PRETREATMENT OF COTTON STALKS WITH IONIC LIQUIDS FOR ENHANCED ENZYMATIC HYDROLYSIS OF CELLULOSE AND ETHANOL PRODUCTION

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PRETREATMENT OF COTTON STALKS WITH IONIC LIQUIDS FOR ENHANCED ENZYMATIC HYDROLYSIS OF CELLULOSE AND ETHANOL PRODUCTION

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ABSTRACT

PRETREATMENT OF COTTON STALKS WITH IONIC LIQUIDS FOR ENHANCED ENZYMATIC HYDROLYSIS OF CELLULOSE AND ETHANOL PRODUCTION

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This study aims efficient conversion of cotton stalks to cellulosic ethanol through ionic liquid pretreatment and enhanced enzymatic hydrolysis. Among several ionic liquids, EMIMAc exhibited the most striking impact on cotton stalks with respect to the changes in biomass structure and digestibility. Cotton stalks, which were subjected to EMIMAc pretreatment at 10% (w cotton stalks/w EMIMAc) of biomass loading and 150°C for 30 minutes, were found to be 9 times more digestible than untreated cotton stalks. Besides, glucose and ethanol yields, which were based on the cellulose content of untreated cotton stalks, were found as 67% and 66%, respectively. These yields were insufficient regarding efficient conversion of the cellulosic portion of cotton stalks to glucose and ethanol which is linked to the superior solvation capability of EMIMAc towards biomass. In order to enhance aforementioned yields, EMIMAc pretreatment was conducted at 30% of biomass loading. Though lignin extracted was much lower, higher yields were obtained compared to the former case since 96% of cellulose was recovered upon EMIMAc pretreatment and reduced crystallinity was observed for pretreated biomass. Glucose yield was achieved as 84% even at a substrate loading of 15% (w/v). Additionally, 76% of ethanol yield and 3% (v/v) of ethanol titer were obtained upon fermentation. Accordingly, reduction in biomass crystallinity was satisfactory to improve enzymatic accessibility of the biomass. Besides, EMIMAc maintained its effectiveness as a pretreatment agent upon recycling since no change in terms of hydrolysis of pretreated samples was observed upon EMIMAc recycling for three times.

Keywords: Cotton stalks, ionic liquid, pretreatment, enzymatic hydrolysis, cellulosic ethanol.

ÖZ

SELÜLOZUN GELİŞTİRİLMİŞ ENZİMATİK HİDROLİZİ VE ETHANOL ÜRETİMİ İÇİN PAMUK SAPININ İYONİK SIVILARLA ÖN İŞLEMİ

Haykır, Nazife Işık
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Şubat 2013, 197 sayfa

Bu çalışma, pamuk saplarının iyonik sıvı ön işlemi ve geliştirilmiş enzimatik hidroliz ile etanole dönüştürülmesini amaçlamaktadır. Kullanılan iyonik sıvılardan, EMIMAc pamuk sapları üzerinde; biyokütlenin yapısı ve enzimatik hidrolizi açısından en çarpıcı etkiyi göstermiştir. %10 (g pamuk sapı/g EMIMAc) biyokütle konsantrasyonu, 150°C ve 30 dakikada EMIMAc ile uygulanan ön işlemin biyokütlenin enzimatik hidrolizini 9 kat arttırdığı gözlemlenmiştir. Ayrıca, ön işlem uygulanmamış pamuk saplarındaki selüloz içeriğine göre belirlenmiş glikoz ve etanol verimleri sırasıyla %67 ve %66 olarak bulunmuştur. Pamuk saplarının selülozik içeriğinin etkili olarak glikoza ve etanole dönüştürülmesi gerektiği dikkate alındığında bu değerler yeterli değildir. Bu bulgu EMIMAc'ın biyokütle üzerindeki üstün çözücülük kapasitesine bağlanmıştır. Sözü edilen değerleri arttırmak için, EMIMAc ile ön işlem %30 biyokütle konsantrasyonunda karıştırmasız olarak uygulanmıştır. Her ne kadar uzaklaştırılan lignin miktarı daha az da olsa, daha yüksek verimler elde edilmiştir çünkü EMIMAc ile ön işlem sonrasında %96 selüloz eldesi sağlanmış ve selülozun kristal yapısının daha düşük olduğu gözlemlenmiştir. %15 (w/v) substrat konsantrasyounda dahi %84 glikoz verimine ulaşılmıştır. Bunlara ilaveten, fermantasyon sonucunda %76 etanol verimi ve %3 (v/v) etanol konsantrasyonu elde edilmiştir. Bunlara göre, selülozun kristal yapısındaki değişim biyokütlenin enzimatik hidrolizini arttırmak için yeterlidir. Ayrıca EMIMAc geri kazanımı sonucunda etkinliğini korumuştur; üç kez ardarda kullanımı sonunda bile ön işlem görmüş örneklerin hidrolizi açısından hiçbir değişiklik gözlenmemiştir.

Anahtar kelimeler: Pamuk sapları, iyonik sıvı, ön işlem, enzimatik hidroliz, selülozik etanol.

To my dearest Dad

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NOMENCLATURE

Symbols

$Y_{P/S}$: yield coefficient for ethanol (g ethanol/g glucose)
$Y_{X/S}$: yield coefficient for dry cell (g cell/g glucose)
π:Dipolarity/polarizability ratio
α: hydrogen bond acidity
β: hydrogen bond basicity
2θ: bragg angle
W_{PRT}: weight of pretreated cotton stalks recovered after pretreatment (g)
W_{UT}: weight of untreated cotton stalks subjected to pretreatment (g)
SR: solid recovery for biomass (%)
C_R: reducing sugar concentration in the enzymatic hydrolyzate (g/L)
C_S: initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L)
C_{UT}: cellulose content of the untreated cotton stalks (%)
C_{PRT}: cellulose content of the pretreated cotton stalks (%)
C_G: glucose concentration of the hydrolyzate (g/L)
C_E: ethanol concentration of the fermentation medium (g/L)
$C_{G,I:}$ initial glucose concentration of the fermentation medium (g/L)
L_{PRT}: lignin content pretreated cotton stalks (%)
L_{UT}: lignin content untreated cotton stalks (%)
AIL: acid insoluble lignin content of biomass (%)
ASL: acid soluble lignin content of biomass (%)

Abbreviations

CS: Cotton stalks
IL: Ionic liquid
HEAF: 2-hydroxy ethyl ammonium formate
AMIMCl: 1-allyl-3-methyl imidazolium chloride
BMIMCl: 1-butyl-3-methyl imidazolium chloride
EMIMCl: 1-ethyl-3-methyl imidazolium chloride
EMIMAc: 1-ethyl-3-methyl imidazolium acetate
YPD: Yeast extract-peptone-dextrose
SEM: Scanning Electron Microscopy
XRD: X- ray Diffraction
ATR-FTIR: Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy
HPLC: High Performance Liquid Chromatography

CHAPTER 1

INTRODUCTION

Cellulosic ethanol as an alternative to conventional transportation fuels has drawn attentions due to its production from abundant, widespread and low cost lignocellulosic feedstocks. Lignocellulosic biomass not only serves as source for cellulosic ethanol, but also has the potential to present numerous kinds of products owing to its unique structure with the main constituents, cellulose, hemicellulose and lignin. Though efficient utilization of each component in the conceptual of biorefinery provides generation of a variety of value-added products, the way that they participate in the biomass structure is puzzling. The components of this heterogeneous structure are associated with each other to such a degree that the biomass accessibility to conversion reactions is significantly hindered (Alvira et al., 2010). The lignin-hemicellulose complex that acts as a shield on cellulose structure and crystalline structure of cellulose have been recognized as the major obstacles that exhibit adverse effects on the digestibility of the cellulose. In order to make the biomass amenable to enzymatic attack, pretreatment processes have been introduced to overcome the recalcitrant structure of the biomass (Hendriks and Zeeman, 2009). There has been rising research interest on pretreatment of lignocellulosic feedstocks to develop effective approaches with minimal environmental impacts. At this point, ionic liquid pretreatment stands out from other conventional methods due to its unique properties.

Ionic liquids are organic solvents that promise numerous advantages in biomass pretreatment. Ionic liquids are regarded as green solvents owing to their negligible vapor pressures at ambient conditions which particularly provide easier handling during biomass pretreatment (Welton, 2004). Ionic liquids have the capability to disrupt the crystalline structure of cellulose (Dadi et al., 2006, H.Wu et al., 2011) and extract lignin as well (S.H. Lee et al., 2009, Sun et al., 2009) during their interaction with lignocellulosic biomass. Ionic liquids offer benefits not only during the pretreatment but also following the pretreatment. Recovery of the pretreated biomass through its precipitation simply with antisolvent addition and utilization of the pretreated sample in enzymatic hydrolysis without pH adjustment have been favorable. Ionic liquids also receive interest due to the convenience in their recovery following the biomass pretreatment and reuse for the subsequent pretreatments. Since ionic liquids do have high costs, their reuse has been critical for development of an economically sustainable process (Stark, 2011).

Ionic liquid pretreatment has been investigated for a wide range of lignocellulosic feedstocks such as switchgrass (Arora et al., 2010; Li et al., 2010; Singh et al., 2009), maple wood flour (S.H. Lee et al., 2009), southern yellow pine (Sun et al., 2009), rice straw (Nguyen et al., 2010), poplar (Samayam and Schall, 2010), corn stover (Li et al., 2011), miscanthus (Shill et al., 2011), corn cob (Bahcegul et al., 2011) and cotton stalks (Haykir et al., 2013).

Though selective delignification has been regarded as an important objective, the most significant influence of ionic liquids on biomass structure has been their capability to disrupt the crystalline structure of cellulose (H.Wu et al., 2011). Ionic liquids are tunable solvents indicating that the possible anion-cation combinations will determine the extent of ionic liquid pretreatment of lignocellulosic biomass (Mora-Pale et al., 2011). The anion nature has been shown to have a pronounced effect on

the interaction of ionic liquids with lignocellulosic biomass since it determines the hydrogen bond acceptance capacity of an ionic liquid (Fukaya et al.,2006, Fukaya et al., 2008, Doherty et al.,2010, Brandt et al., 2010).The higher the hydrogen bond acceptance capacity of an ionic liquid; the more easily it interacts with cellulose through hydrogen bonding and the more efficient it disrupts the crystalline structure of the cellulose in a lignocellulosic biomass (Swatloski et al., 2002, Doherty et al.,2010). Hydrogen bond acceptance capacity of the ionic liquids has been found to correlate well with the reductions in crystalline structure of cellulose in lignocellulosic biomass and enzymatic accessibility of the biomass (Doherty et al., 2010, Mora-Pale et al., 2011). Recently, 1-ethyl-3-methylimidazolium acetate (EMIMAc) has been very successful in reduction of cellulose crystallinity due to high HBA capacity of the acetate anion. EMIMAc has been also found to extract large fractions of lignin from wood samples and corn stover (S.H. Lee et al., 2009, Sun et al., 2009, H.Wu et al., 2011).

The structural modifications derived upon ionic liquid pretreatment of lignocellulosic biomass resulted with very fulfilling findings with respect to the enzymatic accessibility of the lignocellulosic biomass (S.H. Lee et al., 2009, H.Wu et al., 2011, Bahcegul et al., 2012b, Haykir et al., 2013). While the extent of enzymatic hydrolysis, in other words the amount of fermentable sugars released upon enzymatic hydrolysis of cellulose and hemicellulose has been an important objective, the rate of enzymatic hydrolysis has not been considered until it was found that regenerated cellulose upon ionic liquid treatment exhibited higher kinetics compared to its native form (Dadi et al., 2006, Dadi et al., 2007). Ionic liquids were shown to enhance initial hydrolysis rates of lignocellulosic biomass such as switchgrass and cornstover (Li et al., 2010, Li et al., 2011).

While ionic liquids induce promising modifications in biomass structure and biomass accessibility to enzymatic attack, implementation of this novel technology in large scales is not possible due to the very high cost of ionic liquids for now. However, recovery and reuse of ionic liquids motivate the researchers to make ionic liquid pretreatment commercially available through development of cost effective solutions (Shill et al., 2011, H.Wu et al., 2011).

In order to point out the aforementioned issues, cotton stalks were selected as raw material in the present study. Cotton stalks have been regarded as potential sources for cellulosic ethanol production owing to their high cellulose content being almost 37% (Haykir et al., 2013). Pretreatment techniques including alkaline pretreatment (Silverstein et al., 2007, Bahcegul et al., 2012a; Binod et al., 2012) and microbial pretreatment (Shi et al., 2009b) have been successful in utilization of cotton stalk for generation of value-added products. In a recently reported study, imidazolium based ionic liquids, AMIMCl, BMIMCl, EMIMCl, EMIMAc and an alkanolamine ionic liquid, HEAF were screened with respect to their effects on digestibility of cotton stalks and the structural variations in the biomass (Haykir et al., 2013). Among, EMIMAc demonstrated promising findings and thereby, encouraged employment of this technology for conversion of cotton stalks to cellulosic ethanol as it was aimed in this study.

At the initial stages of the present study, enzymatic digestibility of the ionic liquid pretreated cotton stalks has been the primary focus. In addition to the results obtained upon enzymatic hydrolysis of the biomass, structural and compositional changes in the cotton stalks have been also monitored. While scanning electron microscopy (SEM) was used to monitor the morphological changes in the biomass, attenuated total reflectance fourier transform infrared spectroscopy (ATR-FTIR) displayed the structural changes in crystalline cellulose and lignin of cotton stalks. X-ray diffraction (XRD) has exhibited the modifications in the crystalline structure of cellulose in the biomass. On the basis of the results gathered upon compositional and characterization analyses, cellulose degradation has been realized due to the elevated solvation capacity of ionic liquids, especially superior capacity of EMIMAc. Since cellulose is the target component, conversion of the cellulose in the untreated cotton

stalks to ethanol with minimal cellulose loss throughout the process has been important. In this context, the research has been conducted to attain the most effective condition for EMIMAc pretreatment of cotton stalks to improve glucose and ethanol yields based on the cellulose content of the untreated cotton stalks. Hence, EMIMAc pretreatment has been carried out at high biomass loadings to alleviate the cellulose degradation.

Additionally, EMIMAc reuse was assessed whether it demonstrated any adverse effects on the changes in biomass structure and enzymatic digestibility of the cotton stalks. Not only improvements in glucose and ethanol yields have been pointed out, but also enhancements regarding glucose and ethanol concentrations have been considered. Accordingly, enzymatic hydrolysis and fermentation have been also assessed with the aim of attaining high glucose and ethanol concentrations. Lastly, a comparison has been performed between the cotton stalks subjected to EMIMAc and alkaline pretreatment in order to find out which one of the pretreated biomass samples favored high substrate loadings during enzymatic hydrolysis for cellulosic ethanol production.

Consequently, this study addresses the benefits of ionic liquid pretreatment for cellulosic ethanol production from cotton stalks through:

- Identifying the most appropriate pretreatment conditions that enable improved access and high recovery for the cellulosic fraction of the biomass.
- Demonstrating the favorable variations in the structure of the biomass that enhance enzymatic hydrolysis.
- Performing changes in substrate loading during enzymatic hydrolysis to increase glucose concentrations and thus, ethanol concentrations upon fermentation.
- Introducing the advantages of ionic liquid pretreatment over alkaline pretreatment with respect to its effect on biomass structure, enzymatic accessibility of the biomass and ethanol production.
- Last but not least, keeping the environmental and economic aspects of the process in mind, such as reuse of ionic liquid and reductions in the amount of ionic liquid.

CHAPTER 2

LITERATURE SURVEY

2.1 Cellulosic ethanol

Considering the environmental and economic issues, researchers have currently put an emphasis on replacement of the fossil fuels with alternative renewable energies since fossil fuel reserves, which account for 80% of the world's energy supply, will obviously face the problem of depletion. Oil, natural gas and coal were reported to be exhausted within 41, 64 and 155 years, respectively (Goldemberg, 2007). A previously reported study describes the fuel share in global energy supply between years, 1971-2020 (World Energy Assessment report by UNDP, 2000). The related data is demonstrated in Figure 2.1. Though the share of renewables appears to increase, this increase is nonsense when compared to the profile of fossil fuel supply. Energy share by fossil reserves will be much more crucial for the next 10 years. Moreover taking the increase in production costs of the fossil fuels into account, creating advances in the utilization of renewable sources is indeed believed to be essential (Goldemberg, 2007).

Biomass, which provides almost 8% of the total energy supply, represents the largest portion of the renewables (Figure 2.2). In this context, ethanol from biomass appear as a substantial substitute to gasoline for transportation sector since it holds unique properties regarding energy and environmental aspects. Contribution of ethanol to the reductions in the greenhouse gas (GHG) emission is one of the fundamental assets regarding the emissions derived from conventional transportation fuels which represents for one third of the total (Wyman, 2007). Such a reduction was reported to be 19% with utilization of corn based ethanol relative to the emissions derived from gasoline (Figure 2.3). Besides, employment of biomass as a fuel during corn based ethanol production is shown to reduce the GHG emissions even to 52%.

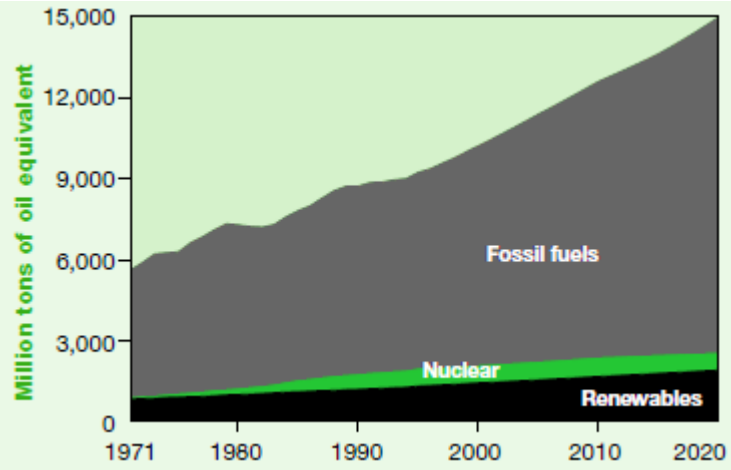


Figure 2.1 Fuels share in global energy supply between the years, 1971-2020 (World Energy Assessment report by UNDP, 2000).

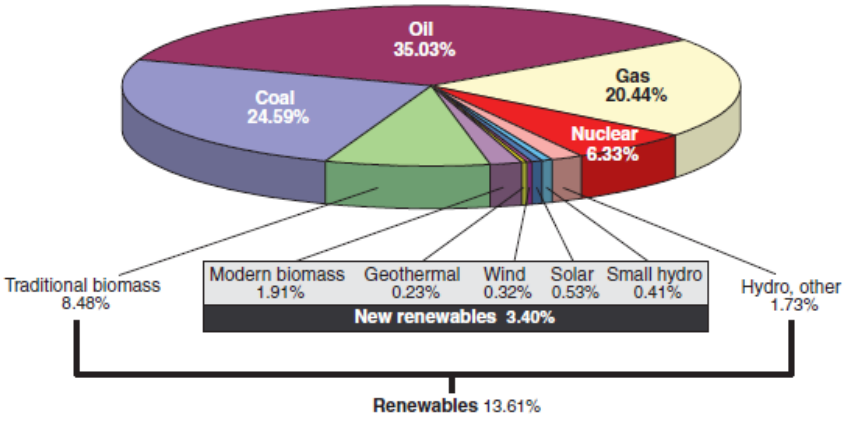


Figure 2.2 World energy supply in 2004 (Goldemberg, 2007).

Table 2.1 Reductions of GHG emissions relative to gasoline due to utilization of alternative liquid fuels (US DOE, 2013).

