Sigma-Aldrich Co (St. Louis, MO). Enzymatic hydrolysis was conducted at 5.1% glucan loading. Glucose and xylose in both monomeric and oligomeric forms were measured. Error bars shown are standard deviations of triplicates.

Mass b

Carboh

After p

were w

and dr

were r

of the

oligom

hemice

anhydr

around

S

Klason

The dr

enzym

matter

concen

and 60

matter

Mass balance construction

Carbohydrate mass balance around pretreatment

After pretreatment, the pretreated solids from both AFEX and dilute acid pretreatment were washed with water at a ratio of 1 g input biomass to 50 mL of water. Total mass and dry matter content (%) of the input and output materials around the pretreatments were recorded. Volumes of the water extracts were recorded. Glucan and xylan content of the dry matters were analyzed using NREL protocol LAP-002. Both monomeric and oligomeric (LAP-014) sugars of the water extract from AFEX-pretreated CS and hemicellulose hydrolysate from dilute acid pretreated CS were analyzed. Total anhydrous equivalent of glucose and xylose were calculated for input and output around both pretreatments. Percent carbohydrate conserved was calculated using Eq. 2.

$$\text{Sugar Preservation}(\%) = \frac{(\text{Anhydrous Equivalent of Sugar})_{output}}{(\text{Anhydrous Equivalent of Sugar})_{input}} \times 100\% \text{--- Eq. 2}$$

Klason lignin mass balance around pretreatment

The dry matter mass of the input and output materials around pretreatment and enzymatic hydrolysis were recorded. The total percent of Klason lignin in the dry matters before and after pretreatment was analyzed using NREL LAP-002. The final acid concentration, temperature and residence time for the assay was 4% sulfuric acid, 121°C and 60 min, respectively. The Klason lignin was calculated by multiplying the total dry matter with the percentage of Klason lignin.

Residual solids analysis and heat value estimation

After enzymatic hydrolysis at 5.1% glucan loading, unhydrolyzed solids were separated by centrifugation, washed twice using distilled water, and dried under vacuum at 55°C. Total dry weight was recorded. Glucan and xylan in the unhydrolyzed solids was analyzed using NREL LAP-002. Residual non-carbohydrate solids and their heating value were estimated is described in Eq. 3 and Eq. 4:

Residual Solids (g) = Total Unhydrolyzed Dry Solids (g)– Carbohydrate in the Solids--Eq. 3

Energy content (kJ) = 0.90 × Residual solids (g) × 25.4 kJ/g----------Eq.4

This is done by assuming 90% of the total residual solids is lignin and the rest of 10% has negligible heat value. The heat value of lignin used (25.4 kJ/g) was as reported [45].

RESULTS

Sugar and lignin preservation during AFEX and dilute acid pretreatment

AFEX pretreatment on CS at 62.5% solids loading preserved all its carbohydrates. Nearly 10% of the AFEX-pretreated CS carbohydrate was water-soluble, of which two-thirds was monomeric or oligomeric xylose (Figure 5A). However, 13% of the xylose sugar was degraded in the dilute acid pretreatment at 5% solids loading. About half of the total input solids was solubilized in the acid solution. While 59% of the total remaining solids after dilute acid pretreatment is glucan, its xylan content was reduced to about 3% (Figure 5B). Forty-two percent of the total output sugars from dilute acid pretreatment was water-soluble, predominantly in monomeric form. The concentration of the total sugars in the acid liquid stream was 14 g/L.

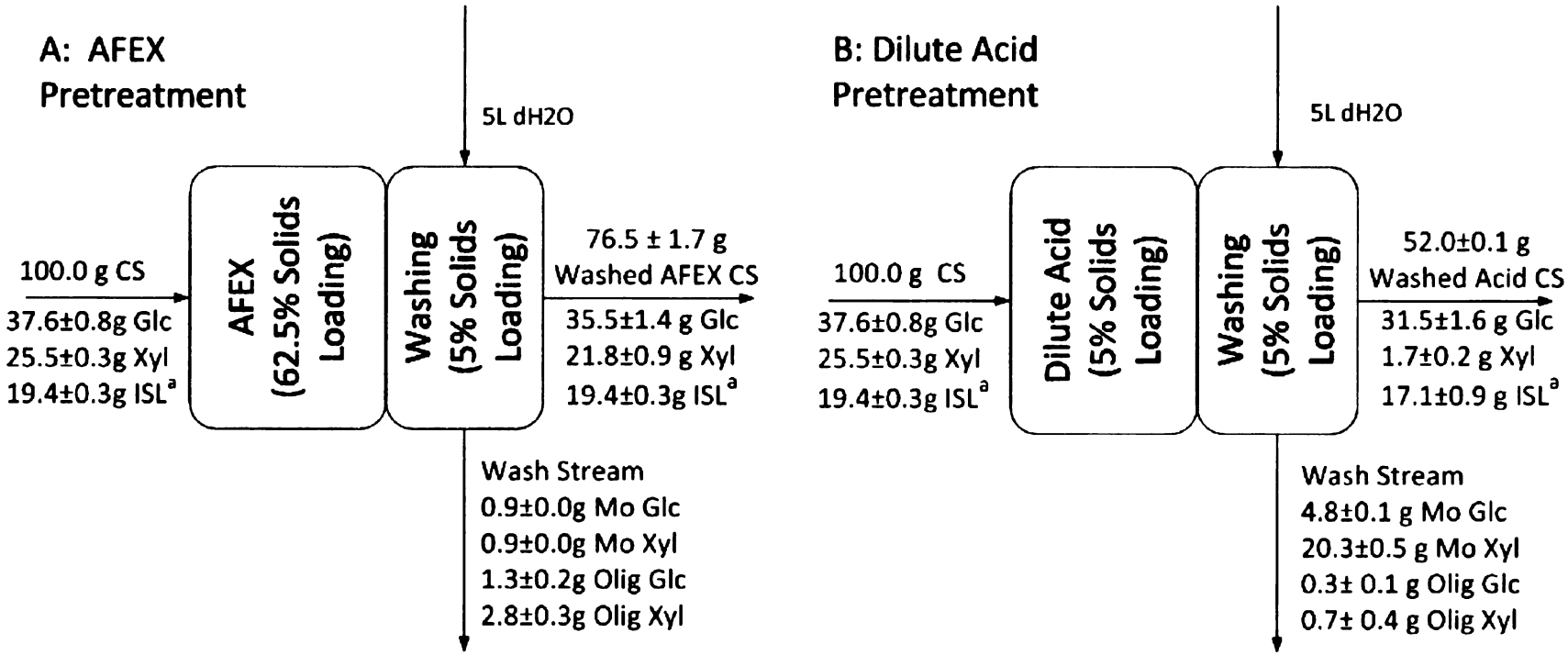


Figure 5 Mass balance comparison between AFEX and dilute acid pretreatment on corn stover (A) AFEX pretreatment and (B) dilute acid pretreatment.

AFEX and Dilute Acid Pretreatment were conducted at 62.5% and 5.0% solids loading, respectively. Washing of pretreated corn stover from both pretreatment was carried out at 5.0% solids loading based on input materials to the pretreatment. ISL: Insoluble lignin (Klason lignin)

[a] The assay condition for the insoluble lignin measure: 4% final H_2SO_4 concentration, 121°C, 60min

Regarding Klason lignin content (at assay condition: 4% sulfuric acid, 121°C, 60 min), AFEX completely preserved the lignin. In the dilute acid pretreatment, 12% of the Klason lignin was not preserved in the remaining solids (Figure 5).

Fermentability of water extracts of the pretreated corn stovers

Categorically, the water extract from AFEX-treated CS exhibited significantly higher fermentability with regards to cell growth, and glucose and xylose consumption in both *S. cerevisiae* 424A(LNH-ST) and *E. coli* KO11. Remarkably, the water extract from dilute acid pretreatment inhibited the growth of KO11 severely in both 7.5% and 15% solids loading (Figure 6) over the tested fermentation period (24hr) (Figure 6). Comparing different dilute acid pretreatment approaches, the water extract of pretreated corn stover from the Sund reactor was more inhibitory than the benchscale, low solid loading pretreatments. The cell density of 424A(LNH-ST) in the Sund-CS water extract was about half of that of acid pretreated CS at laboratory bench-scale.

In contrast, all tested AFEX-CS water extracts were high fermentable. In essence no inhibitory effect on cell growth was observed. Fermentations of AFEX-treated material performed similarly to that of yeast base nutrient (YNB, 13.7 g/L). In the case for KO11 fermentation, xylose consumption in AFEX-CS water extract (7.5% solids loading equivalent) was two-fold higher than that of YNB. Complete glucose (8-10g/L) fermentation was achieved regardless of pretreatments by the *S. cerevisiae* (Figure 7A,B). While better xylose fermentation was achieved in KO11 than 424A(LNH-ST) in

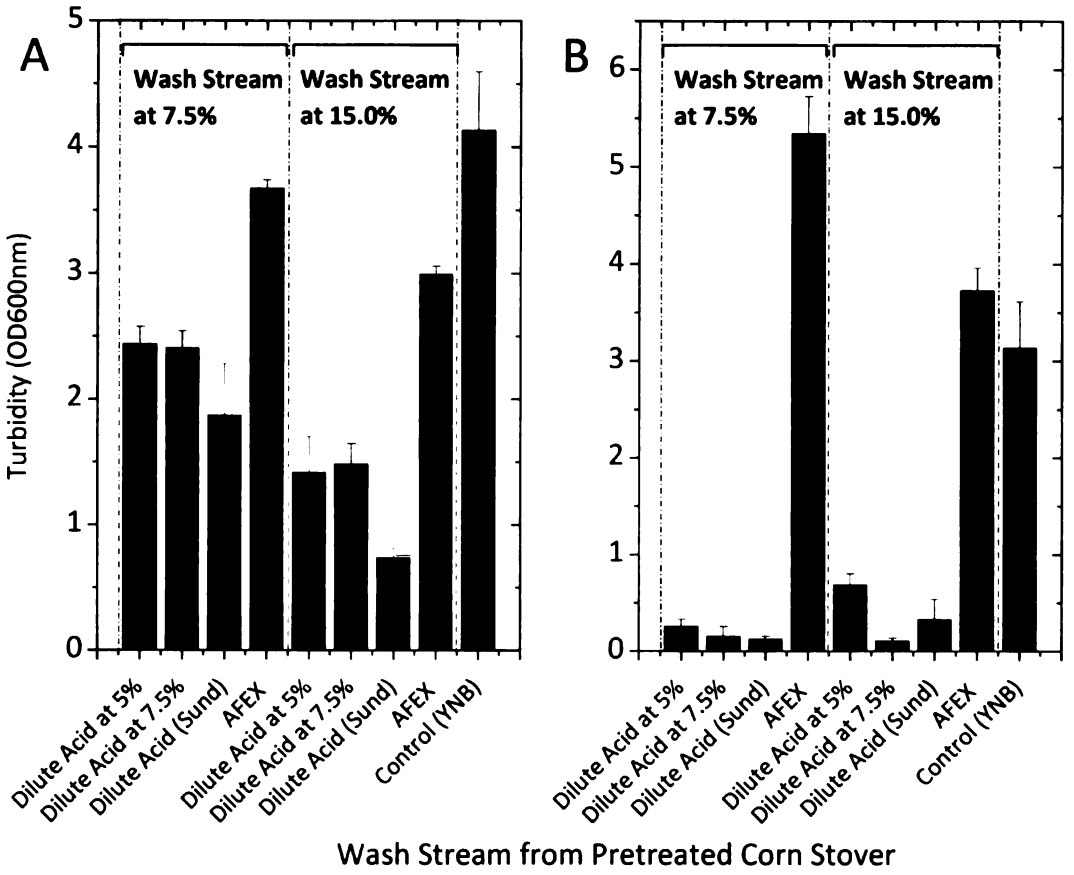


Figure 6 Cell density after 24 hr of fermentation in YNB-supplemented water extracts from dilute acid pretreatment at different solids loading (5%, 7.5%, 30%[Sund]) and AFEX pretreatment. (A) 424A(LNH-ST) and (B) KO11

Fermentation was conducted in 24-well microplate at 2.0 mL working volume with Initial cell density of 0.1 unit of OD600nm. Solids loading equivalent of the water extracts tested was 7.5% and 15.0%.

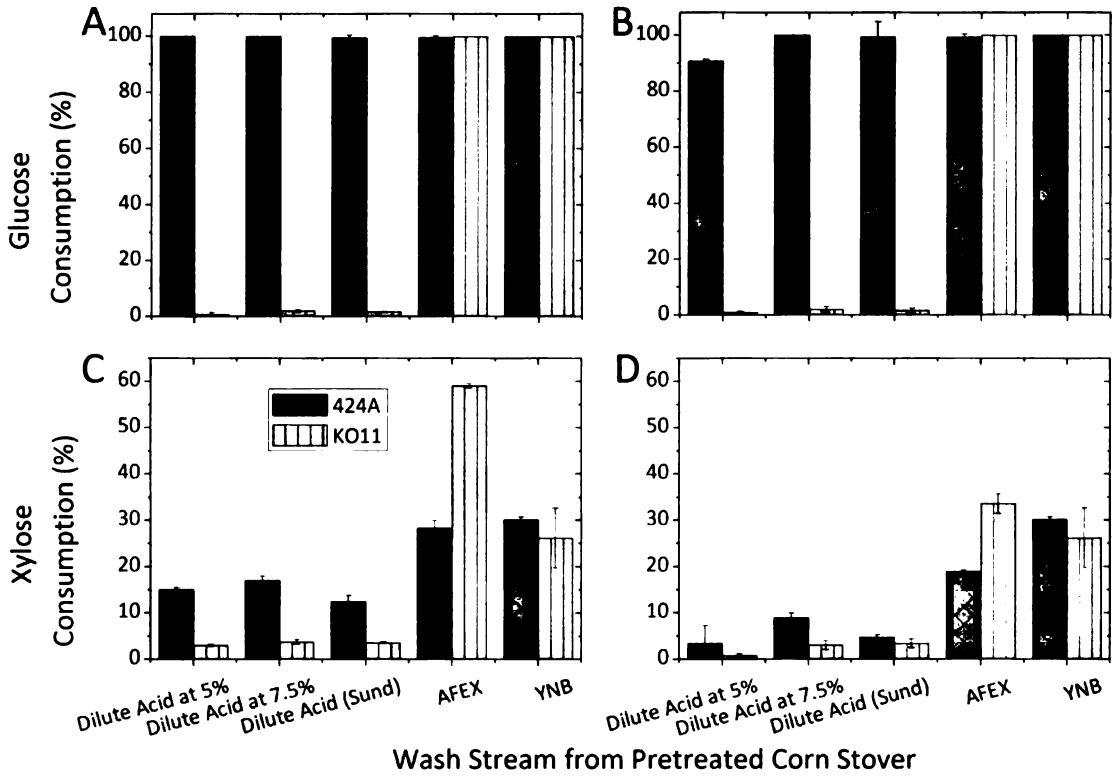


Figure 7 Sugar consumption after 24 hr of fermentation in YNB-supplemented water extracts from dilute acid pretreatment at different solids loading (5%, 7.5%, 30%[Sund]) and AFEX pretreatment in water extract at 7.5% solids loading equivalent (A, C) and 15.0% solids loading equivalent (B, D).

Fermentation was conducted in 24-well microplate at 2.0 mL working volume with Initial cell density of 0.1 unit of OD600nm. Initial glucose and xylose is at 9±1 g/L and 35±2 g/L, respectively.

AFEX-CS water extract, the opposite trend was observed in dilute-acid-CS water extract due to the inhibitory nature of the water extract and the strain robustness toward the inhibitors. The time course of KO11 and 424A(LNH-ST) fermentations in both water extracts also showed similar trends as the microplate fermentations regarding strain robustness of 424A(LNH-ST) and better xylose fermentation of KO11 in AFEX-CS water extract (Figure 8). At the low initial cell density tested, KO11 could not grow in the water extract from the acid-pretreated corn stover over the fermentation period.

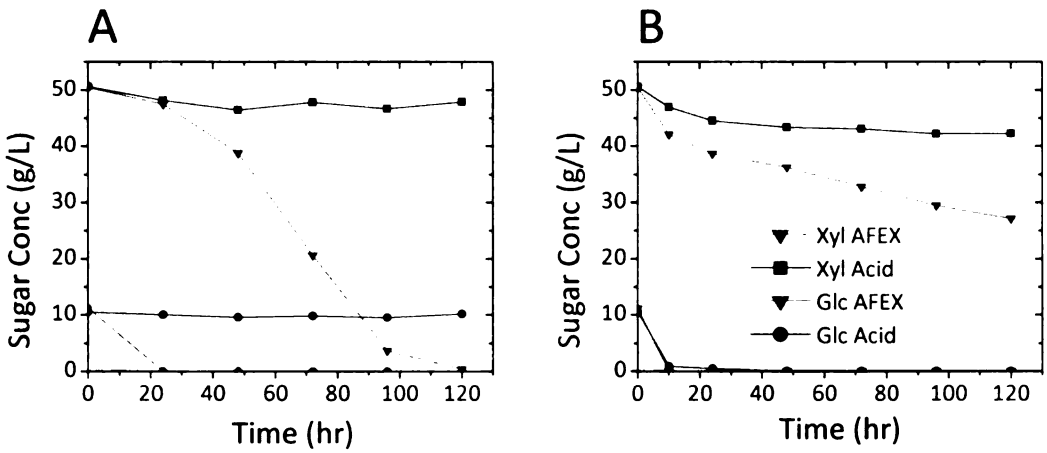


Figure 8 Time course of fermentation of water extract from 7.5% solids loading equivalent of AFEX and dilute acid pretreated corn stover.

Fermentation was initiated with 0.1 unit OD600nm and the water extracts with supplemented with 1 g/L yeast extract and 2 g/L peptone.

Enzymatic hydrolysis of washed pretreated solids

Enzymatic hydrolysis at 5.1% glucan loading of washed solids from both pretreatments achieved similar total glucose yields at 82% when the cellulase-only mixture was used. However, AFEX achieved 6% higher glucose yield when both cellulase and hemicellulase was added (the difference was within the margin of error) (Figure 9). The added hemicellulase mixture also improved xylose yield in AFEX improved from 83% to 91%. In contrast, the hemicellulase mixture does not affect sugar yields in dilute acid pretreated solids. This is probably due to the low xylan content (about 3%) in the solids. The proportion of glucose and xylose oligomers to the total sugars in the hydrolysates is about 12.5% and 25.0%, respectively (Figure 9).

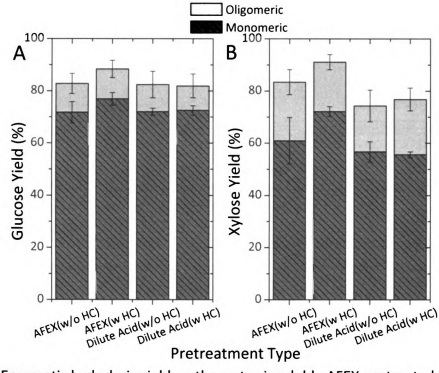


Figure 9 Enzymatic hydrolysis yield on the water-insoluble AFEX-pretreated corn stover and dilute acid pretreated corn stover at 5.1% glucan loading, pH 4.8 and 50°C.

Note: Xylan content in water-insoluble dilute acid pretreated corn stover is very low (3%), HC: Hemicellulases

Energy content of non-carbohydrate residual solids

Non-carbohydrate residual solids from AFEX and dilute acid pretreatment and hydrolysis are 19.1 g and 15.8 g per 100 g of untreated corn stover, respectively. Based on the calculation method listed, biomass processing technology based on AFEX pretreatment is able to generate 737 kJ/kg more energy from the residual solids than that of dilute acid pretreatment. About 23.2% (AFEX) and 19.3% (Dilute Acid) of the heating value in the untreated corn stover remained in the non-carbohydrate residual solids (Figure 10).

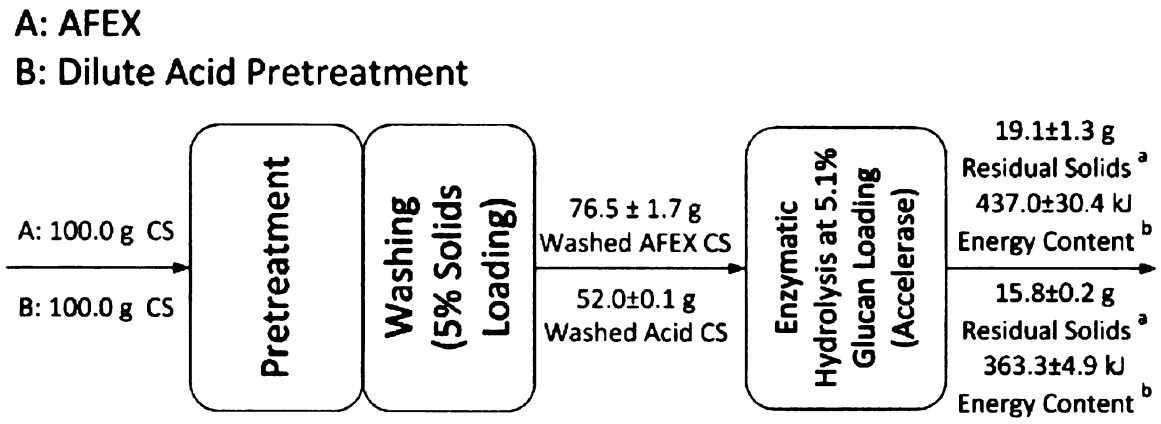


Figure 10 Mass balance and energy content for non-carbohydrate insoluble solids after pretreatment, washing and enzymatic hydrolysis.

[a] Residual solids = (Recorded total dry solids left unhydrolyzed)-(Dry glucan and xylan in the solids)

[b] Energy content = 0.9 × Residual solids (g) × 25.4 kJ/g. This is done by assuming 90% of the total residual solids is lignin and the rest of 10% has negligible heat value. The heat value of lignin used (25.4 kJ/g) was as reported

DISCUSSION

Dilute acid pretreatment reduces maximum possible product yield by 10%

Overall yield has the greatest impact on the profitability or viability of a commercial process. To make cellulosic ethanol a cost-effective fuel, efforts to increase ethanol yield per unit mass of biomass (corn stover) at a given product titer deserve the highest priority. In this regard, AFEX preserves all carbohydrates while effectively increasing the susceptibility of the pretreated corn stover to hydrolytic enzymes. Unlike AFEX, acid-catalyzed pretreatment hydrolyze hemicellulose almost completely. Unfortunately, monomeric pentoses are further degraded to byproducts such as furfural under acid treatment conditions. In this report, about 13% of xylan was lost through chemical degradation. However, a greater degree of degradation (20-30%) was reported at a higher solids loading of dilute acid pretreatment [15]. This reduces the maximum product yield by 10%. In other words, using dilute acid as the catalyst for feedstock pretreatment would require a 10% increase in raw material cost to achieve the same yield compared to AFEX assuming that both technologies achieve 100% bioconversion of carbohydrate. In any mature chemical process for commodities, raw material is the dominant factor in the processing costs [46]. Hence, selection of a pretreatment that highly preserves plant carbohydrates is critical for long term success in this industry.

Pretreatment dictates the fermentability of pretreated biomass

Apart from the preservation of carbohydrate, an ideal pretreatment reduces the generation of inhibitory degradation compounds. AFEX-pretreated CS is highly fermentable using both bacteria and yeast. In certain cases, the soluble fraction of AFEX-pretreated CS has been shown to be beneficial to microbial growth [47]. In contrast, corn stover hydrolysate from dilute acid pretreatment is significantly more inhibitory and difficult to be fermented. The nitrogenous (amides and amines) reaction products formed during ammonia-lignocellulose reactions are generally non-inhibitory toward microbial growth. These degradation products would otherwise be organic (aliphatic and phenolic) acids in acid catalyzed reactions[35]. Fermentation at higher initial cell density, nutrient supplementation and/or detoxification are likely needed to alleviate or overcome their inhibitory effects of acid pretreatment [17, 29] judging from the extensive investigations that on the related subjects centered on dilute acid pretreated materials.

The pretreated biomass, which inherently possesses various nutrients, can serve as the carbon and nitrogen sources for bioconversion. If there is no interference from inhibitory degradation compounds on fermentation, cellulosic ethanol fermentation can be conducted in an approach similar to the corn ethanol industry (where hydrolysate conditioning and high initial cell density are not required).

Pretreatment determines feasible biomass processing configurations

Due to the nature of pretreatment, particularly with respect to the degree of hemicellulose solubilization, inhibitor generation and nutrient preservation, different biomass processing strategies that maximize the advantages of each pretreatment should be exploited. Dilute acid pretreatment effectively hydrolyze hemicellulose, eliminating the need for hemicellulases during enzymatic hydrolysis. Nevertheless, the hemicellulase stream is inhibitory toward enzymes and microorganisms. Therefore, separation of solids and the hemicellulose stream as previously proposed [48] is essential to minimize the adverse effects of the inhibitors from the bioconversion of the remaining solids. However, important technical issues need to be solved in a cost-effective fashion, including (i) separation of solids and liquid with low fresh water use and (ii) effective fermentation of the hemicellulose stream at high sugar concentration without significant conditioning.

AFEX-centered biomass processing can be performed in a straightforward manner where the pretreated biomass (cellulose and hemicellulose) can be converted to ethanol after enzymatic hydrolysis and fermentation without washing or stream separation [47]. In this report, washing was done in AFEX-pretreated corn stover to establish a basis for comparison. Due to high fermentability of AFEX-pretreated biomass, washing, nutrient supplementation and high initial cell density are not required during fermentation stage[47]. In comparison to dilute acid pretreatment, a relatively large portion of oligomeric xylose is present in AFEX hydrolysate. Exploitation of

hemicellulase-secreting strains such as *Thermoanaerobacterium saccharolyticum* to biologically process AFEX-pretreated materials could address this issue without added cost of hemicellulase[49].

AFEX enhances co-product generation and diversity

Reduction in greenhouse gas emissions by cellulosic ethanol E85 relative to petroleum gasoline is projected to be 68-102% and it is largely due to the heating value of residual solids (primarily lignin) to generate steam or electricity as a co-product [50, 51]. Our results indicate that AFEX-centered cellulosic technology is expected to have about 17% more available energy from the insoluble lignin residue compared to dilute acid. This also implies that the selection of pretreatment directly affects the magnitude of environmental benefits brought about by a cellulosic ethanol plant beyond the direct impact of the pretreatment process. Nevertheless, a definitive conclusion on the impact of different pretreatments on various environmental benefits can only be made after careful life cycle analysis based on these experimental data.

Lignin removal is a function of severity in terms of acid concentration, temperature and residence time [52], and part of the solubilized lignin can be recovered [53]. Hence, optimization of acid pretreatment condition that takes into account of the economics of recoverable lignin can be important. However, the recovery process will inevitably increase the processing cost relative to a production where lignin is preserved in the solid residue and not washed out.

Furthermore, a pretreatment that does not produce inhibitors that are detrimental to living cells could well determine the product diversity of the prospective full scale cellulosic biorefinery. AFEX has been shown to improve the rumen digestibility of lignocellulosic materials [54], and thus providing a valuable co-product (animal feed) from the biorefinery [55] through unlocking plant carbohydrate for animal production. This approach can create a scenario where food and fuel production are cooperative rather than competitive.

CONCLUSIONS

Ammonia fiber expansion (AFEX), a dry-to-dry pretreatment process, completely preserves Klason lignin and carbohydrate. In comparison, 13% of xylan was degraded to byproduct and 12% of the Klason lignin was not preserved in the dilute acid pretreated corn stover. Categorically, streams resulting from AFEX-CS displayed significantly better fermentability than those from dilute acid. While dilute acid pretreatment eliminates the need for hemicellulolytic enzymes for hydrolysis, AFEX-centered cellulosic technology simplifies production steps, reduces the requirement for nutrient supplementation, increases the diversity of co-products and potentially enhances the environmental benefits beyond the direct impact of the pretreatment processes.

CHAPTER III STRAIN COMPARISON FOR CELLULOSIC ETHANOL FERMENTATION

INTRODUCTION

Development of microbial platforms has been extensively pursued to achieve cost-competitive ethanol yield, titer and productivity [56, 57]. Among the ethanologenic strains, *Saccharomyces cerevisiae* [29, 58], *Zymomonas mobilis* [31] and *Escherichia coli* [28, 59] have been widely investigated and developed for cellulosic ethanol production. An economically-attractive cellulosic technology requires the strain to achieve ethanol yield, titer and rate higher than 90%, 40 g/L (5.1%v/v), 1.0 g/L/hr, respectively [27].

Despite the wealth of publications on strain development, efforts to compare their performance are often hampered by the variations in experimental conditions such as sugar type and concentration, media nutrient levels, initial cell density, feedstock pretreatment selection and detoxification (if applied)[60-62]. In this work, a common platform to obtain comprehensive fermentation parameters using *S. cerevisiae* 424A(LNH-ST), *Z. mobilis* AX101, and *E .coli* KO11 as the fermenting strains is

established. In addition, the effect of the water-soluble substances (mainly pretreatment-mediated reaction compounds) from AFEX pretreated corn stover on the growth and fermentation of these three strains is investigated. Fermentation of enzymatic hydrolysate from AFEX-pretreated corn stover at high solids loading was also examined.

MATERIALS AND METHODS

AFEX-pretreated corn stover (AFEX-CS)

Corn stover (CS) was obtained from NREL (Golden, Colorado), milled and passed through a 4 mm screen. The untreated corn stover consisted of 33.2% cellulose, 22.4% xylan, 3.3% arabinan and 2.3% protein on a dry weight basis. The pretreatment conditions were as follows: temperature at 110-130 °C; catalyst loading at 1.0 g anhydrous ammonia to 1.0 g dry corn stover ratio; water loading at 0.6g water to 1.0 g dry corn stover; 15 min retention time. Each pretreatment batch contained 150 g corn stover on a dry weight basis. The AFEX apparatus, pretreatment conditions and experiment procedures were as reported on page 14.

Microbial strains

Metabolically-engineered ethanologens used in this investigation are *Saccharomyces cerevisiae* 424A(LNH-ST), *Zymomonas mobilis* AX101 and *Escherichia coli* KO11. Strains *Saccharomyces cerevisiae* 424A(LNH-ST), *Zymomonas mobilis* AX101 were provided by

Purdue University and the National Renewable Energy Laboratory (NREL)respectively.

Strain *E. coli* KO11 was obtained from American Type Culture Collection (ATCC) with designated number 55124. Genetic modification and reported fermentation performance were previously reported [28, 31, 63, 64].

Corn steep liquor (CSL)

FermGold™ Corn Steep Liquor (Lot: 154-07) from Cargill, Inc (Minneapolis, MN) was used as the nitrogen source for fermentation. Technical information from Cargill, Inc indicated that FermGold™ CSL contained 48.0-52.0% dissolved solids and 19.5-23.5% total protein. To prepare 20%w/v CSL, 200 g of FermGold™ CSL was diluted to total volume of 1.0 liter with distilled water after pH was adjusted to 7.0 with regent grade KOH. The insoluble solids were separated from the liquid by centrifugation at 5,000 × g for 30 min. The 20%w/v CSL was sterile-filtered (0.22μm) and used for media preparation.

AFEX-CS water extract preparation

AFEX-pretreated corn stover was washed with distilled water at a ratio of 1 g dry CS to 5 mL of water to produce a water extract at 20% solids loading equivalent as described on page 16. The AFEX-CS water extract was used for the fermentation studies.

