OPTIMIZATION OF BIOETHANOL FUEL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

by

BAŞAK DAYLAN
BS. in Env.E., Yıldız Technical University, 1998
M.S.in E.Sc., Boğaziçi University, 2007

Submitted to the Institute of Environmental Sciences in partial fulfillment of
the requirements for the degree of
Doctor
of
Philosophy

Boğaziçi University
2016

OPTIMIZATION OF BIOETHANOL FUEL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

APPROVED BY:

Assoc. Prof. Dr. Nilgün CILIZ . . . . . .
(Thesis Supervisor)

Prof. Dr. Ayşen ERDİNÇLER . . . . . .

Assoc. Prof. Dr. Burak DEMİREL . . . . . .

Prof. Dr. Ferhan ÇEÇEN . . . . . .

Prof. Dr. Hasancan OKUTAN . . . . . .

DATE OF APPROVAL: 27/01/2016

ACKNOWLEDGEMENTS

First, I would like to express my sincere appreciation and gratitude to my supervisor Assoc. Prof. Nilgün Cılız, for her continuous support and guidance during my research. Her support and inspiring suggestions have been precious for the development of this thesis content.

I would also express my special thanks to the other jury member of my thesis; Prof. Dr. Ayşen Erdinçler, Assoc. Prof. Burak Demirel, Prof. Dr. Ferhan Çeçen and Prof. Dr. Hasancan Okutan for spending their valuable time to evaluate this thesis.

I would also thank to Prof. Dr. Ayşen Erdinçler, Prof. Dr. Işıl Balcıoğlu and Assoc. Prof. Ulaş Tezel for allowing me to use their laboratory equipment for the experiments and the analysis. A special thank goes to my project colleagues Hacer Yıldırım for her constant support, help and encouragement. She helped me at any time to solve any unsolvable problems. I would also thank to the colleagues from BU-SDCPC; Ece İzbul, Şila Temizel, Fulya Kundaklar and Rana Okur for their help and friendship. I would express my special thanks to Aydın Mammadov who help me whenever I need and to my old roommate Emrah Çoraman for patiently answering all my questions. Special thanks to my ESC family; Binnur Aylin Alagöz, Gamze Sözak, Dr. Ceyda Uyguner Demirel, Gül Geyik, Asu Ziylan Yavaş, Elif Hot, Derya Aydın for their friendship, support and encouragement.

The financial support of this thesis by TÜBİTAK 1001 project (Project No: 110Y261) and BU-SRC (Project No: 10Y00D13, Project No: 13Y00P2 and Project No: 09R103) are gratefully acknowledged.

Last, but not least, I would like to thank my family: to my parents for giving me life in the first place, for educating me with aspects from sciences; to my husband for his unconditional support and encouragement to pursue my interests, for listening to my complaints and frustrations, for believing in me and loving me; to my son who is only 3 years old “yet” for his magic cure to take away my all day tiredness.

OPTIMIZATION OF BIOETHANOL FUEL PRODUCTION FROM LIGNOCELLULOSIC BIOMASS

The aim of this research was to enhance the process of bioethanol fuel production from selected lignocellulosic residues – corn stover and wheat straw. In order to obtain a high conversion yield of sugars, dilute sulfuric acid/steam explosion pretreatment was applied, and the process conditions were optimized. After the optimization studies, the pre-treated residue was subjected to enzymatic hydrolysis using Cellic®CTec2 enzyme in order to decrease the optimum temperature of 50°C while still achieving a high glucan recovery. The sugars released by enzymatic saccharification using the selected enzyme loads of 30 and 45 FPU g/cellulose were fermented by recombinant *S. cerevisiae ATCC® 20618™* and *S. cerevisiae* to increase the ethanol yield. The ethanol yields of different batch reactors were also examined, including "separate hydrolysis and co-fermentation (SHCF)" at 50°C for pre-hydrolysis and at 30°C for fermentation, as well as "simultaneous saccharification and co-fermentation (SSCF)" at 32°C. The results indicated that the selected feedstocks contain fermentable sugars at a level of approximately 46-48%. The optimum conditions for dilute sulfuric acid/steam explosion pretreatment of the selected feedstocks almost doubled the cellulose content, indicating efficient sugar hydrolysis. Xylan loss and concentrations of by-products from the pretreatment hydrolysate were found to be linear with pretreatment severity. The ethanol yield for SSCF was similar to SHCF (Pearson's r= 0.99), demonstrating the applicability of the optimum conditions found in this study.

After the laboratory studies, the environmental and economic performance of bioethanol fuel compared to conventional gasoline (CG) was evaluated using Life Cycle Assessment (LCA) and Environmental Life Cycle Cost (ELCC) Analysis for a 1-km travel distance functional unit (FU). The selected ethanol blends were E10 (10% ethanol/90% gasoline by volume) and E85 (85% ethanol/15% gasoline by volume). The results showed that E10 and E85 fueled vehicles can reduce the global warming potential by 4.7% and 47.1% with respect to CG, respectively. According to the ELCC calculations, E85 provided a 23% lower driving cost compared to CG.

LİGNOSELÜLOZİK BİYOKÜTLEDEN BİYOETANOL YAKITI ÜRETİMİNİN OPTİMİZASYONU

Bu çalışmanın amacı seçilmiş olan lignoselülozik biyokütleden (mısır sapı ve buğday samanı) biyoetanol yakıtı üretim prosesi veriminin arttırılmasıdır. Bu kapsamda en yüksek şeker dönüşüm verimi sağlayan seyreltik asit/buharlı patlama ön arıtma koşulunun tespit edilmesi için optimizasyon çalışması gerçekleştirilmiştir. Ön arıtımı yapılan katı malzemede yüksek glukan verimi sağlayabilmek için, Cellic®CTec2 selülaz enziminin kullanıldığı enzimatik hidroliz prosesinin sıcaklığı 50°C' nin altına düşürülmeye çalışılmıştır. İki farklı enzim dozunda (30 ve 45 FPU g/selüloz) gerçekleştirilen enzimatik sakkarifikasyon işlemi ile elde edilen şekerlerin etanol dönüşüm verimi, rekombinant *S. cerevisiae ATCC® 20618™* ve *S. cerevisiae* mayaları ile değerlendirilmiştir. 32°C' de geçekleştirilen "eş zamanlı sakkarifikasyon ve kofermantasyon" ile 50°C' de ön-hidroliz ve 30°C' deki fermantasyon aşamalarını içeren "ayrı hidroliz ve kofermantasyon" reaktörlerinin etanol verimi karşılaştırılmıştır. Elde edilen sonuçlara göre seçilmiş olan lignoselülozik atıklar %46-48 fermente edilebilir şeker seviyesine sahiptir. Belirlenmiş olan optimum ön arıtma şartlarının uygulandığı hammaddelerin selüloz içeriğinde yaklaşık iki kat artış tespit edilmiştir. Hidroliz sıvısındaki ksilan kayıpları ve yan ürün konsantrasyonlarının, ön arıtma şiddeti ile doğru orantılı olduğu gözlemlenmiştir. Eş zamanlı sakkarifikasyon ve kofermantasyon reaktörünün etanol veriminin, ayrı hidroliz ve kofermantasyon reaktöründe elde edilen verim ile benzer olması (Pearson's r= 0.99), bu çalışmada elde edilen optimum şartların uygulanabilir olduğunu göstermiştir.

Farklı lignoselülozik biyoetanol yakıt karışımlarının (E10; %10 biyoetanol/%90 benzin ve E85; %85 biyoetanol/%15 benzin, hacimsel olarak) çevresel ve ekonomik avantajlarının benzin yaktı ile karşılaştırılması için Yaşam Döngüsü Değerlendirmesi (YDD) ve Çevresel Yaşam Döngüsü Maliyet Analizi (ÇYDM) 1 km' lik taşıma mesafesine göre gerçekleştirilmiştir. Elde edilen sonuçlara göre; E10 ve E85 küresel ısınma potansiyelinde benzine göre sırası ile %4.7 ve %47.1 azalma sağlamıştır. Ayrıca ÇYDM hesaplamaları ile E85 yakıtının sürüş maliyetinin benzine göre %23 daha az olduğu tespit edilmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
SHCF	Separate hydrolysis and co-fermentation	
SSCF	Simultaneous saccharification and co-fermentation	
LCA	Life cycle assessment	
ELCC	Environmental life cycle costing	
FU	Functional unit	
FFV	Flexy-fuel vehicle	
SSF	Simultaneous saccharification and fermentation	
SHF	Separate hydrolysis and fermentation	
MPa	Mega pascal	
HMF	Hydroxymethyl furfural	
FPU	Filter paper unit	
WIS	Water insoluble solids	
LCI	Life cycle inventory	
LCIA	Life cycle impact assessment	
GWP	Global warming potential	
TS	Total solids content	%
ODW_{sample}	Oven dried weight	g
R_{starch}	The starch recovered after analysis	%
HPLC	High Performance Liquid Chromatography	
C_{HPLC}	The concentration of sugars as determined by HPLC	mg/mL
NF	Nitrogen Factor	
TKN	Total Kjeldahl Nitrogen	%
SRS	Sugar recovery standards	
AIR	The percent of acid insoluble residue	%
AIL	Acid insoluble lignin	%
ASL	Acid soluble lignin	%
NREL	National Renewable Energy Laboratory	

R_{sugar}	The recovery percentage of a sugar after acid hydrolysis	%
C_x	The concentration of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis	mg/mL
CSF	Combined severity factor	
$Yield_{WIS}$	Yield of water insoluble solids	%
SAC	Hydrolysis of lignocellulosic biomass	
YP	Yeast peptone	
[EtOH]o	The ethanol concentration at the beginning of the fermentation	g/L
[EtOH]f	The ethanol concentration at the end of the fermentation	g/L
f	Cellulose fraction in dry biomass	g/g
E10	10% bioethanol and 90% gasoline, by volume	
E85	85% bioethanol and 10% gasoline, by volume	
CG	Conventional gasoline	
S1	Feed stock acquisition subsystem	
S2	Bioethanol production subsystem	
S3	Combustion of fuel blends subsystem	
WWTP	Wastewater treatment plant	
EDR	Enzyme dilution ratio	
OD	Optical density	
AP	Acidification	m^2 UES
AEP	Aquatic eutrophication	kg NO_3-eqv
TEP	Terrestrial eutrophication	m^2 UES
GWP	Global warming	kg CO_2-eqv
POP	Photochemical oxidant formation	person*ppm*h
SOP	Stratospheric ozone depletion	kg R11-eqv

1. INTRODUCTION

Lignocellulosic biomass from agricultural residues is the largest source of hexose (C6) and pentose (C5) sugars with a potential for the production of bioethanol fuel. The bioconversion of ethanol from lignocellulosic biomass can be accomplished by pretreatment, enzymatic hydrolysis and fermentation. The feasibility of bioethanol production from agricultural residues depends on a high carbohydrate content of lignocellulosic residues and their availability at low cost (Carrasco et al., 2011).

Lignocellulosic material composed of cellulose (40–50%), hemicelluloses (25–35%) and lignin (15–20%) is extremely resistant to enzymatic digestion. Therefore, a thermochemical pretreatment is usually required to remove hemicellulose and to disrupt the lignin content in order to increase enzymatic digestibility (Kaparaju et al., 2009). There are several methods to enhance the enzymatic reaction, including grinding/milling, dilute acid/steam explosion and hot water pretreatment (Sun and Cheng, 2002). Thermal pretreatment of biomass results in two main portions — the solid fraction, consisting mainly of cellulose (hexose: glucose), and the liquid phase (hydrolysate), consisting mainly of hemicellulose (pentose: xylose and arabinose) (Erdei et al., 2013).

Hexoses can be efficiently converted to bioethanol and the process is carried out with a high yield (around 0.4–0.51 g ethanol/g glucose) by *Saccharomyces cerevisiae. S. cerevisiae* strains (commercial baker's yeast) cannot utilize pentose sugars (Ibeto et al., 2014). Several recombinant yeast and bacteria, such as recombinant *Zymomonas mobilis* and *S.cerevisiae* for pentose sugar fermentation, have been described and presented as the future solution. It has been shown that xylose conversion by simultaneous saccharification and co-fermentation (SSCF) enhanced ethanol yield by as much as 7-8% (Ohgren et al., 2006).

Simultaneous saccharification and fermentation (SSF) process combines enzymatic hydrolysis of cellulose with simultaneous fermentation of the obtained sugars to ethanol. SSF process has shown many advantages compared to separate hydrolysis and fermentation (SHF), such as a decrease in processing time, an increase in ethanol

productivity as a consequence of the fast conversion of glucose to ethanol, and a decrease in enzyme inhibition due the presence of sugars. However, the difference between the optimal temperature for cellulose activity and yeast growth is an issue that needs to be resolved for efficient SSF. The optimal temperature for cellulase enzymes (about 50°C) is higher than the tolerance range of yeast used for bioethanol production (about 30-37°C) (Ruiz et al., 2012). Matching the temperature conditions required for the optimum performance of cellulase enzymes and fermenting microorganisms has been the main focus of recent bioethanol studies.

Life Cycle Assessment (LCA) and Environmental Life Cycle Costing (ELCC) have been widely used as decision-making tools to analyze the environmental and economic benefits of products and processes. Spatari et al. (2010) incorporated LCA into the optimization of processes to establish a link between the environmental and economic impacts of different products. Kim and Dale (2005) studied GHG emissions associated with E10 (10% bioethanol/90% gasoline by volume) and E85 (85% bioethanol/15% gasoline by volume). They concluded that using ethanol (E85) fuel in a midsized passenger vehicle can reduce GHG emissions by 41–61% per kilometer driven, when compared to gasoline-fueled vehicles.

In this study, in order to investigate the efficiency of ethanol production, two different enzymatic saccharification and fermentation applications (SSCF by recombinant *S. cerevisiae ATCC® 20618™* and SSF by *S. cerevisiae* at 32°C) were compared. The effects of selected enzyme loads (30 and 45 FPU g/cellulose) on the hydrolysis rate for pre-treated corn stover and wheat straw were also examined. The overall sugar yield of the selected feedstocks was evaluated prior to fermentation experiments, by optimizing dilute sulfuric acid/steam explosion pretreatment conditions. The ethanol yields of SSCF and separate hydrolysis and co-fermentation (SHCF) reactors were evaluated in order to show the technical viability of the optimized conditions in this study. Additionally, LCA and ELCC were used to assess the sustainability of bioethanol and gasoline fuels by comparing the environmental and economic performance of the selected fuel blends.

2. AIM AND SCOPE OF THE STUDY

Recently, many studies have focused on the feasibility of improving the bioconversion process because of the numerous environmental benefits of biomass-based bioethanol relative to fossil fuels. The objective of this research is to evaluate the efficiency of bioethanol fuel production from corn stover and wheat straw.

The main issue in converting lignocellulosic residues to bioethanol fuel is the accessibility of the polysaccharides for enzymatic breakdown into monosaccharides. Dilute sulfuric acid/steam explosion pretreatment was applied to corn stover and wheat straw. The effect of various combinations of pretreatment conditions on sugar conversion yield was examined. The composition of the selected feedstocks was also determined in order to evaluate pretreatment efficiency. The investigated parameters for optimization of dilute sulfuric acid/steam explosion pretreatment were temperature, acid load and retention time. These three parameters were further used in the calculation of a single combined severity factor, CSF, which is an indicator of the pretreatment severity.

In the next step, enzymatic hydrolysis with Cellic®CTec2 cellulase enzyme was performed to decrease the process temperature from 50°C to 32°C to obtain a high glucan recovery. The optimization of process variables such as temperature, enzyme dosage (30 and 45 FPU g/cellulose) and yeast type (recombinant *S. cerevisiae ATCC® 20618*™ and *S. cerevisiae*) was carried out for improved bioethanol production in SSCF and SSF processes. The ethanol yields of pre-treated corn stover for different batch reactor applications, namely SSCF and SHCF, were compared in order to show the applicability of the optimum process conditions specified in this study.

To prepare an environmental sustainability assessment for lignocellulosic bioethanol fuel, comparative LCA and ELCC studies for conventional gasoline (CG) and bioethanol fuel blends (E10 and E85) were performed.

The outline of this thesis can be defined as follows:

- Physical and chemical characterization of selected feedstocks (wheat straw and corn stover),
- Optimization of pretreatment to find the optimum reaction time, temperature and acid concentration to increase the hydrolysis rate of cellulose and hemicellulose sugars,
- Identification of the optimum enzyme dosage and optimum temperature for enzymatic hydrolysis to increase glucan recovery,
- Identification of an efficient yeast type for the optimized fermentation temperature to increase bioethanol fuel yield,
- Verification of optimized saccharification and fermentation conditions, comparing SSCF and SHFP reactors, and
- Comparison of bioethanol fuel blends (E10, E85) with CG in terms of environmental and economic impacts using LCA and ELCC methodologies.

3. THEORETICAL BACKGROUND

3.1. Bioethanol as an Alternative Fuel

Bioethanol (ethyl alcohol, $CH_3–CH_2–OH$ or ETOH) is a liquid biofuel which can be produced from several biomass feedstocks such as sucrose-containing feedstocks (sugar beet, sweet sorghum and sugar cane), starchy materials (wheat, corn, and barley), and lignocellulosic biomass (wood, cereal straw, corn stover, cotton wastes, pruning and sawmill, grasses). Bioethanol is an oxygenated fuel containing 35% oxygen which provides more efficient combustion, as a result, NO_x, hydrocarbons and particulate emissions are reduced. Since CO_2 released during combustion of bioethanol equals the CO_2 tied up by the plant during photosynthesis, the net CO_2 in the atmosphere also does not increase (Cardona and Sánchez, 2007). Fuel property of bioethanol and gasoline fuels is given in Table 3.1 (Balat, 2011).

Table 3.1. Fuel property of bioethanol and gasoline fuels (Balat, 2011).

Property	Ethanol	Gasoline
Chemical Formula	C_2H_5OH	C_4 to C_{12}
Molecular Weight	46.07	100–105
Carbon content (wt %)	52.2	85–88
Hydrogen content (wt %)	13.1	12–15
Oxygen content (wt %)	34.7	0
Density (g/cm^3 at 15.5°C)	0.79	0.72-0.75
Octane number	108	90–100
Energy Density (MJ/L)	20,000	32,200
High heating value (MJ/kg)	29.8	47

Bioethanol is most commonly blended with gasoline in concentrations of 10% bioethanol to 90% gasoline (by volume), known as E10. This blend requires no engine modification and is covered by vehicle warranties. With engine modification, bioethanol can be used at higher levels, for example, E85.

3.2. Potential of Agricultural Wastes in Turkey

Turkey is a country which has a rich agricultural potential with 23.07 million ha agricultural arable land (Acaroğlu and Aydoğan, 2012). The total annual field crops production and residues in Turkey is represented in Table 3.2. Corn stover (4.1 million tons) and wheat straw (3.5 million tons) have the highest ethanol production yield among the other lignocellulosic residues (Başçetinçelik, 2005). The theoretical bioethanol yield is about 431 L/ton for wheat straw and 427 L/ton dry feedstock for corn stover (Sluiter et al., 2004). According to the theoretical bioethanol yields, the potential bioethanol production from wheat straw and corn stover could be 440 and 863 million liters in Turkey, respectively.

Table 3.2. Field crops and quantity of residues in Turkey (Başçetinçelik, 2005).

Crops	**Residue**	**Total residues (ton)**		**Available residue (ton)**	**Availability (%)**	**Calorific value (MJ/kg)**	**Total calorific value (GJ)**
		Theoretic	**Actual**				
Wheat	Straw	29,170,785	23,429,907	3,514,486	15	17.90	62,909,300
Barley	Straw	9,992,948	8,963,012	1,344,452	15	17.50	23,527,908
Rye	Straw	405,188	358,040	53,706	15	17.50	939,855
Corn	Stalk	5,911,902	4,970,259	2,982,155	60	18.50	55,169,873
	Stover	596,592	1,907,307	1,144,384	60	18.4	21,056,667
Rice	Straw	582,555	209,532	125,719	60	16.70	2,099,510
	Hull	88,527	77,747	62,198	80	12.98	807,327

3.3. Evaluation of Lignocellulosic Feedstock

The general structure of lignocellulosic biomass is given in Figure 3.1 (Ratanakhanokchai et al., 2013). The detailed composition of selected feedstocks is presented in Table 3.3 (Linde et al., 2008; Sassner and Zacchi, 2008). Cellulose $(C_6H_{10}O_5)_x$ is the predominant polymer with the range of ~35–50 wt% of dry wood in lignocellulosic biomass. Hemicellulose $(C_5H_8O_4)_m$ and lignin $[C_9H_{10}O_3.\ (OCH_3)_{0.9\text{-}1.7}]_n$ found in the range of 20-35 wt% and 15-20 wt% of dry wood, respectively.

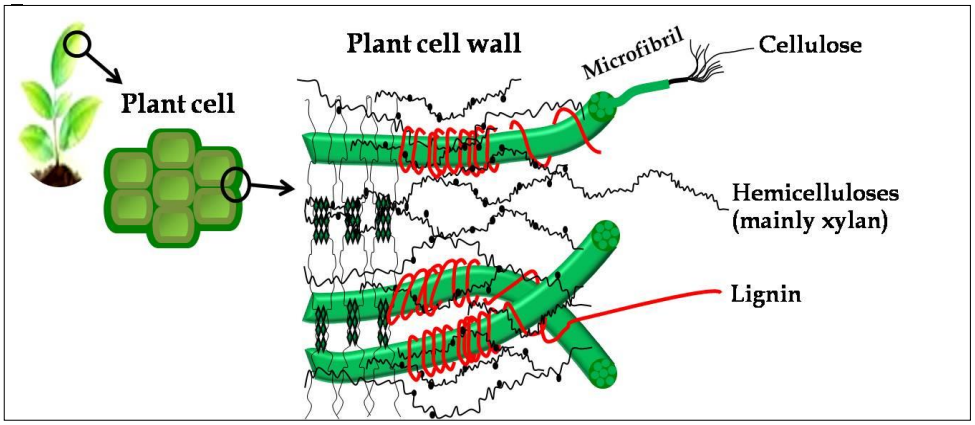


Figure 3.1. General structure of lignocellulosic biomass (Ratanakhanokchai et al., 2013).

Table 3.3. Composition of selected feedstocks (Linde et al., 2008; Sassner and Zacchi, 2008).

Carbohydrates	**Wheat straw (%)**	**Corn stover (%)**
Glucan (C6) (cellulose convertible to sugars)	32.6	40
Hemicellulose convertible to sugars	24.2	28
Xylan (C5)	20.1	21
Galactan (C6)	0.8	2
Arabinan (C5)	3.3	5
Mannan (C6)	0.0	0.0
Protein	3.3	3.8
Ash	4.6	3.5
Lignin	26.5	23

Cellulose is a linear polymer of anhydro D-glucose units linked by β-1,4 glucosidic bonds (Figure 3.2). Cellulose molecules have a strong tendency to form hydrogen bonds. Bundles of cellulose molecules are thus aggregated together in the form of microfibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions. As a consequence of its fibrous structure and strong hydrogen bonds, cellulose is insoluble in most solvents including water (Corredor, 2008; Haghighi et al., 2013). The crystalline cellulose can account for approximately 50–90% of the total cellulose, the remainder being composed of more disorganised amorphous cellulose (Foyle et al., 2007).

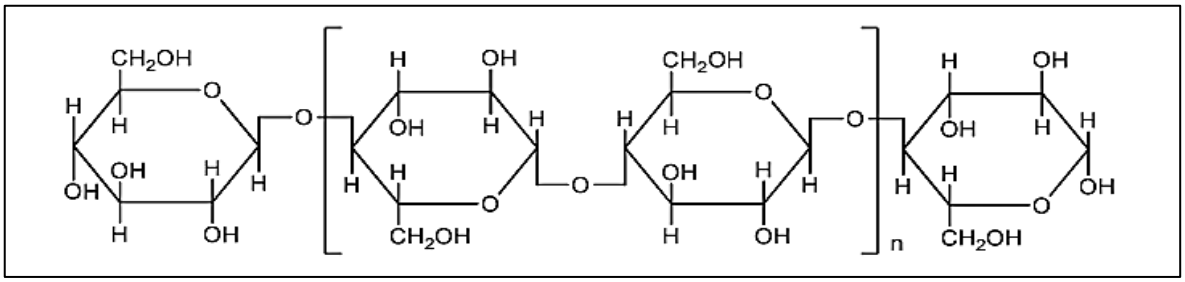


Figure 3.2. The chemical structure of cellulose (Foyle et al., 2007).

Hemicelluloses are heterogeneous polymers of pentoses, hexoses, and sugar acids (Haghighi Mood et al., 2013; Limayem and Ricke, 2012). Hemicellulose, because of its branched, amorphous nature, is relatively easy to hydrolyze (Kim et al., 2008). The hemicellulose forms hydrogen bonds with the cellulose microfibrils, increasing the stability of the cellulose–hemicellulose–lignin matrix. In order to increase the digestibility of cellulose, large amounts of hemicelluloses must be removed as they cover cellulose fibrils limiting their availability for the enzymatic hydrolysis (Haghighi Mood et al., 2013). The chemical structure of hemicellulose is given in Figure 3.3 (Foyle et al., 2007).

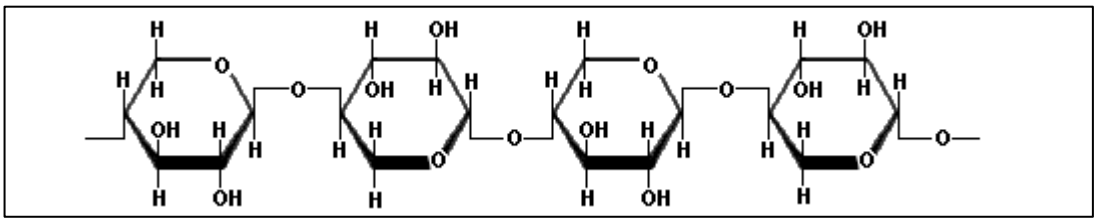


Figure 3.3. The chemical structure of hemicellulose (Weiss et al., 2010).

Lignin effectively protects the plant against microbial attack. Only a few organisms, including rot-fungi and some bacteria can degrade it. Lignin restricts hydrolysis by shielding cellulose surfaces or by adsorbing and inactivating enzymes (Limayem and Ricke, 2012). The close union between lignin and cellulose prevented swelling of the fibers, thereby affecting enzyme accessibility to the cellulose. Any ethanol production process will have lignin as a residue since it is presented in all lignocellulosic biomass. Such by-product can be burned to provide heat and electricity, or used to manufacture various polymeric materials (Ohgren et al., 2007). The chemical structure of lignin is shown in Figure 3.4 (Foyle et al., 2007).

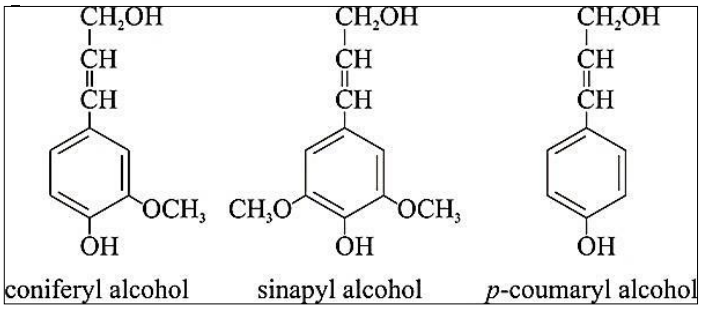
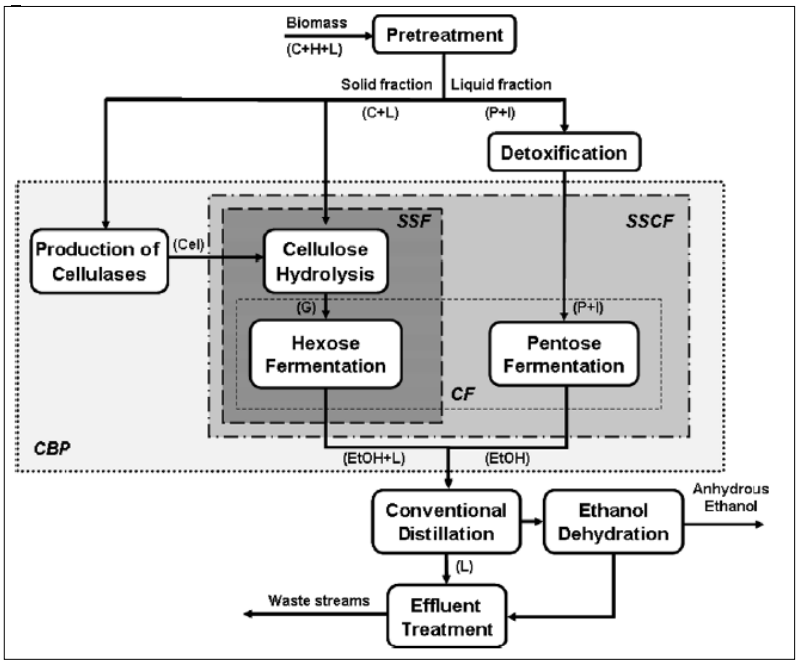


Figure 3.4. The chemical structure of lignin (Foyle et al., 2010).

3.4. Biochemical Conversion of Cellulosic Feedstocks into Bioethanol

Processing of lignocellulosic to ethanol consists of pretreatment, hydrolysis, fermentation, and product separation (Figure 3.5) (Cardona and Sánchez, 2007). Cellulose (C) can be broken down by hydrolysis into glucose (G) either enzymatically by cellulases or chemically by sulfuric or other acids. While hexoses can be fermented readily to ethanol by many naturally occurring organisms, pentoses (P) are fermented to ethanol by only a few native strains, and usually at relatively low yields. Ethanol fermentation can be carried out by three main processes: simultaneous saccharification and co-fermentation (SSCF), simultaneous saccharification and fermentation (SSF) and/or separate hydrolysis and fermentation (SHF) (Limayem and Ricke, 2012). Ethanol is recovered from the fermentation broth by distillation. The residual material containing lignin (L), unreacted cellulose and hemicellulose (H), ash, enzyme, microorganism and other components accumulates in the bottom of the distillation column. These materials may be burned as fuel to power the process, or converted to various co-products (Mosier et al., 2005). The design of cost-effective processes for fuel ethanol production implies the selection of the most appropriate feedstocks, and the definition of a suitable process configuration. Thus, the conversion of raw materials into the end product meeting given specifications can be achieved (Chen et al., 2009).



CBP, consolidated bioprocessing; I, inhibitors

Figure 3.5. Generic block diagram of fuel ethanol production from lignocellulosic biomass (Cardona and Sánchez, 2007).

3.5. Pretreatment of Lignocellulosic Biomass

The most important challenge in production of biofuel is pretreatment of biomass which makes the remaining solid biomass more accessible to further chemical or biological treatment. Utilization of cellulose in native form, not only consumes large amount of enzyme but also results in low enzymatic digestibility yield of cellulose (<20%). The main goal of pretreatment is to break the lignin seal and disrupt the crystalline structure of cellulose which provide more reachable surface area for cellulose enzymes to react with cellulose (Figure 3.6) (Haghighi et al., 2013; Mosier et al., 2005). Therefore, pretreatment of lignocellulosic material is required and recognition of the main structural limiting factors is a critical step. These factors include (1) specific surface area, (2) cellulose crystallinity, (3) degree of polymerization, (4) cellulose sheathing by hemicelluloses and (5) lignin content (Haghighi et al., 2013). The other goals of an effective pretreatment process are (i) to increase sugar conversion yield by hydrolysis (ii) to avoid loss and/ or degradation of sugars formed (iii) to limit formation of inhibitory products (iv) to reduce energy demands and (v) to minimize costs (Sarkar et al., 2012).