Fuel	Energy used	Reduction of GHG emissions relative to gasoline (%)
Corn ethanol	Current average	19
	Natural gas	28
	Biomass	52
Sugar cane ethanol	Biomass	78
Cellulosic ethanol	Biomass	86

Today, ethanol production majorly relies on food based sources. Ethanol from sugar and starch based sources have been recognized as first generation biofuels. US and Brazil, who are the world's top ethanol producers, produce ethanol from corn and sugarcane, respectively. It was previously reported that 14% of world's corn has been converted to ethanol in US (CNN International, 2013). Brazil, which is the world's largest producer of ethanol, produces ethanol from sugarcane for over 30 years (Mousdale, 2008). However, poverty, finite area for plantation and increase in food prices, which have been substantial global concerns, strengthen the interest in cellulosic ethanol production over food based ethanol production.

Ethanol production from the abundant and low cost lignocellulosic biomass offers numerous advantages over the food based ethanol. According to US DOE (US DOE, 2013), cellulosic ethanol derives much less amounts of greenhouse gases compared to ethanol from corn and sugarcane; 86% of reduction in GHG emissions is observed relative to the emissions from gasoline (Table 2.1). This is actually related to the fossil fuel requirements during the production of fuels. According to Figure 2.3, fossil energy requirements during cellulosic ethanol production is estimated to be only 10% of that necessary for production of gasoline and even much less than the energy required for production of corn ethanol (US DOE, 2013). Since unhydrolyzed residues are either burned or gasified for energy supply during cellulosic ethanol production (Wyman, 1999). Based on these findings, production costs for cellulosic ethanol production appear to be less compared to the costs for ethanol production from food based sources. In fact, the opposite scenario is the case since the release of fermentable sugars from lignocellulosic biomass is more complicated compared to the hydrolysis of sugar or starch based biomass.

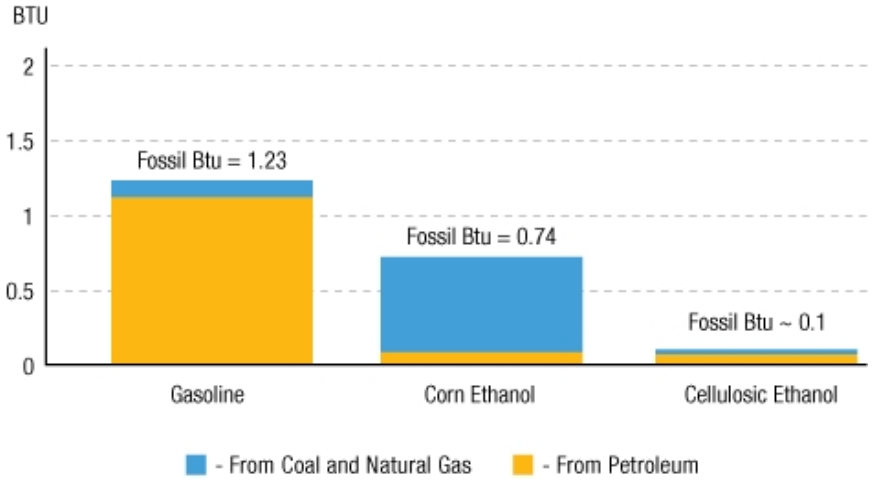


Figure 2.3 Fossil energy requirements for production of liquid fuels (US DOE, 2013).

2.2 Cost analysis for cellulosic ethanol

Lignocellulosic biomass is made up of three major components, cellulose, hemicellulose and lignin. The strong association of these components in the structure challenges its hydrolysis to fermentable sugars (Hendriks and Zeeman, 2009). Thus, conversion of lignocellulosic biomass to ethanol via biochemical routes requires a pretreatment step prior to enzymatic hydrolysis (Figure 2.4). Biomass pretreatment, which introduces chemical and physical changes in the biomass structure, facilitates the conversion of the biomass to fermentable sugars. Several options are introduced to open the robust lignocellulosic biomass structure and enhance its enzymatic digestibility. According to the NREL report on cellulosic ethanol (2007), effective and less expensive pretreatment methods should be developed in order to attain competitiveness with respect to the production costs. In order to accomplish cost competitiveness with the other liquid fuels, Department of Energy of US (DOE) targeted a production cost of $1.07 (in 2002 dollars) for cellulosic ethanol by the year, 2012 (NREL report on Cellulosic Ethanol, 2007). Table 2.2 compares the production costs of ethanol from corn, sugarcane and lignocellulosic feedstocks in 2006 and reveals the necessity to gain cost competitive for cellulosic ethanol. Ethanol from sugarcane holds the lowest production costs which is almost 0.81 $/gallon particularly due to the low cost feedstocks. Ethanol from lignocellulosic feedstocks costs 2.26 $/gallon which is almost 2-3 fold higher than the production costs of ethanol from corn and sugarcane. On the other hand, the close ratio of renewable output to fossil input for cellulosic ethanol and ethanol from sugarcane encourages the utilization of cellulosic ethanol as a transportation fuel (Goldemberg, 2007).

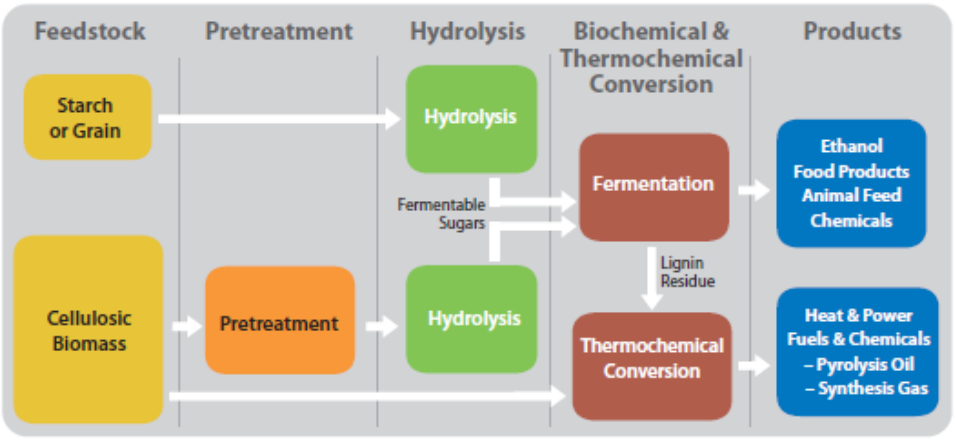


Figure 2.4 Major steps followed for ethanol production from grain, starch and lignocellulosic feedstocks through either biochemical or thermochemical routes (NREL report on Cellulosic Ethanol, 2007).

Table 2.2 Production costs (2006 costs) of ethanol from different raw materials (Goldemberg, 2007).

Feedstock	Cost ($/gallon)	Renewable output to fossil input
Sugarcane, Brazil	0.81	10.2
Corn, US	1.03	1.4
Cellulosic ethanol		10.0
Achieved in 2006	2.25	
Target for 2012	1.07	

National Renewable Energy Laboratory (NREL) has offered dilute acid pretreatment conducted at elevated temperature and pressure as an appropriate pretreatment method in one of its reports published in 2007 (NREL report on Cellulosic Ethanol, 2007). In 2011, NREL reported a study on process design and economics for ethanol production from corn stover which is assumed to be subjected to dilute acid pretreatment (NREL Technical Report on Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol, 2011). This design is currently substantial since it reports the projections on cellulosic ethanol production for 2012. In this report, non-enzymatic conversion was found to account for 1.08 $/gallon for the ethanol which had a minimum selling price of 2.15$/gallon. Besides, enzymes and feedstock were found to contribute 0.34 and 0.74 $/gallon, respectively.

Figure 2.5 demonstrates the two-stage dilute acid pretreatment of corn stover together with the neutralization and conditioning of the pretreated biomass prior to enzymatic hydrolysis. Dilute acid pretreatment, which mainly results with hemicellulose solubilization and lignin deconstruction (Mosier et al., 2005b and Alvira et al., 2010), was found to derive promising results based on the aforementioned design. For instance, the targets for xylan to xylose conversion and xylose degradation were respectively 5% and 90% for corn stover subjected to pretreatment at 30% (w/w)

of solid loading. The slurry derived upon pretreatment was completely subjected to enzymatic hydrolysis after neutralization via ammonia. Utilization of whole slurry provided conversion of both hexoses and pentoses, which were respectively present in the solid and liquid fractions of the slurry in particular, to ethanol during fermentation (NREL Technical Report on Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol, 2011).

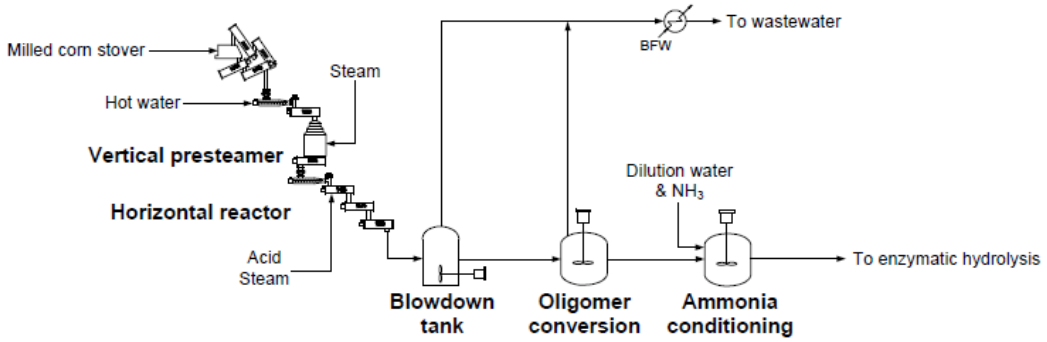


Figure 2.5 Flowsheet for pretreatment and conditioning steps conducted during ethanol production from corn stover (NREL Technical Report on Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol, 2011).

According to the design, separate enzymatic hydrolysis and fermentation was assumed for the conversion of the fermentable sugars in the slurry to ethanol. The slurry was subjected to enzymatic hydrolysis at continuous mode for 84 hours by the enzyme produced on-site. On-site enzyme production was advantageous since it omitted the contribution of the enzyme purchase to the process costs. The hydrolyzate obtained upon hydrolysis was assumed to be fermented via the genetically modified microorganism, *Zymomonas mobilis* for 5 days. In the previous studies, recombinant *Z. mobilis* was found to be able to not only ferment glucose and xylose but also capable of converting arabinose to ethanol. Besides, this recombinant microorganism possesses high tolerance towards the inhibitor compounds derived upon pretreatment (Davis et al., 2005). Accordingly, 5.4% (w/w) of ethanol concentration, which corresponds to 79 gallon ethanol/dry ton corn stover and 76% of the theoretical maximum yield based on the feedstock carbohydrate composition, was assumed to be obtained according to the designed fermentation parameters. As a conclusion, on-site enzyme production, efficient utilization of all carbohydrate components of the feedstock and their conversion to ethanol by the recombinant *Z. mobilis* were considered as chief advances for cellulosic ethanol production from dilute acid pretreated corn stover. Accordingly, the minimum ethanol selling price was predicted to decrease from 2.61 (2008 model) to 2.15$/gallon (Table 2.3). Table 2.3 shows that the model designed in 2008 did not include on-site cellulase production. Moreover, the solid-liquid separation was conducted prior to enzymatic hydrolysis in the same model. Moreover, the proposed model in 2011 demonstrated advances in ethanol yields even separate hydrolysis and fermentation (SHF) was offered in place of simultaneous saccharification and fermentation (SSF) which was shown to provide higher ethanol yields than SHF in the vast majority of literature studies (Öhgren et al., 2007, Olofsson et al., 2008).

Table 2.3 Comparison of the models designed for ethanol production from dilute acid pretreated corn stover in 2008 and 2012 (NREL Technical Report on Process Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to Ethanol, 2011).

	2008	**2012**
Minimum ethanol selling price ($/gallon)	2.62	2.15
Yield (gallon/dry ton corn stover)	73	79
Pretreatment		
Pretreatment temperature (°C)	190	158
Solid loading (%, w/w)	30	30
Xylan to xylose (%)	75	90
Xylan to degradation products (%)	11	5
Solid-liquid separation for hydrolysate	YES	NO
Enzymatic hydrolysis and fermentation		
Enzyme loading (mg/g)	20	20
Total solid loading (% w/w)	20	20
Overall cellulose to ethanol (%)	85	86
Xylose to ethanol (%)	80	85
Arabinose to ethanol (%)	0	85

2.3 Lignocellulosic biomass

Lignocellulosic biomass has been regarded as an attractive source for production of biofuels and bio based products. What makes them attractive is that they are available in large quantities and have much lower cost in comparison with the food crops (Alvira et al., 2010). There are numerous types of lignocellulosic feedstocks and each type is found to possess unique properties with respect to their composition and other feedstock related parameters. Lignocellulosic biomass include forest residues like wood, forest biomass such as poplar, agricultural residues from crops such as cotton, corn, wheat and rice, herbaceous biomass such as switchgrass and miscanthus and municipal wastes like paper and food wastes (Hu et al., 2008) (Figure 2.6).

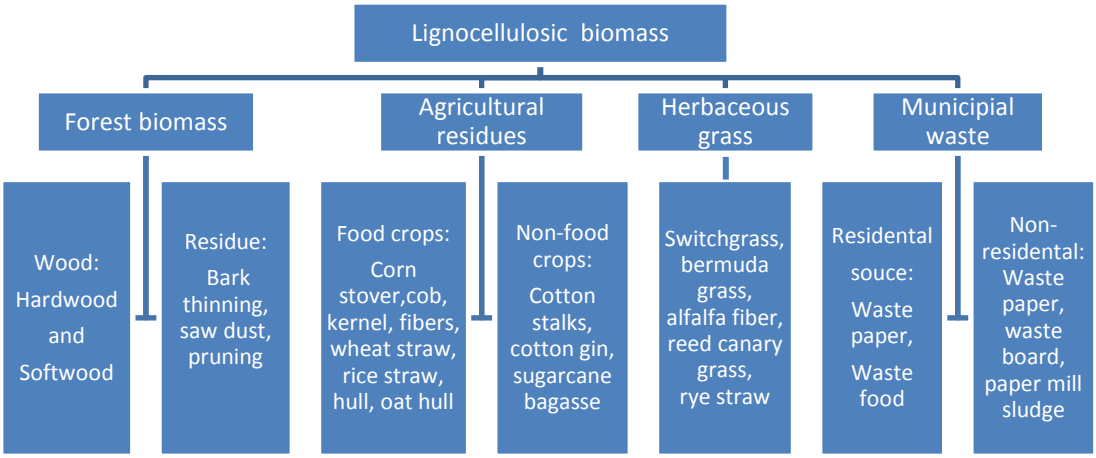


Figure 2.6 Major lignocellulosic biomass (adapted from Hu et al., 2008).

Lignocellulosic biomass consist three major components, cellulose, hemicellulose and lignin which are linked to each other to form a compact and rigid structure that provide stability to the plant cell wall. (Figure 2.7) (Rubin, 2008, Hendriks and Zeeman, 2009). The percentages of these major components were found to change from one type to another as shown in Table 2.4 (Sun and Cheng, 2002). This variation not only occurs in between different species but also observed for the same biomass type considering the geography and environment related factors (Tadesse and Luque, 2011).

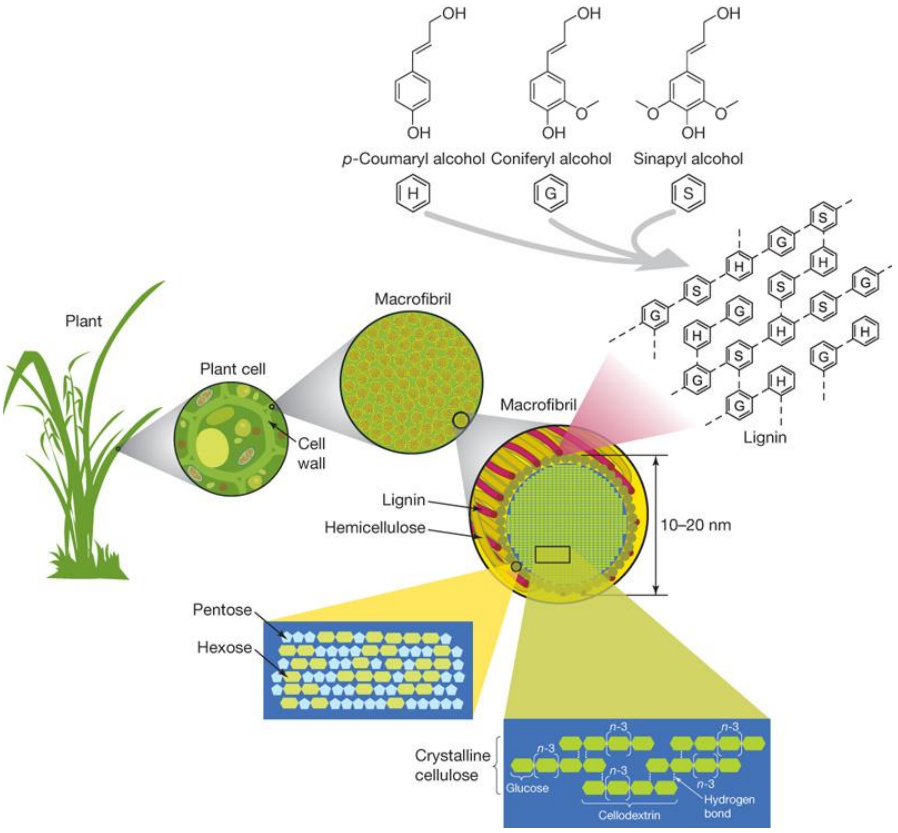


Figure 2.7 Structure of plant cell walls (Rubin, 2008).

Turkey has a production capacity of almost 10 million tons of agricultural wastes in which 3.2 million tons was estimated to account for cotton stalks production (Demirbas, 2008, Saglam et al., 2010). Furthermore, Turkey comes up as one of the eight leading countries producing 85% of the world's cotton stalks (Hepbasli et al., 2007). Cotton stalks together with cotton linters make up the main by-products of cotton. While cotton stalks represent stems and branches, cotton linters are known as the short fibers left on the seed after ginning process (Figure 2.8). According to a previously reported study, almost 2 million tons of cotton stalks are released for each hectare of cotton plantation (Binod et al., 2012). Cotton stalks as an agricultural residue needs to be removed from the field during cotton harvest since it will adversely affect the subsequent plantation (Kaur et al., 2012). Though burning is the most straightforward approach, cotton stalks can be assessed as a potential raw material for cellulosic ethanol production (Shi et al., 2009a) owing to its high cellulose content. Pretreatment techniques including alkaline pretreatment (Silverstein et al., 2007, Bahcegul et al., 2012a, Binod et al., 2012) and microbial pretreatment (Shi et al., 2009a) have been successful in utilization of cotton stalk for generation of value-added products such as cellulosic ethanol and hemicellulose based films.

(a)

(b)

Figure 2.8 Major byproducts of cotton; (a) Cotton stalks (mainly the branches and stems of the plant) and (b) cotton linter (the residue left on cotton seeds) (Save-on-crafts, 2013, Textile exchange, 2013).

Table 2.4 Cellulose, hemicellulose, and lignin contents of common agricultural residues and wastes (Sun and Cheng, 2002, Haykir et al., 2013).