AFEX-CS enzymatic hydrolysate 6% glucan loading (18% solids loading)

Enzymatic hydrolysate from AFEX-CS was hydrolyzed using both cellulase and hemicellulase commercial mixtures. The cellulase mixture consisted of Spezyme CP [86.7 mL/kg CS; 15 FPU/g cellulose] and Novozym 188 [43.7 mL/kg CS; 32 *p*NPGU/g cellulose]. The hemicellulase mixture was Multifect Xylanase [12.7 mL/kg CS] and Multifect Pectinase [8.9 mL/kg CS]. Enzymatic hydrolysis was performed for 96 hr at pH 4.8, 50°C and 250 rpm agitation. Other details were as described previously [47]

Seed culture preparation

The frozen (-80°C) glycerol stock was transferred to 100 mL liquid media (nitrogen source, 50 g/L total sugar, appropriate buffer and antibiotics) in a 250 mL unbaffled flask. The cells were grown overnight under largely anaerobic conditions at their respective temperatures and initial pH, 150 rpm agitation. Details of culture temperature, initial pH, antibiotics, sugar levels and nitrogen source are as listed in Table 3

Table 3 Seed culture media recipe for the three ethanologenic strains

Strain	Temp (°C)	Buffer/pH	Antibiotics	Sugars Conc	Nitrogen Source
KO11	37	0.1 M MOPS/ 7.0	50 mg/L Chloramphenicol	50 g/L for Glucose-only and fermentation; 30 g/L + 20 g/L for co fermentation and xylose-only	2.0% w/v CSL for fermentation in CSL fermentation; 5.0 g/L yeast extract+ 10.0 g/L peptone for water extract and AFEX hydrolysate fermentation
AX101	30	0.05 M Phosphate / 5.5	30 mg/L Ampicillin		
424A-(LNH-ST)	30	0.05 M Phosphate / 5.5	50 mg/L Ampicillin		

Water extract fermentation

Fermentation was conducted on *E. coli* KO11 using 15.0% solids-loadings-equivalent of AFEX-WS with or without addition of commercial enzymes at loadings described in previous section [47]. YEP (5 g/L yeast extract, 10 g/L peptone), 50 g/L glucose and 25 g/L xylose were added into the media mixtures. Fermentation was conducted at 37°C, pH 7.0, in a 125 mL shake flask with a 50 mL working volume. Initial cell density was at 0.5 OD (600nm). Error bars shown in the results are standard deviations of duplicates.

HPLC analysis

The concentrations of glucose, xylose, ethanol, acetate, formate, lactate, glycerol and xylitol in the fermentation and culture experiments were analyzed using HPLC with a Biorad Aminex HPX-87H column (Hercules, CA). The column temperature was maintained at 60°C and the mobile phase (5 mM H_2SO_4) was kept at 0.6 mL/min flow rate. The HPLC system used was as reported [3].

RESULTS

Fermentations using CSL as nutrients supplement

Fermentations using 2% w/v CSL as nitrogen source indicated that these three strains effectively produce ethanol from glucose or a mixture of glucose and xylose. During glucose fermentation, the fermentations were completed within 72 hr (Figure 11A) and ethanol was produced at concentrations higher than 40 g/L. In particular, *S. cereivisiae*

424A(LNH-ST) has the highest rate of glucose utilization at 4.16 g/L/hr (Figure 11A). However, an increase in xylose concentration correlated with a decrease of the overall fermentation rate. Overall sugar consumption rates compared between the glucose and xylose fermentation were closest for *E. coli* KO11 followed by *Z. mobilis* AX101 and then S. cereivisiae 424A(LNH-ST) (Figure 11,Table 4). Remarkably, xylose fermentation in S. cereivisiae 424A(LNH-ST) achieved only 37.9% of xylose consumption after 168hr. Nevertheless, xylose fermentation by *S. cereivisiae* 424A(LNH-ST) was completed when using YEP as the nutrients supplement. Specific ethanol productivities of fermentations using the bacteria (AX101 and KO11) as the fermenting strain were at least twice as great as those for *S. cereivisiae* 424A(LNH-ST), regardless the type of carbon source (Table 4).

Metabolic ethanol yield and byproducts profiles

For *Z. mobilis* AX101 and *S. cereivisiae* 424A(LNH-ST), metabolic ethanol yield appeared to decrease in complete fermentation of xylose-containing CSL media (Table 4). Carbon source (glucose or xylose) did not significantly affect the metabolic yield in *E. coli* KO11 fermentation. This trend is also reflected through the profile of targeted byproducts. The total concentrations of the targeted net-byproducts formation in xylose-containing fermentation increased for *Z. mobilis* AX101 and *S. cereivisiae* 424A(LNH-ST) compared to glucose-only fermentation, but were essentially unchanged for *E. coli* KO11 (Figure 12). In xylose-containing fermentation (both xylose only and cofermentation) by AX101

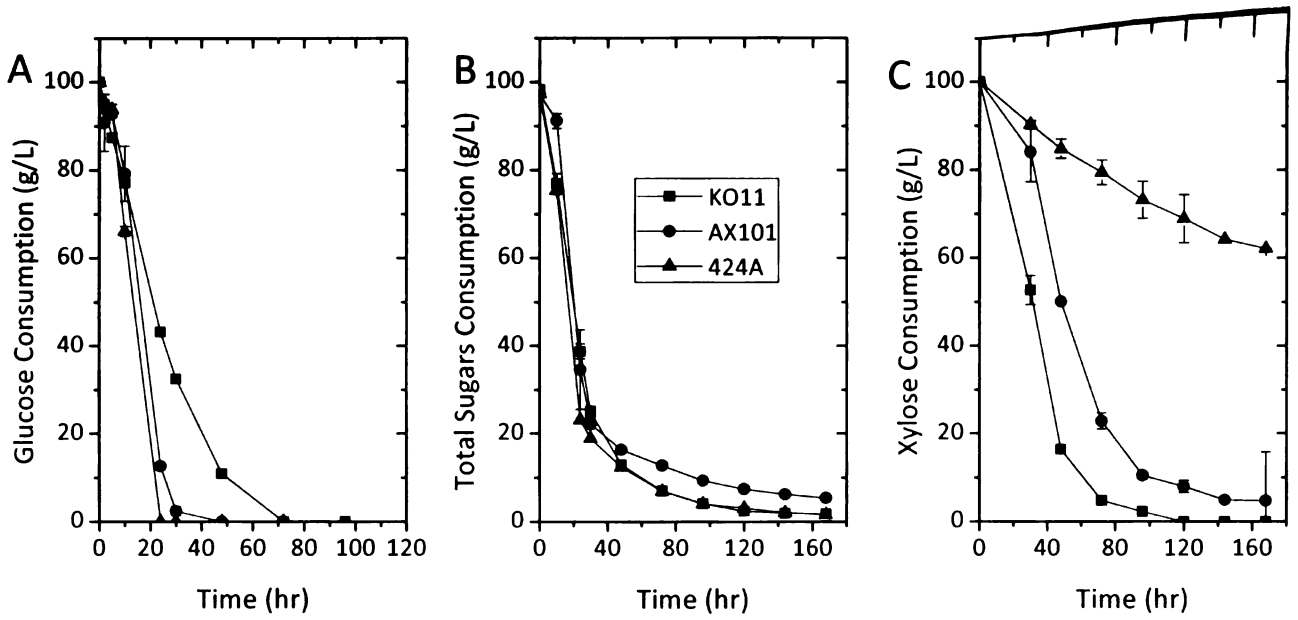


Figure 11 Fermentation using *E. coli* KO11, *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST) in 2% w/v corn steep liquor (CSL) with (A) glucose as sole carbon source, (B) glucose and xylose mixture at ratio 7:3 and (C) xylose as sole carbon source.

Fermentation was conducted in the fleaker fermentor under largely anaerobic condition and initiated at cell density equivalent to 0.5 unit OD600nm. Temperature and pH were controlled at 37°C, 6.8 for *E. coli* KO11, and 30°C, 5.5 for *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST).

Table 4 Results summary for fermentation using *E. coli* KO11, *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST) in 2% w/w corn steep liquor (CSL) or yeast extract peptone (YEP)

Nutrients Source/ Sugar Type	Conc (g/L)	Strain	Sugar Consumption (%)[a]	Metabolic Yield (%)[a,b]	Volumetric Productivity (g/L/hr)[b]			Specific EtOH Productivity (g/L/hr/ g cell)[b] 0-48 hr
					Glc 0-24hr	Xyl 0-48hr	EtOH 0-48hr	
CSL/ Glucose	100	KO11	100.0 ±0.0	87.0 ±0.9	-2.37 ±0.00	NA	0.79	1.50±0.00
		AX101	100.0 ±0.0	93.2 ±0.1	-3.64 ±0.13	NA	0.97	2.61±0.03
		424A(LNH-ST)	100.0 ±0.0	85.2 ±0.5	-4.16 ±0.03	NA	0.87	0.69±0.00
CSL/ Glucose + Xylose	70 + 30	KO11	98.2 ±0.5	85.1 ±1.1	-2.16 ±0.07	-0.38 ±0.03	+0.72 ±0.01	0.55±0.00
		AX101	94.5 ±2.4	88.6 ±0.0	-2.43 ±0.37	-0.29 ±0.05	+0.77 ±0.02	0.65±0.02
		424A(LNH-ST)	98.4 ±0.5	82.4 ±0.5	-2.76 ±0.08	-0.39 ±0.04	+0.73 ±0.01	0.13±0.00
CSL/ Xylose	100	KO11	100.0 ±0.0	85.1 ±0.0	NA	-1.74 ±0.02	+0.72 ±0.01	0.54±0.00
		AX101	95.3 ±0.1	84.9 ±0.2	NA	-1.04 ±0.01	+0.42 ±0.00	0.43±0.01
		424A(LNH-ST)	37.9 ±6.5	89.8 ±1.3	NA	-0.32 ±0.05	+0.09 ±0.02	0.10±0.00
YEP[c]/ Xylose	100	424A(LNH-ST)	100 ±0.0	92.0 ±0.01	NA	-1.77 ±0.02	+0.87 ±0.01	0.11±0.00

[a] Time-span for the calculated data: Glc fermentation 0-72hr; Xyl and co-fermentation 0-168hr

[b] The method to calculate metabolic yields and productivities were as reported [3]

[c] YEP: 10 g/L Bacto Yeast Extract + 20 g/L Bacto Peptone

xylitol is the primary byproduct and it contributed about 70% of the total measured byproducts. In fermentation by *S. cereivisiae* 424A(LNH-ST), net productions of 67 and 81 mg glycerol/g total consumed sugar(s) were observed in glucose and co-fermentation, respectively (Figure 12A & B). In addition, xylitol production during co-fermentation contributed to the lower metabolic yield observed compared to glucose-only fermentation. Organic acids were identified as the predominant group of byproducts from fermentation by *E. coli* KO11 (Figure 12). Although total concentrations of the targeted byproducts were at 6.0-6.5 g/L regardless of carbon source, the byproduct profile varied substantially. While acetate formation increased from 21.4 (glucose-only) to 47.5 mg/g consumed sugar (xylose-only), lactate production diminished during xylose-only fermentation. Of all fermentations, glucose fermentation by *Z. mobilis* AX101 achieved the highest metabolic yield and lowest targeted byproduct formation.

Fermentation using AFEX-CS water extract

AFEX-CS water extract was used to provide a representative compound profile found in the pretreated biomass without the involvement of enzymatic hydrolysis. Fermentations by these three strains exhibited similar patterns; in that moderate levels of AFEX-CS water extract improved cell growth but the degree of improvement decreased as the strength of the water extract increased. However, a greater cell density was achieved in most of the water extract-containing fermentations relative to the control (YEP with no water extract) (Figure 13A).

The rate of xylose fermentation correlated well with the cell growth pattern (Figure 13). *E. coli* KO11 consumed xylose completely at the highest rate (close to 20 g/L/hr) at 5% solids loading equivalent of water extract. However, the rate decreases substantially as the solids loading increased. In the highest tested solids loading, *S. cereivisiae* 424A(LNH-ST) has the greatest xylose consumption rate (12.8 g/L/hr) followed by *E. coli* KO11 and *Z. mobilis* AX101. Although able to ferment at the highest specific rate (g/hr/g cells), *Z. mobilis* AX101 consumed both sugars at the lowest volumetric rate. *Z. mobilis* AX101 also appears to have the lowest tolerance toward water-soluble compounds in AFEX-CS. The cell density of *Z. mobilis* AX101 at 24 hr decreased by 66% when the solids loading was increased from 5% to 15% (Figure 13A). This decrease for both *S. cerevisiae* 424A(LNH-ST) and *E. coli* KO11 was 26%. The effect of AFEX-CS reaction compounds at 15% solids loading on glucose fermentation was practically negligible for *E. coli* KO11 (Figure 14). However, these compounds are shown to be rather inhibitory toward xylose fermentation (Figure 14). The xylose consumption rate within 96hr in water extract fermentation was five times lower than that of the control experiment.

Fermentation using AFEX-CS Hydrolysate (18% Solids Loading) and AFEX-CS Water Extract

All tested strains were able to grow and completely consume glucose on the AFEX-CS hydrolysate without washing of the pretreated biomass, nutrient supplementation or detoxification (Figure 15). Similar to co-fermentation in CSL (Figure 11B, Table 5), xylose fermentation is considerably slower than glucose fermentation. In the hydrolysate

fermentation, xylose fermentation from the tested bacteria (AX101 and KO11) was very poor; less than 20% of the total xylose was consumed (Figure 15A, C; Table 5). Hence, xylose fermentation became the bottleneck for yield, concentration and rate for the bacteria. However, nearly complete xylose consumption was achieved in *S. cereivisiae* 424A(LNH-ST) fermentation at a metabolic yield of 0.47 gram ethanol per gram consumed sugars.

Table 5 Qualitative summary of the relative fermentation performance of *Z. mobilis* AX101, *S. cerevisiae* 424A(LNH-ST) and *E. coli* KO11

Fermentation Parameters		**AX101**	**424A(LNH-ST)**	**KO11**
Glucose Consumption	In Corn Steep Liquor	Very Fast	Very Fast	Fast
	In Lignocellulosic Hydrolysate	Average	Very Fast	Average
Xylose Consumption	In CSL Co-Fermentation	Average	Very Slow	Fast
	In Lignocellulosic Hydrolysate	Very Slow	Average	Very Slow
Nutrient Requirement	Glucose-only	Low	Low	Low
	Co-fermentation	Low	Low	Low
	Xylose-only	Average	High	Low
Growth Robustness		Average	Very High	High
Metabolic Yield		Very High	High	High

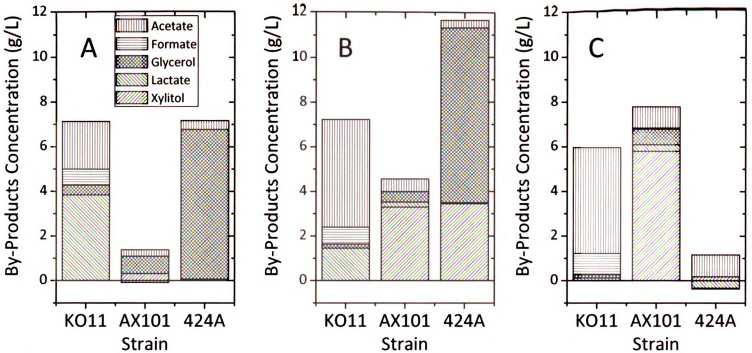


Figure 12 By-products concentration during (A) glucose fermentation at 72 hr, (B) co-fermentation at 168 hr and (C) xylose fermentation at 168 hr, in CSL media using *E. coli* KO11, *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST).

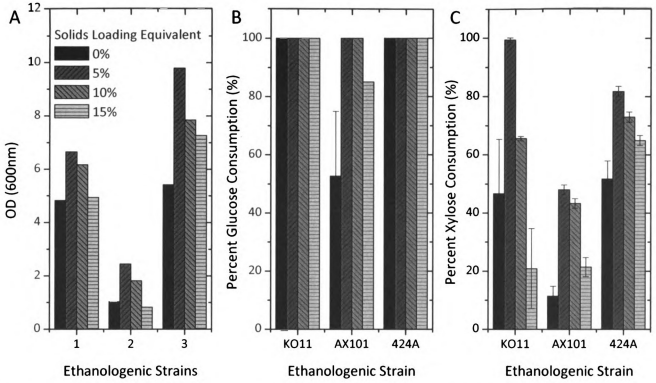


Figure 13 The effect of water-soluble compounds from AFEX-treated corn stover on (A) cell growth (B) percent glucose consumption and (C) percent xylose consumption after 24 hr fermentation.

The experiments were conducted in 24 wells plate at 2.0 mL working volume under largely anaerobic condition. The initial concentration of glucose, xylose and cell density was 30 g/L, 20 g/L and 0.5 unit of OD600nm, respectively. The fermentation media was supplemented with 2.5 g/L yeast extract and 5.0 g/L peptone.

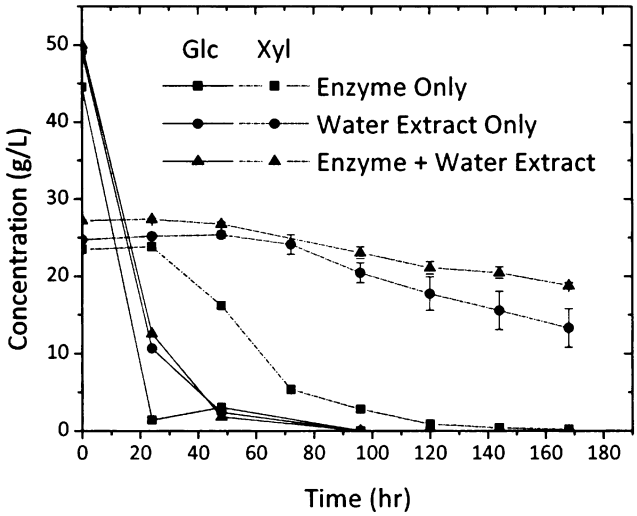


Figure 14 Fermentation using *E. coli* KO11 on YEP media supplemented with commercial enzymes, 15% solids loading equivalent of AFEX-pretreated corn stover water extract or a combination of commercial enzymes and the water extract.

Fermentation was conducted at 37 °C, pH 7.0 (adjusted during fermentation) and was initiated at 0.5 OD600nm.

DISCUSSION

Rationale behind the platform for comparison

In this chapter, I first compared glucose, xylose and co-fermentation in the CSL and followed by co-fermentations on AFEX-CS enzymatic hydrolysate to elucidate its effects on microbial growth pattern and xylose utilization. Fermentations using CSL reveal fermentation performance of respective ethanologens without the interference from

reaction products from the pretreated biomass. CSL has also been regarded as a economical nitrogen source in large scale fermentation [39]. Lignocellulosic hydrolysate from AFEX-CS without washing, detoxification and supplementation provided the actual sugar media for cellulosic ethanol production. This investigation platform would enable us to evaluate the strains based on their intrinsic fermentation ability and robustness for industrial applications.

Benchmarking of the non-cellulase-secreting ethanologenic strains on CSL media

The three tested strains were able to produce ethanol with a metabolic yield between 82.4-93.2% of theoretical maximum in both glucose and co-fermentation at concentrations of 40 g/L or higher, at a rate over 0.72 g/L/hr (0-48hr). These parameters are comparable to those projected to be necessary for a viable cellulosic ethanol industry [27]. The growth robustness of *E. coli* KO11 and *S. cerevisiae* 424A(LNH-ST) should be sufficient to withstand the potential inhibitory effect of AFEX-CS degradation compounds at very high solids loading (greater than 25%). However, in the case for AFEX-hydrolysate, robustness of the strain can be readily increased through cell adaptation, proven for both *Z. mobilis* AX101 (Figure 15A) and KO11 [3]

Higher hexose to pentose ratio improves overall fermentation efficiency and economics

Among all tested strains, regardless of media used, fermentations with higher glucose to xylose ratios yielded better results in term of ethanol yield, concentration and rate;

these are the three most important parameters dictating the overall economics of cellulosic ethanol production. Pentose-only fermentation, even in a naturally-occurring xylose-metabolizing strain such as *E. coli*, has proven to be more difficult than hexose fermentation. One proven cause is the lack of precursors to synthesize products derived from 2-ketoglutarate[65]. In the heterologous pentose metabolic system, further complicating issues are associated with pentose transport [66] and redox balance [57, 58] must be resolved. The increase of hexose (glucose and mannose) to pentose ratio in biomass could be achieved through plant genetic engineering [67].

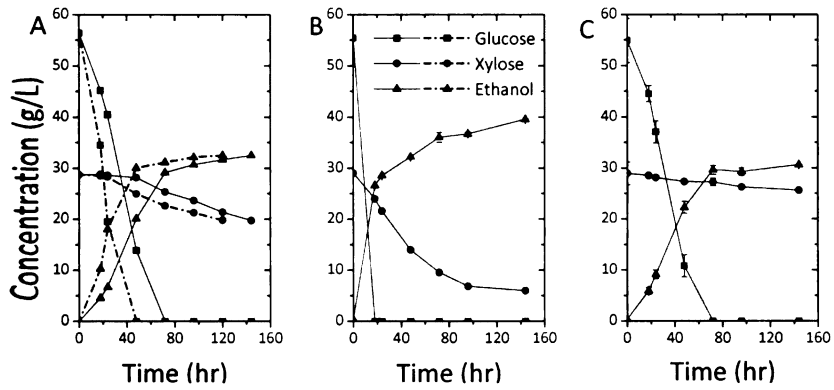


Figure 15 Fermentation using (A) *Z. mobilis* AX101, (B) *S. cerevisiae* 424A(LNH-ST), (C) *E. coli* KO11, in enzymatic hydrolysate from 6.0% glucan loading of AFEX-pretreated corn stover.

Fermentation was conducted under largely anaerobic condition and initiated at cell density equivalent to 0.5 unit OD600nm Temperature and pH were controlled at 37°C, 6-8 for KO11, and 30°C, 5.5 for AX101 and 424A(LNH-ST). Solid lines: Seed culture in YEP; Dotted lines: Seed culture in 3% glucan loading of AFEX-CS hydrolysate.

Degradation products from pretreated biomass complicates xylose fermentation

Xylose fermentation in lignocellulosic hydrolysate is substantially more challenging relative to co-fermentation in CSL. Xylose consumption in the bacteria fermentations was considerably weaker than in *S. cerevisiae* 424A(LNH-ST) fermentation. The selective inhibition, presumably from reaction-degradation products from pretreated biomass, on xylose fermentation is not well understood and deserves extensive investigation.

In most pretreatments of biomass at acidic pH such as dilute acid and steam explosion, a hemicellulose hydrolysate stream is produced and separated from the solids stream for fermentation [43]. Inhibition due to an acidic pH pretreatment was reported to be considerably more extensive than inhibition due to AFEX pretreatment [2]. Fermentation of a hemicellulose hydrolysate can be very difficult; detoxification, nutrient supplementation and/or high initial cell density may be required to increase its fermentability. Improved fundamental understanding of the inhibitory mechanism selectively targets xylose fermentation, presumably by these degradation products, could help alleviate this crucial process bottleneck.

Advantages of the individual fermenting strains

S. cerevisiae 424A(LNH-ST) exhibited the best glucose fermentation rate and highest overall fermentation yield in the presence of AFEX-CS degradation compounds with acceptable metabolic ethanol yield. The bacterial pathways were shown to be effective, achieving higher ethanol production per unit cell biomass and metabolic yields

(compared to the yeast). In particular, *E. coli* KO11 showed robust growth and co-fermentation in AFEX-CS water extract (Figure 13). This is contrary to the general perception that E. coli has low growth robustness [27]. Fermentation pH (near-neutral pH for KO11) might be one of the key influences, as organic acids are less inhibitory in dissociated form [68]. E. coli KO11 was also able to produce and tolerate ethanol concentrations at least up to 45 g/L (Figure 11,Table 4). Although the maximum ethanol tolerance of *E. coli* might not be comparable to *Z. mobilis* or *S. cerevisiae* [27], the ability to produce and tolerate ethanol between 40-70 g/L is probably sufficient for commercial cellulosic ethanol production.

Compatibility between strain selection and biomass processing strategy

The selection of an ethanologenic strain for cellulosic ethanol production is highly dependent on the process design, particularly feedstock pretreatment selection and the configurations of the biologically-mediated processes (whether enzymatic hydrolysis and fermentation are combined).

S. cerevisiae 424A(LNH-ST) is highly robust and able to ferment both glucose and xylose reasonably well, even at high solids loading, but likely requires more nutrients for effective pentose-only fermentation (Table 5). *E. coli* KO11 is able to tolerate a relatively high concentration of AFEX-CS degradation compounds and produces ethanol at a high metabolic yield and rate. Unfortunately, xylose utilization in degradation compound-containing media (high solids loading) is severely affected. *Z. mobilis* AX101 appears to

be an excellent ethanologenic strain for media with low levels of inhibitors due to its superior metabolic yield and glucose fermentation rate (Table 5). Evidently, selection of a pretreatment with low inhibitory compound generation is the crucial factor, particularly for the *Z. mobilis* AX101 microbial platform.

The importance of a low-inhibitor-generating pretreatment

The feedstock pretreatment chemistries and temperatures dictate the profile (type and concentration) of degradation compounds produced. AFEX produces substantially less inhibitory compounds compared to pretreatments at acidic pH [2]. Compatibility between pretreatment, fermenting strain and configurations of bio-mediated processes may well the key for the viability/profitability of this industry. Reducing the production levels of inhibitory degradation compounds would inevitably widen the range of suitable ethanologenic strains and ultimately improve overall economics.

CONCLUSIONS

The tested ethanologens are able produce ethanol from a CSL-supplemented co-fermentation at a metabolic yield, final concentration and rate greater than 0.42 g/g consumed sugars, 40 g/L and 0.7 g/L/hr (0-48hr), respectively. Xylose-only fermentation of the tested ethanologenic bacteria is 5-8 times faster than *S. cerevisiae* 424A(LNH-ST) in the CSL fermentation.

All tested strains grow and co-ferment sugars at 15% solids loading equivalent of AFEX-pretreated corn stover water extract. However, both *E. coli* KO11 and *S. cerevisiae* 424A(LNH-ST) exhibit higher growth robustness than AX101. In 18% solids loading lignocellulosic hydrolysate from AFEX pretreatment, complete glucose fermentations can be achieved at a rate greater than 0.77 g/L/hr. In particular, the glucose consumption by *S. cereivisiae* 424A(LNH-ST) is higher than 3.1 g/L/hr. Furthermore, *S. cereivisiae* 424A(LNH-ST) consumed xylose in lignocellulosic hydrolysate at the greatest extent and rate, leading to its decided advantage in overall ethanol yield and titer over other tested strains.

CHAPTER IV CELLULOSIC ETHANOL PRODUCTION USING AFEX AND *SACCHAROMYCES CEREVISIAE* 424A(LNH-ST) AS PLATFORM TECHNOLOGY

INTRODUCTION

Ammonia fiber expansion (AFEX) has been shown (in Chapter II) to be an effective pretreatment method for generating highly fermentable substract [3]. *Saccharomyces cerevisiae* 424A(LNH-ST) produced ethanol at the highest overall yield and rate compared to other tested ethanologens in Chapter III [57]. In this chapter, I seek to take advantage of the promising features of AFEX pretreatment and *S. cerevisiae* 424A(LNH-ST) to formulate and demonstrate an industrially-relevant strategy for fermentation using lignocellulosic biomass.

The requirements for: i) high starting cell density, ii) hydrolysate conditioning and iii) nutrient supplementation to conduct lignocellulosic fermentation are evaluated. I also construct a comprehensive mass balance based on conversion of carbohydrates in untreated biomass to ethanol to elucidate the current status and the bottlenecks of the technology. To better understand the phenomenon of slow xylose utilization in

hydrolysate, the interactions between degradation products, xylose metabolism, cell growth and media nutrient content are described.

MATERIALS AND METHODS

Corn stover (CS)

The composition of the corn stover was as described on page 14

AFEX pretreatment

The AFEX pretreatment was conducted as described on page 14

AFEX-corn stover (AFEX-CS) water extract

Washing of CS was conducted by spraying distilled water on AFEX-treated CS at a ratio of 1 g dry CS to 5 mL of water (20% solids loading) as described on page 16. Washed CS solids were then enzymatically-hydrolyzed. This water extract was used for fermentation studies.

Enzymatic hydrolysis

The AFEX-treated corn stover was enzymatically-hydrolyzed by commercial enzymes mixtures. The cellulase mixture consisted of Spezyme CP [86.7mL/kg CS; 15 FPU/g cellulose] and Novozyme 188 [43.7 mL/kg CS; 32 *p*NPGU/g cellulose]. The hemicellulase

mixture was Multifect Xylanase [12.7 mL/kg CS] and Multifect Pectinase [8.9 mL/kg CS]. The spectrum of activities for the commercial enzymes were as reported [44]. The Spezyme and Multifect enzymes were obtained from Genencor Inc (Palo Alto, CA) and Novozyme 188 was purchased from Sigma-Aldrich Co (St. Louis, MO). These enzyme mixtures, unless otherwise stated, were used in all hydrolysis experiments. The cellulose loading for the hydrolysis was kept at 6.0% by weight (60 g of cellulose per 1 kg of hydrolysis mixture) which corresponds to 17.6% and 16.2% solids loading, in unwashed and washed AFEX-CS respectively. The reaction was carried out for 96 hr at pH 4.8 (0.05 M phosphate buffer), 50°C and 250 rpm agitation. Each hydrolysis was conducted in a 1.0 L baffled flask with 500 g total saccharification mixture. Chloramphenicol (Cm) was added to a final concentration of 50 mg/L to minimize the risk of contamination. After 96 hr, the hydrolysis mixture was centrifuged twice at 5,000×g for 30 min to separate the liquid solution from unhydrolyzed solids. The supernatant (not sterilized) was used for fermentation.

Microorganism and seed culture preparation

Xylose-fermenting *Saccharomyces cerevisiae* 424A(LNH-ST) was obtained from Dr Nancy W. Y. Ho from Purdue University through MSU Material Transfer Agreement (MTA06-119). Details of the metabolic engineering of this yeast have been reported [64, 69]. To prepare seed culture, the strain was grown on YEP (5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) plate for one to two days at 30°C. The cells were transferred to liquid YEP media supplemented with 50 g/L glucose in an unbaffled flask. The 424A(LNH-ST)

seed was grown overnight at 30°C, 150 rpm agitation, under a largely anaerobic conditions. The cell density of a typical 424A(LNH-ST) overnight seed culture reached 15 units absorbance at 600nm.

Fermentation

Fermentations were conducted at a working volume of 70 mL in a 250 ml unbaffled flask at 30°C, initial pH5.5, 150 rpm agitation. A designated volume of seed culture was centrifuged (15,000×g) for 5 min and the yeast cell pellet was resuspended into the media to initiate fermentation. The flasks were capped with rubber stoppers pierced with a needle to vent carbon dioxide formed during fermentation. Samples were taken during the course of fermentation. Cell density was measured using a spectrophotometer at 600nm (DU Series 700 UV/Vis, Beckman Coulter). One unit of absorbance is approximately equal to 0.55 g (dry-wt-cell)/L. Glucose, xylose, glycerol and ethanol profiles were measured using HPLC as previously described [3].