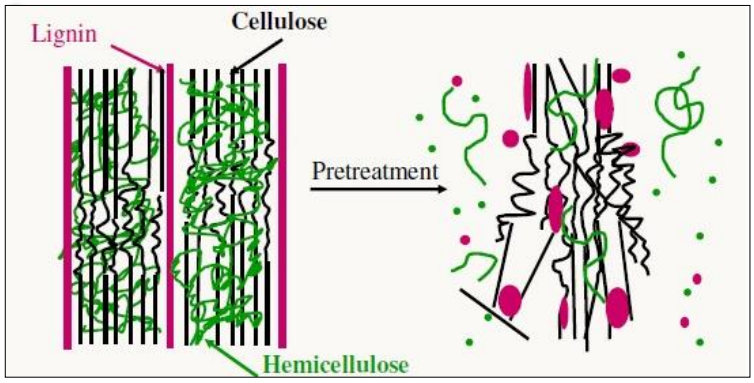


Figure 3.6. Schematic illustration of pretreatment for lignocellulosic biomass (Haghighi et al., 2013).

3.5.1. Physical Pretreatment

3.5.1.1. Mechanical size reduction. The first step for bioethanol production from agricultural wastes is comminution through chipping, grinding and milling to reduce cellulose crystallinity. The size of the materials is usually 10–30 mm after chipping and 0.2–2 mm after milling or grinding (Kazi et al., 2010; Sarkar et al., 2012). Bridgeman et al. (2007) studied the effects of size reduction of switchgrass achieved via ball milling. The study reported that for particle sizes smaller than 90 μm, cellulose content was 13.4% lower than for larger sized particles. The losses in lignin and hemicellulose were 3.43% and 4.74%, respectively. These results indicate that extensive size reduction is undesirable as it causes significant carbohydrate losses which ultimately results in less reducing sugars and reduced ethanol yield (Keshwani and Cheng, 2009).

3.5.2. Physico-chemical Pretreatment

3.5.2.1. Dilute acid-steam explosion. Steam explosion is conducted at temperatures of 160–260°C (corresponding pressure 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process provides hemicellulose degradation and lignin transformation due to high temperature, thus; the potential of cellulose hydrolysis is increased (Sarkar et al., 2012). Acetic acid is generated from hydrolysis of acetyl groups associated with the hemicellulose. It is further used to catalyze hydrolysis of glucose or xylose (Mosier et al., 2005). The steam pretreatment with the addition of an acid catalyst such as H_2SO_4 or SO_2 is a prerequisite to reach high sugar

yields (Balat and Balat, 2008). Mosier et. al. (2005) reported higher hydrolysis yield from lignocellulose pre-treated with diluted H_2SO_4 compared to other acids. A saccharification yield of 74% was obtained from wheat straw when pretreatment conditions of 0.75% v/v of H_2SO_4 at 121 ºC for 1 h were applied. The process conditions for acid catalyzed steam pretreatment are given in Table 3.4.

Table 3.4. Acid catalyzed steam pretreatment conditions (Balat and Balat, 2008).

Two-step pretreatments		**One-step pretreatments**
First step	**Second step**	
180ºC, 10 min, H_2SO_4(0.5%)	200ºC, 2 min, H_2SO_4 (2%)	225ºC, 5 min, H_2SO_4 (0.5%)
190ºC, 2 min, SO_2 (3%)	220ºC, 5 min, SO_2 (3%)	210ºC, 5.5 min, SO_2 (3.5%)

The important parameters for steam explosion pretreatment are residence time, temperature, chip size and moisture content. Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short residence time (270°C, 1 min) or lower temperature and longer residence time (190°C, 10 min). Recent studies shows that lower temperature and longer residence time are more favorable (Sun and Cheng, 2002). Steam pretreatment of corn stover at 190°C for 5 min with SO_2 as acid catalyst has resulted in high sugar yields (almost 90% overall glucose yield and almost 80% overall xylose yield) after 72 h enzymatic hydrolysis (Sassner and Zacchi, 2008). SO_2-impregnated corn stover treated by steam (200°C, 5min) resulted in 80.2% overall ethanol yield based on the glucose content in the raw material (Ohgren et al., 2007).

3.5.3. Formation of Inhibitors and Detoxification of Lignocellulosic Hydrolyzates

Phenolic compounds from lignin degradation, furan derivatives (furfural and 5-hydroxymethyl furfural (HMF) from sugar degradation, and acetic acid, formic acid and levulinic acid are considered as fermentation inhibitors generated from pre-treated lignocellulosics due to high temperature or prolonged pretreatment time (Limayem and Ricke, 2012). Degradation products of hemicellulose are xylose, mannose, acetic acid, galactose and glucose. Cellulose is hydrolyzed to glucose (Figure 3.7) (Corredor, 2008). Xylose is further degraded to furfural at high temperature. Similarly, 5-HMF is formed from hexose degradation. Formic acid is formed when furfural and HMF are broken down.

Levulinic acid is formed by HMF degradation. Phenolic compounds are generated from partial breakdown of lignin (Corredor, 2008). Production of these compounds increases when hydrolysis takes place at severe conditions such as higher temperatures and higher acid concentrations. Inhibitors increase the environmental stress for the fermentative organism due to decreased water activity and increasing ethanol concentrations. Microorganisms can survive stress up to a certain limit, but cell death would occur if the stress exceeds the limit that cell can bear (Olofsson et al., 2010). Depending on the type of employed pretreatment and hydrolysis, detoxification of streams is required. Detoxification methods can be physical, chemical or biological. Alkali treatment is considered one of the best detoxification methods. In this method, furaldehydes and phenolic compounds are mainly removed leading to great improvement in fermentability, especially in the case of dilute-acid hydrolyzates. Treatment with calcium hydroxide (overliming) or ammonia has shown better results than treatment with sodium or potassium hydroxide (Binod et al., 2010).

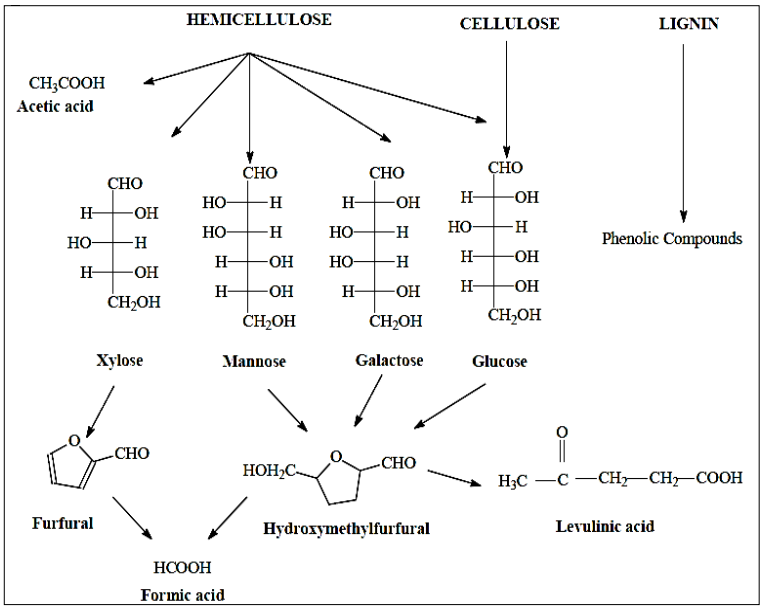


Figure 3.7. Hydrolysis reactions of lignocellulosic biomass (Corredor, 2008).

3.6. Hydrolysis

The success of the hydrolysis step is essential to the effectiveness of a pretreatment operation. During this reaction, the released polymer sugars, cellulose and hemicellulose are hydrolyzed into free monomer molecules readily available for fermentation conversion

to bioethanol (Limayem and Ricke, 2012). As the pretreatment is finished, the cellulose is prepared for hydrolysis, meaning the cleaving of a molecule by adding a water molecule:

$$(C_6H_{10}O_5)_n + nH_2O \longrightarrow nC_6H_{12}O_6 \quad (2.1)$$

This reaction is catalyzed by dilute acid, concentrated acid or enzymes (cellulase). Hydrolysis without conducting pretreatment process yields typically 20% of sugar, whereas sugar yields after pretreatment often exceed 90% (Balat and Balat, 2008). Enzymatic hydrolysis consists of cleaving the polymers of cellulose and hemicellulose using enzymes called cellulases. The main hydrolysis product of cellulose is glucose, whereas the hemicellulose gives rise to several pentoses and hexoses. Cellulases (EC 3.2.1.4) from *Trichoderma reesei* are mostly used, as several mutant strains of this fungus produce high levels of extracellular cellulolytic enzymes, up to 40 g/L (Tabka et al., 2006).

The factors that affect the enzymatic hydrolysis of cellulose include substrates, cellulase activity, and reaction conditions (temperature and pH). At low substrate levels, an increase of substrate concentration normally results in an increase of the yield and reaction rate of the hydrolysis (Hsu et al., 2011). However, high substrate concentration can cause substrate inhibition, which substantially lowers the rate of hydrolysis. The extent of substrate inhibition depends on the ratio of total substrate to total enzyme (Sun and Cheng, 2002). Cellulase enzyme loadings in hydrolysis vary from 7 to 45 FPU/g substrate (FPU, filter paper unit, defined as a micromole of reducing sugar as glucose produced by 1 ml of enzyme per minute), depending on the type and concentration of substrates (Sun and Cheng, 2002).

3.7. Fermentation of Lignocellulosic Biomass

For a viable ethanol production, an ideal microorganism should have broad substrate utilization, high ethanol yield and productivity. It also should have the ability to withstand high concentrations of ethanol and high temperature. In addition, it should be tolerant to inhibitors present in hydrolysate and have cellulolytic activity. Although traditional *Saccharomyces cerevisiae* and *Zymomonas mobilis* can ferment glucose to ethanol rapidly and efficiently, they cannot ferment other sugars such as xylose and arabinose to ethanol

(Keshwani and Cheng, 2009). According to the reactions shown in Equation 2.2 and 2.3, the theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide per kg of xylose and glucose (Balat and Balat, 2008):

$$3C_5H_{10}O_5 \longrightarrow 5C_2H_5OH + 5CO_2 \quad (2.2)$$

xylose ethanol

$$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2 \quad (2.3)$$

glucose ethanol

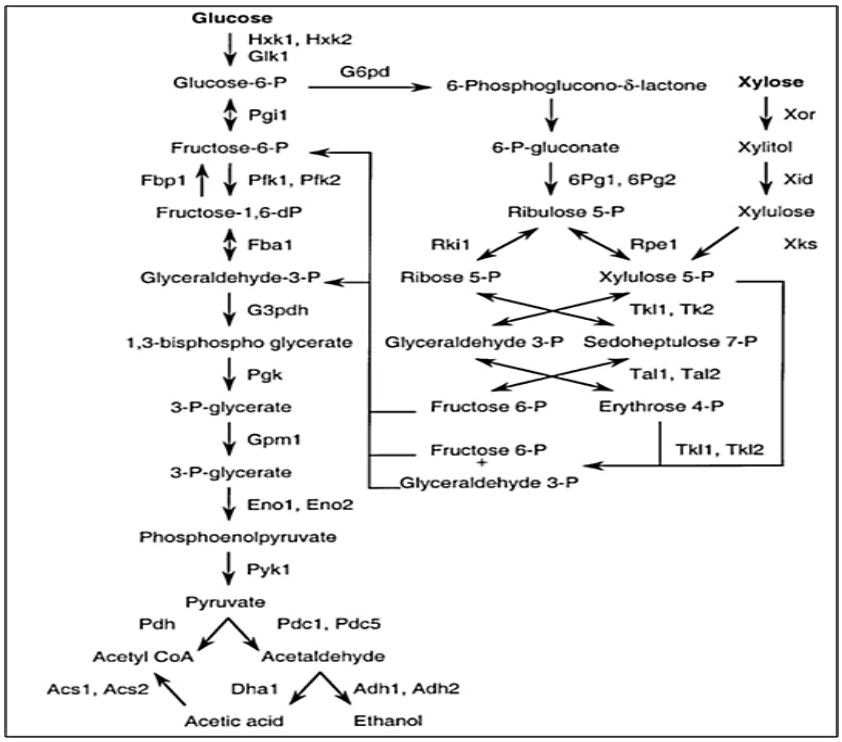


Figure 3.8. Schematic illustration of bioethanol production from glucose and xylose (Binod et al., 2010).

The schematic illustration of bioethanol production from glucose and xylose is given in Figure 3.8 (Balat and Balat, 2008). In glycolysis, glucose is broken down into two three-carbon pyruvate molecules, yielding two molecules of ATP per glucose. For each pyruvate, 2 ATP molecules are formed and 1 electron is harvested as NADH. The net outputs are 2 molecules of ATP and NADH per glucose. In fermentation, pyruvic acid is converted first to acetaldehyde and then to ethanol. Again, the NADH is converted back to NAD^+ in this process (Paulová et al., 2014).

3.7.1. Simultaneous Saccharification and Fermentation (SSF)

Due to the relatively similar process conditions in enzymatic hydrolysis and ethanol fermentation, these two steps are widely carried out together in SSF (Foyle et al., 2007). Simultaneous saccharification (hydrolysis) of cellulose and hemicellulose and fermentation of sugars to ethanol improves the kinetics and economics of biomass conversion by a) decreasing accumulation of hydrolysis products that are inhibitory to enzymes, b) decreasing contamination risk because of the presence of ethanol, and c) decreasing capital equipment investment. An important drawback of SSF is that the reaction has to operate at a compromised temperature around 30°C, instead of the enzyme optimum temperature of 45-50°C (Matsushika et al., 2009). In the SSF, the concentration of monomeric sugars (soluble monosaccharides- arabinose, galactose, glucose, xylose, mannose) is constantly kept low since the microorganism ferments them to ethanol as soon as they are liberated from the polymers. Ethanol has also a noticeable inhibitory effect on *S. cerevisiae* at concentrations above 15 g/L. However, the ethanol producing capability is not completely inhibited until the ethanol concentration reaches 105 g/L (Ohgren et al., 2007).

The microorganisms used in the SSF are usually the fungus *T. reesei (*for enzymatic hydrolysis) and yeast *S. cerevisiae* (for fermentation). The optimal temperature for SSF is around 38°C, which is an average between the optimal temperatures for hydrolysis (45–50°C) and fermentation (30°C) (Sun and Cheng, 2002). Hydrolysis is usually the rate-limiting process in SSF. Ohgren et al. (2007) found that longer prehydrolysis time resulted in a larger decrease in overall ethanol yield than shorter prehydrolysis. The highest ethanol concentration, 33.8 g/L, was reached in the SSF with 11.5% water insoluble solid (WIS) using the developmental thermoactive cellulase complex, and 1.8 g/L compressed baker's yeast. This concentration corresponded to 80.2% overall ethanol yield based on the glucose content in the raw material. However, if the xylose present in the beer at the end of the SSF could be fermented to ethanol, another 12.6 g ethanol/L could theoretically be produced (0.51 g ethanol/g xylose).

Xu et al. (2009) examined different pretreatments with and without addition of low concentration organic acids for corn stover at 195°C for 15 min. The highest xylan recovery of 81.08% was obtained after pretreatment without acid catalyst and the lowest of

58.78% after pretreatment with both acetic and lactic acid. Glucan recovery was less sensitive to the pretreatment conditions than xylan recovery. SSF of WIS showed that a high ethanol yield of 88.7% of the theoretical based on glucose in the raw material was obtained following pretreatment at 195°C for 15 min with acetic acid employed.

Hydrothermal pretreatment on corn stover was investigated at 195°C for different times varying between 10 min and 30 min (Xu et al., 2013). As the pretreatment time increased from 10 min to 30 min, the xylan recovery from liquid phase changed between 39.5% and 45.6% and the total xylan recoveries decreased from 84.7% to 61.6%, while the glucan recovery seemed not to depend on pretreatment time. The glucan recovered from liquid was increased from 4.9% to 5.6% and the total glucan recoveries from all the pretreatments were higher than 98%. HMF, furfural, acetic, lactic, formic acid were also found in the liquid phase. Concentrations of all potential inhibitors were low enough so that they did not affect the activity of *S. cerevisia.* The best pretreatment condition was 195°C for 15 min. The estimated total ethanol production was 201 g/kg corn stover by assuming the fermentation of both C-6 (glucan, galactan and mannan) and C-5 (xylan and arabinan).

Ohgren et al. (2006) investigated ethanol production by *TMB3400* compared with baker's yeast (*S. cerevisia.*) in batch SSF of whole pre-treated corn stover slurry at 0.05 g WIS/g SSF slurry. The co-fermentation of glucose and xylose by *TMB3400* increased the overall ethanol yield from 52 to 64% of theoretical in batch 0.05 WIS. The ethanol yield based on xylose in SSF with *TMB3400* was estimated to 0.39 g/g.

3.7.2. Simultaneous Saccharification and Co-Fermentation

One of the main problems in bioethanol production from lignocellulosics is that *S. cerevisiae* can ferment only certain mono and disaccharides like glucose, fructose, maltose and sucrose. Pentoses obtained during hemicellulose hydrolysis (mainly xylose) cannot be assimilated by this yeast (Cardona and Sánchez, 2007). Yeasts as *Pichia stipitis, Candida shehatae and Pachysolen tannophilus* can assimilate pentoses. However, their ethanol production rate from glucose is at least five times less than that observed for *S. cerevisiae*.

Moreover, their culture requires oxygen and ethanol tolerance is 2-4 times lower (Claassen et al., 1999).

Pentose fermenting yeasts require a careful control for maintaining low oxygen levels in the culture medium needed for their oxidative metabolism. For pentose utilizing microorganisms, if fermentation time is not sufficiently long, pentoses remain in the medium decreasing the utilization rates of the lignocellulosic complex. As a rule, microorganisms prefer glucose over galactose followed by xylose and arabinose (Gong et al., 1999). *Escherichia Coli (E. Coli*) was genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase (adhB) and pyruvate decarboxylase (pdc) from the bacterium *Zymomonas mobilis*. Hemicellulose hydrolyzates of agricultural residues such as corn stover, and corn hulls plus fibers were fermented to ethanol by recombinant *E. Coli strain KO11*. Fermentations were complete within 48 h, achieving 40 g ethanol/L, ethanol yields ranging from 86 to 100% of the maximum theoretical yield (Asghari et al., 1996).

3.8. Distillation

Bioethanol obtained from a fermentation conversion requires further separation and purification of ethanol from water through distillation. Because the boiling point of water (100ºC) is higher than the ethanol-boiling point (78.3ºC), ethanol will be converted to steam before water. Thus, water can be separated via a condensation procedure and ethanol distillate recaptured at a concentration of 95%. Liquid mixtures are heated and allowed to flow continuously along the column. At the top of the column, volatiles are separated as a distillate and residue is recovered at the bottom of the column (Limayem and Ricke, 2012).

3.9. Life Cycle Assessment as a Decision-Making Tool

3.9.1. Methodology of Life Cycle Assessment

The ISO 14040 has defined LCA as a technique for assessing the environmental aspects associated with a product by a) compiling an inventory of relevant inputs and outputs of a product system, b) evaluating the potential environmental impacts associated

with those inputs and outputs and, c) interpreting the results of the inventory analysis and impact assessment phases.

The results of an LCA quantify the potential environmental impacts of a product system over the life cycle, help to identify opportunities for improvement and indicate more sustainable options where a comparison is made. The phases of LCA defined by ISO 14040 standards are a) goal and scope definition, b) inventory analysis, c) impact assessment and d) interpretation (Jensen and Thyø, 2007).

In LCA studies, all relevant inputs and outputs in Life Cycle Inventory (LCI) phase and final impact scores in Life Cycle Impact Assessment (LCIA) phase are expressed with a reference flow, which is called the functional unit (FU) (Malça and Freire, 2011). In the case of motor fuels, the functional unit can be based on mass such as kg of ethanol (Kim and Dale, 2005) or on driven distance e.g. km (González-García et al., 2009; Kim and Dale, 2006; Luo et al., 2009). The results of the studies showed that the selection of the FU considerably affects the final conclusions. In all cases, shifting from gasoline to bioethanol presents environmental advantages in terms of GHG emissions and use of non-renewable energy sources and, environmental disadvantages in terms of acidification, eutrophication and photochemical ozone depletion emissions.

González-García et al. (2009) analyzed the environmental performance of two ethanol-based fuel applications (E10 and E85) produced from *Brassica carinata*. E85 seems to be the best alternative when ethanol production based functional unit is considered in terms of GHG emissions and E10 in terms of non-renewable energy resources use. Nevertheless, E85 offers the best environmental performance when travelling distance oriented functional unit is assumed in both impacts.

Luo et al. (2009) studied the costs for sugarcane based bioethanol and gasoline fuels. Based on the cost data in 2005, the gasoline production cost was assumed as 0.59 $/kg. Ethanol production cost was calculated as 0.26 $/kg in the future case. The results indicate that driving with ethanol fuels is more economical than gasoline, due to the low production cost of ethanol from sugarcane.

4. MAIN STEPS OF THE STUDY

The laboratory studies were conducted within the scope of TÜBİTAK 1001 project (Project No: 110Y261) and Boğaziçi University Scientific Research Project (BU-SRP) (Project No: 10Y00D13). The lignin residue obtained from fermentation in this study was used in production of activated carbon and the process conditions were optimized (Part IV) (TÜBİTAK 1001, Project No: 110Y261 and BU-SRP, Project No: 13Y00P2). This part was not included in this study. LCA and ELCC studies were conducted within the scope of TÜBİTAK 1001, Project No: 110Y261 and BU-SRP (Project No: 09R103). The main stages of the projects are shown in Table 4.1.

Table 4.1. The main stages of the projects.

PART I **Characterization of Feedstock** TÜBİTAK 1001 (Project No: 110Y261)	**PART II** **Optimization of Dilute Sulfuric Acid/Steam Explosion Pretreatment** TÜBİTAK 1001 (Project No: 110Y261)	**PART III** **Optimization of Fermentation** TÜBİTAK 1001 (Project No: 110Y261) BU-SRP (Project No: 10Y00D13)	***PART IV*** ***Activated carbon production from lignin-rich residue*** TÜBİTAK 1001 (Project No: 110Y261) BU-SRP (Project No: 13Y00P2)	**PART V** **LCA and ELCC analysis** TÜBİTAK 1001 (Project No: 110Y261) BU-SRP (Project No: 09R103)
• Determination of total solids, ash, extractives, starch and protein • Determination of lignin • Determination of structural carbohydrate	• Feedstock substrate and chemical preparation • High pressure/Parr reactor applications in different operational conditions • Measurements of hydrolysate and solid part of pre-treated biomass	• Optimization of enzymatic hydrolysis conditions • Optimization of SSF and SSCF conditions • Evaluation of the optimized conditions by SSCF and SHCF reactors	• *Recovery of lignin-rich residues* • *Optimization of chemical activation process* • *Optimization of carbonization process* • *Characterization of produced activated carbons*	• Life cycle inventory analysis • LCA and ELCC through GaBi Software • Life cycle impact assessment

5. MATERIALS AND METHODS

5.1. Optimization of Bioethanol Production

5.1.1. Biomass

During this thesis, wheat straw and corn stover have been selected to evaluate the biomass conversion yield to bioethanol fuel, as they are existing biomass resources with annual production rates in Turkey of almost 3.5 and 4.1 million tons, respectively.

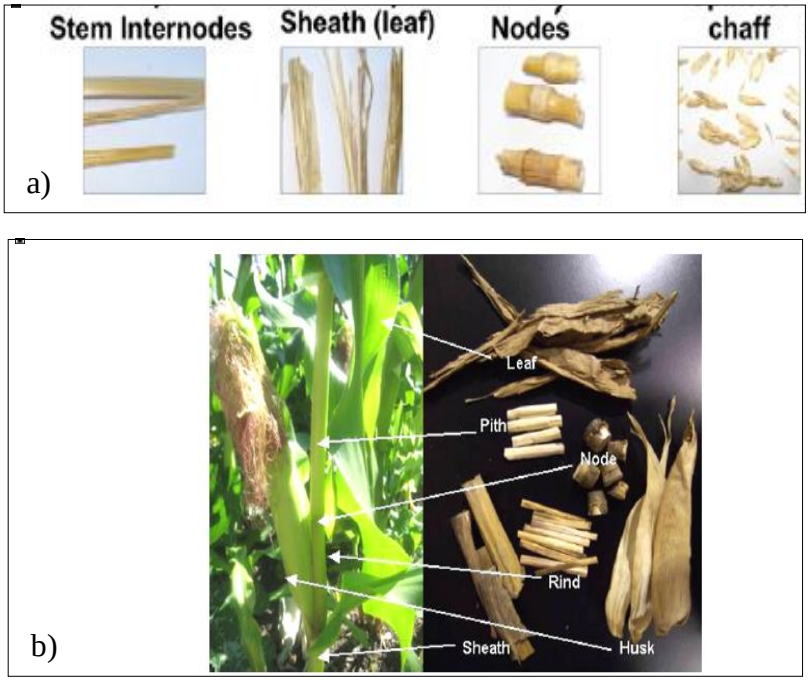


Figure 5.1. Botanical fractions of (a) wheat straw and (b) corn stover (Liu et al., 2010; Silva and Rouau, 2011).

The botanical fractions of wheat straw and corn stover are shown in Figure 5.1 (Liu et al., 2010; Silva and Rouau, 2011). Wheat straw samples were obtained from the Şile district of Istanbul, located in the Marmara region of Turkey in the year 2012. Homogenized wheat straw samples were obtained using fractions of internodes, sheath (leaves) and nodes. Corn stover samples were collected from the city of Ordu, located in

the Black Sea region of Turkey. These samples were also collected in 2012 and composed of botanical fractions of leaves, husk, nodes, sheath, rind and pith.

5.1.2. Characterization of Feedstocks

During characterization of feedstocks, the National Renewable Energy Laboratory (NREL) procedures for biomass analysis were used. The main stages applied for the physical and chemical characterization of lignocellulosic biomass samples are given in Figure 5.2.

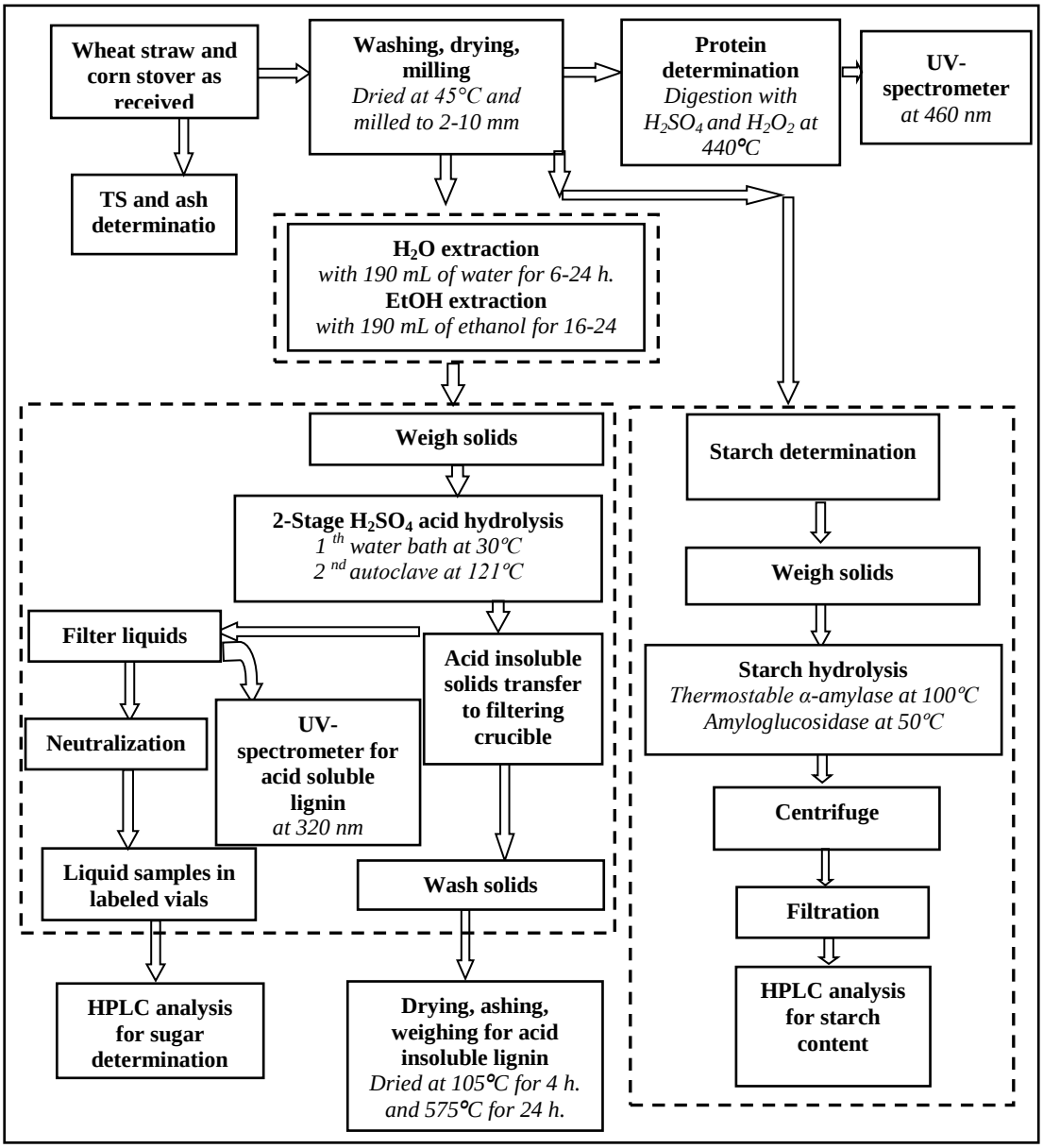


Figure 5.2. Schematic of physical and chemical characterization of lignocellulosic samples.

5.1.2.1. Preparation of samples for compositional analysis. Preparation of biomass sample was carried out according to the NREL/TP-510-2620 procedure (Preparation of samples for compositional analysis). The biomass sample was dried in an oven (Nüve FN500) at 45±3°C for 24 to 48 h and cooled to room temperature. This drying method is suitable for small samples of biomass (<20 g) (Hames et al., 2008). After drying, the material was milled by a laboratory knife-mill. The milling was carried out in a laboratory cutting mill (Tetra Kimya A.Ş) equipped with a sieve with an equivalent pore size of 2-10 mm. The milled samples were stored inside sealed plastic bags and placed into the refrigerator, at 4ºC for short term application. The (a) wheat straw and (b) corn stover samples before and after milling are shown in Figure 5.3.

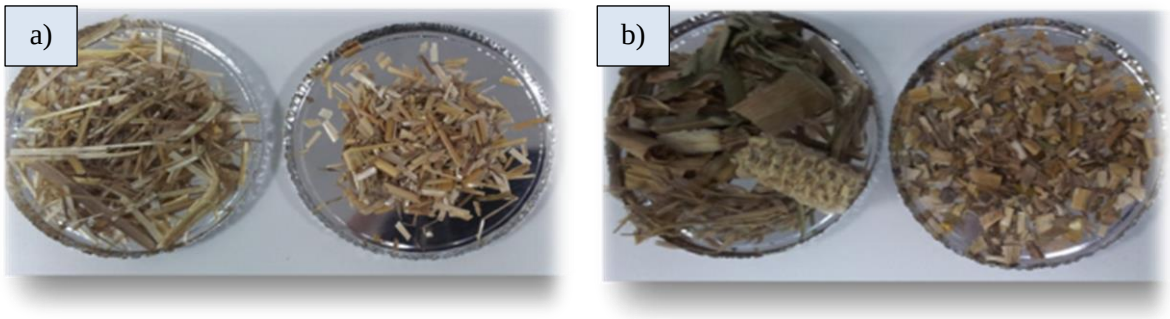


Figure 5.3. (a) Wheat straw and (b) corn stover samples before and after milling.

