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DEPARTMENT OF FOREST INDUSTRY ENGINEERING

BIOETHANOL PRODUCTION VIA ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS: WHEAT STRAW, CORN STALKS AND HAZELNUT HUSKS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AYHAN TOZLUOĞLU

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APPROVAL PAGE

This postgraduate study which was conducted by Ayhan Tozluoğlu under the title of "*Bioethanol Production via Enzymatic Hydrolysis of Lignocellulosic Biomass: Wheat Straw, Corn Stalks and Hazelnut Husks"* was approved as a doctoral dissertation of Forest Industry Engineering by the committee convened upon the provision No. 2012/349 in 01.10.2012 of the Board of Trustees of the Graduate School of Natural and Applied Sciences, Düzce University, Düzce.

This is to certify that we have read this thesis and that in our opinions it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

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This is to certify that Ayhan Tozluoglu has completed doctoral programme, being awarded with a degree of Doctor of Philosophy in the Forest Industry Engineering by the Board of Trustees of the Graduate School of Natural and Applied Sciences, Düzce University, Düzce.

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DECLARATION PAGE

I hereby declare that this dissertation is my own work and effort from draft to the manuscript without any unethical attitude, and all data included have been obtained following academic and ethic values, and where other sources of information have been used, they have been acknowledged, and all the sources of information I have used are listed in the references, and I have no behaviour against any action of breach of register or copyright from draft to the manuscript.

15/10/2012

Ayhan TOZLUOĞLU

This dissertation is dedicated to my dear wife Aylin TOZLUOĞLU for her constant love and support along the way…

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October 2012 **Ayhan TOZLUOĞLU**

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
°C	Celsius
Atm	Atmospheric Pressure
cm	Centimeters
g	Gram
h	Hour
ha	Hectar
K	Kelvin
kg	Kilogram
L	Liter
M	Molar
min	Minute
m^2	Square Meter
ml	Milliliter
MJ	MegaJul
MT	Metric Ton
mM	Milimolar
nm	Nanometer
o.d.	Oven dry
rpm	Revolutions Per Minute
t	Ton
Tg	Teragram
U/g	Unit/Gram
v/v	Volume/Volume
w/w	Weight/Weight
w/v	Weight/Volume
AFEX	Ammonia Freeze/Fiber Explosion
ADH	Alcohol Dehydrogenase
AHP	Alkaline Hydrogen Peroxide
ANOVA	Analysis of Variance

CSTR	Continuous Stirred Tank Reactor
C	Carbon
$CaCO_3$	Calcium Carbonate
$Ca(OH)_2$	Calcium Hydroxide
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
CO_2	Carbon Dioxide
DP	Degree of Polymerization
DDGS	Dried Distillers Grains
DG-DGS	Distillers Grains
DMC	Direct Microbial Conversion
ETOH	Ethanol
EU	European Union
FAO	Food and Agriculture Organization
FFV	Flexible Fuel Vehicle
FPU	Filter Paper Units
GRAS	Generally Regarded as Safe
GL	Gigaliter
GHG	Greenhouse Gas
HCl	Hydrochloric Acid
HBA	Hydroxybenzaldehyde
HNO_3	Nitric Acid
H_2S	Hydrogen Sulfur
H_2O_2	Hydrogen Peroxide
H_2SO_4	Sulfuric Acid
HMF	5-Hydroxymethyl Furfural
ICE	Internal Combustion Engine
K_2HPO_4	Potasium Phosphate
KOH	Potassium Hydroxide
LAP	Laboratory Analytical Procedures
LHW	Liquid Hot Water
$MgSO_4.7H_2O$	Magnesium Sulphate Hepta Hydrate
MTBE	Methyl Tertiary Butyl Ether
MSW	Municipal Solid Waste

$NaBH_4$	Sodium Borohydrate
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
NREL	National Renewable Resources Laboratory
N_2	Nitrogen
NMR	Nuclear Magnetic Resonance
$(NH_4)_2SO_4$	Ammonium Suphate
OTV	Private Consuming Tax
PDC	Pyruvate Decarboxylase
RID	Refractive Index Detector
SC-CO_2	Supercritical Carbon Dioxide
SE	Steam Explosion
SGA	Siringaldedyde
SHF	Separate Hydrolysis and Fermentation
SSF	Simultaneous Saccharification and Fermentation
TUBITAK	Scientific and Technological Research Council of Turkey
US	United States
WDGS	Wet Distillers Grains
XR	Xylose Reductase
XDH	Xylitol Dehydrogenase
XK	Xylulokinase
SO_2	Sulfur Dioxide

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ABSTRACT

BIOETHANOL PRODUCTION VIA ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BİOMASS: WHEAT STRAW, CORN STALKS AND HAZELNUT HUSKS

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Wheat straw, corn stalks and hazelnut husks which are abundant agricultural waste products in Turkey, could be valuable raw materials for bioethanol production. After harvest, these raw materials are usually left in the fields and burned, which decreases the biological activity of the soil and causes air pollution. Through the use of innovative biotechnology, these waste materials may be valuable in generating economic benefits as well as environmental dividends. The main objective of this study was to examine the suitability of these wastes for bioethanol production and to compare pretreatment techniques with regard to their efficiencies. In this study, wheat straw, corn stalks and hazelnut husks were first steam exploded and then chemically treated to achieve efficient hydrolysis. The conventional chemicals of sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and an alternative chemical, sodium borohydrate ($NaBH_4$), were utilized for the first time ever in the chemical treatment procedure.

The obtained results showed that NaOH and $NaBH_4$ treated wheat straw resulted in 87.8% and 83.3% glucan conversion in enzymatic hydrolysis, but H_2O_2 (74.7%) and H_2SO_4 (71.7%) had lower glucan conversion. The highest ethanol yield (115 g/kg wheat straw) was observed for 4% $NaBH_4$ pretreated sample (60 min) and the theoretical yield (86.9%) was also calculated to be highest for the sample.

Results showed that the corn stalks treated with NaOH (83.9%) and $NaBH_4$ (82.9%) gave higher glucan conversion in enzymatic hydrolysis compared to those treated with H_2O_2 (74.5%) and H_2SO_4 (56.6%). The highest ethanol yield (97.4 g/kg corn stalk) was obtained when the stalks were pretreated with 4% $NaBH_4$ for 90 min; the theoretical ethanol yield was found to be 72.5%.

On the other hand, 4% $NaBH_4$ (90 min) delignified the highest amount of lignin (49.1%) from the hazelnut husks structure. Pretreatment with NaOH and $NaBH_4$, compared to H_2O_2 and H_2SO_4, resulted in selective delignification. The highest glucan to glucose conversion (74.4%) and the highest ethanol yield (52.6 g/kg husks) were observed for hazelnut husks treated with 2% NaOH for 90 min.

Among the raw materials, wheat straw was found to be the most suitable for bioethanol production. In addition, results indicated that, pretreatment chemical of $NaBH_4$ was as effective as NaOH.

Keywords: Bioethanol, Corn stalk, Hazelnut husk, Pretreatment, Wheat straw.

ÖZET

ENZİMATİK HİDROLİZ İŞLEMİ İLE EKİN SAPI, MISIR SAPI VE FINDIK ZURUFUNDAN BİYOETANOL ÜRETİMİ

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Ülkemiz dünyanın sayılı tarım üreticisi ülkeleri arasında bulunmaktadır. Ekin sapı, mısır sapı ve fındık zurufu atıklarının mevcut potansiyel durumu göz önüne alındığında bu lignoselülozik biyokütlelerin biyoetanol üretiminde değerlendirilmesi önem arz etmektedir. Gelişmekte olan yeni biyoteknolojik yaklaşımlar sayesinde bu tarz yenilenebilir lignoselülozik biyokütlelerden biyoetanol üretimi, çevresel pozitif katkılarının yanı sıra ülke ekonomisini de olumlu yönde etkileyecektir. Bu çalışmada ekonomik değeri düşük/yok olan lignoselülozik biyokütlelerden ekin sapı, mısır sapı ve fındık zurufunun biyoetanol üretiminde kullanım olanakları araştırılmıştır. Ön muamele işlemlerinde kullanılan geleneksel kimyasallara (H_2SO_4, NaOH, H_2O_2) alternatif olarak $NaBH_4$ kimyasalı bu çalışmada ilk kez ön muamele amacıyla kullanılmıştır.

Elde edilen veriler ekin sapı için değerlendirildiğinde enzimatik hidroliz işleminde NaOH (%87.8) ve $NaBH_4$ (%83.3)'ün H_2O_2 (%74.7) ve H_2SO_4 (%71.7)'e nazaran daha etkin oldukları ve daha yüksek oranda glukan-glukoz dönüşümü sağladıkları gözlemlenmiştir. En yüksek etanol verimi (115 g/kg ekin sapı) %4 $NaBH_4$ ile 60 dak süreyle muamele edilen örnekte belirlenmiş olup teorik verim aynı örnek için %86.9 dur.

Mısır sapında NaOH (83.9%) ve $NaBH_4$ (82.9%) enzimatik hidroliz işleminde en yüksek glukan-glukoz dönüşümü ortaya koymuş olup, en yüksek etanol verimi (97.4 g/kg mısır sapı) mısır sapı % 4$NaBH_4$ ile 90 dak süre ile muamele edildiğinde belirlenmiştir. Bu örnek için teorik verim değeri %72.5 tir.

Fındık zurufu için yapılan çalışmalarda ise $NaBH_4$'ün en yüksek miktarda yapıdan lignini uzaklaştırdığı belirlenmiş ve NaOH ve $NaBH_4$'ün H_2O_2 ve H_2SO_4'e nazaran daha seçici lignin delignifikasyonu sağladıkları tespit edilmiştir. Enzimatik hidroliz işleminde en yüksek glukan-glukoz dönüşümü (%74.4) ve en yüksek etanol verimi (52.6 g/kg fındık zurufu) örnekler %2 NaOH ve 90 dak süre ile muamele edildiğinde gözlemlenmiş olup bu örnek için teorik verim değeri %72.6 olarak belirlenmiştir.

Numuneler kendi aralarında karşılaştırıldığında ekin sapının biyoetanol üretimi için kullanımının daha uygun olduğu görülmektedir. Ayrıca kimyasal ön muamelelerde NaOH ve $NaBH_4$'ün etkin oldukları görülmektedir.

Anahtar sözcükler: Biyoetanol, Mısır sapı, Fındık zurufu, Ön muamele, Ekin sapı.

GENİŞ ÖZET

ENZİMATİK HİDROLİZ İŞLEMİ İLE EKİN SAPI, MISIR SAPI VE FINDIK ZURUFUNDAN BİYOETANOL ÜRETİMİ

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1. GİRİŞ

Dünya nüfusunun hızla artmasına paralel olarak insan ihtiyaçları her geçen gün artmakta ve bu ihtiyaçların karşılanabilmesi için üretimin de artırılması kaçınılmaz olmaktadır. Bu noktada önemli üretim fonksiyonlarından biri olan enerjinin de bol miktarda elde edilmesi gerekmektedir. Giderek artan fiyatlar ve özellikle petrol gereksinimini dış alım yolu ile karşılayan ülkeler için değişik bir enerji kaynağı olarak lignoselülozik maddelerden yararlanmak üzere, biyokütle miktarının yükseltilmesine, kimyasal maddelere dönüştürmeye ve uygun işleme olanaklarına yönelik çalışmalar yapılmaktadır. Bu çalışma kapsamında lignoselülozik maddelerden biri olan ve tarlalarda çürümeye bırakılan veya yakılmak suretiyle yok edilirken çevreye zarar veren fındık zurufundan biyoetanol üretim olanakları araştırılmıştır. Dünyada birçok yıllık bitki sapları ve lignoselülozik atık maddelerinden biyoetanol eldesi konusunda araştırmalar mevcut olmasına karşılık fındık zurufunun bu amaçla kullanımı konusunda herhangi bir literatür mevcut değildir. Fındık zurufuna ek olarak ülkemizde bol miktarda bulunan yıllık bitki atıklarından ekin ve mısır saplarının biyoetanol üretimde kullanım olanakları da bu çalışma kapsamında araştırılmıştır. Ön muamele işlemlerinde kullanılan geleneksel kimyasallara (H_2SO_4, NaOH, H_2O_2) alternatif olarak $NaBH_4$ kimyasalı bu çalışmada ilk kez ön muamele amacıyla kullanılmıştır.

2. MATERYAL VE YÖNTEM

Bu çalışmada hammadde olarak kullanılan ekin ve mısır sapları ile fındık zurufu örnekleri Düzce ili bölgesinden lokal olarak temin edilmiştir. Hammaddeler enzimatik

hidroliz işleminin etkinliğini ve fermentasyon sonrası etanol verimini artırmak amacıyla bir dizi ön muamele işlemine tabi tutulmuş olup bu maksatla numunelere önce buhar patlatma sonra çeşitli kimyasal ön muamele işlemleri uygulanmıştır. Buhar patlatma işlemi 198-200^{o}C sıcaklık ve 15 bar basınç altında 5 dak süreyle reaktör içerisinde gerçekleştirilmiştir. Kimyasal ön muamele işlemi için %0.5, 2 ve 4 (w/v) konsantrasyonlarında hazırlanan NaOH, H_2SO_4, H_2O_2 ve $NaBH_4$ solüsyonlarından 400 ml alınarak içlerinde 40 g (fırın kurusu) numuneler olan poşetlere konulmuştur (%10 (w/v) katı madde oranında). Kimyasal muamele işlemleri 121°C sıcaklık ve 15 psi (103.4 kPA) basınç altında 30, 60 ve 90 dak süreyle otoklav içerisinde gerçekleştirilmiştir. Süre bitiminde numuneler filtrasyona tabi tutularak sıvı ve katı kısma ayrılmıştır. Enzimatik hidroliz işleminde *Celluclast* (700 U/g) ve *Cellobiase* (*Novozym 188*) (250 U/g) enzim karışımları kullanılmış ve enzim reaksiyonu çalkantılı inkübatörde 42°C ve 100 rpm'de 72 sa süreyle gerçekleştirilmiştir. Hidroliz sonrası hidrolizatların fermentasyonu için *Saccharomyces cerevisiae ATCC 26602* mayası kullanılmış ve işlem çalkantılı inkübatörde 100 rpm, 72 sa ve 30°C'de gerçekleştirilmiştir.

3. BULGULAR VE TARTIŞMA

Buhar patlatma işlemi sonrası elde edilen veriler genel olarak incelendiğinde, buhar patlatma ön muamelesi daha fazla hemiselülozik şekeri mısır ve ekin sapından çözmüş, daha fazla lignini ise fındık zurufundan uzaklaştırmıştır. Kimyasal ön muamele işlemlerinde ise ekin sapında en fazla glukan çözünürlüğü (%16.7) %0.5 H_2SO_4 (30 dak), en fazla ksilan çözünürlüğü (%75.0) %4 NaOH (60 dak) ve en fazla lignin redüksiyonu (%66.3) %2 NaOH (90 dak) ön muameleleri sonrası gözlemlenmiştir. Mısır sapında en fazla glukan çözünürlüğü (%42.5) %4 NaOH (90 dak), en fazla ksilan çözünürlüğü (%80.2) %4 NaOH (90 dak) ve en fazla lignin redüksiyonu (%83.0) %4 NaOH (90 dak) ön muameleleri sonrası gözlemlenmiştir. Fındık zurufunda ise en fazla glukan çözünürlüğü (%29.9) %4 $NaBH_4$ (90 dak), en fazla ksilan çözünürlüğü (%78.0) %4 NaOH (90 dak) ve en fazla lignin redüksiyonu (%42.4) %2 $NaBH_4$ (90 dak) ön muameleleri sonrası gözlemlenmiştir. Enzimatik hidroliz işlemi sonucu ekin sapında en yüksek glukan-glukoz dönüşümü NaOH (%83.3) için gerçekleşmiş olup, bunu sırasıyla $NaBH_4$ (%83.3), H_2O_2 (%74.7) ve H_2SO_4 (%71.7) ön muameleleri izlemiştir. Mısır sapında en yüksek glukan-glukoz dönüşümü NaOH (%83.9) için gerçekleşmiş olup,

bunu sırasıyla $NaBH_4$ (%82.4), H_2O_2 (%74.5) ve H_2SO_4 (%56.6) ön muameleleri izlemiştir. Fındık zurufunda ise en yüksek glukan-glukoz dönüşümü NaOH (%74.4) için gerçekleşmiş olup, bunu sırasıyla $NaBH_4$ (%61.8), H_2O_2 (%58.8) ve H_2SO_4 (%54.3) ön muameleleri izlemiştir. Ekin sapında en yüksek etanol verimi (115 g/kg ekin sapı) örnekler %4 $NaBH_4$ ile (60 dak) muamele edildiğinde belirlenmiş olup teorik verim aynı örnek için %86.9'dur. Mısır sapında ise en yüksek etanol verimi (97.4 g/kg mısır sapı) örnekler % 4$NaBH_4$ ile (90 dak) muamele edildiğinde belirlenmiştir. Bu örnek için teorik verim değeri %72.5'dir. Fındık zurufunda en yüksek etanol verimi (52.6 g/kg fındık zurufu) örnekler %2 NaOH ile (90 dak) muamele edildiğinde gözlemlenmiş olup bu örnek için teorik verim değeri %72.6 olarak belirlenmiştir.

4. SONUÇ VE ÖNERİLER

Çalışma kapsamında ekin sapı, mısır sapı ve fındık zurufu numuneleri, buhar patlatma ve takibinde çeşitli kimyasal ön muamele işlemlerine tabi tutulmuş ve bu ön muamele işlemlerinin enzimatik hidroliz işlemi ve etanol verimliliği üzerindeki etkileri belirlenmiştir. Elde edilen veriler genel olarak incelendiğinde, lignoselülozik yapıdan maksimum oranda ksilan ve lignin uzaklaştırıldığında enzimatik hidroliz işleminin olumlu yönde etkilendiği ve takibinde etanol veriminin arttığı tespit edilmiştir. Çalışmada kullanılan üç hammadde türü içinde en yüksek ksilan çözünürlüğü NaOH ve en yüksek lignin redüksiyonu $NaBH_4$ ön muamele işlemlerinden sonra gözlemlenmiştir. Bu ön muame işlemleri sonrasında enzimatik hidroliz işlemine tabi tutulan numunlerde glukan-glukoz dönüşümünün önemli derecede arttığı belirlenmiştir. Özellikle lignin redüksiyonunun ksilan çözünürlüğüne göre enzimatik işlem üzerindeki etkisinin daha yüksek olduğu ve dolayısıyla diğer tüm ön muamele kimyasallarına nazaran $NaBH_4$'ün lignoselülozik biyokütlelerden biyoetanol üretiminde NaOH kadar aktif bir kimyasal olarak kullanılabileceği tespit edilmiştir. Kimyasal ön muamele işlemlerinde farklı süre, sıcaklık, basınç ve konsantrasyon parametrelerinin optimizasyonuyla daha etkin bir ön işlemin gerçekleştirilebileceği, enzimatik hidroliz işlemi sırasında farklı enzim kombinasyonlarının değişen süre ve konsantrasyon parametrelerinde uygulanışı ve fermentasyon işleminde 5C ve 6C'lu karbonhidratları fermente edici farklı maya ve/veya bakteri türlerinin denenmesiyle bu tarz lignoselülozik biyokütlelerden etanol veriminin daha da artırılabileceği düşünülmektedir.

1. INTRODUCTION

World population is expected to be over eight billion by 2030, causing a 50 percent increase in the global energy demand, and this expansion of human population is expected to result in future energy shortages. Currently, energy needs are mostly supplied from conventional fossil fuels; their use pollutes the environment and thus dramatically increases the greenhouse gases in the atmosphere. Consequently, alternative energy sources have recently become of interest to researchers.

Presently, there is the utmost need for alternative energy resources which are cheap, renewable and do not cause pollution. Therefore, attention is being given to alternate and renewable energy sources such as solar, wind, thermal, hydroelectric, biomass, etc. Biomass is the fourth largest source of energy in the world after coal, petroleum and natural gas, and provides about 14% of the world's primary energy consumption. Renewable biomass is being considered as an important energy resource all over the world. Biomass is used to meet a variety of energy needs, including generating electricity, fueling vehicles and providing process heat for industries (Bridgewater et al. 1999). It is the only renewable source of carbon that can be converted into convenient solid, liquid and gaseous fuels through different conversion processes (Ozbay et al. 2001).

The history of ethanol as a fuel dates back to the early days of the automobile era. However, cheap petrol (gasoline) quickly replaced ethanol as the fuel of choice, and it was during the late 1970's, when the Brazilian government launched their ''Proalcool'' Programme, that ethanol made a comeback to the marketplace. Today, fuel ethanol accounts for roughly two-thirds of world ethyl alcohol production (Saxena et al. 2009).

The expansion of the ethanol market has led researchers to investigate alternative low-cost materials and methods to produce bioethanol. Lignocellulosic materials such as wood and agricultural residues make them a valuable resource for energy production. Utilizing abundant lignocellulosic waste is one especially good possible alternative. The usage of several agricultural residues in bioethanol production has already been studied (Balat et al. 2008).

Wheat is the world's most widely-grown crop, and 850 Tg of wheat straw residues are

produced annually (Atwell 2001). Up to 238 GL of bioethanol could be produced from this residue. Wheat straw is also the largest biomass cultivated in Europe (Kim and Dale 2004). In addition, approximately 40-53 million t of straw is produced in Turkey per year (Ergudenler and Isigigur 1994). Burning wheat straw has been a long-time practice and produces a large amount of air pollutants (Li et al. 2008) which result in health problems. The cellulose, hemicellulose and lignin contents of wheat straw are 33-40, 20-25 and 15-20% w/w, respectively (Prasad et al. 2007), with the variation in composition depending on the wheat species, soil, climate conditions, etc.

Corn stalks, rich in natural cellulose (35-50%) (Fei and Hongzhang 2009), are an abundant, renewable, low-cost and widely-available resource in Turkey. Their use as a substrate in bioethanol production may also result in decreasing the soil and air pollution associated with discarding and burning the stalks. Earlier studies have examined the use of corn stover (Kadam and McMillan 2003) and corn kernels (Tang et al. 2011) in ethanol production; however, few studies have been done on corn stalks (Fei and Hongzhang 2009). Corn kernels (Tang et al. 2011), high in glucan and easily broken down to fermented sugars, could be utilized as a raw material in ethanol production; however, it should be taken into consideration that using corn kernels or other food resources might compete with human and animal food needs.

Nearly 70% of the world's hazelnuts are grown in Turkey, which makes it a significant hazelnut producer. Based on this production, the amount of husk waste is estimated to be 200,000 t/year (Midilli et al. 2000). This abundant agricultural waste has had no economic value to date and is usually burned in the fields, causing air pollution and soil erosion. In addition, the burning decreases the biological activity of the soil (Arslan and Saracoglu 2010). Any possible industrial usage of hazelnut husks can be expected to yield economic as well as environmental dividends. The literature on using husk waste in industrial applications has been very limited. In earlier studies, the possible usage of husk waste in particleboard (Copur et al. 2007, Guler et al. 2009) and medium-density fiberboard (Copur et al. 2008) production was examined. The only work using hazelnut shells as a renewable and low-cost lignocellulosic material for bioethanol production was carried out by Arslan and Saracoglu (2010). They were able to achieve a good fermentability when the lignin content of the shells was removed by treatment with 3% NaOH before the hydrolysis step. The ethanol yield was 0.084g ethanol/g of hazelnut

shells.

Similar to other biomass, wheat straw, corn stalks and hazelnut husks consist of cellulose, hemicelluloses, and lignin with a small amount of extractives and ash. The cellulose in these kinds of lignocellulosic materials has a tightly-packed structure that does not allow penetration of water or enzymes (Laureano-Perez et al. 2005). Due to the complex structure of lignocellulosic materials, bioethanol production from them requires at least four major steps: pretreatment, hydrolysis, fermentation and distillation (Talebnia et al. 2010).

The pretreatment methods are classified as physical, physico-chemical, chemical and biological processes. The application of pretreatments is expected to improve the cellulose accessibility of hydrolytic enzymes, while decreasing hemicelluloses and cellulose degradation during the process. If degradation occurs, it may result in a lower ethanol yield. In addition, degradation byproducts may inhibit the effectiveness of the yeast used in the fermentation process (Ohgren et al. 2007).

The physical application consists of size reduction by milling/grinding and chipping. Reduced material size improves the efficiency of the following treatment step due to a higher specific surface area of the material being processed. Physico-chemical methods are practiced to solubilize lignocellulosic components from the material structure based on pH, temperature and moisture content, and make the material easily exposed for the next processing step. Steam explosion is a commonly utilized physio-chemical method for treating lignocellulosic materials. In this method, the materials are exposed to pressurized steam for a time period and then expelled from the vessel. This procedure breaks down the lignocellulosic structure by dissolving some hemicelluloses and cellulose, depolymerizing the lignin components and defibering the cell walls (Cara et al. 2006).

Chemical methods were originally developed and extensively used in the paper industry to produce high-quality paper products by delignifying the cellulosic materials (Fan et al. 1982). Effective and inexpensive chemical treatment techniques have been developed for biomass bioconversion by modifying the chemical pulping processes. Alkali treatments remove lignin and various uronic acid groups of hemicelluloses and improve the accessibility of enzymes to the hemicelluloses and cellulose (Chang and

Holtzapple 2000). Sodium hydroxide (NaOH) breaks the ester linkages between lignin and xylan and deprotonates the phenolic lignin groups. Swelling and partial hemicellulose solubilization results in the distribution of cellulose and hemicelluloses bonds and causes some glucan dissolution (Chen and Sharma-Shivappa 2007). Acid applications improve the accessibility of enzymes to biomass (Balat et al. 2008). Sulfuric acid (H_2SO_4) removes hemicelluloses from cell wall structures and increases the structure porosity. Maximum enzymatic digestibility may be possible with the removal of all hemicelluloses from the structure (McMillan 1994). Hydrogen peroxide (H_2O_2) is a well- known bleaching agent in the paper and cellulose industry. Oxidative delignification is utilized to detach and solubilize the lignin and to loosen the lignocellulosic matrix, thus improving enzymatic digestibility (Rabelo et al. 2011).

Turkey has 71.3% of the world's boron (B_2O_3) reserves. It can be valuable to examine the use of this chemical in other industrial applications. As is known, borohydrate is a powerful reducing agent that degrades the lignin in the structure. On the other hand, it converts the carbonyl group in the reducing end units of the carbohydrate chains to hydroxyl groups, therefore preserving the carbohydrates. Several boron derivates have been studied for pulp production (Copur and Tozluoglu 2007), but there is no literature on boron derivatives in the chemical pretreatment step of bioethanol production. One important disadvantage of $NaBH_4$ is the price of the chemical. The pulping additive sodium borohydrate ($NaBH_4$) is utilized to improve pulping selectivity by preventing peeling reactions and hemicelluloses degradation (Hoije et al. 2005); $NaBH_4$ degrades lignin more selectively (Copur et al. 2012).

1.1. LITERATURE REVIEW

1.1.1. Defining the Resources

Biorenewable resources are usually classified as either waste or dedicated energy crops. Categories of waste materials that qualify as biorenewable resources include agricultural residues, yard waste, municipal solid waste, food processing waste, and manure. Agricultural residues such as corn stover, rice hulls, wheat straw, cotton stalks, and bagasse, are the portion of the crop discarded after harvest. Municipal solid waste (MSW) is waste discarded as garbage, not all of which is suitable as biomass feedstock.

In communities where yard waste is excluded, the important components of MSW are paper (50%), plastics and other fossil-fuel-derived materials (20%), food wastes (10%), and non-flammable materials including glass and metal (20%) (Brown 2003). Food processing waste is the effluent from a variety of industries ranging from breakfast cereal manufacturers to alcohol breweries. One of the major benefits of using waste products for conversion to fuels and chemicals is their low cost. By definition, waste products have minimal economic value and can be acquired for little more than the cost of transporting the material from the point of origin to a processing plant. Sometimes, when a biorenewable resource processing plant is paid by a company to dispose of a waste stream, there is even a negative cost associated with the acquisition of the biomass (Brown 2003).

Dedicated energy crops are the other classification of biorenewable resources. These crops are defined as plants specifically grown for applications other than food or feed. Numerous crops have been proposed or are being tested for commercial energy farming. Potential energy crops include woody crops and grasses/herbaceous plants, starch and sugar crops, and oilseeds. In general, the characteristics of the ideal energy crop are: high yield, low energy input to produce, low cost, composition with fewest contaminants, and low nutrient requirements (McKendry 2002).

1.1.2. Interest in Biomass and Biobased Products

In the past 10 years there has been a renewed world-wide interest in biomass as an energy source (McKendry 2002). Technological developments relating to crop production, conversion, etc., give a double promise of biomass at lower cost along with higher conversion than was previously possible. More advanced options for electricity production also appear promising and would allow a cost-effective use for energy crops in operations such as production of methanol and hydrogen by gasification processes (McKendry 2002).

Air pollution is an important factor motivating interest in alternative fuels at the global level. Carbon dioxide (CO_2) is responsible for more than half of the projected anthropically-mediated climate change. Transportation fuels account for 27% of the 2.2 billion MT of CO_2 released annually in the United States (US) from combustion of fossil fuels. Vehicles account for 4.7% of total worldwide CO_2 emissions, with US

vehicles accounting for 2.5% of the total emissions (Ramamurthi and Bali 2000). The use of biomass to produce energy has the potential to reduce the high emission levels of greenhouse gases. When produced by sustainable means, biomass emits roughly the same amount of carbon during conversion as is taken up during plant growth, so the use of biomass does not contribute to a buildup of CO_2 in the atmosphere (McKendry 2002).

1.1.3. Fuel Ethanol

Bioethanol (ethyl alcohol, grain alcohol, $CH_3–CH_2–OH$ or ETOH) is a liquid biofuel which can be produced from several different biomass feedstocks and conversion technologies. Bioethanol is an attractive alternative fuel because it is a renewable bio-based resource; it is oxygenated, and thereby provides the potential to reduce particulate emissions in compression ignition engines (Hansen et al. 2005). However, corn ethanol production causes more soil erosion and uses more nitrogen fertilizer than any other crop grown. These two environmental limitations also apply to sugar cane production in Brazil (Pimentel 2003).

Bioethanol has a higher octane number, broader flammability limits, higher flame speeds and higher vaporization temperatures than gasoline. These properties allow for a higher compression ratio, shorter burn time and leaner burn engine, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (Balat 2007). Disadvantages of bioethanol include its lower energy density than gasoline (66% of the energy of gasoline), its corrosiveness, low flame luminosity, lower vapor pressure (making cold starts difficult), miscibility with water, and toxicity to ecosystems (MacLean and Lave 2003). Some properties of alcohol fuels are shown in Table 1.1.

Ethanol is an oxygenated fuel that contains 35% oxygen, which reduces particulate and NO_x emissions from combustion. Ethanol has a higher octane number (107) than gasoline, broader flammability limits, higher flame speeds and higher vaporization temperatures. These properties allow for a higher compression ratio and shorter burn time, which lead to theoretical efficiency advantages over gasoline in an internal combustion engine (ICE, invented by Nikolas Otto in 1897). Octane number is a measure of the gasoline quality and can be used for prevention of early ignition, which leads to cylinder knocks. Higher octane numbers are preferred in internal combustion engines. An oxygenate fuel such as bioethanol provides a reasonable anti-knock value.

Also, as it contains oxygen, fuel combustion is more efficient, reducing hydrocarbons and particulates in exhaust gases. Complete combustion of a fuel requires an existing amount of stochiometric oxygen. However, the amount of stochiometric oxygen generally is not enough for complete combustion. The oxygen content of a fuel increases its combustion efficiency. Because of this, the combustion efficiency and octane number of bioethanol are higher than those of gasoline.

Table 1.1. Some properties of alcohol fuels (Balat 2007).

Fuel property	Isoctane	Methanol	Ethanol
Cetane number	-	5	8
Octane number	100	112	107
Auto-ignition temperature (K)	530	737	606
Latent heat of vaporization (MJ/kg)	0.26	1.18	0.91
Lower heating value (MJ/kg)	44.4	19.9	26.7

The presence of oxygen in bioethanol improves combustion and therefore reduces hydrocarbon, carbon monoxide, and particulate emissions, but oxygenated fuels also tend to increase nitrogen oxide emissions. In a gasoline engine, bioethanol is appropriate for the mixed fuel because of its high octane number and its low cetane number. Its high vaporization temperature impedes self-ignition in a diesel engine, so ignition improver, glow-plugs, surface ignition, and pilot injection are applied to promote self-ignition when using a diesel/ bioethanol blended fuel (Kim et al. 2005).The most popular blend for light-duty vehicles is known as E85, and contains 85% bioethanol and 15% gasoline. In Brazil, bioethanol for fuel is derived from sugarcane and is used pure or blended with gasoline in a mixture called gasohol (24% bioethanol, 76% gasoline) (de Oliveria MED et al. 2005). In several states of the US, a small amount of bioethanol (10% by volume) is added to gasoline and called gasohol or E10. Blends having higher concentrations of bioethanol in gasoline are also used, e.g. in flexible-fuel vehicles (FFV) that can operate on blends of up to 85% bioethanol-E85 (Malca and Freire 2006). Some countries have exercised biofuel programs involving both forms of bioethanol/gasoline blend programs, *e.g*. the United States (E10 and for FFV, E85), Canada (E10 and for FFV, E85), Sweden (E5 and for FFV, E85), India (E5), Australia (E10), Thailand (E10), China (E10), Columbia (E10), Peru (E10), Paraguay (E7), and Brazil (E20, E25 and for FFV, any blend) (Kadiman 2005).