Complex media and hydrolysates

Three different fermentation media were prepared: (i) complex media (YEP + 70 g/L glucose+ 40 g/L xylose); (ii) hydrolysate I: AFEX-CS hydrolysate at 6.0% glucan loading; (iii) Hydrolysate II: AFEX-CS hydrolysate at 6.0% glucan loading with supplemental sugars (10 g/L glucose + 12 g/L xylose), which is the monomeric sugar equivalent of the oligomeric sugar content in the hydrolysate. The initial pH of the hydrolysates was adjusted to 5.5 using reagent grade KOH. The starting cell density of fermentation was

at 2.0 (OD600nm) which is approximately 1.1 g dry-cell-wt./L. Hydrolysates were fermented without prior detoxification or nutrient supplementation. Volumetric productivities of glucose, xylose and ethanol were calculated from their concentration gradients over the first 6, 12, 24 hr of fermentation, respectively. Specific productivities (g/L/hr/g cell) were calculated by dividing volumetric productivities by the respective values for dry cell mass. Metabolic ethanol yield was estimated based on total consumed glucose and xylose. The theoretical maximum yield was 0.51 g EtOH/g sugar.

Effect of pH, temperature, initial cell density, nutrient supplementation and washing on xylose fermentation in hydrolysate

Several fermentations were carried out to investigate the effect of (i) pH, (ii) temperature, (iii) initial cell density, (iv) nutrient supplementation and (v) minimal washing on xylose utilization in the cofermentation of hydrolysate. Enzymatic hydrolysis was carried out with the cellulase mixture only. Initial glucose and xylose concentrations in the hydrolysate were 55±2 g/L and 23±1 g/L, respectively. Other experimental details were as listed in Table 6.

Table 6 Summary of parameters for four experiments examining the effects of pH, temperature, initial cell density, and washing and nutrient supplementation on xylose fermentation of corn stover hydrolysate

Experiment / Parameter	Effect of pH	Effect of Temperature	Effect of Initial Cell Density	Effect of Washing and Nutrient Suppl.
Initial pH	3.5, 4.5, 5.5, 6.5	5.5	5.5	5.5
Temperature (°C)	30	25,30,35,37	30	30
Initial Cell Density (OD600 nm)	0.5	0.5	0.5, 6.0, 12.0, 18.0	0.5
Washing on CS	No	No	No	Yes (Minimal Washing)
Nutrient Supplementation	No	No	No	Yes (10 g/L Yeast Extract, 20 g/L Peptone)
Relevant Figure	Figure 16A & B	Figure 16C	Figure 16D	Figure 18

Water extract (With or without YEP supplementation)

The water extract was used to investigate the effects of soluble compounds on AFEX-CS on fermentation. Each fermentation medium contains the water extract, 10 g/L glucose, 40 g/L xylose and 50 mg/L chloramphenicol. YEP (5 g/L yeast extract and 10 g/L peptone), was added to create nutrient-rich conditions. The final concentrations of the water extract in the fermentation media ranged from 0 to 16% solids-loading-equivalent. The reaction was carried out at 15 mL working volume in 20 mL screw-capped vial. Three glass beads (1 cm in diameter) were added into each vial to aid

stirring. Cell density and specific xylose consumption over the first 24 hr of fermentation were estimated.

Corn stover to ethanol analysis

To construct a corn stover to ethanol mass balance analysis, sugars (glucose and xylose in monomeric, oligomeric and polymeric forms) and ethanol content were measured before and after each process, i.e. pretreatment, enzymatic hydrolysis and fermentation.

Around pretreatment

The total weight of dry corn stover before and after pretreatment was recorded. Glucan and xylan contents (as a percentage) of untreated and AFEX-treated corn stover were estimated using the NREL protocol (LAP-002).

Around enzymatic hydrolysis

A known amount of pretreated corn stover was added into the system. Total sugars solubilized in the liquid phase or remaining in unhydrolyzed solids were determined. Soluble oligomeric sugars in the liquid stream were estimated according to the NREL protocol (LAP-014).

Preparation of washed unhydrolyzed corn stover dry matter

Prior to the analysis, unhydrolyzed corn stover was washed twice using distilled water at a ratio of 1 g wet unhydrolyzed corn stover to 5 mL of water. The mixture was centrifuged for 30 min at 6,000 ×g to separate the washed pellet from the supernatant. The volumes of water extracts were recorded, and the concentration

of glucose and xylose were estimated. After washing, a representative sample of unhydrolyzed corn stover was dried under vacuum at 60°C (Vacufuge, Eppendorf) overnight. The glucan and xylan contents in this dry matter were estimated using the NREL protocol (LAP-002). The remaining solids were dried in an oven at 110°C and the total dry weight of the washed solids (from two different dryers) was recorded.

Sugars and ethanol estimation in liquid streams

The liquid streams after saccharification were analyzed to determine ethanol, monomeric glucose and monomeric xylose concentrations. To estimate the concentration of both oligomeric and monomeric sugars in the stream, acid hydrolysis (H_2SO_4) was conducted to hydrolyze sugars from oligomers to monomers. Experimental details were as described in the NREL protocol (LAP-014).

Mass closure analysis:

Mass closure (as a percentage) was calculated by dividing total sugars (glucose or xylose) exiting the unit by total input sugars. Total output sugars were then normalized to 100%.

Around fermentation

The amount of input sugars into the fermentation unit was known. Total ethanol produced and the remaining sugars (both monomeric and oligomeric) after fermentation were estimated. The process yield around fermentation was calculated using Eq.5.

Wh

a

U

AF
Pre

Enzy
Hydro

Ferme

Sourc

$$\text{Yield}(\%) = \frac{[\text{EtOH}]_f - [\text{EtOH}]_i}{[\text{Glc} + \text{Xyl}]_i \times 0.51} \times 100\% \text{--------- Eq 5}$$

Where [EtOH] is concentration of ethanol, [Glc+Xyl] is total concentration of glucose and xylose, f and i denote for final and initial conditions, respectively. Data collected from the analyses were adjusted to the basis of 1.000 kg of dry untreated CS. Detailed experiment procedure on mass balance construction was as described in Table 7.

Table 7 Experimental details on the construction of mass balance

Unit Operation	Recorded Data	Analysis Data
AFEX Pretreatment	Total dry weight of corn stover before and after AFEX	Glucan and xylan contents (in percentage) of untreated and AFEX-treated corn stover were estimated using NREL protocol (LAP-002)
Enzymatic Hydrolysis	Total weight of AFEX-treated CS entering the system	Glucan and xylan contents (in percentage) using NREL protocol (LAP-002)
	Total volume of liquid hydrolysate	Concentration of both oligomeric and monomeric sugars in the stream (NREL LAP-014).
	Total volume of water extract of unhydrolyzed solids	
	Total dry weight of washed unhydrolyzed solids	Glucan and xylan contents (in percentage) using NREL protocol (LAP-002)
Fermentation[a]	Total volume of hydrolysate entering the system	Concentration of both oligomeric and monomeric sugars in the stream before and after fermentation (NREL LAP-014). Final ethanol concentration and cell density (absorbance at 600nm) was measured.

[a] Volume change during fermentation is assumed negligible

RESULTS

Optimization of fermentation conditions

Optimal conditions for fermentation of AFEX-CS-hydrolysate are pH 5.5 and 30°C (among the conditions tested) with respect to overall fermentation yield and rate. While fermentation at pH 6.5 exhibited the highest xylose consumption rate, it had the lowest metabolic yield (79.5%) among tested pH values with the highest level of glycerol (5.0 g/L) formation. Judging from the overall ethanol yield, pH 5.5 was determined to be the optimal pH (Figure 16A &B). Regarding temperature optimization, xylose utilization in fermentations at 35°C and 37°C essentially ceased after 72 hr; 10.9 g/L and 16.1 g/L of xylose were left unconsumed. Xylose fermentation at 25°C was slower than at 30°C (Figure 16C).

The initial rate of xylose consumption is directly correlated to the initial cell density (Figure 16D). However, the difference between the extent of xylose consumption (after 144 hr) was rather small (<3 g/L). Final ethanol yield was almost independent of initial cell density. In order to be more industrially-relevant, relatively low cell densities (≤1.1 g dry-cell-wt./L) were used. With the exception of hydrolysate fermentation at pH 3.5, glucose fermentations were completed within 18 hr (Glucose consumption profiles are not shown in Figure 16).

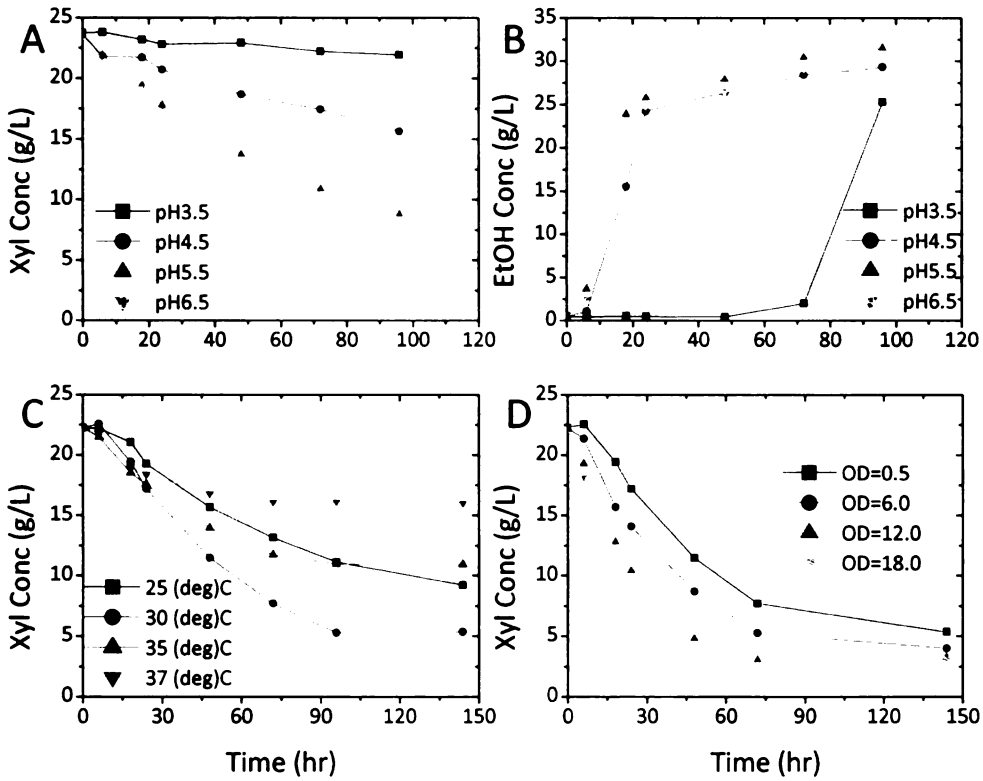


Figure 16 Effect of (A, B) pH, (C) temperature and (D) initial OD on fermentation using hydrolysate from enzymatically-digested AFEX-treated CS using *Saccharomyces cerevisiae* 424A(LNH-ST).

(Glucose consumption profiles are not shown in this graph). Note: Ethanol production from 0 to 25 g/L shown in Panel B is largely due to glucose fermentation

Laboratory media vs AFEX-CS hydrolysate

Fermentations using 424A(LNH-ST) in complex media (YEP) and AFEX-CS-hydrolysates derived from saccharification at 6% glucan loading were compared side-by-side under identical conditions (Figure 17). These hydrolysates were fermented without conditioning (no washing, nutrient supplementation or detoxification) at 1.1 g dry-cell-wt./L starting cell density. The difference between Hydrolysate I and II is that an

additional 10 g/L glucose and 12 g/L xylose were supplemented into Hydrolysate II to examine the scenario in which soluble oligomeric glucose and xylose in the hydrolysate were also utilized.

Strain 424A(LNH-ST) was able to grow well in both complex media and AFEX-CS-hydrolysates and achieved cell densities greater than 6.0 g/L within 12hr of fermentation (Table 8; Figure 19). In complex media, 70 g/L glucose and 40 g/L xylose were completely consumed within 48 hr (Figure 17A). The volumetric glucose consumption rate was 7.3 g/L/hr; 0-6 hr, which was roughly seven-fold higher than the xylose consumption rate. For fermentations in AFEX-CS-hydrolysate, volumetric xylose consumption rates were an order of magnitude lower than for glucose. This indicates that xylose utilization was more susceptible to inhibition. Surprisingly, specific glucose consumption rates in hydrolysate achieved 10.6 g/L/hr/g cell; 0-6 hr, substantially higher than that in complex media (Table 8). Fermentations in the hydrolysates achieved higher metabolic yields with lower xylitol formation (0.46 g ethanol/g consumed sugars; 0.3 g/L xylitol) than complex media (0.43 g ethanol/g consumed sugars; 3.2 g/L xylitol).

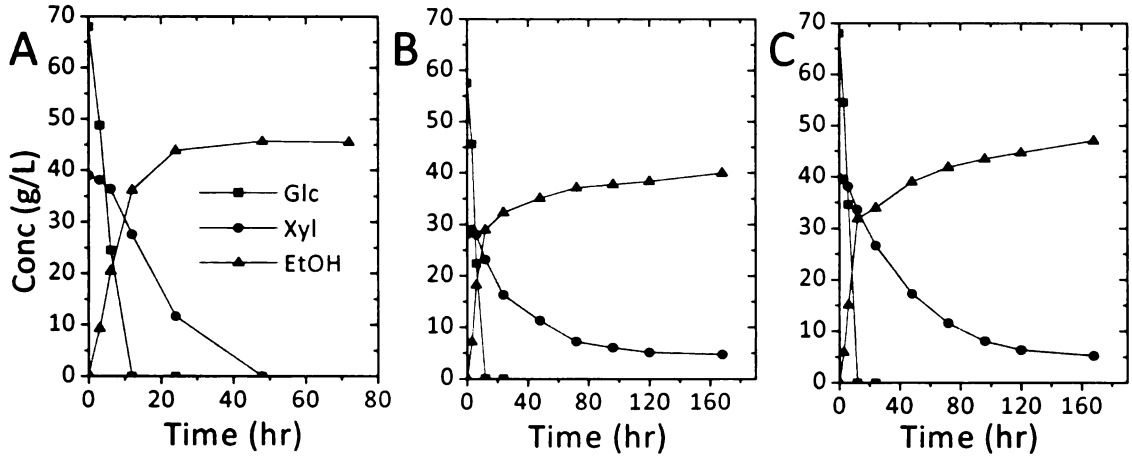


Figure 17 Fermentation of (A) complex media; (B) CS-Hydrolysate I; (C) CS-Hydrolysate II using *S. cerevisiae* 424A(LNH-ST).

Fermentations were initiated with 1.1 g (dry-wt.)/L of 424A(LNH-ST) inoculum, carried out at 30°C, pH5.5 and 150rpm under largely anaerobic condition. The hydrolysates were neither detoxified nor externally nutrient-supplemented.

Washing removes both degradation products and biomass nutrients

The impacts of washing AFEX-pretreated CS (prior to enzymatic hydrolysis) on xylose utilization and the requirements for nutrient supplementation were investigated. Washing of AFEX-CS does not improve xylose utilization unless additional nutrients are provided. Xylose fermentation for unsupplemented washed-CS hydrolysate had the lowest rate (0.12 g/L/hr, 0-96hr) followed by unsupplemented unwashed-CS hydrolysate (Figure 18). This observation is attributed to the loss of nutrients through washing. Besides removing degradation products [70], the water extract of AFEX-CS contains residual ammonia from pretreated materials and other biomass components that are important nutrient sources for fermentation (Table 11, Table 12 in Chapter V). With YEP supplementation, washed-CS hydrolysate had a better xylose fermentation (0.20 g/L/hr,

0-96hr) than unwashed-CS. Evidently, even under nutrient-rich conditions, the presence of degradation products affected xylose utilization.

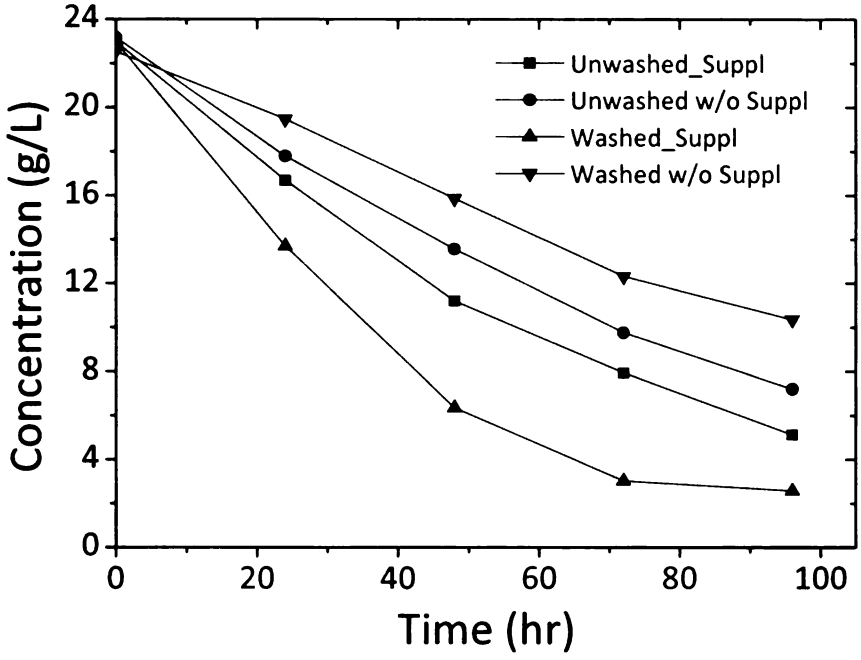


Figure 18 Effect of washing and nutrient supplementation on xylose consumption in the fermentation of hydrolysates from enzymatically-digested AFEX-treated CS.

(Glucose consumption profiles are not shown in this graph)

The effects of soluble substances from AFEX-CS on xylose fermentation

The relationship between xylose fermentation with the levels of degradation products and nutrient content in fermentation media was further investigated using the water extract from AFEX-CS. In nutrient-rich conditions, cell growth decreased as the concentration of AFEX-CS water extract increased. However, the level of specific xylose consumption remained virtually constant at 0.25 g/L/hr/g cell; 0-24hr (Figure 20A). This suggested that xylose utilization was lowered due to the reduced cell mass production

Table 8 Summary of fermentation parameters for complex media and hydrolysates

	Media a	Monomeri Sugar Conc (g/L) [c]		Final EtOH Conc (g/L)	Volumetric Productivity (g/hr/L)			Specific Productivity (g/hr/L/g cells)			Metab. Yield [h] (%)	Process Yield (%)	Cell density [i] (g dry wt/L)
		Glc	Xyl		Glc [e]	Xyl [f]	EtOH [g]	Glc [e]	Xyl [f]	EtOH [g]			
A	YEP [b]	68.0	39.0	45.6 ±0.2	-7.3 ±0.2	-1.1 ±0.1	0.9 ±0.0	-6.5 ±0.3	-2.9 ±0.3	4.9 ±0.1	83.6 ±0.9	83.6 ±0.9	9.5±0.2
B	CS-Hydrolysate I[c]	57.5	28.1	40.0	-5.9	-0.5	0.8	-7.9	-1.8	4.9	92.9	88.5	6.7
C	CS-Hydrolysate II[d]	68.0	39.8	47.0 ±0.3	-5.6 ±0.4	-0.6 ±0.0	0.8 ±0.0	-10.6 ±0.8	-2.0± 0.0	5.7 ±0.1	90.0 ±0.8	85.6 ±0.6	6.5±0.1

[a] Fermentations were conducted at 30 °C, pH5.5 and 150rpm agitation under largely anaerobic conditions, initial cell density was at 1.1 g dry-cell-wt./L;

[b] 5 g/L Bacto Yeast Extract + 10 g/L Bacto Peptone;

[c] Hydrolysate from AFEX-treated CS (enzymatically-digested at 6% glucan loading for 96hr at pH4.8, 50°C);

[d] Hydrolysate from AFEX-treated CS (enzymatically-digested at 6% glucan loading for 96hr at pH4.8, 50°C) with supplemental sugars of 10 g/L glucose and 12 g/L xylose, which is the monomeric sugar equivalent of the oligomeric sugar content in the hydrolysate.

[e,f,g] Productivity over the first 6,12 and 24 hr, respectively;

[h] Metabolic yield calculated based on total consumed glucose and xylose, theoretical EtOH yield was assumed as 0.51 g/g sugar;

[i] Cell density after 24 hr of fermentation, one unit of absorbance at 600nm is approximately equal to 0.55 g dry-cell-wt./L

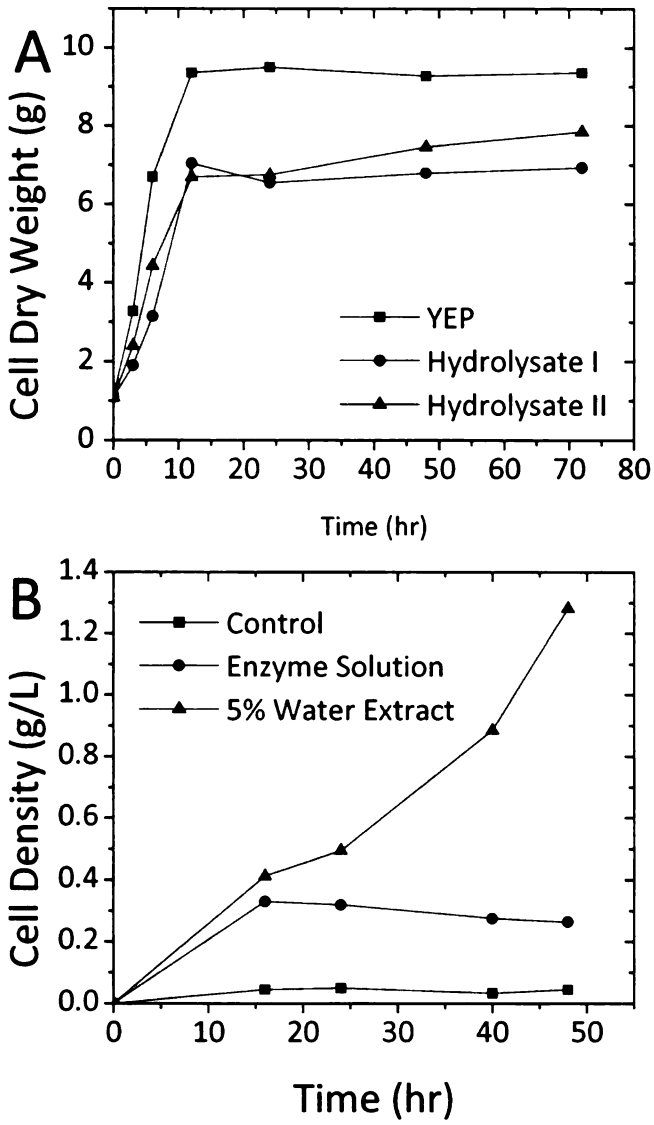


Figure 19 Cell density profile of the fermentation using 424A(LNH-ST)

(A) Cell density profile of the fermentations reported in Figure 17
(B) Fermentation was conducted in 100 mL total volume, at 30 °C, pH5.5 and 150rpm, the initial cell density was at 0.055 g (dry-wt)/L. Control: 50mM phosphate buffer (PB), Enzyme Solution: 50 mM PB+ 0.43 mL Spezyme CP+0.22 mL Novozyme 188+0.064 mL Multifect Xylanase+0.045 mL Multifect Pectinase. 5% Water Extract: 50mM PB in final water extract concentration at 5% solids-loading equivalent. Glucose and xylose (both at 25 g/L) was supplemented to each fermentation.

in the presence of degradation products, while xylose metabolism in cells was apparently not affected. In nutrient-limiting conditions, where the AFEX-CS water extract was the sole source of nutrient, cell growth is proportional to the concentration of the water extract. The relationship between the specific xylose consumption rate and the water extract concentration exhibited a left-skewed curve with the highest rate (0.15 g/L/hr/g cell, 0-24hr) at 4% solids loading water extract (Figure 20B). These results imply that, in nutrient-limiting conditions, (i) a low level of degradation products resulting from AFEX pretreatment stimulated xylose metabolism within cells, but the rate of xylose utilization was limited by cell growth (nutrient availability) and (ii) at high water extract concentration (≥8% solids loading), inhibition of xylose metabolism was the primary factor affecting xylose utilization. Under both conditions, biomass degradation products resulting from the pretreatment (soluble products in the water extract) were shown to increase metabolic ethanol yield (Figure 20A & B). Fermentation using a water extract at 16% solids-loading-equivalent as the sole nutrient source (Figure 20B) achieved comparable cell growth to the YEP-supplementation fermentation (Figure 20A).

Current status and bottlenecks of the technology

Current technology using corn stover as feedstock, AFEX as the pretreatment technology and *S. cerevisiae* 424A(LNH-ST) as the ethanologenic strain in separate hydrolysis and fermentation (SHF) with a starting cell density at 1.1 g dry-cell-wt./L, was able to achieve 191.5 g EtOH/kg untreated CS (Figure 21), 60.8% of the theoretical

maximum yield, at an ethanol concentration of 40.0 g/L (5.1 v/v%) without the need for washing, detoxification and nutrient supplementation.

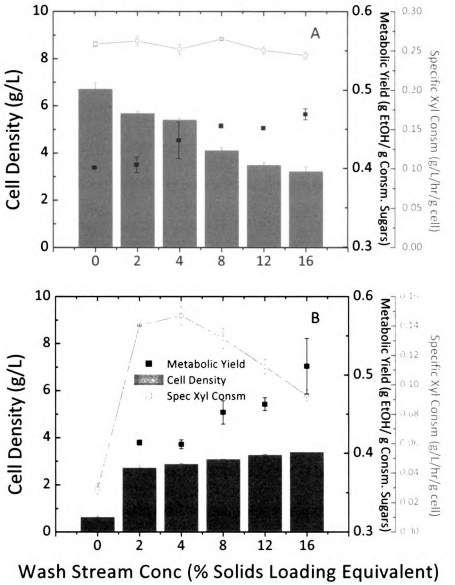


Figure 20 Effect of AFEX-CS water extract concentration on cell growth, metabolic yield and specific xylose consumption rate under (A) nutrient-rich and (B) nutrient-limiting conditions.

Fermentations were initiated with 0.3 g (dry-wt.)/L of 424A(LNH-ST) inoculum, carried out at 30°C and 150rpm. Data points presented were at 24 hr.

During AFEX pretreatment, there was no sugar loss, total material mass was increased by 1-2%, probably due to the ammonia binding onto the biomass [71]. The mass balance around the enzymatic hydrolysis step achieved 98.9% and 107.1% closure for glucose and xylose, respectively. In enzymatic hydrolysis at 17.6% solids loading, 85.8% of the total input sugars (glucose and xylose) were hydrolyzed and solubilized, of which, 78.2% was hydrolyzed to their monomers (Figure 21; Table 9). About two-thirds (62.0%) of the total oligomeric sugars were xylose. Fermentation using 424A(LNH-ST) effectively converted monomeric glucose and xylose with 88.5% ethanol yield. As expected, oligomeric sugars were not utilized by 424A(LNH-ST).

The three process parameters having the strongest influence on ethanol production economics are yield, titer and rate. Enzymatic hydrolysis at high solids loading has been identified as the primary bottleneck affecting overall yield and titer for the cellulose-to-ethanol bioconversion. One-third of the total output sugars were oligomers or polymers which could not be utilized by 424A(LNH-ST). To improve overall ethanol productivity, efforts should focus on increasing the xylose consumption rate during fermentation.

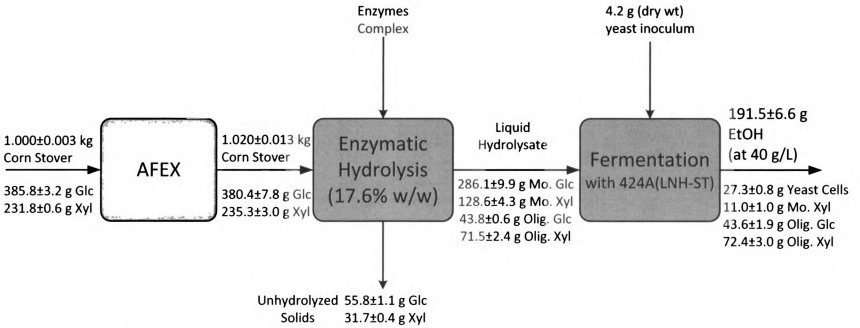


Figure 21 Corn stover to ethanol mass balance analysis.

The analysis was based on AFEX as feedstock pretreatment technology and *S. cerevisiae* 424A(LNH-ST) as the ethanologenic strain; enzymatic hydrolysis was conducted at 6.0% glucan loading (equivalent to 17.6% w/w solids loading). Carbohydrate contents in CS are expressed as the hydrated monomers. Glc: Glucose; Xyl: Xylose; Mo: Monomeric; Olig: Oligomeric.

Table 9 Output sugars analysis from enzymatic hydrolysis of 6.0% cellulose loading AFEX-CS (after normalization)

		Glucose			Xylose			Glucose + Xylose		
		g/L	g	Coverage (%)	g/L	g	Coverage (%)	g/L	g	Coverage (%)
Liquid Hydrolysate	Monomer	58.4±3.1	286.1±9.9	74.2	26.2±1.7	128.6±4.3	55.5	84.6±3.5	414.7±10.8	67.2
	Oligomer	8.9±1.0	43.8±0.6	11.4	14.6±1.4	71.5±3.0	30.8	23.5±1.7	115.3±1.5	18.7
	Subtotal	67.4±2.0	329.9±6.8	85.5	40.8±0.2	200.1±0.0	86.3	108.2±2.0	530.0±6.8	85.8
Unhydrolyzed		NA	55.8±1.1	14.5	NA	31.7±0.4	13.7	NA	87.6±1.2	14.2
Total		NA	385.8±12.1	100	NA	231.8±5.0	100	NA	617.6±13.1	100

NA: Not Applicable

DISCUSSION

Significance of the process integration

Despite abundant published research on ethanol production from lignocellulosic materials, comprehensive system-wide studies with industrially-relevant performance metrics are still lacking. Washing and detoxification steps, though often incorporated to improve the fermentability of the hydrolysate, may well be prohibitively expensive [38]. The straightforward cellulosic ethanol technology reported here, which converts sugars from lignocellulosic materials to ethanol without washing, detoxification and nutrient supplementation, is significant in the search for a highly competitive cellulosic ethanol production strategy. A final ethanol titer of 40 g/L, a benchmark concentration for commercial cellulosic ethanol production, was achieved.

Using AFEX as the pretreatment and *S. cerevisiae* 424A(LNH-ST) as the fermenting strain were the key innovations responsible for achieving this progress. Hydrolysate from lignocellulosic biomass is generally regarded to be nutrient-deficient [72]. However, our results strongly contradict this perception. The perceived nutrient deficiency is likely due to pretreatment at high temperature and acidic pH followed by washing which degrade and/or remove nutrients. In contrast, nutrients provided through AFEX-pretreated corn stover were sufficient to support robust yeast growth. The saccharolytic enzyme preparation provides relatively little nutrient value (Figure 19). Cellulosic ethanol technology therefore need not depend on commercial nutrient

supplements such as yeast extract or corn steep liquor. Yeast cells can also be produced as a valuable co-product using this platform. A recent publication using *E. coli* KO11 to ferment enzymatic hydrolysate from AFEX-treated corn stover has also confirmed the general fermentability of corn stover [3].