5.1.2.2. Determination of total solid and ash contents. The total solid (TS) content was determined by the NREL/TP-510-42621 procedure (Determination of total solids in biomass and total dissolved solids in liquid process samples) (Sluiter et al., 2008). All TS determinations were done in triplicate. The reported results were averages of those values. The TS was calculated using Equation 4.1:

$$\%TS = \left(\frac{W_{\text{dry sample+crucible}} - W_{\text{dry crucible}}}{W_{\text{sample as recieved}}}\right) * 100 \qquad (4.1)$$

where; TS is the total solids content, (%); $W_{\text{sample as received}}$ is the weight of sample as received, (g); $W_{\text{dry sample+crucible}}$ is the weight of dry sample and crucible, (g) and $W_{\text{dry crucible}}$ is the weight of dry crucible, (g).

The ash content was determined according to the NREL/TP-510-42622 procedure (Determination of ash in biomass). The ash content was calculated by Equations 4.2 and 4.3:

$$ODW_{sample} = \left(\frac{W_{sample\ as\ recieved} * \%TS}{100}\right) \quad (4.2)$$

$$\%\ Ash = \left(\frac{W_{\ dry\ sample+crucible} - W_{dry\ crucible}}{ODW_{sample}}\right) * 100 \quad (4.3)$$

where; ODW_{sample} is the oven dried weight of sample, (g) and %Ash is the percent ash content of the sample.

5.1.2.3. Determination of extractives. The samples were analyzed for water and ethanol extractives according to the NREL/TP-510-42619 procedure (Determination of extractives in biomass) (Sluiter et al., 2008b). It is necessary to remove non-structural material from biomass prior to structural components analysis to prevent interference with later analytical steps (Sluiter et al., 2010). Water soluble materials may include inorganic material (soil or fertilizer), non-structural sugars, and nitrogenous material, etc. Ethanol soluble material includes chlorophyll, waxes, or other minor components (Sluiter et al., 2004).

During experiments, batches of five biomass samples (approximately 2-10 g wheat straw and corn stover) were sequentially extracted with water and ethanol using a soxhlet extraction system. The water extraction was carried out first, by addition of 190 mL of HPLC-grade water into the receiving flask. The extraction process was run for about 6-24 hours, adjusting the heating mantles to assure that the liquid inside the Soxhlet tube refluxed 4-5 times per hour. Before stopping the extraction, it was verified that the water inside the Soxhlet tube looked completely clear. The receiving flasks containing the water residue were then removed and replaced by similar flasks containing 190 mL of ethanol. This extraction was carried around 16-24 hours, also having 6-10 refluxes per hour. Likewise, the extraction was only stopped after the liquid present in the Soxhlet tube looked clear.

When reflux time was completed, the extracted solids were transferred onto cellulose filter paper in a Buchner funnel. The solids were washed with approximately 100 mL of fresh 190 proof ethanol, filtered using vacuum filtration, air dried and weighed. The water and ethanol extraction residues had their respective solvents evaporated by placing them in a Heidolph Laborata 4000-Efficient Rotary Evaporator equipped with a water bath at 60ºC, in case of water, and at 40ºC, in case of ethanol. The solvent-free solids were then placed in an oven at 40±2 °C for 24 hours, cooled to room temperature in a desiccator, and weighed to the nearest 0.1 mg. The extractive content (in a dry weight basis) was calculated according to Equation 4.5 and 4.6:

$$ODW_{sample} = \frac{(W_{thimble+sample} - W_{thimble}) * \%TS}{100} \quad (4.5)$$

$$\%\ Extractives = \frac{(W_{flask+extarctives} - W_{flask})}{ODW_{sample}} * 100 \quad (4.6)$$

where; $W_{thimble+sample}$ is the weight of dry sample and thimble, (g); $W_{thimble}$ is the weight of dry thimble, (g); $W_{flask+extractives}$ is the weight of dry flask and extractives, (g) and W_{flask} is the weight of dry flask, (g).

5.1.2.4. Determination of starch in solid biomass samples. The samples were analyzed for starch content according to the NREL/TP-510-42624 procedure (Determination of starch in solid biomass samples) (Sluiter and Sluiter, 2008). 100±10 mg of sample and starch sample of known purity were added to a tared 15 mL plastic centrifuge tube with a tightly fitting screw cap. 0.2 mL of ethanol and 2 mL of dimethyl solfoxide (DMSO) were added and vortex mixing was applied vigorously. The tubes were placed in a boiling water bath for five minutes. 2.9 mL MOPS buffer and 0.1 mL thermostable α-amylase were added and mixed. The tubes were incubated in a boiling water bath (Selecta Precisdig 12 L) for 6 minutes and then, placed in a water bath at 50ºC with addition of 4 mL of the sodium acetate buffer and 0.1 mL (20 units) amyloglucosidase. The tubes were mixed and incubated for 30 minutes at 50ºC. The samples were removed from the water bath and centrifuged by a benchtop centrifuge (Centurion 1020 D) for 10 minutes at 3,000 rpm. The sample has been capped tightly throughout the analysis, the solvent volume was constant,

and the volume of the solvent was assumed to be 9.3 mL. An aliquot was filtered through a 0.2 μm filter (Sartorious Stedim Biotech, Minisart Sterile filter) into an auto sampler vial, sealed and labeled for HPLC analysis. HPLC conditions such as flow rate and concentration of mobile phase, injection volume of sample, column and detector temperature were optimized in order to get a clear glucose peak. The obtained HPLC conditions are presented in Table 5.1 for starch determination.

Table 5.1. The HPLC conditions used in starch determination.

Injection volume (μL)	**Mobile phase**	**Flow rate (mL/min)**	**Column temp. (°C)**	**Detector temp. (°C)**	**Running time (min)**	**Type of column**
20	0.01N H_2SO_4	0.6	65	50	25	Phenomenex Organic acid H^+

For the reagent starch samples, the amount of starch recovered after analysis ($\%R_{starch}$) was calculated with Equation 4.7. The percent of starch value for each sample was calculated using Equation 4.8:

$$\%R_{starch} = \frac{(\text{Concentration detected by HPLC})}{(\text{Known concentration of starch before analysis})} * 100 \quad (4.7)$$

$$\%\text{Starch} = \frac{(C_{HPLC}) * (\frac{\text{Volume solution}}{\text{ODW}})}{1.11 * \%R_{stach}} * 100 \quad (4.8)$$

where; C_{HPLC} is the concentration of glucose as determined by HPLC, (mg/mL); the volume solution is 9.3 mL which is the total volume of liquid added to solids; ODW is the oven dry weight of the sample, (mg) and "1.11" is the glucose to starch oligomer correction factor and $\%R_{starch}$ is the starch recovery.

5.1.2.5. Determination of protein content in biomass. The samples were analyzed for protein content according to the NREL/TP-510-42625 procedure (Determination of protein in biomass) and Hach method (Hames et al., 2008; Hach et al., 1985). The quantity of

protein in biomass was calculated from the amount of nitrogen by the Hach method. The nitrogen factor (NF) of 6.25 was used for estimating the amount of protein to convert nitrogen value to protein (Moon et al., 2011). The assumption related to the calculation of NF is that proteins contain about 16% of nitrogen (16=100/6.25) (Mariotti et al., 2008).

0.5 g of each sample was placed in a digestion flask. 4 mL of H_2SO_4 was added and the mixture was heated at 440°C for 4 min. Then 15 ml 30% H_2O_2 was added through the capillary funnel, maintaining the temperature stable. The digestion was completed after 8-10 minutes. When the digestion flask reached room temperature, the volume was brought to 100 mL with DI water. A 1-3 mL aliquot of the digest was withdrawn and mixed with 1 drop of TKA indicator, 8-10 drops of 8 N KOH (until blue color develops), 3 drops of mineral stabilizer solution, 3 drops of polyvinyl alcohol. Then DI water was added until sample volume reaches 25 mL. 1.00 mL of Nessler's reagent was added. The absorbance vales of each sample were measured using UV-160A Shimadzu UV-Vissible Spectrophotometry at 460 nm. The absorbance was converted into concentration by means of a linear calibration curve (percentage of nitrogen or percentage of protein). The percent of Total Kjeldahl Nitrogen (TKN) and protein content was calculated by Equations 4.9 and 4.10:

$$\% \text{ TKN} = \frac{(0.0075 * UV_{abs})}{W_{sample} * V_{digested\ sample}} \tag{4.9}$$

$$\% \text{ Protein} = \%TKN * NF \tag{4.10}$$

where; % TKN is the percent of Total Kjeldahl Nitrogen; UV_{abs} is the absorbance at 460 nm; W_{sample} is the sample weight, (0.1-0.5 g); $V_{digested}$ sample is the volume of digested sample, (1-3 mL) and NF is the nitrogen factor for biomass, (6.25).

5.1.2.6. Sample preparation and hydrolysis. This analysis step was based on the NREL/TP-510-42618 procedure (Determination of structural carbohydrates and lignin in biomass) (Sluiter et al., 2008). The extractive-free samples obtained from previous experiments (section 5.1.2.4) were used in the carbohydrate and lignin analysis since this procedure is suitable for samples that do not contain extractives.

Triplicates were made for each sample and averages results were reported. Approximately 300 mg of sample was added into tarred pressure tubes (ACE Glass pressure tube; 8648-30 with solid PTFE plug; 5845-47) and their weight was recorded. 3 mL of 72% sulfuric acid (v/v) was added to each pressure tubes. After brief mixing, the tubes were put into a water bath (Selecta Precisdig 12 L) at 30ºC. The mixture was incubated for an hour and stirred every 5 minutes. After this reaction, the acid was diluted to 4% by adding 84 mL of DI water. The tubes were then autoclaved in the Selecta Presoclave-2 autoclave for one hour at 121ºC. To measure the background sugar degradation during this step, a set of sugar recovery standards (SRS) was prepared. The calculated amount of sugars was weighed out to pressure tubes. 10.0 mL DI water and 348 µL of 72% sulfuric acid were added to the tubes. Since the SRS sugar concentrations should be chosen to most closely resemble the concentrations of sugars in the test sample, concentration given in Table 5.2 was used for SRS preparations during sugar analysis. The SRS was used in duplicate and autoclaved along with the samples.

Table 5.2. The selected SRS concentration obtained from literature.

Parameter	**SRS concentrations for corn stover (mg/mL) (Ruth, 2003)**	**SRS concentrations for wheat straw stover (mg/mL) (Linde et al., 2008)**
D (+) Glucose	1.38	1.13
D (+) Xylose	0.72	0.69
D (+) Galactose	0.07	0.03
L (+) Arabinose	0.17	0.11

5.1.2.7. Determination of acid insoluble and acid soluble lignin. Determination of acid soluble and acid insoluble lignin were achieved by NREL/TP-510-42618 procedure (Sluiter et al., 2008). The portion of acid insoluble lignin was determined using the hydrolysate from dilute acid hydrolysis. The autoclaved hydrolysate was vacuum filtered through filtering crucibles. The filtrates were captured and saved for acid soluble lignin and carbohydrate determination. The acid insoluble residues (AIR) remaining in the crucibles were dried at 105ºC for 4 hours, until constant weight was achieved. Their dry weight was recorded. Then, the crucibles were put inside the muffle furnace at 575ºC for

24 hours. After cooling inside a desiccator, the crucibles containing the ash were weighed. The acid insoluble lignin (AIL) was calculated according to Equations 4.11, 4.12 and 4.13:

$$ODW_{sample} = \frac{(W_{air\ dry\ sample}) * \%TS}{100} \quad (4.11)$$

$$\%\ AIR = \frac{(W_{crucible+AIR} - W_{crucible})}{ODW_{sample}} * 100 \quad (4.12)$$

$$\%AIL = \frac{(W_{crucible+AIR} - W_{crucible}) - (W_{crucible+ash} - W_{crucible}) - W_{protein}}{ODW_{sample}} * 100 \quad (4.13)$$

where; %AIR is the percent of acid insoluble residue; $W_{crucible+AIR}$ is the weight of crucible and acid insoluble residue, (g); $W_{crucible+ash}$ is the weight of crucible and ash, (g); %AIL is the percent of acid insoluble lignin; $W_{protein}$ is the amount of protein present in the acid insoluble residue, (g).

Aliquots of the filtrates from the hydrolysate liquors were analyzed for acid soluble lignin by the UV-160A Shimadzu UV-Vis Spectrophotometry at 320 nm, within six hours of the hydrolysis. The samples were diluted to have an absorbance in the range of 0.7-1.0. The percentage of acid soluble lignin (ASL) was determined using Equation 4.14. The total lignin was the sum of these two parameters (Equation 4.15).

$$\%ASL = \frac{(UV_{abs} * Volume\ of\ filtrate * Dilution)}{\varepsilon * ODW_{sample}} * 100 \quad (4.14)$$

$$\%Lignin_{ext\ free} = \%AIL + \%ASL \quad (4.15)$$

where; %ASL is the percent of acid soluble lignin; UV_{abs} is the average UV-Visible absorbance for the sample at 320 nm; the volume of filtrate is 87 mL; ε is the absorptivity of biomass at specific wavelength and the dilution is the $(Volume_{sample} + Volume_{dilutingsolvent})/Volume_{sample.}$

5.1.2.8. Structural carbohydrate determination. The carbohydrate content of the samples was determined according the NREL/TP-510-42618 procedure. An HPLC system was used with automatic sampling composed of a Perkin Elmer Series 200 LC System with 200 autosampler, RI detector and column oven. 20 mL of the hydrolysate and SRS samples were first neutralized to pH 6 by adding calcium carbonate while stirring. After reaching the desired pH, the supernatants were decanted and then filtered through 0.2 μm filters into the autosampler vials for HPLC analysis. The optimized HPLC conditions obtained in this study for sugar analysis are shown in Table 5.3.

Table 5.3. The HPLC conditions used in sugar analysis.

Injection volume (μL)	**Mobile phase**	**Flow rate (mL/min)**	**Column temp. (°C)**	**Detector temp. (°C)**	**Running time (min)**	**Type of column**
20	HPLC grade water	0.35	70	50	35	Phenomenex Monosaccharide Pb^{+2}

The amount of each component sugar recovered after acid hydrolysis was calculated, accounting for any dilution made prior to HPLC analysis according to Equation 4.16. Any replicate ($\%R_{sugar}$) values obtained for each individual sugar were reported as $\%R_{avg,\ sugar}$.

$$\%R_{\ sugar} = \frac{(\text{Concentration detected by HPLC})}{(\text{Known concentration of standard before hydrolysis})} * 100 \qquad (4.16)$$

where; $\%R_{sugar}$ is the recovery percentage of a sugar after acid hydrolysis.

The sugar concentrations (C_x) obtained by HPLC for each of the hydrolyzed samples were then corrected with this factor, according to Equation 4.17, accounting for any dilution made prior to HPLC analysis.

$$C_x = \frac{(C_{HPLC} * \text{dilution factor})}{R_{\ avg,sugar}/100} \qquad (4.17)$$

where; C_{HPLC} is the concentration of a sugar as determined by HPLC, (mg/mL); $\%\ R_{ave.sugar}$ is the average recovery of a specific SRS component; C_x is the concentration of a sugar in the hydrolyzed sample after correction for loss on 4% hydrolysis, (mg/mL).

It is necessary to multiply the calculated concentrations with an anhydro factor to compensate for the water molecules incorporated during polymeric sugar hydrolysis. The anhydro factor for hexoses (glucose, galactose and mannose) is 0.88, and for pentoses (xylose and arabinose) it is 0.9 (Equation 4.18) (Dowe and Mcmillan, 2008).

$$C_{anhydro} = C_x * \text{anhydro correction} \tag{4.18}$$

The percentage of each sugar on an extractive-free basis was then calculated with Equation 4.19. To correct all the above mentioned percentages from an extractive-free basis to an as-received basis, it was necessary to use the percentage of total extractives with Equation 4.20.

$$\%\ \text{Sugar}_{\text{ext. free}} = \frac{(C_{anhydro} * V_{filtrate} * (1g/1000mg)}{ODW_{sample}} * 100 \tag{4.19}$$

$$\%\ \text{Sugar}_{\text{as received}} = \frac{(100\text{-}\%\text{Extractives})}{100} * \%\ \text{Sugar}_{\text{ext.free}} \tag{4.20}$$

where; $V_{filtrate}$ is the volume of filtrate, (87 mL); % Extractives is the percent extractives in the prepared biomass sample.

5.1.3. Optimization of Dilute Sulfuric Acid/Steam Explosion Pretreatment

5.1.3.1. Feedstock substrate and chemicals preparation. The appropriate amount of feedstock which was suitable to be placed in the 500 L Parr reactor was weighed in a 1-L beaker (Hsu, 1995). The moisture content of feedstock was assumed to be 7.35% so, substrate amount was calculated as; 12.9*(100-7.35)/100=12 g. The weight of the acid was determined according to the calculations shown below; (the target pH level of pre-hydrolysate was around 1.3-1.5. NREL experience has shown that, for a hardwood

substrate, 0.73 wt% acid in the liquid phase and for a herbaceous substrate, 0.88 wt% acid is generally acceptable). The acid amount;

$$\frac{12.9x(1\text{-}7.35\%)}{10\%} * \ (1\text{-}10\%)*0.88\%* \frac{1}{72\%} = 1.32 \text{ g} \qquad (4.21)$$

Targeting a 10% solids level in the reactor, the appropriate amount of DI water was weighed according to the below calculations. The total amount of liquid in the substrate was;

$$\frac{12.9*(1\text{-}7.35\%)}{10\%} *(1\text{-}10\%) = 108 \text{ g} \qquad (4.22)$$

$$\underset{\text{(a)}}{\text{The amount of water} = 108} - \underset{\text{(b)}}{(12.9*7.35\%)} - \underset{\text{(c)}}{1.32} - \underset{\text{(d)}}{15} = 91 \text{ g} \qquad (4.23)$$

where; (a) is the total amount of liquid in the substrate; (b) is the moisture content of substrate; (c) is the amount of 72% w/w acid; (d) is the washing water.

5.1.3.2. Pretreatment procedure. The dilute sulfuric acid/steam explosion pretreatment experiments were conducted in a 500 mL high pressure-parr reactor system (Berghof - BR-300). The parr reactor was designed by a heating and cooling part in order to provide rapid temperature transition. The reactor was operated according to the NREL LAP-007 procedure (Preparation of dilute-acid pre-treated biomass) (Hsu, 1995). The calculations were done according to the 10% solids level in the reactor. The calculated amount of 72% (w/w) sulfuric acid was injected by a chemical injection valve and HPLC pump (Shimadzu LC-10AT). The dilute sulfuric acid/steam explosion pretreatment reaction time began with acid solution injection.

To examine the effect of different combinations of variables on hydrolysis rate of sugar, the pretreatment conditions listed in Table 5.4 were used. The sample abbreviations of corn stover and wheat straw samples with corresponding pretreatment conditions are also shown in Table 5.4. The optimal hemicellulose solubilization and hydrolysis can be

achieved by either high temperature and short residence time or lower temperature and longer residence time (Sun and Cheng, 2002). When choosing the pretreatment conditions in Table 5.4, this factor was considered. The pretreatment temperatures were kept within the range of 160–200ºC, with a reaction time of 2, 5 and 10 minutes and H_2SO_4 concentrations from 0.2% to 1.5% (w/w) depending on the pretreatment level.

The investigated pretreatment parameters (temperature, acid load and residence time) were used in the calculation of a single combined severity factor, CSF which is an indicator of the pretreatment severity (Equation 4.24) (Hsu et al., 2011).

$$CSF=\log [t. \exp [(T_H- T_R)/ 14.75]]-pH \quad (4.24)$$

where; t is the reaction time, (minutes); T_H is the reaction temperature, (ºC); T_R is the reference temperature, most often 100ºC and pH is the acidity of aqueous solution.

Table 5.4. The experimental conditions of pretreatment.

Temperature (°C)	**Acid load (% w/w)**	**Time (minutes)**	**CSF**	**Sample abbreviation**
160	0.75	5	2.44	C1 / W1
		10	2.74	C2 / W2
170	0.75	5	2.73	C3 / W3
		10	3.03	C4 / W4
180	0.5	5	2.92	C5 / W5
		10	3.23	C6 / W6
190	0.5	2	2.82	C7 / W7
		5	3.22	C8 / W8
200	0.2	2	2.84	C9 / W9
		5	3.23	C10 / W10

The volume of acid solution and water added to the pretreatment reactor were changed with respect to H_2SO_4 concentration value (% w/w). As a result, the amount of feedstock, acid solution and water added to the parr reactor according to a total reactor volume of 200 mL are given in Table 5.5. The feedstock substrate and water were transferred into the reactor and the reactor contents were mixed manually. The reactor was closed; the mixer

motor was turned on, setting the rpm to be about 175 for 5 minutes. The heater was turned off manually when the reactor temperature reached 50°C, but the temperature continued to rise to about 75°C. The reactor was heated at about 75°C and mixed for 10 minutes. The air vent was opened to remove trapped air. When the reactor temperature reached 159.5°C (for a specific temperature of 160ºC experiment condition), the HPLC pump was turned on at a flow rate of 6 mL/min and the stopwatch was started by opening the chemical injection valve for addition of H_2SO_4 solution. An additional 15 mL volume of water was injected in order to avoid the loss of H_2SO_4 solution remained in the acid injection line. The temperature needs to be 1°C within 160" or the experiment must be repeated. Then, the reactor was put to the cooling system.

Table 5.5. The amount of feedstock, acid solution and water added to the parr reactor.

H_2SO_4 (% w/w)	**H_2SO_4 (g)**	**Solid load (%)**	**Substrate (g)**	**Water (g)**	**Washing water for acid injection line (g)**	**Acid load (g H_2SO_4/ g total load)**
0.2	0.28	10	12.9	92	15	0.002316
0.5	0.7	10	12.9	91	15	0.00579
0.75	1.04	10	12.9	91	15	0.008602
0.88	1.32	10	12.9	91	15	0.010918
1.5	2	10	12.9	90	15	0.016543

5.1.3.3. Pretreatment sample handling. Using a Scienceware® Polypropylene Buchner funnel system, the contents of the reactor was filtered. The pre-hydrolysate was sealed, labeled and stored in a refrigerator until sugar and by-product analysis. The pre-treated solids were washed. Then, pH of the solid was adjusted to 5 and the solid part was sealed, labeled and stored at -20°C (Sluiter et al., 2008).

5.1.3.4. Analysis of the pre-hydrolysate for monomeric sugars. A series of calibration standards containing the compounds that are to be quantified for suggested concentration range (1.2-24.0 mg/mL) was prepared. The pH of the samples was measured and if the pH is less than 5, it was neutralized to pH 5–6 using use calcium carbonate. After reaching pH 5–6, the sample was allowed to settle and the clear liquid was decanted off. The HPLC conditions given in Table 5.2 were used in monomeric sugars analysis.

5.1.3.5. Analysis of the pre-hydrolysate for total sugar content (monosaccharides and oligosaccharides). The pH of the sample was measured and recorded. Based on sample pH, the amount of 72% w/w sulfuric acid required to bring the acid concentration of each aliquot to 4% was added according to the Appendix 1 given in the NREL/TP-510-42623 procedure. 20 mL sample was transferred to the pressure tubes. A set of sugar recovery standards (SRS) was used to correct for losses due to decomposition of sugars during dilute acid hydrolysis. SRS concentration suggestions are given in Table 5.6. The low concentrations given in Table 5.6 were used for SRS preparation since these concentrations were the closest ranges for the selected biomass samples.

Table 5.6. Suggested concentrations for SRS (Sluiter et al., 2008).

SRS type	**Sugar concentrations (mg/mL)**				
	Glucose	**Xylose**	**Galactose**	**Arabinose**	**Mannose**
High	40	100	20	20	10
Medium	20	50	10	10	5
Low	4	10	2	2	1

348 µL sulfuric acid (72% w/w) was added to each sugar recovery standard. The sealed samples and SRS were autoclaved for one hour at 121°C. The HPLC conditions given in Table 5.2 were also used in total sugars analysis.

5.1.3.6. Analysis of the pre-hydrolysate for by-products. The pre-hydrolysate obtained from pretreatment were passed through a 0.2 µm filter into an auto sampler vial. The optimized HPLC conditions obtained in this study for by-product analysis are given in Table 5.7.

Table 5.7. The HPLC conditions used in by-product analysis.

Injection volume (µL)	**Mobile phase**	**Flow rate (mL/min)**	**Column temp. (°C)**	**Detector temp. (°C)**	**Running time (min)**	**Type of column**
25	0.0025 M H_2SO_4	0.6	60	50	50	Phenomenex Organic Acid H^+

5.1.3.7. Determination of insoluble solids in pre-treated biomass. Water insoluble solids (WIS) in pre-treated biomass were determined according to the NREL/LAP-018 procedure (Determination of insoluble solids of pre-treated biomass material) (Eddy, 1998). The weight of the Buchner funnel with filter paper was measured to the nearest 0.01 g on an analytical balance. After filtering the pre-treated slurry, the weights of the Buchner funnel, filter paper and the slurry were measured. WIS content and the yield of glucose and xylose in hydrolysate were calculated according to below formulas (Xu et al., 2009).

$$\text{Yield}_{\text{WIS [g/100 g]}} = \frac{\text{Mass}_{\text{Dry.WIS}}}{\text{Mass}_{\text{Dry.Raw.Material}}} * 100 \quad (4.25)$$

$$\text{Yield}_{\text{Pretreatment[g/100 g]}} = \frac{\text{Mass}_{\text{Xylose.and.Glucose.in.Liquor}}}{\text{Mass}_{\text{Xylose.and.Glucose.in..Raw.Material}}} * 100 \quad (4.26)$$

where; $\text{Yield}_{\text{WIS}}$ is the yield of water insoluble solid, (%); $\text{Mass}_{\text{Dry.WIS}}$ is the weight of dry biomass after pretreatment, (g); $\text{Mass}_{\text{Dry.Raw.Material}}$ is the weight of dry biomass before pretreatment, (g); $\text{Yield}_{\text{Pretreatment}}$ is the yield of pretreatment as % of glucose or xylose recovery; $\text{Mass}_{\text{Xylose.and.Glucose.in.Liquor}}$ is the sugar percentage of the hydrolysate, (%); $\text{Mass}_{\text{Xylose.and.Glucose.in..Raw.Material}}$ is the sugar percentage of the raw material, (%).

5.1.4. Optimization of Fermentation

5.1.4.1. Measurement of cellulase activities. The measurement of cellulase activities procedure (NREL/TP-510-42628) has been designed to measure cellulase activity in terms of "filterpaper units" (FPU) per milliliter of original (undiluted) enzyme solution (Adney and Baker, 2008). The value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper (4% conversion) in 60 minutes has been designated as the intercept for calculating FPU. Commercially available enzyme solution Cellic® CTec2 was kindly supplied from Novozymes A/S (Bagsvaerd, Denmark). During the experiment, enzyme assay tubes, control tubes and glucose standards tubes were used. A rolled filter paper strip (50 mg Whatman No. 1 filter paper; 1.0 x 6.0 cm) was placed into each test tube. 1.0 mL of 0.05 M Na-citrate buffer was added to the tubes and equilibrated to 50ºC. Then, each of enzyme dilution given in Table 5.8 was added to the tubes as 0.5 mL. A stock enzyme solution was

prepared using citrate buffer in a portion of 1/20 (Adney and Baker, 2008; Ghose, 1987).

Table 5.8. The enzyme dilution ratios used in preparation of enzyme control tubes.

Dilution #	**Citrate buffer (mL)**	**1:20 Enzyme (mL)**	**Enzyme dilution ratio (EDR)**
ED 1	1650	350	0.00875
ED 2	1700	300	0.00750
ED 3	1800	200	0.00500
ED 4	1850	150	0.00375
ED 5	1900	100	0.00250

The term enzyme dilution ratio (EDR) was used to represent the proportion of the original enzyme solution present in the dilution added to the assay mixture. For example a 1:10 dilution of the 1:20 working stock of enzyme had an EDR of 0.005. While blank tubes contained 1.5 mL citrate buffer, 1.0 mL citrate buffer and 0.5 mL enzyme dilution solutions were added to enzyme control tubes. 1.5 mL citrate buffer and the filter paper strip were placed to substrate control tubes. 10 mg/mL glucose stock solution and citrate buffer were used to prepare four different glucose dilution solutions. Then 0.5 mL glucose dilution solutions and 1 mL citrate buffer was added to each glucose standards tubes. Blanks, controls and glucose standards were incubated at 50ºC along with the enzyme assay tubes, and then "stopped" at the end of 60 minutes by addition of 3.0 mL of DNS reagent. All tubes were boiled for exactly 5.0 minutes in a vigorously boiling water bath containing sufficient water to cover the portions of the tubes. The color of the tubes turns from yellow to red. After boiling, the tubes were transferred to a cold ice bath (Miller, 1959).

All tubes (assays, blanks, standards and controls) were diluted in water. The color formation was determined by measuring absorbance against the reagent blank at 540 nm. With this dilution, the glucose standards described above should give absorbance in the range of 0.1 to 1.0. A linear glucose standard curve was constructed using the absolute amounts of glucose (mg/0.5 mL) plotted against corresponding absorbance value at 540 nm. Using this standard curve, the amount of glucose released for each sample tube was determined. The concentration of enzyme which would have released exactly 2.0 mg of

glucose was estimated using this curve. The enzyme activity as FPU (filter paper unit) was calculated according to the below formula;

$$\text{Filter Paper Activity} = \frac{0.37}{\text{Enzyme concentration released 2 mg glucose}} = \text{FPU/mL} \qquad (4.27)$$

5.1.4.2. Procedure for hydrolysis of pre-treated biomass (SAC). The NREL/TP-510-42630 procedure (SSF experimental protocols-lignocellulosic biomass hydrolysis and fermentation) was used during optimization of enzymatic saccharification (Dowe and Mcmillan, 2008). The goal of this part is to evaluate the hydrolysis efficiency of the pre-treated substrates during saccharification (SAC) that is catalyzed by Cellic® CTec2 cellulase enzymes. Temperatures of 50ºC (optimum temperature of enzyme) and 32°C (selected temperature for this study to conduct enzymatic saccharification and fermentation simultaneously) were used as variable operating parameters and the glucan recovery was measured. The pre-treated substrate was washed to remove the residual glucose (than 0.1 g/L glucose) and inhibitors before loading the flasks. The TS of the washed substrate were determined to calculate the amount of washed solids to add to each flask during SAC experiment.

Each SAC flask was loaded with 1% w/w cellulose, 1% w/v yeast extract, 2% w/v peptone, 0.05 M citrate buffer (pH 4.8) and Cellic® CTec2 cellulase enzyme. Based on the washed biomass moisture content and cellulose content of pre-treated biomass, the quantity of biomass needed was determined. The correct amount of biomass and DI water were weighed into the flask. Since all the work up to this point had not been done aseptically, the flasks were autoclaved at 121ºC for 30 minutes. After cooling, each flask was re-weighed to the nearest one-hundredth of a gram and lost weight as mL of sterile DI water was added. The amount of enzyme needed for each flask was calculated according to the 15 FPU/g cellulose of enzyme dosage. YP, citrate and enzyme mixture aseptically were added to the flasks in a laminar flow hood and a time zero slurry sample was taken.

The flasks were incubated in a rotary shaker at 130 rpm at the selected temperatures. Appropriate sampling times were 0, 3, 6, 24, 48 and 72 hours. 3 mL slurry sample was aseptically taken by a sterile pipet and stored in capped tubes at -20ºC until HPLC analysis.