Lignocellulosic biomass	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Cotton stalks	41	16	25
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemical pulps	60-70	10-20	5-10
Primary waste water solids	8-15	-	24-29
Swine waste	6	28	-
Coastal Bermuda grass	25	35.7	6.4
Switchgrass	45	31.4	12

2.3.1 Cellulose

Cellulose not only receives its reputation for being the most abundant organic polymer on earth but also for being the major raw material in cellulosic ethanol production (Yang and Wyman, 2008, Yeh et al., 2010). It is composed of glucose monomers which are linked to each other by the β-1,4 glycosidic bonds (Hendriks and Zeeman 2009). The chemical structure of cellulose is shown in Figure 2.9. The interchain (between the chains) and intrachain hydrogen bonds (within the chains) between glucose subunits form an organized network (Figure 2.10). This hydrogen bonded network includes mainly the crystalline regions which hardly get deconstructed by enzymatic attack and some amorphous regions that are more susceptible to the enzymatic attack (Hendriks and Zeeman, 2009) (Figure 2.11). Owing to its adverse effect on enzymatic accessibility of cellulose, crystalline structure of cellulose has been monitored via numerous types of characterization techniques and regarded as one of the major challenges in pretreatment. The degree of polymerization (DP) of cellulose indicating the number of repeating subunits within a chain is found to vary between 300 and 15000 glucose units (Ragauskas et al., 2006).

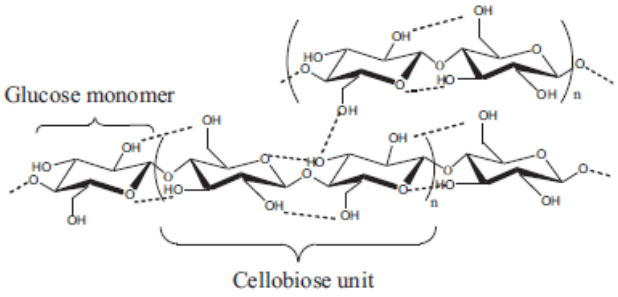


Figure 2.9 Cellulose structure (Cabiac et al., 2011).

Cellulose content of lignocellulosic species mainly including agricultural residues and perennial grasses ranges between 40 to 50% (w/w). Cellulose has been recognized as the most substantial part of the biomass owing to its contribution to biofuels industry. Relying on cellulose for the production of liquid fuels introduces benefits over the petroleum. Besides serving as a low cost and renewable resource, substitution of conventional fuels with cellulosic ethanol will obviously bring less negative environmental impact (Yang and Wyman, 2008).

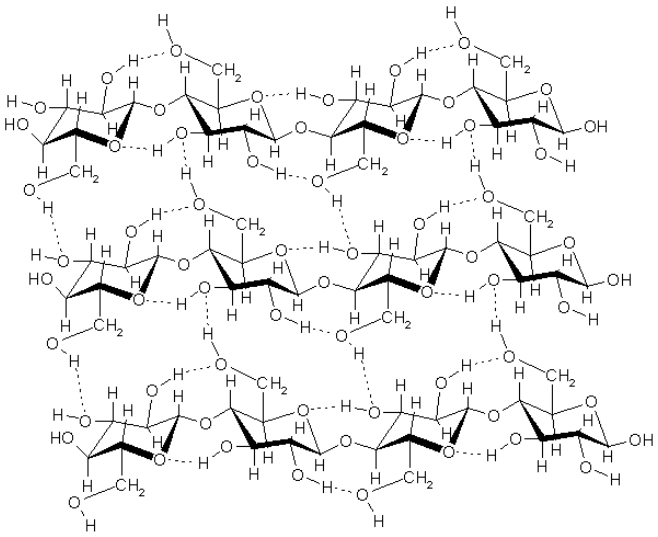


Figure 2.10 Hydrogen bonding in cellulose, the dashed lines demonstrate hydrogen bonds within a cellulose molecule (Chakraborty et al., 2010).

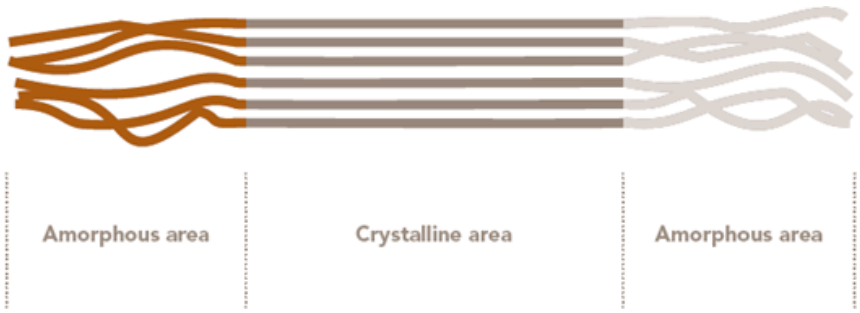


Figure 2.11 Crystalline and amorphous regions of cellulose (Cotton Inc., 2013).

Conversion of cellulose to glucose by means of enzymatic reactions involves utilization of the cellulolytic enzymes (Figure 2.12). Enzymatic hydrolysis of cellulose is fundamentally a heterogeneous reaction in which insoluble cellulose is cleaved through the β-1,4 glycosidic linkages via endoglucanases and cellobiohydrolases. While endoglucanases act on amorphous regions of the polymer, cellobiohydrolases are found to attack the crystalline parts of the cellulose molecule, as shown in the figure. The conversion of cellobiose which is a disaccharide, to glucose, is a homogeneous reaction and conducted by the enzyme, β-glucosidase (RWTH, Chemical Engineering, 2013).

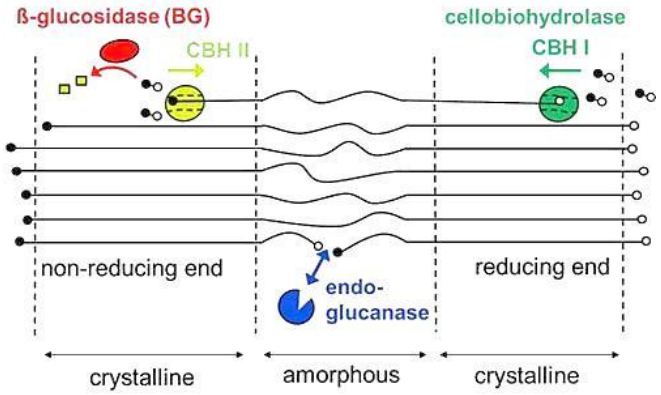


Figure 2.12 Enzymatic hydrolysis of cellulose (RWTH, Chemical Engineering, 2013).

Besides being utilized for cellulosic ethanol production, cellulose applications are extensive. Owing to its biocompatible and chiral structure, cellulose has been shown to form composites with synthetic polymers and biopolymers. Thus, the major products that involve utilization of cellulose derivatives are coatings, laminates, optical films, pharmaceuticals, foods, and textiles (Klemm et al., 2005, Kim et al., 2006).

2.3.2 Hemicellulose

Hemicellulose is identical to cellulose with respect to being a sugar based polymer but it is made up of shorter and branched chains of various subunits compared to the long strands of glucose subunits forming the organized structure of cellulose. Another significant structural difference between cellulose and hemicellulose is that hemicellulose is entirely an amorphous polymer. While possessing six-carbon sugars including, glucose, mannose, galactose and it also consists five-carbon sugars, including xylose and arabinose (Hendriks and Zeeman, 2009; Qing and Wyman, 2011). Its degree of polymerization (DP) varies from 70 to 200 (Ragauskas et al., 2006). Figure 2.13 shows the typical hemicellulose structures.

It contributes to the rigid structure of the biomass particularly by its sheathing effect on the cellulose microfibrils in contribution with lignin (Laureano-Perez et al., 2005). Hemicelluloses exhibit structural variations in biomass depending on whether the species is a hardwood or softwood. Softwoods chiefly comprise galactoglucomannans and arabinoglucuronoxylans whereas hardwoods are composed of glucuronoxylans. As can be understood from the terms, they are complex structures with different side chain groups (Pu et al., 2011). Table 2.5 demonstrates the major hemicellulose components in softwoods and hardwoods (IPST GATECH, Technical review on chemical composition of wood, 2013).

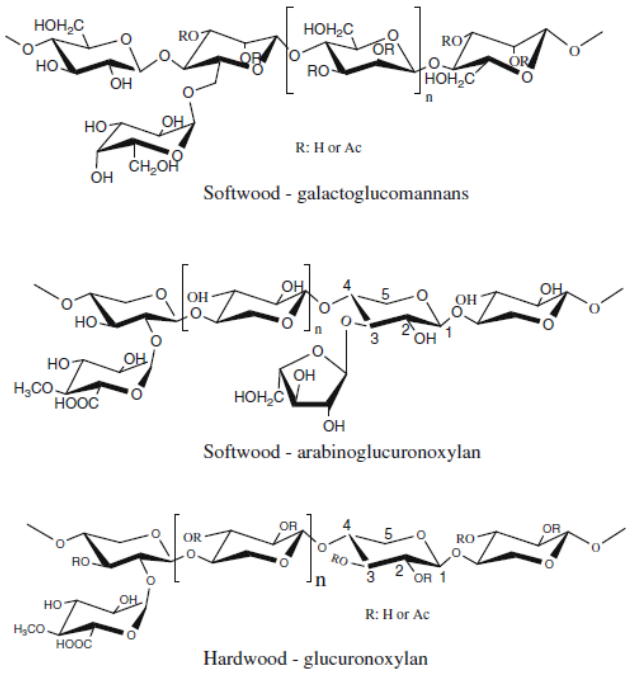


Figure 2.13 Typical hemicellulose structures (Pu et al., 2011).

Table 2.5 Major hemicellulose components in softwoods and hardwoods (IPST GATECH, Technical review on chemical composition of wood, 2013).

Wood	Hemicellulose type	Amount on wood (%)	Composition			DP
			Units	Molar ratios	Linkage	
Softwood	Galacto-glucomannan	5-8	β-D-Man*p* β-D-Glc*p* α-D-Gal*p* Acetyl	3 1 1 1	1→4 1→4 1→6	100
	(Galacto)-glucomannan	10-15	β-D-Man*p* β-D-Glc*p* α -D-Gal*p* Acetyl	4 1 0.1 1	1→4 1→4 1→6	100
	Arabino-glucuronoxylan	7-10	β-D-Xyl*p* 4-O-Me- α-D-Glc*p*A α-L-Araf	10 2 1.3	1→4 1→2 1→3	100
Hardwood	Glucuronoxylan	15-30	β-D-Xyl*p* 4-O-Me- α-D-Glc*p*A Acetyl	10 1 7	1→4 1→2	200

Xylan degradation is mainly achieved by endoxylanases and β- xylosidase which cleave xylose from the non-reducing ends of xylooligosaccharides and xylobiose, respectively. The other essential enzymes, which have been found to attack on the side groups of hemicellulose, together with endoxylanases and β- xylosidase are shown in Figure 2.14 (Collins et al., 2005).

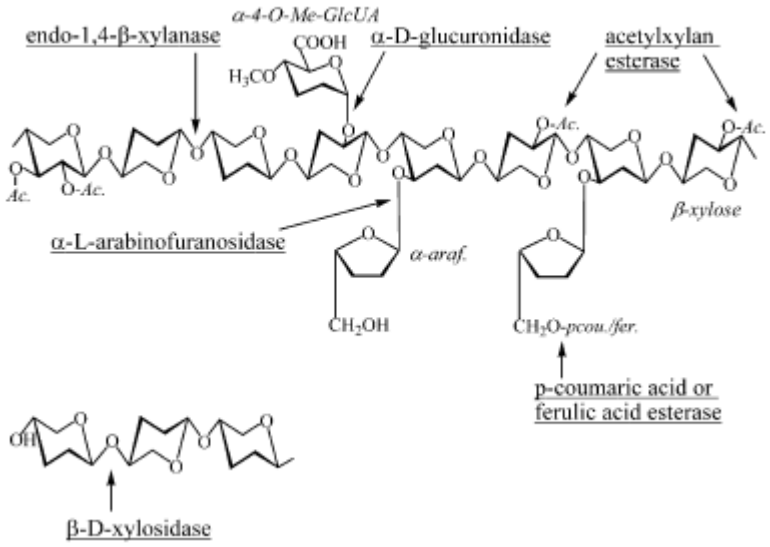


Figure 2.14 Xylan degrading enzymes (Collins et al., 2005).

Owing to its amorphous structure, hemicellulose decomposition is easier compared to cellulose (Hendriks and Zeeman, 2009). Environments at low pH were shown to facilitate its depolymerization (Qing and Wyman, 2011). For instance, steam explosion has been an attractive method for achieving hemicellulose removal from numerous types of lignocellulosic feedstocks. Steam explosion is conducted at elevated temperatures and pressures with or without addition of an acid catalyst in which high degrees of hemicellulose solubilization has been achieved (Sun and Cheng, 2002, Mosier et al., 2005b, Kumar et al., 2009).

In addition to ethanol production, biotechnological applications of hemicellulose mainly include production of the value-added products such as xylitol, 2, 3-butanediol (Saha, 2003), films and coatings (Goksu et al 2007, Hansen and Plackett, 2008, Bahcegul et al., 2012a). Xylitol has received interest due to its use as a natural sweetener in foods (Saha, 2003). It has been previously shown that hemicelluloses derived from wheat straw and sugarcane bagasse were converted to xylitol by *Candida* species. Furthermore, 2, 3-butanediol has been regarded as a value-added liquid product owing to its utilization as a substantial precursor in polymer industry and its high heating value which is almost equivalent to those of ethanol and methanol (Ge et al., 2011). Utilization of hemicellulose based biodegradable films in packaging with the aim of substituting conventional synthetic polymeric materials has gained a growing interest. Studies showed that hemicellulose based films or coatings introduce numerous advantages with respect to their promising physical properties such as high tensile strength and low oxygen permeability (Hansen and Plackett, 2008; Bahcegul et al., 2012a).

2.3.3 Lignin

Compared cellulose and hemicellulose, lignin has utterly a different structure. It is a three-dimensional and complex polymer composed of phenylpropanoid units linked through several major types of carbon-carbon and ether bonds (Figure 2.15). These phenylpropanoid (Figure 2.16) units include coniferyl, sinapyl and p-coumaryl alcohol (Pu et al., 2011). These subunits have phenylpropenoid skeleton in common but they differ from each other with respect to the degree of oxygen substitution on the phenyl ring (Doherty et al., 2011). Accordingly, the structure possessing a single hydroxyl or methoxy group is named as H-structure (p-coumaryl /4-hydroxy phenyl), the one with two such groups is the G-structure (coniferyl/guaiacyl), and the last one with the three aforementioned groups is recognized as the S-structure (sinapyl /syringyl) (Doherty et al., 2011, Pu et al., 2011) (Figure 2.16).

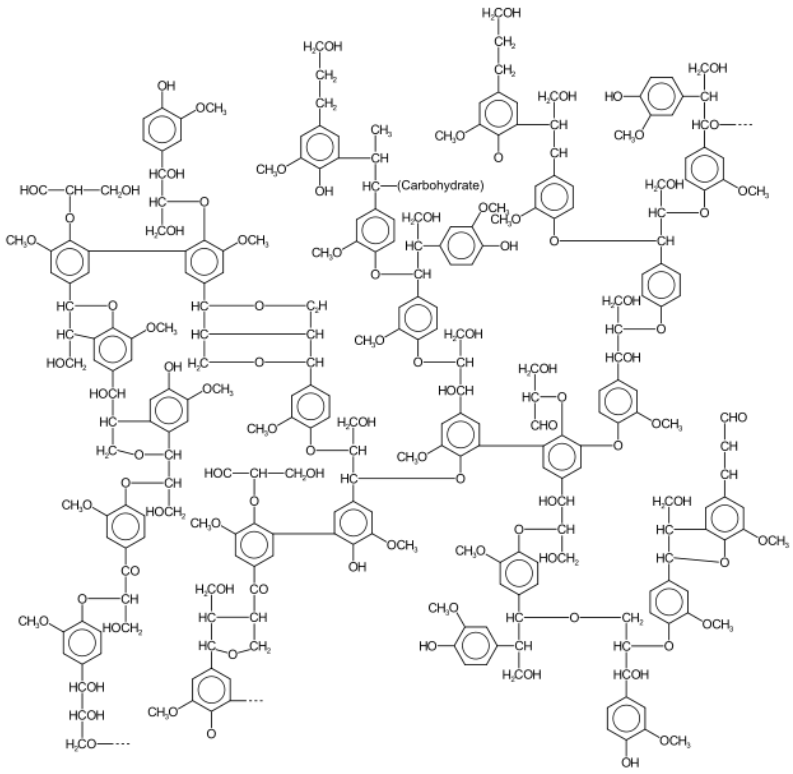
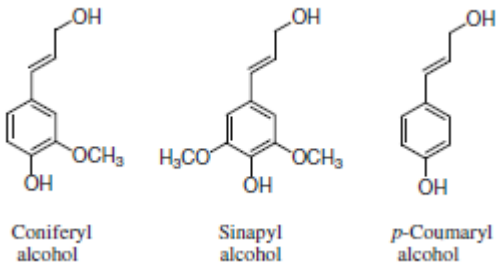


Figure 2.15 Typical lignin structure (Wikipedia, Lignin structure, 2013).



(a) (b) (c)

Figure 2.16 Basic lignin units, (a) coniferyl/guaiacyl alcohol, (b) sinapyl/syringyl alcohol and (c) p-coumaryl /hydroxyphenyl (Pu et al., 2011).

Since lignin does not possess any uniformly distributed repeating units, measurement of its DP is not possible (Doherty et al., 2011). Though it is amorphous, it is not easily got decomposed like hemicellulose. Lignin, which is imbedded into cellulose-hemicellulose matrix, plays a substantial role in biomass structure via providing the robustness. It limits the enzymatic accessibility of the biomass and thus results with insufficient sugar yields upon hydrolysis of the lignocellulosic biomass. Its degradation is possible under various pretreatment conditions particularly in the presence of alkaline agents. "Kraft pulping" was introduced for delignification of wood under alkaline environment in the last 19th century. This patented process has been used for paper making and other related products through pretreatment of wood by sodium hydroxide and sodium sulfide. This way of pretreatment at elevated temperatures (around 170°C) resulted with derivation of black liquor which mainly contained solubilized lignin fragments (IPST GATECH, Technical review on the basics of Kraft Pulping & Recovery Process, 2013). Since then, researchers focused on utilization of alkaline reagents such as sodium hydroxide and potassium hydroxide for degradation and removal of lignin either at low temperature in the presence of concentrated alkaline solutions or at higher temperatures in the presence of dilute alkaline solutions (Silverstein et al., 2007, L.Wu et al., 2011b, Bahcegul et al., 2012a). Besides, alkaline pretreatment, utilization of lignin-degrading microbes such as the white-rot fungi, *Phanerochaete chrysosporium* or treating the biomass with enzymes such as lignin peroxidase, manganese peroxidase and laccase prior or after enzymatic hydrolysis are offered as enviromentally friendly approaches for delignification of biomass (Palonen and Viikari, 2004, Jurado et al.,2009, Sánchez, 2009, Shi et al., 2009b).

Isolation of lignin from wood and utilization of this renewable product in industrial applications rather than burning it in order to provide energy has been an essential asset in the biorefinery context. For instance, its usage as a supplement in cement industry to enhance the mechanical and physical properties of cement has been under investigation. Another substantial area that involves utilization of lignin is polymer industry in which lignin addition to the polymer blends was reported to result with products with good thermal properties (Stewart, 2008, Doherty et al., 2011).

2.4 Pretreatment of lignocellulosic biomass

Development of an efficient pretreatment is necessary for disrupting the recalcitrant structure of lignocellulosic biomass in order to yield fermentable sugars and provide their utilization in cellulosic ethanol production (Wyman et al., 2009). Numerous pretreatment techniques have been developed in order to enhance the enzymatic accessibility of the biomass and are found to target on different structural factors limiting the digestibility of the lignocellulosic biomass (Hendriks and Zeeman, 2009). This part starts out by discussing on the limiting factors that make the pretreatment of biomass necessary prior to the conversion of the biomass to fermentable sugars and ethanol. Next, several types pretreatment techniques will be described. Finally, ionic liquid pretreatment as the major investigated technique in this study will be described in detail.