Important steps in improving yield and titer

However, further improvements in overall yield and titer are required to make this technology more commercially-attractive. Results presented herein suggest that efforts to increase the availability of fermentable sugars at high concentrations deserve the highest priority. This can be done through (i) optimization of the enzyme formulation for effective saccharification of both cellulose and hemicellulose; (ii) development of ethanologenic strains which consume a wider range of substrates (both mono- and oligosaccharides) and (iii) integration of enzymatic hydrolysis and fermentation in the configurations of simultaneous saccharification and co-fermentation (SSCF) or consolidated bioprocessing (CBP) to alleviate sugar inhibition at high solids loading and reduce enzyme dosage. In this report, enzymatic hydrolysis and fermentation have been conducted separately in order to facilitate understanding of the individual processes. Nevertheless, simultaneous saccharification and fermentation (SSF) was shown to be beneficial compared to separate hydrolysis and fermentation (SHF) [73], in spite of a lower reaction temperature that was adopted to allow fermentation at the expense of greater efficiency during enzymatic hydrolysis. The full potential of these configurations will be better realized if both hydrolysis and fermentation share common

optimal conditions. Developing an oligosaccharide-utilizing strain will be particularly advantageous for AFEX-centered cellulose technology as a sizeable portion of sugars in the hydrolysate are oligomers. As a near-future projection, the overall yield could be increased to 251.4 g EtOH/kg untreated CS with final titer of 52.5 g/L or 6.7%(v/v) (Figure 16B) if complete utilization of soluble sugars is achieved (Table 10).

Table 10 Future projections for ethanol yield and titer based on 6% cellulose loading of AFEX-CS

	Label	Areas of Improvement	g/kg	gal/ton	Conc (g/L)	Conc (%v/v)
Current Technology	CT	N.A.	191.46	64.10	40.00	5.07
Near-Future Technology	NFT1	CT + Complete Monomeric Xylose Utilization	196.69	65.86	41.09	5.21
	NFT2	NFT1 + Half of Soluble Oligosaccharides Utilization	224.04	75.01	46.81	5.93
	NFT3	NFT1 + Complete Soluble Oligosaccharides Utilization	251.39	84.17	52.52	6.66
Mature Technology	MT1	NFT3 + Half of Sugars Utilization in Unhydrolyzed Solids	272.15	91.12	56.86	7.21
	MT2	NFT3 + Complete Sugars Utilization in Unhydrolyzed Solids	292.92	98.07	61.20	7.76
Theoretical Maximum		No Yield Loss in Any Stage	314.96	105.46	65.80	8.34

Interaction between degradation products and xylose fermentation

Xylose fermentation was more susceptible than glucose fermentation to inhibition by the degradation products from AFEX pretreatment, resulting in prolonged fermentation. However, as our results indicate, the effects of AFEX degradation products on fermentation are complex. Generally, AFEX degradation products increase metabolic yield by reducing the formation of fermentation by-products. Certain degradation compounds have been postulated to act as electron acceptors to provide redox balance in xylose metabolism [58, 74]. An equally important benefit of the degradation products from AFEX-pretreated biomass is that they increase metabolism of sugars which translates into higher specific ethanol production rates. Degradation compounds such as organic acids and 4-hydroxybenzaldehye have been shown to stimulate fermentation when present at moderate levels [16, 75]. Nonetheless, they inhibit the cells from propagating to a density warranted by available nutrients. In a commercial setting, a two-fold increase from the existing xylose consumption rate is likely required. Instead of supplementing with excess nutrients or detoxifying hydrolysates, improving xylose fermentation through cell recycle to increase cell density might be a more cost-effective solution.

CONCLUSIONS

Current technology using corn stover as feedstock, AFEX as the pretreatment technology and *Saccharomyces cerevisiae* 424A(LNH-ST) as the ethanologenic strain in

separate hydrolysis and fermentation (SHF) was able to achieve 191.5 g EtOH/kg untreated corn stover, at an ethanol concentration of 40.0 g/L (5.1 v/v%) without washing of pretreated biomass, detoxification or nutrient supplementation. Enzymatic hydrolysis at high solids loading was identified as the primary bottleneck affecting overall ethanol yield and titer. Degradation compounds in AFEX-pretreated biomass were shown to increase metabolic yield and specific ethanol production while decreasing the cell biomass generation. Nutrients inherently present in corn stover and those resulting from biomass processing are sufficient to support microbial growth during fermentation. This platform offers the potential to improve the economics of cellulosic ethanol production by reducing the costs associated with raw materials, process water and capital equipment.

CHAPTER V A NEW PARADIGM FOR CELLULOSIC ETHANOL PRODUCTION: UTILIZATION OF AFEX- PRETREATED CORN STOVER AS SELF-SUSTAINED CARBON AND NITROGEN SOURCES

INTRODUCTION

Besides carbohydrates, plant materials contain other extractives including nutrients [76]. Although present at relatively low levels compared to carbohydrate and lignin, plant nutrients can be vital for a cost-effective lignocellulose bioconversion. Further understanding of nutrient characteristics of plant hydrolysate is required to facilitate rational approaches for fermentation optimization.

In this chapter, the nutrient content of enzymatic hydrolysate from AFEX pretreated corn stover at high solids loading, identifying potential excess or limiting nutrients is investigated. I seek to demonstrate biomass processing and bioconversion strategies which feature (i) utilization of AFEX-pretreated corn stover as the self-sustained carbon and nutrient source, (ii) complete and rapid fermentation of AFEX-CS hydrolysate at high solids loading and (iii) conceptual proof of the feasibility to utilize nutrients inherent

from AFEX-pretreated corn stover to support saccharolytic enzyme production for the hydrolysis of lignocellulosic biomass.

MATERIALS AND METHODS

Corn Stover (CS)

The composition of the corn stover was as described on page 14

AFEX pretreatment

The AFEX pretreatment was conducted as described on page 14

Enzymatic hydrolysis at 18% solids loading

AFEX-CS was hydrolyzed using both cellulase and hemicellulase commercial mixtures. The cellulase mixture consisted of Accelerase 1000. The hemicellulase mixture was Multifect Xylanase and Multifect Pectinase. Enzymatic hydrolysis at total mass of 2.0 kg (18.0% solids loading) was performed for 96 hr at pH 4.8, 50°C and 1200 rpm agitation in an bioreactor (Biobundle 2L, Applikon Inc).

Corn stover and enzyme feeding strategies

To achieve proper liquefaction and stirring throughout the enzymatic hydrolysis. Both AFEX-CS and commercial enzymes were fed in batches. AFEX-CS was fed in 5 batches

(6.0%, 3.0%, 3.0%, 3.0%, 3.0% solids loading) with respective intervals of 2, 1, 1, 1.5, 1.5 hr between feeding. Regarding enzyme feeding, two-thirds of the total enzymes was added during the first 8 hr of the feeding (1/3, at 0 hr; 1/6, at 4hr; 1/6, at 8hr). The remaining one-thirds of the enzymes were fed continuously to the reactor for 36 hr (1/6, 8-24 hr; 1/6, 24-48hr). A total protein loading of 6.7 mg protein/g biomass was used. The enzyme loading in volume is 106.1 mL Accelerase 1000, 12.4 mL Multifect Xylanase and 12.4 mL Multifect Pectinase per 1.0 kg of dry CS.

Water extraction of AFEX-pretreated corn stover

AFEX-pretreated corn stover was washed with distilled water at a ratio of 1 g dry CS to 5 mL of water to produce a water extract at 20% solids loading equivalent as described on page 16.

Nutrient analysis

The nutrient content in (i) AFEX-CS enzymatic hydrolysate at 18% solids loading, (ii) enzyme solution (used during the enzymatic hydrolysis) and (iii) water extract of AFEX-CS was analyzed. The nutrient of interest includes ammonium ion, protein, amino acids, trace elements, and vitamins. The method used for each analysis is described as follows.

Ammonia

Free ammonia in the solutions was analyzed through an enzymatic assay from R-biopharm AG (Cat no: 11112732035, Darmstadt, Germany). The solution was diluted to

an appropriate level for assay detection. The level of reduction of NADH, which indicates the concentration of ammonia in the solution, was measured at absorbance wavelength of 340 nm using a spectrophotometer. A standard ammonia solution (control experiment) was tested to ensure the accuracy of the results. Other experimental details and enzymatic chemistry explanation can be found in the manufacturer's instruction manuals.

Amino acids

The analyses for amino acid concentrations were conducted in MSU Macromolecular Structure Facility through a High Performance Liquid Chromatography (HPLC) system equipped with a Nova Pak C18 (3.9mm×150mm; Waters). Operational details of the system were as described [77]. The full range of amino acid was analyzed.

Free Amino Acids

About 500 µL of respective solutions was filtered (Millipore Centricon), 20uL of the filtered elute was derivatized with AccQ Tag (Waters), 10% of the total derivatized sample was injected into the HPLC system.

Protein Amino Acids

The three solutions were dried under vacuum (SpeedVac, Savant) and hydrolyzed with 6N HCl at vapor phase at 100°C for 24 hrs. The hydrolyzed dry samples were solubilized in 100 µL of 20mM HCl and 10µL of the mixture was derivatized with AccQTag (Waters). 10% of the derivatized mixture was injected into a Nova Pak C18 (3.9mm×150mm; Waters).

Trace elements

Trace elements were measured by an inductively-coupled-plasma mass spectrometry (ICP-MS) in the MSU Department of Geological Sciences.

One hundred milligrams of sample were digested in 5 mL of 70% HNO_3 (Optima, Fisher Scientific, Pittsburgh, PA) and placed in an ultrasonic bath for 1 hr to ensure the materials was uniformly distributed in solution. The sample was placed on a hot plate at 70°C for 36 hrs. Then, 5 mL of H_2O_2 were added and the solutions were allowed to be evaporated. Nitric acid (2%, 5mL) was used to dissolve the residue at 70°C for 2 hr. This solution was diluted in distilled water by a factor of 200 and run in the ICP-MS for full mass scan analyses.

Vitamins

Five vitamins important for industrial fermentations were analyzed using a LC/MS/MS (Quattro Micro, Waters) using a Water Symmetry C-18 column. The mobile phase was run at 0.3 mL/min with a gradient of 1 mM perfluoroheptanoic acid and acetonitrile. Mass spectra was acquired for 6 min using electrospray ionization in positive ion mode. The capillary voltage, extractor voltage and RF lens voltage was set at 3.17 kV, 4.00 V and 0.3 V, respectively. The source temperature and desolvation temperature were at 110°C and 350°C. The desolvation gas flow was set at 400L/hr. Collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software. Data was acquired with MassLynx 4.0 and processed with QuanLynx software.

Solids-liquid separation

Remaining solids and liquid hydrolysate after the enzymatic hydrolysis were separated (i) by centrifugation and (ii) an in-house manufactured press using miracloth (Calbiochem, San Diego, CA) as the filter to retain moist unhydrolyzed solids. The moisture content (total weight basis) of the remaining solids after press is 54±1%. The solids-free liquid hydrolysate was used for fermentation.

***Trichoderma reesei* fermentation**

Seed culture

Trichoderma reesei RUT-C30 (provided by Dr. Jonathan Walton from MSU Plant Biology Department) was used to investigate the feasibility of using constituents from AFEX-pretreated corn stover as the source of carbon and nitrogen for protein production. Seed cultures were prepared in YEPG solution (10 g/L yeast extract + 5 g/L peptone + 50 g/L glucose) by inoculating *T. reesei* cells grown on agar plate into the media and was grown in 30°C,150 rpm for 48 hr.

Substrate/Nutrients for the fermentation

Fermentation was conducted in a 500 mL stirred-tank fermentor (Qplus, Sartorius, Germany) with a working volume of 300 mL at 28°C. The fermentation media was a mixture of 10% solids loading equivalent (SLE) AFEX-CS water extract + 1.8% SLE of AFEX-CS enzyme hydrolysate. The dissolved oxygen (DO) level was controlled at 25% saturation and the stirring was at 400-600rpm. The DO was controlled in cascade by

varying air supplementation first and followed by changing stirring speed. About 30mL of sample was taken every 24 hr. The fermentation was stopped after 5 days.

Ethanol fermentation

Seed culture

Enzymatic hydrolysate with 6.0% SLE was diluted at a factor of 3:10 and used as the seed culture media. No other sugar or nutrient supplements were added. Frozen glycerol stock of *Saccharomyces cerevisiae* 424A(LNH-ST) was inoculated into the 6.0% SLE enzymatic hydrolysate at initial cell density corresponding to OD600nm of 0.1. The seed culture was grown at 30°C for 18 hr and used as the inoculum for ethanol fermentation.

Fermentation and cell recycle

Fermentation was conducted in a 250 mL Erlenmeyer flask with 70 mL working volume. The fermentation temperature was kept at 30°C through a water bath regulated by a heater recirculator. A six-spot magnetic stirrer plate was placed underneath the water bath to drive the magnetic bar which was placed in the flask. Fermentation was initiated by inoculating the seed culture at a cell density corresponding to OD600nm of 0.5. Cell increase equivalent to OD600nm of 20 was continuously fed into the fermentation broth in 2 intervals (10 units per each interval, 16-24hr and 40-48hr). Fermentation was allowed for 72hr. Cell density, sugar and ethanol concentration were measured as described [47].

The feasibility of recycling cells without addition of fresh cells was tested for 4 generations of fermentation. The yeast cells generated from previous fermentation were used to initiate fermentation at cell density corresponding to OD600nm of 0.5, and the remaining cells were cultured in 30 mL of 18% solids loading AFEX-CS hydrolysate for 2 hr in 30°C, 150rpm (termed as cell preincubation unit). The concentrated cell-hydrolysate mixture was then stored in an ice-water bath and continuously fed into the hydrolysate in 2 batches (16-24hr and 40-48hr) from the cell preincubation unit.

Mass balance construction and HPLC analyses

Mass balance and HPLC analyses of glucose, xylose, xylitol, glycerol and ethanol were conducted as described in the Chapter IV

RESULTS

Core bioprocessing and bioconversions

Enzymatic hydrolysis

Enzymatic hydrolysis using 6.7 mg protein/g biomass was able to produce sugar stream at 110 g/L which accounted for 85% of the total carbohydrate entering the overall process. About one-seventh of the soluble sugars was oligomers. Among them, 70% is xylooligomers. In essence, the same sugar yield was achieved compared to the previous chapter where Spezyme CP and Novozyme 188 were added instead of Accelerase 1000. Protein concentration of each enzyme is as listed in Table 15 in Appendix A

Fermentation

The first generation of AFEX-hydrolysate fermentation essentially finished after 72 hr with 58 g/L glucose was completely consumed within 16 hrs and xylose was utilized at a rate of 0.37 g/L/hr; 0-60hr. All tested generations of fermentation achieved the same sugar consumption rate at 0.36±0.01 g/L/hr without addition of fresh cells. Metabolic ethanol yield of 0.45-0.48 g/g consumed sugar was achieved throughout these fermentations (Figure 22). This affirmed that the high recyclability of yeast cells in AFEX-CS hydrolysate fermentation. The final cell densities of each generation of fermentation were 29, 32, 39 and 39 unit absorbance at OD 600nm, respectively.

Complementary unit operations

Seed culture production

Low level of fresh inoculation is expected to be necessary in an industrial production. . In our study, enzymatic hydrolysate at 6.0% solids loading equivalent was able to generate cell densities at 7 unit OD600nm within 18 hr. Assuming that fresh cell at 0.5 unit OD600nm is need in each generation of fermentation, an inoculum volume at 7% that of the ethanol fermentor is sufficient to support this demand.

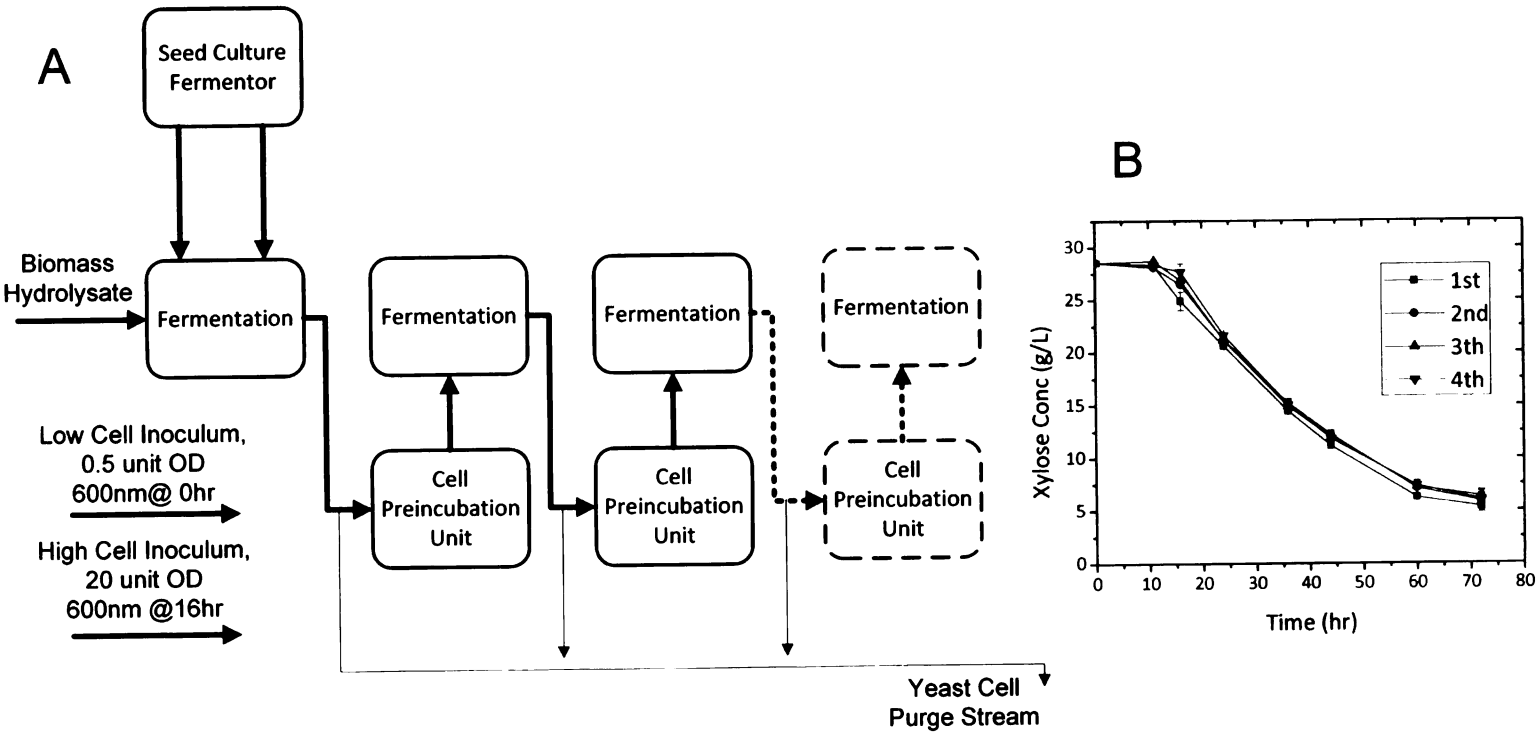


Figure 22 AFEX-corn stover hydrolysate fermentation. (A) fermentation at high cell density using cell recycle and (B) xylose consumption over four generation without fresh cell addition.

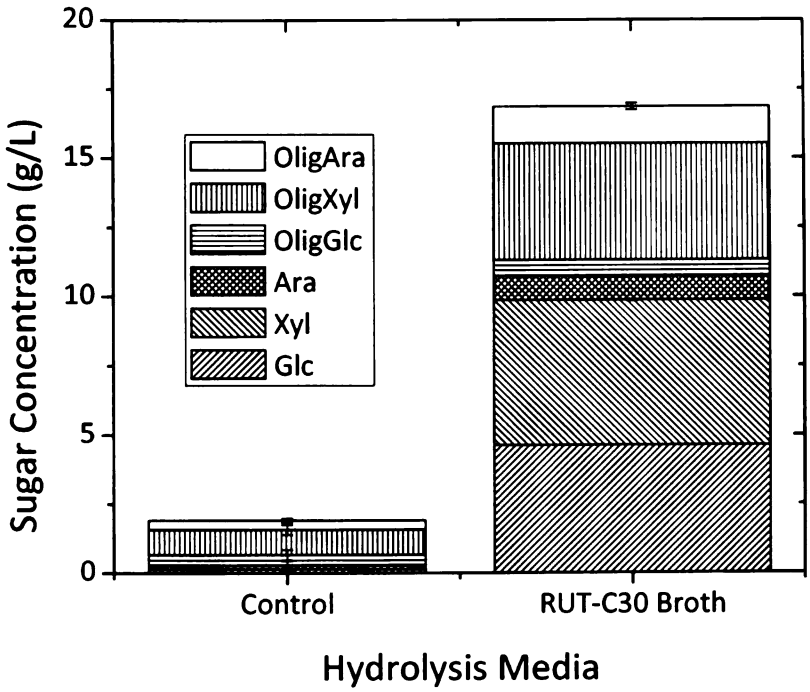


Figure 23 Sugar yield after enzymatic hydrolysis using fermentation broth from *Trichoderma reesei* RUT-C30.

Control: Hydrolysis in water; RUT-C30 broth: Hydrolysis in fermentatio broth of *Trichoderma reesei* RUT-C30. Enzymatic hydrolysis was conducted for 96hr, at pH 4.8, 50°C.

Note: Olig: Oligomeric, Mo: Monomeric, Ara: Arabinose, Glc: Glucose, Xyl: Xylose.

Proof of concept for in-house enzyme production

Enzyme production from *Trichoderma reseei* RUT-C30 was conducted using carbohydrate and nitrogen sources from AFEX-CS water extract (10% SLE) and enzymatic hydrolysate (1.8% SLE). The fermentation broth after 4 days of culture was used to conduct enzymatic hydrolysis on washed AFEX-CS at 5% solids loading. The result confirmed that half of the total sugars in AFEX-CS was hydrolyzed to soluble sugars after

72 hr. About 75% of the total xylan was solubilized. Among the soluble sugars, 36% was oligomers of which 70% is xylooligomers (Figure 23)

Ammonia and protein

AFEX-CS hydrolysate contained 0.80 g/L of ammonium ions, resulting from residual ammonia from pretreatment, this free ammonium ion accounted for 0.1% of the total input ammonia for AFEX pretreatment. After enzymatic hydrolysis, total protein in enzymatic hydrolysate was 0.88 g/L, of which more than one-thirds (0.37 g/L) was in free amino acids (Table 11).

This protein originated from enzyme addition (for saccharification) and the inherent nutrient content from plant biomass. Total added enzyme protein corresponded to 1.8 g/L, if all protein were to remain in liquid phase. Evidently most of the enzyme protein was bound to biomass during enzymatic hydrolysis and removed alongside with unhydrolyzed solids. This conclusion is consistent with the previous report [78].

Minerals

Ten important trace elements for fermentation were analyzed through an ICP-MS. The concentration of these ten minerals exceeded required levels for yeast fermentation suggested by Walker [79]. Plant biomass was the predominant source for trace elements in the AFEX-CS hydrolysate (Table 12). Magnesium, a macroelement which is usually limited in most industrial fermentations [80], is present at sufficient level (99.6

mg/L) in AFEX-CS hydrolysate. However, the concentration of calcium exceeds the reference value by 4 orders of magnitude; potentially undermining the metabolic reaction catalyzed by magnesium presumably due to interference of calcium on magnesium uptake and cellular utilization [81].

Vitamins

Panthothenic acid, pyridoxine and nicotinic acid are available at the respective concentration of 3.0±0.1, 2.1±0.0 and 26.8±0.2 μM, respectively, which are considered of appropriate levels for industrial fermentations (Table 12).The respective concentrations of thiamine and biotin were below 1 μM. Supplementation is needed if the fermenting strains have strict growth requirements for these vitamins. Nevertheless, these vitamin concentrations are comparable to what is commonly present in wort used in the brewery industry [82].

Proposed scheme for integrated cellulosic ethanol production

To fully utilize the nitrogenous compounds generation during AFEX-pretreatment, the proposed scheme features (i) core biomass processing and conversion, (i) cellulase production, (iii) seed culture maintenance based on AFEX-pretreated biomass as the exclusive source for carbon, nitrogen and nutrients (Figure 24). After enzymatic hydrolysis, solids and liquid was separated. The moist residual with 50% moisture content was washed with water (1 g wet solids to 1 mL water). The water extract, solid-free diluted hydrolysate produced after moisture content of the washed solids is

reduced again to 50%, is used for seed culture maintenance. The remaining washed solids were directed to enzyme production. Ethanol fermentation was conducted on solid-free enzymatic hydrolysis and yeast cell can be separated from the broth for recycle or as purge stream before ethanol distillation step (Figure 24).

One-fifth of the total input carbohydrate is projected to channel for enzyme production and 78% was used for ethanol production (Figure 25). Most of the carbon source (71%) provided to enzyme production is from unhydrolyzed solids which contain residual cellulose and hemicellulose. The maximum overall ethanol yield using the proposed carbon partition is 267g EtOH/kg CS or 90 gal/ton. About 60% of the accounted nitrogenous sources (ammonium, amino acid and acetamide) are directed to enzyme production (Table 13) and 40% for ethanol fermentation. Protein bound on unhydrolyzed solid residues is assumed non-bioavailable. Both carbon and nitrogen sources used for seed culture maintenance is less than 2% of the bioavailable nitrogen source (Figure 25). If 20% of the total nitrogen source is assimilated for enzyme production, enzymatic hydrolysis at 10.6 mg/g biomass can be conducted.

Table 11 Amino acid concentration of AFEX-water extract (16.7% Solids Loading) and AFEX corn stover enzymatic hydrolysate (18.0% solids loading)

Components	AFEX-Water Extract (mg/L)		AFEX-Hydrolysate (mg/L)	
	Free	Total	Free	Total
NH^{4+}	750±50		800±50	
Asp	8.7±0.3	48.3±0.2	7.7±0.7	54.5±0.1
Glu	6.9±0.4	73.4±0.3	7.2±0.5	76.0±0.2
Ser	24.2±0.0	50.3±1.9	43.9±2.1	74.0±0.6
Gly	5.8±0.0	72.0±0.0	13.2±0.8	88.8±0.9
His	3.1±0.3	15.0±0.1	6.7±0.4	24.8±0.7
Thr	14.3±0.5	39.1±0.4	40.0±2.7	75.0±0.3
Arg	9.0±0.9	26.6±0.0	34.0±1.5	37.8±0.3
Ala	30.7±1.6	78.7±0.1	32.6±1.7	83.6±0.4
Pro	43.0±5.3	72.3±4.7	51.1±2.4	75.6±3.8
Tyr	16.3±6.0	30.1±0.1	42.8±1.6	23.5±0.3
Val	7.5±1.3	44.0±0.2	19.1±1.4	56.3±0.3
Met	3.2±1.3	9.4±0.7	5.9±1.2	11.8±0.1
Ile	8.2±0.4	26.2±0.0	13.8±0.8	41.9±4.7
Leu	7.8±0.0	50.6±0.2	19.8±0.9	66.8±0.4
Lys	1.3±0.0	10.3±0.0	1.2±0.2	15.0±0.4
Phe	5.7±0.1	50.9±0.2	29.6±1.2	72.1±0.6
Total	195.7±14.5	697.2±3.1	368.6±17.8	877.2±12.8

Table 12 Trace elements and vitamins in AFEX-water extract (16.7% solids loading) and AFEX corn stover enzymatic hydrolysate (18.0% solids loading)

			Unit	AFEX-Water Extract	AFEX-Hydrolysate	Reference Value [a,b]
Trace Elements	K	Potassium	mg/L	166.71±4.81	170.44±0.66	76
	Mg	Magnesium		100.52±1.30	26.63±0.83	48
	Ca	Calcium		271.45±3.02	45.62±1.27	0.02
	Mn	Manganase		2.08±0.08	0.19±0.00	0.1
	Co	Cobalt		0.10±0.00	0.01±0.00	0.0027
	Ni	Nickel		0.38±0.02	0.08±0.00	0.28
	Cu	Copper		0.16±0.02	0.09±0.00	0.0435
	Zn	Zinc		1.12±0.07	0.19±0.01	0.24
	Mo	Molybdenum		0.15±0.01	0.05±0.00	0.063
	Fe	Iron		1.72±0.07	0.32±0.01	0.078
Vitamins		Panthothenic Acid	µM	1.50±0.12	3.01±0.08	0.68-1.14
		Pyridoxine		1.26±0.18	2.14±0.00	0.89-1.18
		Nicotinic Acid		10.87±1.38	26.78±0.22	12.18-20.31
		Biotin		~0.05	~0.10	0.02-0.04
		Thiamine		~0.66	~0.40	0.57-2.83

[a] The reference value for trace elements were as suggested by Walker, 2004 [81],

[b] The typical vitamin concentration present in wort used for brewery industry was used as the reference value for the vitamins [82]

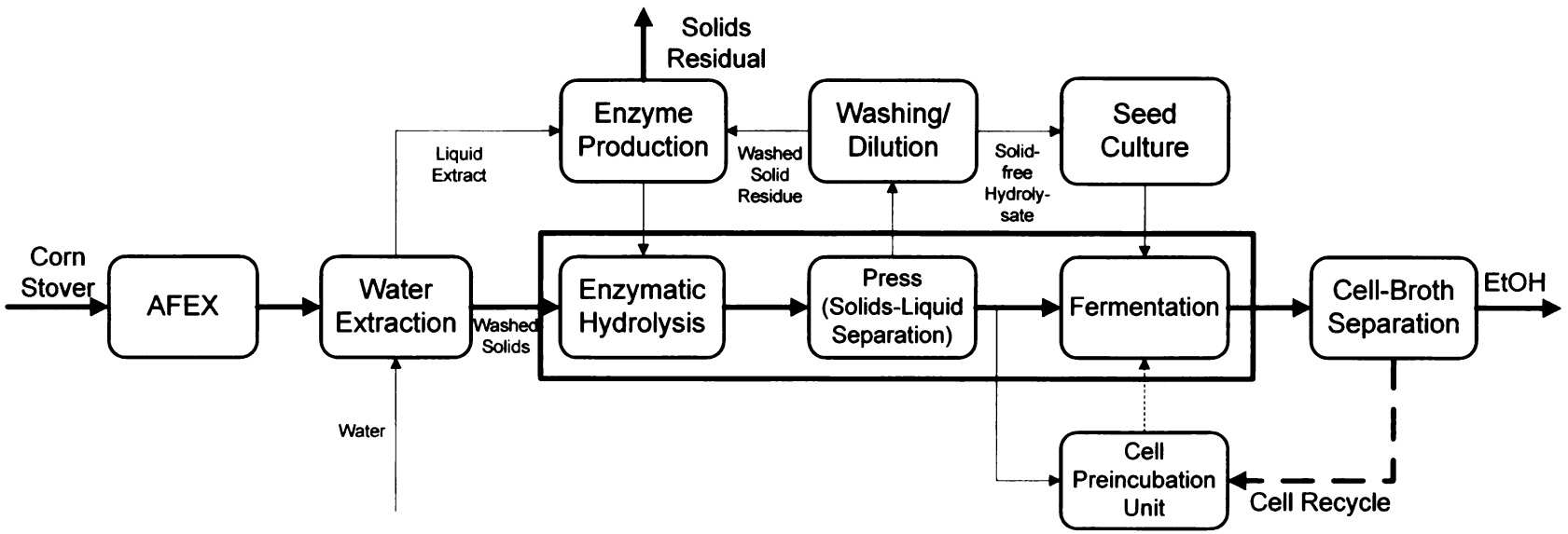
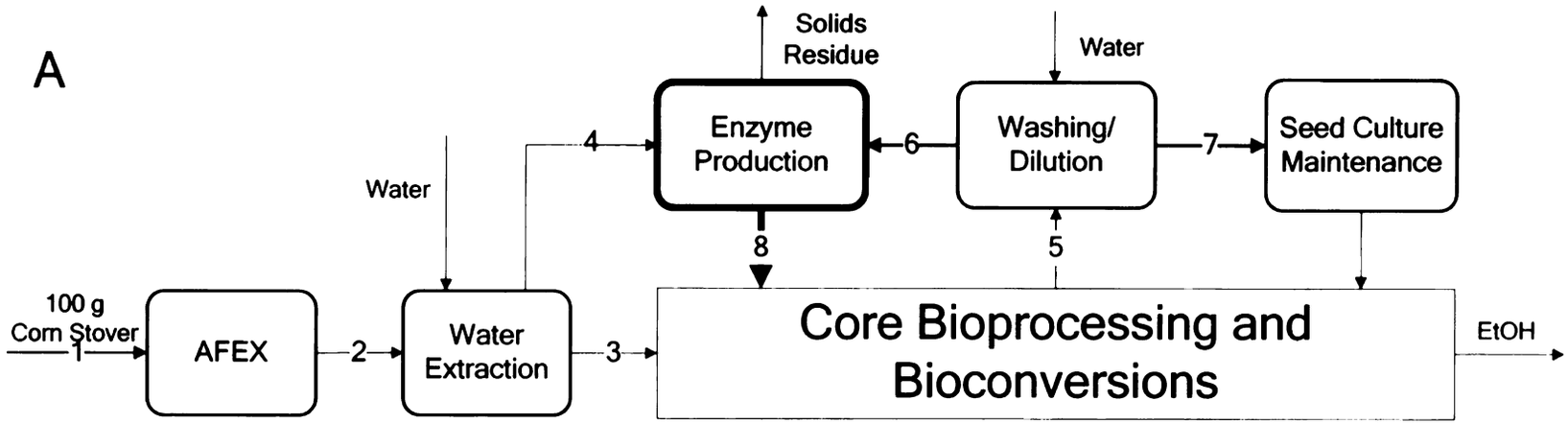


Figure 24 The integrated biomass processing and bioconversion platform using AFEX-pretreated corn stover as exclusive carbon, nitrogen and nutrient source.