Using 0.2 micron cellulose filter, the samples were taken to vials for glucose and cellobiose analysis. The HPLC conditions of sugar analysis (Table 5.2) were used in SAC analysis. The glucan yield as % of the theoretical yield (% digestibility) was calculated by using the following formula:

$$\%\text{Yield} = \frac{[\text{Glucose}] + 1.053 * [\text{Cellobiose}]}{1.111 * f * [\text{Biomass}]} * 100 \qquad (4.28)$$

where; [Glucose] is the residual glucose concentration, (g/L); [Cellobiose] is the residual cellobiose concentration, (g/L); [Biomass] is the dry biomass concentration at the beginning of the fermentation, (g/L); f is the cellulose fraction in dry biomass, (g/g); 1.053 is the multiplication factor which converts cellobiose to equivalent glucose.

5.1.4.3. Procedure for inoculum preparation. This part of the study was also based on the NREL/TP-510-42630 procedure. The growth efficiency of different yeast types and the ethanol concentrations were compared using recombinant *Saccharomyces cerevisiae ATCC® 20618™* (*S.cerevisiae SCXF 138*, obtained from ATCC-LGC Standards Partnership) and *Saccharomyces cerevisiae* (obtained from Pakmaya A.Ş.)

The recombinant *S. cerevisiae ATCC® 20618™* (which was mutant strain of ATCC 24553) provides the conversion of D-xylose to ethanol in high yields. Aerobic and anaerobic conversion yield of D-xylose to ethanol by mutant strain of *S. cerevisiae ATCC® 20618™* were 1.41 and 1.29 (w/v) (ATCC, 2013). *S. cerevisiae* (commonly known as baker's yeast) can only ferment glucose sugars. The frozen cultures of *S. cerevisiae ATCC® 20618™* and *S.cerevisiae* were prepared according to below recipe (Table 5.9) at pH 6.0 ±0.2 and stored at -80°C (ATCC, 2013).

Table 5.9. The recipe of the feed culture *of S. cerevisiae ATCC® 20618™* and *S.cerevisiae* (ATCC, 2013).

Component	**Concentration**	
	***S. cerevisiae* ATCC® 20618™**	***S.cerevisiae***
Yeast Extract (g)	3.0	1.0
Malt Extract (g)	3.0	-
Dextrose (g)	10.0	5.0
Peptone (g)	5.0	2.0
Agar (g)	20.0	-
DI water (mL)	1000	100

A 1:5 working volume to total flask volume ratio was used for inoculum preparation. Each solution was prepared according to 100 mL working volume and pH was adjusted with 1 M NaOH or HCl. After 1% of inoculation of *S. cerevisiae ATCC® 20618™* and *S.cerevisiae*, preculture flasks were incubated at 30°C, 130 rpm for 20-24 hours. Then, the frozen cultures were prepared in a ratio of 500 µL pre-culture/500 mL glycerol stock solution (40% v/v) in Eppendorf tubes and stored at -80ºC.

In the comparison of yeast growths and ethanol concentrations at different temperatures, two parallel culture solutions for each type of yeast were prepared using YP with 5% dextrose medium (Table 5.10) (Dowe and Mcmillan, 2008). The prepared samples were incubated at 30ºC and 32ºC, 130 rpm. The optical density (O.D.) of samples was checked in defined time interval. 1 mL sample was taken from each flask and measured at spectrophotometer at 600 nm using YP solution as blank. Also, 1 mL sample was taken and stored at -20 ºC for glucose/ethanol measurement.

Table 5.10. Growth profile medium recipe (Dowe and Mcmillan, 2008).

Parameter	**5% Dextrose (mL)**	**10X YP (mL)**	**DI water (mL)**	**100 µL frozen culture (mL)**
Feed culture	10	10	80	1

5.1.4.4. Procedure for fermentation of pre-treated biomass. The fermentation experiments were performed according to the NREL/TP-510-42630 procedure. The experiments were conducted in 250 mL Erlenmeyer flasks and each one was equipped with a cotton stopper;

the exterior tip was submerged in glycerol. Glycerol was used as a lock to prevent oxygen back-diffusion to the medium, while permitting evolved CO_2 to leave the flask and to maintain anaerobic conditions. Each flask was loaded with 3% w/w cellulose, 1% w/v yeast extract, 2% w/v peptone, 0.05 M citrate buffer (pH 4.8), Cellic® CTec2 cellulase enzyme and the yeast. The correct amount of biomass and DI water were weighed into the flask. pH was adjusted using 1M NaOH to 5.0±0.7. The flasks were autoclaved at 121ºC for 30 minutes. After cooling, each flask was re-weighed to the nearest one-hundredth of a gram and lost weight was added back as mL of sterile DI water.

The ethanol yields of different applications such as simultaneous saccharification and co-fermentation (SSCF) by recombinant *S. cerevisiae ATCC® 20618*™ and simultaneous saccharification and fermentation (SSF) by *S. cerevisiae* were compared. The selected enzyme loads of 30 and 45 FPU g/cellulose were applied during optimization studies for different feedstocks; corn stover and wheat straw.

The fermentation experiments conducted using two parallel samples. In a laminar flow hood, YP and citrate buffer, 10X concentrated inoculum, the enzyme were aseptically added to the flasks. A time zero slurry sample (4 mL) was taken. The flasks were incubated in a rotary incubator at 130 rpm at for the selected temperature. Appropriate sampling times were 0, 4, 24, 48, 72, 96, 120, 144, and 168. 4 mL slurry sample was aseptically taken by a sterile pipet and the liquid and solid parts were separated by Hettich Rotina 380 Centrifuge. The liquid part was stored in capped tubes at -20°C until HPLC analysis. The samples were taken to vials for glucose and cellobiose, glycerol, lactic acid, and acetic acid analysis. The % theoretical ethanol yield was calculated by using the following formula (Dowe and Mcmillan, 2008):

$$\% \text{ Cellulose conversion} = \frac{[EtOH]f - [EtOH]o}{0.51*(f*[Biomass]*1.111} * 100 \qquad (4.29)$$

where; $[EtOH]_f$ is the ethanol concentration at the end of the fermentation as (g/L) minus any ethanol produced from the enzyme and medium; $[EtOH]_o$ is the ethanol concentration at the beginning of the fermentation (g/L); [Biomass] is the dry biomass concentration at the beginning of the fermentation, (g/L); f is the cellulose fraction of dry biomass, (g/g);

0.51 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast. 1.111 is used to convert cellulose to equivalent glucose.

5.1.4.5. Procedure for bioreactor operation. In order to show the applicability of the optimum process conditions obtained in previous fermentation experiments, the ethanol yields of pre-treated biomass for different batch reactors such as SSCF at 32°C and SHCF at 50°C (optimum temperature of enzyme) for prehydrolysis and at 30°C (optimum temperature of yeasts) for fermentation were compared.

Bioreactor was loaded with the same recipe of fermentation experiments for a total weight of 1500 g. During bioreactor operation, first pH was calibrated. All the solutions except for yeast and enzyme were added to the reactor. Sterilization before addition of yeast and enzyme was achieved at 121ºC for 30 minutes by autoclaving the reactor. O_2 probe calibration was achieved with dry air and N_2. The yeast and enzyme was added and the reactor operation started. The sampling times were 0, 4, 24, 48, 72 and 96 hours. The same parameters were observed with the fermentation experiments.

5.2. Environmental Sustainability Assessment of Bioethanol Fuel

The main purpose of environmental sustainability application was to assess dissemination of the optimization results of bioethanol fuel production in regional basis. Within this scope, Life Cycle Assessment and Environmental Life Cycle Costing were applied as comprehensive decision-making tools. Environmental and economic performances of lignocellulosic bioethanol fuel blends (E10; 10% ethanol/90% gasoline and E85; 85% ethanol/15% gasoline by volume) and conventional gasoline (CG) were compared.

In this study, a-1 km travel distance FU perspective with a flexy fuel vehicle (FFV-having a modified engine for ethanol fuel blends higher than 20% by volume) was applied for LCA and ELCC analyses. Additionally, the driving cost of the selected fuel blends for the same FU was calculated. GaBi4 LCA software, Ecoinvent database and EDIP 2003 methodology were used during LCA and ELCC analysis.

5.3. Statistical Analysis

Single sample t-test was applied to determine the statistical significance of differences between the parallel samples of characterization parameters. Significant difference values of parallel samples were determined as $p>0.05$ meaning that they were statistically the same.

Pearson's ‘r’ correlation test was also applied to compare the yields of hydrolysis and fermentation experiments. The linear correlations between the yields from different experiments were assessed by comparing their values for each time interval. The ‘r’ value results in the range of -1 and 1; an ‘r’ estimate close to these values indicates a either a negative or a positive correlation, respectively (Puth et al., 2014).

6. RESULTS AND DISCUSSION

6.1. Characterization of Feedstock

Since composition of lignocellulosic biomass significantly affects process yield of bioethanol production, characterization of the selected biomass samples were achieved. For example, alkali pretreatment is more effective with low lignin-content biomass (Templeton et al., 2009; Xu et al., 2013). The whole compositional data of corn stover and wheat straw found in this study are given in Table 6.1 and Table 6.2. The total carbohydrate contents of corn stover and wheat straw were observed as 45.8% and 48.3%, respectively. The feedstock composition do not only have an impact on biochemical conversion process and economics, but also on ethanol yield, which is proportional to potential sugar content in feedstocks (Garlock et al., 2009; Tao et al., 2013). Independent of feedstock type, glucan was found to be the most abundant component. The main component of hemicellulose was xylan. Other sugars existed in smaller quantities, whereas mannose was not detected. For bioethanol production, all of the carbohydrates are valuable components.

The levels of both cellulose (33.2%) and hemicellulose (15.1%) existing in wheat straw were found to be quite similar with the percentages of cellulose (31.5%) and hemicellulose (14.3%) in corn stover. In terms of by-product utilization, the lignin content of wheat straw (17.9%) was slightly higher than that of corn stover (16.0%). Among the tested lignocellulosic samples, corn stover yielded higher protein (5.3%) content relative to wheat straw, which had a protein content of 2.4%. Since protein has an inhibiting effect on enzymatic hydrolysis, wheat straw – having low protein content – has an advantage in biofuel production (Wild et al., 2012). The ash content of corn stover (8.2%) was also higher than that of wheat straw samples (5.1%). When comparing feedstocks equal moisture content, lower ash content consequently produced higher bioethanol yields since there is more useable carbon to convert at same feed rate with lower ash content (Kadam and McMillan, 2003).

Table 6.1. The composition of corn stover.

Parameters	**Corn stover**					
	Sample batch no					
	I	**II**	**III**	**IV**	**V**	**Avg.**
Total solids, TS (%)	93.10	91.23	92.35	92.79	94.57	**92.81±1.21**
Moisture (%)	6.90	8.77	7.65	7.21	5.43	**7.19±1.21**
Ash (%)	7.86	8.38	8.30	11.21	5.27	**8.20±2.11**
Extractives (%)	21.15	22.67	20.01	25.41	20.56	**21.96±2.17**
Water extractives (%)	18.16	17.07	17.39	19.76	15.24	**17.52±1.65**
Ethanol extractives (%)	2.99	5.60	2.62	5.65	5.32	**4.44±1.50**
Total solids, TS (%) (extractive-free)	94.74	91.46	91.82	92.82	94.45	**93.06±1.49**
Moisture (%) (extractive-free)	5.26	5.75	5.41	7.18	5.55	**5.83±0.78**
Ash (%) (extractive-free)	1.04	0.90	3.14	4.53	4.32	**2.79±1.74**
Lignin (%) (extractive-free)	18.53	15.43	24.97	15.90	26.20	**20.63±5.75**
Acid insoluble lignin (AIL)	17.29	14.63	23.77	15.07	24.94	**19.60±5.51**
Acid soluble lignin (ASL)	1.26	0.80	1.20	0.83	1.26	**1.02±0.24**
Lignin (%) (as received)	14.63	11.93	19.98	11.86	20.81	**16.15±4.92**
Carbohydrates (%) (extractive-free)						
Cellulose sugars						
Glucan (C6)	26.40	42.09	48.81	29.32	26.40	**36.60±3.20**
Hemicellulose sugars						
Xylan (C5)	10.05	12.82	18.94	10.40	10.05	**12.45±3.81**
Galactan (C6)	0.93	1.25	2.00	1	0.93	**1.22±0.45**
Arabinan (C5)	2.09	2.62	4.34	2.28	2.09	**2.68±0.95**
Carbohydrates (%) (as received)						
Cellulose sugars						
Glucan (C6)	36.33	32.55	37.04	21.87	20.97	**31.75±2.91**
Hemicellulose sugars						
Xylan (C5)	9.88	9.92	15.15	7.76	7.98	**11.15±2.18**
Galactan (C6)	0.90	0.97	1.60	0.74	0.74	**0.99±0.36**
Arabinan (C5)	1.48	2.03	3.47	1.70	1.66	**1.87±0.70**
Protein (%)	4.69	5.58	5.63	5.20	5.16	**5.25±0.38**
Starch (%)	1.19	1.87	2.15	1.27	2.07	**1.71±0.45**

Table 6.2. The composition of wheat straw.

Parameters	**Wheat straw**					
	Sample batch no					
	I	**II**	**III**	**IV**	**V**	**Avg.**
Total solids, TS (%)	91.81	91.93	95.56	92.21	95.01	**93.30±1.82**
Moisture (%)	8.19	8.07	4.44	7.79	4.99	**6.70±1.82**
Ash (%)	5.27	5.59	5.28	5.02	4.38	**5.10±0.45**
Extractives (%)	25.96	20.25	15.05	18.33	12.11	**18.34±5.28**
Water extractives (%)	18.66	15.66	5.77	14.56	9.51	**12.83±5.14**
Ethanol extractives (%)	7.30	4.59	9.28	3.77	2.61	**5.51±2.72**
Total solids, TS (%) (extractive-free)	95.42	93.22	93.48	91.71	91.46	**93.06±1.59**
Moisture (%) (extractive-free)	4.58	6.78	6.52	8.29	8.54	**6.94±1.59**
Ash (%) (extractive-free)	2.09	2.61	3.20	2.99	3.15	**2.81±0.46**
Lignin (%) (extractive-free)	18.91	22.67	20.93	26.58	23.39	**23.39±2.36**
Acid insoluble lignin (AIL)	17.63	21.43	19.64	25.34	21.14	**21.89±2.43**
Acid soluble lignin (ASL)	1.28	1.24	1.29	1.24	1.25	**1.26±0.02**
Lignin (%) (as received)	14	18.08	17.78	21.78	19.67	**19.33±1.83**
Carbohydrates (%) (extractive-ree)						
Cellulose sugars						
Glucan(C6)	40.03	39.36	39.20	40.44	44.20	**40.65±2.05**
Hemicellulose sugars						
Xylan (C5)	13.57	12.36	13.64	13.55	13.90	**13.40±0.60**
Galactan(C6)	1.29	1.12	1.06	0.88	1.55	**1.18±0.25**
Arabinan (C5)	3.62	4.07	3.17	2.78	5.89	**3.91±1.21**
Carbohydrates (%) (as received)						
Cellulose sugars						
Glucan(C6)	29.64	31.39	33.31	33.03	38.85	**33.24±3.46**
Hemicellulose sugars						
Xylan (C5)	10.05	9.86	11.59	11.07	12.21	**10.96±1.00**
Galactan(C6)	0.95	0.90	0.90	0.72	1.36	**0.97±0.24**
Arabinan (C5)	2.68	3.24	2.70	2.27	5.18	**3.21±1.15**
Protein (%)	3.16	2.28	2.22	2.25	2.06	**2.39±0.44**
Starch (%)	1.34	1.77	1.51	0.99	1.57	**1.43±0.29**

Since 3 to 5 parallel samples from batches of five biomass samples were used during characterization studies, the mean values for the soluble-free compositional analysis of corn stover and wheat straw were calculated and presented in Table 6.3. The average data obtained from parallel samples could be accepted as the representative values since there were no statistically significant differences ($p>0.05$) among the parallel sample results of characterization parameters.

Table 6.3. The mean values for compositional analysis of corn stover and wheat straw.

Corn stover	**% Dry weight (soluble-free basis)**								
	Glucan	**Xylan**	**Galactan**	**Arabinan**	**Lignin**	**Ash**	**Starch**	**Protein**	**Extractives**
Minimum	27.9	9.9	0.9	0.5	11.9	5.4	1.3	4.7	20.0
Maximum	36.3	15.2	1.6	3.5	20.8	11.2	2.1	5.6	25.4
Average	31.8	11.2	0.9	1.9	16.0	8.2	1.7	5.3	21.9
SD	2.9	2.2	0.4	0.7	5.6	2.1	0.5	0.4	2.2
Wheat straw	**Glucan**	**Xylan**	**Galactan**	**Arabinan**	**Lignin**	**Ash**	**Starch**	**Protein**	**Extractives**
Minimum	29.6	9.9	0.72	2.3	14.0	4.4	1.0	2.1	15.1
Maximum	38.9	12.2	1.36	5.2	21.7	5.6	1.8	3.2	25.9
Average	33.2	10.9	1.0	3.2	17.9	5.1	1.5	2.4	22.2
SD	3.5	1.0	0.2	1.2	0.2	0.5	0.3	0.4	5.4
SD: Standard deviation									

The compositions of the feedstocks found in this study are in good agreement with previously published results in literature, with the exception of xylan content of corn stover (Table 6.4). In this study, the glucan contents of corn stover and wheat straw were found as 31.5% and 33.2%, which are in the range of 30 to 39.8% and 28.21 to 40.7% in the literature, respectively. However, xylan content of Turkish corn stover (11.2%) sample was lower than literature values, which are in the range of 18.5 to 24.3%. This difference is due to the variation of anatomical parts of the samples used in compositional analyses or the growing location and condition of the feedstocks. Literature values for starch content for both corn stover and wheat straw are limited, however the starch contents of corn stover (1.7%) and wheat straw (1.5%) found in this study are very similar to the values given in Van Eylen et al., (2011) and Adapa et al., (2009), respectively. Galactan, arabinan and protein contents of corn stover and wheat straw found in this study were also very similar to literature values given in Table 6.4.

Table 6.4. Chemical composition (% w/w) of corn stover and wheat straw in comparison to literature.

Corn stover	**% Dry weight (soluble-free basis)**									
	Glucan	**Xylan**	**Galactan**	**Arabinan**	**Mannan**	**Lignin**	**Ash**	**Starch**	**Protein**	**Extractives**
This study	31.5	11.2	1.1	2.0	bdl	16.2	8.2	1.7	5.3	21.9
Templeton et al., 2009	38.9	23.0	1.8	3.4	0.4	16.2	4.8	-	4.5	-
Wolfrum and Sluiter, 2009	33.1	18.9	0.51	0.86	0.15	6.18	4.01	-	3.7	15.65
Liu et al. 2012	33.18	18.94	2.17	3.13	1.12	22.1	3.37	-	-	-
Ruth et al., 2003	37.1	19.2	2.5	1.6	1.3	20.7	5.2	-	3.8	2.6
Philip Ye et al., 2008	33.18	18.94	2.17	3.13	1.12	22.10	3.37	-	-	-
Templeton et al., 2010	34.0	19.2	1.0	2.5	-	12.3	4.7	-	2.2	22.5
Van Eylen et al., 2011	30	19	1.0	2.7	1.1	29	-	0.6	4.2	-
Weiss et al., 2010	35.0	18.5	-	-	-	13.6	3.8	-	-	10.5
Zimbardi et al., 2007	39.8	24.3	1.0	2.3	-	21.0	6.8	-	-	5.1
Wheat straw	**Glucan**	**Xylan**	**Galactan**	**Arabinan**	**Mannan**	**Lignin**	**Ash**	**Starch**	**Protein**	**Extractives**
This study	33.2	10.9	1.0	3.2	bdl	19.3	5.1	1.5	2.4	19.9
Adapa et al., 2009	34.2	23.68	-	-	-	13.88	2.36	2.58	2.33	-
Foyle et al., 2007	35.2	30.48	0.00	4.42	0.00	18.48	4.45	-	-	-
Tomás-Pejó et al., 2009	40.7	23.7	2.6	1.3	-	17.0	4.7	-	-	-
Fu and Mazza, 2011	37.43	18.97	0.77	1.50	0.82	13.38	1.21	-	3.57	11.18
Huijgen et al., 2012	34.6	21.5	0.5	2.1	0.2	16.1	8.5	-	-	13.2
Singh et al., 2011	28.21	11.01	1.09	0.19	-	12.83	-	-	-	16.5
Wild et al., 2012	36.9	19.9	-	1.9	0.2	17.8	6.1	-	-	10.4
Naik et al., 2010	53.6	16.0	1.0	18.6	9.8	21.3	1.3	-	-	12.4

bdl: below detection limits.

6.2. Optimization of Dilute Sulfuric Acid/Steam Explosion Pretreatment

6.2.1. Evaluation of Pretreatment Hydrolysate

The principle aim of pretreatment step is to solubilize the hemicellulose (xylan) and decrease the lignin content while holding as much cellulose (glucan) as possible in the WIS consequently increasing the concentration of monomeric sugars in the enzymatic hydrolysis (Xu et al., 2009). Within this concept, the pretreatment conditions that gave the highest xylose and the lowest glucose yield of pre-hydrolysate were determined. The WIS content and the yield of glucose and xylose of pre-hydrolysate are given in Table 6.5 for corn stover and wheat straw.

The calculated combined severity factor (CSF) values (Table 5.4) showed that high residence time (>5 min) with high temperature and acid load resulted in CSF values higher than 3 indicating an increase in pretreatment severity. Table 6.5 showed that xylose loss due to degradation increased in high CSF values (≥3) for both of the feedstocks.

The effect of different dilute sulfuric acid/steam explosion pretreatment conditions on the recovery of glucan and xylan for corn stover is shown in Figure 6.1. It was found that the maximum xylose yield of 126.5% and the minimum glucose yield of 10.1% (as monomer and oligomer) were achieved when the corn stover was pre-treated at 160ºC, 0.75% acid loading and 5 minutes residence time (optimum pretreatment condition-OPC), which corresponded to a CSF value of 2.44. However, the glucose yields dramatically increased to 15-25% when the temperature of pretreatment was raised to 190- 200ºC with a corresponding CSF range of 2.82-3.23. The glucose release in hydrolysate may result in a decrease of cellulose concentration remaining in the pre-treated solid residue. As a result, the glucose concentration available for the consecutive enzymatic hydrolysis and fermentation process was reduced (Saha et al., 2005). The amount of released xylose and glucose were estimated to be 17.2 g and 3.9 g for each 100 g of raw corn stover under these circumstances, respectively. Zimbardi et al. (2007) have investigated the effect of sulfuric acid/steam explosion of corn stover and the maximum xylose recovery of 16.8 g/100 g feedstock was obtained at 190 ◦C for 5 min and an acid loading of 1.5 wt.%.

Table 6.5. The WIS and the yield of glucose and xylose in pre-hydrolysate for corn stover and wheat straw.

Parameter	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**
CSF	2.44	2.74	2.73	3.03	2.92	3.23	2.82	3.22	2.84	3.23
Glucose $_{\text{Yield Pretreatment}}$ (g/100 g)	10.09	19.71	23.52	22.46	22.88	24.07	16.97	15.35	18.39	14.97
Xylose $_{\text{Yield Pretreatment}}$ (g/100 g)	126.51	127.45	129.18	133.49	110.34	110.92	94.38	89.63	137.45	41.05
% WIS yield	48.22	46.87	58.27	50.60	45.73	47.23	40.12	52.98	49.71	42.01
Parameter	**W1**	**W2**	**W3**	**W4**	**W5**	**W6**	**W7**	**W8**	**W9**	**W10**
CSF	2.44	2.74	2.73	3.03	2.92	3.23	2.82	3.22	2.84	3.23
Glucose $_{\text{Yield Pretreatment}}$ (g/100 g)	8.02	6.21	9.45	8.57	21.24	14.66	12.24	12.36	23.29	28.70
Xylose $_{\text{Yield Pretreatment}}$ (g/100 g)	101.73	91.98	112.62	95.03	73.27	92.57	79.60	96.91	44.15	64.40
% WIS yield	51.74	49.77	53.96	58.35	64.89	42.04	47.05	67.49	54.72	51.70

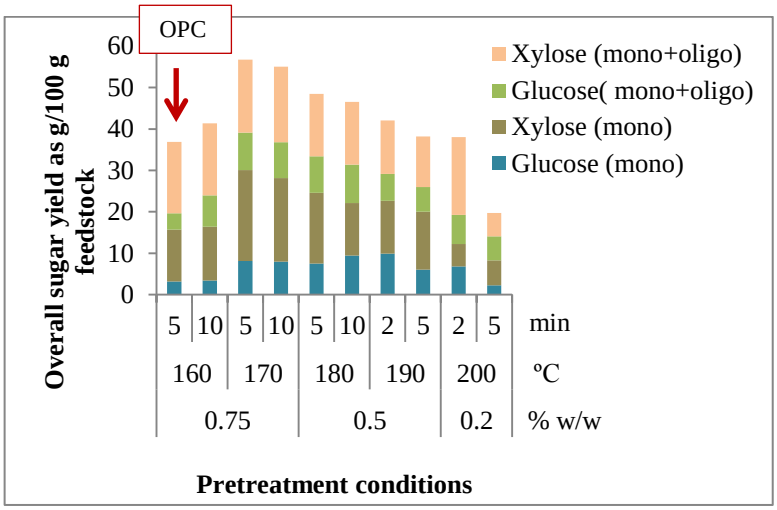


Figure 6.1. The overall sugar yield of corn stover as g/100 g feedstock.

The amount of furfural, HMF and acetic acid in the pre-hydrolysate for corn stover samples were 0.70, 0.59 and 2.76 g/L, respectively for the OPC giving the lowest concentration of by-product among the other pretreatment conditions (Figure 6.2). However, the concentration of furfural and HMF (hydroxymethylfurfural); the furan by-products from the conversion of sugars, were escalated dramatically in the pre-hydrolysate after pretreatment under severe conditions (CSF≥3). Acetic acid also existed in the pre-hydrolysate with a concentration of 2.65 to 4.93 g/L according to the severity of the pretreatment condition because of the release of the acetyl groups present in the hemicelluloses. It has been shown that when the xylose yield was greater than 85%, the maximum concentrations of acetic acid, HMF, and furfural in the pre-hydrolysate were about 1.6 g/L, 0.2 g/L and 1.9 g/L, respectively (Hsu et al., 2011).

The inhibitory effect of these by-products on the growth of *S. cerevisiae* species resulted in a decrease of ethanol yield in fermentation process. The highest concentration of by-products as acetic acid, HMF and furfural were 4.93, 1.13 and 5.85 g/L. These concentrations were observed at 200°C, 0.2% acid loading and 5 minutes residence time with a CSF value of 3.23. Previous studies have indicated that a decline in the bioethanol yield was shown when the concentration of acetic acid, HMF, and furfural were above 2.5 g/L, 1.0 g/L, and 1.0 g/L, respectively (Palmqvist and Hahn-Hägerdal, 2000). As a result, pretreatment conditions with a higher CSF value of 3 will cause a reduction in bioethanol yield because of the inhibitory effect of these by-products on *S. cerevisiae*.

Studies showed that the yield of furfural and HMF, degradation by-product of xylose and glucose, raised as the acidity of pretreatment increased at the same temperature (Xu et al., 2009). In our study, the results showed that the concentration of by-products increased with the increasing residence time at the same temperature and acid load. Additionally, the pretreatment with the highest CSF caused more degradation of sugars and thus all of the by-products in the liquor were higher than those of the other pretreatments with low CSF. The detoxification methods, such as over-liming and vacuum evaporation may be required to reduce the inhibition effect of furfural to increase the ethanol productivity. Increasing the pH value of the pre-hydrolysate in xylose fermentation has also been suggested as a way to reduce the inhibitory effect of acetic acid (Palmqvist and Hahn-Hägerdal, 2000).

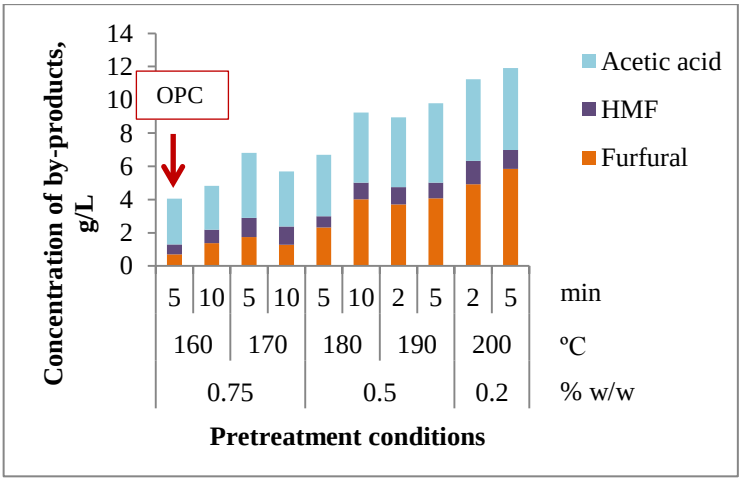


Figure 6.2. The amount of by-products in g/L for corn stover samples.

For wheat straw samples, the maximum xylose yield of 101.7% and the minimum glucose yield of 8.0% were achieved when the wheat straw was pre-treated at 160ºC, 0.75% acid loading and 10 minutes residence time (OPC), which corresponded to a CSF value of 2.74 (Figure 6.3). The concentrations of glucose and xylose for OPC were 2.68 g and 13.60 g for each 100 g wheat straw, respectively. Ohgren et. al. (2007) obtained glucose and xylose recovery of 4.7 g/L and 11.7 g/L in liquid fraction with corn stover sample at 170ºC for 9 min and an acid loading of 3 wt.%

The amount of by-products as furfural, HMF and acetic acid in the wheat straw pre-hydrolysate were 0.38 g/L, 0.07g/L and 1.84 g/L, respectively for the OPC with a corresponding CSF value of 2.74 (Figure 6.4). At the most severe conditions, 200ºC for 5

min, 5% acid load, the concentrations were 0.79 g/L HMF and 2.95 g/L furfural. As a result, the concentration of HMF and furfural were increased by almost ten times at the most severe conditions compared to OPC.

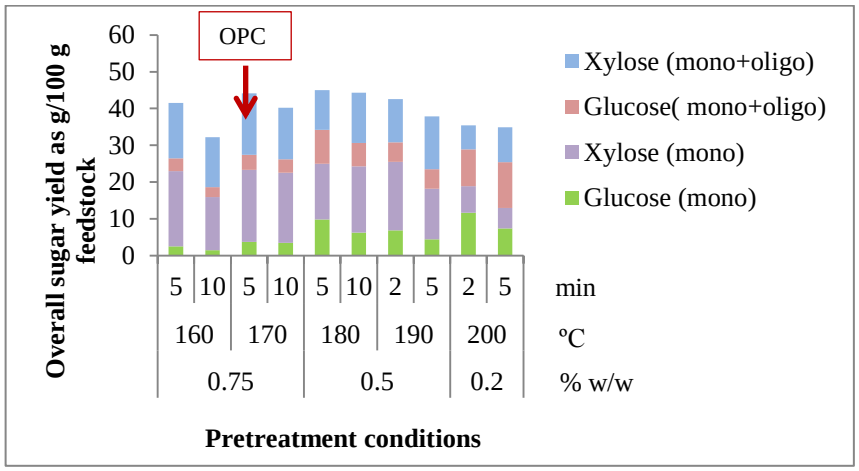


Figure 6.3. The overall sugar yield of wheat straw as g/100 g feedstock.

Increased degradation of the hemicellulose under more severe conditions also increased the yield of acetic acid, which was formed when side chains of acetyl groups were released (Linde et al., 2008). The production of acetic acid, the most abundant volatile, was increased around two times at high temperature/acid loading conditions corresponding CSF values of around 2.9 to 3.3 for wheat straw.