Methanol is produced by a variety of processes, the most common of which is the

distillation of liquid products from wood, coal, natural gas, and petroleum gas. The cost of ethanol is higher than that of methanol because ethanol is produced mainly from biomass bioconversion. The systematic effect of ethyl alcohol differs from that of methyl alcohol. Ethyl alcohol is rapidly oxidized in the body to CO_2 and water, and in contrast to methyl alcohol, no cumulative effect occurs. Methanol is considerably easier to recover than ethanol. Ethanol forms an azeotrope with water, so it is expensive to purify ethanol during recovery. If the water is not removed, it will interfere with the reactions. Methanol recycles easily because it does not form an azeotrope.

1.1.4. Global Liquid Biofuel Production and Main Feedstocks

Bioethanol is the most widely used liquid biofuel. The largest producers in the world are the US, Brazil, and China. Production of bioethanol from sugarcane in Brazil in 2004 accounted for nearly 18% of the country's automotive fuel needs. In Brazil, ethanol-powered and flexible-fuel vehicles are manufactured for operation with hydrated ethanol (around 93% v/v ethanol and 7% water). As a result of this, together with the development of domestic deep-water oil sources, Brazil has achieved complete self-sufficiency in oil (Brown et al. 1998).

World ethanol production (all grades) reached a record $62x10^9$ L in 2007, with the United States and Brazil as dominant producers (approximately 70%) (Licht 2008a). Recently the United States surpassed Brazil as the world's largest producer of bioethanol. In 2009, the US produced $39.5x10^9$ L of ethanol using corn as a feedstock ("first generation" of ethanol production) while the second largest producer, Brazil, created about $30x10^9$ L of ethanol using sugarcane.

Europe is the most important biodiesel producer in the market, with European rapeseed accounting for 58 percent of global biodiesel produced. Germany, France, the US, and Italy are the leading producers of biodiesel.

Over 90% of the world's bioethanol derives from crops (60% from cane sugar and beet sugar and the remainder from grains, mainly cornstarch, using the "first generation of biofuel plants". The US ethanol industry uses corn as its main feedstock (Licht 2008b). The share of the US corn crop that is consumed by the ethanol industry has grown from around 5% to more than 25% in 10 years. Brazilian ethanol is produced from sugarcane

on land that could be used for food production. Practically all biofuels in the world are produced from feedstocks that could be used to produce food or are produced on land that could produce food (Banse et al. 2008a). The expansion of biofuel production in the US, Europe, and South America has coincided with recent sharp increases in prices for food grains, feed grains, oilseeds, and vegetable oils.

Producing biofuels from the "second generation of biofuel plants" out of feedstocks that cannot be used directly for food production or do not reduce the amount of land that can be used to produce food can be accomplished in two ways (Banse et al. 2008b). The most straightforward way is to capture biomass that is currently treated as either waste or that is a co-product of existing production processes with currently very low or negative economic value. Examples of waste streams that could potentially be converted into biofuels include perennial grasses, agricultural wastes (*e.g*. wheat straw), a portion of municipal trash and garbage (*e.g.* waste paper, waste food scraps, used cooking oils), crop residues, in particular corn (maize) stover, wheat and rice straw, wood pulp residues, macroalgae, and forest residues (*e.g.* wood pieces left over after timber extraction). Currently these streams often generate negative value in that consumers and firms must pay for disposal. A recent study estimated that a city of one million people could provide enough organic waste (1300 t/day) to produce 430,000 L of bioethanol a day. Horticultural waste biomass (*e.g.* tree trunks, twigs, and leaves) could also be a potential source of cellulosic feedstock (Koh and Ghazoul 2008). The authors estimated that the 50,000-156,000 t of horticultural biomass collected each year from about 1 million planted trees in Singapore could be used to produce 14-58 million L of bioethanol, enough to replace 1.6-6.5% of the country's transport gasoline demand. New technology that allows for economic conversion of these potential sources of feedstock for biofuels offers the double benefit of a reduction in global waste and the generation of valuable transportation fuels. In addition, tapping waste streams places no burden on the world's ability to produce food. The second way that biomass can be created without competing for food land is to use land that is not suitable for producing food or to grow the biomass without using land. There are large areas in US and Europe that once produced food crops, but are now in pasture or trees. Conversion of these lands to the production of woody biomass to be used for cellulosic biofuels would not affect food prices. The candidate grass species for cellulosic ethanol production include switch grass (*Panicum virgatum*), miscanthus (*Miscanthus* spp.), reed canary (*Phalaris*

arundinacea), and giant reed (*Arundo donax*) (Lewandowski and Kauter 2003, Lewandowski and Schmidt 2006). Most of these crops can be cultivated on marginal or agriculturally degraded lands, and thus would not compete with food production. High diversity mixtures of grassland species can even provide greater bioenergy yields and greenhouse gas (GHG) reductions than certain conventional bioethanol or biodiesel production systems.

Forest plantations and agroforestry systems can also serve as potential sources of cellulosic feedstocks for bioethanol production. Over the past four decades, new forest plantations in the United Kingdom have been increasing at an average rate of 25,000 ha per year, mostly in Scotland, northern England, and Wales (Milne and Cannell 2005). The planted species in these forests include Sitka spruce (*Picea sitchensis*), Scots pine (*Pinus sylvestris*), lodgepole pine (*Pinus contorta*), hybrid larch (*Larix* spp.), Douglas fir (*Pseudotsuga* spp.), and noble fir (*Abies procera*). Although these forests have been planted for timber, they could also be harvested to supply biofuel production.

An example of using the "third-generation of biofuel plants" is to produce biomass without extensive use of land, using macroalgae as another potential source of biofuel feedstock. Aquatic unicellular green algae, such as *Chlorella* spp., are typically considered for biodiesel production owing to their high growth rate, population density, and oil content (Campbell 2008). Algae have much higher productivity (90,000 L of biodiesel per hectare) than soybeans (450 L/ha), rapeseed (1,200 L/ha), or oil palm (6,000 L/ha) (Haag 2007). In addition to their high yields, macroalgae cultures are not land-intensive and may provide further benefits of wastewater remediation or nutrient reduction (Campbell 2008).

1.1.5. Ethanol Demand and Production Perspectives

The demand for bioethanol is expected to increase dramatically until 2020. In 1999 the US signed an executive order specifying a tripling in the production of biobased products and bioenergy by the year 2020. As a consequence, US oil imports will be reduced by nearly 4 billion barrels over that time. Efforts to decrease GHG emissions are expected to spur the production of renewable energy sources by 6% within the European Union (EU) by 2020 (Zaldivar et al. 2001). In France, the approval of a clean air act could increase ethanol production to 500 million L. Similar projects in Spain,

Sweden and the Netherlands are expected to increase the utilization of ethanol to account for 15% of transportation fuels by 2020 (Anonymous 2012c). The EU market for fuel ethanol will grow considerably in the coming years, as a result of the EU policy to substitute 8% of fossil transport fuels by renewable biofuels by the year 2020.

The cost of raw material dominates the cost of total ethanol production. To attain commercial interest, the costs of bioethanol production must be reduced, and a sufficient amount of cheap and readily available raw material is a necessity. Currently, the lowest cost routes are to produce bioethanol from US corn or Brazilian sugarcane. Process options which involve the importation of intermediate products (sugar concentrate) prior to processing are less cost-effective. None of the biofuel options are currently cost-competitive with petrol or diesel on a pre-tax basis. The lowest cost biofuel, bioethanol from Brazilian sugarcane, is about 40% more expensive than gasoline on an energy basis. According to some studies (Anonymous 2010a), by 2020, minimum costs of bioethanol are expected to fall by about 10% compared to the 2002 values. The perspective for the fuel pathways for bioethanol production up to the year 2020 are:

- In the EU countries for bioethanol production from wood, straw, wheat or corn.
- In North America for bioethanol from wood, straw, wheat or corn.
- In South America for bioethanol from sugarcane.
- In Eastern Europe for bioethanol from wood, straw, wheat or corn.

1.1.6. Ethanol Market in Turkey

The Turkish government did not specify any criteria at the initial stages of the establishment of the Turkish biofuels sector. There are currently four bioethanol production facilities established in Turkey. However, only one of them is actively operating. This facility uses mostly corn and very rarely wheat as raw material. The total capacity of the sector is currently 160,000 MT; the total production in 2009 was 40,000 MT. Approximately 150,000 MT of corn was used to produce 40,000 MT bioethanol in 2009 (Erkut 2010). Bioethanol produced from 2004 to date has been mixed only up to 2% to gasoline due to the Private Consuming Tax (OTV) applied in Turkey. The use of bioethanol is expected to increase, depending on the new regulations

in future (Anonymous 2010b).

1.1.7. Feedstocks for Bioethanol Production

Sucrose to Ethanol

The most common disaccharide used for bioethanol production is sucrose, which is composed of glucose and fructose. Sucrose contributed to 48% of the world's fuel ethanol production in 2006 (Licht 2006). Fermentation of sucrose is performed using commercial yeast such as *Saccharomyces cerevisiae*. The chemical reaction is a result of enzymatic hydrolysis followed by fermentation of simple sugars. First, invertase (an enzyme present in the yeast) catalyzes the hydrolysis of sucrose to convert it into glucose and fructose. Then, another enzyme (*zymase*), also present in the yeast, converts the glucose and the fructose into ethanol and CO_2. One ton of hexose (glucose or fructose) theoretically yields 511 kg of ethanol. However, the practical efficiency of fermentation is about 92% of this yield.

In the bioethanol industry, the sucrose feedstock is mainly sugarcane and sugar beet. It may also be sweet sorghum. A significant share of the fuel ethanol worldwide comes from sugarcane juice, Brazil being the main producer. In 2005, Brazil produced 16 billion L of fuel ethanol, 2 billion of which was exported. Another potential large producer of sugarcane to ethanol is India, which together with Brazil are the world leaders of sugarcane production. However, Indian bioethanol production is currently low; around 300 million L were produced in 2005, mainly from sugarcane molasses. The EU is also a potentially large producer of ethanol based on sugar beet juice. Sugar beet currently plays a minor role in the production of ethanol in the EU compared to wheat, but its market share could increase significantly in the future due to the new incentives given by the EU for energy crops. In 2005, around 950 million L of bioethanol were produced in the EU.

Starch to Ethanol

For converting starch to ethanol, the polymer of α-glucose is first broken through a hydrolysis reaction with glucoamylase enzyme. The resulting sugar is known as dextrose, or D-glucose that is an isomer of glucose. The enzymatic hydrolysis is then

followed by fermentation, distillation, and dehydration to yield anhydrous ethanol.

In the fuel bioethanol industry, starch is mainly provided by grains (corn, wheat, or barley). Corn, which is the dominant feedstock in the starch-to-ethanol industry worldwide, is composed of 60 to 70% starch. Conversion to ethanol is achieved in dry or wet mills. In the dry milling process, the grain is ground to a powder, which is then hydrolyzed and the sugar contained in the hydrolysate is converted to ethanol, while the remaining flow containing fiber, oil, and protein is converted into a co-product known as distillers grains (DG), or DGS when it is combined to produce syrup. The co-product is made available either wet (WDGS), or more commonly dried (DDGS), and is sold as animal feed. WDGS is preferably reserved for local markets, while the co-product is usually dried if the feed has to be shipped far away. Another co-product may be carbon dioxide, which can be sold for different applications (e.g. carbonated beverages or dry ice). Dry mills are dominant in the grain-to-ethanol industry. However, in a number of large facilities, the mills are kinds of biorefineries in which the grains are wet-milled first to separate the different components, that is, starch, protein, fiber, and germ, before converting these intermediates into final co-products.

The US is the leading grain-based ethanol producer in the world and the second producer with all feedstocks inclusive. There was a rapid increase of its production of fuel ethanol from 8 billion L in 2002 to 15 billion L in 2005. Corn-to-ethanol mills represented around 93% of the 18.5 billion L of US bioethanol capacity in 2006. The renaissance of fuel ethanol in the US started with the world oil crises of 1973 and 1979, the aim being to improve the security of the energy supply. Later on, ethanol was used as a substitute for lead in gasoline. Finally, the Clean Air Act of the 1990's spurred on the use of bioethanol as an oxygenated compound in the reformulated gasoline, especially in areas where smog was an issue. Ethanol competes with methyl-tertiary-butyl-ether (MTBE) as an oxygenate. The ban on MTBE in several states launched the irresistible rise of ethanol in the US oxygenate market. Besides these uses, fuel ethanol is also marketed as a gasoline extender and octane booster. Gasohol, a blend of 10% ethanol and 90% gasoline by volume, is used in conventional internal combustion engines. FFVs are currently emerging in the new car market. Other major grain-to-ethanol producers are the EU, where wheat is the dominant feedstock. Canada and China are producers as well. South Africa has also launched an ambitious corn-to-

ethanol program (Pandey 2009).

Lignocellulosics to Ethanol

Lignocellulosic biomass, such as agricultural residues, wood and energy crops, is an attractive material for bioethanol fuel production since it is the most abundant reproducible resource on the earth. Lignocellulosic biomass could produce up to 442 billion L per year of bioethanol. Thus, the total potential bioethanol production from crop residues and waste crops is 491 billion L per year, about 16 times higher than the current world bioethanol production (Kim and Dale 2004).

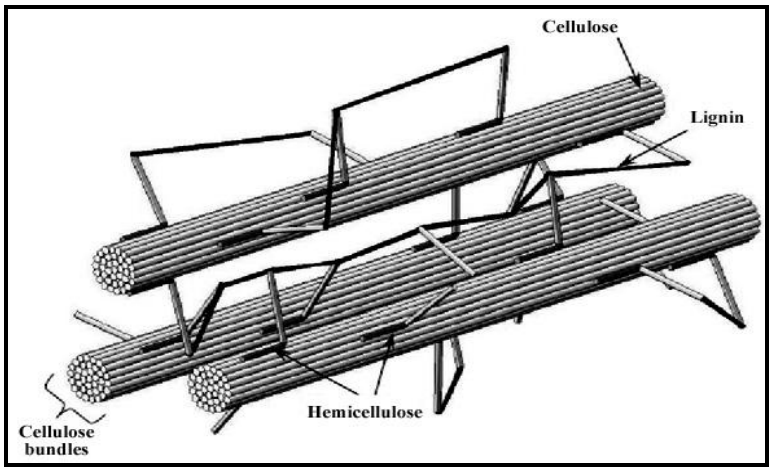

Figure 1.1. Structure of plant cell walls (Shleser 1994).

The basic structure of all lignocellulosic biomass consists of three basic polymers: cellulose $(C_6H_{10}O_5)_x$, hemicelluloses such as xylan $(C_5H_8O_4)_m$, and lignin $[C_9H_{10}O_3(OCH_3)_{0.9\text{-}1.7}]_n$ (Figure 1.1) in trunk, foliage, and bark (Demirbas 2005a, Arin and Demirbas 2004).

The cost of bioethanol production from lignocellulosic materials is relatively high when based on current technologies, and the main challenges are the low yield and high cost of the hydrolysis process (Sun and Chen 2002). Because the feedstock can represent >40% of all process costs, an economical biomass-to-bioethanol process depends critically on the rapid and efficient conversion of all of the sugars present in both its cellulose and hemicellulose fractions (Mohagheghi et al. 2002).

Feedstocks used in this study:

- Wheat straw (*Triticum aestivum* L.): Wheat is the world's most widely-grown crop, and 850 Tg of wheat straw residues are produced annually (Atwell 2001); up to 238 GL of bioethanol could be produced from this residue. Wheat straw is also the largest biomass cultivated in Europe (Kim and Dale 2004). According to the Turkish Statistical Institute's report, Turkey's wheat production was 21.8 million t in 2011 (Anonymous 2012a). Burning wheat straw has been a long-time practice, and this burning produces large amounts of air pollutants (Li et al. 2008) and resulting health problems. Similar to other biomass, wheat straw consists of cellulose, hemicelluloses and lignin with a small amount of extractives and ash. The cellulose, hemicellulose and lignin contents are 33-40, 20-25 and 15-20 % w/w, respectively (Prasad et al. 2007), the variation in composition depending on the wheat species, soil, climate conditions, etc. The cellulose in wheat straw has a tightly-packed structure that is impenetrable to water and enzymes (Laureano-Perez et al. 2005). On the other hand, hemicelluloses could be processed by dilute acids and hemicelluloses enzymes. The complex structure of lignin, connected with cellulose and hemicelluloses in the structure, makes its removal complicated. Due to its complex structure, bioethanol production from wheat straw requires at least four major steps: pretreatment, hydrolysis, fermentation and distillation (Talebnia et al. 2010).

- Corn stalks (*Zea mays* L.): Corn (maize) is a significant crop all around the world. The annual production worldwide is about 520 $x10^9$ kg. The major production regions are North America (42%), Asia (26%), Europe (12%) and South America (9%) (Kim and Dale 2004). According to the Food and Agriculture Organization (FAO), worldwide production of corn in 2002 was $604x10^6$ t cultivated in $1383x10^6$ m^2, 134 of which were cultivated in Europe (Anonymous 2004a). On the other hand, Turkey's corn production was 4.2 million t in 2011 (Anonymous 2012a). Corn stalks, rich in natural cellulose (35-50%) (Fei and Hongzhang 2009), are an abundant, renewable, low-cost and widely available resource in Turkey. However, most corn (about 64% of global production) is used for animal food. The amount for human needs is 19%, while only 5% of global production is lost as waste. Wasted corn can be utilized as feedstock for bioethanol production (Kim and Dale 2004). Its use as a substrate in bioethanol production may also result in decreasing the soil and air pollution associated with discarding the stalks.

For the past 15 years, maize has been used as raw material for bioethanol production, which tripled up to $28x10^6$ t in 2003. Corn residue may contain valuable materials, but the current economic values are less than the apparent cost of collection, transportation and processing for beneficial use (Tsai et al. 2001). Currently, this agricultural waste is being studied as a raw material for energy and active carbon preparation.

- Hazelnut husks (*Corylus colurna* L.): Among the nut species, hazelnuts play a major role in human nutrition and health because of their special composition of fat (mainly oleic acid), protein, carbohydrate, vitamins (vitamin E), minerals, dietary fiber, phytosterols (β-sitosterol) and antioxidant phenolics. The nutritional and sensory properties of hazelnuts make them unique and an ideal ingredient in various food products. Turkey cultivates hazelnuts in an area of about 600,000 ha and produces approximately 550-650,000 t/in-shell a year. Turkey contributes more than 75% of the world's total production of hazelnuts (Anonymous 2012b). Based on the production, the amount of husk waste is approximated to be 200,000 t/year (Midilli et al. 2000).

Hazelnut husks can be one of the most important types of biomass, as they are an abundant and important agricultural and commercial material in Turkey. Burning agricultural residue may cause air pollution, soil erosion and a decrease in the biological activity of the soil (Copur et al. 2007). Therefore, any possible usage of hazelnut husks will yield economic as well as environmental dividends. The conversion of hazelnut husks to useful chemicals such as acetic acid, methanol (Asik et al. 1977), ammonia (Corlett 1975), furfural (Demirbas 2006a) and hydrogen (Midilli et al. 2000) have been investigated. No known effort has been made to utilize hazelnut husks as a renewable and low-cost lignocellulosic material for bioethanol production. The high lignin content of hazelnut husks is a significant obstacle for such a biotransformation.

1.1.8. Feedstock Composition

Cell Wall Organization

Most of the carbohydrate content of plants consists of structural polysaccharides that provide support, strength, and shape for the plant. This complex structural material in the cell wall, known as lignocellulose, is a composite of cellulose fibers embedded in a cross-linked lignin hemicellulose matrix (Brown 2003). The three main components of

lignocellulosic materials are cellulose, hemicellulose, and lignin, with other minor components being ash, protein, and extractives. The distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall is shown below in Figure 1.2. Lignin is most abundant in the middle lamella and decreases with increasing distance into the fiber cell wall, with percentages in the primary cell wall and S1 layer of the secondary cell wall higher than in the S2 and S3 sections of the secondary cell wall. Cellulose is most abundant in the secondary cell wall, as seen in the diagram below. The cellulose microfibrils in the primary cell wall have no specific orientation, while the microfibrils in the secondary cell wall run parallel to each other, but at a different angle for each of the three layers S1, S2, and S3.

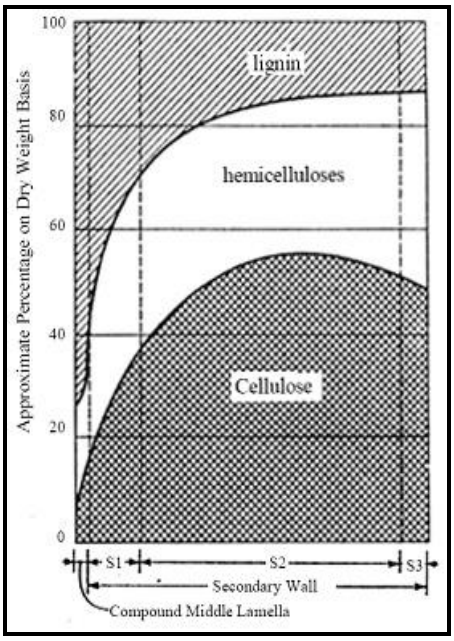

Figure 1.2. Distribution of cellulose, hemicellulose, and lignin in a typical plant cell wall (Panshin and DeZeeuw 1980).

Cellulose

Cellulose is a linear polymer of anhydro D-glucose units connected by β-1,4 glycosidic bonds, as shown below in Figure 1.3. Native cellulose exists in the form of microfibrils, which are paracrystalline assemblies of several dozen (1→4) β-D-glucan chains held together by intermolecular hydrogen bonds (Carpita and McCann 2000). Intramolecular hydrogen bonds also form between two glucose units in the same chain (Fengel and Wegener 1984). The combined bonding energies of the intermolecular and

intramolecular hydrogen bonds increase the rigidity of cellulose and form the crystalline structure that makes it highly insoluble and recalcitrant to most organic solvents. The cellulose microfibrils are imbedded in a matrix of noncellulosic polysaccharides, mainly hemicellulose and pectic substances (Sun 2002), which complicates hydrolysis of cellulose to glucose even further. The cellulose in lignocellulosic biomass feedstocks provides the main source of glucose used during ethanol fermentation.

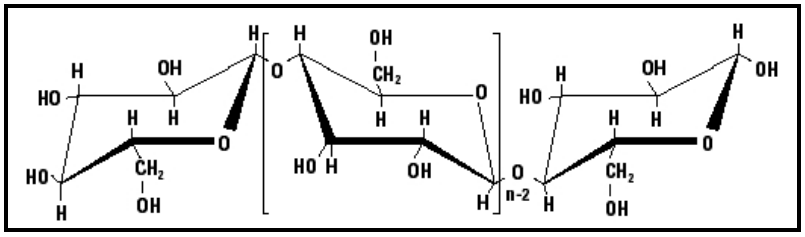

Figure 1.3. The structure of a linear cellulose polymer (Anonymous 2004b).

Hemicellulose

Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids. Unlike cellulose, hemicelluloses are not chemically homogeneous. Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (McMillan 1994). Xylans of many plant materials are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units. Besides xylose, xylans may contain arabinose, glucuronic acid or its 4-O-methyl ether, and acetic, ferulic, and ρ-coumaric acids. The frequency and composition of branches are dependent on the source of xylan (Aspinall 1980). The backbone consists of O-acetyl, α-L-arabinofuranosyl, α-1,2-linked glucuronic or 4-O-methylglucuronic acid substituents. However, unsubstituted linear xylans have also been isolated from guar seed husk, esparto grass, and tobacco stalks (Eda et al. 1976). Xylans can thus be categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan.

Xylans from different sources, such as grasses, cereals, softwood, and hardwood, differ in composition. Birch wood xylan contains 89.3% xylose, 1% arabinose, 1.4% glucose, and 8.3% anhydrouronic acid (Kormelink and Voragen 1993). Rice bran neutral xylan contains 46% xylose, 44.9% arabinose, 6.1% galactose, 1.9% glucose, and 1.1%

anhydrouronic acid (Shibuya and Iwasaki 1985). Wheat arabinoxylan contains 65.8% xylose, 33.5% arabinose, 0.1% mannose, 0.1% galactose, and 0.3% glucose (Gruppen et al. 1992). Corn fiber xylan is one of the complex heteroxylans, containing β-(1,4)-linked xylose residues (Saha and Bothast 1999a); it contains 48-54% xylose, 33-35% arabinose, 5-11% galactose, and 3-6% glucuronic acid (Doner and Hicks 1997). About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose or glucuronic acid linked to O-2 and/or O-3 of xylose residues, and also by oligomeric side chains containing arabinose, xylose, and sometimes galactose residues (Figure 1.4) (Saulnier et al. 1995).

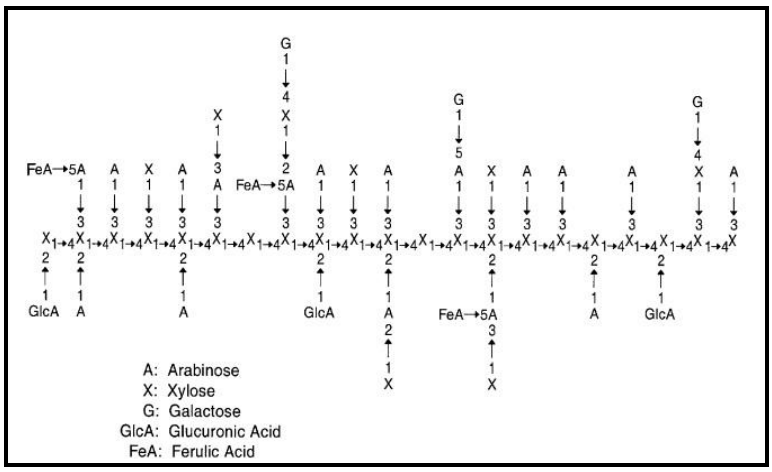

Figure 1.4. Schematic structure of corn fiber heteroxylan (Saulnier et al. 1995).

A model for the corn fiber cell wall is shown in Figure 1.5 (Saha 2003). The heteroxylans, which are highly cross-linked by diferulic bridges, constitute a network in which the cellulose microfibrils may be imbedded. Structural wall proteins might be cross-linked together by isodityrosine bridges and with feruloylated heteroxylans, thus forming an insoluble network (Hood et al. 1991). In softwood heteroxylans, arabinofuranosyl residues are esterified with ρ-coumaric acids and ferulic acids (Mueller-Hartley et al. 1986). In hardwood xylans, 60-70% of the xylose residues are acetylated (Timell 1967). The degree of polymerization of hardwood xylans (150-200) is higher than that of softwoods (70-130).

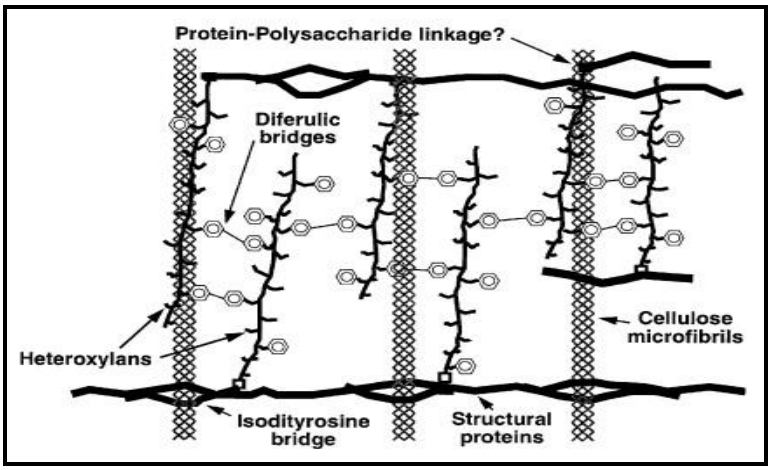

Figure 1.5. Model for corn fiber cell walls (Saha 2003).

Lignin

Lignin is a three-dimensional phenylpropane polymer with phenylpropane units held together by ether and carbon-carbon bonds (Sun 2002). It is constructed of three monomers: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol, each of which has an aromatic ring with different substituents (Brown 2003). The dominant monomeric units in the polymers are benzene rings bearing methoxyl, hydroxyl, and propyl groups that can be attached to other units (Klass 1998). When the plant is mature and the cell growth ceases, the middle lamella (the cement between the primary walls of adjacent cells) and the secondary wall (inside of the primary cell wall) have large amounts of lignin. Lignin strengthens the cell structures by stiffening and holding the fibers of polysaccharides together (Fan et al. 1987). The structure of a small section of a lignin polymer is shown below in Figure 1.6.

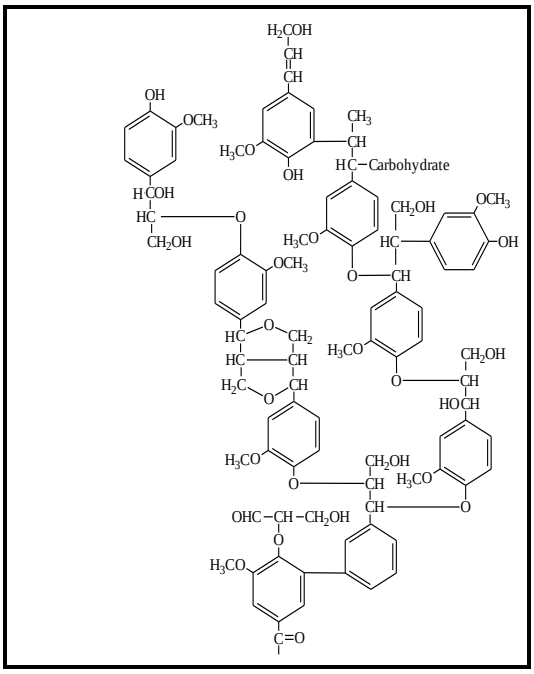

Figure 1.6. Structure of a section of a lignin polymer (Anonymous 2005).

1.1.9. Pretreatment of Lignocellulosic Materials

Pretreatment is the first step required to fractionate lignocellulosic material into its major plant components of lignin, cellulose and hemicellulose. The mechanisms by which pretreatments improve the digestibility of lignocellulose are, however, not well understood (Brown 2003). An important goal of pretreatment is to increase the surface area of the lignocellulosic material, making the polysaccharides more susceptible to hydrolysis. Along with an increase in surface area, pretreatment effectiveness and hydrolysis improvement have been correlated with removal of hemicellulose and lignin and the reduction of cellulose crystallinity (McMillan 1994). The large number of pretreatments used for lignocellulosic materials can be classified into groups as physical, physico-chemical, chemical, and biological processes.

Physical (Mechanical) Pretreatments

Milling (cutting the lignocellulosic biomass into smaller pieces) is a mechanical pretreatment of the lignocellulosic biomass. The objective of a mechanical pretreatment is a reduction of particle size and crystallinity. The reduction in particle size leads to an increase of available specific surface and a reduction of the degree of polymerization (DP) (Palmowski and Muller 1999). The milling also causes shearing of the biomass.

The increase in specific surface area, reduction of DP, and shearing are all factors that increase the total hydrolysis yield of the lignocellulose, in most cases by 5-25% (depending on kind of biomass, kind of milling, and duration of the milling). They also reduce the technical digestion time by 23-59%, and thus increase the hydrolysis rate (Delgenes et al. 2002, Hartmann et al. 2000). A reduction of particle size below 40 mesh, however, has little effect on the hydrolysis yield or hydrolysis rate of the biomass (Chang and Holtzapple 2000).

Milling causes a 5-25% increase in methane yield (Delgenes et al. 2002) and also increases the ethanol yield and hydrolysis rate. As no inhibitors (like furfural and 5-hydroxymethyl furfural-HMF) are produced, milling is suited for both methane and ethanol production. However, it has a high energy requirement (Cowling and Kirk 1976, Ramos 2003) and was therefore, in 1987, found not economically feasible as a pretreatment, taking into account the high energy requirements of milling and the continuous rise of the energy prices (Fan et al. 1987).

Physicochemical Pretreatments

1) Liquid Hot Water (LHW-Thermal Pretreatment)

A different thermal pretreatment is the "liquid hot water" pretreatment. In this case, liquid hot water (LHW) is used instead of steam. The main objective of the LHW is to solubilize the hemicellulose to make the cellulose more accessible and to avoid the formation of inhibitors. To avoid the formation of inhibitors, the pH should be kept between 4 and 7 during the pretreatment. Maintaining the pH between 4 and 7 minimizes the formation of monosaccharides, and consequently also the formations of degradation products that can further catalyze hydrolysis of the cellulosic material during pretreatment (Kohlmann et al. 1995, Mosier et al. 2005a, Weil et al. 1997). If catalytic degradation of sugars occurs, it results in a series of reactions that are difficult to control and lead to undesirable by-products. By keeping the pH between 4 and 7, the autocatalytic formation of fermentation inhibitors is avoided during the pretreatment.

LHW and steam pretreatment/steam explosion differ in the amount and concentration of solubilized products. In a LHW pretreatment, the amount of solubilized products is higher, while the concentration of these products is lower compared to steam

pretreatment (Bobleter 1994). This is probably caused by the higher water input in LHW pretreatment compared to steam pretreatment/steam explosion. The yield of solubilized (monomeric) xylan is generally also higher for LHW pretreatment. However, this result diminishes when the solid concentration increases because (monomeric) xylan is then further degraded by hydrolytic reactions to, for example, xylose and furfural (Laser et al. 2002).