This approach includes a core processing unit (highlighted in box) and complimentary processes i.e. enzyme production and seed culture generation to support enzymatic hydrolysis and fermentation.

A



B

Components		Stream 1 Untreated Corn Stover	Stream 2 AFEX Corn Stover	Stream 3 Washed AFEX Corn Stover	Stream 4 Water Extract (Solid-free)	Stream 5 Moist Unhydrolyzed Solids	Stream 6 Moist Unhydrolyzed Solids	Stream 7 Diluted Sugar Hydrolysate
Acetamide (g)		0.000	2.500[a]	0.250[b]	2.250	0.013	0.007	0.007
Ammonium (g)		0.000	0.400	0.090	0.310	0.002	0.001	0.001
Protein/Amino Acid (g)		2.300	2.300[c]	1.96	0.340	0.020[d]	0.010	0.010
Total Sugar	Insoluble Sugar (g)	66.700	66.700	64.260	0.000	9.640[e]	9.640	0.000
	Soluble Sugar (g)				2.440	2.730[e]	1.365	1.365

Figure 25 Proposed carbon and nitrogen flow for core and complementary units. (A) flow diagram for the proposed unit operations, (B) carbon and nitrogen flow for each stream

Figure 25 con't

Figure 25 con't

Note: The concentration of ammonium ion, protein and amino acids is estimated according to the analysis results from this chapter (Table 11, corn stover composition)

[a] Calculated from [83]

[b] Assumption: 90% of the total acetamide is water-extracted

[c] Assumption: Protein was preserved during AFEX pretreatment

[d] Protein bind on unhydrolyzed biomass is not accounted in the scheme and is assumed not bioavailable for microbial assimilation

[e] Carbohydrate in unhydrolyzed solids is estimated based on Figure 21 (Chapter IV)

Table 13 Total projected carbon and nitrogen entering enzyme production

Components		Total Entering Enzyme Production (g/100g CS)	Total Elemental Nitrogen/Carbon Equivalent (g/100g CS)
Acetamide		2.26	0.85 (Nitrogen)
Ammonium		0.31	
Protein/Amino Acid		0.35	
Total Sugar	Insoluble Sugar	9.64	5.38 (Carbon)
	Soluble Sugar	3.80	

DISCUSSION

A new paradigm for cellulosic ethanol production

Consolidated bioprocessing (CBP) has been widely regarded and developed as the most economical approach to produce cellulosic biofuels. Fundamentally, this advanced technology relies on the microbial platform to conduct complex multi-parametric reactions which include (i) enzyme (cellulase and hemicellulase) production, (ii) enzymatic hydrolysis of biomass (cellulose and hemicellulose) and (iii) bioconversion of both hexoses and pentoses in one reactor. I herein propose a new paradigm where enzyme production, enzymatic hydrolysis and fermentation are conducted in separate reactors and controlled at respective optimal conditions. Nevertheless, ethanologenic CBP strain can play an pivotal role in this processing approach by increasing final ethanol yield and titer through digesting oligomer formed in the enzymatic hydrolysate, a major bottleneck in the overall process [47]. This paradigm also features integrated biomass processing and bioconversion strategies solely based on AFEX-pretreated corn stover as the exclusive source for carbon, nitrogen and nutrients and established ethanologenic strain *Saccharomyces cerevisiae* for fermentation.

The role of ammonia in pretreatment, enzyme production and ethanol fermentation

Ammonia, introduced during AFEX pretreatment, contributes to three major goals (i) overcoming recalcitrance of biomass (releasing plant carbohydrate), (ii) producing inhibitory degradation compounds at low levels, and (iii) preserving and enriching the nitrogenous content of the pretreated biomass. About 1.5 g ammonia was chemically-

bonded in each 100 g of dry corn stover. In the proposed scheme, the ammonium ion and acetamide (a reaction product between acetyl group and ammonia) are the main nitrogen sources for enzyme production. This effectively creates a cost-sharing scenario between pretreatment, enzyme production, and nutrient supplementation.

Xylose fermentation and yeast cells

Xylose consumption has been identified as the primary rate-limiting factor for overall production in Chapter IV [47]. Through increasing cell density during fermentation, xylose fermentation was essentially completed within 72hr (Figure 22). More importantly, fermentation using recycled yeast cells was as effective as using fresh cells at least up to 4 generations, therefore substantially reducing the need for seed inoculum after the first fermentation was initiated. Due to the net increase of cell density in each generation of fermentation, a purge stream of yeast cell as a co-product is expected. Unlike in SSF where cells and solids are in a mixture, yeast cells in the solid-free fermentation broth can be readily separated by sedimentation.

Nutrient is sufficient for ethanol fermentation

Nutrient content, in the form of ammonium ion, protein, trace elements and vitamins, inherently from AFEX-pretreated corn stover was sufficient to support microbial growth for industrial fermentation. Analyzed nutrient content is considered adequate compared to reference value suggested for yeast fermentation [81, 82]. Ethanol fermentation can exclusively rely on AFEX-pretreated corn stover as the source for carbon, nitrogen and

other nutrient requirements. Fermentation using corn steep liquor as the commercial nutrient supplementation was projected to cost 5-8 cent/gal, a significant cost particularly in biofuels production where processing cost is expected to be at 1.0 USD/gal to be commercially-attractive.

Potential self-dependence on enzyme production

Saccharolytic enzymes include a wide range of cellulolytic and hemicellulolytic enzymes are identified as the major part of the processing cost of cellulosic ethanol production. A recent study estimated cellulase cost at 32.2 cents/gal EtOH, a dominant operating cost in the ethanol production [84]. Nevertheless, the actual cost of these enzymes is still uncertain, and would largely depend on the traditional supply and demand dynamics. Hence, the ability to produce enzymes in-house using water extract of AFEX-treated corn stover and residual unhydrolyzed biomass solids is significant in the search for a bioprocessing strategy where biorefinery can be independent from external enzyme sources. Assuming that 20% of the bioavailable nitrogen-equivalent compounds was converted to saccharolytic enzymes optimal for biomass deconstruction, enzymatic hydrolysis at 10.6 mg enzyme/g biomass can be conducted. This enzyme loading is 3.9 mg/g higher than what is utilized in enzymatic hydrolysis in this chapter.

CONCLUSIONS

Cellulosic ethanol production based on AFEX-pretreated corn stover can depend on the biomass as the sole source for carbon, nitrogen and nutrients source for both enzyme production and ethanol production. In this proposed scheme, about 80% of the carbon source is used for ethanol production and 20% is channeled for complementary unit operations i.e. enzyme production and seed inoculum maintenance. Assuming that 20% of the extractable nitrogenous source could be converted to saccharolytic enzymes, enzymatic hydrolysis at 10.6 mg/g dry corn stover enzyme loading can be conducted. Close to 80% of the total xylose can be fermented within 72 hr through high cell density fermentation. The fermentation using yeast cells were as effective as that of fresh cells at least up to 4 generations, effectively reducing the need for fresh cells to conduce fermentation. The nutrient content analysis affirmed that enzymatic hydrolysate from 18% solids loading of AFEX-pretreated corn stover contains sufficient nitrogen, trace elements and vitamins for yeast fermentation.

CHAPTER VI GENERAL CONCLUSIONS AND RESEARCH OUTLOOK

GENERAL CONCLUSIONS

Cellulosic ethanol provides an environmentally-friendly alternative to petroleum-based fuels. By utilizing locally-produced feedstocks as carbon sources for transportation fuel, ethanol can create self-dependence on energy supply and reduce geopolitical implications. However, major technological challenges to produce ethanol in a cost-effective manner must be overcome to realize the commercial potential of cellulosic ethanol.

The levels of overall ethanol yield, concentration and rate indicate the maturity of the technology. The cost associated with hydrolysate conditioning, nutrients, and enzymes have also been the major barriers for its commercialization. Our study directly addresses these issues by investigating and developing AFEX-pretreated corn stover as the model substrate to provide understanding and practical solutions that result in cost reductions in those areas.

To achieve this goal, I focused to identify (i) a pretreatment that generates fermentable pretreated biomass (ii) an ethanologenic strain that produces ethanol at the highest yield, titer and rate and (iii) the major bottlenecks for the overall process. Overcoming biomass recalcitrance without generating inhibitors can eliminate the cost associated with hydrolysate conditioning in terms of detoxification and nutrient supplementation. The nature of pretreatment chemistry dictates the inhibitory effect of the pretreated materials. It was found that degradation compounds generated from AFEX pretreatment is substantially more benign to the growth of ethanologenic *E. coli* and *S. cerevisiae* in comparison to dilute acid pretreatment and this effectively eliminates the need for detoxification, which is projected to cost 20% of the total processing cost. Lignin, an important biomass constituent for co-product generation, is highly preserved in AFEX-pretreated corn stover. It is expected that 13% more energy can be harvested in an AFEX-centered process relative to that of dilute acid pretreatment.

The ability of an ethanologenic strain to utilize all sugars derived from lignocellulosic biomass and convert them to ethanol is the key process that affects overall process economics. In this report, *E. coli* KO11, *Z. mobilis* AX101 and *S. cerevisiae* 424A(LNH-ST) were tested on AFEX-CS hydrolysate from high solids loading hydrolysate without detoxification and commercial nutrient supplementation. These three ethanologens were able to grow and consume glucose completely within 72hr. However, the extent and rate of xylose consumption become the yield- and rate-determining factor in fermentation. Fermentation using *S. cerevisiae* 424A(LNH-ST) yielded the highest level of

ethano

was ob

from lig

From a

gal ton)

rave be

that 20

polymer

ST) was

commer

To furth

ethanolo

digest 60

the overa

has been

72hr. The

fermenta

Lignocellu

nitrogeno

attempt w

ethanol in all tested strains. Nearly 20-fold increase in cell density after fermentation was observed and this confirmed the feasibility of producing yeast cell as the co-product from lignocellulosic fermentation.

From a system-wide perspective, 191 g ethanol can be produced from 1.0 kg (64 gal/ton) of corn stover at a titer of 40 g/L. Enzymatic hydrolysis at high solids loading have been identified as the primary bottleneck that affects the overall process. I found that 20% of the output sugar from hydrolysis unit was oligomers and 15% remained in polymeric and water-insoluble form. Xylose fermentation using *S. cerevisiae* 424A(LNH-ST) was prolonged. Overall fermentation rate must increase by 2-3 fold to suit commercial production.

To further improve the overall yield, a consolidated bioprocessing (CBP)-enabling ethanologen *Thermoanaerobacterium saccharolyticum* ALK2 has shown to be able to digest 60-70% of the total oligomeric sugars in the enzymatic hydrolysate. This brought the overall product yield close to 77 gal/ton (Appendix A). High cell density fermentation has been demonstrated as a viable approach to achieve complete fermentation within 72hr. The ability to recycle the cells at least up to 4 generations while achieving similar fermentation efficacies without fresh cell addition was also confirmed.

Lignocellulosic hydrolysate from AFEX-pretreated corn stover contains 4-5 g/L of nitrogenous source in the form of ammonium, amino acids and acetamide. While no attempt was made in our study to understand the bioavailability of trace elements, it

was e

ferme

at suf

ferme

I prop

straig

units)

sourc

Maxir

gal/to

stove

produ

solubi

assim

condu

This n

as the

ferme

nutrie

opera

was expected that essential minerals are in excess relative to the requirement of most fermentation microorganisms. Regarding vitamins, although most vitamins are present at sufficient levels, biotin and thiamine might need to be supplemented if the utilized fermentation strain has the stringent growth requirements of these two vitamins.

I proposed an integrated biomass processing and conversion approach that features (i) straightforward pretreatment, enzymatic hydrolysis and ethanol fermentation (core units), (ii) in-house cellulase production and (iii) seed inoculum unit. Carbon (sugar) source was divided between core and complimentary units at weight ratio of 4:1. Maximum ethanol yield under this carbon partition scheme is 267 g EtOH/kg CS or 90 gal/ton. About 60% of the extractable nitrogen and nutrients from AFEX-pretreated corn stover was projected to be used for cellulase production. As a proof of concept, enzyme produced from *Trichoderma reseei* RUT-C30 from the proposed scheme was shown to solubilize 47% of the total polymeric plant sugars. If 20% of the available nitrogen is assimilated for enzyme production, enzymatic hydrolysis at 10.6 mg/g biomass can be conducted.

This new paradigm of cellulosic ethanol production utilized AFEX-pretreated corn stover as the exclusive source for carbon, nitrogen and nutrients to ethanol and enzyme fermentation within the integrated biorefinery without detoxification and commercial nutrient supplementation. The potential cost reduction is substantial as 45% of the total operating cost is comprised of raw materials associated these unit processes.

RE

It

so

pr

hy

pr

op

se

Ce

Sac

use

enz

sec

fer

pro

Evi

sho

The

pret

RESEARCH OUTLOOK

It was concluded from our study that 14% of the total sugar remained in unhydrolyzed solids and 15% as soluble oligomers in Chapter IV. To improve overall yield based on the proposed biomass processing scheme, (i) effective sugar solubilization during enzymatic hydrolysis and (ii) utilization of the whole spectrum of soluble sugar for ethanol production are required. Specific to achieving these two primary goals, I suggest that optimization of cellulase production and incorporation of a CBP-enabling (cellulase-secreting) ethanologen to address both challenges.

Cellulase production optimization

Saccharolytic microorganisms such as *Trichoderma sp.* and *Aspergillus sp.* are widely used for industrial cellulase production due to their ability to secrete a wide range of enzymes for biomass deconstruction. Nevertheless, the levels and types of enzyme secreted are highly dependent on strain type, induction method, carbon source and fermentation condition [85]. To maximize sugar solubilization, high secretion level to produce an optimal enzyme mixture from the saccharolytic microorganism is needed. Evidently, detailed optimization of cellulase production with regards to those factors should be pursued.

The ability to utilize acetamide, the most abundant nitrogenous compound in AFEX-pretreated biomass, as the nitrogen source expanded the bioavailable nitrogen source

by

mic

als

Th

du

bio

Inc

Cor

pro

enz

par

The

inte

stra

abil

feas

opt

dev

enzy

by 73% in AFEX-centered process. Native saccharolytic strains such as *Aspergillus nidulans* secretes acetamidase [86]. Expression of *amdS* gene in a heterologous host was also reported to enable growth using acetamide as the sole nitrogen source [87]. Through assimilation of acetamide as nitrogen source and effective enzyme production during fermentation, cellulase can be produced in excess as a potential coproduct in the biorefinery.

Incorporating CBP microorganisms on AFEX-pretreated biomass processing platform

Consolidated bioprocessing is generally regarded as the most economical biomass processing approach due to its projected ability to eliminate the need for exogenous enzyme and sugar inhibition on cellulolytic enzymes. However, the fermentation parameters from reported CBP has not met the levels required for industrial production. Therefore, to achieve an effective CBP-center cellulosic ethanol production, several key interactions must be further investigated, they include (i) the growth robustness of the strain with respect to ethanol and degradation compounds from pretreatment, (ii) the ability and extent of oligomer uptake, (iii) the enzyme profile of the strain and (iv) the feasibility of exploiting directed evolution to engineer enzyme secretory profile toward optimal hydrolysis of AFEX-pretreated biomass. Most CBP-enabling ethanologens developed are anaerobic bacteria and natively saccharolytic. It is expected that the enzyme profile could be directed through the available carbon sources.

Fundar

Most p

fermer

long te

achiev

cells su

fermer

the ob

Identif

negati

of the

ferme

Conc (g/L)

Figure

Fundamental understanding on cell-solids interactions

Most proposed biomass processing configurations involve direct contact between fermenting cells and plant biomass. The cell-solids interaction was found to reduce the long term viability of yeast cells; therefore lower ethanol yield and titer (Figure 26) were achieved in this processing approach. Interactions between insoluble solid residues and cells such as cell adsorption and shear stress can potentially reduce cell viability and the fermentation efficiency in slurries (solid-liquid mixture). They are the likely causes for the observed substantial reduction in xylose utilization in slurry-state fermentation. Identification, understanding and alleviation of the solid-cell interactions which exert negative impacts on overall fermentation deserve further extensive investigation as it is of the greatest significance during the process of integrating enzymatic hydrolysis and fermentation in the configuration SSCF or CBP.

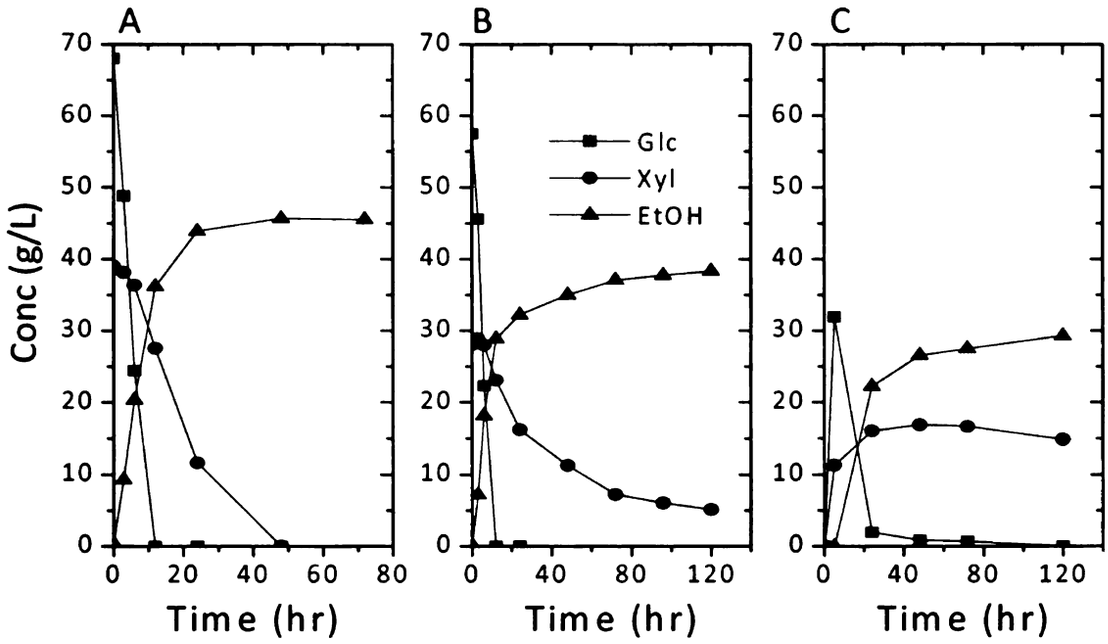


Figure 26 Fermentation using *S. cerevisiae* 424A(LNH-ST) in (A) laboratory media, (B) 18% w/w AFEX-CS liquid hydrolysate (solid-free) and (C) 18% w/w AFEX-CS hydrolysate (slurry, SSCF)

AP

(CE

TH

INT

The

sec

ara

met

[49]

cou

Con

pro

pro

dige

robu

enzy

conc

hydr

APPENDIX A EXPLORATION OF CONSOLIDATED BIOPROCESSING (CBP) ON AFEX-PRETREATED CORN STOVER USING *THERMOANAEROBACTERIUM SACCHAROLYTICUM* ALK2

INTRODUCTION

Thermoanaerobacterium saccharolyticum is a native saccharolytic microorganism which secretes various hemicellulolytic enzymes including endoxylanase, β-xylosidase, arabinofuranosidase and acetyl esterase for xylan digestion [49, 88, 89]. The metabolically-engineered strain ALK2 ferments ethanol close to thereotical maximum [49]. A biomass processing strategy centered on AFEX and *T. saccharolyticum* ALK2 could potentially simplify cellulosic ethanol production in the configuration of Consolidated Bioprocessing (CBP) which is projected to be the most economical production strategy [46]. This research serves as the early efforts to develop CBP processing based on AFEX-pretreated materials. I evaluate (i) the level of oligomer digestion on enzymatic hydrolysate from AFEX-pretreated materials, (ii) the growth robustness of ALK2 in water extract of AFEX-pretreated corn stover and (iii) levels of enzyme loading required to generate enzymatic hydrolysate containing high concentration of oligomeric sugars. Fermentation parameters of ALK2 on lignocellulosic hydrolysate will also be investigated.

MAT

Corr

Corr

Colo

appr

term

2.3%

Corr

whic

preh

was

mL s

pres

reac

130

thro

over

MATERIALS AND METHODS

Corn stover and ammonia fiber expansion (AFEX)

Corn stover was provided by the National Renewable Energy Laboratory (NREL, Golden, Colorado). It was milled and passed through a 4 mm screen. The moisture content was approximately 7% (total weight basis). The milled corn stover was kept at 4°C for long term storage. This corn stover contains 33.2% cellulose, 22.4% xylan, 3.3% arabinan and 2.3% protein on a dry weight basis.

Corn stover was AFEX-pretreated in a 2.0L pressure vessel (Parr Instruments, Moline, IL.) which was equipped with a thermocouple and a pressure sensor. The reactor was preheated to 100-110°C and prewetted corn stover (150 g dry CS + 90 g distilled water) was loaded into the reactor. The reactor was bolted shut and vacuum. A separate 500 mL stainless steel cylinder (Parker Instrumentation, Jacksonville, AL) was heated until its pressure reached 4.48 MPa (650 psi) and the heated ammonia was transferred to the reactor containing biomass. The initial and final temperatures of the pretreatment were 130±5°C and 110±5°C, respectively. The reactor pressure was released after 15 min through an exhaust valve. The AFEX-pretreated CS was air-dried in a fume hood overnight.

P

A

4

(c

C

pl

ar

pr

Ge

(S

ce

fer

En

cor

in t

CS,

wer

of d

Preparation of enzymatic hydrolysate (at 18.1% Solids Loading)

AFEX-CS was enzymatically-hydrolyzed using cellulase and hemicellulase mixtures at pH 4.8, 50°C for 96hr. The solids loading of the enzymatic hydrolysis was at 18.1% w/w (corresponding to 6.0% cellulose loading). The cellulase mixture consisted of Spezyme CP [86.7 mL/kg CS; 15 FPU/g cellulose] and Novozyme™ 188 [87.5 mL/kg CS; 64 *p*NPGU/g cellulose]. The hemicellulase mixture was Multifect Xylanase [12.7 mL/kg CS] and Multifect Pectinase [8.9 mL/kg CS]. The spectrum of activities for the enzymes was previously reported [44]. The Spezyme and Multifect enzymes were obtained from Genencor Inc. (Palo Alto, CA) and Novozyme 188 was purchased from Sigma-Aldrich Co. (St. Louis, MO). After 96 hr of hydrolysis, liquid hydrolysate was separated from solids by centrifugation at 6,000×g for 30 min. Liquid hydrolysate was used for the following fermentation studies. Kanamycin at 30 mg/L was used to reduce risk of contamination.

Enzymatic hydrolysis using Accelerase 1000 (Genencor Inc., Palo Alto, CA) as sole complex enzyme at respective volume loading of 106 and 70 μL/g dry CS was conducted in the similar condition. The corresponding protein loadings are 5.6 and 3.8 mg/ g dry CS, respectively. Oligomeric glucose, xylose and arabinose of all enzymatic hydrolysates were measured using NREL protocol LAP 014. Error bars shown are standard deviations of duplicates.

Det

Pro

188

ana

cen

adc

pre

wa

pre

fol

pre

un

Ni

Ni

is

co

st

TI

M

pr

Determination of protein concentration in complex enzymes

Protein concentration of commercial enzymes Accelerase 1000, Spezyme CP, Novozyme 188, Multifect Xylanase, Multifect Pectinase were determined through nitrogen content analyses of protein precipitate of respective enzymes. Each complex enzyme was centrifuged (13,000 x g) for 5 min, 0.20 mL of clear supernatant of the enzyme was added with 0.25 mL 100% w/v trichloroacetic acid (TCA) and 0.80 mL distilled water to precipitate protein in the enzyme solution. After 5 min of incubation at 4°C, the mixture was centrifuged at 13,000 x g for 5 min and the supernatant was decanted. The precipitate was washed with 1.0 mL cold (4°C) acetone twice, each washing was followed by centrifugation and the removal of acetone water extract. Washed protein precipitate was removed to a crucible (a sample holder for nitrogen analyzer) and dried under vacuum.

Nitrogen content within the precipitate was determined using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). The principle behind the nitrogen analysis is based on Dumas method using EDTA as the standards. Nitrogen content was converted to protein content by multiplying a factor of 6.25. Errors represented are standard deviation of duplicate experiments.

***Thermoanaerobacterium saccharolyticum* ALK2 seed culture preparation**

MTC media (Table 14) supplemented with 10 g/L of glucose and 10 g/L xylose was prepared and 50 mL of this media was sterile-filtered before transferring into a sterile

200 mL serum bottle. The bottle was intermittently purged with nitrogen (filtered through 0.22μm pore) and vacuumed for 5 cycles to create anaerobic condition. Each cycle lasted approximately for 40 sec. The frozen glycerol stock (-80°C) of ALK2 was anaerobically inoculated into the media. The bottle was incubated in a 55°C shaker (Innova 4080, New Brunswick Scientific) at 180 rpm agitation for about 18 hr. The grown cells were used as the inoculum for fermentation studies. To achieve inoculation without adding nutrients from seed culture process, grown cells were centrifuged and supernatant (fermentation broth) was removed carefully under anaerobic condition. Sterile distilled water (at volume equals to removed broth) was added to resuspend the pellet cells. Appropriate volume of inoculum was taken for inoculation.

Table 14 MTC Media for *T. saccharolyticum* ALK2 Growth

		Final Conc.
Solution A	Yeast extract	10 g/L
	Tryptone	5 g/L
	MES (buffer)	10 g/L
Solution B	Citric acid potassium salt	2.00 g/L
	Citric acid monohydrate	1.25 g/L
	Sodium sulfate (Na_2SO_4)	1.00 g/L
	Potassium dihydrogen phosphate (KH_2PO_4)	1.00 g/L
	Sodium bicarbonate ($NaHCO_3$)	2.50 g/L
Solution C	Urea	5.00 g/L
	Ammonium chloride (NH_4Cl)	1.50 g/L
Solution D	Magnesium chloride hexahydrate ($MgCl_2.6H_2O$)	1.00 g/L
	Calcium chloride dehydrate ($CaCl_2.2H_2O$)	0.20 g/L
	Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$)	0.10 g/L
	L-cysteine hydrochloride monohydrate	1.00 g/L
Solution E	Pyridoxamine dihydrochloride	0.020 g/L
	P-Aminobenzoic acid	0.004 g/L
	D-Biotin	0.002 g/L
	Vitamin B_{12}	0.002 g/L

Batch

Liqui

pH-a

final

Tabl

filte

and

by t

carr

per

ana

Fed

Fed

NJ)

ext

sys

of

wa

ml

fer

su

Batch fermentation of enzymatic hydrolysate at low solids loading equivalent

Liquid hydrolysate from 18% solids loading saccharification of AFEX-CS was diluted and pH-adjusted to 6.2 using KOH. MTC media was supplemented to the hydrolysate at the final concentration listed in

Table 14. The final dilution factor was at 1:5. The diluted hydrolysate (50 mL) was sterile filtered and transferred into a 250 mL sterile serum bottle. The bottle was vacuumed and purged with nitrogen as described in the previous section. Inoculation was achieved by transferring 5.0 mL of seed culture into the diluted hydrolysate. Fermentation was carried out in a 55°C shaker at 180 rpm agitation. Samples were taken at designated period. Glucose, xylose and arabinose in monomeric and oligomeric forms were analyzed through HPLC and NREL Protocol LAP-014.

Fed-batch fermentation of enzymatic hydrolysate at high solids loading equivalent

Fedbatch fermentation was conducted in a custom-made fermentor (NDS Technologies, NJ) equipped with a pH probe. Temperature of the fermentor was controlled by an external water bath recirculator. Feeding and pH were controlled by Sartorius Aplus system (Goettingen, Germany). Initial volume of the reactor is 120 mL which consisted of 20 mL enzymatic hydrolysate at 18% solids loading, nutrient supplement and distilled water (for dilution). For rich media fermentation, 1.0 g yeast extract, 0.5 g peptone, 10 mL of concentrated stock for solution B, C, D and E was added; while for lean media fermentation, 0.2 g yeast extract, 0.1 g tryptone and stock for solution D was used as supplement. The fermentation media was pH-adjusted to 6.2 with KOH and sparged

with nitrogen for about 10 min to create anaerobic condition. Seed culture (10mL) was inoculated to initiate fermentation. Undiluted 18% solids loading enzymatic hydrolysate at pH 6.2 (rich media: supplemented with 10 g/L yeast extract and 5 g/L peptone, lean media: no supplementation), was used as the feed. Feeding started 4 hr after inoculation at the rate of 4.0 mL/hr until 180 mL of feed volume was added into the fermentor. Samples were taken at the designated period. Glucose, xylose, arabinose (in monomeric form) and ethanol were analyzed using HPLC. Oligomeric sugars were analyzed through acid hydrolysis based on NREL Protocol LAP-014.

Fermentation using water extract of AFEX-pretreated corn stover (inhibitory testing)

AFEX-CS water extract at 20% solids loading equivalent was produced as previously described [47]. In essence, AFEX-pretreated corn stover was washed with distilled water at a ratio of 1 g dry CS to 5 mL of water and the water extract was removed from the biomass through an in-house manufactured press. The water extract contained 3.7 g/L anhydrous-equivalent glucose (1.7 g/L monomer + 2.0 g/L oligomer) and 8.3 g/L anhydrous-equivalent xylose (1.7 g/L monomer + 6.9 g/L oligomer). The water extract was diluted to final solids-loading-equivalents (SLE) of 3.0, 6.0, 9.0, 12.0 and 15.0%. Each fermentation media contained 7.8 g/L anhydrous-equivalent glucose and 14.6 g/L anhydrous-equivalent xylose for carbon source; 5.0 g/L yeast extract and 2.5 g/L tryptone for nutrient supplementation; and 100 mM phosphate buffer at pH 6.0. Seed culture at 1.5 mL was inoculated to initiate fermentation. Total volume of the each fermentation is 15.0 mL. Sample was taken after designated periods. Sugar and ethanol

was analyzed in a HPLC system. Errors reported were the standard deviations of duplicate experiments.