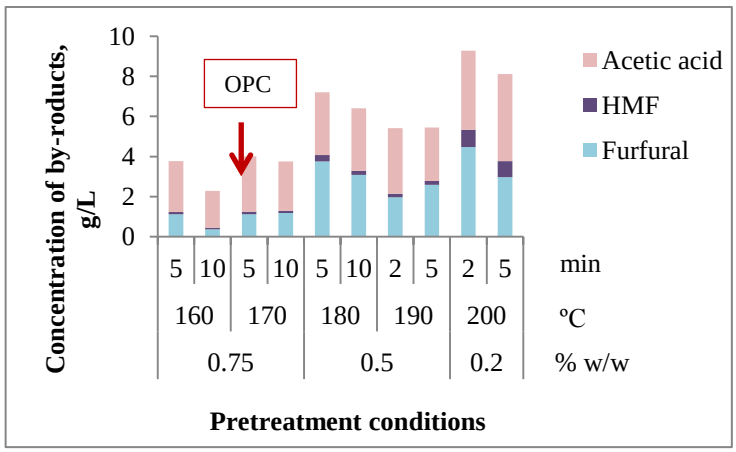


Figure 6.4. The amount of by-products in g/L for wheat straw samples.

6.2.2. Characterization of Pre-treated Solid Residue

The result of characterization for both solid residue and liquid after pretreatment conducted at optimum pretreatment conditions (OPCs) for corn stover and wheat straw are given in Table 6.6.

Table 6.6. The composition of pre-treated solids and liquid fraction.

WIS in slurry (g/g)	**Content in the solid fraction (%)**			**Content in the liquid fraction**				
				as monomer and oligomers (g/100g feedstock)		**(g/L)**		
Corn stover	Glucan	Xylan	Lignin	Glucose	Xylose	Acetic acid	HMF	Furfural
0.342	58.42	2.63	25.12	3.89	17.24	2.76	0.59	0.7
Wheat straw	Glucan	Xylan	Lignin	Glucose	Xylose	Acetic acid	HMF	Furfural
0.377	60.30	7.57	23.66	2.68	13.60	1.84	0.07	0.38

The glucan/xylan contents of pre-treated solid residues for corn stover and wheat straw were 58.4%/2.63% and 60.30%/7.57% for the OPC, respectively. The cellulose fraction was found to be the major component present in both of the pre-treated solid residues. The increase of glucan content could be correlated to the solubilization of hemicellulose components, because the hemicellulose was more amorphous and less stable than cellulose (Ohgren et. al., 2007). As a result, a substantial removal of hemicelluloses during hydrothermal processing was observed.

The glucan content of corn stover increased from 31.5% to 58.42% and xylan content decreased from 11.2% to 2.63% for corn stover (for wheat straw; an increase from 33.2% to 60.30% for glucan and a decrease from 10.9% to 7.57% for xylan) indicating an efficient pretreatment for both of the feedstock. These results are in agreement with the data reported for the same feedstock (Ruiz et al., 2012) with a similar pretreatment method as an increase in glucan content from 37.4% to 63.7% and a decrease in xylan content from 29.4% to 7.55% for raw feedstock to pre-treated feedstock.

The lignin content of the solid residues of corn stover and wheat straw was found to be 25.1% and 23.66% after the OPCs, respectively. The glucan and lignin content in the WIS increased due to hemicellulose removal. Ohgren et al. (2006) have studied 2% SO_2 impregnated/steam pre-treated corn stover at 200ºC and 5 minutes and they found that the glucan, xylan and lignin content of solid fraction were 62.5%, 6.6% and 25.2%, respectively. They also found that the glucose, xylose, acetic acid, HMF and furfural concentration in the liquid fraction as 5.9, 36.2, 2.1, 0.2 and 1.3 g/L, respectively (Ohgren et al., 2006). The concentrations of acetic acid, HMF and furfural given in Table 6.6 should not impose a toxic effect on the selected yeast. Larsson et al. (1999) found no reduction in yield when fermenting glucose to ethanol by *S. cerevisiae* in the presence of 6 g/L HMF and 4.6 g/L furfural.

6.3. Optimization of Fermentation

6.3.1. Measurement of Cellulase Activities

In order to calculate the volume of enzyme corresponding to enzyme doses of 30 and 45 FPU/g cellulose, the activity of Cellic® CTec2 cellulase enzyme was determined. As indicated in Section 5.1.4.1, the color development occurred after the addition of DNS reagent to the glucose stock, enzyme control and assay tubes are shown in Figure 6.5.

Figure 6.5. The color development after addition of DNS reagent to the assay tubes.

The UV absorbance values at 540 nm for glucose stock tubes and the corresponding glucose stock curve are given in Table 6.7 and Figure 6.6, respectively. Each of the glucose concentrations corresponding to a ΔE_n value was calculated using Figure 6.6, and the results are shown in Table 6.8. Using the enzyme dilution ratios (EDR) (Table 5.8) and the glucose concentration of enzyme tubes, the enzyme dilution curve was established and is given in Figure 6.7. The EDR value released 2 mg glucose concentration was then calculated from this figure as 0.0007. The cellulase activity was calculated as 142.31 FPU/mL using Equation 4.26.

Table 6.7. The UV absorbance values at 540 nm for glucose stock tube.

Tube no	**Concentration of glucose stock tubes (mg/0.5 mL)**	**UV $_{abs}$**
GS1	1.00	0.221
GS2	1.65	0.400
GS 3	2.50	0.574
GS 4	3.35	0.750

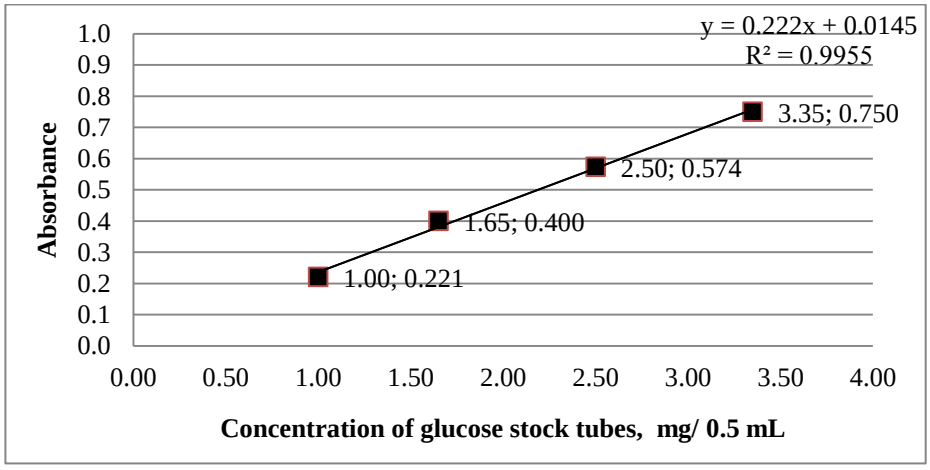


Figure 6.6. Glucose stock curve.

Table 6.8. The glucose values obtained from Figure 5.7 and corresponding enzyme dilutions.

Tube no	UV $_{abs}$	Concentration of glucose (mg/0.5 mL)	Enzyme dilution ratio
ΔE1	1.016	4.511	0.00875
ΔE2	0.928	4.115	0.00750
ΔE3	0.721	3.182	0.00500
ΔE4	0.551	2.417	0.00375
ΔE5	0.450	1.963	0.00250

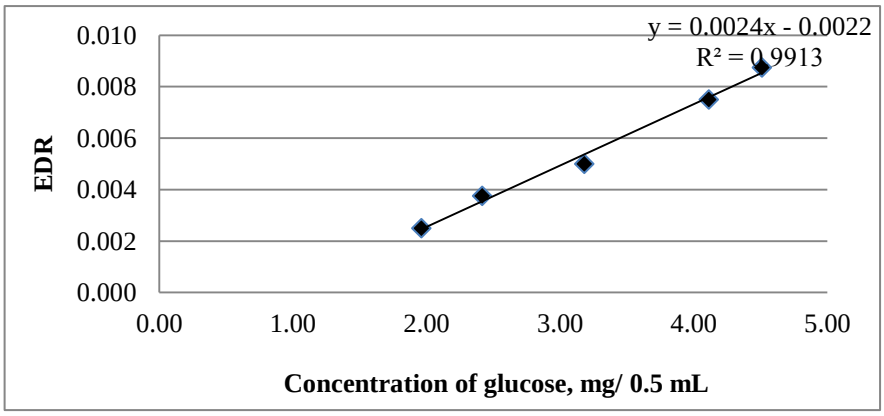


Figure 6.7. Enzyme dilution ratio (EDR) versus glucose concentrations.

6.3.2. Hydrolysis of Lignocellulosic Biomass (SAC)

The aim of the enzymatic saccharification experiments was to measure the enzymatic digestibility of Cellic® CTec2 at 32ºC in order to optimize the temperature of subsequent fermentation processes, in turn, increasing the conversion yield of both enzyme and yeast. Otherwise, enzymatic hydrolysis and fermentation processes should be conducted separately at temperatures between 45ºC and 50ºC for enzyme activity and at 30ºC for yeast growth. SAC experiments were performed using the pre-treated solid residue obtained from pretreatment conducted at OPCs for corn stover and wheat straw samples (C1: 160°C, %0.75 w/w acid load, 5 min residence time; and W2: 160°C, %0.75 w/w acid load, 10 min residence time).

The basic characterization parameters of pre-treated solids and the amount of each solution used during SAC experiments are given in Table 6.9 for corn stover and wheat straw.

Table 6.9. The basic parameters of pre-treated solids and the amount of solutions used in SAC experiments.

Parameter	Wheat straw	Corn stover
Cellulose content of pre-treated solids (%)	60.30	58.42
Cellulose content of 50 g of working weight (1% w/w) (g)	0.50	0.50
Total solids content (%)	20.20	21.10
The weight of pre-treated solids that contains 0.5 g cellulose (g)	0.83	0.86
The weight of the solids with TS content correction (g)	4.10	4.06
Cellulose content (g/100 g)	0.60	0.58
Enzyme dosage (FPU/g)	30.00	30.00
FPU of enzyme	15.00	15.00
Enzyme activity of the cellulase (FPU/mL)	142.31	142.31
The volume of enzyme (mL)	0.11	0.11
10X YP medium (mL)	5.00	5.00
Citrate buffer (mL)	2.50	2.50
DI water (mL)	38.29	38.34

The enzymatic digestibility of pre-treated corn stover, wheat straw and α-Cellulose as % of glucan recovery at 50ºC and 32ºC are shown in Figure 6.8. The highest digestibilities of the treated biomass were 84.4% and 79.37% of glucan for corn stover and wheat straw at 32ºC, respectively (Figure 6.8b). Zimbardi et al. (2007) showed that 84.8% of the glucan in the corn stover was recovered at 40ºC, at best, after the pre-impregnation of 3 wt% H_2SO_4 and steam explosion at 200ºC. As seen in Figure 6.8, the enzymatic digestibility values of studied temperatures – 50ºC and 32ºC – was similar, and there was a strong linear relationship between their trends (Pearson's r= 0.99) for both of the feedstocks. As a result, it has been shown that high glucan recovery could also be achieved at low temperature enzymatic hydrolysis. The digestibilities of pre-treated biomass were substantially higher than those of α-cellulose. The hydrolysis reaction profiles of Figure

6.8 also indicate that the pre-treated samples have much higher initial hydrolysis rates than α-cellulose.

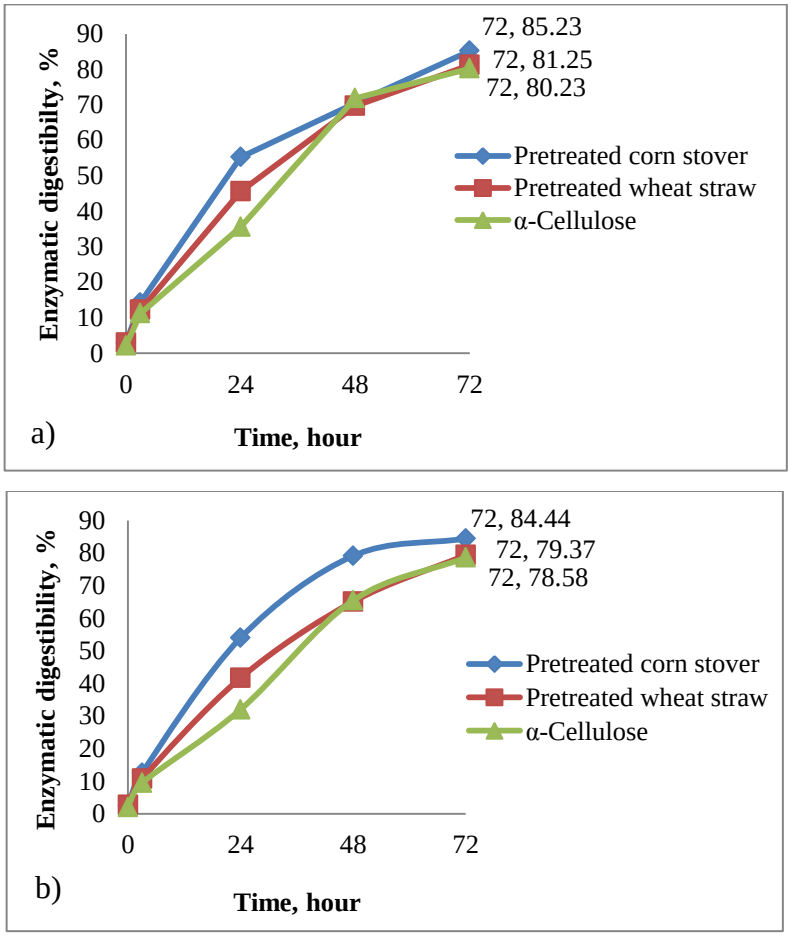


Figure 6.8. Enzymatic digestibility as % of glucan recovery of pre-treated corn stover, wheat straw, and α-Cellulose at (a) 50ºC and (b) 32ºC.

6.3.3. Optimization of Inoculum

In order to examine the growth rate of the selected yeast cultures, inoculum solutions were incubated at 30ºC and 32ºC. The initial (a) and final (b) views of inoculum solutions during experiments are shown in Figure 6.9.

The optical density (OD) values and growth profile of recombinant *S. cerevisiae* ATCC® 20618™ and *S. cerevisiae* at 30ºC for defined time interval are shown in Figure 6.10.

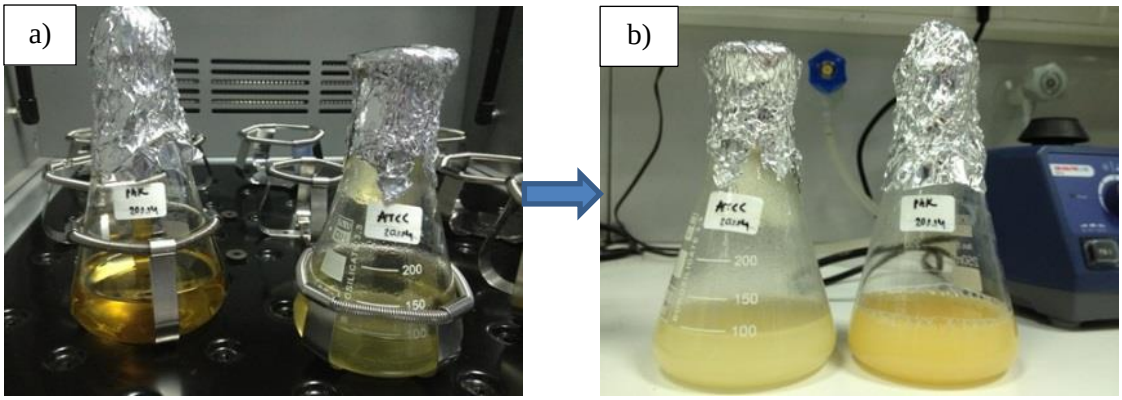


Figure 6.9. (a) The initial and (b) final views of inoculums.

The evaluation of the inoculum studies showed that while the exponential growth rate and doubling time of *S. cerevisiae ATCC® 20618™* were 0.41 and 1.71 h at 30ºC, respectively (Figure 6.10a), these values were 0.51 and 1.34 h for *S. cerevisiae* at 30ºC (Figure 6.10b). The concentration changes of glucose and ethanol versus time in growth medium of recombinant *S. cerevisiae ATCC® 20618™* and *S. cerevisiae* at 30ºC are shown in Figure 6.10. The highest ethanol concentration was obtained at the 10^{th} hour as 13.53 g/L for *S. cerevisiae ATCC® 20618™* and at the 12^{th} hour as 13.95 g/L for *S. cerevisiae*, at 30ºC. The images of *S. cerevisiae ATCC® 20618™* and *S. cerevisiae* at 30ºC by microscopic view with a magnification of x40 are also shown in Figure 6.11.

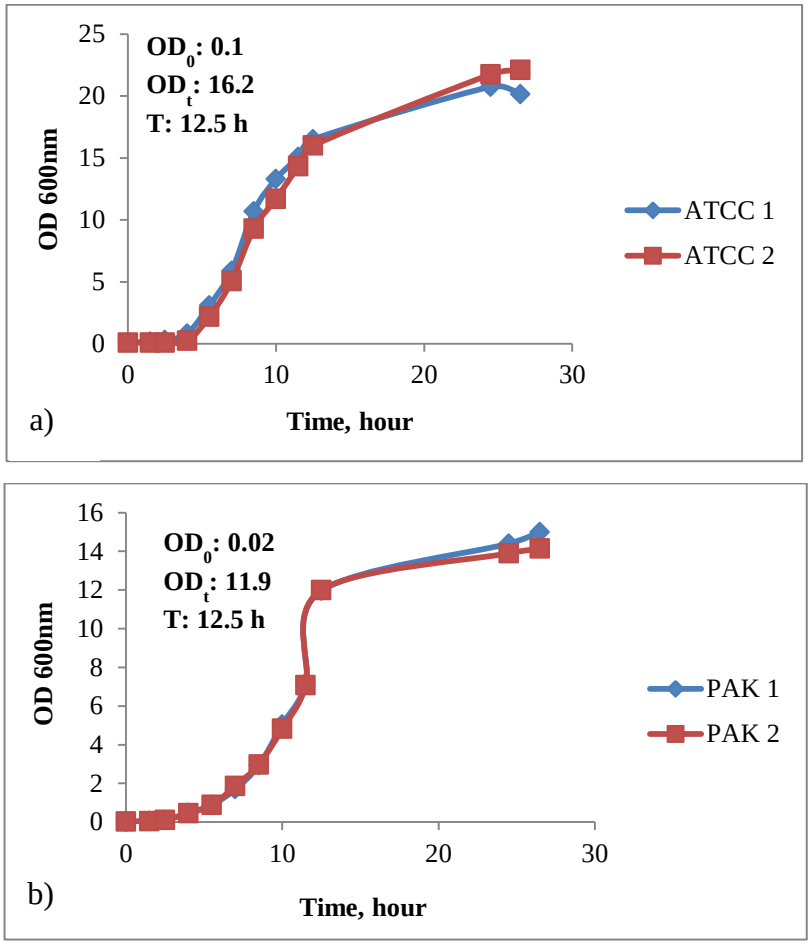


Figure 6.10. The growth rate profile of (a) recombinant *S. cerevisiae ATCC® 20618™* (initial pH: 6.02; final pH: 5.74) and (b) *S. cerevisiae* at 30ºC (initial pH: 6.02; final pH: 5.88) (ATCC: recombinant *S. cerevisiae ATCC® 20618™*, PAK: *S. cerevisiae*).

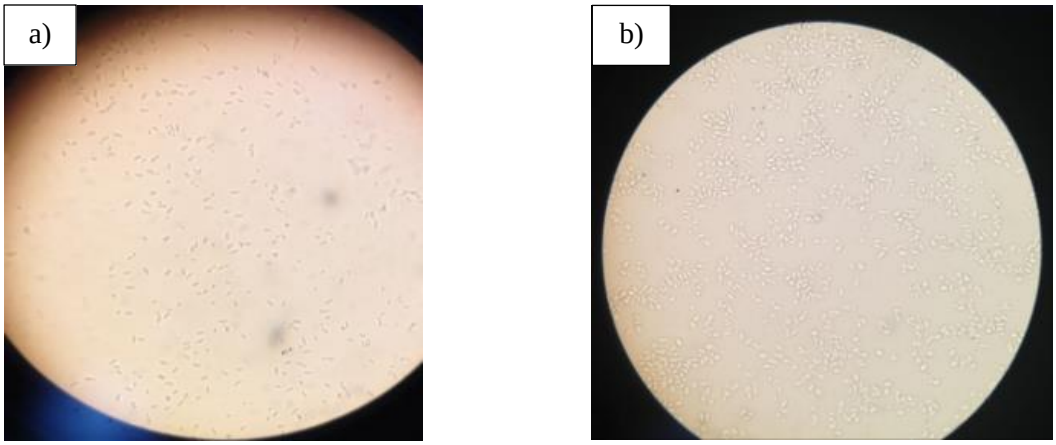


Figure 6.11. The image of (a) recombinant *S. cerevisiae ATCC® 20618™* and (b) *S. cerevisiae* at 30ºC by microscope with magnification of x40.

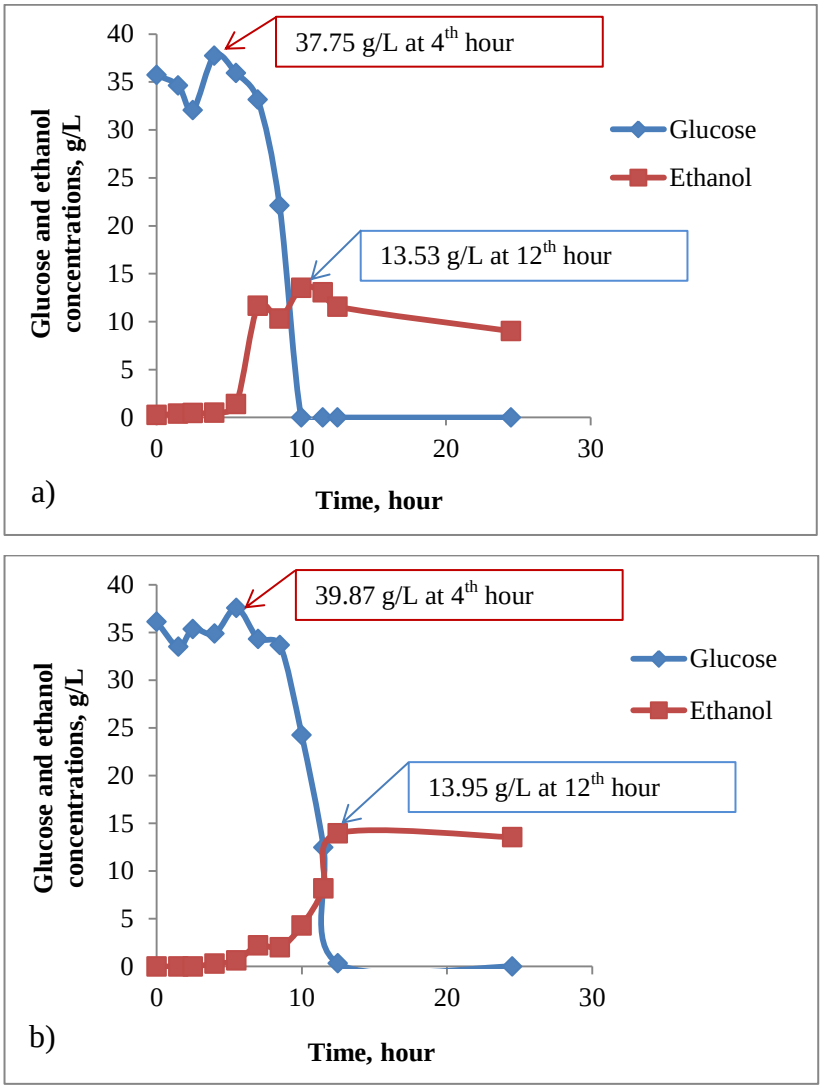


Figure 6.12. Glucose and ethanol concentrations in growth medium of (a) recombinant *S. cerevisiae ATCC® 20618™* and (b) *S. cerevisiae*, at 30ºC.

The OD values and growth profile of recombinant *S. cerevisiae ATCC® 20618™* and *S. cerevisiae* at 32ºC for defined time intervals are also shown in Figure 6.13. The images of the selected yeast as seen through a microscope with magnification of x40 are also given in Figure 6.14. The concentration of glucose and ethanol versus time in growth medium of the selected yeast at 32ºC are shown in Figure 6.15.

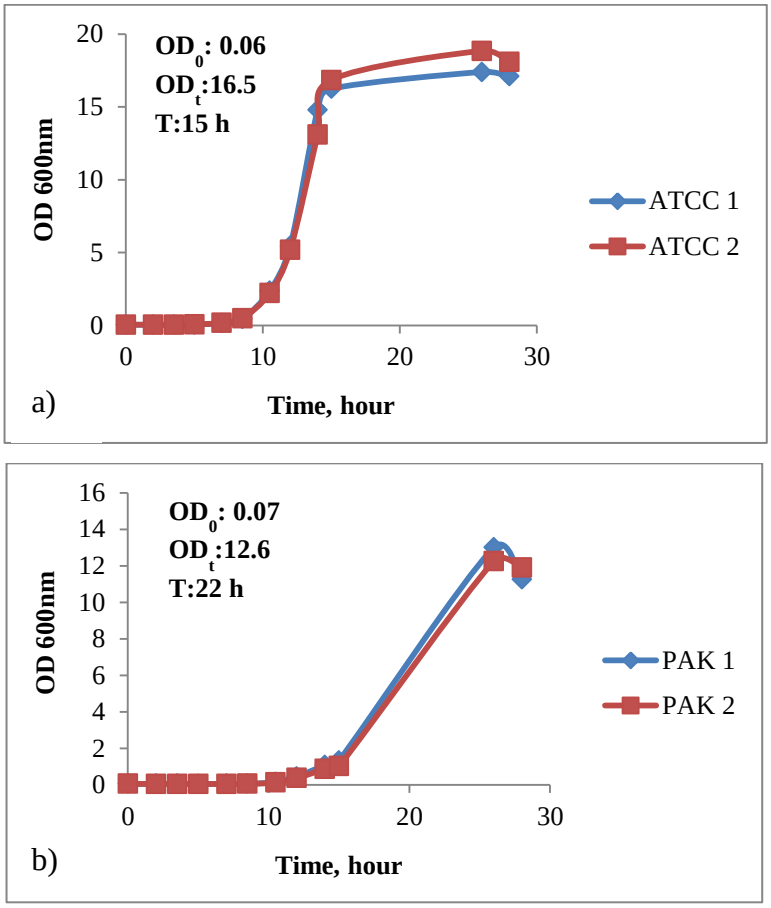


Figure 6.13. The growth rate profile of (a) recombinant *S. cerevisiae ATCC® 20618™* (initial pH: 6.02; final pH: 6.12) and (b) *S. cerevisiae* (initial pH: 6.02; final pH: 5.21) at 32ºC (ATCC: recombinant *S. cerevisiae ATCC® 20618™* PAK: *S. cerevisiae*).

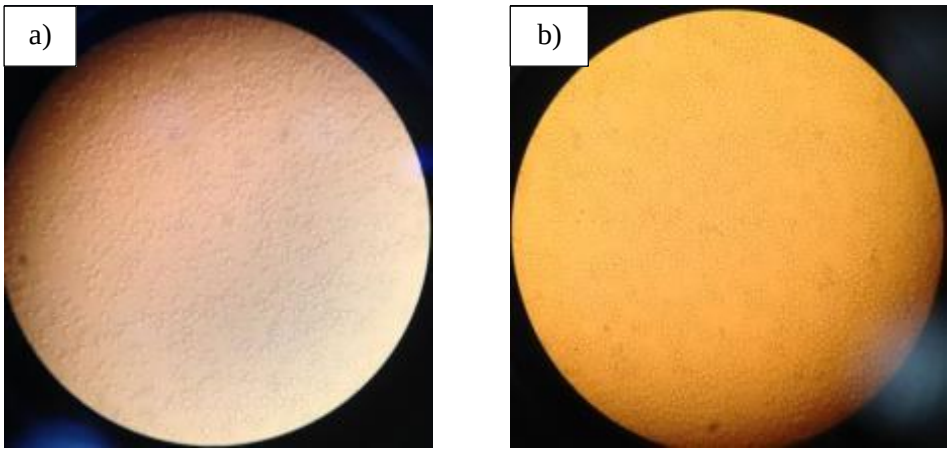


Figure 6.14. The image of (a) recombinant *S. cerevisiae ATCC® 20618™* and (b) *S. cerevisiae* at 32ºC by microscope with magnification of x40.

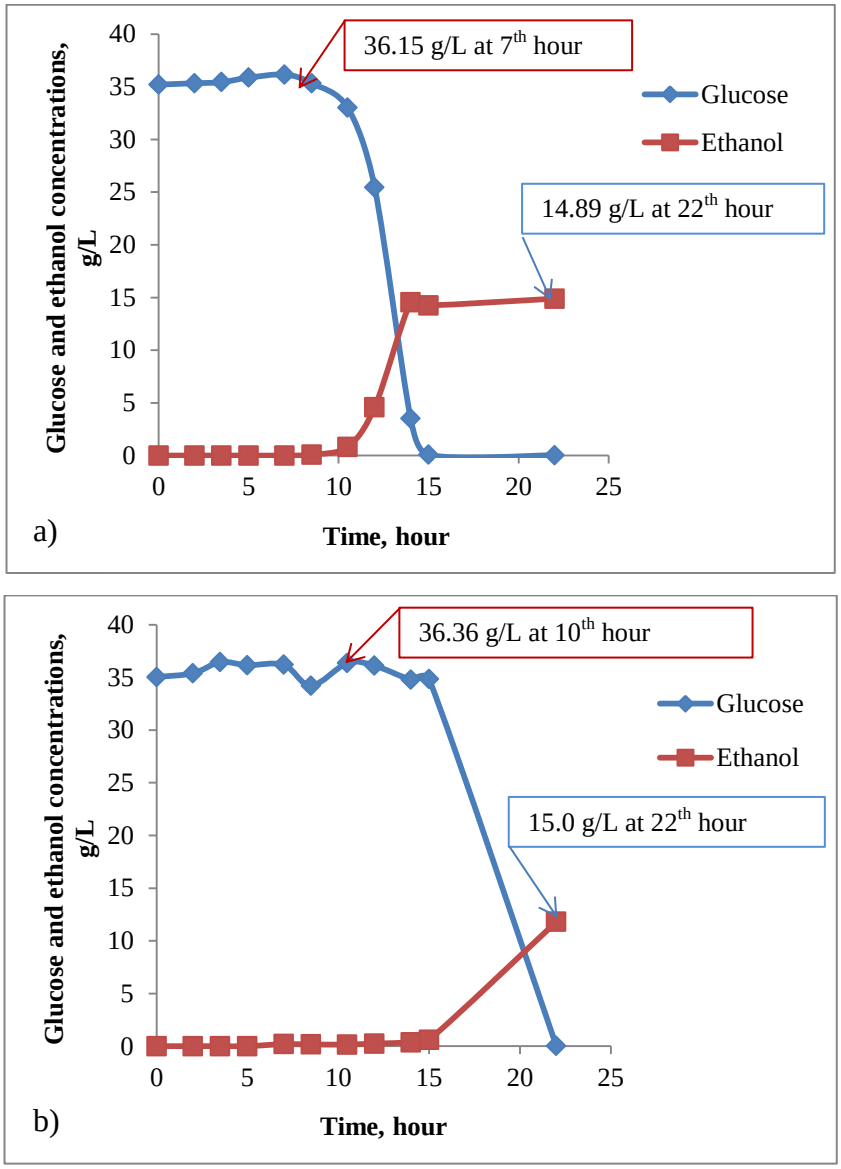


Figure 6.15. Glucose and ethanol concentrations in growth medium of (a) recombinant *S. cerevisiae ATCC® 20618™* and (b) *S. cerevisiae*, at 32ºC.