2.4.1 The factors that limit the digestibility of biomass

Major factors that suppress the accessibility of biomass to enzymatic attack are typically the compact framework formed by hemicellulose and lignin that embed cellulose and crystalline structure of cellulose (Hendriks and Zeeman, 2009). Thus, researchers aim to open up this robust structure efficiently by simply removing lignin and hemicellulose and also disrupting the crystallinity of cellulose (Figure 2.17). It may sound simple but it actually is not. The efficiency of the pretreatment depends on how the major components are separated from each other without losing a considerable fraction from any of them.

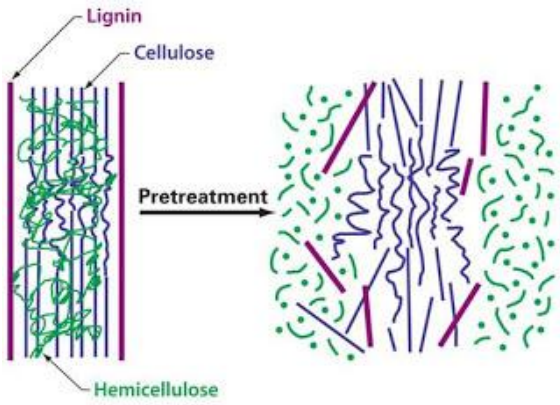


Figure 2.17 Schematic representation of biomass pretreatment (Mosier et al., 2005b)

Presence of lignin in the native form of biomass has been reported to result with nonspecific adsorption of cellulases on lignin (Yang and Wyman, 2006). Lignin not only hinders the enzymatic reaction through non-productive binding of the enzymes onto it, it also physically prevents the enzymes to get in touch with cellulose due to its sheathing effect (Chang and Holtzapple, 2000, Palonen et al., 2004). Palonen et al. (2004) investigated nonspecific binding of two types of cellulases

(cellobiohydrolases, CBHI and endoglucanases, EGII) on the following substrates, steam pretreated softwood, alkali lignin and lignin derived upon hydrolysis of biomass via excessive cellulases. They found that cellulose binding domain (CBD) of enzymes had a substantial effect on nonspecific binding of the cellulases on lignin. At this point, researchers introduced a way to alleviate this negative impact through employment of surfactants during enzymatic reaction. For instance, Kristensen et al. (2007) investigated the effect of surface active additives, BSA (bovine serum albumin), PEG (polyethylene glycol), Tween 80, PEG 2000, 4000 and 6000 and Berol (alcohol ethoxylate) on the efficiency of enzymatic hydrolysis. Utilization of surfactants was shown to increase cellulose conversion up to 70%. Another study showed that addition of BSA prior to enzymatic hydrolysis lowered enzyme loadings during hydrolysis of AFEX pretreated corn stover (Yang and Wyman, 2006). Researchers attributed these promising findings, which were offered by numerous types of surfactants, to the hydrophobic interaction between lignin and surfactants (Eriksson et al., 2002, Börjesson et al., 2007).

Lignin removal has been essential to enhance cellulose accessibility to cellulases. As previously mentioned, alkaline reagents have been beneficial pretreatment agents to remove lignin with the aim of enhancing the digestibility of biomass (Silverstein et al., 2007). The common route to remove lignin from biomass under alkaline conditions has been lignin solubilization. Though it appears as a promising approach, liquid fraction derived upon alkaline pretreatment, which composes solubilized lignin fragments, will be inhibitory for the preceding steps, enzymatic hydrolysis and fermentation (Palmqvist and Hahn-Hagerdal, 2000). At this point, development of a suitable detoxification method such as excessive washing of pretreated biomass prior to enzymatic hydrolysis or adaptation of the yeast to a medium containing inhibitory components prior to fermentation will be beneficial.

Another critical parameter that plays a substantial role in the digestibility of biomass is the crystalline structure of cellulose. Cellulose is composed of glucose subunits that are linked to each other in a well-organized fashion via strong hydrogen bonds. This ordered network, which appears as an obstacle for enzymatic hydrolysis of cellulose, need to be disrupted somehow. Accordingly, researchers put an intensive effort to reduce the crystalline structure of lignocellulosic biomass in order to enhance sugar yields. Studies show that reductions in crystalline structure of biomass not only improved the extent of the enzymatic reaction but also enhanced initial rates of the hydrolysis. Since adsorbed enzymes will progress more conveniently and thus faster on the amorphous substrate (Chang and Holtzapple, 2000, Laureano-Perez et al., 2005, Dadi et al., 2007, Hall et al., 2010).

To determine the crystalline structure of cellulose, X-ray diffraction (XRD) analysis has been applied to characterize biomass samples (Hall et al., 2010, Park et al., 2010). The results derived upon XRD analysis of numerous lignocellulosic feedstocks have been meaningful. Since the findings obtained upon XRD analysis have been in accordance with the results derived from enzymatic hydrolysis of the biomass (Chang and Holtzapple, 2000, Dadi et al., 2007, S.H. Lee et al., 2009).

Other factors that limit enzymatic accessibility of the lignocellulosic biomass could be summarized as particle size, degree of polymerization (DP), available surface area and pore size of the biomass (Kumar et al., 2009, Hendriks and Zeeman, 2009). Reduction in the particle size of the biomass prior to enzymatic hydrolysis is reported to have positive effect on the digestibility of the biomass (Yeh et al., 2010, Khullar et al., 2013). Though reported to be a cost intensive technology, milling has been shown to enhance the specific surface area and reduce the crystallinity of the biomass (Chang and Holtzapple, 2000, Yeh et al., 2010). Chain length of cellulose or in other words, DP of cellulose is believed to affect the glucose yields obtained upon enzymatic hydrolysis (Hendriks and Zeeman, 2009). Reductions in DP of cellulose resulted with improvements in the enzymatic hydrolysis of lignocellulosic biomass (Cateto et al., 2011). Though it was not much introduced in the literature as a chief determinant for efficiency of the enzymatic hydrolysis, researchers discussed that pore size has

a straightforward impact on the functionality of cellulolytic enzymes. According to previously reported studies, compatibility between the pores size of the biomass and the enzyme size has been crucial for the accessibility of the substrate to enzymes (Gregg and Saddler, 1996, Mooney et al., 1998).

2.4.2 Pretreatment techniques

2.4.2.1 Biological pretreatment techniques

Biological pretreatment of lignocellulosic biomass is an environmentally benign approach that employs fungi species with the aim of reducing lignin content of the biomass (Sánchez, 2009). The white-rot fungi, *Phanerochaete chrysosporium,* which secretes lignin degrading enzymes, peroxidases and laccases, has been widely recognized (Alvira, 2010). *P. chrysosporium* has been capable of degrading high amounts of lignin to produce ethanol from various lignocellulosic feedstocks (Sánchez, 2009, Shi et al., 2009b). Though introduced advantages over the conventional chemical pretreatment for being a low cost method and not presenting detrimental effects on environment, the duration of pretreatment, which is almost 14 days, is much longer compared to the other types of pretreatments (Sun and Cheng, 2002).

2.4.2.2 Physical pretreatment techniques

2.4.2.2.1 Mechanical comminution

Mechanical comminution consist milling, grinding and chipping of the lignocellulosic feedstocks. These mechanical methods differ from each other according to the final particle size of the biomass. While chipping reduces the particle size of the biomass to 10-30 mm, milling and grinding result with particles having a much smaller particle size which range between 0.2-2 mm (Sun and Cheng, 2002). Several studies show milling to facilitate the enzymatic hydrolysis of the biomass owing to the fact that biomass with reduced particle size has reduced crystallinity, lower DP and higher surface area (Alvira et al., 2010). Though particle size reduction has been offered by several studies, mechanical comminution requires high energy consumption and lengthy process periods (Mousdale, 2008, Hendriks and Zeeman, 2009). Wood, forestry biomass and straws are the types of biomass that are typically exposed to mechanical pretreatment.

2.4.2.2.2 Extrusion

Another way to pretreat biomass mechanically is the thermomechanical method called extrusion. The pretreatment was conducted in an extruder where biomass is exposed to heating, mixing and shearing. This continues process derives physical and chemical modifications in the fibers which eventually result with improvements in the sugar yields upon enzymatic hydrolysis (Alvira et al., 2010). The major pretreatment related parameters that have been shown to yield substantial effects on the enzymatic hydrolysis of the feedstocks are feedstock size, compression ratio, screw speed and temperature (Karunanithy and Muthukumarappan, 2010). Modification of this continuous technique through exploitation of chemicals during the process may yield better results in terms of enzymatic accessibility of cellulose (S-H. Lee et al., 2009).

2.4.2.3 Chemical pretreatments

2.4.2.3.1 Steam explosion

Steam explosion has been a widely recognized physicochemical method which is conducted either in the presence or absence of chemicals. The simplest description of the process is that biomass is exposed to high pressurized steam at almost 0.69-4.85 MPa and 160-290°C for a few minutes and the reaction is terminated via venting the steam suddenly and lowering the pressure to atmospheric pressure (Sánchez and Cordona, 2008). This sudden explosive decompression that biomass is exposed in the final step of the process brings substantial impacts on the structural features of the biomass. It not only opens up the structure but also removes considerable amounts of hemicellulose. As mentioned previously, hemicellulose is one of the major components of the biomass which is the most easily decomposed one due to its amorphous polysaccharide structure. While the biomass gets exposed to steam under very high pressures, hemicellulose gets fractionated into its subunits (Alvira et al., 2010). Additionally, this type of pretreatment has resulted with partial deconstruction of lignin structure (Sun and Cheng, 2002, Alvira et al., 2010). During uncatalyzed steam explosion, hemicellulose solubilization results with release of the acetyl groups in the form of acetic acid. Though the process is not catalyzed via acid addition, acetic acid formed upon hemicellulose solubilization automatically catalyzes the pretreatment. This method increases accessible surface area for cellulose hydrolysis (Mosier et al., 2005b). Steam explosion may also be coupled by exploitation of acid catalysts such as sulphur dioxide. Such a study was reported by Öhgren et al. (2005) in which steam explosion of sulphur dioxide impregnated corn stover resulted with 89% of the yield based on the glucan content of the untreated biomass. A variety of analyses involved acid catalysts such as sulphur dioxide and sulphuric acid during steam explosion in order to enhance glucose yields from woody biomass (Sassner et al., 2008, Monavari et al., 2009, Wyman et al., 2009). Steam explosion has been also utilized for cellulosic ethanol production from a numerous type of lignocellulosic biomass including Salix (Sassner et al., 2008) and spruce (Monavari et al., 2009).

Solubilization of hemicellulose under high pressure is the main advantage obtained from steam explosion. However solubilization of hemicellulose ends up with formation of inhibitor compounds. The inhibitor, furfural is reported to be formed upon hemicellulose degradation. Even in the case of partial cellulose solubilization, hydroxyl methyl furfural (HMF) can be produced which is considered as a substantial inhibitor for enzymatic hydrolysis and particularly for yeast growth during ethanol production (Alvira et al., 2010). Figure 2.18 shows the conversion reactions of xylose and glucose to furfural and HMF, respectively (Rogalinski et al., 2008).

The major drawbacks presented by these inhibitor (furfural, HMF and acetic acid) compounds have been under investigation (Palmqvist and Hahn-Hagerdal, 2000). In order to minimize their adverse effects on the subsequent steps, insoluble fraction of pretreated slurry could be washed with sufficient amount of water. However, this type of detoxification will be problematic since it will not only increase the process costs but also generate high amounts of waste water. Alternatively, construction of novel yeast strains being tolerant to inhibitors for ethanol production has been presented as an environmentally approach (Yang et al., 2011).

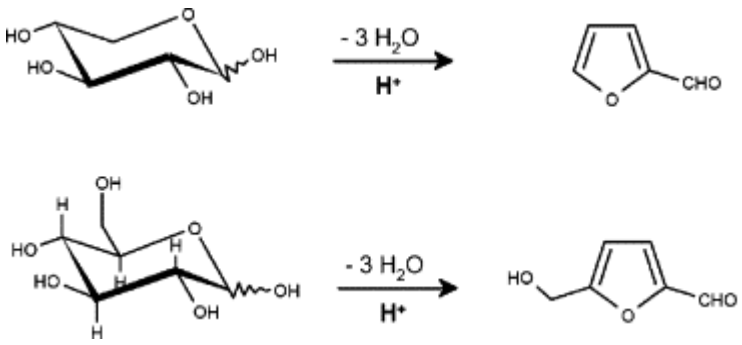


Figure 2.18 Conversion reactions of xylose to furfural (upper reaction) and glucose to hydroxyl methyl furfural (HMF) (lower reaction) (Rogalinski et al., 2008).

2.4.2.3.2 Liquid hot water pretreatment

Liquid hot water (LHW) is an alternative hydrothermal pretreatment in which decompression of the reaction medium or catalyst addition are not applicable as it has been the case in steam explosion. Still high pressures are employed but the aim is to maintain the water in liquid state at temperatures of almost 160-240°C (Alvira et al., 2010). The major finding observed upon LHW is hemicellulose removal mainly through its depolymerization. Owing to the fact that the pretreatment has been conducted under milder conditions (controlled pH) such that catalyst utilization is not provided, hemicellulose recovery is much higher and much less amounts of inhibitors are released compared to steam explosion. LHW is attractive not only due to being an environmentally benign method, but also due to the fact that recovered hemicellulose from the liquid fraction of the pretreatment slurry can be further utilized for ethanol production (Mosier et al., 2005b). Moreover, LHW was found to result with partial lignin degradation (Alvira et al., 2010).

LHW has been applied to various types of lignocellulosic feedstocks such as wheat straw (Perez et al., 2008) and corn stover (Mosier et al., 2005a) in which the glucose yields based on the cellulose content of the untreated material were highly satisfying. Despite possessing advantageous points regarding the environmental issues, commercial scalability of LHW is not possible owing to employment of high volumes of water (Alvira et al., 2010).

2.4.2.3.3 Acid pretreatment

Acid pretreatment has been recognized as the most widely used method for pretreatment of lignocellulosic biomass in industry. In order to minimize the formation of potential inhibitors, dilute acids such as sulfuric acid, hydrochloric acid and nitric acid have been employed (Sun and Cheng, 2002, Saha et al., 2003). Similar to the aforementioned pretreatment types (steam explosion and liquid hot water) dilute acid pretreatment has mainly provided hemicellulose solubilization (Sun and Cheng, 2002). It is conducted through interaction of the biomass with dilute acids at a temperature ranging between 120–200°C for a period ranging from minutes to seconds. Besides its considerable effect on hemicellulose, elevated temperatures are shown to result with cellulose hydrolysis. Despite being favorable for fermentable sugar formation, dilute acid pretreatment has less impact on lignin

such that it only resulted with redistribution of lignin (Sánchez and Cordona, 2008). The dilute acid pretreated lignocellulosic feedstocks include sugarcane tops (Sindhu et al., 2011), corn stover (Lloyd and Wyman, 2005, Zhu et al., 2009) and switchgrass (Li et al., 2010).

2.4.2.3.4 Ammonia fiber explosion (AFEX)

In ammonia fiber explosion (AFEX), the biomass is subjected to liquid ammonia (1-2 kg ammonia/kg biomass) at a temperature ranging between 70-100°C and pressure ranging between 150-400 psi for 5 to 30 minutes. The progression of AFEX is identical to steam explosion since the reaction between liquid ammonia and biomass is terminated upon ammonia release. The sudden release of ammonia enabled recovery of ammonia in vapor phase and thereby, provided reutilization of ammonia in the subsequent pretreatments (Li et al., 2011). Since it has been conducted at milder conditions compared to steam explosion, it has presented inhibitor concentrations close to zero and much less corrosive environments. However, it is not as efficient as steam explosion regarding hemicellulose solubilization and not suitable for biomass possessing high lignin (Sun and Cheng, 2002). In a previously reported study, glucose yields of almost 90% were obtained upon enzymatic hydrolysis of AFEX pretreated switchgrass at low enzyme loadings of 15 FPU/g glucan (Alizadeh et al., 2005).

2.4.2.3.5 Supercritical CO_2 pretreatment

Owing to being environmentally benign and low-cost, supercritical CO_2 is regarded as an alternative extraction solvent for lignocellulosic biomass pretreatment. At elevated pressures and milder temperatures that correspond to the operating conditions exceeding the critical point of CO_2, CO_2, which possesses a density similar to liquids, effectively penetrates into the pores of the biomass. The sudden pressure release at the end of the processs is reported to restructure cellulose and hemicellulose and thus, enhance the sugar yields (Alvira et al., 2010). For instance, supercritical CO_2 pretreatment, which was conducted at 3100 psi and 165°C for 30 min, was shown to enhance the enzymatic digestibility of Aspen to 84% of the theoretical maximum glucose yield (Kim and Hong, 2001). Though this pretreatment technique provided nontoxic environments, high pressure requirements make the process economically unattractive.

2.4.2.3.6 Alkaline pretreatment

The major effect of alkaline pretreatment on biomass is lignin removal which enhances the enzymatic accessibility of cellulose. Various alkaline agents such as sodium hydroxide, calcium hydroxide (lime) and hydrogen peroxide have been employed (Sánchez and Cardona, 2008). While being very effective in lignin removal, alkaline pretreatment is also able to make modifications in hemicellulose by removing the acetyl groups (Chang and Holtzapple, 2000). Alkaline agents also found to present swelling effects on the biomass which accordingly result with an increase in the surface area of the biomass (Hendriks and Zeeman, 2009). Besides, utilization of alkaline reagents at high concentrations was shown to reduce the crystalline structure of cellulose. For instance, sweet sorghum bagasse subjected to sodium hydroxide pretreatment at a molarity of 5 M demonstrated an amorphous structure compared to the biomass in its native form (L.Wu et al., 2011a).

In contrast to steam explosion and dilute acid pretreatment, less corrosive effects have been observed in alkaline pretreatment. However, either neutralization or excessive washing of the pretreated biomass following the pretreatment are essential since biomass itself consumes significant amounts of alkali during pretreatment (Hendriks and Zeeman, 2009). This will obviously increase process costs and generate high amounts of waste water.

Alkaline pretreatment has been effective towards herbaceous plants and agricultural residues (Silverstein et al., 2007). Such that, cotton stalks (Silverstein et al., 2007, Binod et al., 2012, Kaur et al., 2012), sweet sorghum bagasse (L.Wu et al., 2011a) and sugarcane stalks (L.Wu et al., 2011b) have been subjected to alkaline pretreatment under different conditions. The effectiveness of alkaline pretreatment has relied on the lignin content of the lignocellulosic biomass (Sun and Cheng, 2002). Recent studies that employed sodium hydroxide for biomass pretreatment have been mostly conducted via dilute alkaline solutions at concentrations ranging between 0.5-4% (w/v). Besides, solid loadings were reported to range between 5-10% (w/v). The pretreatment temperature and period have been at most 121°C and 90 minutes, respectively (Silverstein et al., 2007, Kaur et al., 2012). Based on the conditions stated, almost 50-60% of lignin removal has been attained upon sodium hydroxide pretreatment of cotton stalks in a previously reported study (Silverstein et al., 2007). Additionally, almost 60% of glucose yield has been achieved regarding the cellulose content of the untreated cotton stalks in the same study. It has been also shown that low temperature alkali pretreatment (25-50°C) yielded better results regarding lignin removal in which at least 60% of lignin removal has been achieved. Because low temperature alkali pretreatment has been conducted at much higher sodium hydroxide concentrations (0.5-5 M) and for longer periods of pretreatment (up to 24 h) compared to the former alkali pretreatment (L.Wu et al., 2011a, L.Wu et al., 2011b). Furthermore, exploitation of high sodium hydroxide concentrations almost 5M has been beneficial for deconstruction of crystalline structure of cellulose. It has been also reported that almost 100% glucose yield was achieved within 24 hours of enzymatic hydrolysis of sweet sorghum bagasse subjected to alkaline pretreatment via 2.5 M NaOH at 50°C for 2 hours (L.Wu et al., 2011a).