HPLC analysis

Sugars and ethanol in this work was analyzed by a HPLC system (Waters 2695) equipped with an autosampler unit. The column was Biorad Aminex HPX-87H column, and is maintained at 60°C. The mobile phase used was 5mM H_2SO_4. Other details about the analysis were as reported previously [47].

RESULTS AND DISCUSSION

Batch fermentation

Fermentation using *Themoanerobacterium saccharolyticum* ALK2 in undetoxified AFEX-CS at 3.6 solids loading equivalent achieved complete fermentation of monomeric glucose, xylose and arabinose within 24 hrs (Figure 27A). In addition, over 50% of the oligomeric sugars were consumed (Figure 27B). In essence, all consumed sugar was converted to ethanol (0.50 g EtOH/g consumed sugars). Due to unconverted oligomeric sugars, the process yield is 0.44 g EtOH/g initial sugars. Xylooligomers consisted of the largest unconsumed sugars (64%) (Figure 27). Arabinose-containing oligomers were not further enzymatically-hydrolyzed indicating that *T. saccharolyticum* lacks of related enzymes.

Hemicellulose, highly complex and heterogenous in nature, comprises of linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. Hence, the lacking of complete enzyme system for hydrolysis of the xylo- and arabino-oligomers were not surprising. Examination of the structure of the residual oligomers could help identify the enzyme activity which is lacking in the *T. saccharolyticum* enzyme secretory system.

ALK2, a genetically-engineered homoethanologenic strain, produced ethanol at theoretical maximum and practically eliminated organic acid production during fermentation. These results are consistent with the previous fermentation report [90] based on synthetic media.

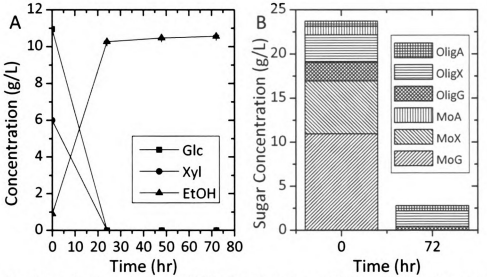


Figure 27 Batch fermentation of AFEX-CS hydrolysate at 3.6% solids loading equivalent using *T. saccharolyticum* ALK2.(A) fermentation profile and (B) initial and final sugar composition.

Inoculum size used to initiate fermentation was 10% (by volume) of fermentation volume. Fermentation was carried out in 55°C, 180rpm under strictly anaerobic condition. Olig: Oligomeric, Mo: Monomeric, A: Arabinose, G: Glucose, X: Xylose.

Fedbatch fermentation

In rich nutrient-supplemented fermentation, close to 90% of the total sugars in the hydrolysate was consumed, metabolic yield of 0.45 g EtOH/ g consumed sugars was achieved (Figure 28A). Fermentation was completed within 64 hr after inoculation; 15 hr after feeding was concluded. Total oligomeric sugar utilization has achieved 60%. This result (the extent of oligomer consumption) is in agreement with the results from batch fermentation at low solids loading equivalent liquid hydrolysate. I demonstrated that ALK2 is able to grow and produce ethanol to 30 g/L at 0.45 g/L/hr (0-64hr) from the hydrolysate containing degradation compounds equivalent to 11.7% solids loading of AFEX-CS (Figure 28B).

In lean-nutrient-supplemented fermentation, the levels of both total sugar consumption and metabolic yield were 80% of those in rich-nutrient-supplemented fermentations. Ethanol production apparently ceased 40 hr after inoculation. This is likely due to (i) the lack of important nutrients for the fermentation and/or (ii) *T. saccharolyticum* cells were not fully adapted to the lignocellulosic hydrolysate environment (Figure 28C).

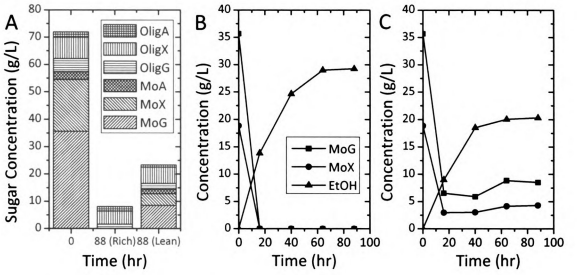


Figure 28 Fedbatch fermentation of AFEX-pretreated corn stover using *T. saccharolyticum* ALK2. (A) initial (if no fermentation occurs) and final sugar concentration with rich and lean nutrient supplementation, (B) fermentation using rich nutrient supplementation and (C) fermentation using lean nutrient supplementation.

Note: Olig: Oligomeric, Mo: Monomeric, A: Arabinose, G: Glucose, X: Xylose.

The inability to utilize oligomers for fermentation was identified as one of the main primary bottlenecks for cellulosic ethanol production. Hence, the results presented herein suggested that integration of CBP microorganisms like ALK2 into AFEX-centered biomass processing technology can increase overall ethanol yield from 64.1 gal/ton; 191.4 g EtOH/kg (as previously reported [47]) to 76.8 gal/ton; 229.5 g EtOH/kg; 270 g EtOH/kg. However, ALK2 fermentation was supplemented with rich nutrients in this work; further adaptation of the strain to lignocellulosic hydrolysate environment might able to mitigate this requirement. Final ethanol was also lower than the previous report [47] due to fedbatch strategy to keep sugar levels low throughout the fermentation.

Table 15 Concentration of nitrogenous compounds in commercial enzymes

	With TCA Protein Precipitation (Protein Precipitate)		Without TCA Protein Precipitation (Whole Enzyme Solution)		Ammonium Content/Total Nitrogen (Whole Enzyme Solution)
	Total Nitrogen	Protein Equivalent	Total Nitrogen	Protein Equivalent	
	mg/mL				
Accelerase 1000	8.5±0.1	53.1±0.6	14.0±0.6	106.6±4.5	0.64/0.53
Spezyme CP	13.4±1.1	83.5±6.8	17.1±0.7	87.6±3.5	0.14/0.12
Novozyme 188	10.6±0.1	66.3±0.7	34.1±2.0	212.9±12.3	8.32/6.85
Multifect Xylanase	5.0±0.3	31.0±1.7	7.2±1.0	45.3±5.8	0.61/0.50
Multifect Pectinase	8.3±0.0	51.9±0.2	12.2±0.2	75.9±1.3	0.28/0.23

The nitrogen content of enzyme solution, its protein precipitate and ammonium ion was analyzed to show the protein concentration and other non-protein nitrogen source such as ammonium in the commercial enzyme complexes (Table 15). Protein content in all the tested enzymes ranged from 31-84 g/L. The protein nitrogen to total nitrogen ratio was between 60-80% for all tested enzymes with exception of Novozyme 188, in which about 70% of the total nitrogen is from non-protein sources. Approximately 30% of the total non-protein nitrogen of Novozyme 188 comprises of ammonium ions, a commonly-used nitrogen supplement in commercial fermentation. To accurately quantify protein content of a complex media, protein precipitation followed by nitrogen content analysis has been demonstrated to be effective to avoid interference due to soluble impurities in the enzyme solution.

Enzymatic hydrolysis at different protein loadings

Enzymatic hydrolysis at 18.1% solids loading using a complex of commercial enzymes (Spezyme CP, Novozyme 188, Multifect Xylanase and Multifect Pectinase) yielded sugars at 110 g/L using protein loading at 11.1 mg protein/g biomass. Enzyme hydrolysis utilizing Accelerase only achieve a hydrolysis with sugar concentration at 102 g/L and 96 g/L; at respective protein loading of 5.6 and 3.8 mg/g biomass (Figure 29). Protein concentration of respective enzymes were analyzed and listed in Table 15. Oligomers to monomers ratio increased from 33% (EH3 in hydrolysis uses 4 complex enzymes) to 80% (EH2, in hydrolysis uses 3.8 mg/g protein loading). Total oligomers concentration also increased from 27 g/L (EH3) to 43 g/L (EH2). Xylooligomers are the largest (60%) group of oligomeric sugar presented across the hydrolysates (Figure 29).

This suggests that carbohydrates in AFEX-pretreated corn stover can be hydrolyzed readily to a mixture of oligomers and monomer at high solids loading and low enzyme loading (3.8-5.6 mg protein/g biomass). Assuming that 70% of the total protein in Accelerase is cellulolytic enzymes, cellulase loading responsible for these hydrolyses ranged from 2.7-3.9 mg cellulase/g biomass. This level of enzyme loading is close to what utilized in the existing corn ethanol industry [91].

ALK2 have been shown to hydrolyze oligomers present in the enzymatic hydrolysate from AFEX-pretreated corn stover. Further integrating a CBP ethanologen such as *T. saccharolyticum* ALK2 that secrete various cellulolytic enzymes particularly for xylooligomer hydrolysis can be highly advantageous due to reduction in requirement

and all

enzym

Figu

Not

and alleviation of sugar inhibition through simultaneous sugar consumption and enzymatic hydrolysis.

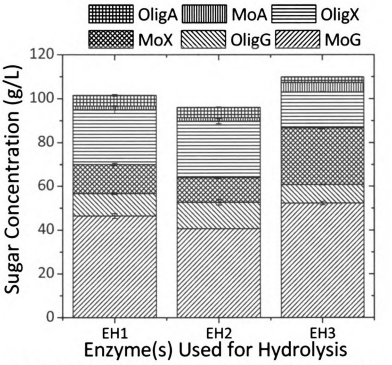


Figure 29 Sugar composition of enzymatic hydrolysate from AFEX-pretreated corn stover utilizing Accelerase at 5.6 (EH1) and 3.8 (EH2) mg protein/g biomass and a combination of four complex enzymes (Spezyme CP, Novozyme 188, Multifect Xylanase and Multifect Pectinase; EH3).

Note: Olig: Oligomeric, Mo: Monomeric, A: Arabinose, G: Glucose, X: Xylose.

Wate

Ferm

gluco

betw

wate

degr

Neve

30).

lign

con

The

the

hy

a s

of

su

ur

Al

pr

Water extract fermentation

Fermentation media consists of AFEX-CS water extract supplemented with additional glucose and xylose. The total sugars are equal in all the fermentations, but the ratios between oligomeric and monomeric sugars are different because most of the sugars in water extract of AFEX-CS was oligomers. At low solids loading (3-9% SLE, low levels of degradation compounds), glucose was consumed at a higher rate than xylose, Nevertheless, xylose was consumed faster than glucose at SLE of 12% and 15% (Figure 30). This feature is distinctively different from most reported co-fermentation of lignocellulosic fermentation where xylose is inhibited when fermentation was conducted in high SLE [3, 47].

The primary challenge in utilizing ALK2 in industrial cellulosic ethanol production is on the growth robustness of this strain in sub-optimal growth media such as lignocellulosic hydrolysate. Fermentation at media containing 15% of SLE was prolonged (Figure 30). At a similar SLE and nutrient level, *S. cerevisiae* 424A(LNH-ST) was able to consume 30 g/L of glucose completely within 24 hr [47]. Provided that overall growth robustness can be substantially improved through adaptation as demonstrated [3], by exploiting the unique feature of ALK2 on carbon source preference and CBP ability, *T. saccharolyticum* ALK2 could provide a highly economical processing approach for cellulosic ethanol production.

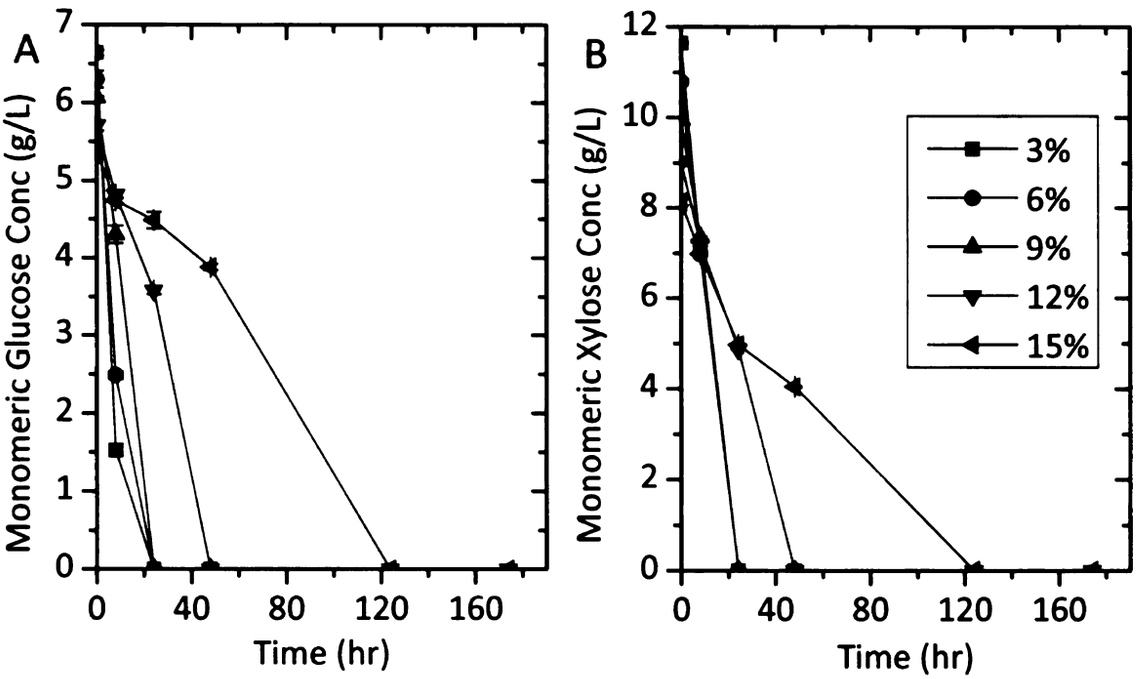


Figure 30 Sugar consumption pattern for (A) glucose and (B) xylose fermentation in water extract of AFEX-pretreated corn stover at varying solids loading equivalent

CONCLUSIONS

Fermentation of enzymatic hydrolysate from AFEX-pretreated corn stover using *T. saccharolyticum* ALK2 consumed 90% of the total sugars (monomers and oligomers) to produce ethanol close to the theoretical maximum with productivity of 0.62 g/L/hr. About 60% of the oligomers were metabolized by *T. saccharolyticum* ALK2, a genetically-engineered ethanologen which is capable of secreting hemi-/cellulolytic enzymes. Xylooligomers were the largest group of unconsumed sugars. Hydrolysates containing high concentrations of oligomeric sugars can be generated from AFEX pretreated corn stover at low enzyme loading (3.8-5.6 mg protein/g corn stover). ALK2 has unique

carbon source preference; xylose was favored relative to glucose in a media containing high levels of degradation compounds. However, higher growth robustness at lower nutrient levels is likely required for a commercial production. Utilizing *T. saccharolyticum* ALK2 as the fermentation platform could increase the overall yield to 76 gal/ton due to oligosaccharides utilization.

APPE

AMM

DISTI

INTR

Depe

serio

etha

prom

Amo

cellu

estin

achi

APPENDIX B ETHANOLIC FERMENTATION OF HYDROLYSATES FROM AMMONIA FIBER EXPANSION (AFEX) PRETREATED CORN STOVER AND DISTILLERS GRAIN

INTRODUCTION

Dependence on petroleum as the nearly exclusive source of transportation fuel poses a serious threat to our economy, environment and energy security [9]. Recently, cellulosic ethanol has gained wide attention from both governmental and private sectors as a promising alternative fuel option due to the abundance of cellulosic materials [92]. Amongst cellulosic materials, corn stover (CS) is considered an important feedstock for cellulosic ethanol production. The National Renewable Energy Laboratory (NREL) estimated that a harvest of 80-100 million dry tonnes per year of corn stover can be achieved in a sustainable fashion [8].

Unfortunately, cellulosic substrates are perceived to be nutrient-deficient. Hence, extra costs due to external nutrient supplement are deemed unavoidable [72]. Unlike most cellulosics, corn distiller's dried grains with solubles (DDGS) is a nutrient-rich feedstock derived from the corn-based ethanol industry [93]. DDGS is used for animal feed due to its high nutrient content [94]. Nonetheless, the potential to utilize DDGS for nutrient supplementation in cellulosic ethanol production has apparently not been recognized. Instead, rich laboratory media were supplemented to conduct efficient fermentations on hydrolysates from corn stover treated by various pretreatment technologies [15, 29, 95, 96]. In addition, cellulosic hydrolysates contain degradation products which could inhibit both enzymatic hydrolysis and fermentation [35, 97]. Fermentation at high solids loading and recycle of liquid streams in industry will probably lead to further build-up of inhibitors. Thus, various methods have been investigated to remove the toxins [20, 36, 98, 99]. However, the cost of detoxification can be as high as 22% of the total ethanol production cost [38]. To avoid detoxification, efforts have been made to increase through genetic engineering the tolerance toward inhibitors of ethanologens [100]. The technique of selective evolution had been exploited to increase the ethanol tolerance limit of *Escherichia coli* KO11 without altering the genetic map [59]. It is expected that a similar technique can be utilized to enhance ethanologenic strains in terms of robustness and fermentation performance.

Escherichia coli KO11 is one of the very first ethanologens engineered for fermentation of sugars from cellulosic materials. *E. coli* B, the parental strain of *E. coli* KO11, has the natural ability to utilize several pentoses and hexoses found in cellulosic hydrolysates. The integration of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) from *Zymomonas mobilis* into *E. coli* B resulted in high ethanol yield [28]. However, its ethanol tolerance and growth robustness are relatively low compared to other ethanologens [27].

Ammonia Fiber Expansion (AFEX), recognized as one of the leading pretreatment technologies [2], offers several advantages including reduced production of inhibitory compounds and nutrient addition due to residual ammonia [22]. Unlike other pretreatments such as dilute acid pretreatment and steam explosion, AFEX is a dry-to-dry process (no liquid stream produced after pretreatment) with essentially complete solids recovery. I believe that the AFEX pretreatment could make possible a new paradigm of cellulosic ethanol fermentation: a paradigm in which neither extensive detoxification nor significant nutrient supplementation is required. This paper represents an early step toward realizing this new paradigm. Therefore, hydrolysis and fermentation on AFEX-treated feedstocks under the stringent baseline conditions (low initial cell density, without washing the pretreated materials, detoxifying or supplementing the hydrolysate with nutrients) are investigated. The key questions investigated here are: i) the feasibility of ethanol fermentation on AFEX-treated corn

stover hydrolysates at industrially-relevant concentrations under the baseline conditions, ii) the value of nutrient-rich feedstocks such as DDGS in fermentation and iii) the usefulness of selective evolution in adapting ethanologenic strains for fermentation. Engineered ethanologen *E. coli* KO11 is used to conduct these investigations in Separate Hydrolysis and Fermentation (SHF). In some ways this represents a "worst case" for hydrolysis and fermentation of AFEX-treated CS and DDGS.

M

M

Co

Na

of

re

pr

Bu

pe

ce

et

(A

AF

AF

pr

m

kg

wit

kg

th

MATERIALS AND METHODS

Materials

Corn stover (CS), premilled and passed through a 10 mm screen, was a kind gift from the National Renewable Energy Laboratory (NREL, Golden, Colorado). The moisture content of the untreated CS was about 7% (total weight basis). Feedstock analysis by NREL revealed an estimated composition of 34.1% cellulose, 22.8% xylan, 4.2% arabinan, 2.3% protein in the corn stover. The DDGS, obtained from Big River Resources (West Burlington, IA), was dried to roughly 11.5% (total weight basis). According to analysis performed at the Purdue University, the content of the DDGS consists of 16.0±6.6% cellulose, 8.2±3.3% xylan and 5.2±3.3% arabinan, 5.2% starch and 26.4 % protein. The ethanologenic strain *E. coli* KO11 was obtained from American Type Culture Collection (ATCC) with designated number 55124.

AFEX pretreatment

AFEX pretreatment was conducted on CS and DDGS under the optimal conditions as previously described [101, 102]. For CS, pretreatment temperature was 90°C with moisture content of prewetted CS at 60% (dry weight basis) and ammonia loading of 1.0 kg anhydrous ammonia/1.0 kg dry corn stover. The pretreatment of DDGS was at 70 °C with moisture content of DDGS at 13.0% (dry weight basis) and ammonia loading of 0.8 kg anhydrous ammonia/1.0 kg dry DDGS. For both feedstocks, after holding the vessel at the target temperatures for five minutes, pressure was rapidly released to accomplish

the e

[102

air-d

Enzy

The

LAP

CA)

cell

50°

Hig

The

gra

co

AF

de

ad

sp

en

in

en

the expansion. AFEX-apparatus description and operational details were as reported [102]. After the pretreatment, the materials were left under a fume hood overnight to air-dry the treated feedstock.

Enzymatic hydrolysis

The enzymatic hydrolysis of AFEX-treated CS and DDGS was carried out based upon the LAP-009 protocol from NREL. Commercial enzymes, Spezyme CP (Genencor, Palo Alto, CA) and β-glucosidase (Novozyme 188, Bagsvaerd, Denmark), were loaded at 15 FPU/g cellulose and 64 *p*NPGU/g cellulose respectively. The reaction was conducted at pH 4.8, 50°C and 200 rpm shaking.

High solids loading enzymatic hydrolysis

The solids loading for each biomass sample was approximately 18% by weight (180 grams of dry solids per liter of hydrolysis mixture). Due to the difference in cellulose contents, the corresponding cellulose loadings were 6% for CS and 3% for DDGS. The AFEX-treated feedstocks were enzymatically hydrolyzed without any prior washing or detoxification. For CS, the feedstock and the corresponding enzyme loadings were added fed-batch wise with 9% solids loading each for two consecutive days. To be specific, in an initial saccharification mixture of 448 g, 44 g of dry CS and corresponding enzyme solutions were added to 396 mL of buffer solution (0.06 M citrate buffer, pH4.8) in a 1L unbaffled Erlenmeyer flask. After 24 hr, another 44 g of dry CS and corresponding enzymes solutions were added into the reaction flask to complete the fed-batch

addition with a final saccharification mixture of 500 g. Chloramphenicol (Cm) was added to a final concentration of 50 mg/L to prevent microbial growth. The total enzymatic reaction time was 144 hr. The hydrolysis of DDGS was conducted in single batch for 72 hr. The final hydrolysates were centrifuged and the supernatants were sterile-filtered for further fermentation studies.

1% cellulose loading enzymatic hydrolysis

The 1% cellulose loading hydrolyses were conducted to compare the effect of solids loading on sugar yields for both AFEX-treated CS and DDGS. The experiments were conducted in a total 15 g saccharification mixture (AFEX-treated CS or DDGS in buffered enzyme solution) in screw-capped vials with a total volume of 25 mL. Both feedstocks were added in a single-batch, the reaction times for CS and DDGS were six days and three days, respectively.

Mass balance for enzymatic hydrolysis

After enzymatic hydrolysis, reaction mixtures were centrifuged to separate the pellet from the supernatant. Only glucose and xylose were determined during the analysis because they are the predominant monomeric sugars present in CS-hydrolysate which are also fermentable by *E. coli* KO11. To obtain accurate sugar yield estimates, monomeric sugars content in supernatants, pellets and reaction flasks (due to irretrievable hydrolysate left in the flasks) were taken into account in the mass balance calculation. The pellets formed after centrifugation were washed with distilled water at

fixe

was

was

mo

con

and

sug

not

Cul

All

vol

sha

car

Sel

KO

hyc

ser

gro

und

fixed proportions (1 g wet pellet in 10 mL water). The sugar concentrations of the washed streams and the moisture content of the washed pellets were analyzed. The washed solids dry matter was calculated based on the moisture content. To estimate monomeric sugars retained in the pellets, it was assumed that 1) the monomeric sugar concentration in washed stream was equal to that of the solution contained in the pellet and 2) the density of the solution in the pellets was 1.0 g/mL. Theoretical possible sugars are calculated based on cellulose and hemicellulose i.e. starch from DDGS was not included in the calculation.

Culture and fermentation

All cultures and fermentations, if not stated otherwise, were carried out at a working volume of 100 mL solution in 250 mL unbaffled Erlenmeyer flask at 37°C, 100 rpm shaking. The flasks were capped with rubber stoppers pierced with a needle to vent carbon dioxide.

Selective evolution

KO11 was selectively evolved in two stages, first on solid medium consisting of hydrolysate resulting from 3% cellulose loading of CS-hydrolysis mixture and followed by serial culture in liquid hydrolysates. The ethanologenic strain, *E. coli* KO11, was first grown overnight at 35°C in Luria-Bertani (LB) agar plate supplemented with 2% xylose under 50 mg/L chloramphenicol (Cm) selective pressure. The raised colonies were

tra

Cm

In s

cel

Init

37°

was

in t

suc

cell

3.5

wer

loac

inoc

KO1

The

strai

mg/l

med

over

transferred to solid medium (CS-hydrolysate) and grown successively at two alternate Cm concentrations (50 mg/L and 600 mg/L) for three cycles.

In serial culture, the concentration of antibiotic Cm was maintained at 100 mg/L. The cells on solid medium were inoculated into hydrolysate to an initial OD_{550nm} of 0.1. Initially, the hydrolysates were kept at neutral pH. Each culture was grown for 24 hr at 37°C before successive transfer to a more concentrated hydrolysate. No pH adjustment was made during these cultures. The corresponding cellulose loading of the hydrolysate in the first liquid culture was 3.5% and this was followed by 0.5% increment in each successive transfer until reaching 6%. Culture transfer to the hydrolysate with higher cellulose loading was conducted only when the culture achieved an OD_{550nm} greater than 3.5 within 24 hr. Mutants adapted to the CS hydrolysate at each stage of serial culture were stored in 30% glycerol at -70°C. The mutant strain isolated from 6% cellulose-loading-CS-hydrolysate was termed ML01. This preculture of ML01 was used as the inoculum for the designated fermentation experiments described below.

KO11 seed preparation

The KO11 strain was grown on LB plate (2% xylose) under Cm selective pressure. The strain was grown successively under alternate Cm concentrations i.e. 50 mg/L and 600 mg/L at 35°C. This cycle was repeated three times before the cells were inoculated to LB media (5% xylose, 100 mg/L Cm) to an initial OD_{550nm} of 0.1. The KO11 seed grown overnight was used as the inoculum for the designated fermentations.

Fermentation media preparation

Hydrolysates from enzymatic saccharification of 6% cellulose-loading-CS (Hydrolysate A) and 3% cellulose-loading-DDGS (Hydrolysate C) were the principal media used in the fermentation. Other fermentation media were formulated based on these two hydrolysates. They are listed as follows:

- Hydrolysate B: A mixture of Hydrolysate A (CS-hydrolysate) and distilled water at 7:3 volume ratio.
- Hydrolysate D: A mixture of Hydrolysate C (DDGS-hydrolysate) and externally added reagent grade glucose (9 g/L) and xylose (22 g/L). These sugars were added into Hydrolysate C to increase their concentrations to levels comparable with Hydrolysate A.
- Hydrolysate E: A mixture of Hydrolysate A and Hydrolysate C at 7:3 volume ratio. Hydrolysate E was used to elucidate the effect of DDGS-hydrolysate on fermentation with Hydrolysate B serving as the control experiment to determine the effect of dilution of the Hydrolysate A on fermentation.

Antibiotic Cm was added at a concentration of 50 mg/L. Total Cm concentration accumulated from both enzymatic hydrolysis and fermentation steps was 100 mg/L. All hydrolysates were pH-adjusted using NaOH and sterile-filtered.

Ethanol fermentation

Fermentation was carried out under semi-sterile conditions. The initial OD_{550nm} of fermentation was predetermined as 0.05 (approximately 16 mg dry-weight/L). Designated inocula were centrifuged and the cell pellets were resuspended into the fermentation medium. Growth was at 37°C with 100 rpm shaking. NaOH (5 M) was manually added to adjust the pH to neutral every twelve hours. Before and after each pH measurement and adjustment, the pH probe was thoroughly rinsed sequentially in four test tubes filled with sterile distilled water and dried with sterile wipes. Samples (1.0 mL) were taken manually at defined periods. The volume changes due to pH adjustment and sampling were recorded. No detoxification step and no external nutrient addition were used. Every fermentation experiment was conducted in duplicate.

Compositional analysis

The glucose and xylose concentrations in hydrolysates during the course of fermentation were determined by HPLC with a Biorad Aminex HPX-87P column (Hercules, California). The HPLC system was equipped with a Waters 410 Differential Refractometer (Milford, Massachusetts), HPLC pump (Waters 515) and aided by Waters 717plus autosampler. The temperature of the column was maintained at 85°C and the eluant (water) flow rate was 0.6 mL/min. Glucose and ethanol concentrations were measured using a YSI 2700 Select Biochemistry Analyzer (Yellow Springs, Ohio) to

confirm the accuracy of the HPLC analysis. Operational details were as described in the user manual.

Ethanol yield and volumetric ethanol productivity calculations

In this report, ethanol yields were expressed as grams of ethanol produced per gram of sugars consumed to assess the efficiency of the fermenting strains in converting the sugars to ethanol. The theoretical yield of ethanol for all consumed monomeric sugars was 0.51 g EtOH/g sugar. Volumetric ethanol productivity was calculated based on ethanol produced at the time available glucose was completely consumed. This parameter along with cell density data were used to evaluate the fermentability of cellulosic hydrolysates for ethanol production.

Moisture content analysis and cell density measurement

Moisture content analysis was performed to determine the dry weight of solid materials. In this report, moisture content was measured by an A & D Moisture analyzer MX-50 (Milpitas, California) to determine the weight of feedstocks for hydrolysis experiments. In addition, this analysis was carried out to determine the liquid retained in pellet for calculation of monomeric sugar yields. The optical density of the cell culture was measured using a Turner SP-890 UV Visible Scanning Spectrophotometer (Dubuque, Iowa). The cell density of fermentation broth was measured at a wavelength of 550nm

(1 cm light path) after the sample was taken and cooled to 4°C. Readings were corrected with the dilution factors as necessary.

RESULTS

AFEX pretreatment and enzymatic hydrolysis

AFEX did not significantly change the physical appearance of treated materials except that they become slightly darker. The moisture contents of the air-dried AFEX-CS and DDGS were 10.5% and 6% (total weight basis) respectively. CS was added in two batches to ensure proper liquefaction and mixing of the hydrolysate. After addition of each CS batch, it was observed that the slurry-like mixture liquefied within two hours. Thus, mixing was not a limiting factor for enzymatic hydrolysis at 18% solids loading of AFEX-treated corn stover. After six days of reaction, a hydrolysate containing 52.0 g/L of glucose and 22.2 g/L of xylose were obtained. The monomeric sugar yields were 65.0% for glucose and 42.1% for xylose. In 1% cellulose loading saccharification, glucose and xylose yields for CS after six days were 95% and 70% respectively. Thus, sugars obtainable from AFEX-pretreated CS are limited more by enzyme properties than by inherent reactivity of the AFEX-treated material.

DDGS hydrolysis at 18% solids loading was carried out in a single batch. After three days of reaction, the resulting DDGS hydrolysate contained 40.0 g/L of glucose, corresponding to 97% glucose yield. Xylose yields were minimal (<1.0 g/L). The cellulase

enzyme complex apparently lacks an effective xylanase for DDGS xylans while this xylanase is somewhat effective in hydrolyzing AFEX-treated CS. In 1% cellulose loading saccharification, the glucose yield after 3 days was 105%. Thus essentially, complete cellulose conversions were achieved in both hydrolyses.