According to the inoculum studies conducted at 32ºC, it has been shown that the exponential growth rate and doubling time of *S. cerevisiae ATCC® 20618™* were 0.38 and 1.82 h (Figure 6.13a), and these values were calculated as 0.20 and 3.42 h for *S. cerevisiae* (Figure 6.13b). The growth rate results indicated that *S. cerevisiae ATCC® 20618™* was less sensitive to temperature change relative to traditional baker's yeast, having almost the same exponential growth rate value for both of the studied temperatures. Both of the yeasts, *S. cerevisiae ATCC® 20618™* and *S. cerevisiae*, also had a high ethanol concentrations at 32ºC — 14.89 g/L and 15.00 g/L, respectively. As a result, 32ºC was selected as the temperature for fermentation studies.

6.3.4. Optimization of Fermentation

To optimize the enzyme dose and to choose the most efficient yeast culture for bioethanol production, fermentation studies were conducted with different pre-treated feedstocks. The simultaneous saccharification and co-fermentation (SSCF) and simultaneous saccharification and fermentation (SSF) experiments were conducted at 32ºC, which was determined to be the optimum simultaneous saccharification and fermentation temperature based on previous enzymatic hydrolysis and inoculum experiments.

The amount of pre-treated solids according to the TS content and the amount of each solution used during SSCF experiments (with recombinant *S. cerevisiae ATCC® 20618™*) are given in Table 6.10. The dilute sulfuric acid/steam explosion pre-treated corn stover consisted of 58.41% glucan and 2.63% xylan by weight, demonstrating that there were 31.4 g/L of total carbon source during the SSCF assays in 3% w/w cellulose loading. The pre-treated wheat straw consisted of 60.30% glucan and 7.57% xylan by weight, with a total carbon source of 33.78 g/L based on glucan and xylan contents.

The co-fermentation of glucose and xylose to ethanol was investigated in the solids fraction from the dilute acid/steam explosion pre-treated corn stover with a xylose-fermenting yeast strain of *S.cerevisiae ATCC® 20618™* and compared to the ethanol yield performance of traditional baker's yeast, *S.cerevisiae.* The fermentation experiments were performed to compare the ethanol yield and ethanol concentration using the SSCF process (performed in duplicate) with the selected yeast cultures for different enzyme loadings of 30 and 45 FPU/g cellulose of Cellic® CTec2.

Table 6.10. The basic parameters of pre-treated solids and the amount of solutions used in SSCF experiments.

Parameters	**Wheat straw**	**Corn stover**
Xylose content of pre-treated solids (%)	7.57	2.63
Cellulose content of pre-treated solids (%)	60.30	58.42
Cellulose content of 100 g of working weight, (3% w/w) (g)	3.00	3.00
Total Solids Content, TS (%)	23.26	28.81
The weight of pre-treated solids that contains 3 g cellulose (g)	4.98	5.14
Experiment 1		
The weight of the solids with TS content correction (g)	21.39	17.82
Cellulose content (g/100 g)	0.60	0.58
Enzyme dosage (FPU/g)	45.00	45.00
FPU of enzyme	135.00	135.00
Enzyme activity of the cellulase (FPU/mL)	142.31	142.31
The volume of enzyme (mL)	0.95	0.95
The volume of inoculum (mL)	1.00	1.00
10X YP medium (mL)	10.00	10.00
Citrate buffer (mL)	5.00	5.00
DI water (mL)	62.66	66.23
Experiment 2		
The weight of the solids with TS content correction (g)	21.39	17.82
Cellulose content (g/100 g)	0.60	0.58
Enzyme dosage (FPU/g)	30.00	30.00
FPU of enzyme	90.00	90.00
Enzyme activity of the cellulase (FPU/mL)	142.31	142.31
The volume of enzyme (mL)	0.63	0.63
The volume of inoculum (mL)	1.00	1.00
10X YP medium (mL)	10.00	10.00
Citrate buffer (mL)	5.00	5.00
DI water (mL)	61.98	65.54
Concentration of biomass (g/L)	49.80	51.40
f (g/g)	0.68	0.61

.

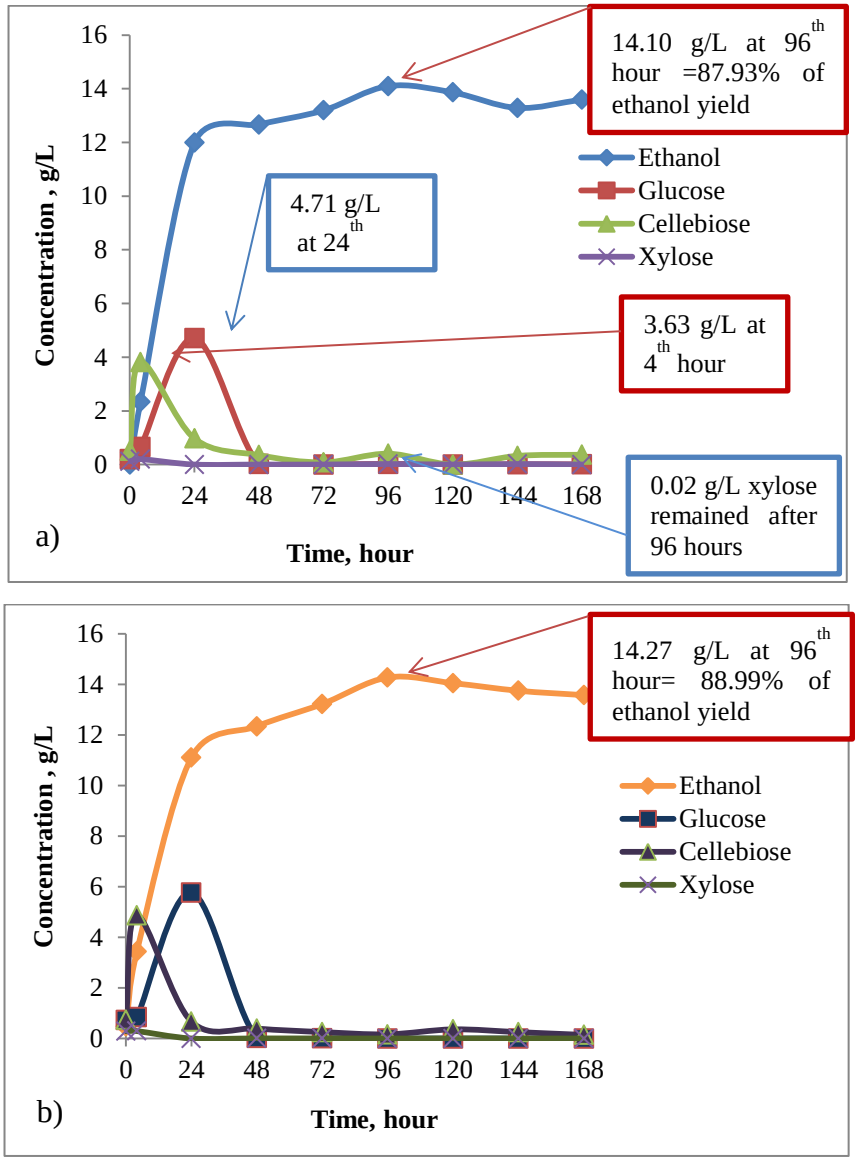


Figure 6.16. The concentrations of sugars and ethanol during SSCF experiment with enzyme loadings of (a) 30 FPU and (b) 45 FPU for corn stover.

The concentrations of sugars and ethanol during SSCF experiments with two different enzyme loadings of 30 and 45 FPU/g cellulose of Cellic® CTec2 for corn stover and wheat straw were plotted versus time (hour), and the results are shown in Figure 6.16 and Figure 6.17, respectively.

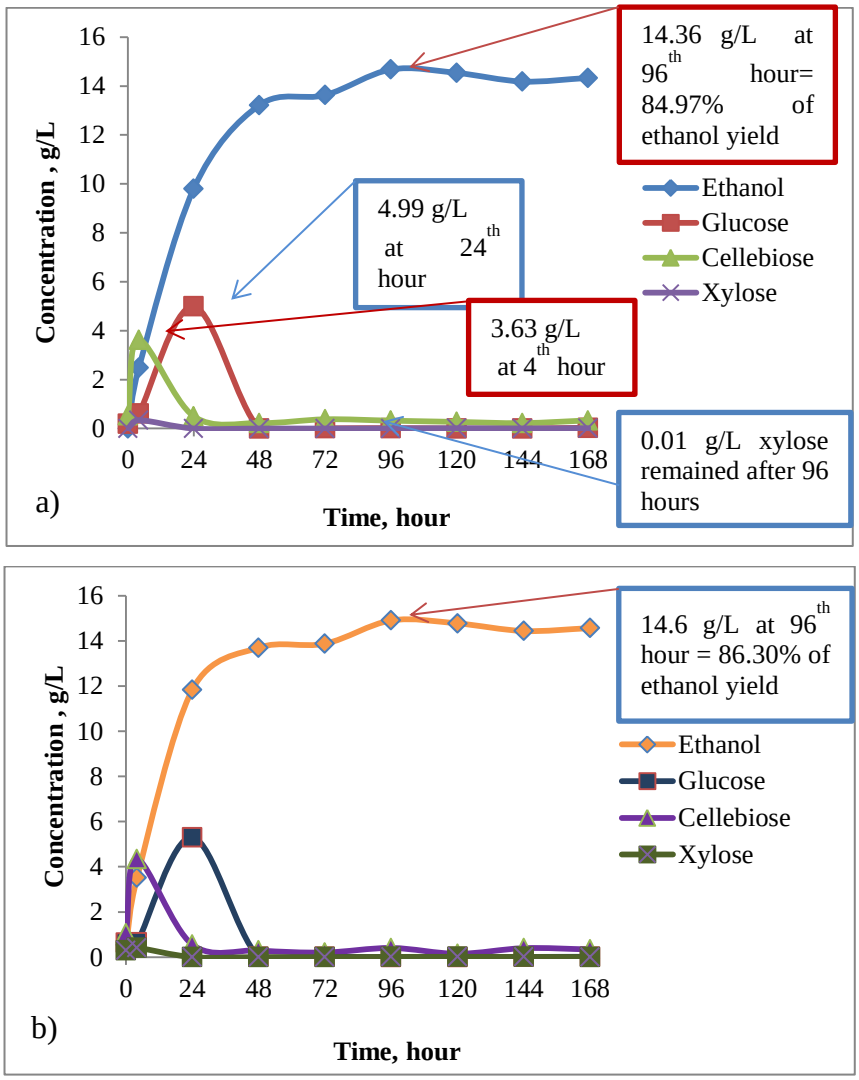


Figure 6.17. The concentrations of sugars and ethanol during SSCF experiment with enzyme loadings of (a) 30 FPU and (b) 45 FPU for wheat straw.

In the enzymatic hydrolysis of cellulose to glucose, the initial phase resulted in the production of a disaccharide, cellobiose, which was then hydrolyzed to a monosaccharide, glucose. For corn stover, although the SSCF experiments had a similar pattern for glucose and cellobiose concentrations, the glucose concentration reached its highest level (4.99 g/L) at the 24th hour, while the cellobiose concentration reached its highest level (3.63 g/L) at the 4th hour for the 30 FPU enzyme load (Figure 6.16a). No cellobiose accumulation was observed throughout fermentations, as it was hydrolyzed to glucose continuously, indicating sufficient b-glucosidase activity in the cellulase preparation and low inhibition by glucose.

Glucose and xylose from enzymatic hydrolysis were rapidly consumed by *S.cerevisiae ATCC® 20618™* after 24 hours for both enzyme loadings. The highest ethanol concentration from the pre-treated corn stover was 14.27 g/L for an enzyme loading of 45 FPU/g cellulose, corresponding to an ethanol yield of 88.99% at the 96th hour (Figure 6.16b). At the same point in time, the ethanol concentration and ethanol yield were 14.10 g/L and 87.93% (Figure 6.16a) for an enzyme loading of 30 FPU/g cellulose based on glucose and xylose contents, giving similar bioethanol yields for both of the enzyme loads (Pearson's r=0.99).

Ruiz et al. (2012) studied hydrothermal pre-treated wheat straw with a high cellulose content (>60%) at 180ºC for 30 min as a substrate in the SSF process using a thermotolerant flocculating strain of *Saccharomyces cerevisiae CA11* for bioethanol production. They found that the highest ethanol conversion yield (85.71%) was obtained at 30ºC, with 2% substrate and 30 FPU of enzyme loading. For wheat straw in this study, the highest ethanol concentration was obtained at the 96th hour, corresponding to 86.3% ethanol yield at a 45 FPU enzyme load (Figure 6.17b). However, the sugar-to-ethanol conversion trends for the selected enzyme loads observed during the fermentation experiments had a strong positive correlation (Pearson's r value=0.99) for both feedstock. As a result, it was demonstrated that high ethanol concentrations can also be obtained at low enzyme applications.

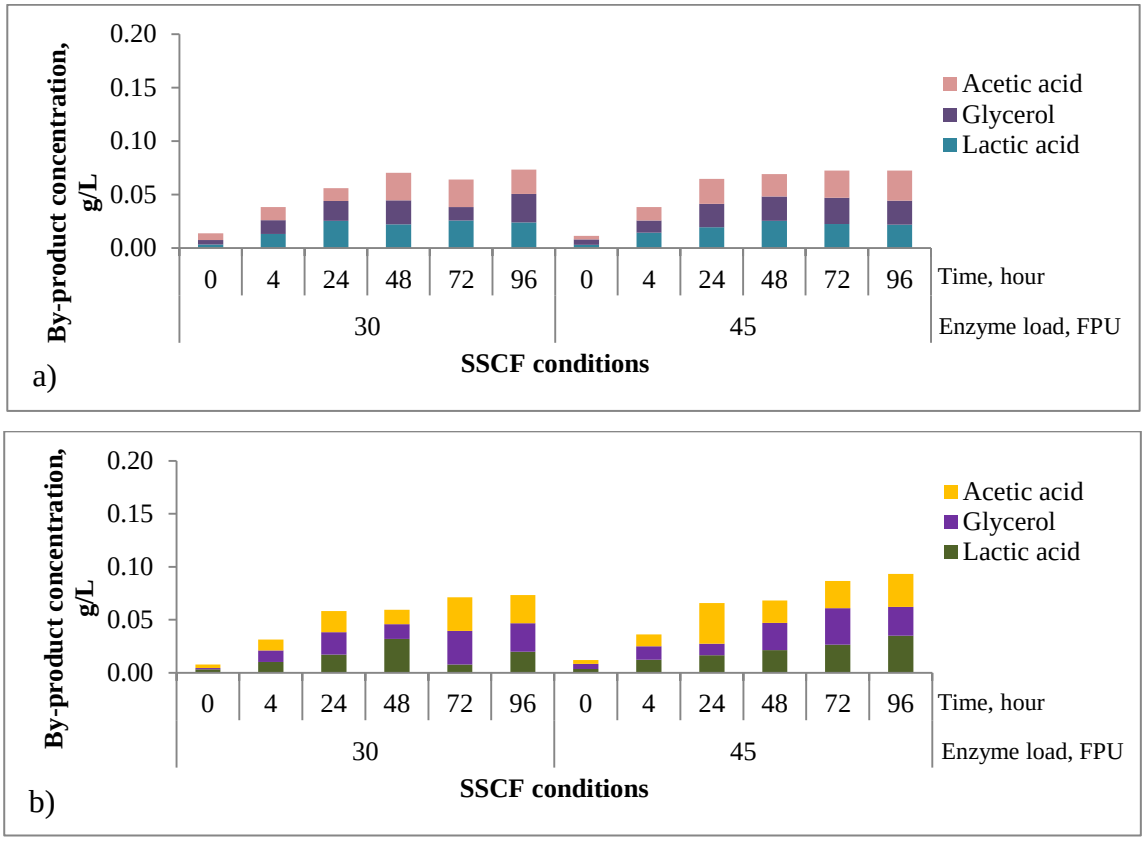


Figure 6.18. The concentration of by-products during SSCF for (a) corn stover and (b) wheat straw.

By-product concentrations for lactic acid, glycerol and acetic acid were within the range of 0.02 to 0.04 g/L at the 96th hour for both feedstock, and the ethanol production efficiency of *S.cerevisiae ATCC® 20618™* was not affected by these by-product concentration levels.

The amount of pre-treated solids according to the TS content and the amount of each solution used during SSF experiments are given in Table 6.11 for corn stover and wheat straw. The dilute sulfuric acid/steam explosion pre-treated corn stover and wheat straw contained about 58.41% and 60.30% glucan by weight, meaning that there were 30.03 g/L of total carbon source based on glucan content in 3% w/w cellulose loading, respectively.

Table 6.11. The basic parameters of pre-treated solids and the amounts of solutions used in SSF experiments.

Parameters	**Wheat straw**	**Corn stover**
Xylose content of pre-treated solids (%)	7.57	2.63
Cellulose content of pre-treated solids (%)	60.30	58.42
Cellulose content of 100 g of working weight, (3% w/w) (g)	3	3
Total solids content (%)	21.17	20.42
The weight of pre-treated solids that contains 3 g cellulose (g)	4.98	5.14
Experiment 1		
The weight of the solids with TS content correction (g)	23.50	25.15
Cellulose content (g/100 g)	0.60	0.58
Enzyme dosage (FPU/g)	45	45
FPU of enzyme	135	135
Enzyme activity of the cellulase (FPU/mL)	142.31	142.31
The volume of enzyme (mL)	0.95	0.95
The volume of inoculum (mL)	1	1
10X YP medium (mL)	10	10
Citrate buffer (mL)	5	5
DI water (mL)	60.55	58.90
Experiment 2		
The weight of the solids with TS content correction (g)	23.50	25.15
Cellulose content (g/100 g)	0.60	0.58
Enzyme dosage (FPU/g)	30	30
FPU of enzyme	90	90
Enzyme activity of the cellulase (FPU/mL)	142.31	142.31
The volume of enzyme (mL)	0.63	0.63
The volume of inoculum (mL)	1	1
10X YP medium (mL)	10	10
Citrate buffer (mL)	5	5
DI water (mL)	59.87	58.22
Concentration of biomass (g/L)	49.8	51.4
f (g/g)	0.60	0.58

The fermentation of glucose to ethanol was investigated in the pre-treated solids residue with traditional baker's yeast, *S.cerevisiae.* The fermentation experiments were performed to compare the ethanol yield and ethanol concentration using the SSF process (performed in duplicate). The concentrations of sugars and ethanol during SSF experiments with enzyme loadings of 30 and 45 FPU/g cellulose of Cellic® CTec2 for corn stover and wheat straw were plotted versus time (hour), and the results are given in Figures 6.19 and 6.20, respectively.

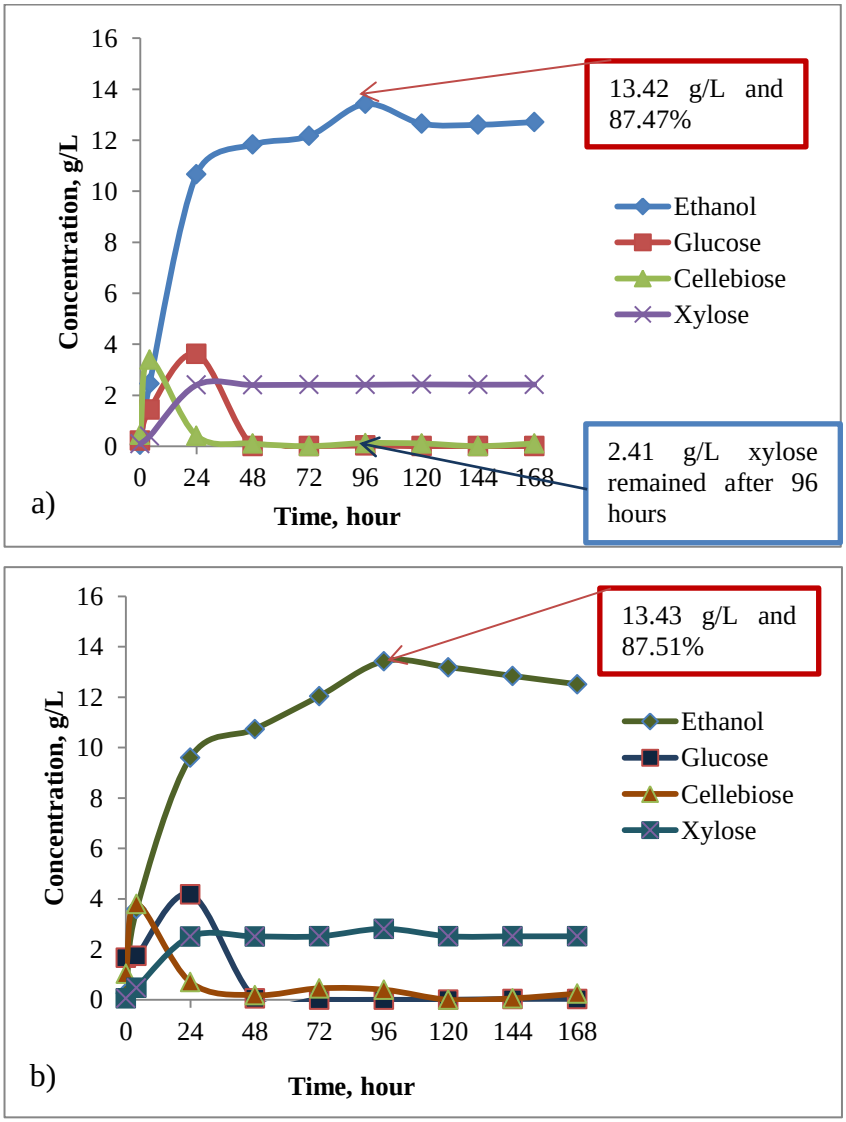


Figure 6.19. The concentrations of sugars and ethanol during SSF experiments with enzyme loadings of (a) 30 FPU and (b) 45 FPU for corn stover.

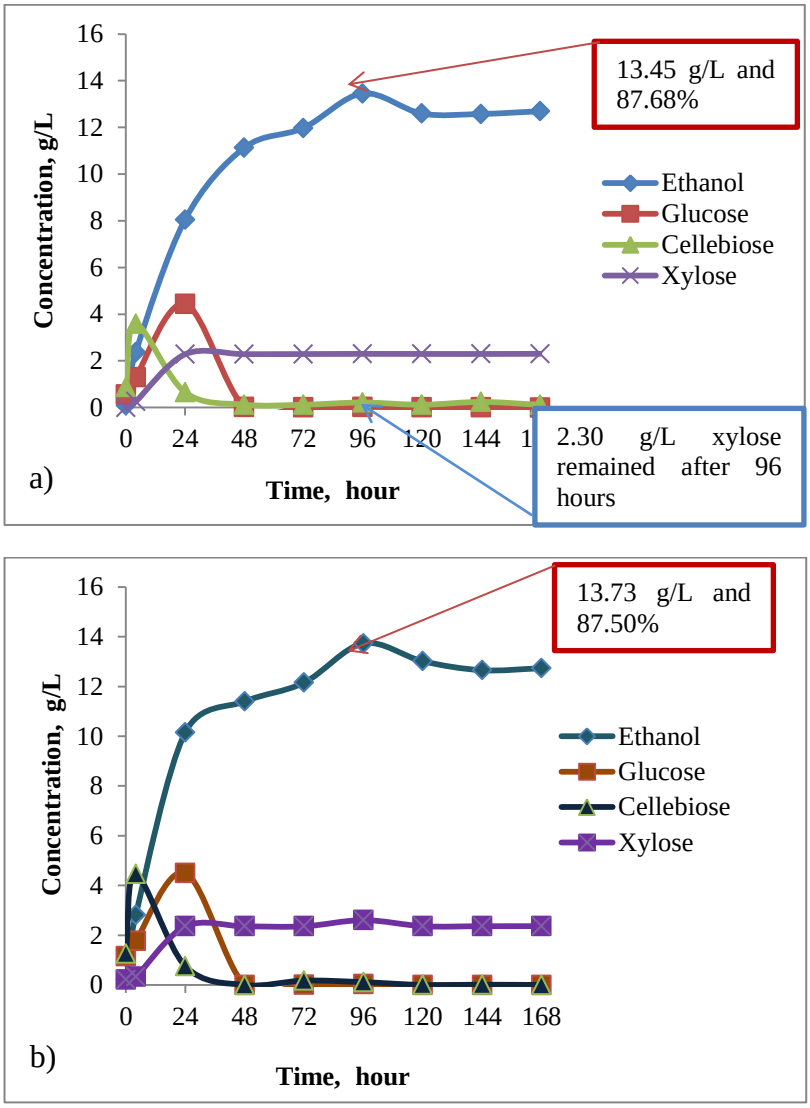


Figure 6.20. The concentrations of sugars and ethanol during SSF experiments with enzyme loadings of (a) 30 FPU and (b) 45 FPU for wheat straw.

The highest ethanol concentration for pre-treated corn stover was 13.42 g/L (Figure 6.19a) and 13.43 g/L (Figure 6.19b) for enzyme loadings of 30 and 45 FPU/g cellulose, respectively, corresponding to ethanol yields of 87.51% and 87.47% at the 96th hour. For wheat straw, 87.68% (Figure 6.20a) and 87.50% (Figure 6.20b) ethanol yields were obtained at the 96th hour for enzyme loadings of 30 and 45 FPU/g cellulose, respectively. The Pearson's r value of the ethanol yields for different enzyme load applications was 0.99, indicating that both enzyme applications gave similar ethanol yields. This result showed that increasing the enzyme load of the SSF assay will not provide a higher ethanol yield for traditional baker's yeast. SSF with solid content using baker's yeast conformed well with earlier studies (Ohgren et al., 2006; Ohgren et al., 2007).

According to the xylose and glucose concentration profiles of SSCF with *S.cerevisiae ATCC® 20618™* and SSF with *S.cerevisiae* for corn stover presented in Figure 6.16 and 6.19, glucose accumulation is noticeable in the early phase of experiments where the cells are in the growth phase. However, after 24 hours, glucose was undetectable in all experiments, indicating that the process proceeds under glucose-limited conditions. The advantage of the SSF is that it eliminates the glucose inhibition of the cellulase enzyme. However, while 0.02 g/L xylose remained in SSCF experiments with *S.cerevisiae ATCC® 20618™* (Figure 6.16a), the same figure with *S.cerevisiae* was 2.41 g/L in SSF experiments after 96 hours (Figure 6.19a) for 30 FPU enzyme load for corn stover. The reason for this is because pentoses (xylose), mainly in the form of xylan, were not fermented to ethanol by *S. cerevisiae.* However, the recombinant *S. cerevisiae ATCC® 20618™* is capable of fermenting hexose and pentose into ethanol, giving a residual xylose concentration at the end of the experiment.

The recombinant strain of *S.cerevisiae ATCC® 20618™* showed an increased ethanol yield relative to *S.cerevisiae*. In the case of 30 FPU enzyme application after 96 hours, 0.01 g/L xylose remained in *S.cerevisiae ATCC® 20618™* for wheat straw samples (Figure 6.17a). The same figure for SSF with *S.cerevisiae* was 2.30 g/L (Figure 6.20a). Thus, 2.29 g/L xylose had been fermented to an additional 0.91 g/L ethanol (14.36-13.45 g/L), which corresponded to a yield of 0.42 g ethanol/g xylose compared with the theoretical 0.51 g/g. At the 24 hour point, the ethanol concentration of the SSCF process with a 45 FPU enzyme load reached 11.10 g/L, higher than the 9.61 g/L observed for the SSF process for corn stover. This trend continued during the remainder of the SSF experiments.

The concentrations of by-products during SSF are shown in Figure 6.21. The level of by-product concentrations at 96 hours were between 0.02-0.03 g/L for lactic acid, glycerol and acetic acid when the fermentation was conducted with *S.cerevisiae.* No lag phase was observed for any of the fermentations (SSCF and SSF) with pre-treated materials, indicating that the level of inhibitors produced in all the pretreatments was low enough not to affect the yeast's activity (Xu et al., 2009).

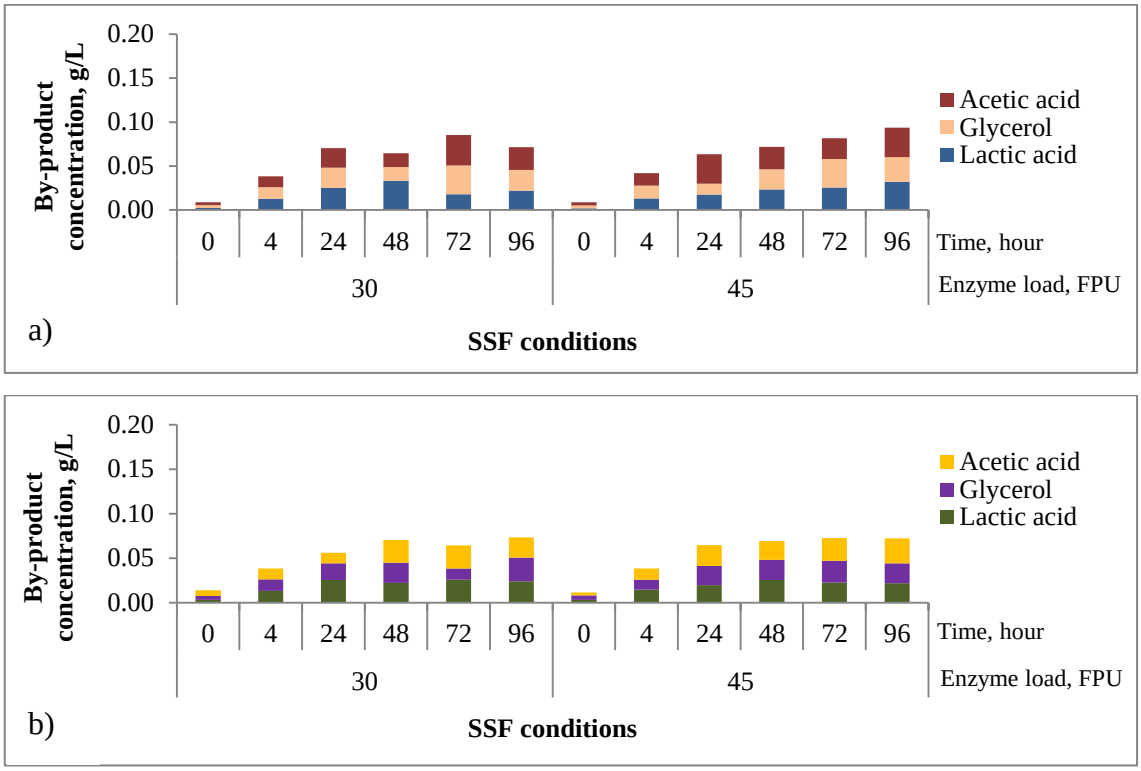


Figure 6.21. The concentration of by-products during SSF for (a) corn stover and (b) wheat straw.

6.3.5. Comparison of SSCF and SHCF Reactors

Bioreactor experiments were conducted to examine and compare the ethanol yield of SSCF at 32°C with separate hydrolysis and co-fermentation (SHCF), which was conducted using a pre-hydrolysis step for 24 hours at 50°C following fermentation for 72 hours at 30°C (Figure 6.22). The pre-treated corn stover that gave the highest ethanol yield of 88.99% at 32ºC with *S.cerevisiae ATCC® 20618™* and 30 FPU/g cellulose loading was used among the other conditions and yeast culture. The reactor vessel was equipped with a water jacket to maintain a constant temperature and a pH controller to keep the pH of the reactor at the desired level, as seen in Figure 6.20. The fermenter was equipped with sensors for control and data sampling of temperature, pH, and pO_2 (partial pressure of oxygen).