Yang and Wyman (2004) discovered that flow-through systems removed more hemicellulose and lignin from corn stover than batch systems did, at the same severity factors. Moreover, the addition of an external acid during the flow-through process caused higher hemicellulose and lignin removal, while in batch tests, the addition of an external acid caused less lignin removal; at increased reaction times at temperatures above 200°C, Klason lignin (acid insoluble lignin) removal decreased. It was concluded that the external acid caused the lignin to solubilize faster, but also to condensate faster. The higher hemicellulose and lignin removal with the addition of an external acid during flow-through experiments is in conflict with the conclusions of Bobleter et al. (1991), Jacobsen and Wyman (2002) and Liu and Wyman (2003), which state that acids have no real effect or are not the only factors affecting the solubilization of hemicellulose and lignin, and that there should be other reasons for the solubilization of hemicellulose and lignin during flow-through experiments.

LHW has the major advantage that the solubilized hemicellulose and lignin products are present in lower concentrations, when compared to steam pretreatment, due to higher water input. With these lower concentrations, the risk of degradation products like furfural and the condensation and precipitation of lignin compounds is reduced. Weil et al. (1998) had a 2 to 5-fold increase in enzymatic hydrolysis of their substrate after LHW pretreatment.

Thermal Pretreatment in Combination with Acid Pretreatment: One way to improve the effect of thermal steam or LHW pretreatment is to add an external acid. This addition of an external acid catalyzes the solubilization of the hemicellulose, lowers the optimal pretreatment temperature and gives a better enzymatic hydrolyzable substrate (Brownell et al. 1986, Gregg and Saddler 1996). The lignocellulose is often impregnated (soaked) with sulfur dioxide (SO_2) or H_2SO_4. During steam pretreatment the SO_2 is converted to H_2SO_4 in the first 20 seconds of the process; after that, the catalytic

hydrolyzation of the hemicellulose starts. Another important point is that gradual removal of hemicellulose and lignin can trigger reorientation of cellulose to a more crystalline form (Gregg and Saddler 1996). The latter is true for every pretreatment that gradually removes hemicellulose and lignin. The effect of the added acid is still not clear, however. Tengborg et al. (1998) showed a significant inhibition in the ethanol production step at a severity factor of 3 and higher with the addition of an external acid. This is in line with the conclusion of Grohmann et al. (1985) that during steam pretreatment at temperatures of 160°C and higher with the addition of 0.5% H_2SO_4, an appreciable production of furfural occurs.

Soderstrom et al. (2002) investigated the use of a two-step steam pretreatment of softwood with SO_2. The first step was carried out at lower severities for the recovery of hemicellulose sugars, and the second step was done at higher severities to improve the digestibility of the cellulose. The highest hemicellulose recovery in the first pretreatment step was achieved at a severity of about 3, and the highest sugar yield in the second pretreatment step at a severity between 3.5 and 4.3. However, pretreatments at temperatures of 220°C did not give satisfactory yields because of the formation of inhibiting compounds. Wu et al. (1999) got the highest amount of monomeric sugars from hemicellulose at a severity factor of approximately 3 (175°C, 7.5 min, 4.5% SO_2). At higher severities, the monomeric sugars were probably degraded to furfural and other compounds.

Thermal Pretreatment in Combination with Alkaline Pretreatment: Another way to improve the thermal pretreatment is to add an external alkali instead of an acid to the process. A very common alkaline thermal pretreatment is lime pretreatment. This pretreatment is usually carried out at temperatures of 100-150°C with lime addition of approximately 0.1 g calcium hydroxide [$Ca(OH)_2$] g substrate^{-1} (Chang et al. 2001a). Chang and Holtzapple (2000) attribute the effectiveness of lime pretreatment to the opening of the "acetyl valve" and partial opening of the "lignin valve", making the substrate more accessible to hydrolysis. According to Kaar and Holtzapple (2000), lime pretreatment (with heating) is sufficient to increase the digestibility of low-lignin-containing biomass, but not for high-lignin-containing biomass. Chang et al. (2001a) mention that lime pretreatment of switchgrass and corn stover did not inhibit the enzymatic saccharification and fermentation steps. Pretreated softwood, however, was

washed before the enzymatic saccharification and fermentation step to prevent possible inhibiting by (the large amount of) solubilized lignin. A benefit of lime is that it is relatively cheap and safe (Gandi et al. 1997) and the calcium can be regained as insoluble calcium carbonate ($CaCO_3$) by the reaction with CO_2. This $CaCO_3$ can be converted to lime again using the lime kiln technology (Chang et al. 1998).

Thermal Pretreatment in Combination with Oxidative Pretreatment: Ando et al. (1988) mention that the saccharification of cedar, soaked in peracetic acid and steam treated at 231°C for 10 min, was directly proportional to the amount of peracetic acid adsorbed in the chips. Wet-oxidation is another oxidative pretreatment method which uses oxygen as an oxidator. The soluble sugars produced during wet-oxidation pretreatment of wheat straw are mainly polymers opposite to the monomers produced during steaming or acid hydrolysis as a pretreatment. Phenolic monomers are not end products during wet-oxidation, but are further degraded to carboxylic acids. In addition, the production of furfural and HMF was low during wet-oxidation, but part of the hemicellulose was lost through reaction to carbon dioxide and water (Klinke et al. 2002).

Thermal Pretreatment in Combination with Alkaline Oxidative Pretreatment: According to Chang et al. (2001a), thermal lime pretreatment is not capable of removing enough lignin of high-lignin biomass to enhance the enzymatic digestibility, and therefore oxygen as an oxidant must be included during the pretreatment. Low sugar degradation was observed, probably as a result of the relative low temperature of 150 degrees applied during the pretreatment. The enzymatic digestibility of the treated biomass was 13 times higher than for the untreated biomass; however, the pretreated biomass was washed to remove probable inhibiting soluble lignin compounds produced (Chang et al. 2001a). After the oxidative lime pretreatment, about 21% of the added lime could be recovered by CO_2 carbonation (Chang et al. 2001b).

2) Steam Pretreatment/Steam Explosion (SE)

During steam explosion (SE) the biomass is put in a large vessel with steam at a high temperature (temperatures up to 240°C) and pressure is applied for a few minutes. After a set time, the steam is released and the biomass is quickly cooled down. The objective of SE is to solubilize the hemicellulose to make the cellulose more accessible for enzymatic hydrolysis and to avoid the formation of inhibitors. The difference between

"steam" pretreatment and "steam explosion" pretreatment is the quick depressurization and cooling down of the biomass at the end of the SE pretreatment, which causes the water in the biomass to "explode". During steam pretreatment, parts of the hemicellulose hydrolyze and form acids, which could catalyze the further hydrolysis of the hemicellulose. This process, in which the acids formed *in situ* catalyze the process itself, is called "auto-cleave" steam pretreatment. The role of the acids is probably not to catalyze the solubilization of the hemicellulose, but to catalyze the hydrolysis of the soluble hemicellulose oligomers (Bobleter et al. 1991, Mok and Antal 1992).

A common term used in steam pretreatment is the so-called "severity factor" ($\log R_0$), which is a measure of the severity of the pretreatment. In this severity factor the temperature of the pretreatment and the duration of the pretreatment are combined in the following way:

$$\log R_0 = \log(t * e^{((T-100)/14.75)}) \qquad (1.1)$$

In the equation, t and T show minutes and Celsius degrees, respectively (Overend and Chornet, 1987). During steam pretreatment, the moisture content of the biomass influences the needed pretreatment time. The higher the moisture content, the longer the optimum steam pretreatment times (Brownell et al. 1986). Low pressure steam pretreatment (2 bars, 120°C, and pretreatment times up to 300 min) did not have a significant effect on the composition of the wheat straw, according to Lawther et al. (1996), though no enzymatic conversion step was carried out for determining the effect on the digestibility.

Steam pretreatment includes the risk of producing furfural, HMF, and soluble phenolic compounds. These compounds inhibit the ethanol fermentation and methane production. The methane-producing bacteria are, however, capable of adapting to such compounds, at least to a certain concentration. Benjamin et al. (1984), Fox et al. (2003), and Noike and Niigata Eng (2001) demonstrated the adaption to these compounds and sometimes even the conversion by anaerobic bacteria after a certain period. Grous et al. (1986) reported a six-fold increase in enzymatic digestibility of the biomass after steam

pretreatment.

The hemicellulose degradation during steam pretreatment may be minimized by separating the biomass from the condensate during the pretreatment (Allen et al. 2001), keeping the pH between 5 and 7 during the pretreatment by the addition of an external alkali (Li et al. 2005, Weil et al. 1998), or by applying a two-step steam pretreatment. However, it is not clear if the higher ethanol or methane yield outweighs the additional cost of a second pretreatment step (Shahbazi et al. 2005, Soderstrom et al. 2002).

The positive effect of steam pretreatment is mostly due to the removal of a large part of the hemicellulose, causing an increase in cellulose fiber reactivity, probably because the cellulose is more easily accessible for the enzymes (Converse et al. 1989, Grohman et al. 1986, Laser et al. 2002). Some yeast species can also convert pentoses to ethanol (Kuyper et al. 2005). Consequently, the degradation of pentoses to, furfural, for example, during the steam pretreatment results in a loss of carbon for the ethanol production. Steam pretreatment includes the risk of condensation and precipitation of soluble lignin components, making the biomass less digestible, and reducing the ethanol as well as the methane production.

The advantages of SE are: 1) its ability to separate the three components of wood lignocelluloses are modified to allow fractionation of hemicellulose in autohydrolysis steam, lignin in aqueous alcohol or alkali, and cellulose as insoluble biomass; 2) cellulose is highly susceptible to acid or enzymatic hydrolysis; 3) lignin is in a suitable form for conversion to chemicals; 4) hemicellulose is easily converted to liquid fuels; and 5) inhibitors are easily extractable. On the other hand, the disadvantages of SE are: 1) it produces substrates with low bulk density; 2) it does not always break down the lignin completely; 3) it requires small particle size; and 4) some of the compounds produced can be inhibitory to the subsequent ethanol fermentation.

Acid-Catalyzed SE: The addition of dilute acid in the SE can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of the hemicellulose. Acid-catalyzed SE is one of the most cost-effective processes for hardwood and agricultural residues, but it is less effective for softwoods. It is possible to recover around 70% xylose potentially as a monomer. The lignin redistribution may explain the reason that dilute acid and SE are effective as a

pretreatment process. Although the lignin is not removed, it is believed that lignin melts during the pretreatment and coalesces upon cooling such that its properties are substantially altered (Lynd et al. 2002). Limitations include destruction of a portion of the xylan fraction, incomplete disruption of the biomass structure, and the generation of inhibitory compounds. The necessary water wash decreases the overall sugar yields.

Ammonia and SE: Lignocellulosic materials can also be exploded using liquid ammonia and SE. This method is considered one of the leading biomass pretreatments. Ammonia freeze/fiber explosion (AFEX) treats the biomass with concentrated ammonia under pressure and at temperatures up to nearly 100°C. After a few minutes under these conditions, the pressure is rapidly released (the "explosion"). The ammonia evaporates and is recovered. AFEX disrupts the lignocellulose and reduces the cellulase requirement, but removes neither hemicellulose nor lignin. The treated biomass is now much more easily converted by enzymes to sugars, and then to ethanol. In a comparative economic evaluation of advanced pretreatments, AFEX performed better than all the other pretreatments studied, except for the dilute acid process. More complete understanding of the morphological changes and chemical compounds formed during AFEX may further improve the pretreatment performance. Ammonia explosion does not produce products that may inhibit fermentation, but it requires that the ammonia be recycled for economic and environmental reasons (Sun and Cheng 2002).

CO_2 Catalyzed SE: CO_2 explosion is similar to steam and ammonia explosion. The glucose yields in the later enzymatic hydrolysis are lower compared to steam and ammonia explosion. The SE of wheat straw, bagasse, and eucalyptus wood chips in the presence of CO_2 at 200°C increases the digestibility above 75%. In addition, CO_2 explosion is more cost-effective than ammonia explosion and does not cause the formation of inhibitors as in steam explosion.

SO_2 Catalyzed SE: Martin et al. (2002) investigated SO_2 and H_2SO_4 impregnation during SE (205°C, 10 min) of sugarcane bagasse and their influence on the enzymatic hydrolysis. The SO_2-impregnated bagasse gave the highest yields of xylose, arabinose, and total sugar on hydrolysis. The hydrolysates of SO_2-impregnated and nonimpregnated bagasse showed similar fermentability with *S. cerevisiae*, whereas the fermentation of the hydrolysate of H_2SO_4-impregnated bagasse was considerably poor. Corn fiber that was steam exploded in a batch reactor at 190°C for 5 min with 6%

SO_2 resulted in 81% conversion of all the polysaccharides in the corn fiber to monomeric sugars on enzymatic hydrolysis, which was subsequently converted to ethanol very efficiently by *S. cerevisiae*, yielding 90 to 96% of the theoretical conversion (Bura et al. 2002).

Supercritical Carbon Dioxide (SC-CO_2): SC-CO_2 is CO_2 above its critical point of 31°C and 73 atm. This pretreatment has many advantages, such as nontoxicity, low solvent cost, low pretreatment temperatures, easy recovery of CO_2, and high solids concentrations in the pretreated materials. However, the low effectiveness for softwood and the high capital cost for the high-pressure equipment may be obstacles for its commercialization. SC-CO_2 had no significant effect on the yield of reducing sugars or enzymatic digestibility of aspen lignocellulosic biomass (Williams 2006). SC-CO_2 explosion enhanced the accessible surface area and increased glucose yield by 50% (Zheng et al. 1995).

Chemical Pretreatments

1) Acid Pretreatment

Pretreatment of lignocellulose with acids at ambient temperatures is done to enhance the anaerobic digestibility. The objective is to solubilize the hemicellulose, thus making the cellulose more accessible. The pretreatment can be done with dilute or strong acids. The main reaction that occurs during acid pretreatment is the hydrolysis of hemicellulose, especially xylan, as glucomannan is relatively acid-stable. Solubilized hemicelluloses (oligomers) can be subjected to hydrolytic reactions producing monomers, furfural, HMF and other (volatile) products in acidic environments (Fengel and Wegener 1984, Ramos 2003). During acid pretreatment, solubilized lignin will quickly condensate and precipitate in acidic environments (Liu and Wyman 2003, Shevchenko et al. 1999). The solubilization of hemicellulose and precipitation of solubilized lignin are more pronounced during strong acid pretreatment compared to dilute acid pretreatment. Xiao and Clarkson (1997) showed that the addition of nitric acid (HNO_3) during acid pretreatment has a tremendous effect on the solubilization of lignin in newspaper.

The advantage of acid pretreatment is the solubilization of hemicellulose, thus making the cellulose more easily accessible for the enzymes. There is, however, a risk of the

formation of volatile degradation products, and this carbon is in many cases lost for the conversion to ethanol. On the other hand, volatile products can be converted to methane. The condensation and precipitation of solubilized lignin components is an unwanted reaction, as it decreases digestibility. Strong acid pretreatment is not preferred for the production of ethanol because there is a risk of the formation of inhibiting compounds. Dilute acid pretreatment, however, is considered one of the promising pretreatment methods because secondary reactions during the pretreatment can be prevented in dilute acid pretreatment.

Acid pretreatment is more suitable for methane production than for ethanol production because methanogens can handle compounds like furfural and HMF to a certain concentration with an acclimatization period. For ethanol as well as methane production, the chance of soluble lignin components is a risk, because soluble lignin compounds are often inhibiting for both processes. Methanogens are nevertheless capable of adapting to such inhibiting compounds (Benjamin et al. 1984, Xiao and Clarkson 1997). When H_2SO_4 or HNO_3 are used in the acidic pretreatment, the methane production during anaerobic treatment will be reduced as a result of the reduction of sulphate and nitrate to hydrogen sulfur (H_2S) and nitrogen (N_2) respectively.

2) Alkaline Pretreatment

During alkaline pretreatment, the first reactions taking place are solvation and saphonication. These cause the biomass to swell and thus make it more accessible to enzymes and bacteria. At strong alkali concentrations, dissolution, peeling of end-groups, alkaline hydrolysis and degradation, and decomposition of dissolved polysaccharides can take place. Loss of polysaccharides is mainly caused by peeling and hydrolytic reactions (Fengel and Wegener 1984). This peeling is an advantage for later conversion, but, because lower molecular compounds are formed as a result, the risk of degradation and loss of carbon, in the form of CO_2, also increases. Xylan can be selectively removed with aqueous potassium hydroxide (KOH). The temperature is kept low during extraction (room temperature or lower) to prevent peeling (Hon and Shiraishi 2001). Glucomannans and xylans can both be subject to the peeling reaction. This in itself is not a problem, but the higher the monomeric hemicellulose fraction, the lower the total recovery of the hemicellulose (Laser et al. 2002). The monomeric forms of hemicellulose are probably easily degradable to other (volatile) compounds.

Furfural, for example, leads to losses of digestible substrate for the ethanol process (Bobleter 1994). An important aspect of alkali pretreatment is that the biomass itself consumes some of the alkali. The residual alkali concentration after the alkali consumption by the biomass is the alkali concentration left over for the reaction (Gossett et al. 1982). Alkali extraction can also cause solubilization, redistribution and condensation of lignin and modifications in the crystalline state of the cellulose. These effects can lower or counteract the positive effects of lignin removal and cellulose swelling (Gregg and Saddler 1996). Another important aspect of alkaline pretreatment is the change of the cellulose structure to a form that is denser and thermodynamically more stable than the native cellulose (Pettersen 1984).

Alkaline pretreatment causes hemicellulose and parts of lignin to solubilize. The removal of hemicellulose has a positive effect on the degradability of cellulose. There is, however, often a loss of hemicellulose to degradation products, and the solubilized lignin components often have an inhibitory effect. Gossett et al. (1982), for example, concluded that alkaline heat-treated lignin in concentrations over 1 g/L gave a major inhibitory effect to the methanogenic microorganisms. This was probably caused by the products formed from the lignin during the alkaline heat pretreatment. The loss of fermentable sugars and production of inhibitory compounds make the alkaline pretreatment less attractive for ethanol production. The production of inhibitors is less severe for methanogens as compared to yeasts for ethanol production. Methanogens are often capable of adapting to such compounds. Pavlostathis and Gossett (1985) mentioned a 100% increase in methane production after an alkaline pretreatment of wheat straw.

3) Oxidative Pretreatment

The rate and extent of lignocellulose digestion by microorganisms present in the stomachs of ruminants are both greatly enhanced when the lignocellulose is first treated with an alkaline (pH 11.5) solution of H_2O_2. The increase in digestibility has been attributed not only to oxidative delignification, but also to a possible decrease in cellulose crystallinity (Gould 1985). Martel and Gould (1990) concluded from their study on wheat straw and kenaf that alkaline hydrogen peroxide (AHP) treatment loosened the lignocellulosic matrix and caused a more open three-dimensional relationship between lignin, cellulose, and hemicellulose at the molecular level. They

also observed that there was either no change or an increase in cellulose crystallinity after AHP treatment, thus supporting the contention that the main effect of AHP treatment is that it detaches and makes soluble the lignin, thus increasing the amount of cellulose available for hydrolysis by enzymes (Martel and Gould 1990), while it does not decrease cellulose crystallinity as previously hypothesized by Gould (1985).

During an oxidative pretreatment, a lot of sugars are often lost because of non-selective oxidation. Also, there is a formation of soluble lignin compounds, which can be inhibiting in the subsequent conversion step of the (hemi) cellulose to ethanol or methane.

4) Ozone

Ozone has been used to degrade lignin and hemicellulose in lignocellulosic materials such as cotton stalks (Ben-Ghedalia et al. 1980, Ben-Ghedalia and Shefet 1983, Yosef et al. 1994), corn stover (Quesada et al. 1999), wheat straw (Ben-Ghedalia and Miron 1981), bagasse, and poplar sawdust. One of the benefits of ozone pretreatment is the fact that no toxic residues are formed, since ozone can easily be decomposed to oxygen using a catalytic bed or an increase in temperature, thus eliminating the need for extensive downstream processing; ozonation reactions take place at ambient temperatures and pressure, so energy and investment costs are minimized (Quesda et al. 1999). Ben-Ghedalia et al. (1980) pretreated cotton straw with ozone to examine the effect on the composition of the cell wall fractions and on in vitro organic matter digestibility. The most notable effects of ozone treatments were demonstrated by the 50% decrease in both lignin and hemicellulose (Ben-Ghedalia et al. 1980). The pH of ozone-treated cotton stalks was considerably more acidic and it was concluded that the low pH values were probably the result of the release of a mixture of formic, acetic, glyoxylic, or other acids from the oxidized lignin. Quesda et al. (1999) later confirmed this by showing the appearance of glycolic, oxalic, malonic, glyoxylic, glyceric, and malic acids in a chromatographic analysis of the aqueous extract of oxidized, extractive-free corn stover, due to the generation of carboxylic acids from extensive lignin degradation. Yosef et al. (1994) showed through Nuclear Magnetic Resonance (NMR) analysis that lignin degradation by ozone is the result of ring cleavage, directly evidenced by the decline in aromatic carbon (C) from 13.0% in untreated cotton stalks to 7.40% in ozone-treated stalks. The rate of enzymatic hydrolysis increased by a

factor of 5 following removal of 60% of the lignin from wheat straw during ozone pretreatment (Binder et al. 1980). As the lignin content of poplar sawdust decreased from 29 to 8% after ozonolysis, enzymatic conversion increased from 0 to 57% (Vidal and Molinier 1988). The optimal moisture content of 60% was found to provide the highest degree of solubilization during ozone treatment of corn stover (Quesda et al. 1999). Results from the same study showed that lignin was the most affected polymer, followed by hemicelluloses and then cellulose.

Biological Pretreatments

In these pretreatments, the natural wood-attacking microorganisms that can degrade lignin are allowed to grow on the biomass, resulting in lignin degradation. The main biological pretreatments include fungi and their enzymes. There is significant loss of the xylan and mannan components of the hemicellulose during the lignin hydrolysis. Reductions up to 65% in the lignin content of cotton straw have been reported using white-rot fungi. This is the most promising organism for biological pretreatment of lignocellulose. The various means to use these organisms are: use of naturally-occurring white-rot fungi; use of celluloseless mutants as efficient lignin degraders and/ or to repress the enzymes that degrade wood carbohydrates.

A white-rot fungus was used to remove 42% lignin, 2% glucan (including cellulose), and 30% hemicellulose of birch wood (Fan et al. 1982). The degradation of wood lignin by white-rot is oxidative and needs an accompanying carbohydrate such as cellulose or hemicellulose. *Phanerochaete chrysosporium*, a white-rot fungus, is the most commonly used organism for delignification. It degraded 48.6% of lignin, 5.30% of cellulose, and 19.7% of hemicellulose in grape cluster stems over the course of 10 to 12 days. Phanerochaete did not have any effect on the enzyme digestibility of raw corn stover. However, another fungus, *Cyathus* sp., increased the digestibility by 3 to 6.9 times the control values over 29 days (Williams 2006). A 17% delignification was achieved by exposing birch wood to celluloseless mutants of Polyporus adustus for 6 weeks. Similarly, 72% conversion of cellulose to glucose by enzymatic hydrolysis after biological delignification of wheat straw using *Pleurotus ostreatus* has also been reported.

Other organisms used for biological treatment are *Ceriporiopsis subvermispora* and

Trametes versicolor. The rate of lignin and hemicellulose breakdown is very slow and still needs optimization in most cases to make it an effective pretreatment method (Sun and Cheng 2002). The advantages of these biological pretreatments are that they require little energy input and are environmentally friendly. The economic feasibility of a nonoptimized biological pretreatment process is still poor due to the long cultivation time of 10 to 14 days. This method can be considered cost-effective only if applied in conjunction with other physical and/or chemical methods such as thermomechanical pulping and SE. In both cases, the removal of resins and other extractable materials can also play an important role in improving the accessibility of the lignocellulosics to bioconversion (Ramos 2003). Sometimes biological treatments are used in combination with chemical treatments (Hamelinck et al. 2005).

Table 1.2. Effects of the different pretreatments on the physical/chemical composition or structure of lignocellulose (Mosier et al. 2005b).

Pretreatment Method	Increase accessible surface area	Decrystallization cellulose	Solubilization hemicellulose	Solobilization lignin	Formation furfural/ HMF	Alteration lignin structure
Mechanical	+	+				
SE	+		+	-	+	+
LHW	+	ND	+	-	-	-
Acid	+		+	-	+	+
Alkaline	+		-	+/-	-	+
Oxidative	+	ND		+/-	-	+
Thermal+acid	+	ND	+	+/-	+	+
Thermal+alkaline	+	ND	-	+/-	-	+
Thermal+oxidative	+	ND	-	+/-	-	+
Thermal+alkaline+ Oxidative	+	ND	-	+/-	-	+
AFEX	+	+	-	+	-	+
CO_2 explosion			+			

+ =major effect.

- =minor effect

ND =not determined.

Although all pretreatment methods have some advantages (Table 1.2), a successful pretreatment must meet the following requirements: (1) improve formation of sugars or the ability to subsequently form sugars by hydrolysis; (2) avoid the degradation or loss of carbohydrate; (3) avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation processes; and (4) be cost-effective (Sun 2002).

1.1.10. Hydrolysis

After the pretreatment is finished, the cellulose is prepared for hydrolysis, meaning the

cleaving of a molecule by adding a water molecule (Vessia 2010):

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6$$

This reaction is catalysed by using dilute acid, concentrated acid or enzymes (cellulase). The latter method has many advantages as the very mild conditions (pH=4.8 and temperature 318-323 K) give high yields, and the maintenance costs are low compared to alkaline and acid hydrolysis, due to the absence of corrosion problems (Vessia 2010). Hydrolysis without preceding pretreatment yields typically <20%, whereas yields after pretreatment often exceed 90% (Hamelinck et al. 2005). A number of processes for hydrolyzing cellulose into glucose have been developed over the years. The vast majority of processing schemes utilize either cellulolytic enzymes or H_2SO_4 of varying concentrations. In the past, enzymes have been too expensive for economical production of fuel ethanol from biomass. H_2SO_4 itself is less expensive than cellulolytic enzymes, although disposal costs associated with the use of H_2SO_4 significantly increase its cost. However, the single largest drawback to using H_2SO_4 is that it also readily degrades glucose at the high temperatures required for cellulose hydrolysis (Mosier et al. 2002).

Lignocellulose biomass may be hydrolyzed by gamma ray or electron-beam irradiation, or microwave irradiation (Demirbas 2004). Hydrolysis of lignocellulosic biomass is more complicated than that of pure cellulose, due to the presence of nonglucan components such as lignin and hemicellulose (Zhang and Lynd 2004).

Acid hydrolysis

In research studies it has been revealed that under controlled treatment conditions, acid hydrolysis of lignocellulosic biomass mainly produces xylose from xylan with the cellulosic and lignin fractions remaining unaltered. Xylan is more susceptible to hydrolysis by mild acid treatment due to its amorphous structure compared to cellulose, which needs severe treatment conditions for its crystalline nature (Rahman et al. 2007). The acid hydrolyzate from sugarcane bagasse contains xylose as the main component. During acid hydrolysis, xylose is degraded rapidly to furfural and other condensation byproducts. These degradation products are inhibitory to microorganisms. The inhibitory effect of different compounds like furfural, HMF, acetate, hydroxybenzaldehyde (HBA), siringaldedyde (SGA) and vanillin on yeast growth is

well documented (Rao et al. 2006).

Acid-catalyzed cellulose hydrolysis is a complex heterogeneous reaction. It involves physical factors as well as the hydrolytic chemical reaction. Monosaccharide products can be further degraded into undesirable chemicals. The number of possible side reactions depends upon, among other things, the permeate composition. As such, evaluation of acid hydrolysis as a means to generate monosaccharides from lactose in whey permeate must be carried out within the context of the intended use of the hydrolysis products (Cote et al. 2004). The acid-hydrolyzed substrates were then subjected to enzyme hydrolysis to give vastly improved yields, as high as 100% for corn stover and 90% for oak wood (Jeoh 1998). There are two basic types of acid hydrolysis processes commonly used: dilute acid and concentrated acid.

Dilute Acid Hydrolysis

This is the oldest technology for converting cellulose biomass to bioethanol (Graf and Koehler 2000). In dilute acid hydrolysis, the hemicellulose fraction is depolymerized at a lower temperature than the cellulosic fraction. Dilute H_2SO_4 is mixed with biomass to hydrolyze hemicellulose into xylose and other sugars (Chandel et al. 2007). The dilute acid process involves a solution of about 1% H_2SO_4 concentration in a continuous-flow reactor at a high temperature (about 488 K) (Graf and Koehler 2000). Most dilute acid processes are limited to a sugar recovery efficiency of around 50% (Badger 2002). The primary challenge for dilute acid hydrolysis processes is to raise glucose yields higher than 70% in an economically viable industrial process, while maintaining a high cellulose hydrolysis rate and minimizing glucose decomposition. Strong acids can reduce the crystalline region, but they degrade glucose (Lee 2005).

Dilute acid hydrolysis occurs in two stages in order to take advantage of the differences between hemicellulose and cellulose. The first stage is performed at a low temperature to maximize the yield from the hemicellulose; the second, higher-temperature stage is optimized for hydrolysis of the cellulose portion of the feedstock (Farooqi and Sam 2004). The first stage is conducted under mild process conditions to recover the 5C sugars while the second stage is conducted under harsher conditions to recover the 6C sugars (Demirbas 2006b).

The biggest advantage of dilute acid processes is their fast rate of reaction, which facilitates continuous processing. Their biggest disadvantage is their low sugar yield. For rapid continuous processes, in order to allow adequate acid penetration, feedstocks must also be reduced in size so that the maximum particle dimension is in the range of a few millimeters (Badger 2002).

Concentrated Acid Hydrolysis

The concentrated acid process provides a complete and rapid conversion of cellulose to glucose and hemicelluloses to 5C sugars with little degradation. The critical factors needed to make this process economically viable are to optimize sugar recovery and to cost-effectively recover the acid for recycling (Demirbas 2004). The concentrated acid process uses relatively mild temperatures, and the only pressures involved are those created by pumping materials from vessel to vessel. Reaction times are typically much longer than for the dilute acid process (Graf and Koehler 2000). The concentrated acid process uses 70% H_2SO_4 at 313-323 K for 2-4 h in a reactor. The low temperatures and pressure will lead to minimization of the sugar degradation. The hydrolyzed material is then washed to recover the sugars. In the next step, the cellulosic fraction has to be deploymerized. The solid residue from the first stage is de-watered and soaked in 30-40% H_2SO_4 for 50 min at 373 K for further cellulose hydrolysis (Chandel et al. 2007). The primary advantage of the concentrated acid process is the potential for high sugar recovery efficiency (Demirbas 2005b). The concentrated acid process offers more potential for cost reductions than the dilute H_2SO_4 process (Farooqi and Sam 2004). Concentrated H_2SO_4 or hydrochloric acid (HCl) is difficult to work with, and essentially all of the acid must be recovered and reconcentrated in order for the process to be economical (Jeffries and Jin 2000).

Enzymatic Hydrolysis

Following the pretreatment of lignocelluloses, enzymatic hydrolysis is carried out to break down cellulose and hemicelluloses into fermentable sugars such as glucose and xylose. Strong acids such as H_2SO_4 and halogen acids are capable of hydrolyzing a wide variety of lignocelluloses into simple fermentable sugars (Wyman 1994). However, high acid concentrations and extreme conditions make this approach environmentally and economically unsound (Wright and Dagincourt 1984). Enzymatic hydrolysis is an

environmentally friendly alternative that involves using carbohydrate-degrading enzymes (cellulases and hemicellulases) to hydrolyze lignocelluloses into fermentable sugars.

Enzyme cost is considered to be a major impediment in extensive commercialization of enzymatic cellulose hydrolysis (Walker and Wilson 1991). Enzyme cost is estimated to represent approximately 50% of the total hydrolysis process cost. A study conducted by Lee in 1981 (cited in Walker and Wilson 1991) puts the enzyme cost into stark monetary terms. The study showed that cellulose free of hemicellulose and lignin could be produced for 55 US dollars/mg^{-1} while the cellulase cost was 2,665 US dollars/mg^{-1}. The cost of enzymes has decreased over the last twenty years, but is still considered to be very high.

Enzymatic Hydrolysis of Cellulose

Enzymatic hydrolysis of cellulose is typically carried out by cellulases. Unlike conventional hydrolysis using concentrated acid or alkaline reagents, enzymatic hydrolysis requires mild conditions (pH of 4.5 and temperature of approximately 50°C). Although cellulases are also produced by several bacterial species (Table 1.3) such as *Clostridium*, *Cellulomonas*, and *Bacillus* (Bisaria 1998), fungal cellulases have the best potential for commercial-scale use (Duff and Murray 1996).

Cellulases are a complex system of three enzymes that act synergistically to hydrolyze cellulose. The three enzyme components are: 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.3), 1,4-β-D-glucan cellobiohydrolyase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Ladisch et al. 1983, Wright et al. 1988). These enzymes are commonly referred to as endoglucanase, exoglucanase, and cellobiase, respectively. Endoglucanase randomly cleaves cellulose chains to form glucose, cellobiose, and cellotriose. Exoglucanase attacks the nonreducing end of cellulose to release cellobiose units. Cellobiase cleaves cellobiose units into fermentable glucose units. Most fungal cellulases are deficient in β-glucosidase activity, which must be supplemented, since cellobiose accumulation results in cellulase inhibition. A cellulase dosage of 10 filter paper units (FPU) per gram of biomass is often used in studies, as it enables high glucose yields in 48-72 h (Gregg and Saddler 1996). However, a range of dosage and hydrolysis conditions have been reported depending on the composition of the

substrates and the pretreatment used.