Lime (calcium hydroxide) pretreatment has been also beneficial to enhance the digestibility of various lignocellulosic feedstocks. Lime pretreatment introduces the advantage of being less expensive and safer over NaOH pretreatment. Additionally, recovery of calcium in the form of calcium carbonate through reaction of calcium and carbon dioxide at the end of the pretreatment is another advantage of the process (Hendriks and Zeeman, 2009). In a previously reported study, lime pretreatment of corn stover at a loading of 0.075 g $Ca(OH)_2$/ g biomass and 120°C for 4 hours resulted with 60% of cellulose to glucose conversion at 10 FPU/g biomass enzyme loading and even better, 88% at 25 FPU/g biomass enzyme loading (Kaar and Holtzapple, 2000). In another study, lime pretreated coastal bermuda grass (0.1 g $Ca(OH)_2$/ g biomass loading, 100°C, 15 minutes) yielded 87% and 83% of the theoretical maximum glucose and ethanol yields, respectively based on the cellulose content of the untreated biomass (Wang and Cheng, 2011). Despite exhibiting positive aspects for the safety and process costs, $Ca(OH)_2$ is not as effective as NaOH with respect to lignin removal since NaOH is a stronger base than $Ca(OH)_2$.

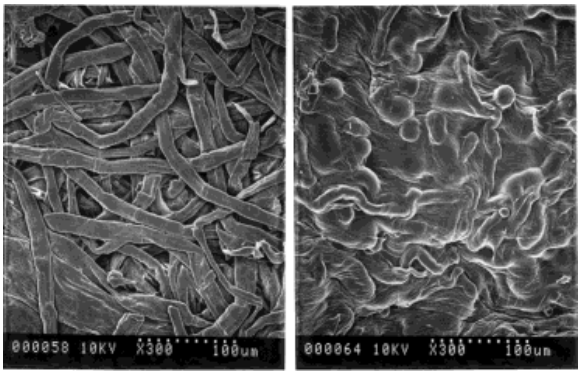
Table 2.6 shows the effects of aforementioned pretreatment techniques on biomass.

Table 2.6 Structural effects demonstrated by the major pretreatment techniques, ●●●: High effect, ●●: Moderate effect, ●:Low effect (adapted from Mosier et al., 2005b and Alvira et al., 2010).

	Increases accessible	**Decrystallizes cellulose**	**Removes hemicellulose**	**Removes lignin**	**Alters lignin**	**Generation of inhibitory**
Biological				●●●	●●●	
Physical	●●●	●●●				
Steam explosion	●●●		●●●	●●	●●●	●●●
Liquid hot water	●●●		●●●	●	●●	●
Acid	●●●		●●●	●●	●●●	●●●
AFEX	●●●	●●●	●●	●●●	●●●	●
Supercritical CO_2	●●●		●●●			
Alkaline		●		●●	●●	

2.5 Ionic liquid pretreatment

Ionic liquid (IL) pretreatment has attracted researchers as a promising pretreatment method from the time when Swatloski and his co-workers (2002) have found out that cellulose could be dissolved in ionic liquids under certain heating conditions. They were able to obtain a solution of cellulose in BMIMCl at 10% (w/w) loading of cellulose and 100°C and similarly, they attained different cellulose-IL solutions with microwave or conventional heating. They demonstrated the significant morphological variation in the cellulose dissolving pulp upon dissolution in BMIMCl via SEM. SEM images below shows the native cellulose dissolving pulp and the material after dissolved in BMIMCl and regenerated into water (Figure 2.19). Based on these findings, the major conclusion derived was that ionic liquids are capable of destroying the well-organized hydrogen bond network of cellulose (Swatloski et al., 2002).



(a) (b)

Figure 2.19 SEM images of the (a) native cellulose dissolving pulp and (b) after it is dissolved in BMIMCl and regenerated into water (Swatloski et al., 2002).

This conclusion was the starting point of the studies that involved interaction of lignocellulosic biomass and ionic liquids. Several parameters have been investigated till now and very remarkable and promising results have been derived.

2.5.1 Properties of ionic liquids

Ionic liquids are room temperature salts that consist solely of anions and cations (Welton, 2004). No solvent addition is required for the ions to get apart from each other however such a solvent addition is necessary for sodium chloride as seen in the figure which puts an emphasis on the distinction between an ionic liquid and ionic solution (Figure 2.20). Ionic liquids usually have low melting points, below 100°C and low vapor pressures at ambient conditions (Welton, 2004). They also have high thermal stability; Huddlestone et al. (2001) reported the decomposition temperature of numerous imidazolium based ionic liquids which ranged between 253-455°C. Owing to their non-volatile characteristics, they are regarded as environmentally benign solvents compared to organic

solvents and highly promising for sustainable processes (Rogers and Seddon, 2003). They are described as “tunable solvents” due to the convenience of making modifications in their anion or cation types according to specific targets (Olivier-Bourbigou et al., 2010). Figure 2.21 shows the possible combinations of major anions, cations and cation substituents that form ionic liquids.

A description of the ionic liquids for being found in liquid state at room temperature is scarce. The large cations for instance, imidazolium with low charge density possess weak molecular interactions within the structure and result with low lattice energy. This low lattice energy eventually confirms the low melting points of the ionic liquids (Welton, 2004, Dupont and Suarez, 2006).

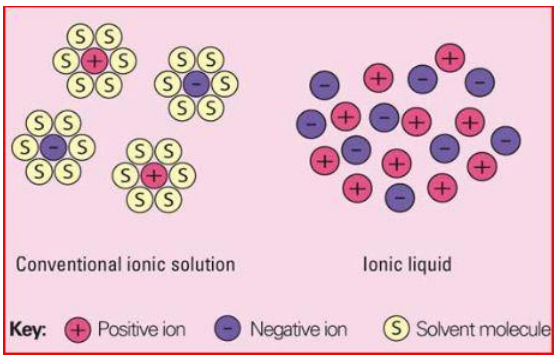


Figure 2.20 The basic difference between an ionic liquid and conventional ionic solution (BP, 2009).

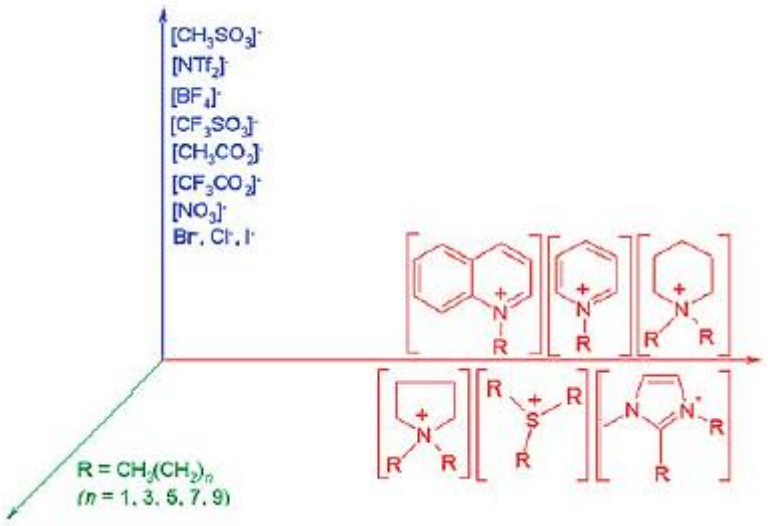


Figure 2.21 Possible combinations of anions, cations and cation substituents that form ionic liquids (Stark, 2011).

The most crucial parameters that need to be discussed in this section are viscosity, cationic, anionic properties and hydrogen bonding properties of ionic liquids such as dipolarity/polarizability ratio and hydrogen bond basicity. Each parameter plays a substantial role in biomass pretreatment via ionic liquids (Mäki-Arvela et al., 2010). The discussions will be more focused on imidazolium based ionic liquids since they have presented promising results for pretreatment of numerous lignocellulosic feedstocks.

The viscosity of ionic liquids has been regarded as a drawback which limits their handling. High viscosity of ionic liquid was reported to introduce mass transfer limitations during biomass processing (Mora-Pale et al., 2011). This will obviously result with inefficient pretreatment of the biomass. The viscosity of ionic liquids has been reported to vary from 10 cP to 500 cP which is comparable to that of oil (Huddlestone et al., 2001). The viscosity of alkyl imidazolium ionic liquids coupled with chloride anion (almost >2000 cP) have higher viscosity compared to those possessing phosphonate, carboxylate, formate and acetate as anions (Fukaya et al., 2006, Zhao et al., 2009, Mora-Pale et al., 2011). Accordingly, one can understand that the types of anions and cations as well as their sizes are determinants of the viscosity of ionic liquids. Moreover, the viscosity of imidazolium based ILs is associated with the length of the subunits present on the imidazolium cation. It was reported that the longer the alkyl chains present on imidazolium cation, the more viscous the ionic liquid (Huddlestone et al., 2001).

Dipolarity/polarizability ratio (π), hydrogen bond acidity (α) and basicity (β) are other tunable parameters that have an influence on the interaction of ionic liquids with biomass. Hydrogen bond acidity (α) and basicity (β) are the indicators of the solvent to donor and accept hydrogen bonds, respectively. In fact, these three parameters are closely associated to each other and correlated via series of equations derived by Kamlet and Taft (1976) (Welton, 2004). The magnitudes of each parameter indeed give an idea about how effective the ionic liquid deconstructs the hydrogen bond network of cellulose present in a biomass (Mora-Pale et al., 2011). The magnitude of these solvation parameters is strongly associated to the type of the cation and anion that the ionic liquid composes. While hydrogen basicity of an ionic liquid depends on the anion type, hydrogen bond acidity is related to the cation type, in particular (Welton, 2004). Dipolarity/polarizability (π) is generally high for all ionic liquids (Brandt et al., 2010).

For biomass processing, ionic liquids that possess high hydrogen bond basicity (β) are preferred since they are more effective in disrupting the hydrogen bond network of cellulose (Fukaya et al., 2006). For instance, Fukaya et al. (2006) designed 1,3-dialkylimidazolium formate ionic liquids, which had higher β compared to AMIMCl and BMIMCl, exhibited much better solvation capability for cellulose. The strong correlation between ionic liquid anion and biomass solubility has been comprehensively discussed in another study (Brandt et al., 2010). They have examined several ionic liquids that contained [BMIM] cation in common and different anions. The ionic liquids with β higher than 0.8 exhibited better swelling effects on pine wood chips. Particularly, BMIMAc with hydrogen bond basicity of 1.2 yielded the most effective results on pine wood chips regarding the dissolution and swelling of the biomass in the ionic liquids. It was also shown that there was a considerable decrease in hydrogen bond acidity (α) at high hydrogen bond basicity (β).

2.5.2 Ionic liquid pretreatment of lignocellulosic biomass

This section comprises information about the effective role of ionic liquids in cellulose and biomass processing together with their significant contribution to biomass conversion into fermentable sugars. Before discussing their effects on lignocellulosic biomass, ionic liquid interaction with cellulose and lignin as an essential subject will be discussed separately. Later on, literature studies on ionic liquid pretreatment of a variety of lignocellulosic biomass will be given in order to appreciate the role of ionic liquids in enhancing the enzymatic digestibility of biomass.

2.5.2.1 Interaction of ionic liquids with cellulose

The interaction of ionic liquids with cellulose is based on cellulose dissolution. The mechanism of cellulose dissolution in ionic liquids involves mainly the disruption of the hydrogen bond network of cellulose. Figure 2.22 shows how the typical ionic liquid AMIMCl interacts with cellulose. For convenience, only hydroxyl groups between two strands of cellulose are involved in the demonstration. It is observed that cation $[AMIM]^+$ attacked on the oxygen atom and the anion $[Cl]^-$ linked to the hydrogen atom of the hydroxyl. This simple mechanism reveals the deconstructive effect of ionic liquids on cellulose structure and thus, cellulose dissolution in ionic liquids. This impact resulted with reduction in cellulose crystallinity and enhancement in the enzymatic digestibility of cellulose (Zhang et al., 2005).

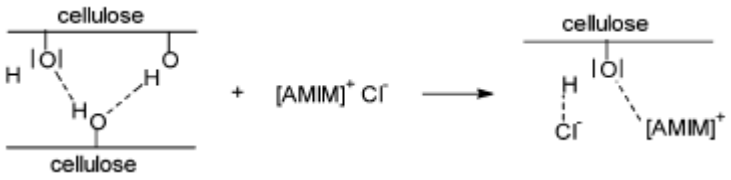


Figure 2.22 Dissolution mechanism of cellulose in AMIMCl (Zhang et al., 2005).

Together with the studies that discussed cellulose dissolution in variety of ionic liquids (Swatloski et al., 2002, Fukaya et al., 2006, Remsing et al., 2006, Zhu et al., 2006, Fukaya et al., 2008), a comprehensive study was conducted by Zavrel et al. (2009) in which twenty one ionic liquids were screened with respect to their performances in the extent of cellulose dissolution and as well as cellulose dissolution rate. Accordingly, AMIMCl, BMIMCl, EMIMCl, ECOENG and EMIMAc were found to be capable of dissolving 5% (w/w) of cellulose completely within 12 hours at 90°C. Among them, EMIMAc was found to be the most effective one regarding cellulose dissolution. Dissolution temperature and melting points of ionic liquids were also shown to be crucial parameters affecting cellulose dissolution.

In another study, the attentions were drawn to the structural changes in cellulose obtained upon its dissolution in AMIMCl (Zhang et al., 2005). The most striking conclusion derived from this study was that regenerated cellulose, which could be conveniently obtained via water addition to the AMIMCl-regenerated cellulose solution, possessed good mechanical properties and reduced crystallinity. Besides, DP of cellulose was found to decrease with an increase in dissolution time at 110°C.

Vitz et al. (2009) investigated cellulose dissolution in a variety of imidazolium based ionic liquids in which tailor-made ionic liquids were included as well. The researchers concluded up with very interesting results. Imidazolium based ionic liquids with chloride as the counter ion possessing even numbered alkyl chains dissolved cellulose better than those having odd numbered alkyl chains. However, this effect could not be observed in those having bromide as the counter ion. The water content of the ionic liquids prior to dissolution was measured since water uptake of an ionic liquid has been a critical factor that adversely affected cellulose dissolution. Though EMIMAc has been the most hygroscopic one, it revealed promising findings with respect to cellulose dissolution; 8% (w/w) of cellulose was completely dissolved in EMIMAc at 100°C within one hour. Imidazolium based ILs with chloride and acetate have been effective in cellulose dissolution, however the color change during their interaction with cellulose (from transparent to dark brown) was associated with cellulose degradation. More interestingly, the tailor-made ionic liquid, $EMIMEt_2PO_4$, which did not result with any cellulose degradation (no color change), was able to dissolve 14% (w/w) of cellulose at 100°C within one hour.

The major conclusion derived from the above findings is that either anion or cation choice exhibits straightforward effect on the cellulose dissolution. Their types and as well as their sizes determine how effective the ionic liquids generate bonds with the hydroxyl groups of the cellulose and thereby, disrupt the crystalline cellulose structure. Reduced crystallinity of cellulose upon dissolution in ionic liquids has been previously reported and linked to the enhanced enzymatic accessibility of the cellulose (Dadi et al., 2006, Dadi et al., 2007). Dadi and his co-workers (2007) showed that regenerated cellulose which was obtained upon cellulose dissolution in either AMIMCl or BMIMCl, had reduced crystallinity compared to its native form. The reduction in crystallinity substantially enhanced the initial hydrolysis rate of cellulose compared to its native structure (at least 50 fold). Regenerated cellulose obtained upon dissolution of cellulose at loadings of 5% and 10% (w/w) in either AMIMCl or BMIMCl resulted with much higher initial rate enhancement (71-82 fold) compared to those dissolved at loading of 15 and 30% (w/w) (27-63 fold). While cellulose completely dissolved in ionic liquids at 5 and 10% of loading, those incubated in ionic liquids at higher loadings were partially dissolved. Furthermore, BMIMCl pretreated samples at 15-30% loading yielded much higher initial rate enhancement than those incubated in AMIMCl (Dadi et al., 2007).

2.5.2.2 Interaction of ionic liquids with lignin

One major advantage that ionic liquids exhibit with respect to lignocellulosic biomass processing is their capability to dissolve lignin. In a previously reported study, several ionic liquids were screened with respect to their effect on solubility of kraft pulp lignin isolated from softwood (Pu et al., 2007). It was shown that anion nature affected lignin solubility; [BMIM] based ionic liquids exhibited lignin solubility in the decreasing order, $MeSO_4$>Cl>Br>PF_6. Interestingly, $BMIMMESO_4$ was capable of dissolving 344 g/L of lignin at 50°C. It was also reported that ionic liquids possessing bulky anions were poor in dissolving lignin.

Solubility of lignin in ionic liquids not only offers benefits with respect to enhancement of the enzymatic accessibility of cellulose in biomass but also conversion of lignin to high-value added products. Binder et al. (2009) investigated lignin depolymerization in ionic liquids with catalysts. Cleavage of aryl-alkyl and aryl ether linkages was reported to result with the release of valuable aromatic compounds. Eugenol (phenylpropene), which was used as a model compound instead of an isolated lignin molecule, was depolymerized to guaiacol with 11.6% of yield and 100% of conversion in EMIMOTf (1-ethyl-3-methylimidazolium triflate) in the presence of Nafion as catalyst. Depolymerization of eugenol to guaiacol was also found to depend on the anion type. Imidazolium based ionic liquids containing the less basic anions such as triflate performed better than those containing anions with moderate basicity such as chloride, acetate and bromide.

Besides, ionic liquids were shown to extract significant amounts of lignin upon pretreatment of various lignocellulosic feedstocks. The dissolution of pine wood in BMIMCl at 100°C resulted with lignin extraction. Recovered biomass, which was obtained upon acetonitrile addition to the ionic liquid-biomass slurry, resembled to pure cellulose samples subjected to dissolution at identical dissolution conditions (Fort et al., 2007). In another study, BMIMCl and EMIMAc were compared with respect to their effect on lignin extraction from triticale straw (Fu et al., 2010). EMIMAc resulted with better delignification in which almost 52% of alkali insoluble lignin was extracted upon triticale dissolution in EMIMAc at 150°C for 90 minutes. Accordingly, cellulose digestibility of almost 95% was achieved which was in accordance with the percentage lignin extracted. S.H. Lee et al. (2009) examined delignification of maple wood flour samples. It was reported that 40% of delignification by EMIMAc has been sufficient for attaining at least 90% of cellulose digestibility for the maple wood flour. This study was also favorable owing to the findings derived upon EMIMAc reuse. EMIMAc preserved its effectiveness upon reuse for four times with respect to delignification of maple wood flour since similar percentages of lignin extraction were achieved upon each EMIMAc reuse.

EMIMAc was also shown to yield better results compared to BMIMCl in terms of wood dissolution (southern yellow pine and red oak) and resulted with 31% of lignin extraction from southern yellow pine. Thus, the regenerated material obtained upon EMIMAc dissolution was enriched in cellulose which was almost 76% (w/w) of the regenerated yellow pine. The recovery of the extracted lignin present in EMIMAc was also shown to be possible through acid precipitation (Sun et al., 2009). In another study, lignin extraction of at least 93% was achieved upon dissolution of sugarcane bagasse in the ionic liquid, EMIMXS (1-ethyl-3-methyl-imidazolium xylene sulfonate) at elevated temperatures and atmospheric pressure. The cellulose pulp and recovered lignin were obtained from the ionic liquid liquor by their subsequent precipitation via addition of sodium hydroxide and hydrochloric acid, respectively. Thereby, it was possible to fractionate the biomass into its major components and reuse the ionic liquid for the following biomass dissolutions (Tan et al., 2009).