The catheterization parameters of pre-treated solids and the amount of each solution used in experiments are given in Table 6.12 for corn stover. The pre-treated corn stover contains about 58.41% glucan and 2.63% xylan by weight, meaning that there were

47.03 g/L of total carbon source during the bioreactor assays in 3% w/w cellulose loading with a total working weight of 1500 g.

Figure 6.22. Application of bioreactor experiments with pre-treated corn stover.

Table 6.12. The basic parameters of pre-treated solids and the amount of solutions used in bioreactor experiments.

Parameters	**Corn stover**
Xylose content of pre-treated solids (%)	2.63
Cellulose content of pre-treated solids (%)	58.42
Cellulose content of 1500 g of working weight (3% w/w) (g)	45.00
Total solids content (%)	20.42
The weight of pre-treated solids that contains 3 g cellulose (g)	77.03
The weight of the solids with TS content correction (g)	377.22
Cellulose content (g/100 g)	0.58
Enzyme dosage (FPU/g)	30.00
FPU of enzyme	2025.00
Enzyme activity of the cellulase (FPU/mL)	142.31
The volume of enzyme (mL)	14.23
The volume of inoculum (mL)	15.00
10X YP medium (mL)	150.00
Citrate buffer (mL)	75.00
DI water (mL)	383.55
f (g/g)	0.61
Concentration of biomass (g/L)	77.03

The concentrations of sugars/ethanol and by-products during bioreactor experiments for the SSCF process were plotted versus time (hour), and the results are given in Figure 6.23. In SSCF, the experiments were run for 96 hours. The glucose consumption and ethanol production were rapid during the first 24 hours. The free glucose in the enzymatic hydrolysis residue was observed to be exhausted at 24 hours, where the ethanol concentration was 19.13 g/L. The high glucose concentration prevented xylose uptake, likely due to competition for common transporters (Meinander et al., 1999); the xylose concentration remained almost constant at approximately 2 g/L, whereas the glucose concentration decreased swiftly during first 12 hours.

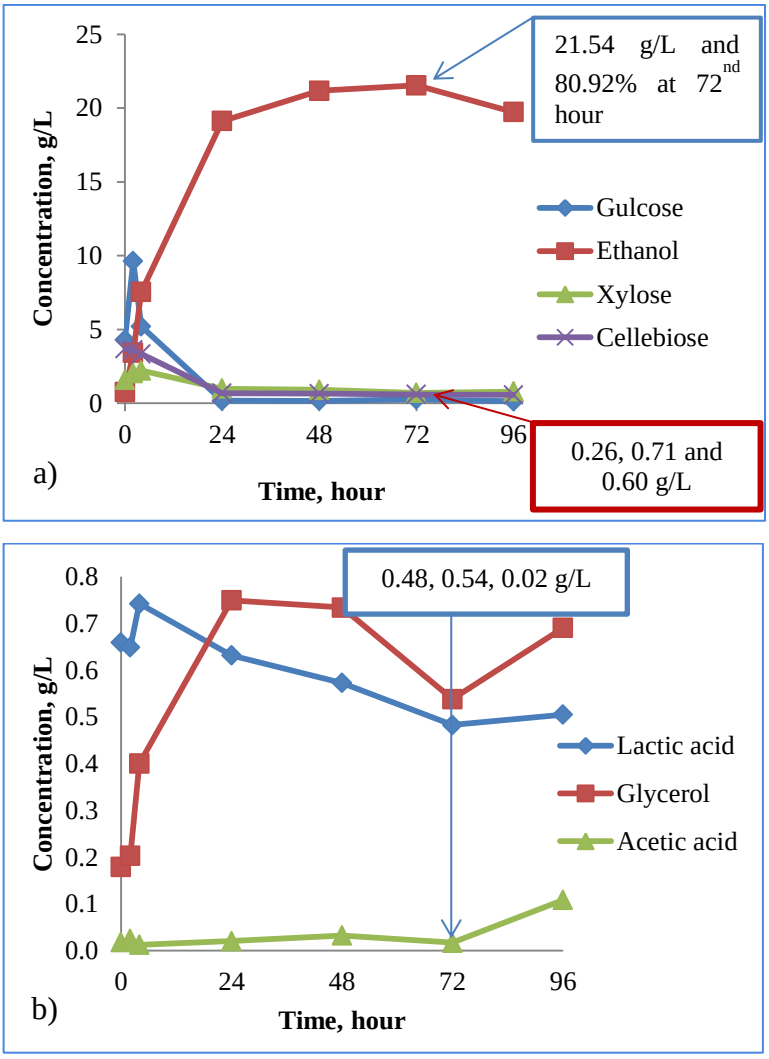


Figure 6.23. The concentrations of (a) sugars/ethanol and (b) by-products for SSCF.

Xylose was co-fermented only after 24 hours when the glucose concentration had been reduced to 0.17 g/L. Co-utilization of xylose commenced and continued throughout the experiment when the glucose concentration decreased. Finally, glucose and xylose were co-fermented giving the highest ethanol concentration of 21.54 g/L, which corresponded to an ethanol yield of 80.92% of the theoretical yield based on the glucose and xylose contents in the raw material (Figure 6.23a).

In SHCF, the pre-hydrolysis stage was run for 24 hours, and the time at which the yeast was added was referred to as "time zero". The experiments were run for another 96 hours, and the pH was maintained at 5.0 by addition of 1M NaOH or 1M H_2SO_4. Samples were withdrawn during pre-hydrolysis (at time −24, −4, −2, −0) and after (at 0, 4, 24, 48, 72 and 96 hours of fermentation). These samples were analyzed for ethanol, sugars, glycerol, acetic acid and lactic acid. The concentrations of sugars/ethanol and by-products during SHCF are given in Figure 6.24.

During SHCF experiments, the glucose concentration was 23.64 g/L after 24 hours of pre-hydrolysis (Figure 6.24a). Addition of yeast resulted in an immediate consumption of glucose by the yeast and a concomitant increase in ethanol concentration. After 24 hours, the ethanol concentration had reached 11.28 g/L. The highest ethanol concentration was obtained at the 72^{nd} hour as 20.82 g/L, corresponding to an overall ethanol yield of 78.19% based on the glucose content of the raw material. The concentration of by-products (lactic acid, acetic acid and glycerol) remained constant during prehydrolysis and started to increase with the addition of yeast in the fermentation process due to hemicellulose degradation (Figure 6.24b).

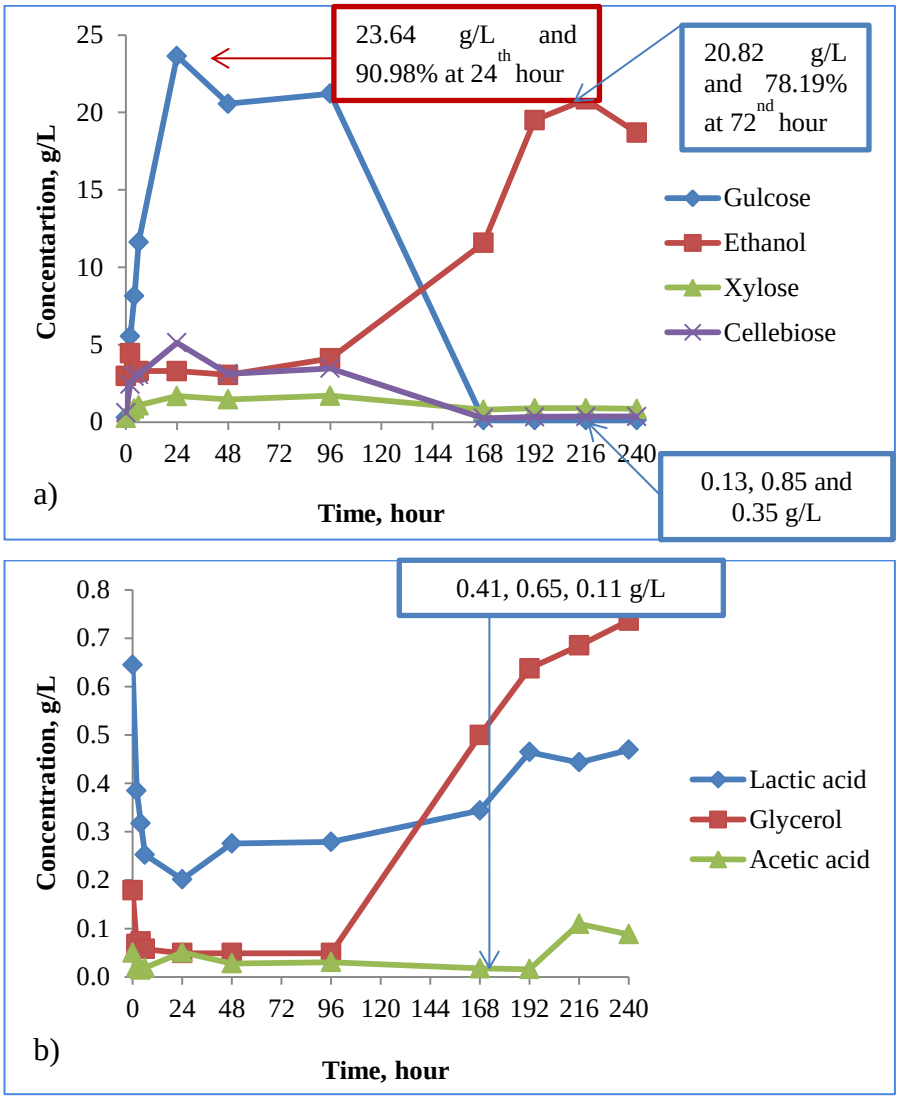


Figure 6.24. The concentrations of (a) sugars/ethanol and (b) by-products for SHCF.

The highest ethanol concentration obtained by SHCF (20.82 g/L) was similar with the concentration obtained by SSCF (21.54 g/L) [(Pearson's r= 0.99) (Figures 6.23a and 6.24a)]. There was a slight decrease in the yield of ethanol for the SHCF reactor. Sudden changes in the osmotic pressure in the surroundings could be the reason for the lower ethanol yield in the experiment with pre-hydrolysis, since this causes yeast cells to make physiological changes. This response is often referred to as the osmostress response. Production of glycerol is one way for the yeast cells to adapt to the change in osmotic pressure. Ohgren et al. (2007) studied the effect on overall ethanol yield of enzymatic pre-hydrolysis prior to SSF of steam pre-treated corn stover at 200°C for 5 minutes with 2% SO_2. Pre-hydrolysis with the commercial enzyme mixture at 48°C and fermentation at

35ºC resulted in a 26.4 g/L ethanol concentration, corresponding to an overall ethanol yield of 75.0%, which is lower than the yields reached in this study (Ohgren et al., 2007).

6.4. Environmental Sustainability Assessment of Bioethanol Fuel

The objective of this part was to evaluate environmental and economic performance of lignocellulosic bioethanol fuel compared to gasoline using Life Cycle Assessment (LCA) and Environmental Life Cycle Costing (ELCC) analyses for E10, E85 and CG fuels. The scope of the study included the whole life cycle of gasoline and bioethanol fuel use, consisting raw material acquisition, transport of raw materials and fuels, production and combustion of fuel blends. The potential environmental impacts of the selected fuel blends of E10, E85 and CG were compared for a 1-km travel distance functional unit (FU).

6.4.1. System Boundaries

The system boundaries of the bioethanol production and consumption were divided three subsystems; “feed stock acquisition” (S1), “bioethanol production” (S2) and “combustion of fuel blends” (S3). These subsystems are shown in detail in Figure 6.25. The main sources of data used in the LCI of the defined boundary are summarized in Table 6.13. The feedstock acquisition subsystem (S1) consists of baling of straw/stover in the field, transportation of bales to a temporary storage area, interim storage of the bales and transportation of bales to the bioethanol plant. All emissions arising from the use of machinery, such as baler machine, tractor and truck were considered in the S1 (Table 6.13). In this part of the study, an agricultural subsystem for cereal harvesting has not been included within the system boundary since straw/stover has been accepted as by-products of cereal production.

The raw material, process chemicals, the energy used for the bioethanol production and theoretical ethanol yield (427 L/ton dry feedstock) are based on the bioethanol conversion process data reported by the National Renewable Energy Laboratory for corn stover (Humbird and Aden, 2009). It has been assumed that the given bioethanol production efficiency is equivalent to other lignocellulosic feedstocks, as well.

The feedstock preparation and bioethanol production subsystem (S2) has been defined with seven processes: (1) feedstock storage; (2) dilute sulfuric acid/steam explosion pretreatment; (3) saccharification and co-fermentation; (4) distillation to refine ethanol up to 99.5%; (5) storage of ethanol; (6) wastewater treatment plant (WWTP), where the wastewater from the distillation and evaporator are treated; and (7) energy production (electricity and process heat) from lignin residue and solids from WWTP (Figure 6.25) (Borrion et al., 2012; Humbird and Aden, 2009). The renewable resources such as the lignin fraction of the biomass and sludge from WWTP were assumed to be used as fuel to produce energy in the bioethanol plant; as a result, there was no fossil fuel consumption to produce the energy requirements including electricity consumption from the grid (González-García et al., 2009).

The distribution of bioethanol from a bioethanol plant to a petrol station has been considered within the combustion of fuel blends (S3) subsystem. Combustion of fuels in a FFV was evaluated for E10, E85 and CG.

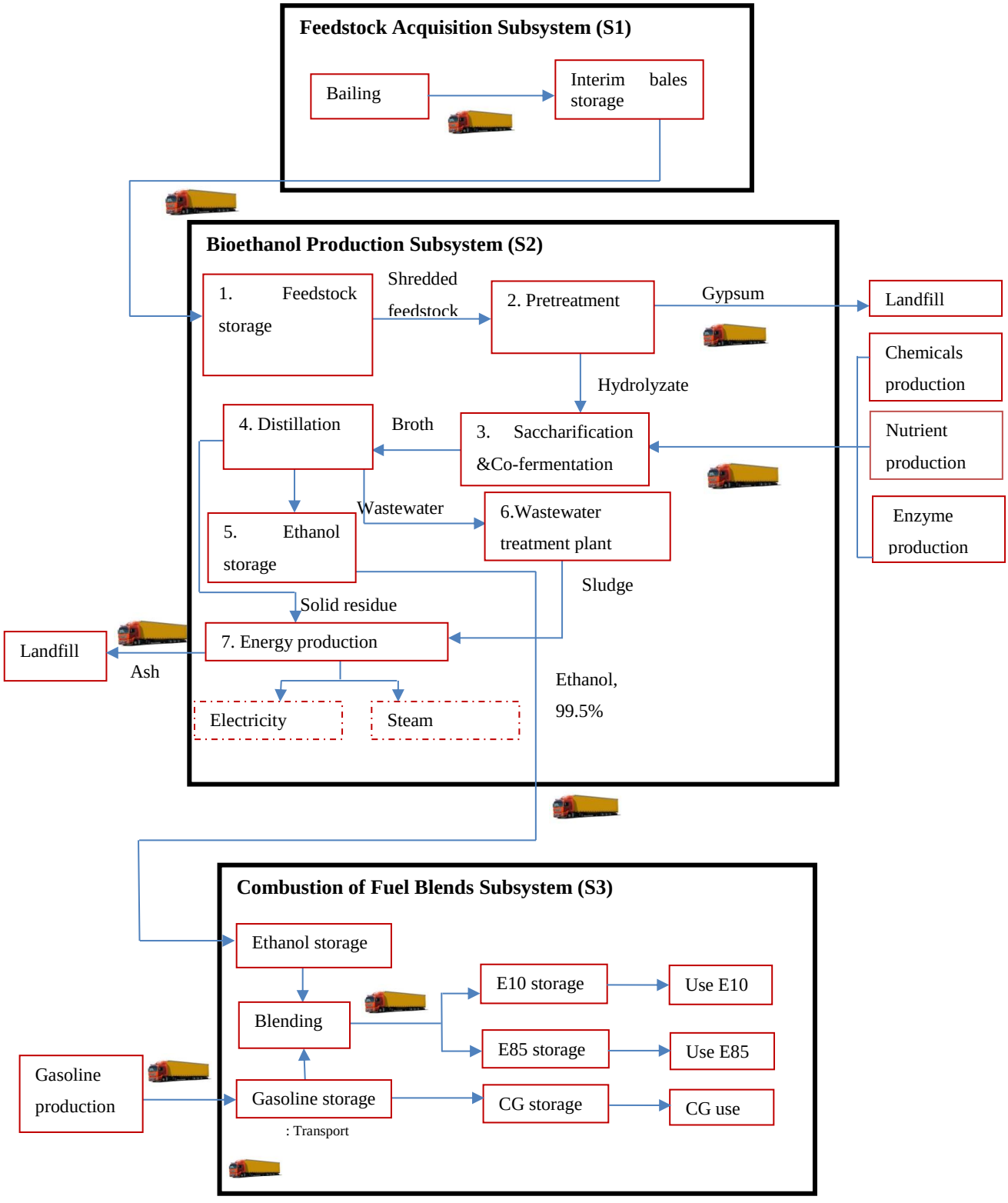


Figure 6.25. System boundaries for lignocellulosic bioethanol fuel blends (E10 and E85).

Table 6.13. The inventory data sources for corn stover based bioethanol life cycle.

Subsystem	Required data	Data sources
S1	Fuel use for baler machine, chemical production and consumption for baling	Ecoinvent report (Nemecek and Kägi, 2007) and the Institute of Environmental Sciences report (Voet et al., 2008; Quintero et al., 2013; Spatari et al., 2010)
	Fuel consumption, distance, capacity for biomass transport	Assumptions (see Table 6.15)
	Fuel production (diesel, gasoline, etc.)	GaBi 4-Ecoinvent database
S2	The theoretical ethanol yield, production capacity of plant and biomass use, chemical, nutrient and enzyme production and use	National Renewable Energy Laboratory (NREL) Technical Report (Humbird and Aden, 2009), Scientific papers (González-García et al., 2009; Voet et al., 2008; Jensen et al., 2007), Master thesis (Bawa, 2008; Corredor, 2008) and GaBi 4-Ecoinvent database
	Energy requirements for the machinery of bioethanol plant	Scientific paper (Luo et al., 2009a; Hu et al., 2004)
	Wastes that recovered, treated or disposed	Scientific paper (González-García et al., 2009)
	WWTP	Research reports (Humbird and Aden, 2009), GaBi 4-Ecoinvent database
	Landfill operation	GaBi 4-Ecoinvent database
	Fuel use, distance, capacity for chemical transport	Assumptions (see Table 6.15)
S3	Fuel use, distance, capacity for gasoline and bioethanol transport, bioethanol, gasoline and blends storage	Assumptions (see Table 6.15)
	Fuel economy values of E10, E85 and CG	Scientific papers (González-García et al., 2010)
	Emission data of car driving	Scientific papers (Kim and Dale, 2005; Kim and Dale, 2006)

6.4.2. Life Cycle Inventory (LCI) Analysis

The inventory data used in S1 is given in Table 6.14 (Nemecek and Kägi, 2007). In feedstock acquisition (S1), diesel fuel is consumed as a result of transportation and loading/uploading of straw/stover bales. Material and energy inventories for diesel fuel production which was taken from GaBi 4 software Ecoinvent database have also been included in the study. Before the bales are fed into the plant, they are stored in a so-called interim straw/stover storage facility. The bales are unloaded from the tractor-trailer using a telescopic handler when they arrive at the interim storage area. Diesel fuel was assumed to be consumed during unloading of the bales into the stack and the loading from the stack. The stack is covered by a low-density polypropylene sheet having an approximate sheet thickness of 1 mm. The assumptions related to transport activities of bioethanol life cycle is given in Table 6.15. In Table 6.14, 3.125 is the conversion factor of [500 kg/160 kg], the fuel consumption for baling round bales of 160 kg is 6.8 L/h, the specific weight of diesel was assumed to be 0.84 kg/L, the operation time for baling is 0.23 times 'silage baling' (0.13 h/bale), 32.9 MJ is the electricity consumption of telescopic handler for baling a round bale and 0.258 L/MJ is the diesel consumption of the telescopic handler in Ecoinvent (Nemecek and Kägi, 2007; McKechnie et al., 2015).

Table 6.14. The inventory data used at S1 (Nemecek and Kägi, 2007).

Process	Flow	Class	Value	Remark
Baling and loading bales	Diesel	Fuel	0.534 kg/bale	0.84 kg/L * 0.13 h/bale *0.23 * 6.8 L/h * (3.125)
	Straw/stover	Material	500 kg	
	Bale	Material	1 unit	
Interim storage and loading bales	Polyethylene	Material	0.9936 kg/bale	The density of polyethylene 920 kg/m^3
	Diesel	Material	0.713 kg/bale	For telescopic handler [32.9 MJ/ bale] / [0.258 L/MJ] * [0.84 kg/L]
	Bale	Material	1 unit	

The inventory data of the bioethanol plant is given in Table 6.16. The global inventory table for lignocellulosic bioethanol production published by González-García et al. (2009) was used to calculate these input and output data for 1000 kg bioethanol production.

Table 6.15. The assumptions related to transport activities of bioethanol life cycle.

Materials	**Transport mode**	**Capacity (tones)**	**Average distance (km)**
Biomass from farm to temporary storage	Diesel tractor	8	50
Biomass from temporary storage area to bioethanol production plant	Diesel truck	16	100
Pretreatment and SSFF chemicals from wholesalers to bioethanol plant	Diesel lorry	16	15
Enzyme from wholesalers to bioethanol plant	Diesel lorry	16	30
Solid wastes from bioethanol plant to landfill area	Diesel lorry	16	25
Ethanol from bioethanol plant to blending refinery	Diesel lorry	26	20
Bioethanol blends to regional storage	Diesel lorry	26	30

Table 6.16. Input/output evaluation of the bioethanol plant (Daylan and Ciliz, 2016).

<table>
<tr><th>Inputs</th><th>Value (kg)</th><th>Energy</th><th>Value (MJ)</th><th colspan="2">Outputs</th></tr>
<tr><td rowspan="2">Corn stover (12.5% moisture content)</td><td rowspan="2">4200</td><td>Electricity [a]</td><td>3840</td><td>Materials</td><td>Value</td></tr>
<tr><td>Steam [a]</td><td>7167</td><td>Ethanol (99.5%) (kg)</td><td>1000</td></tr>
<tr><td>Well water</td><td>7700</td><td rowspan="2">Transport</td><td rowspan="2">Value (km)</td><td>Electricity (MJ)</td><td>3840</td></tr>
<tr><td>Vinyl acetate</td><td>1.1</td><td rowspan="3">16 ton lorry</td><td rowspan="3">15</td><td>Steam (MJ)</td><td>7167</td></tr>
<tr><td>Sulfuric acid</td><td>133</td><td rowspan="2">Wastes to treatment</td><td rowspan="2">Value (kg)</td></tr>
<tr><td>Lime</td><td>97</td></tr>
<tr><td>Diammonium phosphate (DAP)</td><td>5.7</td><td rowspan="2">16 ton lorry</td><td rowspan="2">15</td><td>Lignin (to boiler)</td><td>450</td></tr>
<tr><td>Corn steep liquor (CSL)</td><td>44</td><td>Gypsum (to landfill)</td><td>295</td></tr>
<tr><td>Enzyme</td><td>38</td><td rowspan="3">16 ton lorry</td><td rowspan="3">30</td><td>Waste water[b] (to treatment plant)</td><td>980</td></tr>
<tr><td>Urea</td><td>16</td><td rowspan="2">Ash (to landfill)</td><td rowspan="2">96</td></tr>
<tr><td>Ammonia</td><td>12</td></tr>
<tr><td colspan="6">[a] From energy production process
[b] The 87% of process water was recovered from the WWTP and sent back to the process.</td></tr>
</table>

The energy balance of the bioethanol plant is given in Table 6.17 (Luo et al., 2009). The electrical energy consumed for the machinery in SSCF processes are supplied by the boiler through lignin combustion. 1000 kg of ethanol (up to 99.5% pure) is obtained and sent to the ethanol storage area (20 km from plant to ethanol storage area as given in Table 6.15). A mass of 450 kg of lignin is obtained as solid residue from the distillation process and used as boiler fuel (as well as sludge from the WWTP) to produce steam and electricity. As a result; the bioethanol plant was assumed as an energy self-sufficient process which was also considered in lignocellulosic residue based bioethanol studies published in the literature (González-García et al., 2010; González-García et al.,2012). In the boiler, electrical energy and steam are produced by lignin combustion, and ash is produced as process residue. The produced ash is sent to the landfill area.

Table 6.17. The energy balance of the bioethanol plant (Luo et al., 2009).

Process	**Electricity (MJ)**	**Steam (MJ)**
Milling and washing	250	-
Steam pretreatment	270	5330
SSCF	2727	
Distillation	245	1837
WWTP	240	-
Total	3732	7167

The combustion of selected fuel blends has been considered for an average passenger car. The combined fuel economy values for CG, E10 and E85 fueled vehicles were calculated using the data in Table 6.18 as 10.91, 10.51 and 8.29 km/L, respectively (González-García et al., 2010; González-García et al., 2009). According to these values, the amount of bioethanol and gasoline fuels consumed as g/km for the selected fuel blends were calculated and given in Table 6.18.

Table 6.18. The average fuel economy considered in the FFV for the selected fuel blends (González-García et al., 2010).

Fuel category	**The fuel economy value (km/L)**	**Fuel requirement (g/km)**	
		Bioethanol	**Gasoline**
CG	10.91	-	65
E10	10.51	6.89	62.01
E85	8.29	77.95	13.76
Density of gasoline and ethanol : 0.710 kg/L and 0.789 kg/L respectively			

The distribution of E10 and E85 to regional storage area (30 km by a 26 ton lorry as given in Table 6.15) and combustion of fuel blends in a passenger car have been included in the S3. The data related to CG production was taken from GaBi4 software's Ecoinvent database (Table 6.13).

6.4.3. Life Cycle Impact Assessment

The data summarized in the inventory phase have been interpreted through classification and characterization using GaBi4 LCA software. LCIA has been conducted using characterization factors from the EDIP 2003 methodology. In this step, the impact categories have been defined, and the input/output data in the inventory have been assigned to the environmental impact categories according to their capacity for contribution to different problem areas.

The emission types resulting from the various stages of the life cycles of bioethanol and their associated environmental impact categories are given in Table 6.19. The air emissions emitted during the life cycle of each fuel blend based on a travel distance oriented FU perspective were calculated by the GaBi 4 LCA software and given in Table 6.20.

Table 6.19. Classifications of emissions to impact categories.

Impact Category	Emissions	Unit
Acidification (AP)	SO_2, NO_x, HCI, HF, NH_3, HS	m^2 UES
Aquatic eutrophication (AEP)	NO_x, NH_3, NH_4^+, NO_3^-, PO_4^{-2}	kg NO_3-eqv
Terrestrial eutrophication (TEP)	NH_3, NO_x	m^2 UES
Global warming (GWP)	CO_2, CH_4, CO, NMVOC, N_2O	kg CO_2-eqv
Photochemical oxidant formation (POP)	CO, CH_4, NMVOC, NO_x	person * ppm * h
Stratospheric ozone depletion (SOP)	Halocarbons (CFCs, HCFCs)	kg R11-eqv

Table 6.20. Air emissions over the life cycle of each fuel based on 1 km FU perspective (Daylan and Ciliz, 2016).

Fuel	**CO_2 (kg/km)**	**CO (mg/km)**	**NMVOC (mg/km)**	**NO_x (mg/km)**	**NH_3 mg/km)**	**SO_2 (mg/km)**	**N_2O (mg/km)**	**CH_4 (mg/km)**
CG	0.27	522	107	198	0.43	420	0.65	135
E10	0.26	284	109	224	1.70	411	0.67	185
E85	0.13	341	63	237	15	227	0.84	670

According to the results given in Table 6.20, CO_2 emissions for E85 and E10 are lower by 52.7% and 5.1% relative to CG, respectively; and further, CO emissions for E85 and E10 are lower by 34.7% and 45.6%, respectively. Regarding the CG and E10 fuel life cycle, the main CO_2 emission releases result from fuel combustion and petrol refinery stages.

SO_2 emissions are lower by about 46% for E85 and 2.1% for E10 when compared to CG. With respect to the E10 fuel life cycle, the petrol refinery and fuel combustion stages are the sources of 85% and 10% of SO_2 emissions, respectively.

The highest N_2O emissions occur when E85 is used. N_2O emissions decrease by 20.2% and 22.6% when E10 and CG are used, respectively. The reason for the high emissions rate of NH_3, N_2O and NO_3^- for E85 is the nitrogen-based chemical consumption in the SSCF process, especially the cellulase production stage and the high exhaust gas emission of fuel combustion.

Regarding organic emissions, petrol refinery and fuel combustion stages are the main processes accounting for NMVOC emissions from CG. The SSCF (especially cellulase production) stage, together with the above mentioned processes, is also responsible for NMVOC emission releases from E10 and E85. Compared to E10 and CG, a reduction of approximately 42% of NMVOC emissions is obtained by E85. However, CH_4 emissions are lower by 73% and 80% for E10 and CG, respectively, compared to E85 since bioethanol production (especially SSCF and pretreatment) accounts for 80% of CH_4 emissions for E85.

During characterization, the contribution of emissions to each impact category was calculated by GaBi4 LCA software. Table 6.21 summarizes the quantified LCA characterization results for E10, E85 and CG for a 1-km travel distance FU.

Table 6.21. Potential environmental impacts of E10 and E85 and CG life cycle (Daylan and Ciliz, 2016).

Category	**Unit**	**E10**	**E85**	**CG**
GWP	kg CO_2-eqv	0.27	0.15	0.28
SOP	kg R11-eqv	$2.96x10^{-8}$	$1.05x10^{-8}$	$3.06x10^{-8}$
AP	m^2 UES	$9.29x10^{-3}$	$6.49x\ 10^{-3}$	$9.20x10^{-3}$
TEP	m^2 UES	$5.86x10^{-3}$	$7.59x10^{-3}$	$5.08x10^{-3}$
AEP	kg NO_3-eqv	$1.25x10^{-4}$	$3.34x10^{-4}$	$0.94x10^{-4}$
POP	pers*ppm*hours	$4.31x10^{-8}$	$5.50x10^{-8}$	$3.99x10^{-8}$

According to characterization results, using bioethanol blends in the form of E10 and E85 as gasoline substitutes leads to a 4.7% and 47.1% reduction of global warming potential (GWP) relative to CG. According to the data given at Morales et al, (2015), GHG emissions reduction is less than 10% for E10 blend and higher than 40% for E85 and upper blends compared to CG.

While the acidification potential (AP) of CG is the almost same with E10, the AP of E85 is lower than CG by 29.5%. Generally, the main acidifying impacts result from the agricultural subsystem in the case of first generation bioethanol. In this study, since lignocellulosic feedstocks were considered as by-products of wheat and corn production,

the agricultural subsystem was not included to the system boundary; as a result, the AP of bioethanol used in the form of E85 is favorable compared to that of CG.

Due to the higher NH_3 emissions from the N-based chemical consumption for the SSCF process and the higher NO_x emissions from the combustion of fuel blends subsystem, using CG offers improved performance in terms of terrestrial eutrophication (TEP) relative to the bioethanol blends. CG provides TEP reductions of 13.3% and 33.1% compared to E10 and E85, respectively. Similarly, the aquatic eutrophication potential (AEP) of CG is lower than those of E10 and E85 by 24.8 and 71.9%, respectively.

CG yields reductions of 7.4% and 27.5% for photochemical ozone depletion (POP) compared to E10 and E85, respectively, due to the higher NO_x emissions of E10 and E85 from combustion of fuel blends subsystem.