Enzymatic Hydrolysis of Hemicelluloses

Complete hydrolysis of xylan involves three main enzymes: endo-β-1-4-xylanase, which primarily targets the internal β-1-4 bonds between xylose units; exoxylanase, that releases xylobiose units; and β-xylosidase, which releases xylose from xylobiose and short chain xylooligosachharides (Saha and Bothast 1999b).

Table 1.3. List of bacteria fungi with the highest specific activity (μmol.min^{-1}.mg^{-1}) for cellulases (Howard et al. 2003).

Bacteria					
Enzyme	Organism	Substrate	Specific activity	Opt.temp. (°C)	Opt.pH
mannan endo-1,4-β-mannosidase	*Bacillus subtilis*	Galactoglucomannan/glucomannans/ mannans	514	50-60	5-7
cellulase	*Clostridium thermocellum*	Avicel/carboxymethylcellulose/cellulose cellopentaose/cellotetraose/cellotriose/	428	75	7
1,3-β-glucan glucohydrolase	*Streptomyces murinus*	laminarin	6.7	50	6
1,3-1,4-β-D-glucan glucanohydrolase	*Bacillus macerans*	β-D-glucan/ lichenan	5030	60-65	6
1,3-β-D-glucan glucanohydrolase	*Bacillus sp.*	3-O-β-D-Glc-D-Glc-D-Glc-D-Glc/ laminarin	369.6	60	9
Fungi					
Enzyme	Organism	Substrate	Specific activity	Opt.temp. (°C)	Opt.pH
mannan endo-1,4-β-mannosidase	*Sclerotium rolfsii*	Galactoglucomannan/ galactomannans/ glucomannans/ mannans	475	72-74	3.3
cellulase	*Aspergillus niger*	Carboxymethylcellulose/ cellohexaose/ cellopentaose/ cellotetraose/ cellotriose/ cellulose	194	70	5
1,3-β-glucan glucohydrolase	*Achlya bisexualis*	Glucan/ laminarin/ neutral glucan/ phosphoglucan	7840	30	6
1,3-1,4-β-D-glucan glucanohydrolase	*Orpinomyces sp.*	β-D-glucan/ lichenin	3659	45	5.8
1,3-β-D-glucan glucanohydrolase	*Rhizopus chinensis*	β-glucan	4800	NA	NA
1,6-β-D-glucan glucanohydrolase	*Penicillium brefeldianum*	β-glucan/ gentiobiose/ pachyman	405	50	4.2

While these enzymes are primarily involved in depolymerization, there are also several ancillary enzymes that are responsible for cleaving side-groups. These include α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid esterase, and ρ-coumaric acid esterase (Saha and Bothast 1999b).

Penicillium capsulatum and *Talaromyces emersonii* have been identified as microorganisms that have complete enzyme systems that degrade xylan (Filho et al. 1991). Other microorganisms (Table 1.4) that have been reported as sources for

hemicelluloses-degrading enzymes are Aureobasidium pullulans (Christov et al. 1997) and several Fusarium species (Saha 2001).

Table 1.4. List of bacteria fungi with the highest specific activity (μmol.min^{-1}.mg^{-1}) for hemicellulases (Howard et al. 2003).

Bacteria					
Enzyme	Organism	Substrate	Specific activity	Opt.temp. (°C)	Opt.pH
Feruloyl esterase	*Clostridium stercorarium*	Ethyl ferulate	88	65	8
Endo-1,4-β-xylanase	*Bacillus pumilus*	B-1,4-D-xylan	1780	40	6.5
β-1,4-xylosidase	*Thermoanaerobacter ethanolicus*	o-nitrophenyl-β-D-xylopyranoside	1073	93	6
Exo-β-1,4-mannosidase	*Pyrococcus furiosus*	p-nitrophenyl-β-D-galactoside	31.1	105	7.4
Endo-β-1,4-mannanase	*Bacillus subtilis*	Galactoglucomannan/ glucomannans/mannan	514	50	5/7
Endo-α-1,5-arabinanase	*Bacillus subtilis*	1,5-α-L-arabinan	429	60	6/8
α-Larabinofuranosidase	*Clostridium stercoarium*	alkyl-α-arabinofuranoside/ aryl-α-arabinofuranoside/ Larabinogalactan/ L-arabinoxylan/ methylumbelliferyl-α-Larabinofuranoside	883	70	5
α-Glucuronidase	*Thermoanaerobacterium saccharolyticum*	4-O-methyl-glucuronosyl-xylotriose	9.6	50	6
α-Galactosidase	*Escherichia coli*	raffinose	27350	60	6.8
Endo-galactanase	*Bacillus subtilis*	arabinogalactan	1790	48	6
β-Glucosidase	*Bacillus polymyxa*	4-nitrophenyl-β-D-glucopyranoside	2417	NA	NA
Acetyl xylan esterase	*Fibrobacter succinogenes*	Acetylxylan/ α-naphthyl acetate	2933	47	7
Fungi					
Enzyme	Organism	Substrate	Specific activity	Opt.temp. (°C)	Opt.pH
Feruloyl esterase	*Aspergillus niger*	Methyl sinapinate	156	55	5
Endo-1,4-β-xylanase	*Trichoderma longibrachiatum*	1,4-β-D-xylan	6630	45	5
β-1,4-xylosidase	*Aspergillus nidulans*	p-nitrophenyl-β-D-xylopyranoside	107.1	50	5
Exo-β-1,4-mannosidase	*Aspergillus niger*	β-D-Man-(1-4)-β-D-GlcNAc-(1-4)-β-DGlcNAc-Asn-Lys	188	55	3.5
Endo-β-1,4-mannanase	*Sclerotium rolfsii*	Galactoglucomannan/mannans galactomannans/glucomannans/	380	72-74	2.9/3.3
Endo-α-1,5-arabinanase	*Aspergillus niger*	1,5-α-L-arabinan	90.2	50-55	4.5-5.0
α-L-arabinofuranosidase	*Aspergillus niger*	1,5-α-L-arabinofuranohexaose/ 1,5-α-L-arabinotriose/ 1,5-L-arabinan/ α-Larabinofuranotriose	396.6	50-60	3.4-4.5
α-Glucuronidase	*Phanerochaete chrysosporium*	4-O-methyl-glucuronosyl-xylobiose	4.5	50	3.5
α-Galactosidase	*Mortierella vinacea*	melibiose	2000	60	4
Endo-galactanase	*Aspergillus niger*	NA	6593	50-55	3.5
β-glucosidase	*Humicola insolvens*	(2-hydroxymethylphenyl)-β-Dglucopyranoside	266.9	50	5
Acetyl xylan esterase	*Schizophyllum commune*	4-methylumbelliferyl acetate/ 4-nitrophenyl acetate	227	30	7.7

As in cellulase systems, xylan-degrading systems also exhibit synergism (Bachmann and McCarthy 1991). While the number of enzymes required for xylan hydrolysis is

much greater than for cellulose hydrolysis, accessibility to the substrate is easier, since xylan does not form tight crystalline structures (Gilbert and Hazlewood 1993). To date, no comprehensive effort has been reported to optimize hydrolysis of switchgrass using hemicelluloses-degrading enzymes.

1.1.11. Fermentation

Lignocellulose is often hydrolyzed by acid treatment; the hydrolysate obtained is then used for bioethanol fermentation by microorganisms such as yeast. Because such lignocellulose hydrolysate contains not only glucose, but also various monosaccharides, such as xylose, mannose, galactose, arabinose, and oligosaccharides, the microorganisms should be required to ferment these sugars efficiently for the successful industrial production of bioethanol (Katahira et al. 2006). According to the reactions, the theoretical maximum yield is 0.51 kg bioethanol and 0.49 kg carbon dioxide per kg of xylose and glucose (Vessia 2005):

$$3C_5H_{10}O_5 \rightarrow 5C_2H_5OH + 5CO_2$$
$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Fermentation involves microorganisms that use the fermentable sugars for food, and in the process produce ethyl alcohol and other byproducts. These microorganisms can typically use the 6C sugars, one of the most common being glucose. Therefore, cellulosic biomass materials containing high levels of glucose or precursors to glucose are the easiest to convert to bioethanol. Microorganisms, termed ethanologens, presently convert an inadequate portion of the sugars from biomass to bioethanol (Demirbas 2005b). There are a number of microorganisms that produce significant (greater than 1% w/v) quantities of bioethanol (Stewart and Russell 1987).

Xylose-fermenting microorganisms are found among bacteria, yeast and filamentous fungi (Hahn-Hagerdal et al. 2006). Today, xylose-fermenting bacteria include both native and genetically-engineered organisms, and many have characteristics useful for simultaneous saccharification and fermentation (Jeffries and Jin 2000). One of the most effective bioethanol-producing yeasts, *S. cerevisiae*, has several advantages, owing to its high bioethanol production from hexoses and high tolerance to bioethanol and other inhibitory compounds in the acid hydrolysates of lignocellulosic biomass. However,

because wild-type strains of this yeast cannot utilize pentoses, such as xylose and arabinose, and celloligosaccharides, bioethanol production from a lignocellulose hydrolyzate is inadequate (Katahira et al. 2006). Using *S. cerevisiae*, high bioethanol yields from xylose also require metabolic engineering strategies to enhance the xylose flux (Hahn-Hagerdal et al. 2006).

The ethanologenic bacteria that currently show the most promise for industrial exploitation are *E. coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* (Dien et al. 2003). *Zymomonas* is well recognized for its ability to produce bioethanol rapidly and efficiently from glucose-based feedstocks, and comparative performance trials have shown that *Z. mobilis* can achieve 5% higher yields and up to a five-fold higher volumetric productivity when compared with traditional yeast fermentations. *Z. mobilis* has demonstrated theoretical bioethanol yields of up to 97%, and bioethanol concentrations of up to 12% (w/v) in glucose fermentations (Mohagheghi et al. 2002). *Z. mobilis* also efficiently produces bioethanol from the hexose sugars glucose and fructose, but not from pentose sugars, although a xylose-fermenting *Z. mobilis* was generated by introducing a xylose-metabolizing pathway from *E. coli* (Hahn-Hagerdal et al. 2006). Despite its advantages as an ethanologen, *Z. mobilis* is not well suited to biomass conversion because it ferments only glucose, fructose and sucrose. However, over the last decade, researchers at the National Renewable Resources Laboratory (NRRL, US Department of Energy) have successfully engineered strains capable of fermenting xylose and arabinose (Dien et al. 2003). *E. coli* and *K. oxytoca* naturally metabolize arabinose, such that the ethanologenic strains ferment all lignocelluloses-derived sugars (Hahn-Hagerdal et al. 2006).Under aerobic conditions, succinate is not produced as a by-product in *E. coli* and acetate is the main by-product. Numerous metabolic engineering strategies to enhance succinate production in *E. coli* have met with success (Lin et al. 2005). *K. oxytoca* is an enteric bacterium found growing in paper and pulp streams as well as around other sources of wood. The microorganism is capable of growing at a pH as low as 5.0 and temperatures as warm as 308 K. *K. oxytoca* will grow on a wide variety of sugars including hexoses and pentoses, as well as on cellobiose and cellotriose. E. coli and *K. oxytoca* have wider substrate ranges than *Z. mobilis* (Dien et al. 2003). Natural xylose-fermenting yeasts, such as *Pichia stipitis*, *Candida shehatae*, and *Candida parapsilosis*, can metabolize xylose via the action of xylose reductase (XR) to convert xylose to xylitol, and of xylitol dehydrogenase (XDH)

to convert xylitol to xylulose. Therefore, bioethanol fermentation from xylose can be successfully performed by recombinant *S. cerevisiae* carrying heterologous XR and XDH from *P. stipitis*, and xylulokinase (XK) from *S. cerevisiae* (Katahira et al. 2006).

Microorganisms for bioethanol fermentation can best be described in terms of their performance parameters and other requirements, such as compatibility with existing products, processes and equipment. The performance parameters of fermentation are temperature range, pH range, alcohol tolerance, growth rate, productivity, osmotic tolerance, specificity, yield, genetic stability, and inhibitor tolerance (Demirbas 2004). All the recombinant strains are mesophilic organisms and function best between 303 and 311 K (Hettenhaus 1998). An organism must maintain a fairly constant pH balance to survive. Most bacteria grow best in a narrow range of pH from 6.5 to 7.5 (Aminifarshidmehr 1996). Yeast and fungi tolerate a range of pH 3.5-5.0. The ability to lower pH below 4.0 offers a method for present operators using yeast in less than aseptic equipment to minimize loss due to bacterial contaminants. The majority of organisms cannot tolerate bioethanol concentrations above 10-15% (w/v) (Hettenhaus 1998).

Fermentation can be performed as a batch, fed batch or continuous process. The choice of the most suitable process depends upon the kinetic properties of the microorganisms and the type of lignocellulosic hydrolysate in addition to the economic aspects of the process (Chandel et al. 2007). Fed-batch reactors are widely used in industrial applications because they combine the advantages of both batch and continuous processes (Saarela et al. 2003). The major advantage of fed-batch, compared to batch, is the ability to increase maximum viable cell concentration, prolong culture life, and allow product accumulation to a higher concentration (Frison and Memmert 2002). A typical fed-batch fermentation process consists of three technological stages: batch-feeding-batch. Process optimization requires determination of the feed start and finish time points and the feed-rate time profile during the feeding time interval. An optimal feed-rate time profile is usually close to exponential; however, simplified time profiles such as a constant rate or a ramp-shaped profile can give process results close to optimal (Levisauskas and Tekorius 2005). This process allows for the maintenance of critical process variables (*e.g.*, temperature, pH, and dissolved oxygen) at specific levels through feedback control (Gunther et al. 2006).

Separate Hydrolysis and Fermentation (SHF)

The SHF process uses separate pretreatment, enzymatic hydrolysis and fermentation steps. The primary advantage of this approach is that by separating these steps, undesirable interactions are avoided. Using separate reactors allows each step to be carried out at its optimum temperature: 40-50ºC for enzymatic hydrolysis and 30ºC for fermentation (Philippidis 1996, Brown 2003). The disadvantage of this method is the inhibition of cellulase and β-glucosidase enzymes by glucose released during hydrolysis, which calls for lower solids loadings and higher enzyme loadings to achieve reasonable yields (Philippidis 1996, Brown 2003). Lower sugar yields result in lower ethanol concentrations, and thus increase the cost of fermentation and ethanol recovery.

Simultaneous Saccharification and Fermentation (SSF)

Extensive research has shown that SSF is a promising way to biochemically convert cellulose into ethanol (Philippidis 1996) and according to Wyman (1996) it is generally accepted as the most effective and economical way to convert cellulose to ethanol. The process combines the enzymatic hydrolysis of cellulose to glucose by cellulolytic enzymes with the catabolism of glucose to ethanol by fermentative microorganisms. By combining cellulose and glucose in the same reactor, glucose is rapidly removed before it can inhibit the cellulase enzymes during hydrolysis. The optimum temperature for the reaction (37-38ºC) is a compromise between the optimum temperatures for the enzymes in hydrolysis and the yeast in fermentation.

Direct Microbial Conversion (DMC)

Direct microbial conversion combines cellulase production, cellulose hydrolysis and glucose fermentation into a single step. The process is attractive in that it reduces the number of reactors, simplifies operation, and reduces the cost of chemicals (Brown 2003). However, the ethanol yields are low, several metabolic byproducts are produced, and the organisms usually have a low tolerance to ethanol (Philippidis 1996). The organism most investigated for DMC of cellulose is *Clostridium thermocellum* (Johnson et al. 1982). Studies on this microorganism have shown ethanol tolerance in the range of 2.9 to 3.6% ethanol, while the typical tolerance of ethanologenic yeast ranges from 8-10% ethanol. In addition, a large fraction of the catabolized carbon goes into acetic and

lactic acid during DMC, which reduces ethanol yield and increases the cost of production (Klapatch et al. 1994).

Fermentation Improvement

Physiological Approach

Improvements to fermentation productivity can be made by understanding that the parameters involved in productivity, namely, the specific rate of ethanol production, and the growth yield coefficient, are affected by environmental factors. Finding the ideal chemical/nutrient composition of the fermentation medium and the optimal temperature and pH can improve product yield. (Lawford 1988).

Yeast vs. Bacteria

Yeasts such as *S. cerevisiae* have been traditionally used to ferment glucose to ethanol. *S. cerevisiae* ferments glucose through the Embden-Meyerhoff-Parnas pathway (Picataggio et al. 1994). This yeast is a facultative anaerobe that prefers aerobic growth, but is capable of growing in anaerobic environments. The ability of the yeast to ferment sugars at a low pH provides protection against bacterial contamination during cultivation. The high indigenous levels of glucose-inducible pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) help insure high fermentation rates and specific productivities, while providing resistance to glucose catabolite repression during fermentation of mixed-sugar hydrolyzates (Picataggio et al. 1994). The gram-negative bacterium, *Z. mobilis* has attracted considerable attention due to its superior kinetic and yield characteristics, and unlike *S. cerevisiae*, there are no oxygen requirements for lipid synthesis. *Z. mobilis* ferments glucose through the Entner-Douderoff pathway (Picataggio et al. 1994). Like yeasts, *Zymomonas* is acid-tolerant and is resistant to bacterial contamination. In addition, the bacterium is able to grow at high sugar concentrations (>25% glucose) and to produce and tolerate ethanol at concentrations up to 13% (w/v) (Rogers et al. 1979).

The main disadvantage of these microorganisms is their limited substrate utilization ranges. Their inability to ferment xylose, the primary pentose present in hemicellulose, as well as all other monosaccharides in lignocellulosic materials makes producing ethanol from lignocellulose less efficient and therefore, less attractive from an economic

perspective. Current research, however, aims to use genetic engineering to modify these organisms, as well as others, to increase ethanol yields.

Genetically Modified Organisms

One important requirement for improving ethanol production from lignocellulose is the use of an efficient microorganism that is able to ferment both pentoses and hexoses as well as to tolerate stress conditions (Zaldivar et al. 2001). Through metabolic engineering, bacterial and yeast strains have been constructed which have desirable traits for producing ethanol from lignocellulose. Essential traits include a broad substrate utilization range, high ethanol yields, minimal by-product formation, high ethanol tolerance, increased tolerance to inhibitors, and tolerance to sudden changes in environmental conditions. Some other traits that are desirable, but not required are: simultaneous sugar utilization, hemicellulose and cellulose hydrolysis, Generally Regarded as Safe (GRAS) status, recyclablility, minimal nutrient supplementation, and tolerance to low pH and high temperatures (Zaldivar et al. 2001). The three main microorganisms that have been investigated are *S. cerevisiae*, *Z. mobilis*, and *E. coli*.

Continuous vs. Batch Fermentation

Batch processes are closed systems where nothing is added after inoculation except possibly acid or alkali for pH control or air for aerobic fermentations. Continuous culture, on the other hand, is an open system where fresh medium is continuously added and the product is removed at the same rate, thus resulting in a constant volume system. Continuous fermentation with cell recycle involves separating the yeast or bacteria cells from the effluent and recycling them back to the fermentor, thus minimizing cell removal from the reactor. One of the first steps taken to improve ethanol productivity from yeast was switching from batch mode to operating in continuous mode. This change increased productivity three-fold, from about 2 to 6 g EtOH/L/h (Cysewski and Wilke 1977). In addition, operating continuously at higher cell densities using cell recycle reactors was another effective way to increase productivity. A single-stage continuously-stirred tank reactor (CSTR) operating at high biomass loadings (50-80 g yeast/L) has an ethanol productivity of 30-40 g EtOH/L/h (Cysewski and Wilke 1978).

1.2 OBJECTIVE OF THE THESIS

Turkey, having rich agricultural potential and large agricultural areas, is an important wheat, corn and hazelnut producer. Approximately 40-53 million t of straw (Ergudenler and Isigigur 1994), 4.2 million t of corn stalks (Anonymous 2012a) and 550-650,000 t of hazelnut (Anonymous 2012b) were produced in Turkey per year. The objective of this thesis was to examine the usage of large quantities and inexpensive wheat straw, corn stalks and hazelnut husks for bioethanol production.

Turkey has 71.3% (B_2O_3) of world boron reserves. It can be valuable to examine the use of this chemical in industrial applications. As is known, borohydrate is a powerful reducing agent that degrades the structure lignin. On the other hand, it converts the carbonyl groups in the reducing end units of carbohydrate chains to hydroxyl groups and therefore preserves the carbohydrates. A hypothesis could be made that selective lignin delignification of $NaBH_4$ may improve the process yield and may result in better enzymatic digestibility in bioethanol production. For this reason, $NaBH_4$ was utilized for the first time ever in the chemical pretreatment step. To compare the obtained results, the conventional chemicals NaOH, H_2SO_4 and H_2O_2 were also investigated in this study.

2. MATERIALS AND METHODS

2.1. MATERIALS

Wheat straw, corn stalks and hazelnut husks were obtained from field directly after harvest in Duzce province in Turkey. Firstly, straw and stalks were cut to suitable sizes (3-5 cm) for the next process using a garden chopper. And then, the chopped materials and hazelnut husks were air dried at room temperature and stored in the plastic bags separately. The moisture contents were determined (Tappi T 412 om-06) and the materials were stored at -5°C.

2.2. METHODS

The raw samples were first steam exploded and then pretreated with NaOH, H_2SO_4, H_2O_2 and $NaBH_4$. The treated samples were enzymatically hydrolyzed and fermented to produce ethanol.

Pretreatments

The SE was carried out in a 20 L reaction vessel. Samples of 1000 g (oven dry-o.d.) were treated for 5 min at 198-200°C (15 psi-103.4 kPA). The steam exploded samples were filtrated using 200 mesh wire screen and the liquid and solid parts were separately collected from each. Extractives, holocellulose, ash, acid soluble/insoluble lignin and sugar contents were determined in the solid material prior to chemical treatment.

The steam exploded solid samples of 40 g (o.d.) each were chemically treated using NaOH, H_2SO_4, H_2O_2 or $NaBH_4$. The treatments were made at 0.5, 2 and 4% (w/v) concentrations. The solid loading applied was 10% (w/v). Duplicate samples were treated at 121°C (15 psi-103.4 kPA) for residence times of 30, 60 and 90 min with each raw material. After treatment, the liquid part was filtrated and the solid part was stored in sealed plastic bags at 4°C for enzymatic hydrolysis. Treatment yield, acid soluble/insoluble lignin and sugar contents were determined in each of the solid samples. The optimum chemical pretreatments for further enzymatic hydrolysis were determined based on the highest ratio of glucan and lignin.

Enzymatic Hydrolysis

The enzymatic hydrolysis was accomplished on 5 g (o.d.) chemically-treated samples using a mixture (50% v/v) of *Celluclast* 1.5 L (700 U/g) and *Cellobiase* (*Novozym 188*) (250 U/g). Hydrolysis was carried out at 5% solid loading in 100 ml of 50 mM sodium acetate buffer at pH 5.0. In addition, sodium azide (NaN_3, 0.0001 M) was used in this study to prevent microbial contamination. The enzyme reaction was performed in a rotary shaker at 42 ºC for 100 rpm. Samples of 1.5 ml were taken at 0, 6, 24, 48, and 72 h. The samples taken were first put into boiling water for 10 min to stop the enzymatic activity. Then, the samples were centrifuged at 10.000 rpm for 5 min. The glucose and xylose contents of the samples were determined.

Fermentation and Ethanol Production

The enzymatically treated hydrolyzates were centrifuged at 5.000 rpm for 10 min. The supernatant samples of 20 ml each were transferred to 100 ml serum bottles for fermentation. Yeast extract (5 g/L) was added to the samples. Minerals required for microbial growth, 3.75 g/L $(NH_4)_2SO_4$, 2.1 g/L K_2HPO_4, 0.375 g/L $MgSO_4.7H_2O$ and 0.5 g/L $CaCl_2.2H_2O$ were added to the samples. *Saccharomyces cerevisiae ATCC 26602* (5% v/v) from overnight cultures was added and then the samples were incubated in an orbital shaker at 100 rpm for 72 h at 30°C. Periodically taken samples (at 6, 24, 48, and 72 h) were centrifuged at 10.000 rpm for 10 min and then the supernatants were filtered through 0.45 µ pore sized filters. The collected materials were stored at -20°C and the glucose, xylose and ethanol concentrations were determined in HPLC.

Analytical Methods

The yield of the samples was determined by gravimetric measurements. The chemical composition of the samples was obtained using appropriate methods: hot and cold water (Tappi T 207 om-88), 1% NaOH (Tappi T 212 om-88) solubility, extractives content (Tappi T 204 om-88), ash (Tappi T 211 om-85) and holocellulose (Wise 1952).

Laboratory Analytical Procedures (LAP) from the NREL (Sluiter et al. 2004) was used to determine sugar and lignin contents of the samples. The sugar contents were determined by utilizing HPLC (Agilent 1200 system) equipped with Shodex 1011 column (mobile phase: 5 mM H_2SO_4, flow rate: 0.5 ml/min, column temperature: 60°C)

and the refractive index detector (RID). The acid insoluble lignin was obtained by weighing the solid samples. The acid soluble lignin was analyzed at the adsorption of 320 nm against blank deionized water.

The percentage of solids recovered was calculated on an oven dry basis as follows:

$$\text{The percentage of solids recovered} = \left(\frac{W_2}{W_1}\right) * 100 \quad (2.1)$$

W_1 is the dry weight of the sample before pretreatment (g); W_2 is the dry weight of the treated sample (g).

The reduction in lignin was calculated regarding the initial dry weight of lignin in the untreated material (LU) and the dry weight of lignin in the remaining solids after treatment (LP). The percentage of lignin reduction was calculated with the following equation:

$$\text{The percentage of lignin reduction} = \left(\frac{\text{LU-LP}}{\text{LU}}\right) * 100 \quad (2.2)$$

LP is the dry weight of lignin in the pretreated sample and LU is the dry weight of lignin in the untreated biomass. In addition, the solubilization of glucan and xylan in the pretreated samples was calculated in the same manner by substituting the appropriate percentage for glucan and xylan.

The percentage of glucan conversion in enzymatically hydrolyzed samples was calculated as follows:

$$\text{The \% of glucan conversion} = \left(\frac{\%GH}{\%GP}\right) * 100 \quad (2.3)$$

The %GH is the dry weight percentage of glucose in the enzyme hydrolysis supernatants and the %GP is the dry weight percentage of glucose in the treated samples. The conversion of xylan during enzymatic hydrolysis was also calculated in the same manner by substituting the appropriate percentage for xylan.

During fermentation, the ethanol yield was calculated as a percentage of the theoretical maximum yield (Kim and Lee 2005):

$$\text{The \% of theoretical ethanol yield} = \left(\frac{E}{G * 0.511} \right) * 100 \qquad (2.4)$$

E and G represent ethanol (g) produced during fermentation and glucose (g) in the hydrolyzates, respectively. The constant 0.511 is the theoretical yield of ethanol produced from glucose.

The obtained data were statistically analyzed by the SPSS 16.0 packet program. To identify significant differences, we used One-way ANOVA for effects of pretreatments on enzymatic hydrolysis and Univariate ANOVA for effects of chemical, time and concentration on glucan, xylan and lignin. Significant differences between groups were identified using the Duncan test. The SE effect on glucan, xylan and lignin for all raw materials was determined by the Levene (F test) and t tests.

3. RESULTS AND DISCUSSION

3.1. CHAPTER 1: WHEAT STRAW

3.1.1. Composition of Wheat Straw

The results of chemical composition for wheat straw obtained in this study and hardwoods and softwoods were given in Table 3.1. The total sugar content of the straw was found to be 59.9%. Glucan was the main component (36.6%) of the structure. Xylan, major hemicellulose constituent, was found to be 19.7%. Arabinan consisted of only a small portion (3.63%). On the other hand, mannan and galactan was not able to be detected in HPLC, in this study. The holocellulose content of straw was 68.9% and compared to hardwoods, it was slightly lower and was in the range of softwood. The high sugar content of straw indicated that wheat straw is a convenient lignocellulosic substrate for ethanol production. In addition, it could be mentioned that the difference between holocellulose content by Wise and total sugar determined in HPLC is probably due to the sugar degradation during the intense hydrolysis of sulfuric acid in HPLC procedure (Badger 2002). Compared to hardwoods and softwoods, the total lignin and extractives content of straw was almost similar. On the other hand, hot, cold and 1% NaOH solubility values were much higher than hardwoods and softwoods. The high ash content was also noticed for straw.

The chemical composition of wheat straw obtained in this study was also compared with earlier literature findings. It is observed that the determined sugar content in this study was within the range reported by other researchers (Heiss-Blanquet et al. 2011). On the other hand, the lignin content observed in this study (35.2%) was found to be slightly higher compared to the literature (Heiss-Blanquet et al. 2011, Kristensen et al. 2008, Deniz 1994). This could be explained by region, crop maturity, harvest times, plant parts, etc. of straw used in this study. On the other hand, the lignin content of straw in general was comparable with herbaceous species and other agricultural residues (McMillan 1994). The solubility values in this study were also similar to reference observation (Moore 1996).

3.1.2. Effects of Pretreatments

Steam Explosion

The straw was first steam exploded and then chemically pretreated to enhance the enzymatic digestibility and the ethanol fermentability. The chemical compositions of steam exploded straw of solid material was presented in Table 3.1. Solid material recovered after SE was observed to be 92.2% (w/w). 7.80% of material was solved and was in the liquid portion. Results indicated that glucose portion was relatively increased by SE and this could be explained mainly by xylan (16.7%) solubilization and some lignin (5.86%) degradation. After SE, 98.4% of glucan was recovered in solid material. In addition, statistical analysis showed that SE had significant ($p<0.001$) effects on glucan, xylan and lignin.

Table 3.1. Composition of untreated and steam exploded wheat straw.

Chemical composition, (%)	Raw straw, (%)*	Steam exploded straw, (%)*	Hardwoods (Fengel and Wegener 1984)	Softwoods (Fengel and Wegener 1984)
Extractives	3.66±0.34		2-6	2-8
Hot water solubility	13.0±0.05		2-7	3-6
Cold water solubility	9.30±0.05		4-6	2-3
1 % NaOH solubility	45.5±0.20		14-20	9-16
Holocellulose	68.9±0.19	52.1±1.07	70-78	63-70
Acid insoluble lignin	34.0±0.05	34.5±1.04		
Acid soluble lignin	1.22±0.02	1.46±0.09		
Total lignin	35.2	36.0	30-35	25-35
Ash	10.1±0.01	8.73±0.15		
Glucose	36.6±1.67	39.0±2.05		
Xylose	19.7±1.23	17.7±0.78		
Arabinose	3.63±0.29	1.43±0.39		
Total sugar	59.9	58.1		

* Composition percentages are dry-weight basis and values are average of triplicate measurements.

Compared with the literature (Tomas-Pejo et al. 2008), the glucan solubility observed in this study was approximately equal, but the xylan solubility and lignin reduction were slightly lower. On the other hand, Martin et al. (2008) found very high amount of xylan solubilization (60%) and lignin degradation (35%) for sugarcane bagasse. Martin accomplished SE at 205°C for 10 min (up to 40 bar). The work of Martin indicated that intensive SE conditions removed more xylan and lignin from the structure, but glucan recovery was also lower. Consequently, mild SE conditions applied in this study were seems to be convenient for bioethanol production.

Chemical Pretreatments

In this study, common pretreatment chemicals of NaOH, H_2SO_4 and H_2O_2 were compared with the chemical of $NaBH_4$. The percent solid recovered after treatment was shown in Table 3.2 for each treatment step. The statistical test results for factors of four chemicals, three treatment time and three chemical concentrations and their interactions on glucan, xylan and lignin were presented in Table 3.3 and Table 3.4.

Table 3.2. Solids recovery after pretreatments.

	Solids recovered after chemical pretreatments (%)[a,b]			
Time (min), Conc. (%)	Sulfuric acid	Sodium hydroxide	Hydrogen peroxide	Sodium borohydrate
30, 0.5	81.9±0.50	79.8±0.50	86.1±0.93	81.3±0.76
30, 2	81.4±0.57	75.6±1.55	80.1±0.29	79.4±0.28
30, 4	80.0±0.46	64.2±0.27	80.4±0.21	77.2±0.44
60, 0.5	81.8±0.25	77.0±0.41	82.7±0.15	78.0 ±0.10
60, 2	79.9±0.12	58.9±0.50	82.0±0.51	77.1 ±0.08
60, 4	80.5±0.43	58.3±0.13	79.8±0.34	76.1±0.06
90, 0.5	73.0±0.95	76.7±0.10	80.6±0.64	78.9±0.40
90, 2	73.9±0.58	58.9±0.20	79.9±0.31	72.6±0.31
90, 4	71.4±0.00	61.4±0.03	75.4±0.38	70.3±0.36

[a] Percentages calculated from value on a dry-weight basis. [b] Data are averages of three replicates.

Table 3.3. Interactions between chemicals, time and concentrations on glucan, xylan and lignin.