2.5.2.3 Interaction of ionic liquids with lignocellulosic biomass

In this part, the literature studies, which mainly targeted on ionic liquid pretreatment of the biomass rather than biomass dissolution, will be discussed. In literature, various lignocellulosic feedstocks were subjected to ionic liquid pretreatment with the goal of enhancing biomass conversion to fermentable sugars. The major findings were found to comprise the modifications gathered in the biomass structure and increased biomass accessibility to enzymatic hydrolysis. Besides, recovery and reuse of ionic liquids were examined with respect to their effect on the hydrolysis yields.

Though not attended biomass pretreatment, the studies reported by Kilpaleinen et al (2007) and Zavrel et al. (2009) were highly significant. Kilpaleinen et al (2007) investigated the structural properties and enzymatic accessibility of regenerated wood samples. The regenerated spruce sawdust samples obtained upon dissolution in AMIMCl and BMIMCl exhibited reduced crystallinity and enhanced glucose yields compared to biomass in its native structure. It was also revealed that increased water content of ionic liquids and biomass with bigger particle sizes exhibited adverse effects on the dissolution of biomass in ionic liquids. Zavrel et al. (2009) screened a variety of ionic liquids with respect to their effect on dissolution of softwoods and hardwoods. Among, AMIMCl was found to dissolve all softwood and hardwood samples, silver fir, spruce, common beach, chestnut completely. EMIMAc was capable of dissolving all of them completely except silver fir which was dissolved partially.

The study performed by S.H. Lee et al. (2009) has been a substantial and comprehensive one. The researchers investigated the effect of pretreatment period and temperature on structural changes (crystallinity and amount of lignin extracted) and enzymatic accessibility of the maple wood flour subjected to EMIMAc pretreatment. According to the findings, the amount of lignin extracted (%) was found to increase from 16% to 86% with an increase in pretreatment period from 30 minutes to 70 hours. However, the crystallinity index (CrI) was not affected much from an increase in pretreatment time after 5 hours. Cellulose digestibility was achieved as 91% for the biomass subjected to EMIMAc pretreatment at 90°C for 5 hours. Increase of pretreatment time from 5 to 70 hours resulted with an increase of cellulose digestibility from 91 to 96%, only. Accordingly, the changes in the crystalline structure of the biomass introduced a more profound impact on the cellulose digestibility compared to the changes in the amount of lignin extracted (%) from the biomass. On the contrast, the variation in pretreatment temperature from 50 to 130°C not only had an effect on the amount lignin extracted but also had a considerable effect on the reduction in crystallinity. Thus, cellulose digestibility was found to increase 46% to 95% with an increase in pretreatment temperature from 50 to 130°C. In summary, cellulose digestibility exhibited a more intense correlation with cellulose crystallinity compared to the amount of lignin extracted from maple wood flour. Furthermore, EMIMAc reuse was investigated in terms of its effect on cellulose digestibility and the amount of lignin extracted. During recovery of EMIMAc, no additional step was conducted for purification of the ionic liquid; it was directly used for the following pretreatment after evaporation of the water (antisolvent) from the aqueous EMIMAc solution. Though lignin accumulated in each ionic liquid reuse, EMIMAc preserved its effectiveness as a pretreatment agent since the amount of lignin extracted and cellulose digestibility remained unaffected from EMIMAc reuse even in the 4^{th} batch of the process.

Simmons and his coworkers reported two substantial studies (Li et al., 2010, Li et al., 2011) which compared ionic liquid pretreatment with dilute acid pretreatment and AFEX (ammonia fiber explosion) with respect to their effects on the lignocellulosic feedstocks, switchgrass and corn stover, respectively. The most striking impact of ionic liquid pretreatment on both biomass types was that EMIMAc pretreated biomass was hydrolyzed much faster than the dilute acid and AFEX pretreated samples. Initial enzymatic hydrolysis rates for EMIMAc pretreated switchgrass and corn stover were 16.7 and 15.2 fold higher than those observed for untreated switchgrass and cornstover. These promising findings on cellulose digestibility were attributed particularly to the reduction in cellulose crystallinity and as well as to the enhancements in surface area of the biomass and delignification.

Nguyen et al. (2010) investigated the effect of ionic liquids, BMIMCl, EMIMCl, EMIMAc and EMIMSu (1-ethyl-3-methyl-imidazolium hydrogen sulfate) on cellulose recovery and cellulose digestibility of rice straw. Cellulose digestibility of the pretreated rice straw was found to decrease in the following order; EMIMAc>EMIMCl>BMIMCl>EMIMSu. EMIMAc pretreatment was shown to yield better cellulose digestibility than ammonia pretreatment for rice straw. EMIMAc pretreatment was also

examined with respect to particle size effect on cellulose digestibility. Though cellulose recovery was attained the lowest for EMIMAc pretreated rice straw having the smallest particle size prior to pretreatment, the highest cellulose digestibility was achieved for the same particles. They also showed the effect of EMIMAc recycling on cellulose recovery and digestibility. There has been a slight increase in cellulose recovery due to the cellulose accumulated in the recovered EMIMAc in each reuse. Cellulose digestibility was unaffected until 5^{th} reuse of EMIMAc however it decreased considerably after that point.

Shill et al. (2011) conducted an investigation which dealt with utilization of basic antisolvents during biomass recovery in order to achieve efficient recycle of EMIMAc and obtain higher enzymatic digestibility for the pretreated biomass. Miscanthus and corn stover were subjected to EMIMAc pretreatment and recovered via addition of aqueous solutions of 40% (w/w) K_3PO_4, 40% (w/w) K_2HPO_4 and water. Among, pretreated miscanthus recovered via 40% (w/w) K_3PO_4 resulted with much higher cellulose digestibility (100%) compared to those recovered via addition of 40% (w/w) K_2HPO_4 (74%) and water (68%). Utilization of kosmotropic salt solutions for recovery of the pretreated biomass generated three-phase system in which the upper, middle and lower phases were enriched in ionic liquid, pretreated biomass, kosmotropic salt, respectively. This system provided high recovery for the ionic liquid and major biomass components, particularly cellulose. Accordingly, the extent of ionic liquid reuse will be enhanced and improved cellulose to glucose conversion will be achieved.

EMIMAc pretreatment was also investigated with respect to its effect on surface area and pore volume of switchgrass which were measured by BET (Brunauer-Emmet-Teller) (Arora et al., 2010). Switchgrass, which was subjected to EMIMAc pretreatment at 160°C for 3 hours, had surface area and pore volume of 30 and 50 fold higher compared to those obtained for untreated biomass, respectively. Accordingly, enzymatic hydrolysis rate of pretreated switchgrass was found to be 40 fold higher compared to untreated biomass.

The literature studies which were discussed up to this point, composes ionic liquid pretreatments which has been conducted at a biomass loading of 3 to 5% (w/w). Lower biomass loadings were found to result with biomass dissolution rather than biomass pretreatment. Though the capability of ionic liquids to dissolve biomass effectively has been regarded positively, excessive dissolution may cause cellulose degradation and thus result with lower cellulose recovery. A few studies pay attention to the cellulose recovery and effective utilization of cellulose present in the untreated biomass to produce fermentable sugars. In the study conducted by H.Wu et al. (2011), corn stover was subjected to EMIMAc pretreatment at biomass loadings higher than 10% (w biomass/w slurry). Utilization of high biomass loadings makes the process more economically viable since there is an opportunity to pretreat higher amounts of biomass in a specific amount of ionic liquid. Interestingly, EMIMAc was found to be capable of reducing the crystallinity of the biomass and resulting with 80% of cellulose to glucose conversion at a biomass loading of 33% (w/w). Though much less lignin was extracted, sufficient sugar yields were attained due to the reduction of the crystalline structure. Besides, reuse of EMIMAc for pretreatment of corn stover was assessed for 10 batches of pretreatment. The results indicated that EMIMAc preserved its effectiveness upon being recycled since identical hydrolysis yields and similar biomass structure with reduced crystallinity were attained at each batch of EMIMAc pretreatment.

2.6 Enzymatic hydrolysis

Conversion of the pretreated biomass, which is less recalcitrant and thus, more prone to enzymatic attack, to fermentable sugars has been a crucial step in cellulosic ethanol production. The major factors that affect the enzymatic hydrolysis of lignocellulosic biomass include substrate (solid) loading, enzyme loading and operational conditions such as temperature, pH (Sun and Cheng, 2002, Alvira et al., 2010). In this part, discussions will be made in order to understand the impact of each parameter on the enzymatic hydrolysis.

Substrate loading determines the amount of sugar released upon enzymatic hydrolysis. The higher the substrate loading the higher the concentration of fermentable sugars released. High fermentable sugar concentration will provide high ethanol concentration during fermentation. Higher ethanol concentration obtained upon fermentation will obviously facilitate the product recovery during distillation and reduce the process costs since less energy will be utilized to attain the final ethanol concentration during downstream processing (Wingren et al., 2003, Jørgensen et al., 2007). However, utilization of high biomass loadings can be challenging owing to the mass transfer limitations and presence of high inhibitor concentration during enzymatic hydrolysis (Kristensen et al., 2009). Though elevated glucose concentration were obtained, lower cellulose conversions were reported in literature owing to the aforementioned challenges. For instance, the substrate loading for the enzymatic hydrolysis of steam exploded wheat straw varied from 10 to 30% (w/w) (Lu et al., 2010). The cellulose conversion was found to decrease much more with an increase in substrate loading for the pretreated biomass subjected to hydrolysis without being washed prior to enzymatic hydrolysis compared to that washed steam exploded wheat straw which was found to possess lower concentrations of inhibitors (acetic acid and furfural) than the former one. Besides, the initial viscosity of the hydrolysis solution containing 30% of substrate (unwashed) loading was found to be 10000cP, whereas it was 4000 cP for the hydrolysis solution containing 10% of substrate loading. This viscosity difference could have also created mass transfer problems during enzymatic hydrolysis. In another study, the negative impact of increased substrate loadings on cellulose digestibility up to 20% (w/w) was linked to the presence of inhibitors, whereas the decrease in cellulose digestibility was related to mass transfer limitations at substrate loadings higher than 20% (w/w) (Hodge et al., 2008).

Enzyme loading during enzymatic hydrolysis of lignocellulosic biomass is another crucial parameter which needs investigation since enzymes contribute significantly to the process costs in cellulosic ethanol production (Kumar and Wyman, 2009a). Accordingly, the efforts have been mainly on conducting the enzymatic hydrolysis at lower enzyme loadings. However, it has not been always the case since the nature of the pretreated biomass, which is an important factor determining the appropriate enzyme loading during hydrolysis, does not always permit utilization of lower enzyme loadings. For instance, presence of residual lignin at high fractions in biomass structure will require higher enzyme loadings owing to the non-productive adsorption of cellulases on lignin (Chang and Holtzapple, 2000, Van Dyk and Pletschke, 2012). At this point, utilization of surfactants during the enzymatic reaction has been offered and been very effective for minimization of the interaction of cellulases with lignin and thus, lowering the enzyme loadings (Yang and Wyman, 2006). Though cellulose crystallinity plays a significant role in determination of initial hydrolysis rates of cellulose (Hall et al., 2010), no data related to any decisive effect of cellulose crystallinity on enzyme loading has been reported. In addition to lignin content of the biomass, the cellulose surface area accessible to enzymatic attack was also found to be a decisive factor for enzyme loading (Sathitsuksanoh et al., 2010, Rollin et al., 2011). Bamboo, which was subjected to COSLIF (cellulose solvent-and organic solvent-based lignocellulose fractionation) pretreatment, was investigated in terms of cellulose accessibility to cellulase (m^2/g). Pretreated bamboo, which hold 33 fold higher cellulose accessibility to cellulase compared to its native structure, was hydrolyzed at very low cellulase loadings; as low as

1 FPU/ g glucan and resulted with almost identical cellulose digestibility (88% at t=72 h) with the samples hydrolyzed at higher enzyme loadings (Sathitsuksanoh et al., 2010). In another study, Rollin et al. (2011) compared COSLIF and aqueous ammonia pretreatments with respect to their effects on the structural changes in switchgrass and enzymatic accessibility of the biomass. COSLIF pretreated switchgrass possessed higher cellulose accessibility to cellulase (16 fold higher than that of untreated biomass) compared to the biomass subjected to aqueous ammonia pretreatment. Thus, it was possible to hydrolyze COSLIF pretreated biomass at low enzyme loadings such that 3 FPU/g glucan was sufficient to attain cellulose digestibility of switchgrass over 80% within 24 hours of enzymatic hydrolysis. On the other hand, cellulose digestibility of aqueous ammonia pretreated switchgrass at 3 FPU/ g glucan of enzyme loading was only 58% even in the presence of BSA (bovine serum albumin) blocking.

In addition to substrate and enzyme related factors, pH and temperature have been also considered as substantial parameters that affect enzymatic hydrolysis. Each enzyme has an optimal range of pH and temperature at which it is active. Researchers have found an optimal pH and temperature range for the cellulolytic enzymes, celulases and β-glucosidase. Owing to their synergy between these enzymes (Zhang and Lynd, 2004), they have been employed simultaneously and thus, at the same pH and temperature. According to vast majority of the laboratory scale studies, the optimum pH and temperature employed for enzymatic hydrolysis of lignocellulosic biomass has been 4.8 and 50°C respectively. Novozymes, which is a leading enzyme supplier, have reported the optimal ranges of pH and temperature for one of its latest product, Cellic Ctec2 as 5.0-5.5 and 45-50°C, respectively. (Novozymes application sheet on Cellic Ctec2).The investigations regarding the optimal pH and temperature are particularly crucial for simultaneous saccharification and fermentation (SSF) in which enzymatic hydrolysis and fermentation are carried out simultaneously for cellulosic ethanol production. Since optimal conditions sustaining both enzyme activity and microbial growth should be employed during SSF (Olofsson et al., 2008, Van Dyk and Pletschke, 2012).

Mixing has been essential particularly for enzymatic hydrolysis conducted at high substrate loadings to provide an effective interaction between the enzymes and biomass. As previously discussed, low cellulose digestibilities, which observed during the hydrolysis at high substrate loadings, have been attributed the increased viscosity of the hydrolysis media (Lu et al., 2010, Kristensen et al., 2009). Therefore, the efforts were made on investigation of the variations in cellulose digestibility with mixing. For instance, Samaniuk et al. (2011) showed that mixing accelerated the cellulose digestibility at 20% substrate loading and linked this finding to the reduced mass transfer limitations and increased surface area of the biomass due to the shortening of the biomass fibers by mixing throughout the hydrolysis. They also discussed about the synergy between mixing and cellulose digestibility. As cellulose was converted to glucose, the amount of insoluble material in the hydrolysis medium decreased, mixing became much easier, thus less energy was required during mixing after a certain period. In another study, the decrease in the cellulose digestibility was related to the lower adsorption capacity of the cellulase at high loadings of cellulose during hydrolysis (W. Wang et al., 2011). They monitored variation of the cellulose conversion with mixing and found out that high stirring at high substrate loadings provided similar cellulose digestibility (almost 70%) with the low stirring condition at low substrate loading. Though stirring appeared to facilitate the enzymatic reaction between cellulases and biomass at high substrate loadings, enzyme deactivation or activity loss may be observed as a result of high shear rates (Samaniuk et al., 2011).

2.6.1 Cellulose degrading enzymes

Cellulases have been recognized as the major enzymes of the cellulolytic system which play significant roles in conversion of lignocellulosic biomass to cellulosic ethanol. The potential producer microorganisms of cellulose degrading enzymes are the fungi namely, *Trichoderma reseei* and *Aspergillus niger* (Zhang and Lynd, 2004). *T. reseei* has been reported to secrete the endoglucanases; Cel7B, Cel5A, Cel12A, Cel61A and Cel45A, exoglucanases; Cel7A, Cel6A and as well as a few xylanases and β-xylosidases. Additionally, β-glucosidase produced by *A. niger* has been utilized as a substantial supplementary to the celluloytic enzymes specified above.

Cellulases are composed of a variety of enzymes, endoglucanases or 1,4-β-D-glucan-4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4- β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and also β-glucosidases or β -glucoside glucohydrolases (EC 3.2.1.21) (Lynd et al, 2002). Each enzyme was found to act on different regions of the cellulose with a synergy that facilitates the degradation of hydrolysis of cellulose (Figure 2.17) (Lynd et al., 2002, Van Dyk and Pletschke, 2012). Endoglucanases generate different lengths of oligosaccharides by acting on the random internal amorphous regions of the cellulose chain. Exoglucanases, which target on the crystalline regions of the cellulose, hydrolyze the reducing and non-reducing ends of the polymer and liberate either glucose (cellodextrinases) or cellobiose (cellobiohydrolases). Furthermore, β–glucosidases hydrolyze soluble cellobiose to generate glucose (Lynd et al., 2002, Andric et al., 2010).

The synergism between cellulose degrading enzymes has been recognized by the researchers and several models have been proposed to describe the mechanisms. The sum of the activities of endoglucanases and exoglucanases employed separately for the hydrolysis of a substrate was found to be low when compared to their combined performance (Bansal et al., 2009). The ratio of the product yields derived upon each case has been recognized as the degree of synergy. (Kumar and Wyman, 2009a, Van Dyk and Pletschke, 2012). Based on the representation shown (Figure 2.23), one could understand that endoglucanases provide end regions on the cellulose chains for the exoglucanases through attacking amorphous internal regions on the same chain. Similarly, cellobiohydrolases liberate soluble cellobiose molecules which are then converted to glucose via β–glucosidases. This mechanism obviously reveals the reason for attaining higher yields observed upon the combined action of the cellulolytic enzymes. In this context, investigations have been carried out in order to determine an optimal exoglucanases to endoglucanases ratio in the cellulases (Van Dyk and Pletschke, 2012).

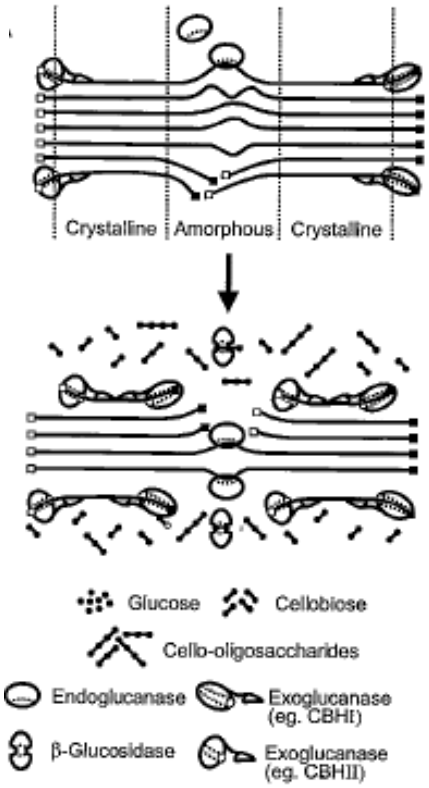


Figure 2.23 Hydrolysis of cellulose by cellulases (Lynd et al., 2002).

Owing to the heterogeneous structure of the substrate, classical models do not fit for the hydrolysis of the insoluble cellulose. Bansal et al. (2009) described cellulose hydrolysis on the basis of adsorption model according to the following steps (Figure 2.24):

1-Cellulases are adsorbed onto substrate through cellulose binding domain (CBD) of the enzyme.
2-Enzyme is placed onto a bond that is prone to hydrolysis.
3-Enzyme-susbtrate complex is formed.
4- 1,4-β-glycosidic bond is cleaved and enzyme is moved forward along the cellulose chain for further hydrolysis.
5-Cellulase is desorbed from the substrate or step 4 or 2 and 3 are repeated if the CBD of the enzyme is removed from cellulose chain.
6-If present, cellobiose is converted to glucose by β-glucosidase.