Selective evolution of KO11

A serial culture of KO11 on solid medium followed by liquid medium was successfully utilized to fully adapt the ethanologen to the AFEX-treated-CS-hydrolysate environment while maintaining its efficient ethanol production metabolic pathway. KO11 was transferred to the solid medium (3% cellulose loading CS hydrolysate) with 50 mg/L Cm. The cell growth was relatively poor but several colonies were able to thrive. These colonies were transferred to similar solid medium with 600 mg/L Cm. Cell growth was still poor. However, with each subsequent transfer cycle, the cell growth significantly improved. After three cycles of culture, the cells from the solid medium were inoculated into CS-hydrolysate. The cell density (OD_{550nm}) after overnight culture at 37°C was 4.2. Each of the successive transfer cultures in hydrolysates at higher cellulose loadings was able to obtain similar densities (OD550nm between 3.8 and 4.2).

Fermentation of corn stover hydrolysate

The fermentation performance of KO11 and adapted mutant ML01 were compared in Hydrolysate A. Both fermentations were carried out successfully with approximate optical density (550 nm) of 4.0 at stationary phase. Glucose (52.0 g/L) was completely

consumed within 72 hr of fermentation using ML01, resulting in 24.9 g/L ethanol concentration, for an ethanol yield of 93.1% based on total consumed sugars (Figure 31 A, B). A prolonged lag phase of about 24 hr was observed in KO11 fermentation while no observable lag phase was seen for ML01 (Figure 31 D). Ethanol productivity doubled for ML01 compared to KO11 based fermentation (Table 16). Surprisingly, xylose was not consumed by either strain during the course of fermentation of Hydrolysate A. However, 58.2% of the xylose in Hydrolysate B was utilized by ML01 (Figure 31C). Control experiments at comparable sugar concentrations (50 g/L glucose, 20 g/L xylose) using Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1.0 L) showed similar cell densities as that of Hydrolysate A with complete utilization of both sugars (results not shown).

Table 16 Summary of fermentation parameters by ethanologenic strains E. coli KO11 and ML01

Table 16 Summary of fermentation parameters by ethanologenic strains *E. coli* KO11 and ML01

Hydrolysate		Strain	Ethanol produced			Xylose Consump-tion	Cell Density at Stationary Phase (OD550nm)
Label	Identity		Conc[a] (g/L)	Yield[b] (%)	Maximum Productivity (g/L/hr)		
A	CS hydrolysate (6% cellulose loading)	KO11	24.9±0.0	92.2±0.0	0.25±0.01	6.1±0.1	3.8-4.4
		ML01	25.6±2.4	93.2±1.1	0.44±0.01	9.6±1.1	4.2-4.5
B	Mixture of Hydrolysate A and distilled water at ratio 7:3	ML01	19.8±0.8	86.5±2.2	0.44±0.02	61.5±19.2	4.3-4.5
C	DDGS hydrolysate (3% cellulose loading)	ML01	19.6±0.4	102.9±0.2	1.20±0.01	N.A.	7.9-8.9
D	Sugars + Hydrolysate C	ML01	32.8±0.3	96.7±0.4	1.06±0.03	95.8±0.1	6.4-8.0
E	Mixture of Hydrolysate A and C at ratio 7:3	KO11	24.5±0.9	94.4±6.8	0.59±0.02	23.0±11.2	5.0-6.8
		ML01	24.8±1.2	99.2±6.4	0.63±0.01	11.5±5.1	4.2-5.8

Conc: Concentration; N.A.: Not applicable

Errors presented were the standard deviation of the duplicate experiments

[a] Ethanol concentration was corrected for dilution

[b] Yield based on consumed glucose and xylose, theoretical yield of ethanol for both sugars was 0.51 g EtOH/g consumed sugar

Fermentation of DDGS hydrolysate

Protein content in DDGS-hydrolysate was substantially higher than CS-hydrolysate and hence higher fermentability was expected in DDGS-hydrolysate. As illustrated in Figure 32, the fermentation using ML01 yielded high cell density (OD_{550nm} between 8 and 9) for Hydrolysates C and D. However, the cell density of hydrolysate with supplemental sugars was consistently lower than that of DDGS hydrolysate (Figure 32 A). The cell densities of both hydrolysates were more than two-fold that of the control experiment in LB media. Glucose in both hydrolysates was consumed within 24 hr with ethanol productivity exceeding 1.0 g/L/hr . Nearly 80% of the xylose in Hydrolysate D was utilized after 48 hr of fermentation. Ethanol yields based on consumed sugars in Hydrolysates C and D were 103% and 97% respectively (Table 16).

Fermentation of mixed hydrolysate

KO11 and ML01 were tested for fermentation using mixed hydrolysates of CS and DDGS at volume ratio of 7:3 (Hydrolysate E). Both strains obtained similar cell densities with OD_{550nm} between 5 and 6 at the stationary phase (Figure 33 A). However, ML01 exhibited better performance in terms of the rate of glucose consumption and ethanol productivity (Table 16). Glucose was completely consumed within 30 hr by ML01 compared to 40 hr when using KO11 (Figure 33 B, C). The ethanol productivity of KO11 and ML01 were 0.59 and 0.63 g/L/hr, respectively. Ethanol yields close to 100% of theoretical yield were achieved. Xylose consumption was slightly improved to 10-20% of total xylose compared to CS-hydrolysate fermentation (<10% total xylose consumed). As

for the control experiment, Hydrolysate B (70% Hydrolysate A and 30% distilled water) fermented by ML01 exhibited a lower ethanol productivity (0.44 g/L/hr) and cell density (4.3-4.5) at stationary phase (Table 16, Figure 31C).

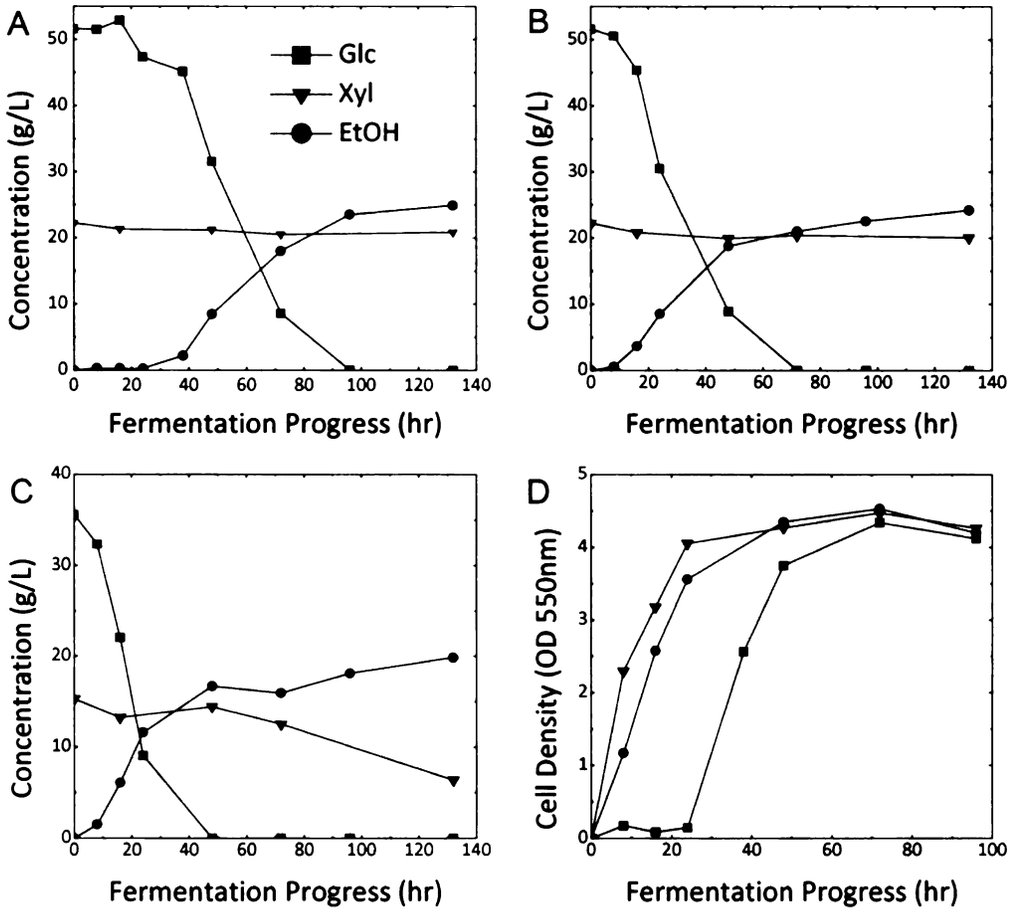


Figure 31 (A) Fermentation of Hydrolysate A (6% cellulose loading CS-hydrolysate) by *E. coli* KO11, (B)Fermentation of Hydrolysate A by *E. coli* ML01,(C) Fermentation of Hydrolysate B (7:10 dilution of Hydrolysate A) by *E. coli* ML01, and (D) Time course of cell density during fermentation in Hydrolysate A and B

The starting cell density of the fermentations was set at 0.05 (OD550nm) and the data presented are averages of the duplicate experiments.

Feedstock-to-ethanol mass balance

In AFEX-pretreatment, a solid-to-solid operation, no loss of mass was expected and none was observed. The residual ammonia on treated-materials was negligible (<0.5% of the total treated material). Under listed enzyme loading and solids loading, 100 g of dry CS yielded 24.6 g of glucose and 11.5 g xylose. About 37.0 g of the dry solid residual material remained after the hydrolysis. Fermentation with KO11 and ML01 produced 12.0 g ethanol with similar yield (93%). The final ethanol concentrations were about 25 g/L. However, the xylose consumption was below 10% in both cases.

For DDGS, 17.2 g of glucose was produced from 100 g dry DDGS with hydrolysis of 3% cellulose-loading. Half of the initial dry matter content of the DDGS was solubilized into the hydrolysate (Figure 35). The ethanol yield in both DDGS fermentations was at the theoretical limit with ethanol concentrations of 19.6 g/L and 32.8 g/L for Hydrolysate C and D, respectively. Glucose and xylose utilizations in Hydrolysate D were essentially complete.

DISCUSSION

High solids loading enzymatic hydrolysis

As expected, one of the significant limiting steps in converting CS to ethanol production was enzymatic hydrolysis using the selected enzymes and loadings. The sugar yields

decreased 30% when the cellulose loadings were increased from 1% to 6%. At higher solids loadings, the concentrations of end products (sugars) and degradation products increase. Monomeric sugars, particularly glucose, dramatically inhibit the cellulase and β-glucosidase activities. As suggested by previous studies, simultaneous saccharification and fermentation (SSF) can be utilized to reduce end product inhibition [23, 103]. The inhibitory effect of degradation products released during pretreatment (wet oxidation) of wheat straw on cellulases and hemicellulases has been reported [97]. Even though AFEX produced degradation compounds at a reduced level [2], it is not surprising that similar inhibitory effects can be found in CS-hydrolysate at high solids loading. In addition, xylose yields were lower than glucose yields in both hydrolyses at 1% and 6% cellulose loadings. Evidently, an optimal enzyme cocktail which contains sufficient activities for both cellulose and hemicellulose is needed to improve xylose yield in AFEX-treated CS.

Similarly, the enzymatic hydrolysis of cellulose in DDGS at 1% cellulose loading achieved higher yield than that of 6% cellulose loading. However, the difference was statistically insignificant due to relatively large margin of error in cellulose content determination. A more precise analysis of composition is required to calculate sugar yield accurately for DDGS. Due to insufficient hemicellulolytic activity of commercial enzymes toward DDGS, the xylose yield was expected to be minimal, as indeed occurred.

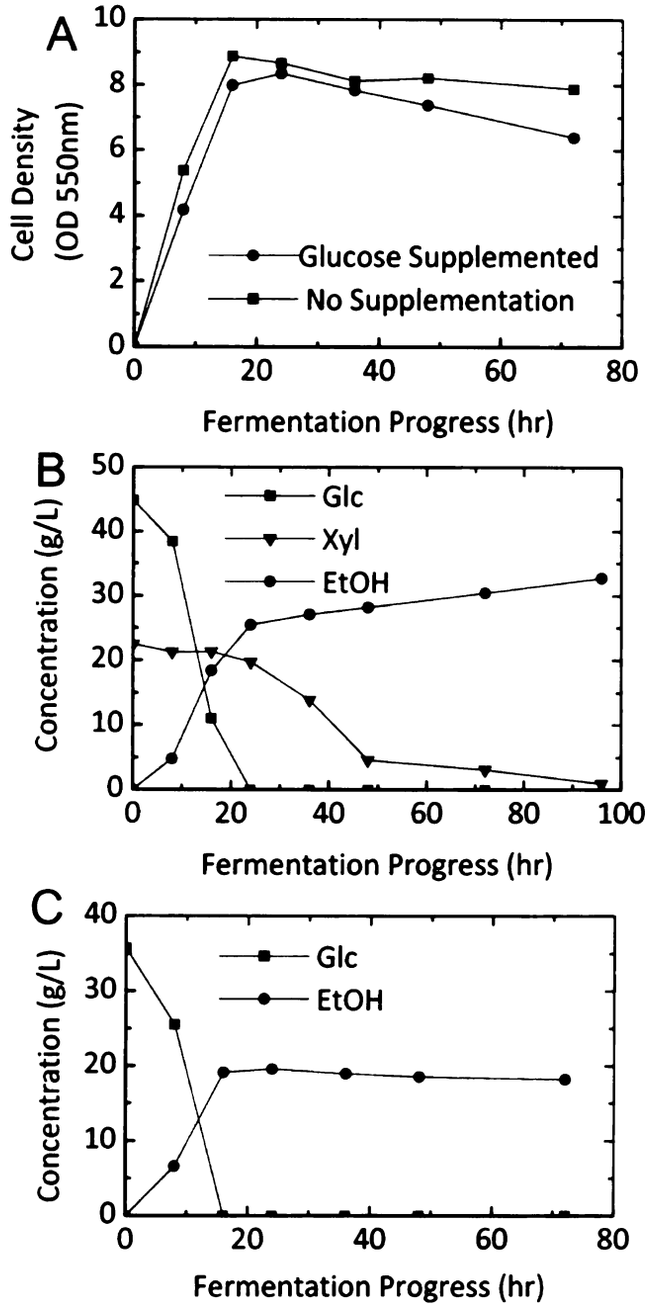


Figure 32 (A) Time course of cell density during fermentation in Hydrolysate C (3% cellulose loading DDGS hydrolysate) and D (DDGS hydrolysate with sugars addition), (B) Fermentation of Hydrolysate C by *E. coli* ML01, (C) Fermentation of Hydrolysate D by *E. coli* ML01.

The starting cell density of the fermentations was predetermined at 0.05 (OD550nm) and the data presented are averages of the duplicate experiements.

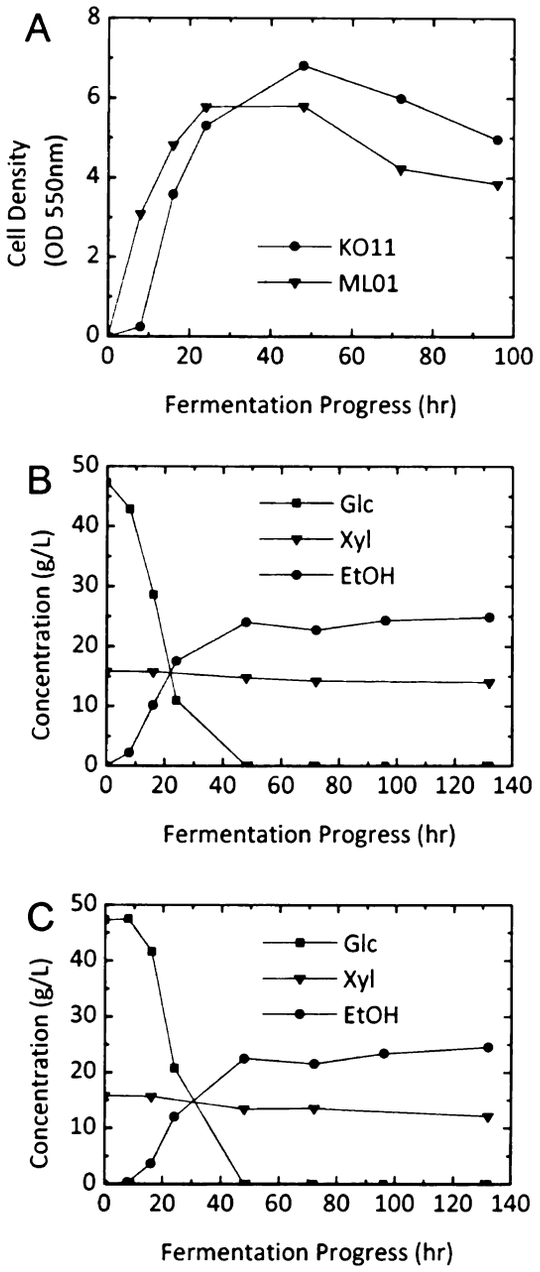


Figure 33 (A) Time course of cell density during fermentation in Hydrolysate E (Mixture of CS and DDGS hydrolysates at ratio 7:3), (B) Fermentation of Hydrolysate E by *E. coli* KO11, (C) Fermentation of Hydrolysate E by *E. coli* ML01.

The starting cell density of the fermentations was set at 0.05 (OD550nm) and the data presented are averages of the duplicate experiments.

Fermentation

Fermentation of corn stover hydrolysate

The hydrolysates of AFEX-treated CS showed high fermentability. Under the baseline conditions (low initial cell density, without washing the pretreated materials, detoxifying or supplementing the hydrolysate with nutrients), AFEX-hydrolysates were benign to the growth of *E. coli*, the least robust genetically-engineered ethanologen amongst its counterparts [27]. Nevertheless, I am fully aware that pH of the hydrolysate is crucial factor that affects the toxicity of certain compounds, e.g. organic acids. This result indicated that the nutrient content of the CS-hydrolysate is not as poor as generally perceived because cell densities similar to culture in LB media were achieved.

Fermentation of DDGS and mixed hydrolysates

DDGS hydrolysate exhibited very high fermentability. There are two general trends observed in the fermentations using hydrolysate mixture of CS and DDGS. Compared to control experiment (Hydrolysate B) which indicating the effect of dilution on the fermentability of Hydrolysate A, ethanol productivity and cell density at stationary phase both increased when DDGS-hydrolysate was part of the fermentation mixture. DDGS improves the fermentability of cellulosic hydrolysates presumably due to high nutrient content in DDGS. It is noteworthy that 25% of the crude protein and 42% of the fat from the original DDGS were solubilized in the hydrolysate during saccharification [101].

DDGS as the source for nutrient supplement

DDGS is a nutrient-rich cellulosic feedstock, which can potentially be used as the source for fermentable sugars and nutrient supplement. The difference in fermentation profiles of Hydrolysate A and E indicates that DDGS enhance glucose utilization and cell growth in the fermentation (Figure 34 A & B). Complete glucose utilization was achieved within 48 hr in Hydrolysate E compared to 96 hr in Hydrolysate A. Fermentation in LB-supplemented CS-hydrolysate (Hydrolysate F) exhibited similar improvements i.e. increased in cell density and glucose consumption rate (Figure 34 A & B). This further affirmed the feasibility to replace external nutrient supplements using DDGS.

Xylose utilization

Regardless of the strains used, xylose consumption was low in Hydrolysate A and E. Both of the hydrolysates produced comparable final ethanol concentrations even though Hydrolysate E exhibited better fermentability. Interestingly, Hydrolysate B (diluted from hydrolysate A) produced lower ethanol concentration with more than 60% xylose consumption. In contrast, xylose consumption was almost complete in DDGS hydrolysate (Hydrolysate D) with the highest final ethanol concentration amongst tested hydrolysates. One plausible interpretation of these results is that both the ethanol concentration and inhibitory compounds present in CS-hydrolysate could reduce xylose utilization but the levels of these inhibitory compounds were low enough in DDGS hydrolysate so as not to interfere with fermentation. It is worth pointing out that the nature of the lignocellulosic content in each feedstock influences the formation of toxic

degradation products. In particular, the higher lignin content in CS increases the potential production of phenolic-based-compounds which are highly inhibitory [35]. Overall, this finding presents a challenge for efficient xylose utilization in co-fermentation of unconditioned CS-hydrolysate with low initial cell density.

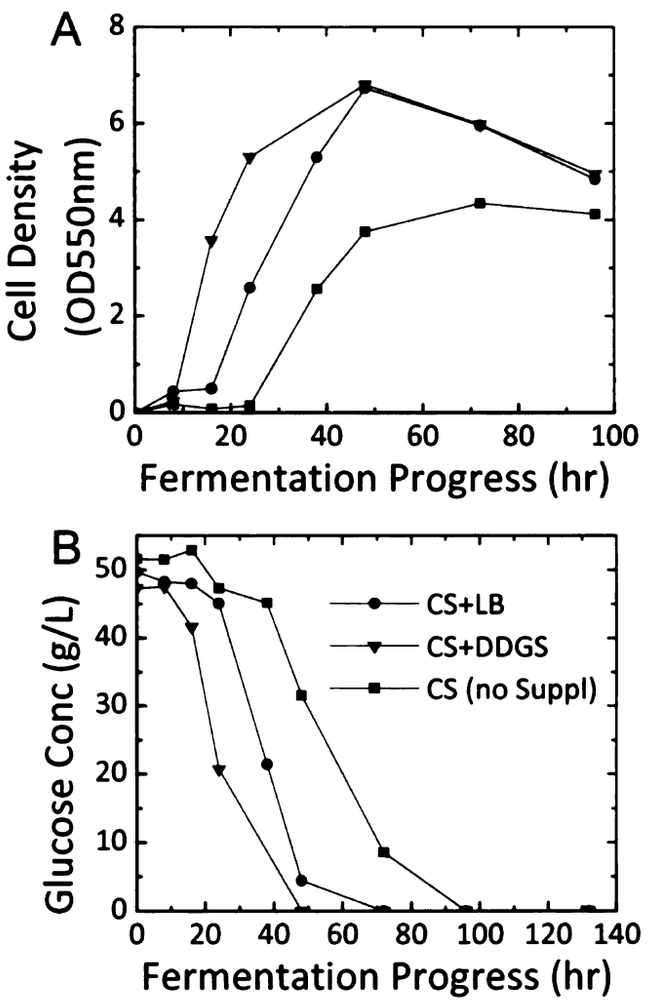


Figure 34 (A) Time course of cell density, (B) Glucose consumption profile in fermentation of corn stover hydrolysates with or without nutrient supplementation.

The starting cell density of the fermentations was predetermined at 0.05 (OD550nm) and the data presented are averages of the duplicate experiments.

Comparison between KO11 and ML01

The mutant strain ML01 exhibited higher ethanol productivity and the difference was more pronounced in CS-hydrolysate which has lower fermentability. ML01 has a higher tolerance and adaptability to the CS-hydrolysate environment and thus performed better than KO11. In terms of ethanol yield and xylose utilization, both KO11 and ML01 displayed similar performance. However, this technique can be very useful to generate suitable strains for fermentation at higher solids loadings.

Significance of the results

To our knowledge, this was the first successful ethanolic fermentation of cellulosic hydrolysates at low initial cell density, without washing, detoxification and external nutrient supplement. This breakthrough was largely made possible by the nature of the AFEX pretreatment. AFEX produces relatively low levels of degradation compounds (Chundawat, personal communication). Thus, the technological challenges to improve the fermentability of hydrolysates are significantly decreased. Various physical, chemical and biological detoxification methods have been explored, primarily for acid pretreated biomass [35, 99, 104, 105]. Nevertheless, detoxification is a significant economic burden for large-scale production of cellulosic ethanol. According to analysis done at the Lund University, 22% of the total cost of cellulosic ethanol production was estimated to be due to detoxification processes [38].

The utilization of DDGS, a feedstock with high nutrient and low lignin content, can help achieve efficient fermentation of cellulosic materials. The fermentation of mixed hydrolysate sugars from nutrient-poor (CS) and nutrient-rich (DDGS) feedstocks could provide a viable option for large-scale industrial production of cellulosic ethanol.

CONCLUSIONS

Hydrolysates from both AFEX-pretreated corn stover and DDGS showed high fermentability with excellent ethanol yield in both KO11 and adapted strain ML01. Nonetheless, incomplete utilization of xylose in CS-hydrolysates was observed. The technique of selective evolution and the incorporation of nutrient-rich feedstock greatly enhance fermentation performance. Cellulosic hydrolysates based on AFEX are not as nutrient-deficient as frequently perceived; the need for external nutrient supplementation and detoxification can be reduced through the AFEX pretreatment method and the utilization of nutrient-rich feedstocks such as DDGS.

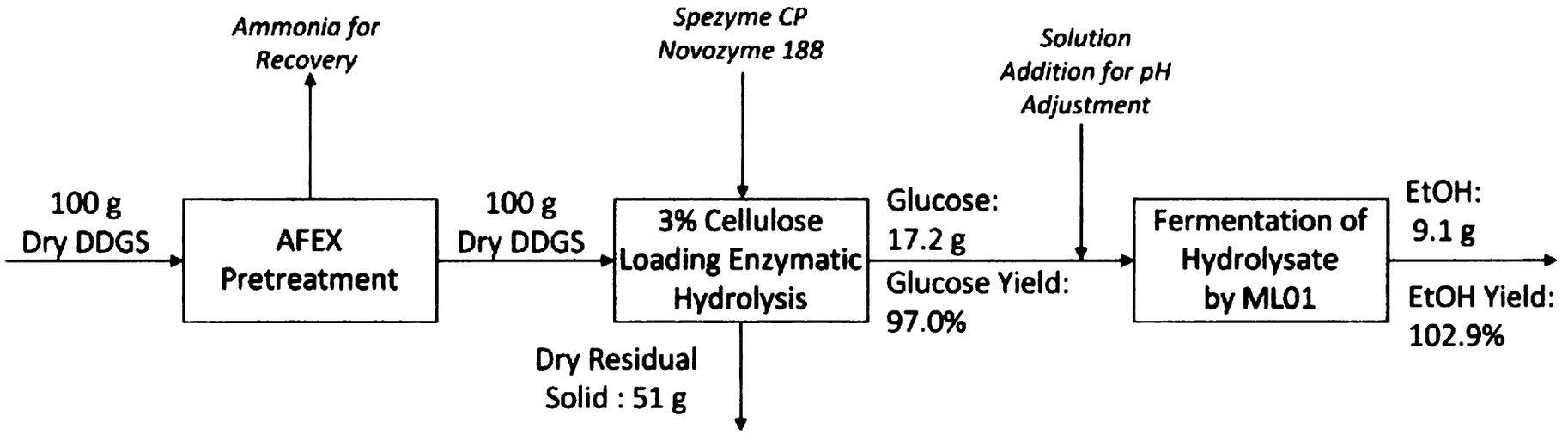


Figure 35 DDGS to ethanol analysis. DDGS was AFEX-pretreated and enzymatically hydrolyzed using Spezyme CP and Novozyme 188 for three days before fermentation using adapted strain *E. coli* ML01

REFERENCE

1. Mosier, N., et al., *Features of promising technologies for pretreatment of lignocellulosic biomass.* Bioresource Technology, 2005. **96**(6): p. 673-686.

2. Lynd, L.R., *Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, economics, the environment, and policy.* Annual Review of Energy and the Environment, 1996. **21**: p. 403-465.

3. Lau, M.W., B.E. Dale, and V. Balan, *Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation.* Biotechnology and Bioengineering, 2008. **99**(3): p. 529-539.

4. Dien, B.S., et al., *Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass.* Biomass & Bioenergy, 2006. **30**(10): p. 880-891.

5. Chang, V., M. Nagwani, and M. Holtzapple, *Lime pretreatment of crop residues bagasse and wheat straw.* Applied Biochemistry and Biotechnology, 1998. **74**(3): p. 135-159.

6. Kabel, M.A., et al., *Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw.* Bioresource Technology, 2007. **98**(10): p. 2034-2042.

7. Luo, C., D.L. Brink, and H.W. Blanch, *Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol.* Biomass and Bioenergy, 2002. **22**(2): p. 125-138.

8. Kadam, K.L. and J.D. McMillan, *Availability of corn stover as a sustainable feedstock for bioethanol production.* Bioresource Technology, 2003. **88**(1): p. 17-25.

9. Greene, N., et al., *Growing energy: how biofuels can help end America's oil dependence.* Nature Resources Defense Council, 2004.

10. Wyman, C.E., *Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power.* Biotechnol Prog, 2003. **19**(2): p. 254-62.

11. Hsu, T.-A., *Pretreatment of Biomass*, in *Handbook on Bioethanol, Production and Utilization*, C.E. Wyman, Editor. 1996, Taylor & Francis: Washington, DC.

12. Wenzl, H.R.J., *The Chemical Technology of Wood*. 1970, Academic Press. p. 32-245.

13. Mosier, N., et al., *Features of promising technologies for pretreatment of lignocellulosic biomass.* Bioresource Technology, 2005. **96**(6): p. 673-86.

14. Wyman, C.E., et al., *Coordinated development of leading biomass pretreatment technologies.* Bioresource Technology, 2005. **96**(18): p. 1959-1966.

15. Schell, D.J., et al., *Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor - Investigation of yields, kinetics, and enzymatic digestibilities of solids.* Applied Biochemistry and Biotechnology, 2003. **105**: p. 69-85.

16. Zaldivar, J., A. Martinez, and L.O. Ingram, *Effect of selected aldehydes on the growth and fermentation of ethanologenic Escherichia coli.* Biotechnology and Bioengineering, 1999. **65**(1): p. 24-33.

17. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification.* Bioresource Technology, 2000. **74**(1): p. 17-24.

18. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.* Bioresource Technology, 2000. **74**(1): p. 25-33.

19. Zaldivar, J. and L.O. Ingram, *Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01.* Biotechnology and Bioengineering, 1999. **66**(4): p. 203-210.

20. Martinez, A., et al., *Effects of Ca(OH)(2) treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates.* Biotechnology and Bioengineering, 2000. **69**(5): p. 526-536.

21. Berson, R.E., et al., *Detoxification of actual pretreated corn stover hydrolysate using activated carbon powder.* Appl Biochem Biotechnol, 2005. **121-124**: p. 923-34.

22. Teymouri, F., et al., *Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover.* Bioresource Technology, 2005. **96**(18): p. 2014-2018.

23. Takagi, M., et al. *A method for production of alcohol direct from cellulose using cellulase and yeast.* in *Proceedings of Bioconversion of cellulosic substances into energy, chemicals and microbial protein*. 1977. IIT, New Delhi.

24. Xiao, Z.Z., et al., *Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 1115-1126.

25. Saha, B.C., *Hemicellulose bioconversion.* Journal of Industrial Microbiology and Biotechnology, 2003. **30**(5): p. 279-91.

26. Lynd, L.R., et al., *Consolidated bioprocessing of cellulosic biomass: an update.* Curr Opin Biotechnol, 2005. **16**(5): p. 577-83.

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

28. Ohta, K., et al., *Genetic improvement of Escherichia coli for ethanol production - chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II.* Applied and Environmental Microbiology, 1991. **57**(4): p. 893-900.

29. Sedlak, M. and N.W.Y. Ho, *Production of ethanol from cellulosic biomass hydrolysates using genetically engineered Saccharomyces yeast capable of cofermenting glucose and xylose.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 403-416.