Source		Type III Sum of Squares	df	Mean Square	F	P
Glucan solubilization	Chemical	1093.4	3	364.5	142.6	*
	Time	162.7	2	81.3	31.8	*
	Concentration	331.1	2	165.5	64.8	*
	Chemical * Time	162.6	6	27.1	10.6	*
	Chemical * Concentration	221.7	6	36.9	14.5	*
	Time * Concentration	40.9	4	10.2	4.00	**
	Chemical * Time * Concentration	148.7	12	12.4	4.85	*
Xylan solubilization	Chemical	11.5	3	3.85	1.64	NS
	Time	66.2	2	33.1	14.1	*
	Concentration	39.4	2	19.7	8.36	*
	Chemical * Time	83.3	6	13.9	5.90	*
	Chemical * Concentration	74.0	6	12.3	5.24	*
	Time * Concentration	3.98	4	0.99	0.42	NS
	Chemical * Time * Concentration	21.4	12	1.78	0.76	NS
Lignin reduction	Chemical	1952.5	3	650.8	500.6	*
	Time	544.8	2	272.4	209.5	*
	Concentration	103.9	2	51.9	40.0	*
	Chemical * Time	501.3	6	83.6	64.3	*
	Chemical * Concentration	388.0	6	64.7	49.7	*
	Time * Concentration	74.2	4	18.6	14.3	*
	Chemical * Time * Concentration	50.1	12	4.18	3.21	**

[P] Significance level. [*] Significant at 0.001 for ANOVA. [**] Significant at 0.01 for ANOVA. [NS] None significant for ANOVA.

Table 3.4. Effects of chemicals, time and concentrations on glucan, xylan and lignin.

Factor	Treatment	Glucan (%)	Xylan (%)	Lignin (%)
Chemical	NaOH	52.1 a [A]	11.1 a	29.8 a
	H_2SO_4	44.7 b	10.7 a	41.5 b
	H_2O_2	41.7 c	11.8 a	29.2 a
	$NaBH_4$	48.1 d	10.9 a	29.3 a
Time	30 min	44.6 a	12.2 a	35.1 a
	60 min	47.2 b	11.3 a	33.6 b
	90 min	48.2 c	9.87 b	28.7 c
Concentration	0.5%	43.9 a	12.1 a	34.0 a
	2%	47.0 b	11.1 b	32.3 b
	4%	49.1 c	10.2 b	31.1 c

[A] Means within each column and factor followed by the same letter are not significantly different by Duncan test ($p<0.05$).

NaOH is an alternative method to acid treatment. In this study, results showed that higher residency time and alkali concentrations diminished the solid recovery (Table 3.2). The glucan content of pretreated solids ranged from 44.2% (0.5%, 90 min) to 59.9% (2%, 90 min). The solubilization of glucan during NaOH treatment was found to be between 5.34% (0.5%, 30 min) and 19.0% (2%, 60 min) (Figure 3.1a). Glucan solubilization was explained by disruption of cellulose and hemicellulose bonds resulting in cellulose swelling and partial hemicellulose solubilization (Chen and Sharma-Shivappa 2007). Higher residency times and alkali concentrations resulted in higher glucan solubilization. On the other hand, lower glucan solubilization for higher treatment concentrations at higher residency time (for 90 min) could be explained by much more xylan solubilization and lignin degradation (Figure 3.1b and Figure 3.1c) in this treatment conditions. Silverstein et al. (2007) reported nearly 21.0% glucan solubilization for cotton stalks at 2% NaOH concentration (121°C/60 min).

The xylan content of pretreated solids ranged from 7.60% (4%, 60 min) to 15.6% (0.5%, 90 min). NaOH treatment resulted in 32.8% (0.5%, 90 min) to 75.0% (4%, 60 min) of xylan solubilization and this finding could be explained by amorphous, low molecular weight, heterogeneous and branched structure of xylan. Xylan solubilization of NaOH treatment was higher compared to other chemical pretreatments (Figure 3.1b).

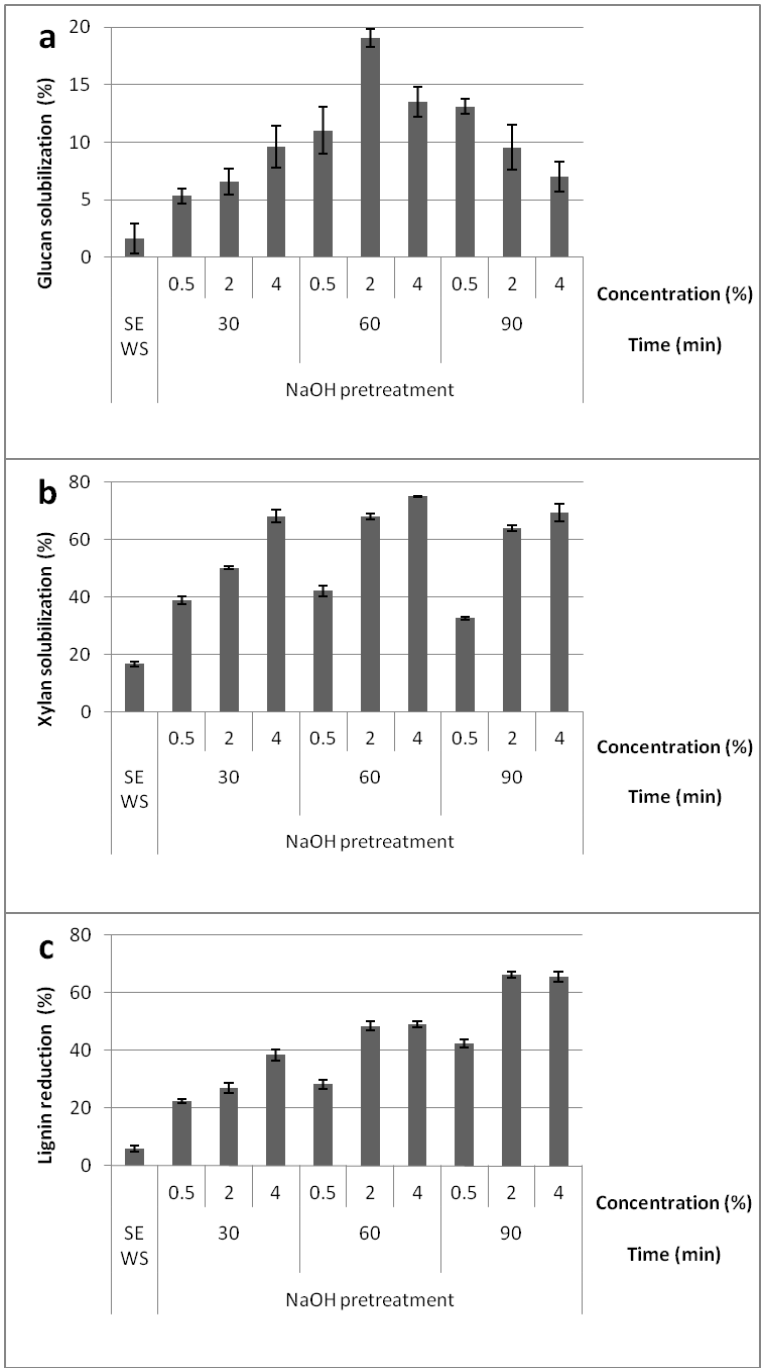

Figure 3.1. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium hydroxide pretreated samples as a function of residence time and concentration (SE WS: steam exploded wheat straw).

Accessibility of hydrolytic enzymes to carbohydrates are limited by lignin because lignin; three dimensional complex aromatic polymer, cover up cellulose and hemicellulose (Fan et al. 1987) and its reduction in the structure is crucial for biomass digestibility. Results in this study showed that NaOH treatment was an effective approach in lignin removal from the structure and lignin degradation in NaOH treatment

could be explained by breakage of ester linkages between lignin and xylan and deprotonation of lignin phenolic groups (Sun et al. 2005a). The amount of total lignin in the solids after NaOH treatment ranged from 35.0% (30 min) to 27.0% (90 min) for 0.5% NaOH, 34.7% (30 min) to 20.6% (90 min) for 2% NaOH, 34.5% (30 min) to 20.2% (90 min) for 4% NaOH. Increase in alkali concentration from 0.5% to 4% resulted in more lignin reductions (Figure 3.1c). The obtained data is also comparable with literature (Chen and Sharma-Shivappa 2007) that reported 84.5% lignin reduction in wheat straw treated with 2% NaOH at 121°C/60 min. Consequently, solubilization of xylan in conjunction with substantial lignin removal in alkali treatment could improve the next process step, enzymatic hydrolysis. The optimum NaOH treatment was determined in this study taking into regard the glucan to lignin ratio. Results showed that glucan to lignin ratio of NaOH treated samples were increased by 2.93% (4%, 90 min). This finding indicated selective delignification of NaOH in which the ratio was 1.08% for steam exploded material. As a result, NaOH treatment accomplished at 4% concentration (90 min) was selected as optimum and this sample was processed further for enzymatic hydrolysis.

H_2SO_4 treatment is one effective treatment method for lignocellulosic biomass because removing hemicellulose from cell wall structure increases porosity and expected to improve enzymatic digestibility and maximum enzymatic digestibility is possible when all hemicelluloses removed from the structure (McMillan 1994). The glucan and xylan solubilizations and lignin reduction during H_2SO_4 treatment of straw were shown in Figure 3.2a-c. The solids recovery after treatment is presented in Table 3.2 and results showed that higher residence times and acid concentrations diminished the solid recovery.

Higher glucan content in acid treated straw was observed for higher acid concentrations and residence times. Glucan content was ranged in this study from 39.7% (0.5%, 30 min) to 52.5% (4%, 90 min). Compared to the steam exploded feedstock, 83.3% and 96.1% of the glucan was preserved when the straw was treated with acid at 0.5% (30 min) and 4% (90 min), respectively (Figure 3.2a). Although, it is very desirable for the cellulose portion to be unaffected by acid treatment, slightly more glucan reduction was observed in this study.

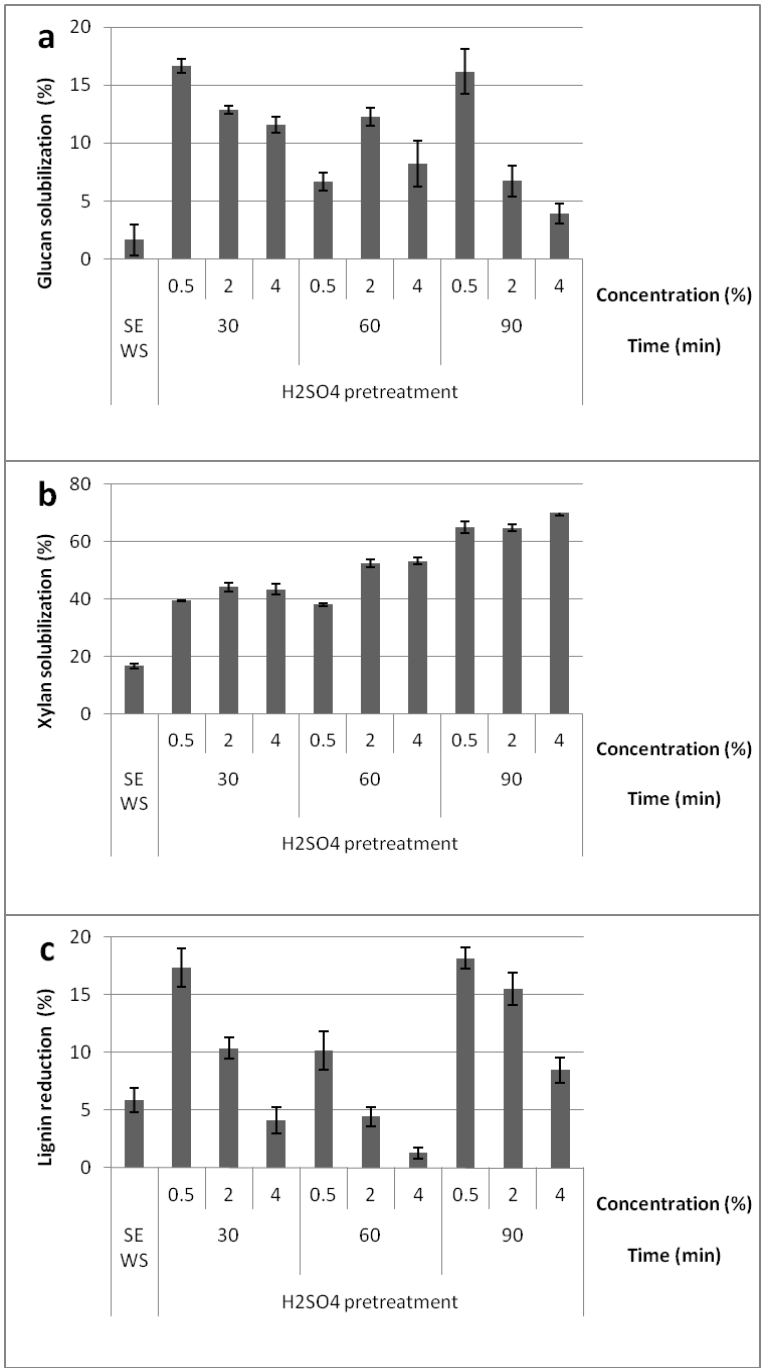

Figure 3.2. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sulfuric acid pretreated samples as a function of residence time and concentration (SE WS: steam exploded wheat straw).

Acid treatments in higher temperature results in hemicellulose hydrolysis by releasing monomeric sugars and soluble oligomers from the cell wall structure. Xylan was the largest portion of hemicellulose of straw. The xylan content of pretreated solids ranged from 7.43% (4%, 90 min) to 13.4% (0.5%, 60 min) (Figure 3.2b). In acid treatment, concentration had a significant effect on xylan reduction and acid treatment resulted

39.5% (0.5%, 30 min) and 70.1% (4%, 90 min) xylan solubilization. Compared to the glucan, more xylan solubilization could be attributed to the fact that xylan is more labile to solubilize (McMillan 1994) due to its non-crystalline structure. The results obtained in this study was comparable to the literature findings and Schell et al. (2003) reported 77% xylan reduction in corn stover at 190°C/60 min with 1.35% acid and Grohmann et al. (1985) reported more than 80% xylan solubilization of wheat straw treated with dilute sulfuric acid at 140°C for 1 h.

Compared with untreated straw, the lignin content increased after acid treatment and the lignin content of straw varied from 36.3% (30 min) to 40.3% (90 min) for 0.5% H_2SO_4, 39.6% (30 min) to 41.1% (90 min) for 2% H_2SO_4, 43.1% (30 min) to 46.1% (90 min) for 4% H_2SO_4. The reduction of lignin, based on the weight of lignin in the steam exploded material and the weight of lignin remaining after acid treatment ranged from 1.28% to 18.2% (Figure 3.2c). Silverstein et al. (2007) was also observed similar results and they found 24.2% lignin reduction in cotton stalks when treated with 2% H_2SO_4 at 121°C/90 min. This finding showed that acid treatment had minimal effect on lignin degradation and thus has no substantial effect to improve enzymatic digestibility (McMillan 1994). Although, the ratio of glucan to lignin contents of steam exploded material was 1.08%, it was 1.20% after acid treatment (2%, 90 min). Consequently, straw treated with 2% H_2SO_4 (90 min) was further processed for enzymatic hydrolysis.

H_2O_2 is well known bleaching agent in paper and cellulose industry. This chemical decomposes into oxygen and water and do not leave residues in materials and form secondary products (Rabelo et al. 2011). H_2O_2 pretreatment utilizes oxidative delignification to detach and solubilize the lignin and loosens the lignocellulosic matrix thus improving enzymatic digestibility (Martel and Gould 1990). The glucan and xylan solubilizations and lignin reduction, due to H_2O_2 pretreatment of straw were shown in Figure 3.3a-c and the solids recovered after treatment was presented in Table 3.2.

The percentage of glucan in treated straw ranged from 38.2% for (2%, 90 min) to 45.5% for (4%, 60 min). Average glucan solubilization varied from 6.90% (4%, 60 min) to 22.7% (4%, 90 min) (Figure 3.3a). Comparable results were also observed by Silverstein et al. (2007), who observed 30.6% xylan and 29.1% glucan solubilization in cotton stalks with 2% H_2O_2 at 121°C/30 min.

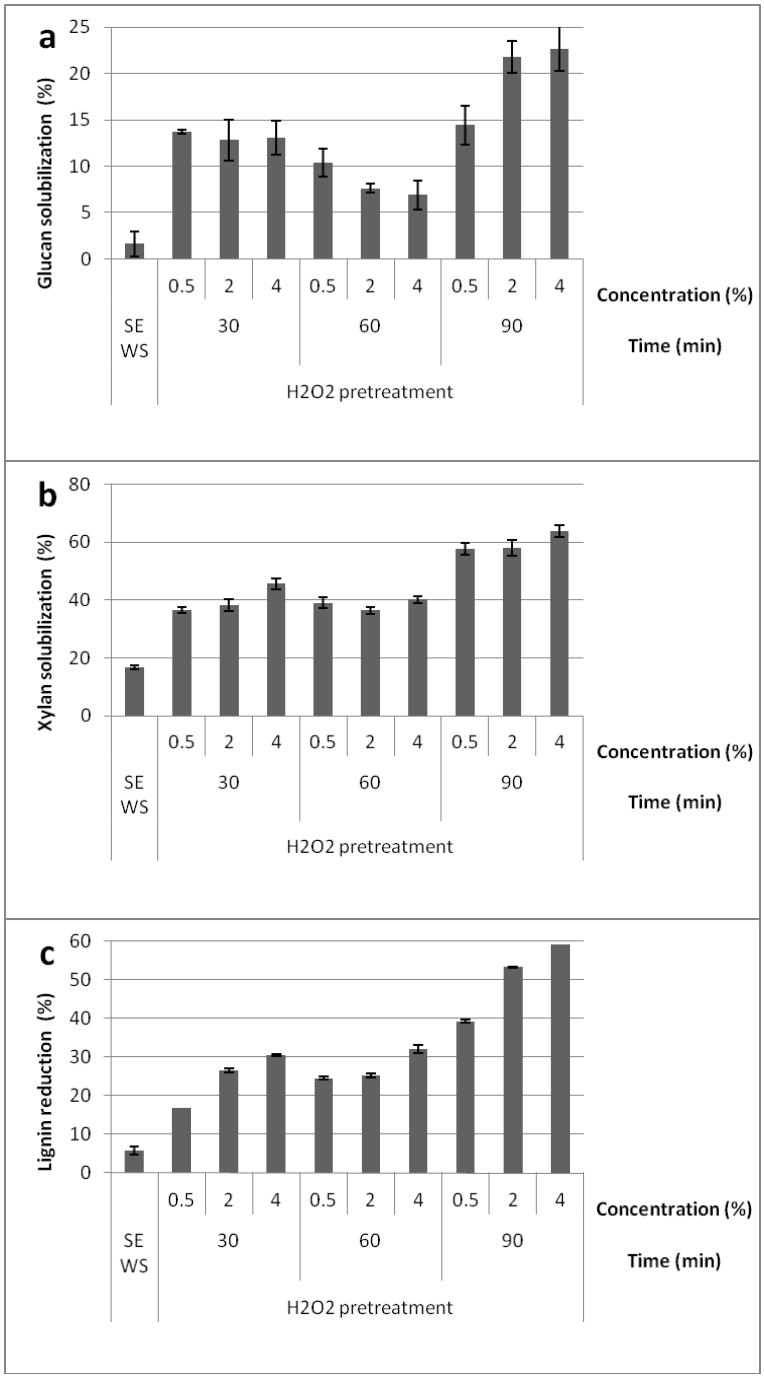

Figure 3.3. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in hydrogen peroxide pretreated samples as a function of residence time and concentration (SE WS: steam exploded wheat straw).

The amount of xylan remained after treatment was 8.50% (4%, 90 min) and 13.8% (2%, 60 min). Solubilized xylan was found to be between 36.5% (0.5%, 30 min) and 63.9% (4%, 90 min) (Figure 3.3b). Increase in concentration and residence time resulted in higher lignin reductions such as 16.9% (0.5%, 30 min) and 59.1% (4%, 90 min) (Figure 3.3c). Azzam (1989) and Sun et al. (2005b) reported 50% lignin reduction in sugarcane

bagasse at 30°C/8 h with 2% alkaline H_2O_2 and more than 80% reduction of lignin in wheat straw treated with 2% H_2O_2 at 50°C for 5 h. Lignin degradation occurred in this study was not as high as expected and this could be explained by decomposition of H_2O_2 to water at high temperatures. The glucan to lignin ratio was 1.08% for steam exploded material and H_2O_2 treatment improved that ratio to 2.05% (4%, 90 min). Consequently, straw treated with 4% H_2O_2 (90 min) was further enzymatically hydrolyzed in this study.

$NaBH_4$ is an additive commonly used to improve the pulping selectivity (Copur and Tozluoglu 2007). This chemical prevents peeling reactions and hemicellulose degradation (Hoije et al. 2005) compared to other traditional pulping chemicals. The aim of using $NaBH_4$ in this study was to preserve more glucan and delignify lignin more selectively. The effect of $NaBH_4$ as pretreatment agent have not drawn much attention yet. The glucan and xylan solubilization and lignin reduction during $NaBH_4$ treatment of straws was shown in Figure 3.4a-c. Higher residency time and chemical concentration resulted in lower solid recovery (Table 3.2). $NaBH_4$ treatment gave solid recovery ranged from 81.3% (0.5%, 30 min) to 70.3% (4%, 90 min).

The glucan content of treated straw ranged from 44.7% (0.5%, 30 min) to 52.0% (2%, 90 min). The solubilization of glucan during $NaBH_4$ treatment was between 2.07% (4%, 60 min) and 7.27% (2%, 30 min) (Figure 3.4a) and as expected, glucan solubilization was found to be lower compared to the other treatment chemicals examined in this study. For all treatment conditions of $NaBH_4$, the glucan solubility was around 6%. This showed that $NaBH_4$ prevented peeling reactions and preserved more glucan in the structure.

The xylan content of treated straw ranged from 9.46% (0.5%, 90 min) to 13.4% (4%, 30 min) and treated straw had 41.8% (4%, 30 min) and 58.7% (4%, 90 min) xylan solubilization (Figure 3.4b). Therefore, compared to the other treatment chemicals used in this study, the xylan solubilization observed with $NaBH_4$ was little lower. $NaBH_4$ dissolved almost 60% of the xylan, which is the main hemicelluloses of wheat straw. Results also indicated that treatment times of 60 and 90 min gave similar xylan solubility. It showed that $NaBH_4$ preserved more xylan compared to NaOH.

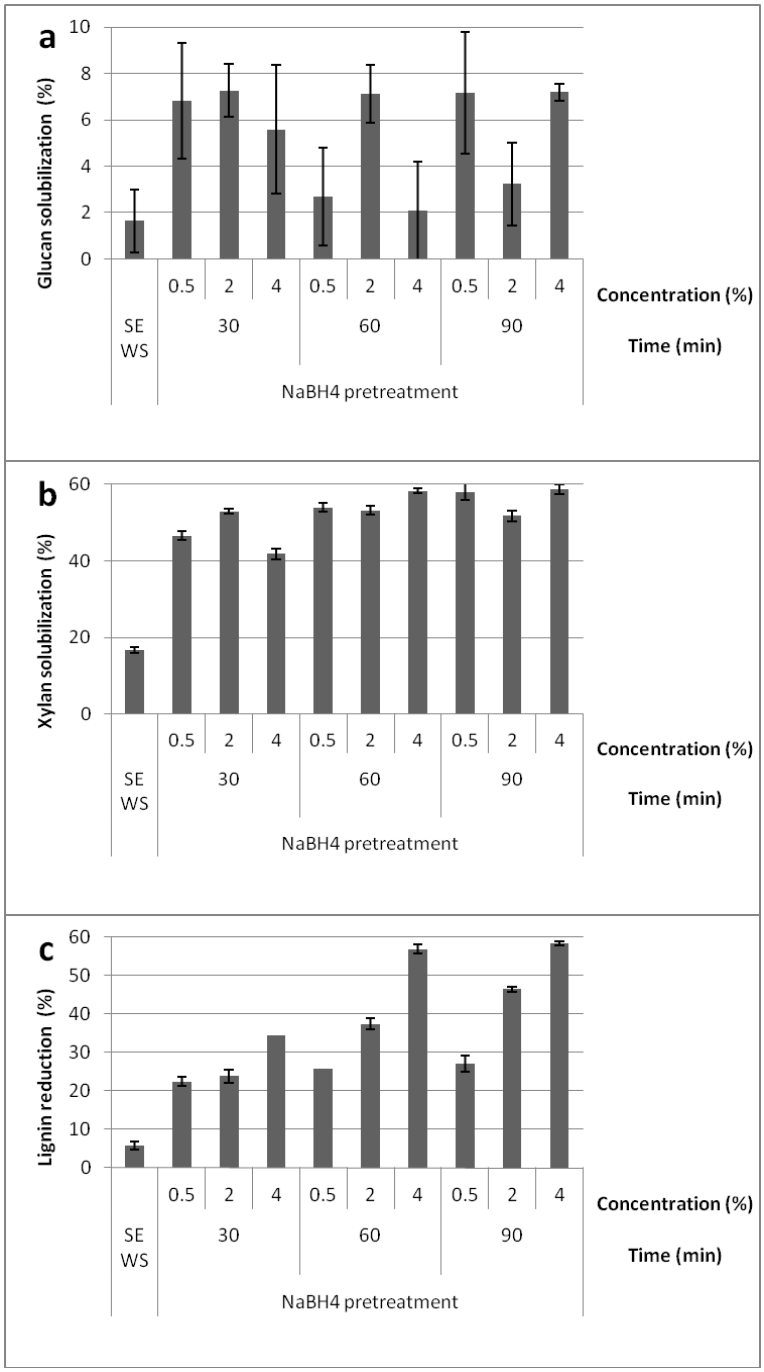

Figure 3.4. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium borohydrate pretreated samples as a function of residence time and concentration (SE WS: steam exploded wheat straw).

$NaBH_4$ treatment significantly decreased the lignin content and the lignin content of the straw varied from 34.3% (30 min) to 33.2% (90 min) for 0.5% $NaBH_4$, 34.5% (30 min) to 26.5% (90 min) for 2% $NaBH_4$, 30.5% (30 min) to 21.3% (90 min) for 4% $NaBH_4$. The maximum lignin reduction was observed with 4% $NaBH_4$ at 90 min (58.4%) (Figure 3.4c). The obtained data was almost similar to 4% NaOH treatment at 90 min

(65.5%). Consequently, $NaBH_4$ removed more lignin from the structure compared to xylan and this could be stated that removal of lignin is much important for better enzymatic digestibility. It could be concluded that $NaBH_4$ was also an effective chemical for selective delignification and pretreatment studies should be continued in bioethanol production. The glucan to lignin ratio of $NaBH_4$ treated straw was 2.46% (4%, 60 min) and it was 1.08% before treatment. Consequently, straw treated with 4% $NaBH_4$ (60 min) was selected for further enzymatic hydrolysis.

3.1.3. Enzymatic Hydrolysis

Samples for enzymatic hydrolysis were determined based on glucan to lignin ratio of the samples after chemical pretreatments. Samples to enzymatic hydrolysis were NaOH 2%/90 min, H_2SO_4 2%/90 min, H_2O_2 4%/90 min and $NaBH_4$ 4%/60 min. Enzymatic hydrolysis were performed to 72 h of treatment time. Sugar content, glucose and xylose were determined using the supernatant liquid. The concentrations of arabinose and galactose were below the detection limit and were not reported in this study. The glucan conversions for each hydrolysis were shown in Figure 3.5. The highest glucan conversion was observed with NaOH treated straw (87.8%) and it was lower for samples of $NaBH_4$ (83.3%), H_2O_2 (74.7%) and H_2SO_4 (71.7%). Statistical analysis indicated that the mean glucan conversions for all test were significant ($p<0.001$).

The differences in glucan conversion during enzymatic hydrolysis could be due to the type/amount of lignin found in samples structure. The lignin content of samples after chemical pretreatments was 41.1%, 20.2% and 20.4% for H_2O_2, NaOH and $NaBH_4$, respectively. The H_2O_2 treated sample had 2.03 times of higher lignin on the other hand had 1.22 times of lower glucan when compared to the NaOH. As a result, lower amount of lignin in sample structure could be likely to have great impact on glucan conversion compared to the xylan in sample structure. In addition, results of enzymatic digestibility showed that $NaBH_4$ was an effective pretreatment chemical as well as conventional chemical NaOH.

Wheat straw contain higher hemicellulose (mainly xylan) compared to woody biomass (Wiselogel et al. 1996) and addition of xylanase during hydrolysis is expected to increase monomeric sugar yields (Mosier et al. 2005a). Duarte et al. (2004) reported that *Celluclast* 1.5 L had *β-xylanase* and *β-xylosidase* activities of 100 U/g and 0.53 U/g

xylose, respectively. Although, in this study no xylanase was added into the *Celluclast* 1.5 L–*cellobiase* enzyme mixture, significant amount of xylose was detected in the hydrolyzates (Figure 3.5). Results indicated that $NaBH_4$ pretreatment resulted in the highest xylan to xylose conversion (44.2%). On the other hand, xylan conversion was slightly lower for NaOH (43.8%). Xylan conversion for all treatments were statistically significant ($p<0.001$). Chemical pretreatments removed some hemicellulose (mainly xylan) from the structure. The results of this study indicated that enzyme application of *Celluclast* 1.5 L and *cellobiase* mixture hydrolyzed the residual xylan and it could be assumed that xylanolytic activity of these enzyme mixtures was enough for hydrolysation.

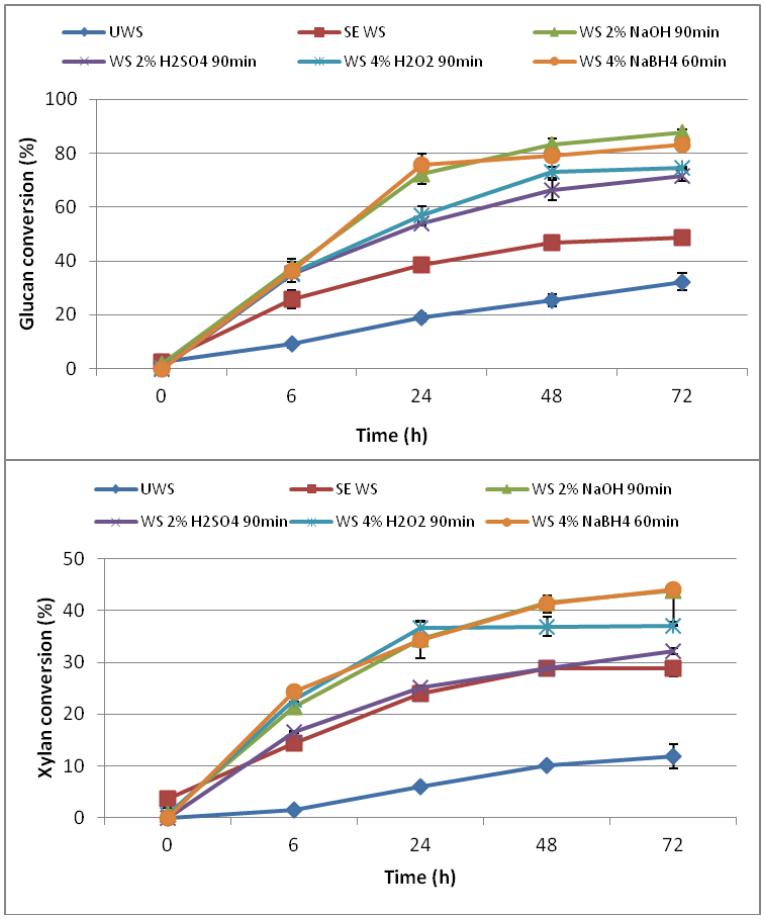

Figure 3.5. Glucan and xylan conversions after enzymatic hydrolysis (UWS: untreated wheat straw, SE WS: steam exploded wheat straw).

3.1.4. Fermentation of Hydrolyzates

The findings of this study showed that pretreatment of straw prior to hydrolysis resulted in a better fermentability of hydrolyzates (Table 3.5). The highest ethanol concentration

of 10.3 g/L was observed with 2% NaOH (90 min) pretreated sample. However, the highest ethanol yield of 115 g/kg (based on untreated straw) was obtained when the material was pretreated with 4% $NaBH_4$ (60 min and the theoretical yield (86.9%) was also calculated to be highest for the sample (Figure 3.6). Consequently, it could be concluded that $NaBH_4$ could be an effective pretreatment chemical for fermentation applications.

A wide range of theoretical ethanol yields (31-84%) for wheat straw (Zhu et al. 2006) was reported in the literature depending on the methods that the material was treated. Theoretical ethanol yields of 66.5-86.9% were observed in this study regarding different treatment methods. Variation in ethanol yields could be due to the raw material, geographic location, cultivar and harvest time, and the yeast strain used in the study and treatment methods. Lower ethanol yields could be explained by the formation of by-products such as furfural, HMF and phenolic compounds due to the chemical pretreatments of straw and this also inhibits the fermentation of sugars by yeasts (Bjerre et al. 1996). Even very low amount used in this study, sodium azide employed in enzymatic hydrolysis to prevent microbial contaminations may also result in some reduction in yeast activity (Fales 1953).