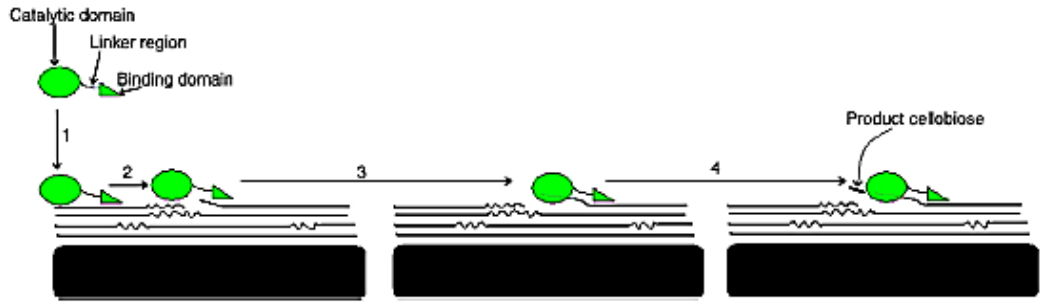


Figure 2.24 Hydrolysis of the cellulose on the basis of adsorption model (Bansal et al., 2009).

Accordingly, vast majority of the literature work describes hydrolysis of the cellulose on the basis of adsorption kinetics (Andric et al., 2010). Michaelis-Menten kinetics, which is the classical approach in enzyme kinetics, has been applicable for the hydrolysis of soluble oligosaccharides. For instance, conversion of cellobiose to glucose by β-glucosidases can be described via Michaelis-Menten kinetics (Bansal et al., 2009). In the previously reported literature studies, Langmuir-Hinshelwood kinetics has been proposed for the adsorption of cellulases on cellulose and subsequent hydrolysis of cellulose (Zhang and Lynd, 2004, Kumar and Wyman, 2009a). Kumar and Wyman (2009a) showed that a strong correlation between the rates at the 24th hour of the hydrolysis and cellulase adsorption capacity of native Avicel and corn stover samples subjected to various leading pretreatments; ammonia recycle percolation (ARP), ammonia fiber explosion (AFEX), controlled pH, dilute acid, lime, SO_2. Cellulase adsorption capacity of the substrates was determined according to the following Langmuir expression:

$$[CE]=\frac{\sigma[S_t][E_f]}{K_d+[E_f]} \tag{2.1}$$

where [CE] is the amount of adsorbed enzyme concentration (mg/ml), $[E_f]$ is the free enzyme concentration (mg/ml), σ is the maximum adsorption capacity (mg protein/g substrate), $[S_t]$ is the substrate concentration (mg/ml) and K_d is the equilibrium constant (mg enzyme/ml) (Kumar and Wyman, 2009a).

Cellulose hydrolysis has been reported to be inhibited by various factors. Product inhibition, in which the hydrolysis products hold adverse effects on cellulases, is believed to be highly substantial (Lynd et al., 2002, Xiao et al., 2004, Bommarius et al., 2008, Andric et al., 2010). Among the hydrolysis products that hold adverse effects on hydrolysis, glucose and cellobiose were recognized in particular. For instance, the inhibitory effect of glucose on cellulase was reported in which this effect was found to be more pronounced than its effect on β-glucosidase (Xiao et al., 2004). Besides, monomeric sugars, xylose, mannose, galactose were found to have inconsiderable effects on the hydrolysis compared to glucose. Interestingly, utilization of higher substrate conditions alleviated the inhibitory effect of glucose on cellulase and β-glucosidase which was offered as a practical solution

to the product inhibition. Recently, simultaneous saccharification and fermentation (SSF) has been regarded as a strategy that reduces the adverse effects of glucose on enzymatic hydrolysis. Since, glucose released during hydrolysis is simultaneously metabolized by the microorganisms and converted to ethanol during SSF process (Andric et al., 2010). In another study, inhibitory effects of cellobiose on enzymatic hydrolysis of acid, alkaline and organosolv pretreated cellulose were investigated (Bommarius et al., 2008). The researchers found out that β-glucosidase supplementation to cellulase remarkably enhanced the rates of cellulose hydrolysis owing to the lower cellobiose concentrations and thus, preservation of cellulase activity throughout the enzymatic reaction. According to the results, complete Avicel hydrolysis was achieved within 96 hours in the presence of β-glucosidase. However, 30% of cellulose conversion could be achieved even after seven days of hydrolysis period without β-glucosidase utilization.

The other factors which were found to cause reductions in hydrolysis rates during enzymatic hydrolysis were reported as enzyme deactivation, biphasic composition of cellulose and jamming (Bansal et al., 2009, Bommarius et al., 2008). Enzyme deactivation may occur as a result of the shear forces and also blockage of the enzymes due to the surrounding cellulose chains that slow down their action (Bansal et al., 2009, Samaniuk et al., 2011). Biphasic composition of cellulose, or in other words, presence of crystalline and amorphous regions on the same cellulose chain, is another factor that decreases the hydrolysis rates. Since cellulases favor amorphous sites of the cellulose chains at the initial periods of the hydrolysis, the remaining unhydrolyzed regions are mainly crystalline parts of the molecule. Accordingly, the hydrolysis rates would decrease due to the slower action of the cellulases on crystalline cellulose (Chen et al., 2007). Jamming, which was defined as the overcrowding of the enzyme on substrate surface, restricted the action of the enzymes along cellulose chains and thus, decreased the hydrolysis rates (Bansal et al., 2009). Exoglucanases, Cel7A and Cel6A, which possess identical dimensions with the distance between parallel cellulose chains, attack crystalline regions on cellulose molecule. At high enzyme concentrations, the action of cellobiohydrolases has been constrained due to the jamming (Bommarius et al., 2008). Figure 2.25 shows the jamming effect of enzymes on cellulose substrate.

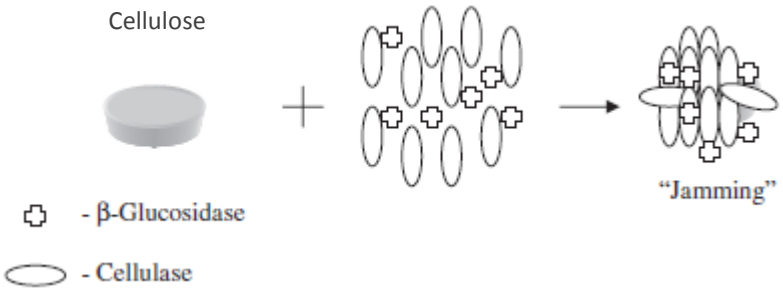


Figure 2.25 Jamming effects of enzymes on cellulose (Bommarius et al., 2008).

2.6.2 Hemicellulose degrading enzymes

Conversion of hemicellulose to fermentable sugars and other value-added products (xylitol, 2,3-butanediol) relies on the efficient mechanisms presented by hemicellulose degrading enzymes, hemicellulases. As mentioned in section 2.3.2, hemicellulose is made up of a backbone (xylan in hardwoods, glucomannan in softwoods) and shorter branched chains of various subunits. It also interacts with cellulose and lignin through covalent and non-covalent linkages in lignocellulosic biomass. Based on the complex structure of hemicellulose and its interaction with the other major constituents of the biomass, hemicellulose degradation requires a variety of enzymes acting with synergism on different structural groups of hemicellulose (Saha, 2003). Figure 2.26 shows the major hemicellulases and hemicellulose structures that the enzymes act on.

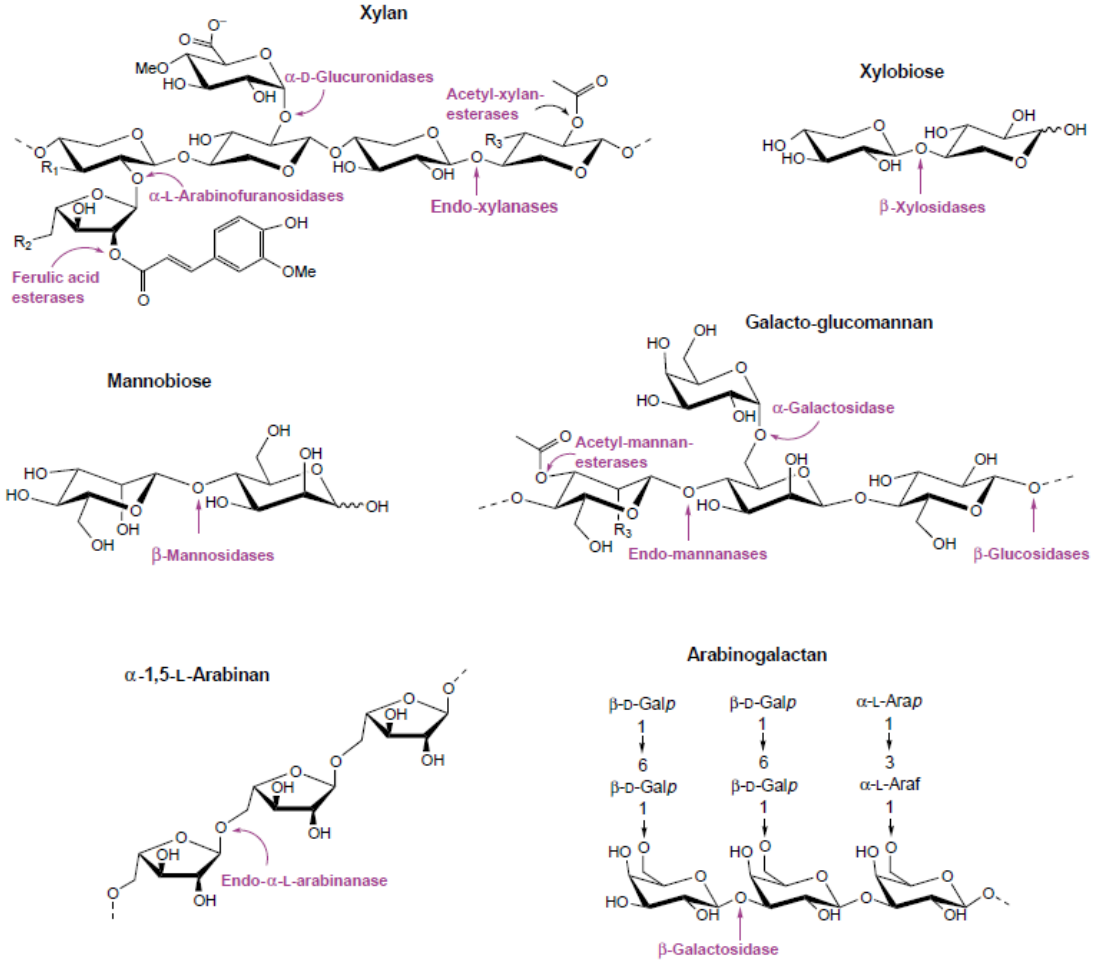


Figure 2.26 Major hemicellulases and hemicellulose structures that the enzymes act on (Shallom and Shoham, 2003).

Xylanases mainly compose endo-1,4-β-D-xylanases (EC 3.2.1.8) that randomly act on the xylan backbone, β-D-xylosidases (EC 3.2.1.37) that cleave xylose from the non-reducing end of xylo-oligosaccharides and xylobiose. The cleavage of the side groups are catalyzed via α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.2.1.72), ferulic acid esterases (EC 3.1.1.73) and p-coumaric acid esterases (EC 3.1.1.-) (Collins et al., 2005). Table 2.7 and Figure 2.27 also summarize the enzymes involved in the hydrolysis of xylan.

Table 2.7 Xylan degrading enzymes (Saha, 2003).

Enzyme	**Mode of action**
Endo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
Exo-xylanase	Hydrolyzes mainly interior β-1,4-xylose linkages releasing xylobiose
β-Xylosidase	Releases xylose form xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidases	Hydrolyzes terminal nonreducing α-Arabinofuranose from arabinoxylans
α-Glucuronidases	Releases glucuronic acid from glucoronoxylans
Acetylxylan esterases	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterases	Hydrolyzes feruloylester bonds in xylans
p-Coumaric acid esterases	Hydrolyzes p-coumaryl ester bonds in xylans

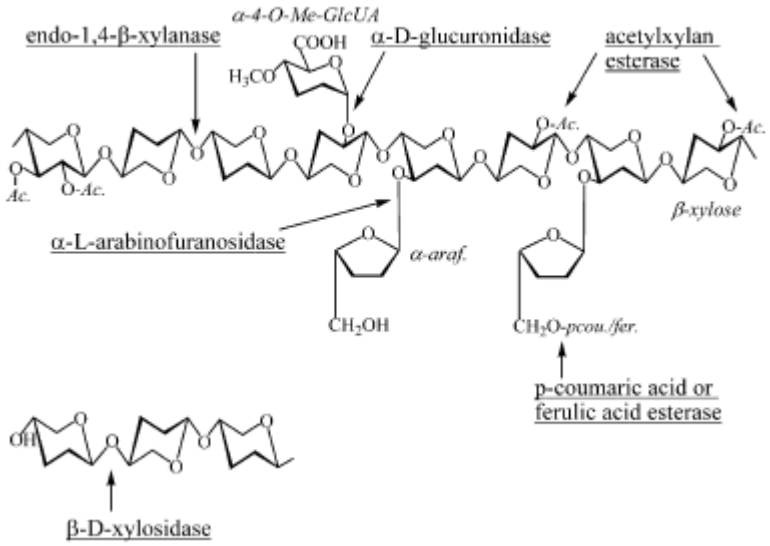


Figure 2.27 Enzymes involved in the hydrolysis of xylan and their mode of action (Collins et al., 2005).

Production of hemicellulases has been widely recognized among the bacteria, *Bacillus* species, *Acidobacterium* species, *Thermotoga* species and also fungi, *Trichoderma* species and *Aspergillus* species (Collins et al., 2005).

Though commercial cellulase cocktails do possess sufficient hemicellulase activity, supplementation of cellulases with xylanase will obviously serve benefits for cellulose hydrolysis. In addition to the production of hemicellulose derived sugars, utilization of xylanases during cellulose hydrolysis has been reported to enhance glucose yields since selective removal of xylan residues from biomass will evidently improve cellulose accessibility to cellulases (Yang and Wyman, 2008). In a previous study, the effects of xylanase supplementation (prior to cellulases) on glucose yields derived from AFEX, ARP, lime, controlled pH, SO_2, dilute acid, flow through pretreated poplar were reported (Kumar and Wyman, 2009b). The researchers found out that xylanase supplementation enhanced glucose yields for all types of pretreated poplar. However the effect of xylanase utilization was found to be less pronounced at increased cellulose/β-glucosidase loadings. Hemicellulose removal via xylanases prior to enzymatic hydrolysis is obviously attractive but contribution of the enzymes to the process costs has been high enough to limit additional enzyme utilization for cellulosic ethanol production.

Enzyme suppliers have introduced novel enzyme blends for laboratory and industrial use in order to reduce enzyme use and thus, contribution to the costs in cellulosic ethanol production. According to the technical data reported by Novozymes, 40% of cost reduction has been achieved with utilization of Cellic Ctec and Cellic Ctec2 in cellulosic ethanol production (Novozymes, Information sheet on Fuel Ethanol, 2013). Besides possessing high activities of cellulase and β-glucosidase, these products were found to contain very satisfactory hemicellulase activity and high protein concentrations (Alvira et al., 2011, Van Eylen et al., 2011). After introduction of Cellic Ctec and Cellic Ctec2 in the market in 2009 and 2010, respectively, the latest product Cellic Ctec3 has been reported to result with much better hydrolysis yield which is almost 1.5 times compared to its former version owing to its revised content; cellulase and β-glucosidase with increased activities and higher variety of hemicellulases (Novozymes, Application sheet on Cellic Ctec3, 2013).

2.7 Fermentation

Fermentation, in other words conversion of the sugars derived upon enzymatic hydrolysis to ethanol, is the third major step of cellulosic ethanol production. In this section, the discussions have been made regarding the biochemical aspects of the fermentation.

Fermentation is a biochemical conversion process which is conducted in the absence of oxygen. In fact, fermentation has been defined as energy generation without electron transport chain. The metabolic pathway shown in the figure represents the anaerobic reaction mechanisms conducted during ethanol production from glucose (Figure 2.28). In the absence of oxygen, pyruvate generated through glycolysis accepts hydrogen from NADH. The produced NAD^+ is then converted to NADH again with the formation of ethanol and CO_2 from pyruvate (Shuler and Kargi, 2002). Figure 2.29 points out how glycolysis is followed in the presence of oxygen, in other words how cellular respiration differs from fermentation.

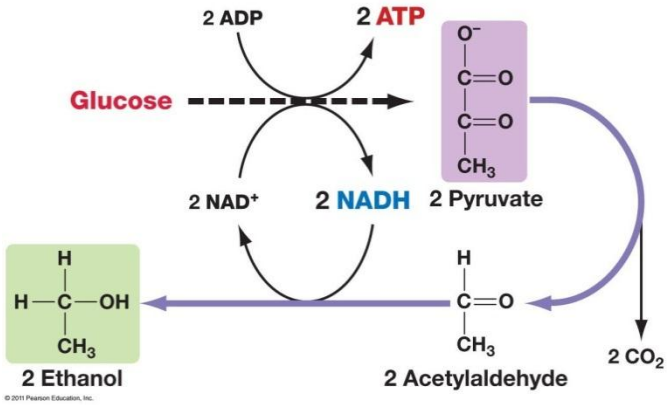


Figure 2.28 Ethanol fermentation in yeast and many bacteria (University of Illinois at Chicago, Lecture notes of BIOS 100 course, 2013).

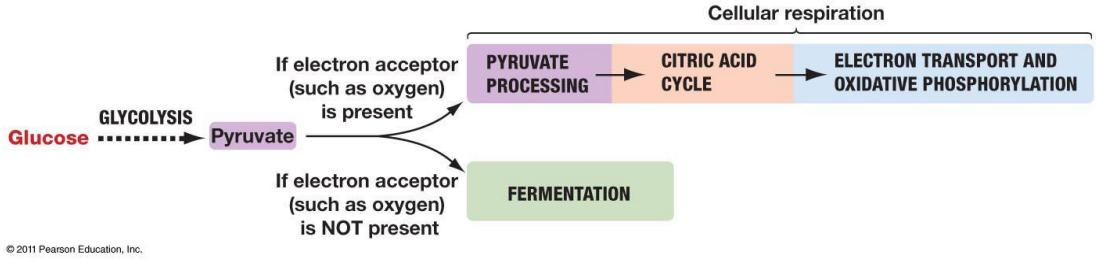


Figure 2.29 The difference between fermentation and respiration (University of Illinois at Chicago, Lecture notes of BIOS 100 course, 2013).

This straightforward mechanism given (Figure 2.28 and equation 2.2) accounts for the main reaction carried out by the yeast, *Saccharomyces cerevisiae* (Figure 2.30). The wild type *S. cerevisiae,* which metabolizes the hexoses in the hydrolyzate medium, has been the most utilized microorganism in production of ethanol from lignocellulosic feedstocks owing to its well-known properties.