30. Mohagheghi, A., et al., *Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of Zymomonas mobilis AX101.* Appl Biochem Biotechnol, 2002. **98-100**: p. 885-98.

31. Zhang, M., et al., *Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic Zymomonas mobilis.* Science, 1995. **267**(5195): p. 240-243.

32. Mohagheghi, A., et al., *Performance of a newly developed integrant of Zymomonas mobilis for ethanol production on corn stover hydrolysate.* Biotechnology Letters, 2004. **26**(4): p. 321-325.

33. Aden, A., et al., *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, N.R.E. Lab, Editor. 2002: Golden, CO.

34. Lee, Y.Y., P. Iyer, and R.W. Torget, *Dilute-Acid Hydrolysis of Lignocellulosic Biomass*, in *Advances in Biochemical Engineering*, T. Scheper, Editor. 1999, Springer-Verlag: New York, NY.

35. Klinke, H.B., A.B. Thomsen, and B.K. Ahring, *Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass.* Applied Microbiology and Biotechnology, 2004. **66**(1): p. 10-26.

36. Nilvebrant, N.O., et al., *Detoxification of lignocellulose hydrolysates with ion-exchange resins.* Applied Biochemistry and Biotechnology, 2001. **91-3**: p. 35-49.

37. Berson, R.E., et al., *Detoxification of Actual Pretreated Corn Stover Hydrolysate Using Activated Carbon Powder*, in *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*. 2005. p. 923-934.

38. VonSivers, M., et al., *Cost analysis of ethanol production from willow using recombinant Escherichia coli.* Biotechnology Progress, 1994. **10**(5): p. 555-560.

39. Lawford, H.G. and J.D. Rousseau, *Corn steep liquor as a cost-effective nutrition adjunct in high-performance Zymomonas ethanol fermentations.* Applied Biochemistry and Biotechnology, 1997. **63-5**: p. 287-304.

40. Humbird, D. and A. Aden, *Biochemical Production of Ethanol from Corn Stover: 2008 State of Technology Model*. 2009: Golden, Colorado.

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

42. Wyman, C.E., et al., *Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover.* Bioresource Technology, 2005. **96**(18): p. 2026-2032.

43. Lloyd, T.A. and C.E. Wyman, *Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids.* Bioresource Technology, 2005. **96**(18): p. 1967-1977.

44. Dien, B.S., et al., *Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol.* Bioresource Technology, 2008. **99**(12): p. 5216-5225.

45. Pordesimo, L.O., et al., *Variation in corn stover composition and energy content with crop maturity.* Biomass and Bioenergy, 2005. **28**(4): p. 366-374.

46. Lynd, L.R., et al., *How biotech can transform biofuels.* Nature Biotechnology, 2008. **26**(2): p. 169-72.

47. Lau, M.W. and B.E. Dale, *Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST).* Proc Natl Acad Sci U S A, 2009. **106**(5): p. 1368-73.

48. Sticklen, M.B., *Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol.* Nat Rev Genet, 2008. **9**(6): p. 433-43.

49. Shaw, A.J., et al., *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, 2008. **105**(37): p. 13769-13774.

50. Wang, M., H. Lee, and J. Molburg, *Allocation of energy use in petroleum refineries to petroleum products - Implications for life-cycle energy use and emission inventory of petroleum transportation fuels.* International Journal of Life Cycle Assessment, 2004. **9**(1): p. 34-44.

51. Wang, M., C. Saricks, and D. Santini, *Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions*, U.S.-Department-of-Energy, Editor. 1999, Argonne National Laboratory: Chicago.

52. Yang, B. and C.E. Wyman, *Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose.* Biotechnology and Bioengineering, 2004. **86**(1): p. 88-95.

53. Gilarranz, M.A., et al., *Acid precipitation and purification of wheat straw lignin.* Separation Science and Technology, 1998. **33**(9): p. 1359-1377.

54. Bals, B., et al., *Ammonia fiber expansion treatment of eleven different forages: Improvements to fiber digestibility in vitro.* Animal Feed Science and Technology, 2009. **[Manuscript Submitted]**.

55. Carolan, J.E., S.V. Joshi, and B.E. Dale, *Technical and Financial Feasibility Analysis of Distributed Bioprocessing Using Regional Biomass Pre-Processing Centers.* Journal of Agricultural and Industrial Organization, 2007. **5**: p. 1-27.

56. Ingram, L.O., et al., *Metabolic engineering of bacteria for ethanol production.* Biotechnology and Bioengineering, 1998. **58**(2-3): p. 204-214.

57. Jeffries, T.W. and Y.S. Jin, *Metabolic engineering for improved fermentation of pentoses by yeasts.* Applied Microbiology and Biotechnology, 2004. **63**(5): p. 495-509.

58. Karhumaa, K., et al., *Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant Saccharomyces cerevisiae.* Microbial Cell Factories, 2007. **6**: p. -.

59. Yomano, L.P., S.W. York, and L.O. Ingram, *Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production.* Journal of Industrial Microbiology & Biotechnology, 1998. **20**(2): p. 132-138.

60. Saha, B.C., et al., *Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol.* Process Biochemistry, 2005. **40**(12): p. 3693-3700.

61. Ohgren, K., et al., *Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400.* J Biotechnol, 2006. **126**(4): p. 488-98.

62. Moniruzzaman, M. and L.O. Ingram, *Ethanol production from dilute acid hydrolysate of rice hulls using genetically engineered Escherichia coli.* Biotechnology Letters, 1998. **20**(10): p. 943-947.

63. Deanda, K., et al., *Development of an arabinose-fermenting Zymomonas mobilis strain by metabolic pathway engineering.* Applied and Environmental Microbiology, 1996. **62**(12): p. 4465-70.

64. Ho, N.W., et al., *Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol.* Adv Biochem Eng Biotechnol, 1999. **65**: p. 163-92.

65. Underwood, S.A., et al., *Flux through citrate synthase limits the growth of ethanologenic Escherichia coli KO11 during xylose fermentation.* Applied and Environmental Microbiology, 2002. **68**(3): p. 1071-81.

66. Sedlak, M. and N.W. Ho, *Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant Saccharomyces yeast.* Yeast, 2004. **21**(8): p. 671-84.

67. Pauly, M. and K. Keegstra, *Cell-wall carbohydrates and their modification as a resource for biofuels.* Plant J, 2008. **54**(4): p. 559-68.

68. Lohmeier-Vogel, E.M., C.R. Sopher, and H. Lee, *Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeasts.* Journal of Industrial Microbiology & Biotechnology, 1998. **20**(2): p. 75-81.

69. Chen, J.H. and C.T. Ho, *Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds.* Journal of Agricultural and Food Chemistry, 1997. **45**(7): p. 2374-2378.

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

71. Solaiman, S.G., G.W. Horn, and F.N. Owens, *Ammonium Hydroxide Treatment on Wheat Straw.* J. Anim Sci., 1979. **49**(3): p. 802-808.

72. Kadam, K.L. and M.M. Newman, *Development of a low-cost fermentation medium for ethanol production from biomass.* Applied Microbiology and Biotechnology, 1997. **47**(6): p. 625-629.

73. Ohgren, K., et al., *A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover.* Process Biochemistry, 2007. **42**(5): p. 834-839.

74. Sonderegger, M., et al., *Fermentation performance of engineered and evolved xylose-fermenting Saccharomyces cerevisiae strains.* Biotechnol Bioeng, 2004. **87**(1): p. 90-8.

75. Pampulha, M.E. and M.C. Loureirodias, *Combined Effect of Acetic-Acid, Ph and Ethanol on Intracellular Ph of Fermenting Yeast.* Applied Microbiology and Biotechnology, 1989. **31**(5-6): p. 547-550.

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

77. Bergman, T., M. Carlquist, and H. Jornvall, *Amino Acid Analysis by High Performance Liquid Chromatography of Phenylthiocarbamyl Derivatives* in *Advanced Methods in Protein Microsequence Analysis*, B. Wittmann-Liebold, Editor. 1986, Springer Verlag: Berlin. p. 45-55.

78. Xu, F., et al., *Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases.* Journal of Molecular Catalysis B: Enzymatic, 2008. **51**(1-2): p. 42-48.

79. Walker, G.M., *Yeast physiology and biotechnology*. 1998, New York, NY: John Wiley & Sons, Inc. .

80. Saltokoglu, A. and J.C. Slaughter, *The effect of magnesium and calcium on yeast growth.* Journal of the Institute of Brewing, 1983. **89**: p. 81-83.

81. Walker, G.M., *Metals in Yeast Fermentation Processes*, in *Advances in Applied Microbiology*, A.I. Laskin, J.W. Bennet, and G.M. Gadd, Editors. 2004, Elsevier Acadamic Press: New York, NY.

82. Boulton, C. and D. Quain, *Brewing Yeast and Fermentation*. 2001, Ames, Iowa: Iowa State University Press. 638.

83. Chundawat SPS. , et al., *Major cell wall decomposition products produced during ammonia fiber expansion pretreatment of corn stover.* [Manuscript In Preparation], 2009.

84. Aden, A., *Biochemical Production of Ethanol from Corn Stover: 2007 State of Technology Model*. 2008: Golden, Colorado.

85. Mach, R.L. and S. Zeilinger, *Regulation of gene expression in industrial fungi: Trichoderma.* Applied Microbiology and Biotechnology, 2003. **60**(5): p. 515-522.

86. Kelly, J.M. and M.J. Hynes, *Transformation of Aspergillus-Niger by the Amds Gene of Aspergillus-Nidulans.* Embo Journal, 1985. **4**(2): p. 475-479.

87. van den Hondel, C.A., P.J. Punt, and R.F. van Gorcom, *Production of extracellular proteins by the filamentous fungus Aspergillus.* Antonie Van Leeuwenhoek, 1992. **61**(2): p. 153-60.

88. Lee, Y.E., S.E. Lowe, and J.G. Zeikus, *Regulation and Characterization of Xylanolytic Enzymes of Thermoanaerobacterium saccharolyticum B6A-RI.* Applied and Environmental Microbiology, 1993. **59**(3): p. 763-771.

89. Lowe, S.E., M.K. Jain, and J.G. Zeikus, *Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates.* Microbiol Rev, 1993. **57**(2): p. 451-509.

90. Shaw, A.J., et al., *Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield.* International Sugar Journal, 2009. **111**(1323): p. 164-171.

91. Kwiatkowski, J.R., et al., *Modeling the process and costs of fuel ethanol production by the corn dry-grind process.* Industrial Crops and Products, 2006. **23**(3): p. 288-296.

92. Schubert, C., *Can biofuels finally take center stage?* Nature Biotechnology, 2006. **24**(7): p. 777-784.

93. Spiehs, M.J., M.H. Whitney, and G.C. Shurson, *Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota.* Journal of Animal Science, 2002. **80**(10): p. 2639-2645.

94. Ham, G.A., et al., *Wet corn distillers by-products compared with dried corn distillers grains with solubles as a source of protein and energy for ruminants.* Journal of Animal Science, 1994. **72**(12): p. 3246-3257.

95. Mosier, N., et al., *Optimization of pH controlled liquid hot water pretreatment of corn stover.* Bioresource Technology, 2005. **96**(18): p. 1986-1993.

96. Kim, T.H., et al., *Pretreatment of corn stover by low-liquid ammonia recycle percolation process.* Applied Biochemistry and Biotechnology, 2006. **133**(1): p. 41-57.

97. Panagiotou, G. and L. Olsson, *Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates.* Biotechnology and Bioengineering, 2006. **96**(2): p. 250-258.

98. Varga, E., et al., *High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol.* Biotechnology and Bioengineering, 2004. **88**(5): p. 567-574.

99. Berson, R.E., et al., *Detoxification of actual pretreated corn stover hydrolysate using activated carbon powder.* Applied Biochemistry and Biotechnology, 2005. **124**(1-3): p. 923-934.

100. Petersson, A., et al., *A 5-hydroxymethyl furfural reducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene conveys HMF tolerance.* Yeast, 2006. **23**(6): p. 455-464.

101. Bals, B., B. Dale, and V. Balan, *Enzymatic hydrolysis of distiller's dry grain and solubles (DDGS) using ammonia fiber expansion pretreatment.* Energy & Fuels, 2006. **20**(6): p. 2732-2736.

102. Teymouri, F., et al., *Ammonia fiber explosion treatment of corn stover.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 951-963.

103. Stenberg, K., et al., *Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production.* Biotechnology and Bioengineering, 2000. **68**(2): p. 204-210.

104. DeMancilha, I.M. and M.N. Karim, *Evaluation of ion exchange resins for removal of inhibitory compounds from corn stover hydrolyzate for xylitol fermentation.* Biotechnology Progress, 2003. **19**(6): p. 1837 -1841.

105. Nichols, N.N., et al., *Bioabatement to remove inhibitors from biomass-derived sugar hydrolysates.* Applied Biochemistry and Biotechnology, 2005. **121**: p. 379-390.

REFERENCE

1. Mosier, N., et al., *Features of promising technologies for pretreatment of lignocellulosic biomass.* Bioresource Technology, 2005. **96**(6): p. 673-686.

2. Lynd, L.R., *Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, economics, the environment, and policy.* Annual Review of Energy and the Environment, 1996. **21**: p. 403-465.

3. Lau, M.W., B.E. Dale, and V. Balan, *Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation.* Biotechnology and Bioengineering, 2008. **99**(3): p. 529-539.

4. Dien, B.S., et al., *Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass.* Biomass & Bioenergy, 2006. **30**(10): p. 880-891.

5. Chang, V., M. Nagwani, and M. Holtzapple, *Lime pretreatment of crop residues bagasse and wheat straw.* Applied Biochemistry and Biotechnology, 1998. **74**(3): p. 135-159.

6. Kabel, M.A., et al., *Effect of pretreatment severity on xylan solubility and enzymatic breakdown of the remaining cellulose from wheat straw.* Bioresource Technology, 2007. **98**(10): p. 2034-2042.

7. Luo, C., D.L. Brink, and H.W. Blanch, *Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol.* Biomass and Bioenergy, 2002. **22**(2): p. 125-138.

8. Kadam, K.L. and J.D. McMillan, *Availability of corn stover as a sustainable feedstock for bioethanol production.* Bioresource Technology, 2003. **88**(1): p. 17-25.

9. Greene, N., et al., *Growing energy: how biofuels can help end America's oil dependence.* Nature Resources Defense Council, 2004.

10. Wyman, C.E., *Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals, and power.* Biotechnol Prog, 2003. **19**(2): p. 254-62.

11. Hsu, T.-A., *Pretreatment of Biomass*, in *Handbook on Bioethanol, Production and Utilization*, C.E. Wyman, Editor. 1996, Taylor & Francis: Washington, DC.

12. Wenzl, H.R.J., *The Chemical Technology of Wood*. 1970, Academic Press. p. 32-245.

13. Mosier, N., et al., *Features of promising technologies for pretreatment of lignocellulosic biomass.* Bioresource Technology, 2005. **96**(6): p. 673-86.

14. Wyman, C.E., et al., *Coordinated development of leading biomass pretreatment technologies.* Bioresource Technology, 2005. **96**(18): p. 1959-1966.

15. Schell, D.J., et al., *Dilute-sulfuric acid pretreatment of corn stover in pilot-scale reactor - Investigation of yields, kinetics, and enzymatic digestibilities of solids.* Applied Biochemistry and Biotechnology, 2003. **105**: p. 69-85.

16. Zaldivar, J., A. Martinez, and L.O. Ingram, *Effect of selected aldehydes on the growth and fermentation of ethanologenic Escherichia coli.* Biotechnology and Bioengineering, 1999. **65**(1): p. 24-33.

17. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification.* Bioresource Technology, 2000. **74**(1): p. 17-24.

18. Palmqvist, E. and B. Hahn-Hagerdal, *Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition.* Bioresource Technology, 2000. **74**(1): p. 25-33.

19. Zaldivar, J. and L.O. Ingram, *Effect of organic acids on the growth and fermentation of ethanologenic Escherichia coli LY01.* Biotechnology and Bioengineering, 1999. **66**(4): p. 203-210.

20. Martinez, A., et al., *Effects of Ca(OH)(2) treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates.* Biotechnology and Bioengineering, 2000. **69**(5): p. 526-536.

21. Berson, R.E., et al., *Detoxification of actual pretreated corn stover hydrolysate using activated carbon powder.* Appl Biochem Biotechnol, 2005. **121-124**: p. 923-34.

22. Teymouri, F., et al., *Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover.* Bioresource Technology, 2005. **96**(18): p. 2014-2018.

23. Takagi, M., et al. *A method for production of alcohol direct from cellulose using cellulase and yeast.* in *Proceedings of Bioconversion of cellulosic substances into energy, chemicals and microbial protein.* 1977. IIT, New Delhi.

24. Xiao, Z.Z., et al., *Effects of sugar inhibition on cellulases and beta-glucosidase during enzymatic hydrolysis of softwood substrates.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 1115-1126.

25. Saha, B.C., *Hemicellulose bioconversion.* Journal of Industrial Microbiology and Biotechnology, 2003. **30**(5): p. 279-91.

26. Lynd, L.R., et al., *Consolidated bioprocessing of cellulosic biomass: an update.* Curr Opin Biotechnol, 2005. **16**(5): p. 577-83.

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

28. Ohta, K., et al., *Genetic improvement of Escherichia coli for ethanol production - chromosomal integration of Zymomonas mobilis genes encoding pyruvate decarboxylase and alcohol dehydrogenase II.* Applied and Environmental Microbiology, 1991. **57**(4): p. 893-900.

29. Sedlak, M. and N.W.Y. Ho, *Production of ethanol from cellulosic biomass hydrolysates using genetically engineered Saccharomyces yeast capable of cofermenting glucose and xylose.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 403-416.

30. Mohagheghi, A., et al., *Cofermentation of glucose, xylose, and arabinose by genomic DNA-integrated xylose/arabinose fermenting strain of Zymomonas mobilis AX101.* Appl Biochem Biotechnol, 2002. **98-100**: p. 885-98.

31. Zhang, M., et al., *Metabolic Engineering of a Pentose Metabolism Pathway in Ethanologenic Zymomonas mobilis.* Science, 1995. **267**(5195): p. 240-243.

32. Mohagheghi, A., et al., *Performance of a newly developed integrant of Zymomonas mobilis for ethanol production on corn stover hydrolysate.* Biotechnology Letters, 2004. **26**(4): p. 321-325.

33. Aden, A., et al., *Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover*, N.R.E. Lab, Editor. 2002: Golden, CO.

34. Lee, Y.Y., P. Iyer, and R.W. Torget, *Dilute-Acid Hydrolysis of Lignocellulosic Biomass*, in *Advances in Biochemical Engineering*, T. Scheper, Editor. 1999, Springer-Verlag: New York, NY.

35. Klinke, H.B., A.B. Thomsen, and B.K. Ahring, *Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass.* Applied Microbiology and Biotechnology, 2004. **66**(1): p. 10-26.

36. Nilvebrant, N.O., et al., *Detoxification of lignocellulose hydrolysates with ion-exchange resins.* Applied Biochemistry and Biotechnology, 2001. **91-3**: p. 35-49.

37. Berson, R.E., et al., *Detoxification of Actual Pretreated Corn Stover Hydrolysate Using Activated Carbon Powder*, in *Twenty-Sixth Symposium on Biotechnology for Fuels and Chemicals*. 2005. p. 923-934.

38. VonSivers, M., et al., *Cost analysis of ethanol production from willow using recombinant Escherichia coli.* Biotechnology Progress, 1994. **10**(5): p. 555-560.

39. Lawford, H.G. and J.D. Rousseau, *Corn steep liquor as a cost-effective nutrition adjunct in high-performance Zymomonas ethanol fermentations.* Applied Biochemistry and Biotechnology, 1997. **63-5**: p. 287-304.

40. Humbird, D. and A. Aden, *Biochemical Production of Ethanol from Corn Stover: 2008 State of Technology Model*. 2009: Golden, Colorado.

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

42. Wyman, C.E., et al., *Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover.* Bioresource Technology, 2005. **96**(18): p. 2026-2032.

43. Lloyd, T.A. and C.E. Wyman, *Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids.* Bioresource Technology, 2005. **96**(18): p. 1967-1977.

44. Dien, B.S., et al., *Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers' grains and their conversion to ethanol.* Bioresource Technology, 2008. **99**(12): p. 5216-5225.

45. Pordesimo, L.O., et al., *Variation in corn stover composition and energy content with crop maturity.* Biomass and Bioenergy, 2005. **28**(4): p. 366-374.

46. Lynd, L.R., et al., *How biotech can transform biofuels.* Nature Biotechnology, 2008. **26**(2): p. 169-72.

47. Lau, M.W. and B.E. Dale, *Cellulosic ethanol production from AFEX-treated corn stover using Saccharomyces cerevisiae 424A(LNH-ST).* Proc Natl Acad Sci U S A, 2009. **106**(5): p. 1368-73.

48. Sticklen, M.B., *Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol.* Nat Rev Genet, 2008. **9**(6): p. 433-43.

49. Shaw, A.J., et al., *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, 2008. **105**(37): p. 13769-13774.

50. Wang, M., H. Lee, and J. Molburg, *Allocation of energy use in petroleum refineries to petroleum products - Implications for life-cycle energy use and emission inventory of petroleum transportation fuels.* International Journal of Life Cycle Assessment, 2004. **9**(1): p. 34-44.

51. Wang, M., C. Saricks, and D. Santini, *Effects of fuel ethanol use on fuel-cycle energy and greenhouse gas emissions*, U.S.-Department-of-Energy, Editor. 1999, Argonne National Laboratory: Chicago.

52. Yang, B. and C.E. Wyman, *Effect of xylan and lignin removal by batch and flowthrough pretreatment on the enzymatic digestibility of corn stover cellulose.* Biotechnology and Bioengineering, 2004. **86**(1): p. 88-95.

53. Gilarranz, M.A., et al., *Acid precipitation and purification of wheat straw lignin.* Separation Science and Technology, 1998. **33**(9): p. 1359-1377.

54. Bals, B., et al., *Ammonia fiber expansion treatment of eleven different forages: Improvements to fiber digestibility in vitro.* Animal Feed Science and Technology, 2009. **[Manuscript Submitted]**.

55. Carolan, J.E., S.V. Joshi, and B.E. Dale, *Technical and Financial Feasibility Analysis of Distributed Bioprocessing Using Regional Biomass Pre-Processing Centers.* Journal of Agricultural and Industrial Organization, 2007. **5**: p. 1-27.

56. Ingram, L.O., et al., *Metabolic engineering of bacteria for ethanol production.* Biotechnology and Bioengineering, 1998. **58**(2-3): p. 204-214.

57. Jeffries, T.W. and Y.S. Jin, *Metabolic engineering for improved fermentation of pentoses by yeasts.* Applied Microbiology and Biotechnology, 2004. **63**(5): p. 495-509.

58. Karhumaa, K., et al., *Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant Saccharomyces cerevisiae.* Microbial Cell Factories, 2007. **6**: p. -.

59. Yomano, L.P., S.W. York, and L.O. Ingram, *Isolation and characterization of ethanol-tolerant mutants of Escherichia coli KO11 for fuel ethanol production.* Journal of Industrial Microbiology & Biotechnology, 1998. **20**(2): p. 132-138.

60. Saha, B.C., et al., *Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol.* Process Biochemistry, 2005. **40**(12): p. 3693-3700.

61. Ohgren, K., et al., *Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400.* J Biotechnol, 2006. **126**(4): p. 488-98.

62. Moniruzzaman, M. and L.O. Ingram, *Ethanol production from dilute acid hydrolysate of rice hulls using genetically engineered Escherichia coli.* Biotechnology Letters, 1998. **20**(10): p. 943-947.

63. Deanda, K., et al., *Development of an arabinose-fermenting Zymomonas mobilis strain by metabolic pathway engineering.* Applied and Environmental Microbiology, 1996. **62**(12): p. 4465-70.

64. Ho, N.W., et al., *Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol.* Adv Biochem Eng Biotechnol, 1999. **65**: p. 163-92.

65. Underwood, S.A., et al., *Flux through citrate synthase limits the growth of ethanologenic Escherichia coli KO11 during xylose fermentation.* Applied and Environmental Microbiology, 2002. **68**(3): p. 1071-81.

66. Sedlak, M. and N.W. Ho, *Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant Saccharomyces yeast.* Yeast, 2004. **21**(8): p. 671-84.

67. Pauly, M. and K. Keegstra, *Cell-wall carbohydrates and their modification as a resource for biofuels.* Plant J, 2008. **54**(4): p. 559-68.

68. Lohmeier-Vogel, E.M., C.R. Sopher, and H. Lee, *Intracellular acidification as a mechanism for the inhibition by acid hydrolysis-derived inhibitors of xylose fermentation by yeasts.* Journal of Industrial Microbiology & Biotechnology, 1998. **20**(2): p. 75-81.

69. Chen, J.H. and C.T. Ho, *Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds.* Journal of Agricultural and Food Chemistry, 1997. **45**(7): p. 2374-2378.

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

71. Solaiman, S.G., G.W. Horn, and F.N. Owens, *Ammonium Hydroxide Treatment on Wheat Straw.* J. Anim Sci., 1979. **49**(3): p. 802-808.

72. Kadam, K.L. and M.M. Newman, *Development of a low-cost fermentation medium for ethanol production from biomass.* Applied Microbiology and Biotechnology, 1997. **47**(6): p. 625-629.

73. Ohgren, K., et al., *A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover.* Process Biochemistry, 2007. **42**(5): p. 834-839.

74. Sonderegger, M., et al., *Fermentation performance of engineered and evolved xylose-fermenting Saccharomyces cerevisiae strains.* Biotechnol Bioeng, 2004. **87**(1): p. 90-8.

75. Pampulha, M.E. and M.C. Loureirodias, *Combined Effect of Acetic-Acid, Ph and Ethanol on Intracellular Ph of Fermenting Yeast.* Applied Microbiology and Biotechnology, 1989. **31**(5-6): p. 547-550.

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

77. Bergman, T., M. Carlquist, and H. Jornvall, *Amino Acid Analysis by High Performance Liquid Chromatography of Phenylthiocarbamyl Derivatives* in *Advanced Methods in Protein Microsequence Analysis*, B. Wittmann-Liebold, Editor. 1986, Springer Verlag: Berlin. p. 45-55.

78. Xu, F., et al., *Partition of enzymes between the solvent and insoluble substrate during the hydrolysis of lignocellulose by cellulases.* Journal of Molecular Catalysis B: Enzymatic, 2008. **51**(1-2): p. 42-48.

79. Walker, G.M., *Yeast physiology and biotechnology*. 1998, New York, NY: John Wiley & Sons, Inc. .

80. Saltokoglu, A. and J.C. Slaughter, *The effect of magnesium and calcium on yeast growth.* Journal of the Institute of Brewing, 1983. **89**: p. 81-83.

81. Walker, G.M., *Metals in Yeast Fermentation Processes*, in *Advances in Applied Microbiology*, A.I. Laskin, J.W. Bennet, and G.M. Gadd, Editors. 2004, Elsevier Acadamic Press: New York, NY.

82. Boulton, C. and D. Quain, *Brewing Yeast and Fermentation*. 2001, Ames, Iowa: Iowa State University Press. 638.

83. Chundawat SPS. , et al., *Major cell wall decomposition products produced during ammonia fiber expansion pretreatment of corn stover.* [Manuscript In Preparation], 2009.

84. Aden, A., *Biochemical Production of Ethanol from Corn Stover: 2007 State of Technology Model*. 2008: Golden, Colorado.

85. Mach, R.L. and S. Zeilinger, *Regulation of gene expression in industrial fungi: Trichoderma.* Applied Microbiology and Biotechnology, 2003. **60**(5): p. 515-522.

86. Kelly, J.M. and M.J. Hynes, *Transformation of Aspergillus-Niger by the Amds Gene of Aspergillus-Nidulans.* Embo Journal, 1985. **4**(2): p. 475-479.

87. van den Hondel, C.A., P.J. Punt, and R.F. van Gorcom, *Production of extracellular proteins by the filamentous fungus Aspergillus.* Antonie Van Leeuwenhoek, 1992. **61**(2): p. 153-60.

88. Lee, Y.E., S.E. Lowe, and J.G. Zeikus, *Regulation and Characterization of Xylanolytic Enzymes of Thermoanaerobacterium saccharolyticum B6A-RI.* Applied and Environmental Microbiology, 1993. **59**(3): p. 763-771.

89. Lowe, S.E., M.K. Jain, and J.G. Zeikus, *Biology, ecology, and biotechnological applications of anaerobic bacteria adapted to environmental stresses in temperature, pH, salinity, or substrates.* Microbiol Rev, 1993. **57**(2): p. 451-509.

90. Shaw, A.J., et al., *Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield.* International Sugar Journal, 2009. **111**(1323): p. 164-171.

91. Kwiatkowski, J.R., et al., *Modeling the process and costs of fuel ethanol production by the corn dry-grind process.* Industrial Crops and Products, 2006. **23**(3): p. 288-296.

92. Schubert, C., *Can biofuels finally take center stage?* Nature Biotechnology, 2006. **24**(7): p. 777-784.

93. Spiehs, M.J., M.H. Whitney, and G.C. Shurson, *Nutrient database for distiller's dried grains with solubles produced from new ethanol plants in Minnesota and South Dakota.* Journal of Animal Science, 2002. **80**(10): p. 2639-2645.

94. Ham, G.A., et al., *Wet corn distillers by-products compared with dried corn distillers grains with solubles as a source of protein and energy for ruminants.* Journal of Animal Science, 1994. **72**(12): p. 3246-3257.

95. Mosier, N., et al., *Optimization of pH controlled liquid hot water pretreatment of corn stover.* Bioresource Technology, 2005. **96**(18): p. 1986-1993.

96. Kim, T.H., et al., *Pretreatment of corn stover by low-liquid ammonia recycle percolation process.* Applied Biochemistry and Biotechnology, 2006. **133**(1): p. 41-57.

97. Panagiotou, G. and L. Olsson, *Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates.* Biotechnology and Bioengineering, 2006. **96**(2): p. 250-258.

98. Varga, E., et al., *High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol.* Biotechnology and Bioengineering, 2004. **88**(5): p. 567-574.

99. Berson, R.E., et al., *Detoxification of actual pretreated corn stover hydrolysate using activated carbon powder.* Applied Biochemistry and Biotechnology, 2005. **124**(1-3): p. 923-934.

100. Petersson, A., et al., *A 5-hydroxymethyl furfural reducing enzyme encoded by the Saccharomyces cerevisiae ADH6 gene conveys HMF tolerance.* Yeast, 2006. **23**(6): p. 455-464.

101. Bals, B., B. Dale, and V. Balan, *Enzymatic hydrolysis of distiller's dry grain and solubles (DDGS) using ammonia fiber expansion pretreatment.* Energy & Fuels, 2006. **20**(6): p. 2732-2736.

102. Teymouri, F., et al., *Ammonia fiber explosion treatment of corn stover.* Applied Biochemistry and Biotechnology, 2004. **113-16**: p. 951-963.

103. Stenberg, K., et al., *Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production.* Biotechnology and Bioengineering, 2000. **68**(2): p. 204-210.

104. DeMancilha, I.M. and M.N. Karim, *Evaluation of ion exchange resins for removal of inhibitory compounds from corn stover hydrolyzate for xylitol fermentation.* Biotechnology Progress, 2003. **19**(6): p. 1837 -1841.

105. Nichols, N.N., et al., *Bioabatement to remove inhibitors from biomass-derived sugar hydrolysates.* Applied Biochemistry and Biotechnology, 2005. **121**: p. 379-390.