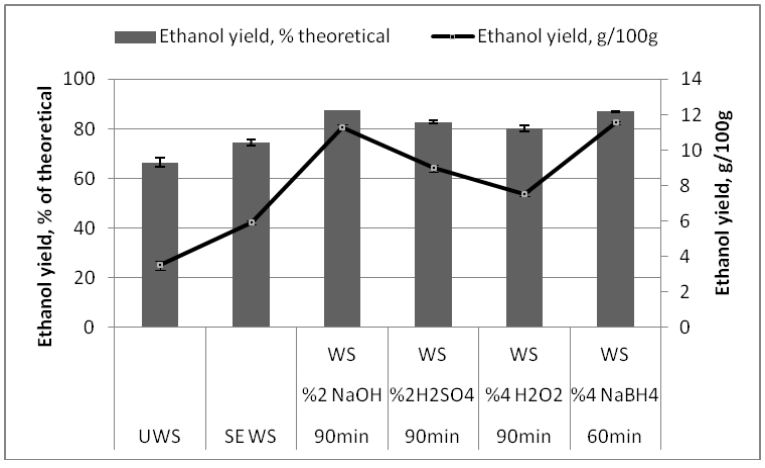

Figure 3.6. Ethanol yield (percent of theoretical and g/100 g raw material) (UWS: untreated wheat straw, SE WS: steam exploded wheat straw).

Table 3.5. Glucose, xylose and ethanol concentrations during fermentation with *S. cerevisiae* in untreated and pretreated straws.

Pretreatment conditions	0 h			6 h			24 h			48 h			72 h		
	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l
UWS*	5.18±0.51	1.02±0.10	0.21±0.04	4.12±0.37	0.96±0.09	1.56±0.15	1.01±0.12	0.62±0.06	1.66±0.11	0.92±0.11	0.49±0.05	1.72±0.14	0.68±0.06	0.48±0.05	1.76±0.13
SE WS	8.37±0.00	2.24±0.01	0.37±0.12	6.64±0.07	2.08±0.02	2.68±0.09	1.21±0.04	1.37±0.00	2.99±0.03	1.02±0.03	1.08±0.01	3.08±0.10	0.95±0.07	1.05±0.01	3.18±0.06
WS %2 NaOH 90 min	23.1±0.28	2.10±0.02	0.97±0.01	18.4±0.39	1.96±0.02	7.90±0.12	1.84±0.03	1.29±0.00	9.88±0.13	1.29±0.23	1.01±0.01	10.2±0.04	1.05±0.13	0.99±0.01	10.3±0.13
WS %2 H_2SO_4 90 min	15.5±0.46	1.20±0.03	0.72±0.01	12.3±0.17	1.06±0.05	4.96±0.16	1.69±0.03	0.74±0.00	6.19±0.07	1.31±0.02	0.58±0.01	6.43±0.22	1.23±0.01	0.56±0.01	6.55±0.15
WS %4 H_2O_2 90 min	13.1±0.03	1.39±0.01	0.60±0.07	10.7±0.25	1.29±0.01	4.22±0.04	1.79±0.06	0.85±0.00	5.19±0.03	1.48±0.01	0.67±0.00	5.24±0.06	1.22±0.01	0.65±0.00	5.38±0.09
WS %4 $NaBH_4$ 60 min	18.4±0.13	1.91±0.28	0.81±0.05	14.8±0.01	1.78±0.26	5.97±0.04	1.45±0.31	1.18±0.19	7.90±0.03	1.17±0.08	0.91±0.13	8.06±0.07	1.06±0.05	0.90±0.13	8.16±0.07

*UWS: untreated wheat straw, SE WS: steam exploded wheat straw.

3.2. CHAPTER 2: CORN STALKS

3.2.1. Composition of Corn Stalks

The chemical composition of corn stalks is shown in Table 3.6. The sugar fraction was found to be 63.8% on dry weight. It was observed that glucan (38.2%) was the main component of the structure. The hemicelluloses component xylan made up 23.1% of the stalks. Arabinan composed only a small portion, and mannan and galactan could not be detected in this study. The holocellulose content was 60.7% on dry weight.

The high carbohydrate content of the stalks indicated that the material is appropriate for bioethanol production. The total sugar (63.8%) and lignin (33.0%) contents determined in this study were parallel to earlier findings (Anonymous 2007, Donghai et al. 2006, Usta et al., 1990). In general, corn stalks had a higher lignin content compared to herbaceous species (10-20%) and some agricultural residues (McMillan 1994, Zhao et al. 2009). The ash content found in this study was also comparable to that found in other references (Donghai et al. 2006, Zhao et al. 2009).

Table 3.6. Composition of untreated and steam exploded corn stalks

Component	Raw stalk, (%)*	Steam exploded stalk, (%)*
Total sugar	63.8	70.6
Glucan	38.2±2.33	44.0±0.34
Xylan	23.1±1.12	24.2±1.77
Arabinan	2.50±0.32	2.40±0.66
Holocellulose	60.7±0.29	51.0±0.12
Acid-insoluble lignin	31.6±1.07	33.3±0.09
Acid-soluble lignin	1.41±0.04	1.33±0.07
Alcohol-benzene solubility	11.9±0.21	20.1±2.10
Hot water solubility	22.2±0.35	
Cold water solubility	25.3±1.05	
1% NaOH solubility	51.7±0.18	
Ash	4.18±0.05	4.73±0.16

*Composition percentages are dry-weight basis and values are average of triplicate measurements.

3.2.2. Effects of Pretreatments

Steam Explosion

In this study, the stalks were steam exploded and then pretreated with chemicals to improve the enzymatic digestibility and the ethanol fermentability. Results showed that SE dissolved and removed some material from the structure, and the total solid recovered after SE was 85.3% (w/w). The amounts of glucan (44.0%), xylan

(24.2%), arabinan (2.40%), insoluble acid (33.3%) and soluble lignin (1.33%), ash (4.73%), alcohol-benzene solubility (20.1%) and holocellulose (51.0%) were determined in the steam exploded stalks (Table 3.6).

Compared to untreated stalks (38.2%), steam exploded stalks had higher glucan content (44.0%). This finding could be explained by the xylan (10.5%) and lignin (10.6%) removal from the structure. The glucan recovery was calculated at 98.2%, and statistical analyses showed significant difference ($p<0.001$) between the untreated and the steam exploded samples. Schultz et al. (1984) also observed similar results for corn stalks. The moderate amount of xylan and lignin solubility in our study could be due to the mild treatment conditions. On the other hand, intensive treatment conditions (205°C, 10 min and up to 40 bar) for sugarcane bagasse dissloved higher lignin (35%), xylan (60%) and glucan (19%) from the structure (Martin et al. 2008). Our results showed that mild SE conditions were more suitable to preserve the glucan. The glucan to lignin ratio was improved from 1.16 to 1.27 when the samples were steam exploded.

Chemical Pretreatments

The solid recovery data after chemical pretreatment are presented in Table 3.7. Results showed that after chemical pretreatment the recovered solids varied depending on the chemicals utilized, pretreatment times, and chemical concentrations.

Table 3.7. Solids recovery after pretreatments

	Solids recovered after chemical pretreatments (%)[a,b]			
Time (min), Conc. (%)	Sulfuric acid	Sodium hydroxide	Hydrogen peroxide	Sodium borohydrate
30, 0.5	77.0±0.72	75.8±0.87	81.2±0.84	85.9±0.61
30, 2	78.2±4.56	64.3±0.36	76.7±0.68	75.0±0.93
30, 4	76.2±0.63	55.3±0.26	76.9±0.42	71.7±0.82
60, 0.5	76.8±0.93	72.9±0.52	76.2±0.74	74.3±0.79
60, 2	76.7±0.84	61.5±0.31	74.7±0.39	70.5±0.33
60, 4	75.2±0.90	54.5±0.30	71.2±0.88	69.5±0.25
90, 0.5	77.0±0.73	71.2±0.30	75.0±0.00	74.2±0.90
90, 2	76.6±0.91	55.4±0.51	72.2±0.48	71.4±0.30
90, 4	75.2±1.21	50.8±0.61	68.9±0.61	70.7±0.39

[a] Percentages calculated from value on a dry-weight basis. [b] Data are averages of three replicates.

Anaylsis indicated that the weight loss could be principally explained by the solubilization of xylan, lignin and extractives from the structure. Table 3.8 and Table 3.9 present the statistical interactions on glucan and xylan solubility and lignin reduction for the four different chemicals used, the three different pretreatment times,

and the three different chemical concentrations.

Table 3.8. Interactions between chemicals, time and concentrations on glucan, xylan and lignin

Source		Type III Sum of Squares	df	Mean Square	F	P
Glucan solubilization	Chemical	573.7	3	191.2	68.0	*
	Time	21.9	2	11.0	3.90	***
	Concentration	276.2	2	138.1	49.1	*
	Chemical * Time	219.0	6	36.5	13.0	*
	Chemical * Concentration	48.6	6	8.09	2.88	***
	Time * Concentration	111.1	4	27.8	9.88	*
	Chemical * Time * Concentration	34.6	12	2.88	1.02	NS
Xylan solubilization	Chemical	372.6	3	124.2	54.8	*
	Time	105.9	2	53.0	23.4	*
	Concentration	87.8	2	43.9	19.4	*
	Chemical * Time	68.2	6	11.4	5.02	**
	Chemical * Concentration	138.2	6	23.0	10.2	*
	Time * Concentration	3.29	4	0.82	0.36	NS
	Chemical * Time * Concentration	27.5	12	2.29	1.01	NS
Lignin reduction	Chemical	2897.3	3	965.8	1251.0	*
	Time	233.8	2	116.9	151.4	*
	Concentration	201.5	2	100.8	130.5	*
	Chemical * Time	586.0	6	97.7	126.5	*
	Chemical * Concentration	331.2	6	55.2	71.5	*
	Time * Concentration	10.2	4	2.55	3.30	***
	Chemical * Time * Concentration	89.8	12	7.48	9.70	*

[P] Significance level. [*] Significant at 0.001 for ANOVA. [**] Significant at 0.01 for ANOVA. [***] Significant at 0.05 for ANOVA. [NS] None significant for ANOVA.

Table 3.9. Effects of chemicals, time and concentrations on glucan, xylan and lignin

Factor	Treatment	Glucan (%)	Xylan (%)	Lignin (%)
Chemical	NaOH	47.0 a [A]	14.1 a	21.8 a
	H_2SO_4	49.2 b	14.7 a	38.5 b
	H_2O_2	47.1 a	19.7 b	33.4 c
	$NaBH_4$	54.0 c	17.8 c	26.8 d
Time	30 min	48.6 a	17.8 a	32.6 a
	60 min	49.7 b	17.0 a	29.5 b
	90 min	49.7 b	14.9 b	28.3 c
Concentration	0.5%	46.9 a	17.8 a	32.4 a
	2%	49.5 b	16.8 b	29.6 b
	4%	51.7 c	15.1 c	28.4 c

[A] Means within each column and factor followed by the same letter are not significantly different by Duncan test ($p<0.05$).

Pretreatment with NaOH, an alternative to acid, disrupts the cellulose and hemicellulose H-bonds, breaks the ester linkages between lignin and xylan, deprotonates the phenolic groups and results in cellulose swelling and partial hemicellulose and lignin solubilization (Sun et al. 2005a, Simpson et al. 2003, Akin et al. 1992). NaOH degrades amorphous, low molecular weight and heterogeneous hemicelluloses. Results in this study showed that more material was removed from the structure when higher residency times and alkali concentrations were applied (Table 3.7).

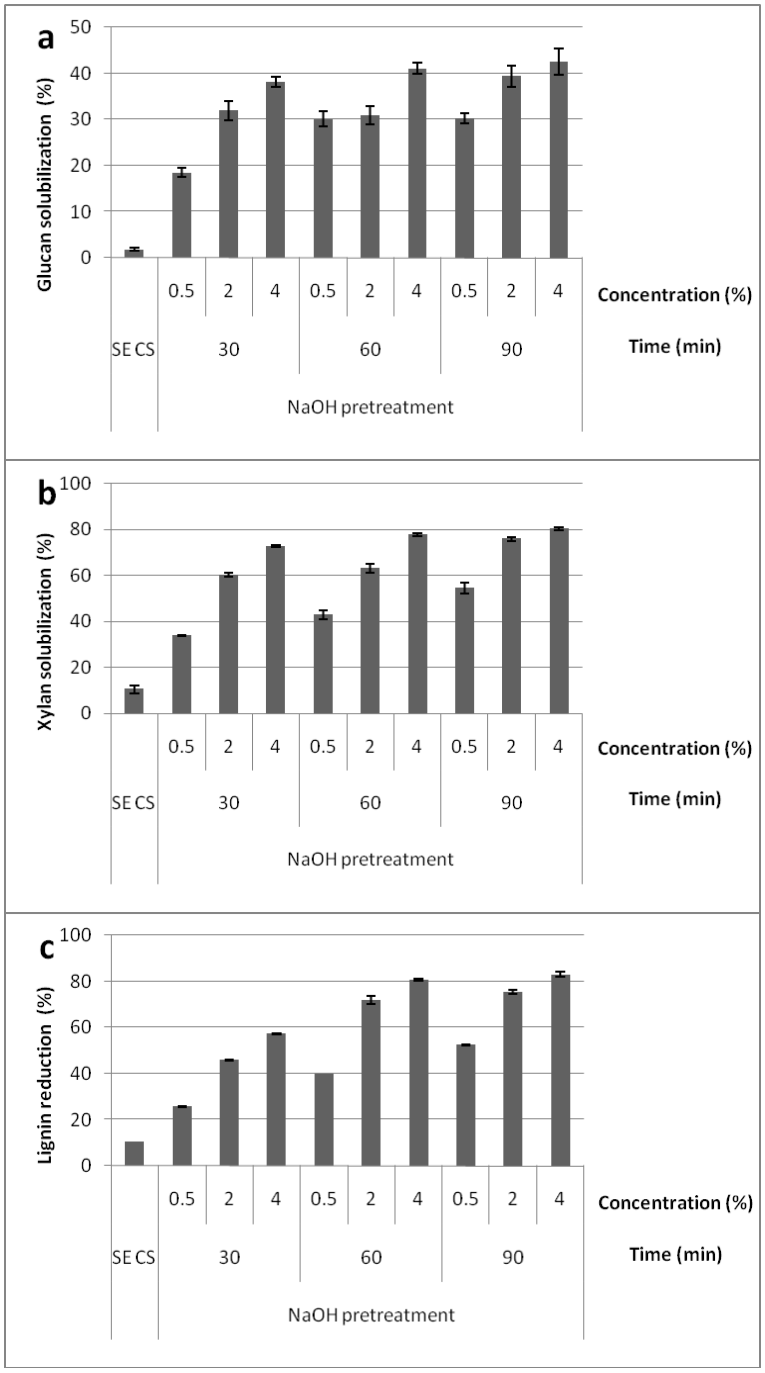

Figure 3.7. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium hydroxide pretreated samples as a function of residence time and concentration (SE CS: steam exploded corn stalks).

The glucan content of pretreated samples ranged from 42.2% (0.5% NaOH, 60 min) to 49.8% (4% NaOH, 90 min). The glucan solubilization of NaOH-pretreated stalks was between 18.5% (0.5%, 30 min) to 42.5% (4%, 90 min) (Figure 3.7a). In another study, Silverstein et al. (2007) reported nearly 21.0% glucan solubilization when cotton stalks

were treated with 2% NaOH at 121°C for 60 min. In similar conditions, the higher glucan solubility (30.1%) in this study could be explained by the steam explosion applied to the stalks.

The xylan content of NaOH pretreated solids ranged from 9.43% (4%, 90 min) to 21.1% (0.5%, 30 min). The xylan solubility of NaOH pretreated stalks (Figure 3.7b) ranged from 33.9% (0.5%, 30 min) to 80.2% (4%, 90 min). The removal of xylan with lignin from the structure is expected to improve the enzymatic digestibility. Chen and Sharma-Shivappa (2007) reported 40%, 35% and 35% xylan solubilizations for barley straw, triticale straw and wheat straw, respectively (2%, 121°C, 60 min). In this study, 55-60% of the xylan removal was observed with similar treatment conditions.

Lignin (Fan et al. 1987), a three-dimensional complex aromatic polymer surrounding cellulose and hemicellulose, limits the enzyme's accessibility to carbohydrates, and its removal is important for efficient enzymatic digestibility. NaOH was an effective pretreatment chemical and removed a high amount of lignin from the structure. NaOH-pretreated stalks at varying times and concentrations had lignin contents that ranged from of 33.9% (0.5%, 30 min) to 11.6% (4%, 90 min). An increase in alkali concentrations and times removed more lignin from the cell structure and reductions of 83.0% (4%, 90 min) and 25.7%, (0.5%, 30 min) were observed in this study (Figure 3.7c). Gaspar et al. (2005) using 2.5% NaOH and Varga et al. (2002) 10% NaOH concentrations (121°C, 60 min) observed almost 95% lignin reduction for corn fiber and corn stover, respectively. In this study, slightly lower lignin reduction was observed when samples were treated with 2% NaOH (75.3%, 90 min). The optimum treatment condition was selected based on lignin removal and glucan avalibility of the samples; the 4% NaOH treated sample at 90 min had the highest glucan to lignin ratio (4.30%).

As expected, the results in this study showed that higher pretreatment concentrations and times removed more material from the structure. In general, regarding treatment times and concentrations, more material was removed at 30 to 60 min. and less at 60 to 90 min., and more from 0.5 to 2% concentrations and less from 2 to 4% concentrations, respectively.

Dilute acids degrade hemicelluloses and release monomeric sugars while solving the oligomers of the cell wall matrix. This treatment increases the porosity of the structure

and that is expected to result in efficient enzymatic digestibility (McMillan 1994).

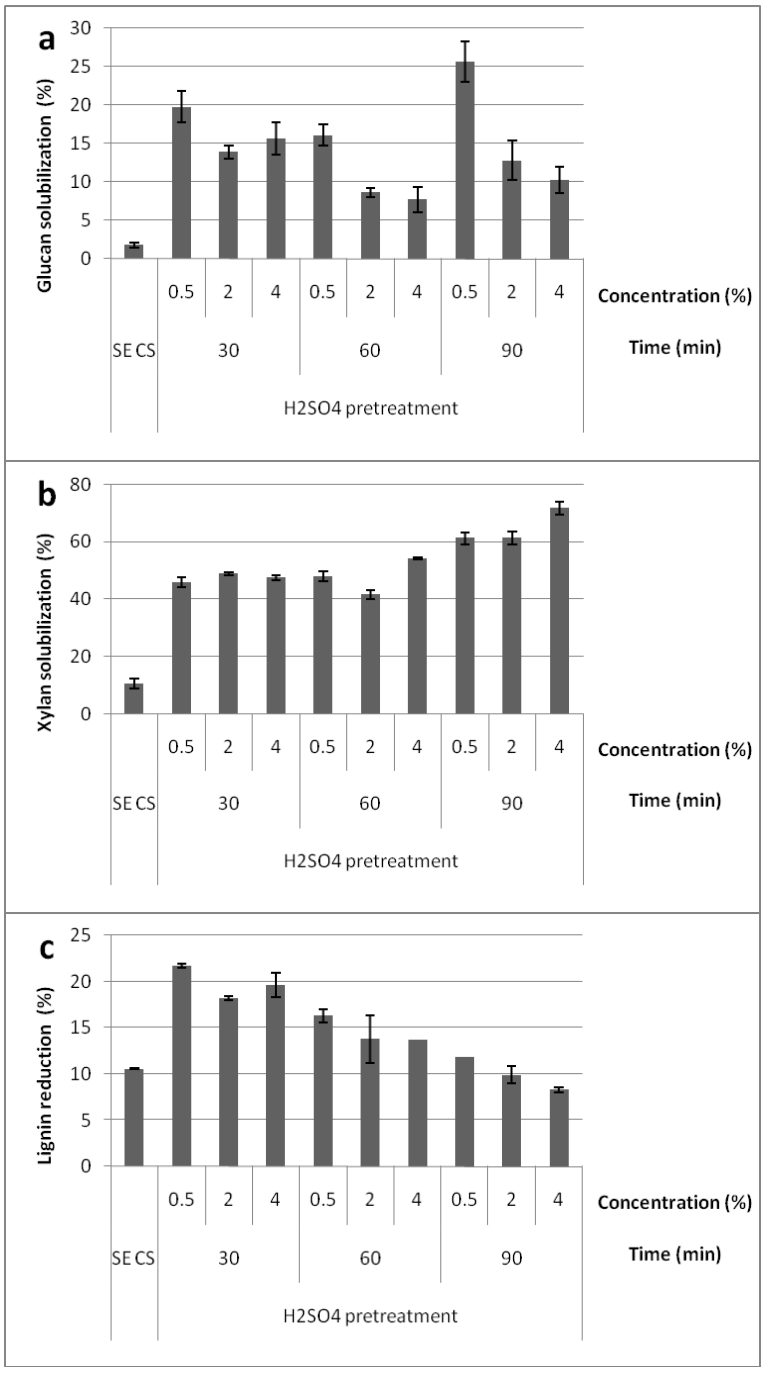

Figure 3.8. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sulfuric acid pretreated samples as a function of residence time and concentration (SE CS: steam exploded corn stalks).

The glucan and xylan solubilization and lignin reduction of the sulfuric acid pretreatment of corn stalks are shown in Figure 3.8a-c, respectively. The solids recovered after pretreatment are presented in Table 3.7. Results showed that higher

residency times and acid concentrations lowered the recovered solids.

The glucan content in the pretreated stalks was more when the acid concentrations and residence times were higher. Depending on the times and concentrations, the glucan contents ranged from 42.5% (0.5%, 90 min) to 54.0% (4%, 60 min). Compared to the steam exploded feedstock, up to 92.3% of the glucan was conserved when samples were treated with 4% acid for 60 min (Figure 3.8a).

The xylan content in treated samples ranged from 9.11% (4%, 90 min) to 18.5% (2%, 60 min). The acid concentration had a significant effect on xylan solubility and 71.7% of xylan was removed when stalks were treated with 4% H_2SO_4 for 90 min (Figure 3.8b). Xylan, a heterogeneous cell wall component, was more liable to be removed from the structure compared to glucan, a crystalline polymer (McMillan 1994). Comparable findings have also been reported in other studies: 77% and 80% of xylan reductions were reported by Schell et al. (2003) for corn stover (1.35%, 190°C, 60 min) and by Grohmann et al. (1985) (0.5%, 140°C, 60 min) for wheat straw, respectively.

The lignin content of H_2SO_4 pretreated stalks varied from 35.2% (0.5% at 30 min) to 42.2% (4%, 90 min). Lignin reduction was found to be up to 21.7% (0.5%, 30 min) (Figure 3.8c). Our results were comparable to other findings,and Silverstein et al. (2007) observed 24.2% (2%, 121°C, 90 min) for cotton stalks and Chen and Sharma-Shivappa (2007) detected as much as 20% (2%, 121°C, 60 min) lignin reduction for barley straw, triticale straw and wheat straw. The optimum glucan to lignin ratio (1.36%) was observed when the sample was treated with 4% H_2SO_4 for 60 min and this sample was further processed with enzymes.

H_2O_2, by oxidizing and reducing the lignin, improves enzymatic digestibility and is a selective bleaching agent (Rabelo et al. 2011). The glucan and xylan solubilization and lignin reduction of H_2O_2 -pretreated samples are shown in Figure 3.9a-c. The solids recovered after pretreatment are shown in Table 3.7.

The recovered glucan after treatment varied from 45.1% (2% H_2O_2, 60 min) to 51.0% (4% H_2O_2 , 90 min). The dissolved glucan varied from 8.30% (0.5%, 30 min) to 16.9% (0.5%, 90 min) (Figure 3.9a). Silverstein et al. (2007) observed 29.1% (2%, 121°C, 30 min) glucan solubility for cotton stalks. In this present study, a significantly lower

glucan solubilization of 16.6% was observed for similar treatment conditions.

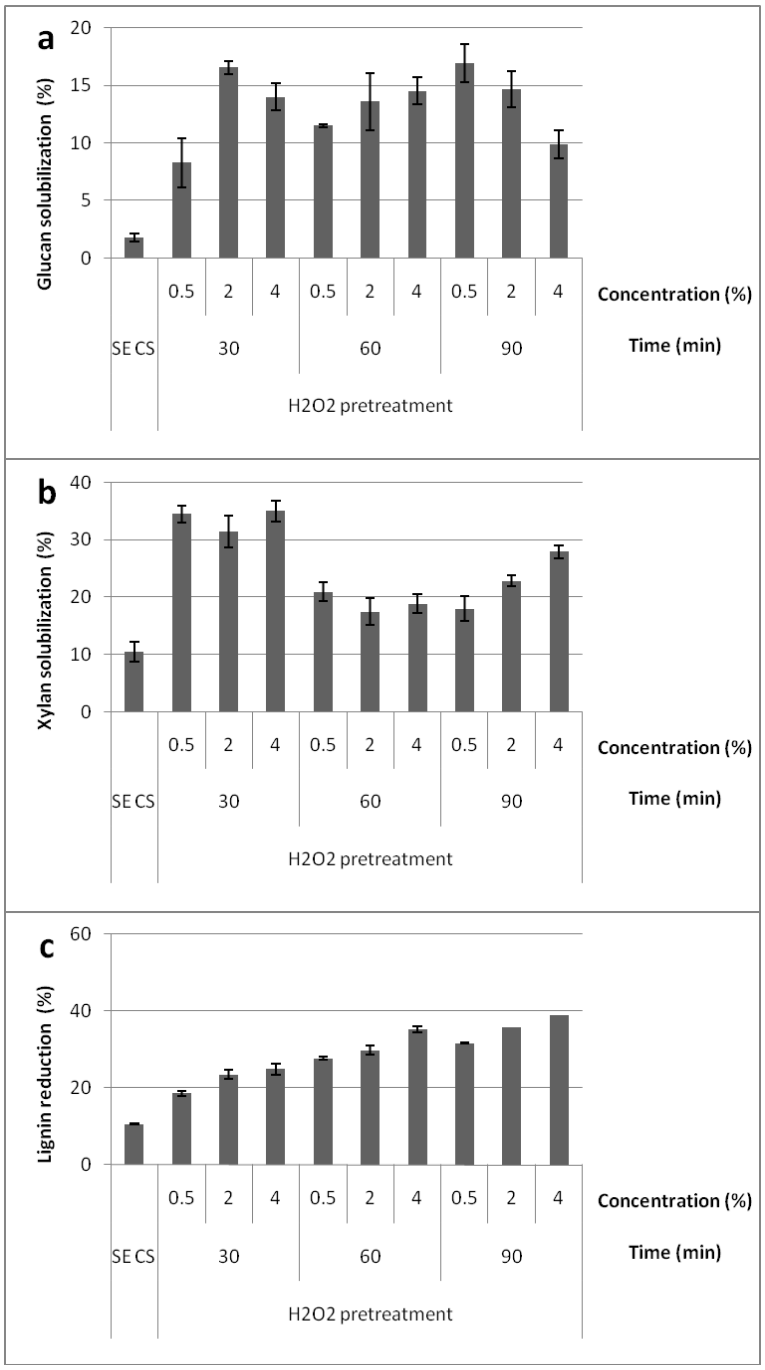

Figure 3.9. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in hydrogen peroxide pretreated samples as a function of residence time and concentration (SE CS: steam exploded corn stalks).

The xylan recovered from solids was of 18.6% (4% H_2O_2, 90 min) and 21.7% (2% H_2O_2, 30 min) and the solubilization of xylan was between 17.5% (2% H_2O_2, 60 min) and 34.4% (0.5% H_2O_2, 30 min) (Figure 3.9b). Similar to our findings in this study,

Silverstein et al. (2007) reported 30.6% xylan solubilization (2% H_2O_2, 121°C, 30 min) for cotton stalks.

An increase in concentration and residence time resulted in more lignin reduction and up to 39.1% (4%, 90 min) of lignin was removed from the structure (Figure 3.9c). Other studies have shown different lignin reductions; Azzam (1989) and Sun et al. (2005b) reported 50% (2%, 30°C, 8 h) lignin reduction for sugarcane bagasse and more than 80% (2%, 50°C, 5 h) reduction for wheat straw. It seems that higher lignin reduction in samples could be possible with intensive treatment conditions. Samples pretreated with 4% H_2O_2 for 90 min had the highest glucan to lignin ratio (1.60%) and this sample was further enzymatically hydrolyzed in this study.

$NaBH_4$ is a bleaching agent, and in addition is used as an additive to improve the pulping selectivity (Copur and Tozluoglu 2007). Figure 3.10a-c show the glucan and xylan solubilization and lignin reduction of $NaBH_4$ pretreated samples. The solids recovered after pretreatment are presented in Table 3.7. Higher residence time and treatment concentrations resulted in lower solid recovery.

Treated solids consisted of varying amounts of glucan. Results showed samples had preserved as much as 60.2% of glucan when they were treated at 4% concentrations for 90 min. The highest glucan solubility (18.9%) was observed when stalks were treated at 2% concentrations for 30 min (Figure 3.10a).

The xylan content of pretreated solids ranged from 16.1% (4%, 30 min) to 19.0% (0.5%, 30 min) and the xylan solubilization was 32.5% (0.5% $NaBH_4$, 30 min) and 49.4% (4% $NaBH_4$, 90 min) (Figure 3.10b).

Compared with untreated material, the total lignin content decreased significantly when samples were treated with $NaBH_4$. The lowest lignin content (18.5%) was observed when samples were treated at 4% concentration for 90 min. Results showed that treatment with $NaBH_4$ significantly decreased the lignin content of the stalks; up to 62.2% of lignin removal was observed when samples were treated at 4% concentratios for 90 min (Figure 3.10c). The optimum glucan to lignin ratio was found to be 3.26% for the treatment condition of 4% $NaBH_4$ (121ºC, 90 min) and this sample was selected for further enzymatic hydrolysis.

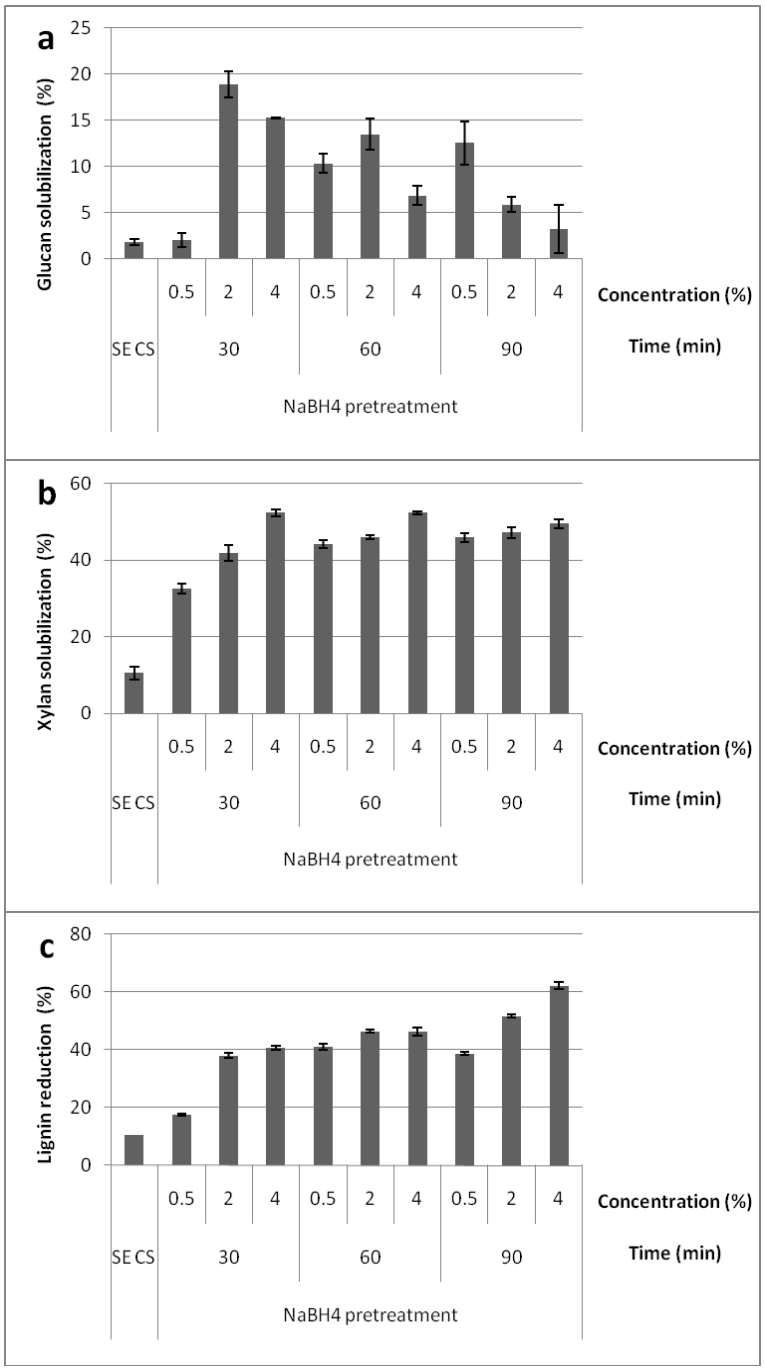

Figure 3.10. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium borohydrate pretreated samples as a function of residence time and concentration (SE CS: steam exploded corn stalks).