Table 6.22 summarizes the detailed GWP emissions for E10, E85 and CG fuels. The LCIA results showed that the levels of emissions that contribute to GW were considerably reduced when shifting from CG to the bioethanol blends E10 and E85. Since the selected feedstocks were considered by-products of cereal production, agricultural production subsystem (fertilizer consumption) was not included in this study. As a result, N_2O emissions calculated in this study were found to be lower than those in literature (Tomás-Pejó et al., 2009).

While total GHG emissions resulting from CG fuel life cycle based on a-1 km driving distance was 0.277 kg CO_2-eqv, total GHG emissions for E10 and E85 life cycle were 0.265 kg CO_2-eqv and 0.147 kg CO_2-eqv, respectively. For E10 and CG, the main contributor to global warming is fuel combustion stage (approximately 85% of total contributions), followed by petrol refinery, which causes the primary GHG emissions such as CO_2 and CH_4. The elevated GHG emissions from E10 are due to the high proportion of gasoline in the blend (i.e. 90% by volume). Feedstock related activities present a negligible contribution (approximately 3% of the total) in E10 fuel life cycle.

When E85 is used as a transport fuel, results change significantly, and bioethanol production stage (approximately 48% of the total) becomes the key area of concern in

terms of GHG emissions, followed by the fuel combustion stage (~45% of total). Cellulase production and pretreatment stages of E85 fuel life cycle are the stages mainly responsible for GWP arising from the bioethanol plant, accounting for 43% and 42% of total GHG emissions, respectively. Many studies related to LCA of lignocellulosic bioethanol production have been published in the literature (Luo et al., 2009b; Borrion et al., 2012; González-García et al., 2010; González-García et al., 2012) however, in most of the cases it is not possible to compare the results due to differences on the allocations, focus or assumptions made by the LCA analysts.

Gonzalez-García et al. (2009) studied the environmental aspects of ethanol-based fuels from *Brassica carinata* and the results showed that using ethanol blends in form of E10 and E85 as a gasoline substitute leads to a reduction of GHG emissions. However, for POP, AP and AEP, CG offers better environmental performance than using ethanol blends. Luo et al. (2009b) evaluated the comparison of LCA results using different allocation methods for gasoline and corn stover based ethanol fuels. The results showed that the level of GWP and SOP are reduced when replacing gasoline by bioethanol fuel irrespective of the allocation method applied. For the rest of the impact categories (POP, AP and AOP), applying bioethanol fuel leads to worse environmental performance. These results were consistent with our study except for AP since agricultural production subsystem was not included in this study.

Table 6.22. GHG emissions of E10, E85 and CG fuels (Daylan and Ciliz, 2016).

GHG emission	**Unit**	**CG**	**E10**	**E85**
CO_2	kg CO_2-eqv/FU	0.27	0.26	0.13
CO	g CO_2-eqv/FU	1.04	0.57	0.68
CH_4	g CO_2-eqv/FU	3.38	4.62	16.80
N_2O	g CO_2-eqv/FU	0.21	0.21	0.27
NMVOC	g CO_2-eqv/FU	0.33	0.32	0.19

6.4.4. Life Cycle Costing

Using GaBi 4 LCA software, the bioethanol production cost was calculated as 0.32 €/kg (tax excluded) for the year 2010. The gasoline production cost has been accepted as

0.69 €/kg based on the reference year of 2009 (Piccolo and Bezzo, 2009).

The production cost of bioethanol from corn stover calculated in this study is with the range of minimum ethanol selling price which is calculated in different studies as $0.24 and $1.21 (Chovau at al., 2013; Langdon, 2007) and it is lower than the gasoline cost, almost half the price. This correlation has previously been shown by Luo et al. (2009) for sugarcane-based bioethanol and gasoline fuels production cost which was consistent with this study.

Table 6.23 gives the calculated cost for the fuel alternatives (i.e. E10, E85 and CG) for a-1 km travel distance FU. According to the fuel economy values given in Table 6.18, the fuel consumption of bioethanol per kilometer is higher than gasoline because bioethanol possesses a lower energy value than gasoline. For this reason, although the bioethanol production cost is lower than the gasoline production cost by 56%, the E10 driving cost compensates for the CG cost because of the higher fuel consumption ratio of bioethanol fuel. As a result, the driving cost is equal (0.047 V/km) for both E10 and CG. However, if the ethanol blend E85 is used, the driving cost for the bioethanol blend is 23% less compared to that of CG.

Table 6.23. Costs of 1 km driving for all the fuel alternatives (Daylan and Ciliz, 2016).

Case	**Gasoline**	**E10**	**E85**	**Unit**
Cost	0.047	0.047	0.036	€/km
Fuel economy	15.39	14.50	10.90	km/kg

7. CONCLUSIONS

Since there is no chemical composition data in the literature for Turkish corn stover and wheat straw to evaluate the yield of pretreatment and fermentation, characterization studies were conducted. The results showed that the selected feedstocks contain fermentable sugars at a level of approximately 46-48%, making them valuable feedstock for bioethanol production.

The pretreatment method applied with the optimum pretreatment condition (OPC) for corn stover from raw feedstock to pre-treated feedstock resulted in an increase in glucan content from 31.5% to 58.42% and a decrease in xylan content from 11.2% to 2.63%. For wheat straw samples, the glucan content increased from 33.2% to 60.30% and the xylan content decreased from 10.9% to 7.57%. These results indicated that optimized conditions obtained in this study provided an efficient sugar hydrolysis for the selected feedstocks, increasing the glucan content two-fold. The glucan content in the WIS increased due to hemicellulose removal.

High CSF values (3.03-3.23) of pretreatment conditions indicated that there was an increase in pretreatment severity, leading to high xylose loss and by-product concentrations. Pretreatment with the highest CSF values caused more degradation of sugars. As a result, all of the by-products in the hydrolysate were higher than those of the other pretreatments with low CSF.

During the enzymatic digestibility studies, the digestibility of pre-treated corn stover, and wheat straw at 50ºC and 32ºC were compared. The results demonstrated that the peak digestibility values of the pre-treated biomass at 32ºC were 84.4% and 79.37% glucan for corn stover and wheat straw, respectively. The enzymatic digestibility values at 32°C and 50°C were quite similar (Pearson's r= 0.99), indicating that high glucan recovery can also be accomplished at low temperature enzymatic hydrolysis.

Both of the yeasts, *S. cerevisiae ATCC® 20618™* and *S. cerevisiae*, produced high ethanol concentrations at 32ºC, which were 14.89 g/L and 15.00 g/L, respectively. As a

result, the temperature was selected as 32ºC, which was the optimized temperature for SSF studies. This is the first study that has shown a distinct decrease in the SSF (32ºC) relative to literature values, which refer to temperatures around 45-50ºC for hydrolysis.

The optimum enzyme dosage for enzymatic saccharification was selected as 30 FPU/g cellulose enzyme load for both the yeast and feedstock applications, since the ethanol yields obtained at 30 and 45 FPU/g cellulose enzyme load applications were similar (Pearson's r value=0.99). The recombinant strain of *S.cerevisiae ATCC® 20618™* showed an increased ethanol yield compared to *S.cerevisiae,* giving an additional yield of 0.29 g ethanol/g xylose (compared with theoretical 0.51 g/g) for corn stover. This value was 0.42 g ethanol/g xylose for wheat straw.

The results of bioreactor experiments indicated that the ethanol yield obtained at 32ºC was similar to the ethanol yield obtained at optimum temperatures of enzymatic saccharification (50ºC) and fermentation (30ºC) (Pearson's r value=0.99). This demonstrates the applicability of the optimized conditions (30 FPU/g cellulose enzyme load at 32ºC with recombinant *S.cerevisiae ATCC® 20618™* for corn stover).

After the optimization of bioethanol fuel production by laboratory-scale experiments, the environmental and economic performance of bioethanol fuel relative to gasoline were evaluated by LCA and ELCC. It has been shown that one kilometer driven by E10 and E85 fueled vehicles can reduce GHG emissions by 4.7% and 47.1% with respect to CG, respectively. It was calculated that the bioethanol production cost was lower than the gasoline production cost by 56%. Additionally, E85 can provide a 23% lower driving cost compared to CG.

Optimization results found in this study will have definite implications for large-scale bio-refinery applications since laboratory-scale systems are important early-stage tools for scaling new biofuels technology. Moreover, the calculated cost reductions made cellulosic bioethanol production cost-competitive with gasoline production. Furthermore, emerging studies for bioethanol production may assist to lower both the environmental impacts and the costs of bioethanol fuels for future case scenarios.

8. RECOMMENDATIONS FOR FUTURE WORK

According to the results obtained in this study, the following recommendations can be made for future research:

1) In the literature, a limited number of studies were observed for characterization of Turkish lignocellulosic material. Since compositional analysis is the first step for evaluation of biomass bioconversion efficiency, chemical characterization of different types of feedstocks (e.g., garden wastes, hard wood, etc.) should be considered.

2) The ethanol yield can be compared in detailed for feedstocks with different levels of lignin and ash contents since ethanol yield is highly influenced by ash content. Ultimate and proximate analysis of feedstocks should also be incorporated into yield studies.

3) In this study, the ethanol yield was investigated using the solids residue obtained from dilute acid/steam explosion pretreatment. Yield studies can be further conducted for solid-liquid state fermentation that uses hydrolysate of pretreatment with different ratios. Inhibition of yeast cultures by the by-products of hydrolysate can also be investigated in detailed for better understanding of the effect of by-product concentration on yeast performance.

4) Microbiological techniques can be integrated into fermentation experiments to produce new cultures of recombinant yeast to increase the ethanol yield by fermenting xylose sugars in high yields.

REFERENCES

Acaroğlu, M., Aydoğan, H., 2012. Biofuels energy sources and future of biofuels energy in Turkey. Biomass and Bioenergy, 36, 69–76.

Adapa, P., Tabil, L., Schoenau, G., 2009. Compaction characteristics of barley, canola, oat and wheat straw. Biosystems Engineering, 104, 335–344.

Adney B., and Baker, J., 2008. Laboratory analytical procedure for measurement of cellulase activities technical report, National Renewable Energy Laboratory. NREL/TP-510-42628. Golden, CO.

Asghari, A, Bothast, R.J., Doran, J., Ingram, L.O., 1996. Ethanol production from hemicellulose hydrolysates of agricultural residues using genetically engineered *Escherichia coli strain KO11*. Journal of Industrial Microbiology, 16, 42-47.

American Type Culture Collection (ATCC), 2013. Product Sheet; *Saccharomyces cerevisiae (ATCC® 20618™).*

Bawa, N., 2008. Improvement of bioethanol production using *Saccharomyces cerevisiae*, M.S. Thesis, University of Saskatchewan Saskatoon, the Department of Chemical Engineering.

Balat, M., 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion Management, 52, 858–875.

Balat, M., Balat, H., Öz, C., 2008. Progress in bioethanol processing. Progress in Energy and Combustion Science 34, 551–573.

Başçetinçelik, A., Öztürk, H.H., Karaca, C., Kaçira, M., Ekinci, K., Kaya, D., Baban, A., Güneş, K., Komitti, N., Barnes, I., Nieminen, M., 2005. A guide on exploitation of agricultural residues in Turkey. Project No. LIFE03TCY/TR/000061. European Commission Life Programme, Europe.

Binod, P., Sindhu, R., Singhania, R.R., Vikram, S., Devi, L., Nagalakshmi, S., Kurien, N., Sukumaran, R. K., Pandey, A., 2010. Bioethanol production from rice straw: An overview. Bioresource Technology, 101, 4767–74.

Borrion, A.L., McManus, M.C., Hammond, G.P., 2012. Environmental life cycle assessment of lignocellulosic conversion to ethanol: A review. Renewable and Sustainable Energy Reviews, 16, 4638–4650.

Cardona, C., Sánchez, O.J., 2007. Fuel ethanol production: process design trends and integration opportunities. Bioresource Technology, 98, 2415–57.

Carrasco, C., Baudel, H., Peñarrieta, M., Solano, C., Tejeda, L., Roslander, C., Galbe, M., and Lidén, G., 2011. Steam pretreatment and fermentation of the straw material "Paja Brava" using simultaneous saccharification and co-fermentation simultaneous saccharification and co-fermentation. Journal of Bioscience and Bioengineering, 111, 167–174.

Chen, M., Zhao, J., Xia, L., 2009. Comparison of four different chemical pretreatments of corn stover for enhancing enzymatic digestibility. Biomass and Bioenergy, 33, 1381–1385.

Chovau, S., Degrauwe, D., Van der Bruggen, B., 2013. Critical analysis of techno-economic estimates for the production cost of lignocellulosic bio-ethanol. Renewable and Sustainable Energy Reviews, 26, 307–321.

Claassen, P.M., Lier, J.B., Lopez Contreras, M., Niel, E.W.J., Sijtsma, L., Stams, J.M., Vries, S.S., Weusthuis, R., 1999. Utilization of biomass for the supply of energy carriers. Applied Microbiology and Biotechnology 52, 741–755.

Corredor, D.Y., 2008. Pretreatment and enzymatic hydrolysis of lignocellulosic biomass, PhD. Thesis, Kansas State University, Department of Biological & Agricultural Engineering.

Daylan, B., and Cılız, N., 2016. Life cycle assessment and environmental life cycle costing analysis of lignocellulosic bioethanol as an alternative transportation fuel. Renewable Energy, 89, 578-587.

Dowe, N, Mcmillan, J., 2008. Laboratory analytical procedure for SSF experimental protocols-lignocellulosic biomass hydrolysis and fermentation technical report, National Renewable Energy Laboratory. NREL/TP-510-42630. Golden, CO.

Eddy, F., 1998. Laboratory analytical procedure for determination of insoluble solids of pretreated biomass material technical report, National Renewable Energy Laboratory. LAP-018. Golden, CO.

Erdei, B., Frankó, B., Galbe, M., Zacchi G., 2013. Glucose and xylose co-fermentation of pretreated wheat straw using mutants of *S. cerevisiae TMB3400*. Journal of Biotechnology, 164, 50– 58.

Foyle, T., Jennings, L., Mulcahy, P., 2007. Compositional analysis of lignocellulosic materials: evaluation of methods used for sugar analysis of waste paper and straw. Bioresource Technology, 98, 3026–36.

Fu, D., Mazza, G., 2011. Optimization of processing conditions for the pretreatment of wheat straw using aqueous ionic liquid. Bioresource Technology, 102, 8003–10.

Garlock, R.J., Chundawat, S.P., Balan, V., Dale, B.E., 2009. Optimizing harvest of corn stover fractions based on overall sugar yields following ammonia fiber expansion pretreatment and enzymatic hydrolysis. Biotechnology for Biofuels 2, 29-35.

Ghose, T.K., 1987. Measurement of cellulase activities, Pure and Applied Chemistry, 59, 257-268.

Gong, C., Cao, N., Du, J., Tsao, G., 1999. Ethanol production from renewable resources. Advances in Biochemical Engineering / Biotechnology, 65, 207-241.

González-García, S., Gasol, C.M., Gabarrell, X., Rieradevall, J., Moreira, M.T., Feijoo, G., 2010. Environmental profile of ethanol from poplar biomass as transport fuel in Southern Europe. Journal of Renewable Energy 35, 1014–1023.

González-García, S., Gasol, C.M., Gabarrell, X., Rieradevall, J., Moreira, M.T., Feijoo, G., 2009. Environmental aspects of ethanol-based fuels from *Brassica carinata*: A case study of second generation ethanol. Renewable and Sustainable Energy Reviews. 13, 2613–2620.

González-García, S., Iribarren, D., Susmozas, A., Dufour, J., Murphy, R.J., 2012. Life cycle assessment of two alternative bioenergy systems involving Salix spp. biomass: Bioethanol production and power generation. Applied Energy, 95, 111–122.

González-García, S., Luo, L., Moreira, M.T., Feijoo, G., Huppes, G., 2009. Life cycle assessment of flax shives derived second generation ethanol fueled automobiles in Spain. Renewable and Sustainable Energy Reviews, 13, 1922–1933.

González-García, S., Moreira, M.T., Feijoo, G., 2010. Comparative environmental performance of lignocellulosic ethanol from different feedstocks. Renewable and Sustainable Energy Reviews, 14, 2077–2085.

Hach, C.C., Brayton, S.V., Kopelove, A.B., 1985. A powerful Kjeldahl nitrogen method using peroxymonosulfuric acid. Journal of Agricultural and Food Chemistry, 33, 1117–1123.

Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H., Gholami, M., Ardjmand, M., 2013. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renewable and Sustainable Energy Reviews, 27, 77–93.

Hames, B., Ruiz, R., Scarlata, C., Sluiter, A., Sluiter, J., Templeton, D., 2008. Laboratory analytical procedure for preparation of samples for compositional analysis technical report, National Renewable Energy Laboratory. NREL/TP-510-42620. Golden, CO.

Hames, B., Scarlata, C., Nrel, A.S., 2008. Laboratory analytical procedure for determination of protein content in biomass technical report, National Renewable Energy Laboratory. NREL/TP-510-42625. Golden, CO.

Hsu, D., 1995. Laboratory analytical procedure for preparation of dilute-acid pretreated biomass, Technical Report, National Renewable Energy Laboratory. LAP-007, Golden, CO.

Hsu, C.L., Chang, K.S., Lai, M.Z., Chang, T.C., Chang, Y.H., Jang, H.D., 2011. Pretreatment and hydrolysis of cellulosic agricultural wastes with a cellulase-producing Streptomyces for bioethanol production. Biomass and Bioenergy, 35, 1878–1884.

Hu, Z., Fang, F., Ben, D., Pu, G., Wang, C., 2004. Net energy, CO_2 emission, and life-cycle cost assessment of cassava-based ethanol as an alternative automotive fuel in China. Applied Energy 78, 247–256.

Huijgen, W.J.J., Smit, T., Wild, P., Uil, H., 2012. Fractionation of wheat straw by prehydrolysis, organosolv delignification and enzymatic hydrolysis for production of sugars and lignin. Bioresource Technology, 114, 389–98.

Humbird, D., Aden, A., 2009. Biochemical production of ethanol from corn stover : 2008 state of technology model technical report NREL/TP-510-43205. Golden, CO.

Ibeto, C.N., Okoye, C.O.B., Ofoefule, A.U., 2014. Bio-ethanol production from thermally pre-treated corn chaff and cassava waste water, International Research Journal of Pure & Applied Chemistry, 4, 227–233.

Jensen, K.H., Thyø, K.A., 2007. 2nd generation bioethanol for transport : the IBUS concept-boundary conditions and, MS. Thesis, Technical University of Denmark, Department of Manufacturing Engineering and Management.

Kadam, K.L., McMillan, J.D., 2003. Availability of corn stover as a sustainable feedstock for bioethanol production. Bioresource Technology, 88, 17–25.

Kazi, F.K., Fortman, J., Anex, R., 2010. Techno-economic analysis of biochemical scenarios for production of cellulosic ethanol technical report, NREL/TP-6A2-46588, Golden, CO.

Keshwani, D.R., Cheng, J.J., 2009. Switchgrass for bioethanol and other value-added applications: a review. Bioresource Technology, 100, 1515–23.

Kim, S., Dale, B.E., 2006. LCA Case Studies Ethanol Fuels : E10 or E85 – Life Cycle Perspectives. International Journal of Life Cycle Assessment, 11, 117–121.

Kim, S., Dale, B.E., 2005. Life cycle assessment of various cropping systems utilized for producing biofuels: Bioethanol and biodiesel. Biomass and Bioenergy 29, 426–439.

Kim, T.H., Taylor, F., Hicks, K.B., 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. Bioresource Technology, 99, 5694–5702.

Langdon, D., 2007. Life Cycle Costing (LCC) as a contribution to sustainable construction: a common methodology Final Report, Davis Langdon Management Consulting. UK.

Larsson, S., Reimann, A., Nilvebrant, N. & Jonsson, L.J., 1999. Comparison of different methods for the detoxification of lignocellulose hydrolysates of spruce. Applied Biochemical Biotechnology, 77, 91–103.

Limayem, A., Ricke, S.C., 2012. Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects. Progress in Energy and Combustion Science, 38, 449–467.

Linde, M., Jakobsson, E., Galbe, M., Zacchi, G., 2008. Steam pretreatment of dilute H_2SO_4-impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. Biomass and Bioenergy, 32, 326–332.

Liu, L., Ye, X.P., Womac, A. R., Sokhansanj, S., 2010. Variability of biomass chemical composition and rapid analysis using FT-NIR techniques. Carbohydrate Polymers, 81, 820–829.

Liu, Z.S., Wu, X.L., Kida, K., Tang, Y.Q., 2012. Corn stover saccharification with concentrated sulfuric acid: effects of saccharification conditions on sugar recovery and by-product generation. Bioresource Technology, 119, 224–33.

Luo, L., Voet, E., Huppes, G., 2009a. An energy analysis of ethanol from cellulosic feedstock–Corn stover. Renewable and Sustainable Energy Reviews, 13, 2003–2011.

Luo, L., Voet, E., Huppes, G., Haes, H., 2009. Allocation issues in LCA methodology: a case study of corn stover-based fuel ethanol. International Journal of Life Cycle Assessment, 14, 529–539.

Luo, L., Voet, E., Huppes, G., 2009b. Life cycle assessment and life cycle costing of bioethanol from sugarcane in Brazil. Renewable and Sustainable Energy Reviews, 13, 1613–1619.

Malça, J., Freire, F., 2011. Uncertainty Analysis of the Life-Cycle Greenhouse Gas Emissions and Energy Renewability of Biofuels. http://cdn.intechweb.org/pdfs/19177.pdf. (accessed June 2015).

Mariotti, F., Tome, D., Mirand, P.P., 2008. Converting nitrogen into protein- beyond 6.25 and Jones' factors. Critical Reviews in Food Science and Nutrition, 48, 177-184.

Matsushika, A., Inoue, H., Murakami, K., Takimura, O., Sawayama, S., 2009. Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting Saccharomyces cerevisiae. Bioresource Technology, 100, 2392–8.

McKechnie, J., Pourbafrani, M., Saville, B., MacLean, H.L., 2015. Exploring impacts of process technology development and regional factors on life cycle greenhouse gas emissions of corn stover ethanol. Renewable Energy, 76, 726–734.

Meinander, N.Q., Boels, I., Hahn-Hägerdal, B., 1999. Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1 and XYL2 from *Pichia stipitis* with and without overexpression of TAL1. Bioresource Technology 68, 79–87.

Miller, G.L., 1959. Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar. Analytical chemistry, 31, 426-428.

Morales, M., Quintero, J., Conejeros, R., Aroca, G., 2015. Life cycle assessment of lignocellulosic bioethanol: Environmental impacts and energy balance. Renewable and Sustainable Energy Reviews, 42, 1349–1361.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M.R., 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresource Technology, 96, 1986–93.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresource Technology, 96, 673–86.

Naik, S., Goud, V.V., Rout, P.K., Jacobson, K., Dalai, A.K., 2010. Characterization of Canadian biomass for alternative renewable biofuel. Renewable Energy, 35, 1624–1631.

Nemecek, T., Kägi, T., 2007. Life Cycle Inventories of Agricultural Production Systems. http://db.ecoinvent.org/reports/15_Agriculture.pdf. (accessed May 2015).

Ohgren, K., Bengtsson, O., Gorwa-Grauslund, M.F., Galbe, M., Hahn-Hägerdal, B., Zacchi, G., 2006. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with Saccharomyces cerevisiae TMB3400. Journal of Biotechnology, 126, 488–98.

Ohgren, K., Bura, R., Saddler, J., Zacchi, G., 2007. Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresource Technology, 98, 2503–10.

Ohgren, K., Vehmaanperä, J., Siika-Aho, M., Galbe, M., Viikari, L., Zacchi, G., 2007. High temperature enzymatic prehydrolysis prior to simultaneous saccharification and fermentation of steam pretreated corn stover for ethanol production. Enzyme and Microbial Technology, 40, 607–613.

Olofsson, K., Palmqvist, B., Lidén, G., 2010. Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnology for Biofuels, 3, 17-26.

Palmqvist, E. and Hahn-Hägerdal B. 2000. Fermentation of lignocellulosic hydrolysates. I : inhibition and detoxification. Bioresource Technology, 74, 17-24.

Paulová, L., Patáková, P., Rychtera, M., Melzoch, K., 2014. High solid fed-batch SSF with delayed inoculation for improved production of bioethanol from wheat straw. Fuel, 122, 294–300.

Philip Ye, X., Liu, L., Hayes, D., Womac, A., Hong, K., Sokhansanj, S., 2008. Fast classification and compositional analysis of corn stover fractions using Fourier transform near-infrared techniques. Bioresource Technology, 99, 7323–32.

Piccolo, C., Bezzo, F., 2009. A techno-economic comparison between two technologies for bioethanol production from lignocellulose. Biomass and Bioenergy, 33, 478–491.

Puth, M.T., Neuhäusera, M., Ruxton, G.D., 2014. Effective use of Pearson's product–moment correlation coefficient. Animal Behaviour, 93, 183–189

Quintero, J., Moncada, J., Cardona, C., 2013. Techno-economic analysis of bioethanol production from lignocellulosic residues in Colombia: a process simulation approach. Bioresource Technology, 139, 300–307.

Ratanakhanokchai, K., Waeonukul, R., Sakka, K., Kosugi, A., Mori, Y., 2013. Paenibacillus curdlanolyticus Strain B-6 multienzyme complex : a novel system for biomass utilization. In Matovic, M. D. (Eds.), Biomass Now – Cultivation and Utilization, 369-394, InTeOpP.

Ruiz, H., Silva, D.P., Ruzene, D.S., Lima, L.F., Vicente, A., Teixeira, J., 2012. Bioethanol production from hydrothermal pretreated wheat straw by a flocculating Saccharomyces cerevisiae strain – Effect of process conditions. Fuel, 95, 528–536.

Ruth, M.F., Thomas, S.R., Renewable, N., 2003. The effect of corn stover composition on ethanol process economics. http://www1.eere.energy.gov/bioenergy/pdfs/34040.pdf. (accessed March 2014).

Saha, B.C., Iten, L.B., Cotta, M., Wu, Y.V., 2005. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochemistry, 40, 3693–3700.

Sarkar, N., Ghosh, S.K., Bannerjee, S., Aikat, K., 2012. Bioethanol production from agricultural wastes: An overview. Renewable Energy, 37, 19–27.

Sassner, P., Zacchi, G., 2008. Integration options for high energy efficiency and improved economics in a wood-to-ethanol process. Biotechnology for Biofuels, 1, 4-15.

Silva, G.G.D., Rouau, S.G.X., 2011. Successive centrifugal grinding and sieving of wheat straw. Powder Technology, 208, 266–270.

Singh, D., Zeng, J., Laskar, D.D., Deobald, L., Hiscox, W.C., Chen, S., 2011. Investigation of wheat straw biodegradation by *Phanerochaete chrysosporium*. Biomass and Bioenergy, 35, 1030–1040.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J., 2008. Laboratory analytical procedure for determination of total solids in biomass and total dissolved solids in liquid process samples technical report. National Renewable Energy Laboratory. NREL/TP-510-42621. Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Laboratory analytical procedure for determination of ash in biomass technical report. National Renewable Energy Laboratory. NREL/TP-510-42622. Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008. Laboratory analytical procedure for determination of sugars, byproducts, and degradation products in liquid fraction process samples technical report. National Renewable Energy Laboratory. NREL/TP-510-42623. Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Laboratory analytical procedure for determination of structural carbohydrates and lignin in biomass technical report. National Renewable Energy Laboratory. NREL/TP-510-42618. Golden, CO.

Sluiter, A., Hyman, D., Payne, C., Wolfe, J., 2008. Laboratory analytical procedure for determination of ınsoluble solids in pretreated biomass material technical report. NREL/TP-510-42627. Golden, CO.

Sluiter, A., Sluiter J., 2008. Laboratory analytical procedure for determination of starch in solid biomass samples by HPLC technical report. NREL/TP-510-42624. Golden, CO.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Laboratory analytical procedure for determination of extractives in biomass technical report. National Renewable Energy Laboratory. NREL/TP-510-42619. Golden, CO.

Sluiter, A., Sluiter, J., Wolfrum, E.J., 2004. Methods for Biomass Compositional Analysis. In Behrens, M., Datye, A.K., (Eds.), Catalysis for the Conversion of Biomass and Its Derivatives, Germany, ISBN: 9783844242829.

Sluiter, J.B., Ruiz, R.O., Scarlata, C.J., Sluiter, A.D., Templeton, D.W., 2010. Compositional analysis of lignocellulosic feedstocks: Review and description of methods. Journal of Agricultural Food Chemistry, 58, 9043–53.

Spatari, S., Zhang, Y., Maclean, H.L., 2010. Life cycle assessment of switchgrass- and corn automobiles. Environmental Science and Technology, 39, 9750–9758.

Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic materials for ethanol production : a review. Bioresource Technology, 83, 1–11.

Tabka, M.G., Herpoël-Gimbert, I., Monod, F., Asther, M., Sigoillot, J.C., 2006. Enzymatic saccharification of wheat straw for bioethanol production by a combined cellulase xylanase and feruloyl esterase treatment. Enzyme and Microbial Technology, 39, 897–902.

Tao, L., Templeton, D.W., Humbird, D., Aden, A., 2013. Effect of corn stover compositional variability on minimum ethanol selling price (MESP). Bioresource Technology, 140, 426–430.

Templeton, D.W., Scarlata, C.J., Sluiter, J.B., Wolfrum, E.J., 2010. Compositional analysis of lignocellulosic feedstocks. Journal of Agricultural Food Chemistry, 58, 9054–9062.

Templeton, D.W., Sluiter, A.D., Hayward, T.K., Hames, B.R., Thomas, S. R., 2009. Assessing corn stover composition and sources of variability via NIRS. Cellulose, 16, 621–639.

Tomás-Pejó, E., Oliva, J.M., González, Ballesteros, I., Ballesteros, M., 2009. Bioethanol production from wheat straw by the thermotolerant yeast *Kluyveromyces marxianus CECT 10875* in a simultaneous saccharification and fermentation fed-batch process. Fuel, 88, 2142–2147.

Van Eylen, D., Van Dongen, F., Kabel, M., De Bont, J., 2011. Corn fiber, cobs and stover: enzyme-aided saccharification and co-fermentation after dilute acid pretreatment. Bioresource Technology, 102, 5995–6004.

Voet, E. Van Der, Oers, L. Van, Davis, C., Nelis, R., 2008. Greenhouse gas calculator for electricity and heat from biomass Technical Report, CML Institute of Environmental Sciences, Leiden University. CML-197. Leiden.

Weiss, N.D., Farmer, J.D., Schell, D.J., 2010. Impact of corn stover composition on hemicellulose conversion during dilute acid pretreatment and enzymatic cellulose digestibility of the pretreated solids. Bioresource Technology, 101, 674–678.

Wild, P.J., Huijgen, W.J.J., Heeres, H.J., 2012. Pyrolysis of wheat straw-derived organosolv lignin. Journal of Analytical and Applied Pyrolysis, 93, 95–103.

Wolfrum, E.J., Sluiter, A. D., 2009. Improved multivariate calibration models for corn stover feedstock and dilute-acid pretreated corn stover. Cellulose, 16, 567–576.

Xu, F., Yu, J., Tesso, T., Dowell, F., Wang, D., 2013. Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: A mini-review. Applied Energy, 104, 801–809.

Xu, J., Thomsen, M.H., Thomsen, A.B., 2009. Enzymatic hydrolysis and fermentability of corn stover pretreated by lactic acid and/or acetic acid. Journal of Biotechnology, 139, 300–305.

Zimbardi, F., Viola, E., Nanna, F., Larocca, E., Cardinale, M., Barisano, D., 2007. Acid impregnation and steam explosion of corn stover in batch processes. Industrial Crops and Products, 26, 195–206.