3.2.3. Enzymatic Hydrolysis

The selected samples for enzymatic hydrolysis regarding the glucan to lignin ratio were of 4% NaOH (90 min), 4% H_2SO_4 (60 min), 4% H_2O_2 (90 min) and 4% $NaBH_4$ (90 min). Samples were enzymatically processed up to 72 h. Sugar analyses on

periodically taken samples were measured in the supernatant liquid, and the percentages of glucose and xylose were determined for the samples. Results showed that glucose and xylose were the predominant monosaccharides in the hydrolysates. This indicated that both cellulose and hemicellulose were degraded simultaneously during hydrolysis. The concentration of arabinose was below the detection limit and was not reported in this study.

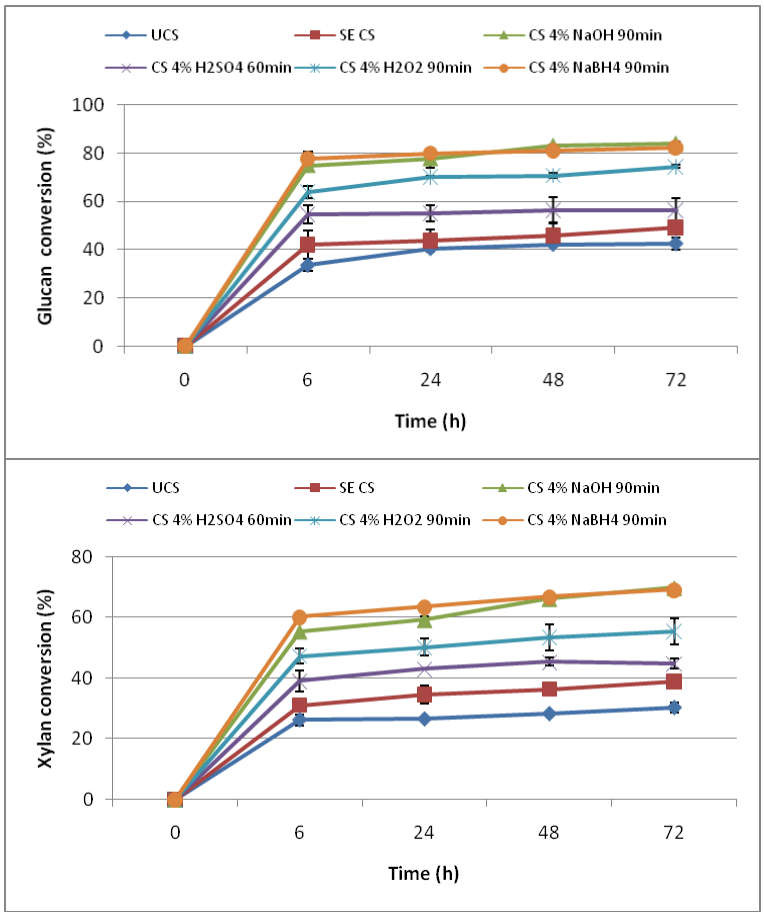

Figure 3.11. Glucan and xylan conversions after enzymatic hydrolysis (UCS: untreated corn stalks, SE CS: steam exploded corn stalks).

The glucan conversion for each hydrolysis treatment is depicted graphically in Figure 3.11. Results showed that stalks pretreated with NaOH (83.9%) and $NaBH_4$ (82.4%) had similar glucan conversions. The lowest glucan conversion was observed with H_2SO_4 (56.6%). Statistical results showed that the mean glucan conversion for all samples was statistically significant ($p<0.001$). The variation of glucan conversions could be explained by the lignin content of the samples after chemical pretreatment. Results showed that NaOH and $NaBH_4$-treated samples, having 11.6% and 18.5% lignin

respectively in their structure, resulted in the highest glucan conversions. The lowest glucan conversion, observed for H_2SO_4, had 39.7% of lignin. Compared to NaOH, the H_2SO_4-treated stalk had almost 3.42 times more lignin and resulted in 1.48 times lower glucan conversion. Raw corn stalks had 1.50 times more lignin compared to xylan, and this also indicated that removal of lignin from the structure was more crucial than xylan removal. Similar findings have also been published (Lu et al. 2002) and Yang and Wyman (2004) explained that condensed lignin could adsorb protein from aqueous solutions, and that lignin removal may improve the hydrolysis performance. Thus, removal of both lignin and xylan from the structure before enzymatic hydrolysis is important, but removal of lignin seems to be much more crucial for efficient glucan conversion.

Although xylanase was not utilized in this study, a significant amount of xylose was detected in the hydrolyzates (Figure 3.11). Duarte et al. (2004) reported that the *Celluclast* 1.5 L utilized in this study had *β-xylanase* (100 U/g) and *β-xylosidase* (0.53 U/g) activities of xylose. Samples treated with NaOH (69.9%) and $NaBH_4$ (69.2%) had higher xylan to xylose conversions compared to those treated with H_2O_2 and H_2SO_4. Differences in mean xylan conversion for all treatments were found to be statistically significant ($p<0.001$). Results showed that chemical pretreatment diminished the hemicellulose (mainly xylan) in the samples and it could be considered that the xylanolytic activity in the *Celluclast* 1.5 L and *cellobiase* utilized in this study was high enough to hydrolyze the xylan in the structure of the stalks.

3.2.4. Fermentation of Hydrolyzates

The fermentation performance of *S. cerevisiae* in treated hydrolysates is shown in Table 3.10. In this study, the maximum theoretical ethanol yield of 72.5% (97.4 g ethanol/kg corn stalks) was found for corn stalks pretreated with 4% $NaBH_4$ for 90 min (Figure 3.12). The theoretical ethanol yields of 85% (for corn fiber) and 93% (for corn stover) was observed by Sedlak and Ho. (2004) The slightly lower ethanol yield in this study may be due to the raw material characteristics of the geographic location, cultivars, harvest time, various parts of samples, etc. Chemical treatment may result in the by-product formation of furfural, HMF and phenolic compounds which could inhibit the sugar fermentation of yeasts (Bjerre et al. 1996). By-products in the hydrolyzates were not detected due to their low concentrations in the samples. Sodium azide was used to

prevent microbial contamination and it may also have reduced the yeast activity (Fales 1953). In our study, results showed that, compared to untreated samples, chemically pretreated samples improved the ethanol yields.

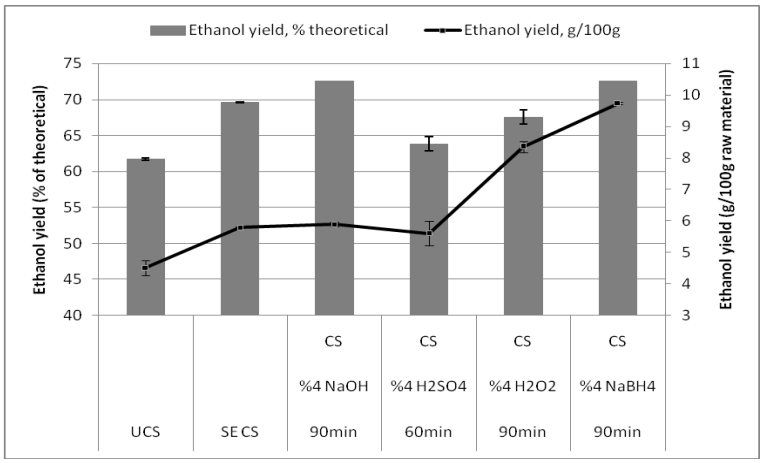

Figure 3.12. Ethanol yield (percent of theoretical and g/100g raw material) (UCS: untreated corn stalks, SE CS: steam exploded corn stalks).

Table 3.10. Glucose, xylose and ethanol concentrations during fermentation with *S. cerevisiae* in untreated and pretreated stalks

Pretreatment conditions	0 h			6 h			24 h			48 h			72 h		
	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l
UCS*	7.17±0.41	3.08±0.16	0.47±0.02	5.80±0.28	2.87±0.16	2.13±0.10	2.35±0.08	1.97±0.11	2.23±0.13	1.50±0.07	1.55±0.11	2.24±0.13	1.00±0.02	1.42±0.08	2.26±0.12
SE CS	9.56±0.04	4.14±0.02	0.67±0.06	7.43±0.07	3.83±0.01	3.22±0.06	3.03±0.02	2.62±0.02	3.27±0.05	1.96±0.03	2.02±0.02	3.36±0.03	1.26±0.02	1.93±0.05	3.39±0.01
CS %4 NaOH 90 min.	18.4±0.23	2.90±0.00	1.35±0.07	13.9±0.02	2.63±0.03	6.51±0.19	5.64±0.01	1.77±0.01	6.66±0.01	3.35±0.10	1.38±0.02	6.71±0.00	2.16±0.09	1.33±0.00	6.80±0.09
CS %4 H_2SO_4 60 min.	13.4±1.13	2.93±0.11	0.97±0.06	10.4±0.81	2.73±0.12	4.24±0.27	4.38±0.42	1.84±0.07	4.32±0.36	2.60±0.12	1.43±0.01	4.34±0.33	1.84±0.20	1.37±0.02	4.37±0.30
CS %4 H_2O_2 90 min.	16.7±0.13	4.55±0.35	1.19±0.01	13.1±0.06	4.23±0.33	5.41±0.18	5.53±0.09	2.83±0.22	5.68±0.19	3.26±0.07	2.21±0.14	5.72±0.14	2.01±0.02	2.09±0.16	5.76±0.13
CS %4 $NaBH_4$ 90 min.	21.8±0.08	5.27±0.09	1.67±0.07	16.4±0.24	4.79±0.10	7.70±0.03	6.57±0.12	3.22±0.03	7.84±0.01	3.90±0.04	2.53±0.04	8.03±0.04	2.41±0.03	2.42±0.04	8.07±0.03

*UCS: untreated corn stalks, SE CS: steam exploded corn stalks.

3.3. CHAPTER 3: HAZELNUT HUSK

3.3.1. Composition of Hazelnut Husk

The chemical composition of the raw hazelnut husks used in this study is presented in Table 3.11. HPLC results showed that the sugar fraction of the material was 44.2% of the dry biomass. Glucan, the major cell wall component, made up 24.2% of the material. Xylan, the major hemicellulose constituent found in the structure, was 16.8%. Arabinan accounted for only 3.15% of the biomass. Mannan and galactan were not detected in this study. The holocellulose fraction, determined by the method of Wise (1952), was 41.1±0.10% of the total biomass. The lignin content was observed to be 39.0%.

Compared to wheat straw and hard/softwood, the husks had relatively lower carbohydrate content (Table 3.11). The lignin content of the husks was higher compared to wheat straw, hard/softwood and herbaceous species (10-20%) (McMillan 1994). On the other hand, the hazelnut husks had higher solubility values (Table 3.11).

Table 3.11. Chemical composition of raw and steam exploded hazelnut husk (current) and wheat straw (Copur et al. 2012).

Component	Raw husk, (%)*	Steam exploded husk, (%)*	Wheat straw, (%)	Hardwoods (Fengel and Wegener, 1984)	Softwoods (Fengel and Wegener, 1984)
Total sugar	44.2	45.4	58.1		
Glucan	24.2±1.27	25.3±1.25	36.6		
Xylan	16.8±1.79	17.7±0.79	19.7		
Arabinan	3.15±0.44	2.36±0.79	3.63		
Holocellulose	41.1±0.10	29.8±0.39	68.9	70-78	63-70
Acid-insoluble lignin	38.6±1.81	36.8±1.09	34.0		
Acid-soluble lignin	0.37±0.05	0.55±0.02	1.22		
Total lignin	39.0	37.4	35.2	30-35	25-35
Alcohol-benzene solubility	16.9±0.40	21.0±0.60	3.66	2-6	2-8
Hot water solubility	29.4±0.00	-	13.0	2-7	3-6
Cold water solubility	23.9±0.09	-	9.30	4-6	2-3
1% NaOH solubility	62.8±0.20	-	45.5	14-20	9-16
Ash	5.43±0.04	5.92±0.00	10.1		

* Percentages are dry-weight basis and values are average of triplicate measurements.

In our earlier work (Copur et al. 2007), the slightly higher holocellulose (55.1%) and lower lignin (35.1%) content of the husks could be explained by the growing location, stage of harvest, harvesting methods, analysis procedures, etc. The chemical composition of the husks and higher lignin in the structure indicated that the process should be optimized to improve the hydrolysis efficiency in converting almost all sugars

to ethanol in order to increase the production yield. Therefore, in this study, samples were first steam exploded and then chemically pretreated.

3.3.2. Effects of Pretreatments

Steam Explosion

The yield after SE was found to be 90.5% (w/w). As can be noted, the glucose proportion (25.3%) after SE increased in relation to the untreated material (24.2%). This increase could be due to the solubilization of hemicelluloses and removal of other materials from the husk structure. Chen et al. (2011) observed similar results and the glucose content (34.5%) of rice straw was increased up to 46.9% when the material was steam exploded at 180°C for 20 min. After SE, 95.5% of the glucose remained in the solid fraction. SE removed 4.57% of xylan and 13.3% of lignin from the structure. On the other hand, results indicated that the glucan, xylan and lignin contents of untreated and steam exploded samples were statistically significant ($p<0.001$).

Martin et al. (2008) found 60% of xylan solubilization and 35% lignin reduction with sugarcane bagasse steam exploded at 205°C for 10 min. In addition, Jeoh and Agblevor (2001) reported complete solubilization of mannan, galactan, and arabinan, and considerably higher xylan removal compared to glucose for cotton gin wastes steam exploded at 185–238°C for 20–265 seconds. It could be concluded that denser SE conditions remove almost all hemicelluloses from the biomass structure. Consequently, the mild SE conditions applied in this study degraded less glucose, and almost all glucose fractions remained in the husk structure. These mild conditions removed moderate amounts of hemicelluloses, and the holocellulose content of the material was 29.8% after SE (Table 3.11).

Chemical Pretreatments

The solids recovered after chemical pretreatments are presented in Table 3.12. Lower yields after pretreatment could be explained mainly by the removal of lignin, hemicellulose and other solubles from the structure. Samples treated with 0.5% $NaBH_4$ for 30 min resulted in the highest solid recovery (81.7%). On the other hand, those treated with 4% NaOH for 90 min gave the lowest solid recovery (64.0%). Factors of chemicals (4 different), times (3 different) and concentrations (3 different) and their

interactions on glucan and xylan solubility and lignin reduction are shown in Table 3.13 and Table 3.14.

Table 3.12. Solids recovery after pretreatments.

	Solids recovered after chemical pretreatments (%)[a,b]			
Time (min.), Conc. (%)	Sulfuric acid	Sodium hydroxide	Hydrogen peroxide	Sodium borohydrate
30, 0.5	79.1±0.26	67.5±0.28	66.0±0.86	81.7±0.64
30, 2	77.9±0.23	68.7±0.27	63.2±0.06	71.7±0.31
30, 4	77.9±0.39	65.1±0.39	63.3±0.44	69.6±0.43
60, 0.5	74.1±0.27	71.7±0.15	75.6±0.47	72.2±0.52
60, 2	73.6±0.22	71.9±0.42	76.2±0.27	71.5±0.47
60, 4	73.8±0.46	71.7±0.36	75.3±0.24	69.9±0.55
90, 0.5	74.9±0.77	69.1±0.30	76.4±0.16	74.9±0.52
90, 2	73.6±0.15	68.2±0.26	72.0±0.72	69.2±0.71
90, 4	66.9±0.99	64.0±0.97	68.7±0.50	68.8±0.30

[a] Percentages calculated from value on a dry-weight basis. [b] Data are averages of three replicates.

Table 3.13. Interactions between chemicals, time and concentrations on glucan, xylan and lignin.

Source		Type III Sum of Squares	df	Mean Square	F	P
Glucan solubilization	Chemical	87.7	3	29.2	18.8	*
	Time	16.5	2	8.25	5.32	**
	Concentration	25.3	2	12.7	8.17	**
	Chemical * Time	47.9	6	7.99	5.15	**
	Chemical * Concentration	61.8	6	10.3	6.64	*
	Time * Concentration	35.3	4	8.83	5.69	**
	Chemical * Time * Concentration	18.1	12	1.51	0.97	NS
Xylan solubilization	Chemical	112.4	3	37.5	65.7	*
	Time	6.85	2	3.42	6.00	**
	Concentration	38.6	2	19.3	33.8	*
	Chemical * Time	35.2	6	5.86	10.3	*
	Chemical * Concentration	24.9	6	4.15	7.27	*
	Time * Concentration	14.4	4	3.61	6.33	**
	Chemical * Time * Concentration	18.0	12	1.50	2.63	***
Lignin reduction	Chemical	2575.3	3	858.4	1415.0	*
	Time	15.2	2	7.62	12.6	*
	Concentration	18.4	2	9.20	15.2	*
	Chemical * Time	154.9	6	25.8	42.6	*
	Chemical * Concentration	119.6	6	19.9	32.9	*
	Time * Concentration	2.08	4	0.52	0.86	NS
	Chemical * Time * Concentration	19.1	12	1.59	2.63	***

[P] Significance level. [*] Significant at 0.001 for ANOVA. [**] Significant at 0.01 for ANOVA. [***] Significant at 0.05 for ANOVA. [NS] None significant for ANOVA.

NaOH disrupts H-bonds in cellulose and hemicellulose, breaks ester linkages between lignin and xylan, and deprotonates phenolic lignin groups (Sun et al. 2005a, Simpson et al. 2003, Akin et al. 1992). It causes the cellulose to swell and dissolves some hemicelluloses and lignin (Chen and Sharma-Shivappa 2007). Chemically treated samples indicated that higher residency times and alkali concentrations resulted in higher solid loss (Table 3.12). The glucan content of treated solids ranged from 27.1%

(0.5%, 60 min) to 33.3% (2%, 90 min). The highest glucan solubility (23.9%) was observed when the sample was treated at 0.5% NaOH for 60 min (Figure 3.13a). An almost similar amount of glucan solubility was observed by Silverstein et al. (2007), who reported nearly 21.0% of solubilization for cotton stalks pretreated with 2% NaOH at 121°C for 60 min. On the other hand, lower glucan solubility was reported by Wang et al. (2010) who observed 2.03-9.77% of solubilizations for bermuda grass pretreated with 0.5-3% NaOH at 121°C for 15-90 min. This indicates that raw material and pretreatment conditions affect the glucan solubility.

Table 3.14. Effects of chemicals, time and concentrations on glucan, xylan and lignin.

Factor	Treatment	Glucan (%)	Xylan (%)	Lignin (%)
Chemical	NaOH	30.1 a [A]	7.84 a	35.9 a
	H_2SO_4	30.1 a	9.90 b	47.5 b
	H_2O_2	30.5 a	11.3 c	37.6 c
	$NaBH_4$	27.7 b	10.1 b	31.1 d
Time	30 min	29.1 a	10.2 a	38.4 a
	60 min	29.4 a	9.42 b	38.2 a
	90 min	30.2 b	9.75 ab	37.4 b
Concentration	0.5%	29.3 a	10.8 a	38.5 a
	2%	30.4 b	9.56 b	38.2 a
	4%	29.0 a	9.02 c	37.3 b

[A] Means within each column and factor followed by the same letter are not significantly different by Duncan test ($p<0.05$).

The xylan content of pretreated solids ranged from to 11.1% (0.5%, 90 min) to 5.81% (4%, 60 min). Results showed that more severe pretreatment conditions dissolved more xylan from the husk stucture (Figure 3.13b). This finding could be explained by the nature of the hemicelluloses, which are amorphous, low molecular weight, heterogeneous and branched polysaccharides. They are more susceptible to alkaline attacks. Severe pretreatment conditions lead to lower xylose yields due to the greater solubilization during the processes. The NaOH pretreatment dissolved 56.6% (0.5%, 90 min) to 77.9% (4%, 90 min) of xylan from the husk structure. Among chemical pretreatment methods, NaOH resulted in the highest xylan solubility (Figure 3.13b). Therefore, solubilization of xylan in conjuction with lignin reduction is expected to improve the following enzymatic hydrolysis. The xylan solubility obtained in this study was comparable with that obtained by Wang et al. (2010), who reported 60.5% xylan solubilization in bermuda grass (3% NaOH/121°C/90 min).

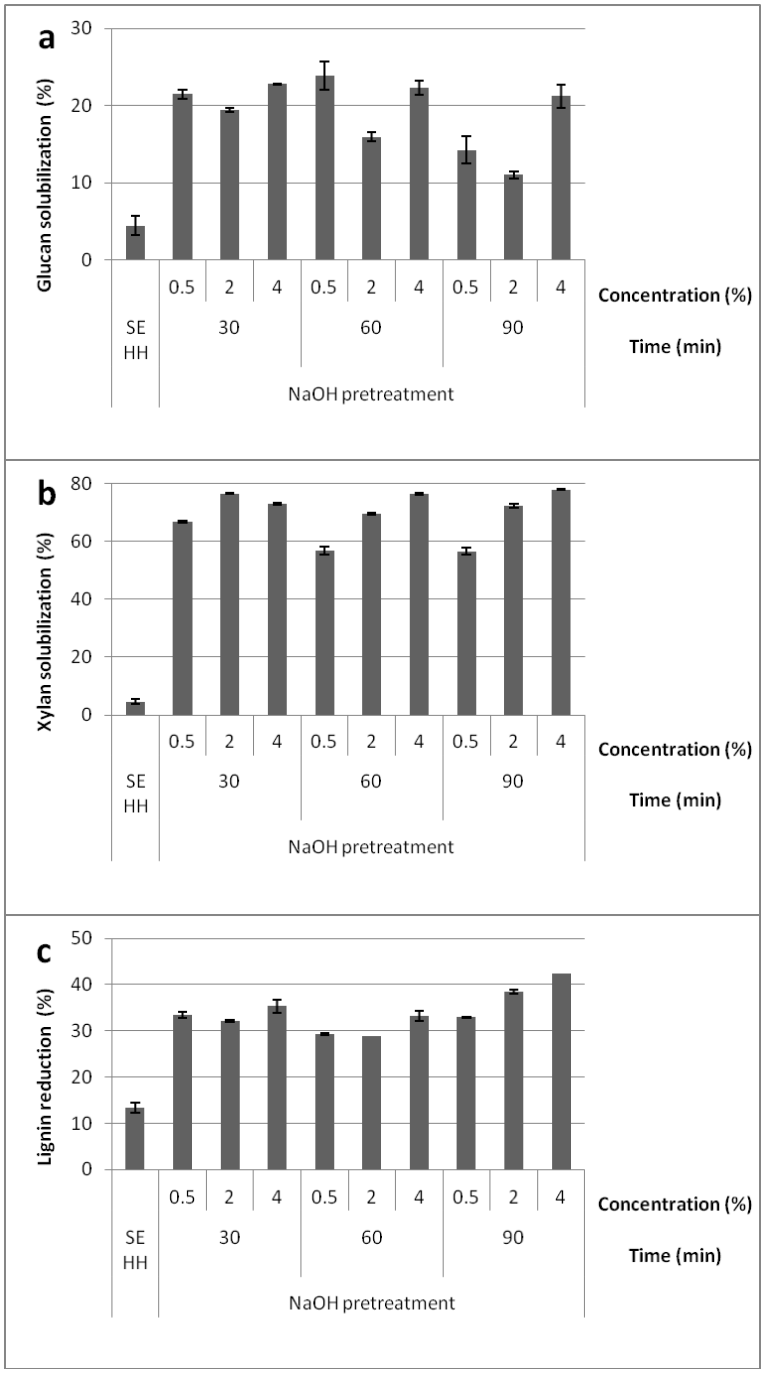

Figure 3.13. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium hydroxide pretreated samples as a function of residence time and concentration (SE HH: steam exploded hazelnut husk).

On the other hand, slightly lower xylan solubility values were reported by Chen and Sharma-Shivappa (2007) for barley straw (40%), triticale straw (35%) and wheat straw (35%) using 2% NaOH at 121°C for 60 min. Results showed that NaOH pretreatment removed more xylan compared to glucan; this could be explained by the vulnerablity of hemicelluloses to the chemicals (Schmidt and Thomsen 1998).

Lignin is a three-dimensional, complex aromatic polymer which forms a sheath surrounding cellulose and hemicellulose and stiffens and holds the fibers together (Fan et al. 1987). This limits the accessibility of carbohydrates to hydrolytic enzymes and its removal from the structure is crucial to improve the digestibility of biomass. Results of this study showed that the NaOH pretreatment was highly effective in removing lignin from the husk structure. The amount of total lignin in the solids after NaOH pretreatment ranged from 36.8% (0.5%, 30 min) to 33.6% (4%, 90 min). Increase in alkali concentrations from 0.5 to 4% resulted in higher lignin reductions of 28.8% (2%, 60 min) to 42.5% (4%, 90 min) (Figure 3.13c). Therefore, results indicated that the major solid loss during NaOH pretreatment was due to the delignification and xylan degradation of the hazelnut husks. Much higher lignin removal was reported by Gaspar et al. (2005) and Wang et al. (2010). They obtained 95% lignin reduction in corn fiber at 121°C for 60 min with 2.5% NaOH and up to 86% reduction of lignin in bermuda grass treated with 3% NaOH at 121°C for 90 min, respectively. Varga et al. (2002) observed almost 95% lignin reduction by treating corn stover with 10% NaOH for 1 h. The high reduction levels may be attributed to the higher NaOH concentration of 10%. On the other hand, the NaOH concentration was limited to 4% in this study. The optimum pretreatment condition of NaOH for the following enzymatic hydrolysis was determined based on the ratio of the glucan and lignin left in the solid material. The optimum ratio was 0.99% when the sample was treated with 2% NaOH for 90 min. This indicated a selective delignification of NaOH when the results were compared to the steam-exploded material with a ratio of 0.68%.

In high temperatures, pretreatment with dilute acids hydrolizes the hemicelluloses by releasing monomeric sugars and soluble oligomers from the cell wall matrix into the hydrolyzate. Acid pretreatment is one of the most effective methods applied for lignocellulosic biomass. Removing hemicelluloses increases the structure porosity and therefore improves the enzymatic digestibility. Literature (McMillan 1994) indicates that maximum enzymatic digestibility is obtained when all hemicellulose is removed from the structure.

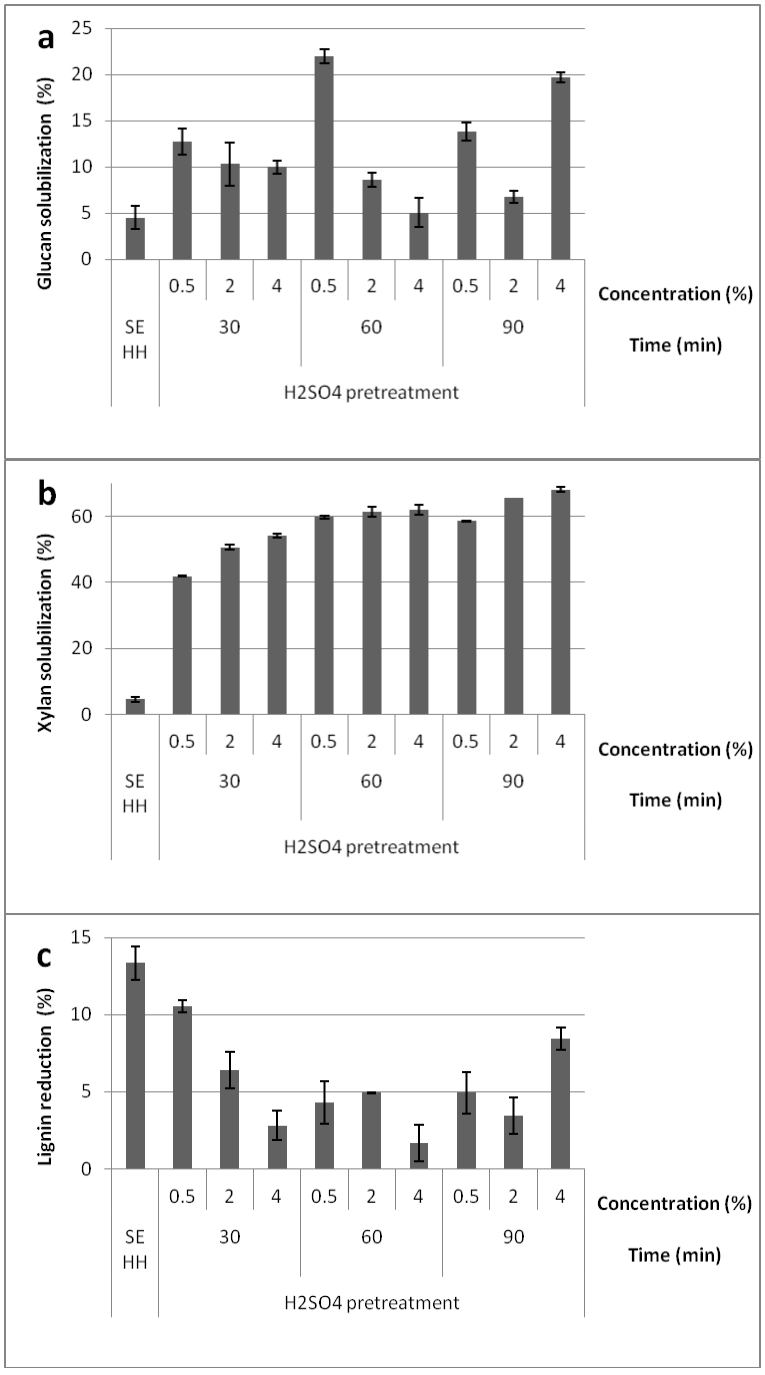

Figure 3.14. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sulfuric acid pretreated samples as a function of residence time and concentration (SE HH: steam exploded hazelnut husk).

The results of H_2SO_4 pretreatment of hazelnut husks for glucan and xylan solubilization and lignin reduction, respectively, are shown in Figure 3.14a-c. The solid recovery after pretreatment is presented in Table 3.12. The results indicate that elevated residency times and acid concentrations lowered the recovered solids.

The glucan content of H_2SO_4 pretreated husks increased as the acid concentrations and

residence times were increased. The glucan content of the pretreated husks ranged from 28.1% (0.5%, 30 min) to 32.8% (4%, 60 min) when treated with 0.5 to 4% of H_2SO_4, respectively. Compared with steam exploded husks, 78.0% and 93.2% of the glucan was preserved when samples were treated at 0.5% (60 min) and 2% (90 min) of H_2SO_4, respectively. (Figure 3.14a). Chen and Sharma-Shivappa (2007) reported 71.9, 92.8 and 76.8% (dry basis) of glucan preservation when samples were treated with 2.0% acid at 121°C for 60 min for barley, pearl millet and sweet sorghum hays, respectively. During pretreatment, it is desirable for the cellulose portion of the biomass to be virtually unaffected. However, during acid pretreatment of the hazelnut husks, a slightly higher glucan reduction was observed in this study.

Xylan was the largest portion of hemicellulose in the untreated husks. The xylan content in pretreated husks ranged from 8.27% (2%, 90 min) to 13.0% (0.5%, 30 min) depending on the treatment conditions. Results showed that acid concentration had a significant effect on xylan reduction and H_2SO_4 pretreatment dissolved up to 68.1% (4%, 90 min) xylan from the husk structure (Figure 3.14b). The higher xylan dissolution compared to glucan could be attributed to the fact that xylan is more labile due to its heterogeneous, non-crystalline structure (McMillan 1994). Results from this study are comparable to those obtained by Schell et al. (2003), who reported 77% xylan reduction in corn stover treated at 190°C for 60 min with 1.35% acid, and Grohmann et al. (1985), who reported more than 80% reduction of xylan in wheat straw which was treated with dilute H_2SO_4 at 140°C for 1 h. In addition, Sun and Cheng (2005) found 66% xylan reduction for rye straw treated with 1.5% acid at 121°C for 90 min, and 62% xylan reduction in Bermuda grass treated with 1.5% acid at 121°C for 60 min.

Compared with untreated samples, dilute acid treatments resuted in an increase in the total lignin content of the material due to the removal of carbonhydrates from the structure. The lignin content of the samples varied from 42.2% (0.5% H_2SO_4, 30 min) to 51.1% (4% H_2SO_4, 90 min) depending on the treatment conditions. On the other hand, the dissolved lignin ranged from 1.67% to 10.5% (Figure 3.14c). The findings of this study are comparable to Silverstein et al. (2007), who observed 24.2% lignin reduction for cotton stalks treated with 2% H_2SO_4 at 121°C for 90 min. In addition, Chen and Sharma-Shivappa (2007) observed as much as 20% lignin reduction for barley straw, triticale straw and wheat straw treated with 2% H_2SO_4 at 121°C for 60 min. The highest

glucan to lignin ratio of the treated samples was 0.67% (0.5% H_2SO_4, 30 min). The ratio was similar to the steam-exploded sample (0.68%). This finding indicates that the H_2SO_4 treatment removed a similar amount of lignin as well as glucan from the structure. Samples treated with 0.5% H_2SO_4 (30 min) were processed for further enzymatic hydrolysis.

Alkaline H_2O_2 is a well-known bleaching agent used in the paper industry. Its main advantage is that it does not leave any residue (secondary products) in the material after treatment as it is decomposed into oxygen and water (Rabelo et al. 2011). It loosens the lignocellulosic matrix while oxidatively delignifying and dissolving the lignin, thus improving the enzymatic digestibility (Martel and Gould 1990).

The glucan and xylan solubilization and lignin reduction of the H_2O_2 treated samples are shown in Figure 3.15a-c, respectively. The solids recovered after pretreatment are presented in Table 3.12. The glucan in pretreated solids was up to 32.4% (0.5% H_2O_2, 30 min) and glucan solubilization was up to 27.6% (2% H_2O_2, 30 min) based on the treatment conditions (Figure 3.15a). The xylan remaining in treated samples ranged from 9.30% (4% H_2O_2, 60 min) to 13.5% (0.5% H_2O_2, 90 min) and the xylan solubilization ranged from 41.8% (0.5% H_2O_2, 90 min) to 60.2% (4% H_2O_2, 60 min) (Figure 3.15b). Results of this study are comparable to other findings; Silverstein et al. (2007) observed 29.1% glucan and 30.6% xylan solubilization for cotton stalks when samples were treated with 2% H_2O_2 at 121°C for 30 min.

Increases in treatement concentration and residence time resulted in higher lignin reductions, and up to 36.3% lignin reduction was observed when the sample was treated with 4% H_2O_2 for 90 min (Figure 3.15c). Results from this study are comparable to those obtained by Azzam (1989) and Sun et al. (2005b), who reported 50% lignin reduction in sugarcane bagasse at 30°C for 8 h with 2% alkaline H_2O_2, and more than 80% reduction of lignin in wheat straw treated with 2% H_2O_2 at 50°C for 5 h. Selig et al. (2009) reported 78.6% lignin reduction for corn stover when the sample was treated with 30 % H_2O_2 at 65°C for 3 h. The extent of lignin reduction observed in this study was comparatively lower and this finding could be due to the shorter treatment time that may have limited the oxidative delignification.

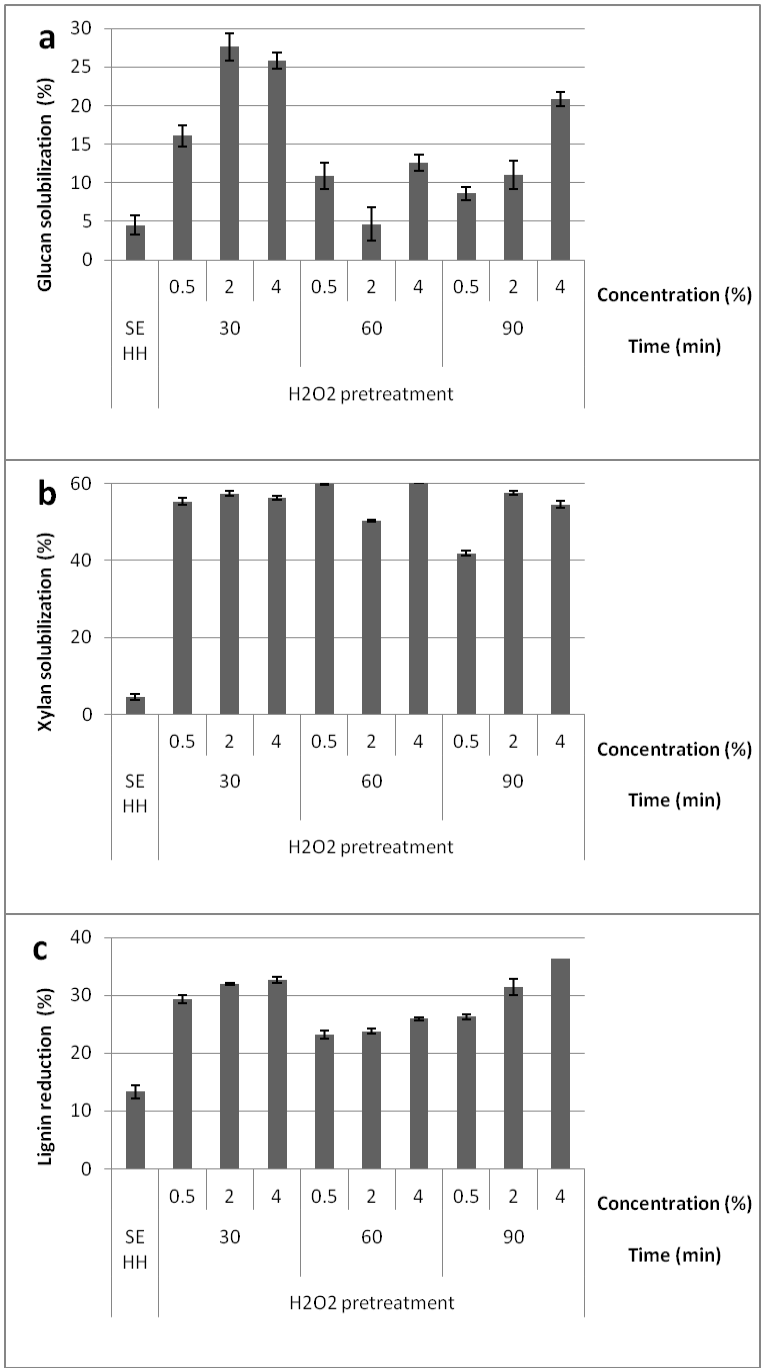

Figure 3.15. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in hydrogen peroxide pretreated samples as a function of residence time and concentration (SE HH: steam exploded hazelnut husk).

Among H_2O_2 treated samples, compared to steam exploded material (0.68%), the highest glucan to lignin ratio was 0.89% when the sample was treated with 2% H_2O_2 for 90 min; this sample was further used for enzymatic hydrolysis. Results indicated that H_2O_2 removed much more lignin compared to carbohydrates from the structure.

$NaBH_4$ is an additive that improves the pulping selectivity of conventional kraft

pulping. It reacts as a catalyst in pulping operations and results in selective delignification (Copur and Tozluoglu 2007). It improves the pulping yield by preventing carbohydrate degradations (Hoije et al. 2005). Literature has very limited information on $NaBH_4$ utilized as a pretreatment chemical in bioethanol production. In our earlier study, the effect of $NaBH_4$ on wheat straw was studied (Copur et al. 2012) and results showed that it is as effective as NaOH as a pretreatment chemical.

The glucan and xylan solubilization and lignin reduction during $NaBH_4$ treatment of hazelnut husks are displayed in Figure 3.16a-c, respectively. The solids recovered after treatment are presented in Table 3.12. Results showed that elevated residency times and treatment concentrations diminished the recovered solids.

The glucan content of $NaBH_4$-treated husks ranged from 25.1% (4%, 30 min) to 31.2% (2%, 60 min) and the glucan solubilization was found to be as much as 31.6% (4%, 30 min) (Figure 3.16a). The xylan content of $NaBH_4$- treated husks ranged from 8.60% (4%, 30 min) to 11.4% (2%, 60 min) and xylan solubilization ranged from 51.9% (0.5%, 90 min) to 66.2% (4%, 30 min) (Figure 3.16b). Results indicated that $NaBH_4$ treated husks removed as much xylan as NaOH. The unexpectedly higher carbohydrate solubitiy of $NaBH_4$ treatments could be due to the raw material characteristics.

The lignin content of the samples ranged from 35.6% (0.5% $NaBH_4$, 30 min) to 27.6% (4% $NaBH_4$, 90 min). Results showed that the $NaBH_4$ treatment significantly decreased the lignin content and up to 49.1% of lignin was removed when the sample was treated with 4% $NaBH_4$ for 90 min (Figure 3.16c). The higher removal results of the $NaBH_4$ treatment was obvious compared to results using NaOH (42.5%) under the same treatment conditions.

The glucan to lignin ratio of steam exploded samples was 0.68%, and the highest ratio of 0.99% was observed when the sample was treated with 2% $NaBH_4$ for 60 min. The results were the same as the sample treated with 2% NaOH (90 min). Consequently this sample was further processed for enzymatic hydrolysis.

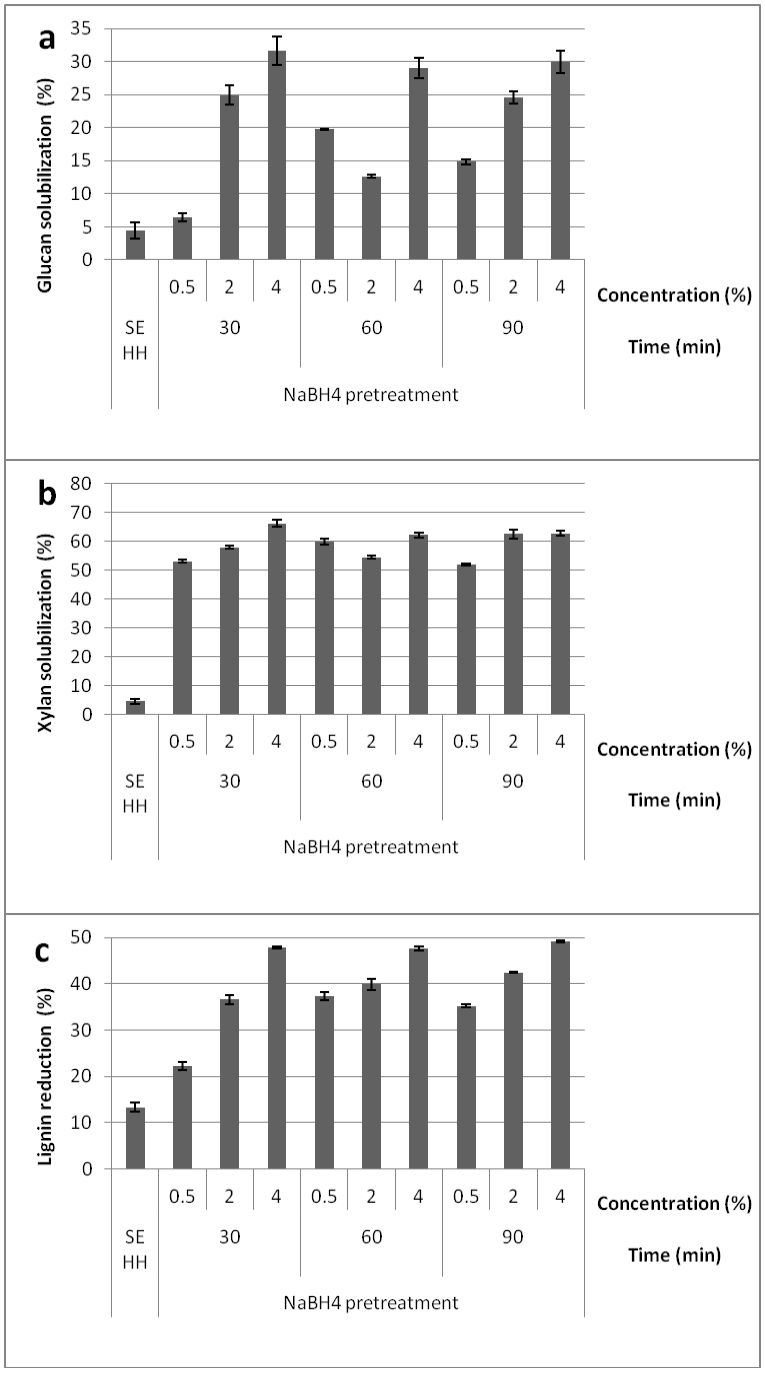

Figure 3.16. (a) glucan solubilization, (b) xylan solubilization and (c) lignin reduction in sodium borohydrate pretreated samples as a function of residence time and concentration (SE HH: steam exploded hazelnut husk).

3.3.3. Enzymatic Hydrolysis

The selected samples based on the highest glucan to lignin ratio (2% NaOH, 90 min; 0.5% H_2SO_4, 30 min; 2% H_2O_2, 90 min; and 2% $NaBH_4$,60 min) were further enzymatically hydrolysed in this study. Results showed that glucose and xylose were the predominant monosaccharides in enzymatic hydrolysates, indicating simultaneous

degradation of cellulose and hemicellulose during the hydrolysis process. The concentrations of arabinose and galactose were below the detection limit and were not reported in this study.

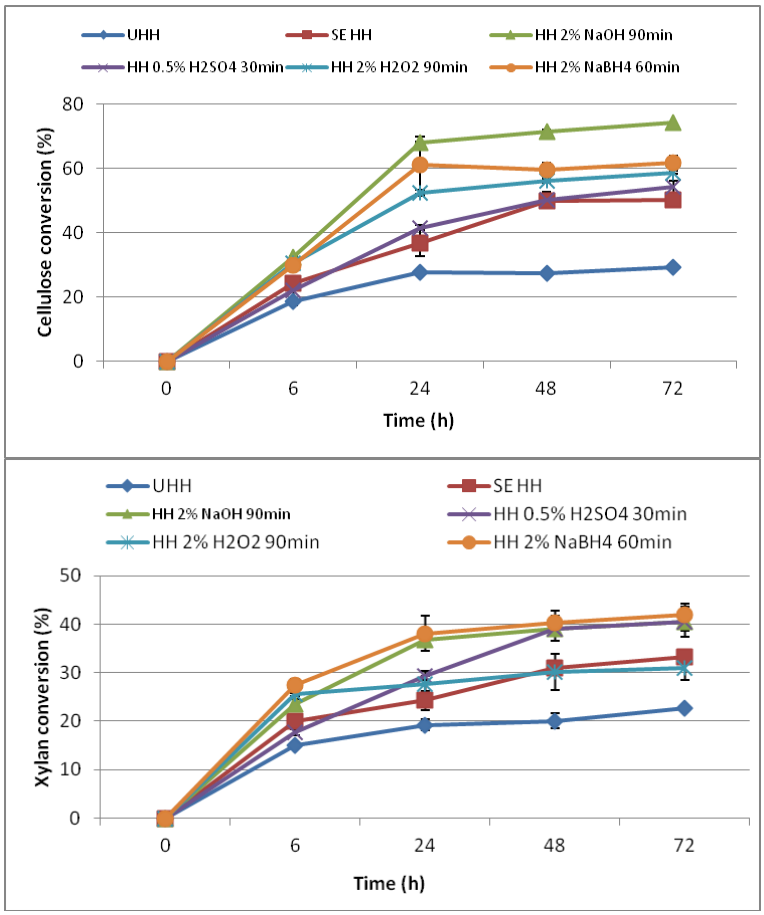

Figure 3.17. Glucan and xylan conversions after enzymatic hydrolysis (UHH: untreated hazelnut husk, SE HH: steam exploded hazelnut husk).

The glucan conversion of each hydrolysis treatment is shown in Figure 3.17. The NaOH treatment had the highest glucan conversion of 74.4%, which was followed by $NaBH_4$ (61.8%), H_2O_2 (58.8%) and H_2SO_4 (54.3%). Differences in mean glucan conversion for all treated samples were found to be statistically significant ($p<0.001$). A higher glucan conversion of 91.7% was reported by Wang et al. (2010) when bermuda grass treated with 3% NaOH at 121°C for 15 min was hydrolyzed by *cellulase* (*NS50013 cellulase complex*) and *cellobiase* (*NS50010 β-glucosidase*). The lower glucan conversion in this study could be due to the specific characteristics of the hazelnut husks utilized in this study. The lignin and xylan contents of the samples could be the main factor. The H_2SO_4 treated samples had the highest lignin content (42.2%) and the $NaBH_4$ treated

samples had the lowest lignin content (31.4%). Consequently, the H_2SO_4 treated samples had 1.25 times more lignin compared to the $NaBH_4$ treated samples. Therefore, the glucan conversion of the H_2SO_4 treated samples was 1.40 times lower compared to the $NaBH_4$-treated samples. On the other hand, the H_2SO_4 treated samples had the highest xylan content (13.0%) and the NaOH-treated samples had the lowest xylan content (6.11%). The H_2SO_4 treated samples had 2.13 times higher xylan in the structure and the glucan conversion of the H_2SO_4 treated samples was 1.37 times lower compared to the NaOH-treated samples. This indicated that both lignin and xylan removal had a significant effect on the enzymatic digestibility of the hazelnut husks. Similar findings were reported earlier by Lu et al. (2002) and Yang and Wyman (2004) found that the effect of lignin on hydrolysis was due to protein adsorption of lignin in aqueous solutions.

Even when no xylanase was added to the enzyme combination in this study, a significant amount of xylose was detected in the hydrolyzates (Figure 3.17). Duarte et al. (2004) reported that *Celluclast* 1.5 L had *β-xylanase* and *β-xylosidase* activities. Therefore, the enzyme combination used in this study reduced the xylan in the hazelnut structure. The $NaBH_4$ treated sample had the highest xylan conversion (41.9%), whereas NaOH gave the lowest (40.5%). Statistical data indicated that differences in mean xylan conversions for all treatments were statistically significant ($p<0.01$). A lower xylan conversion of 37.6% was reported by Wang et al. (2010) when bermuda grass was treated with 3% NaOH at 121°C for 15 min.

3.3.4. Fermentation of Hydrolyzates

Arslan and Saracoglu (2010) studied hazelnut shells and reported 44.9 % of theoretical ethanol yield when they used *Pichia stipitis* in fermentation. The shells had a much higher lignin and lower glucose content compared to hazelnut husks. Therefore, the ethanol yields obtained in this study were much higher compared to hazelnut shells, and this showed the effect of feedstock content. It could be stated that SE and chemical treatments improved the fermentability of hazelnut husks. The fermentation performance of S. *cerevisiae* in treated hydrolysates is compared in Table 3.15. In this study, the highest ethanol concentration (4.26 g/L) was observed when the husks were treated with 2% NaOH for 90 min. The highest ethanol yield (52.6 g/kg husks) was observed for samples treated with 2% NaOH for 90 min. This corresponds to a

maximum theoretical amount of 76.7% ethanol (Figure 3.18).

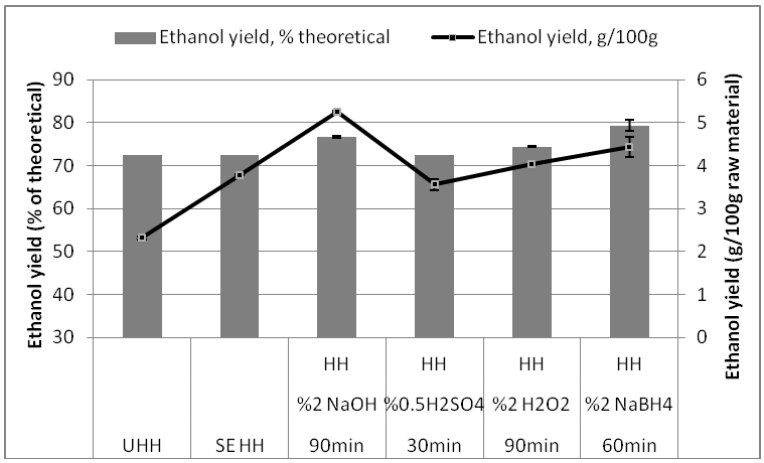

Figure 3.18. Ethanol yield (percent of theoretical and g/100g raw material).

Table 3.15. Glucose, xylose and ethanol concentrations during fermentation with *S. cerevisiae* in untreated and pretreated husk

Pretreatment conditions	0 h			6 h			24 h			48 h			72 h		
	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l	Glucose g/l	Xylose g/l	Ethanol g/l
UHH*	3.13±0.05	1.67±0.03	0.14±0.02	2.67±0.04	1.63±0.04	1.10±0.03	0.83±0.02	0.96±0.02	1.13±0.02	0.47±0.01	0.75±0.02	1.15±0.01	0.33±0.03	0.74±0.01	1.16±0.02
SE HH	5.65±0.02	2.58±0.01	0.29±0.01	4.75±0.03	2.49±0.02	2.02±0.01	1.45±0.02	1.46±0.01	2.04±0.01	0.80±0.02	1.16±0.00	2.07±0.03	0.49±0.01	1.14±0.00	2.09±0.01
HH %2 NaOH 90 min	10.9±0.01	1.28±0.10	0.56±0.01	9.02±0.02	1.21±0.09	4.03±0.02	2.63±0.04	0.71±0.05	4.07±0.09	1.26±0.07	0.58±0.04	4.18±0.04	0.83±0.04	0.56±0.04	4.26±0.01
HH %0.5 H_2SO_4 30 min	6.71±0.25	2.31±0.11	0.33±0.01	5.65±0.19	2.21±0.1	2.35±0.09	1.68±0.06	1.28±0.05	2.42±0.09	0.95±0.05	1.04±0.05	2.46±0.07	0.55±0.02	1.02±0.05	2.48±0.09
HH %2 H_2O_2 90 min	8.15±0.06	1.42±0.12	0.41±0.00	6.82±0.01	1.36±0.11	2.98±0.02	2.01±0.02	0.79±0.06	3.04±0.03	1.09±0.01	0.64±0.05	3.07±0.04	0.64±0.00	0.62±0.05	3.09±0.02
HH %2 $NaBH_4$ 60 min	8.49±0.32	2.08±0.12	0.45±0.03	6.96±0.24	1.95±0.12	3.18±0.07	1.95±0.06	1.13±0.06	3.27±0.07	0.93±0.02	0.94±0.05	3.38±0.13	0.59±0.03	0.92±0.05	3.43±0.18

*UHH: untreated hazelnut husk, SE HH: steam exploded hazelnut husk.

3.4. CHAPTER 4: EFFECTS OF PRETREATMENT CHEMICALS

The effects of pretreatement chemicals were briefly discussed in this chapter. The highest glucan solubility was found when H_2SO_4 was used in the pretreatment of wheat straw, but the NaOH pretreatment gave the highest glucan solubility for corn stalks. In addition, there was substantial solubilization of glucan during the $NaBH_4$ pretreatment of hazelnut husks. The direct exposure of free husk fibers to the pretreatment chemical probably contributed to the higher percentages of glucan solubilization, and the unexpected glucan removal of $NaBH_4$ could be due to the characteristics of the husks.

The highest xylan solubility was observed when all raw materials were treated with NaOH. Therefore, the most substantial effect of the H_2SO_4 pretreatment on the feedstocks was the solubilization of xylan. The H_2O_2 pretreatment resulted in lower xylan solubilization than expected. There was a linearly increasing relationship between the solubilization of xylan and pretreatment severity.

In general, $NaBH_4$-treated raw materials had the lowest lignin contents. The most substantial effect of the NaOH pretreatment was delignification. There was a linearly increasing relationship between lignin reduction and pretreatment severity. On the other hand, the lowest lignin reductions were observed in the H_2SO_4-treated samples. The H_2O_2 pretreatment also resulted in lower lignin solubilization than expected. This was probably due to the decomposition of H_2O_2 into water at high temperatures. The NaOH and $NaBH_4$ pretreatments were more effective at delignification than H_2O_2. For NaOH and $NaBH_4$, the concentration of the pretreatment chemicals had the most significant effect on lignin reduction.

Results indicated that pretreatment with $NaBH_4$ was as effective as NaOH in terms of removing xylan and lignin from the structure. The obtained data indicated that using NaOH and $NaBH_4$ as pretreatment chemicals, compared to others, improved selective delignification. Delignification appears to have more effect on enzyme digestibility than xylan solubilization. The selective capability of $NaBH_4$ in delignification improved the process yield and resulted in better enzymatic digestibility of wheat straw, corn stalks and hazelnut husks in bioethanol production. The NaOH and $NaBH_4$ pretreatments of raw materials resulted in significantly higher glucan conversions during enzymatic

hydrolysis than the H_2O_2 and H_2SO_4 pretreatments.

It could be concluded that it is possible to utilize NaOH in the chemical pretreatment step of raw materials like hazelnut husks, which have a high amount of lignin in the structure. On the other hand, better enzymatic digestibility was observed when raw materials like wheat straw and corn stalks, with low lignin content, were treated with $NaBH_4$ in the chemical pretreatment step.

4. CONCLUSIONS AND RECOMMENDATIONS

4.1. CONCLUSIONS

Bioethanol production from agricultural residues (wheat straw, corn stalks and hazelnut husks) was investigated in this study. The materials were pretreated with steam and chemicals and then enzymatically hydrolyzed and fermented. Due to the complex nature of wheat straw, the material was first steam exploded; this process removed 16.7% xylan and 5.86% lignin from wheat straw, but the glucan removed was observed to be insignificant (1.60 %) . Among the chemically pretreated samples, the highest glucan recovery (more than 90%) was observed when wheat straw was treated with $NaBH_4$. On the other hand, the amount of lignin removed by $NaBH_4$ was almost 60%. Enzymatic hydrolysis resulted in 87.8% and 83.3% glucan conversion when wheat straw was pretreated with NaOH and $NaBH_4$, respectively. The highest ethanol yield (115 g/kg) was observed for the wheat straw sample pretreated with 4% $NaBH_4$ for 60 min and the theoretical yield (86.9%) was also calculated to be highest for the sample.

SE removed 10.5% xylan, 10.6% lignin and a small amount of glucan (1.80%) from the corn stalk structure. Among the chemically pretreated samples, the highest glucan recovery was observed when corn stalks were treated with $NaBH_4$. More than 85% of the glucan was preserved in the $NaBH_4$-treated samples. Pretreatment with NaOH and $NaBH_4$ removed substantially more lignin from the corn stalk structure; this improved the accessibility and, consequently, the digestibility of the glucan. The amount of lignin removed from the structure by $NaBH_4$ was similar to the amount removed by the conventional chemical NaOH. The glucan conversions of NaOH and $NaBH_4$ were found to be 83.9% and 82.9%, respectively. Results showed that the highest ethanol yield (97.4g/kg) was observed when corn stalks were pretreated with 4% $NaBH_4$ for 90 min. The theoretical ethanol yield for the sample was 72.5%.

On the other hand, SE removed 4.57% xylan, 13.3% lignin and 4.50% glucan from the hazelnut husk structure. Chemical treatments showed that NaOH dissolved the highest amount of xylan. The highest glucan solubility and lignin reduction were observed when $NaBH_4$ was used in the chemical treatment. The lowest lignin reduction was found when the husks were treated with H_2SO_4. Conclusively, results indicated that

NaOH and $NaBH_4$, compared to H_2SO_4 and H_2O_2, improved selective delignification. $NaBH_4$ dissolved the most glucan, but also removed the most lignin from the structure. The glucan to glucose conversions of NaOH and $NaBH_4$ were found to be 74.7% and 61.8%, respectively. Chemical treatment improved the selectivity, and the highest ethanol yield was 52.6 g/kg when the husks were treated with 2% NaOH for 90 min. The theoretical ethanol yield of the sample was found to be 76.7%.

When pretreatment chemicals were compared, using NaOH in the chemical pretreatment step could be advantageous for raw materials like hazelnut husks, which have a relatively higher lignin content in the structure. On the other hand, better enzymatic digestibility could be possible when $NaBH_4$ is used in the chemical pretreatment step for raw materials like wheat straw and corn stalks, which have a lower lignin content in the structure.

4.2. FUTURE WORK

- Various commercial borate products and minerals like borax pentahydrate, borax, sodium perborate, boric acid, colemanite and ulexite could be examined for bioethanol production. They have been utilized as bleaching agents in the pulp and paper industry.

- Using $NaBH_4$ in a variety of concentrations, temperatures and pressures should be investigated in order to optimize the ethanol yield of raw materials with low and high lignin contents.

- Enzymatic hydrolysis could be optimized to achieve efficient cellulose conversion, based on the pretreatment parameters.

- Parameters of time, enzyme, and solid loadings could be examined to minimize the processing costs by maximizing the glucose yield and optimizing enzymatic hydrolysis..

- The economic feasibility of ethanol production from wheat straw, corn stalks and hazelnut husks should be investigated, since it could not be addressed in this study.

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6. APPENDICES

6.1. APPENDIX-1. STEAM EXPLOSION EFFECT

Table A1.1. SE effect on glucan, xylan and lignin for all raw materials.

Wheat Straw					
Factor	Levene's Test for Equality of Variances		t-test for Equality of Means		
	F	Significance	t	df	Significance (2-tailed)
lignin	$3.587E^{16}$	0.000	-1.10	1	0.47
xylan		0.000	1.91	2	0.22
glucan	$8.405E^{14}$	0.000	-1.26	2	0.34
Corn Stalks					
Factor	Levene's Test for Equality of Variances		t-test for Equality of Means		
	F	Significance	t	df	Significance (2-tailed)
lignin	$1.678E^{16}$	0.000	-2.10	1	0.28
xylan		0.000	-0.74	2	0.55
glucan	$9.327E^{15}$	0.000	-3.47	1	0.17
Hazelnut Husk					
Factor	Levene's Test for Equality of Variances		t-test for Equality of Means		
	F	Significance	t	df	Significance (2-tailed)
lignin	$4.750E^{15}$	0.000	1.14	2	0.39
xylan		0.000	-0.65	1	0.61
glucan		0.000	-1.03	2	0.41

6.2. APPENDIX-2. GLUCAN AND XYLAN CONVERSIONS

Table A2.1. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of glucan conversions for wheat straw pretreated with different methods.

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	4643.1	5	928.6	343.3	0.000
Within Groups	16.2	6	2.71		
Total	4659.4	11			

Table A2.2. After 72 h enzymatic hydrolysis, Duncan test results of glucan conversions for wheat straw pretreated with different methods.

Methods	Subset For Alpha= 0.05				
	1	2	3	4	5
UWS	32.2				
SE WS		48.8			
WS 2% H_2SO_4 90 min.			71.7		
WS 4% H_2O_2 90 min.			74.7		
WS 4% $NaBH_4$ 60 min.				83.3	
WS 2% NaOH 90 min.					87.8

Table A2.3. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of xylan conversions for wheat straw pretreated with different methods.

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	1457.2	5	291.4	41.1	0.000
Within Groups	42.6	6	7.09		
Total	1499.8	11			

Table A2.4. After 72 h enzymatic hydrolysis, Duncan test results of xylan conversions for wheat straw pretreated with different methods.

Methods	Subset For Alpha= 0.05			
	1	2	3	4
UWS	11.8			
SE WS		28.8		
WS 2% H_2SO_4 90 min.		32.2	32.2	
WS 4% H_2O_2 90 min.			37.2	
WS 2% NaOH 90 min.				43.9
WS 4% $NaBH_4$ 60 min.				44.2

Table A2.5. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of glucan conversions for corn stalks pretreated with different methods.

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	3135.8	5	627.2	124.8	0.000
Within Groups	30.1	6	5.02		
Total	3165.9	11			

Table A2.6. After 72 h enzymatic hydrolysis, Duncan test results of glucan conversions for corn stalks pretreated with different methods.

Methods	Subset For Alpha= 0.05				
	1	2	3	4	5
UCS	42.7				
SE CS		49.4			
CS 4% H_2SO_4 60 min			56.6		
CS 4% H_2O_2 90 min.				74.5	
CS 4% $NaBH_4$ 90 min.					82.4
CS 4% NaOH 90 min.					84.0

Table A2.7. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of xylan conversions for corn stalks pretreated with different methods

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	2646.4	5	529.3	126.2	0.000
Within Groups	25.2	6	4.19		
Total	2671.6	11			

Table A2.8. After 72 h enzymatic hydrolysis, Duncan test results of xylan conversions for corn stalks pretreated with different methods.

Methods	Subset For Alpha= 0.05				
	1	2	3	4	5
UCS	30.3				
SE CS		38.9			
CS 4% H_2SO_4 60 min			45.0		
CS 4% H_2O_2 90 min.				55.7	
CS 4% $NaBH_4$ 90 min.					69.3
CS 4% NaOH 90 min.					69.9

Table A2.9. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of glucan conversions for hazelnut husks pretreated with different methods

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	2225.7	5	445.1	283.4	0.000
Within Groups	9.43	6	1.57		
Total	2235.1	11			

Table A2.10. After 72 h enzymatic hydrolysis, Duncan test results of glucan conversions for hazelnut husks pretreated with different methods.

Methods	Subset For Alpha= 0.05				
	1	2	3	4	5
HH	29.4				
SE HH		50.3			
HH 0.5% H_2SO_4 30 min.			54.3		
HH 2% H_2O_2 90 min.				58.8	
HH 2% $NaBH_4$ 60 min.				61.8	
HH 2% NaOH 90 min.					74.4

Table A2.11. After 72 h enzymatic hydrolysis, variation analysis results (ANOVA) of xylan conversions for hazelnut husks pretreated with different methods.

Variation Source	Sum of Squares	Degree of Freedom	Mean Square	F	Significance
Between Groups	557.5	5	111.5	26.1	0.001
Within Groups	25.6	6	4.27		
Total	583.1	11			

Table A2.12. After 72 h enzymatic hydrolysis, Duncan test results of xylan conversions for hazelnut husks pretreated with different methods.

Methods	Subset For Alpha= 0.05		
	1	2	3
HH	22.6		
HH 2% H_2O_2 90 min.		31.0	
SE HH		33.2	
HH 0.5% H_2SO_4 30 min.			40.4
HH 2% NaOH 90 min.			40.5
HH 2% $NaBH_4$ 60 min.			41.9

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Publications

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1. Copur Y., Ozyurek O., **Tozluoglu A.**, Kutuk S., Enzymatic digestibility of tomato, pepper, and eggplant stalks mixture, *BioRes*., 7 (3) (**2012**) 3252-3261.

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Projects

1. Bioethanol Production Via Enzymatic Hydrolysis of Lignocellulosic Biomass: Wheat Straw, Corn Stalks and Hazelnut Husks, TUBITAK 109O200, Yalçın ÇÖPÜR (Project Leader), Mualla Balaban UÇAR (Researcher), Melek ÖZKAN (Researcher), **Ayhan TOZLUOĞLU, (Scholarship Holder)**, Ömer ÖZYÜREK (Scholarship Holder), Selva KÜTÜK (Scholarship Holder), 2009-2012.

2. A Study on Kraft, Bio-kraft, Bio-kraft-AQ, Bio-polisulfur and Bio-kraft-sodium borhidrür Pulping Using Pinus brutia, TUBITAK 106O461, Yalçın ÇÖPÜR (Project Leader), Mehmet AKGÜL (Researcher), **Ayhan TOZLUOĞLU, (Scholarship Holder)**, 2006-2008.

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