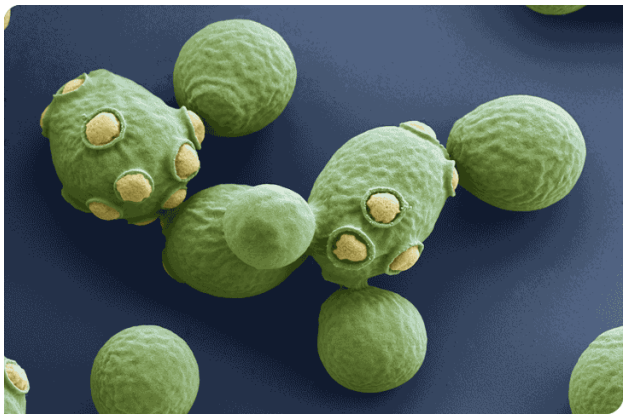


Figure 2.30 The yeast, *Saccharomyces cerevisiae* (Microbiology Online, 2013).

S. cerevisiae has been generally regarded as safe (GRAS). It is recognized for being one of the first eukaryotic organisms whose genome has been completely sequenced. It has been also shown to be more tolerant to the growth inhibitors released upon pretreatments which are conducted at severe conditions such as steam explosion. Besides *S. cerevisiae,* the typical yeast species that were found to produce ethanol are *Kluyveromyces* species, *Pichia* species and *Candida* species. Additionally the bacteria, *Escherichia* species and *Zymomonas* species have received interest due to their capability of fermenting pentoses and their employment as donor organisms which provide pentose metabolism in the yeast through metabolic manipulations (Mousdale, 2008).

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2 + 2\ ATP \qquad (2.2)$$

Based on the stoichiometry of the reaction given above, the theoretical ethanol yield over glucose is 0.51 g/g. Ethanol yields, which have been reported in the vast majority of literature studies, are calculated on the basis of this conversion factor.

2.7.1 Fermentation of lignocellulosic hydrolyzates

Due to the variations in the biomass composition, pretreatment conditions, sugar yields upon enzymatic hydrolysis; different approaches have been developed for conversion of lignocellulosic feedstocks to ethanol. These approaches have been recognized as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and co-fermentation (SSCF)

2.7.1.1 Separate hydrolysis and fermentation (SHF)

This is the most straightforward approach in which enzymatic hydrolysis and fermentation take place separately. Fermentable sugars, which are released upon enzymatic hydrolysis of the insoluble fraction of the pretreated biomass, are converted to ethanol in the following step, fermentation. Each step has to be conducted under its optimum conditions. Product inhibition will be the case in SHF since glucose and cellobiose released during hydrolysis will remain in the reaction medium without being consumed and thus, decrease the hydrolysis rates (Öhgren et al., 2007, Sánchez and Cardona, 2008). Besides, separation of the enzymatic hydrolyzate from the unhydrolyzed fraction of the pretreated biomass (lignin enriched) was found to result with sugar loss (Olofsson et al., 2008). Employment of fed-batch operation mode in SHF offers advantages over batch mode with respect to utilization of high substrate loading during enzymatic hydrolysis. Accordingly, it enables production of high glucose concentrations and thus high ethanol concentration during fermentation.

Vast majority of literature studies on cellulosic ethanol production involve SHF since it is much more convenient to be exploited in laboratory scales when compared to SSF. However, performing enzymatic hydrolysis and fermentation simultaneously offer advantages particularly with respect to process costs in large scale operations. Thus, SSF has been introduced and replaced the conventional SHF in large scale operations due to economic aspects.

2.7.1.2 Simultaneous saccharification and fermentation (SSF)

In simultaneous saccharification and fermentation, enzymatic hydrolysis and fermentation are conducted simultaneously under exactly the same conditions (Figure 2.31). The starting point of this type of fermentation is the product inhibition which was expressed by a group of researchers in 1976. The adverse effect of glucose and cellobiose on the cellulolytic enzymes produced by the fungus, *Trichoderma reseei* was realized and shown to be alleviated by SSF (Gauss et al., 1976, Olofsson et al., 2008). This major finding is one of the substantial advantages of SSF since glucose and cellobiose, which lowers hydrolysis rates, are consumed by the yeast simultaneously and converted to ethanol. One positive aspect of SSF is the detoxifying effect of the yeast. Adaptation of the yeast to the hemicellulose hydrolyzate prior to fermentation was shown to result with metabolization of the inhibitor compounds, which generate adverse effects on enzymatic hydrolysis, by the yeast during SSF (Öhgren et al., 2007).

The product inhibition has been a substantial issue but SSF introduces additional advantages with respect to process safety and costs. Such that employment of the same reactor for enzymatic hydrolysis and fermentation lowers the process costs. Utilization of the pretreated slurry entirely in the fermentation medium minimizes the sugar loss which was observed in the case of SHF. Thus, SSF has been reported to provide higher ethanol yields than SHF. Presence of ethanol at higher concentrations in the medium thereby alleviates the risk of contamination compared to SHF.

Despite the fact that it offers numerous benefits over SHF, maintaining identical operation conditions for both enzymatic hydrolysis and fermentation has been challenging since the optimized conditions for yeast growth are noticeably different compared to those for enzymatic hydrolysis. While enzymatic hydrolysis has been conducted at 50°C, the yeast prefers milder conditions for growth; such that 30°C was reported to be the optimal growth temperature of *S. cerevisiae* (Xiao, 2006). Together with the typical operation conditions (pH and temperature), substrate-related parameters play significant roles in SSF. Presence of lignin in the pretreated biomass was found to be

problematic for the separation of the yeast from the insoluble pretreated biomass and thus, for the reuse of yeast in the subsequent fermentation. Also substrate loading or in other words, concentrations of water insoluble solids (WIS) were reported to affect the ethanol yields and thus, this should not exceed certain limits (Öhgren et al., 2007).

In previously reported study, SHF and SSF conducted for steam pretreated corn stover at 8% loading were compared with respect to ethanol yields (Öhgren et al., 2007). SSF resulted with higher ethanol yields, which was almost 72%, compared to SHF (almost 59%) even in the presence of inhibitors. This major finding was linked to the alleviation of the inhibitory effects of the hydrolysis products and products derived upon degradation of hemicellulose during steam pretreatment such as acetic acid, furfural and HMF during SSF. Table 2.8 comprises the most recent studies reported on SSF of lignocellulosic biomass.

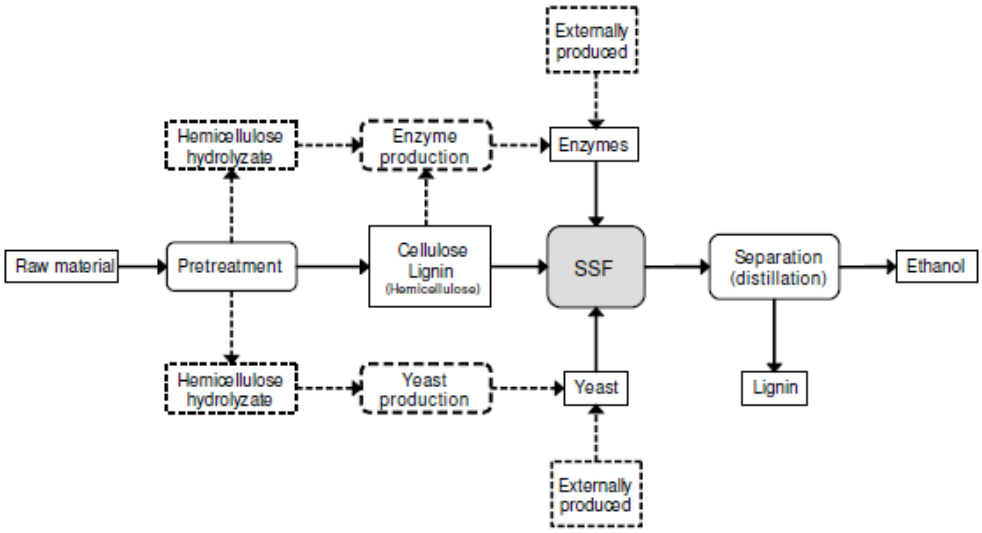


Figure 2.31 Schematic representation of an SSF process (Olofsson et al., 2008).

Table 2.8 SSF studies conducted for various lignocellulosic feedstocks

Raw material	Pretreatment type	Strain	Solid loading	Temperature	Ethanol concentration	Reference
Poplar, eucalyptus, wheat straw, sweet sorghum bagasse, Brassica carinata residue	Steam pretreatment	*Kluyveromyces fragilis*	10% (w/v)	42°C	16-19 g/L	Ballesteros et al. (2004)
Spruce	Steam pretreatment	*Saccharomyces cerevisiae*	10% (w/w)	37°C	45 g/L	Rudolf et al. (2005)
Salix	Steam pretreatment	*Saccharomyces cerevisiae*	11% (w/w)	37°C	33 g/L	Sassner et al. (2006)
Barley straw	Steam pretreatment	*Saccharomyces cerevisiae*	7.5 (w/w)	35°C	22 g/L	Linde et al. (2007)
Aspen	SPORL (Sulfite pretreatment to overcome recalcitrance of lignocellulose)	*Saccharomyces cerevisiae*	18% (w/v)	35°C	59 g/L	Zhu et al. (2011)
Aspen	Dilute acid	*Saccharomyces cerevisiae*	18% (w/v)	35°C	53 g/L	Zhu et al. (2011)

2.7.1.3 Simultaneous saccharification and co-fermentation (SSCF)

While only hexoses are converted to ethanol in SSF, pentoses are also fermented during simultaneous saccharification and co-fermentation (SSCF) in addition to hexoses. Accordingly, SSCF provides utilization of the hemicellulose derived sugars and production of higher ethanol concentrations (Olofsson et al., 2008). Though this strategy appears straightforward, the number of xylose fermenting microorganisms is limited. Conversion of xylose to ethanol by any microorganism depends on the possession of the genes responsible for xylose utilization. These genes encode the production of the enzymes, xylose reductase (XR) and xylitol dehydrogenase (XDH) which play significant roles in xylose metabolism. While XR is responsible for the conversion of xylose to xylitol, XDH catalyzes the reaction for conversion of xylitol to xylulose. Thus, the absence of the genes, XR and XDH in the wild type *Saccharomyces cerevisiae*, clarifies the incapability of the yeast to metabolize xylose. However, genetic engineering offers opportunities for utilization of xylose by the yeast. Such that *Escherichia coli*, *Zymomonas mobilis* and *Pichia stipitis,* which possess the genes encoding the aforementioned enzymes, are used for the expression of the xylose metabolism in the wild type yeast, *Saccharomyces cerevisiae* (Mousdale, 2008).

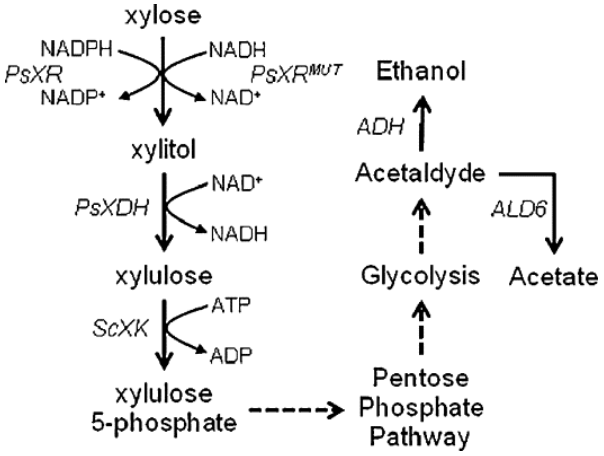


Figure 2.32 Metabolic pathways for xylose utilization (Lee et al., 2012).

Öhgren et al. (2006) employed the genetically modified *Saccharomyces cerevisiae* TMB3400 to co-ferment glucose and xylose in the production of cellulosic ethanol from corn stover. Co-fermentation of glucose and xylose present in the non-detoxified slurry derived upon steam pretreatment of corn stover by the genetically modified microorganism enhanced the overall ethanol yield in batch mode operation. The fed-batch operation mode provided utilization of higher substrate loadings and thus, production of higher ethanol concentrations, almost 40 g/L.

In another study, Rudolf et al. (2008) compared performances of the following xylose fermenting microorganisms, recombinant *S. cerevisiae* TMB3400 and wild-type *P. stipitis* CBS6054. The non-detoxified steam pretreated sugar cane bagasse was used at 5 to 7.5% of substrate loadings during SSF. *S. cerevisiae* TMB3400, which consumed all glucose and almost all xylose (88%) in the hydrolyzate at 5% substrate loading and 32°C under anaerobic conditions, yielded higher ethanol concentration (almost 21 g/L) compared to *P. stipitis* CBS6054. Whereas, *P. stipitis* CBS6054, which required micro aeration during SSF, resulted with almost 19 g/L of ethanol and 92% of xylose consumption at 7.5% substrate loading, 35°C and pH 6.

2.7.2 Fermentation of ionic liquid pretreated biomass

Though numerous studies have been reported on the potential advantages of ionic liquids in biomass processing, the research reported on conversion of the ionic liquid pretreated biomass to cellulosic ethanol is scarce. This should be attributed to the high cost of ionic liquids which obviously constraints implementation of this technology in commercial scales for now.

A few number of laboratory scale investigations on ethanol production from ionic liquid pretreated biomass were reported and found to derive promising findings. For instance, EMIMAc and BMIMCl pretreated wood samples were converted to ethanol in a recently reported study (Shaifei et al., 2013). EMIMAc pretreatment yielded better results such that, the ethanol yield, which was obtained upon fermentation of the spruce wood samples subjected to EMIMAc pretreatment at 120°C for 15 hours, was almost 81%. On the other hand, only 9% of ethanol yield was achieved upon fermentation of untreated spruce wood powder. In another study (Li et al., 2009), EMIMDEP (1-ethyl-3-methyl imidazolium diethyl phosphate) was employed for pretreatment of wheat straw at 130 °C for 30 min. The glucose in the hydrolyzate obtained upon hydrolysis of the pretreated biomass was converted to ethanol with a yield of 0.43 g/g glucose within 26 h.

Besides, inhibitory effect of ionic liquids on biological systems has been regarded as critical for employment of this technology in large scale production of cellulosic ethanol. Turner et al. (2003) demonstrated cellulase (from *Trichoderma reseei*) deactivation during hydrolysis in the presence of BMIMCl. They attributed the inhibitory effect of BMIMCl on cellulase activity not only to the high chloride ion concentrations in the hydrolysis medium and but also to the protein unfolding. In another study, MMIMDMP (1-3-dimethylimidazolium dimethyl phosphate) was shown to decrease the activity of the purified cellulolytic enzymes, endoglucanase, cellobiohydrolase and β-glucosidase and thus, lower the conversion of regenerated cellulose to glucose (Engel et al., 2012). Similarly, the effects of MMIMDMP, EMIMAc, BMIMAc and EMIMlactate on cellulase activity were monitored. Among MMIMDMP and EMIMlactate exhibited less adverse effect on the cellulose conversion yields. Cellulases from *Trichoderma reseei* preserved their activity in the presence of 40% (w/w) of either MMIMDMP or EMIM lactate in the hydrolysis buffer (Wolski et al., 2011). Furthermore, Y.Wang et al (2011) examined compatibility of enzymes with ionic liquids. They found out that commercial cellulolytic enzymes (Celluclast 1.5L and β-glucosidase) retained their activity up to 15% (v/v) of EMIMAc in the hydrolysis medium in which 91% of cellulose conversion was attained.

In addition to the unfavorable effects on cellulolytic enzymes, ionic liquids were reported to hold adverse effects on the yeast, *Saccharomyces cerevisiae* (Ouellet et al., 2011). The researchers showed that $EMIM^+$ cation has been the primary cause for growth inhibition of the yeast. Washing of the pretreated biomass to remove the residual ionic liquid was regarded as a reasonable solution, yet excessive water consumption would not be a cost saving approach. At this point, genetic engineering comes into play to obtain enzymes and microorganisms possessing higher tolerance towards ionic liquids. For instance, the thermophilic endoglucanases from *Thermatoga maritima* and *Pyrococcus horikoshii* were expressed in E. coli and showed higher tolerance to EMIMAc compared to industrially available cellulases from *Trichoderma viride* (Datta et al., 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All chemicals are analytical grade except otherwise stated and listed in Appendix A.1

3.2 Laboratory Equipment

The list of laboratory equipment used in the study is given in Appendix A.2.

3.3 Buffers and stock solutions

Preparation methods of the buffers, the stock solutions, DNS reagent and the ionic liquid, HEAF are given in Appendix B.

3.4 Enzymes

The cellulase cocktails, Celluclast 1.5L and Cellic Ctec2, which were provided by Novozymes (Bagsværd, Denmark), were used at a loading of 3% (v/v) during enzymatic hydrolysis of cotton stalks. Cellic Ctec2 is one of the recent products of the Danish company in the area of cellulosic ethanol production. Though both enzyme blends were found and reported to possess the following enzymes, cellulase, β-glucosidase and xylanase (Alvira et al., 2011, Canella et al., 2012), Cellic Ctec2 provided with more efficient conversion of the pretreated biomass to fermentable sugars compared to Celluclast 1.5L. As given in Table 3.1, Cellic Ctec2 exhibited 3-fold higher cellulase activity (225 FPU/ml) compared to Celluclast 1.5L (75 FPU/ml). It also possessed 2-fold higher total amount of protein compared to Celluclast 1.5L. Cellic Ctec2 also demonstrated much higher β-glucosidase activity, which has been essential for hydrolysis of cellobiose molecules to glucose, compared to Celluclast 1.5L (Canella et al., 2012). Additionally, xylanase activity of Cellic ctec2 was higher than that found for Celluclast 1.5L.

Table 3.1 Protein content and enzyme activities of Celluclast 1.5L and Cellic Ctec2 (a: Canella et al., 2012).

	Protein content (mg/ml)	Cellulase (FPU/ml)	β-glucosidase (U/ml)	Xylanase (U/ml)
Celluclast 1.5L	60	75	15[a]	45
Cellic Ctec2	120	225	2731[a]	60

3.5 Microorganism and culture media

The wild type yeast, *Saccharomyces cerevisiae* NRRL Y-132 supplied from USDA, ARS (United States Department of Agriculture, Agricultural Research Service) Culture Collection. The constituents of the culture media used for yeast growth and fermentation are given in Table 3.2. The yeast was incubated on petri plates containing YPD agar for 2 days in incubator at 30°C. One loop from the freshly grown microorganism agar plate was then transferred to liquid YPD medium of 50 ml where it was aerobically precultivated in Erlenmeyer flask of 250 ml placed in a shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) for 24 hours at 30°C and 150 rpm. The precultivated cultures were finally transferred to the fermentation media (fermentation medium adapted from Bawa, 2008) with 10% (v/v) inoculation for ethanol production in shaking incubator. The calibration curve for the yeast and other growth-related preliminary investigations are given in Appendix C.

Table 3.2 The culture media used for the growth and fermentation of S*accharomyces cerevisiae* NRRL Y-132

YPD agar		Liquid YPD		Fermentation	
Component	**Concentration (g/L)**	**Component**	**Concentration (g/L)**	**Component**	**Concentration (g/L)**
Yeast extract	10	Yeast extract	10	Yeast extract	10
Peptone	20	Peptone	20	Urea	6
Glucose	20	Glucose	20	$Na_2HPO_4.7H_2O$	3
Agar	20			KH_2PO_4	3
				$MgSO_4.7H_2O$	0.25
				$CaCl_2.2H_2O$	0.08

3.6 Pretreatments

3.6.1 Ionic liquid pretreatment

Ionic liquid pretreatment was carried out under different conditions throughout the study since one of the major tasks of this work was to perform an optimization for pretreatment conditions. For convenience, operation conditions for ionic liquid pretreatments are summarized in Table 3.3. All cotton stalks samples were dried at 105°C overnight prior to ionic liquid pretreatments.

Table 3.3 Conditions used for pretreatment of cotton stalks via ionic liquids

Part 4.1			**Part 4.2**			**Part 4.3**		
Ionic liquid	EMIMCl	Solvionic	**Ionic liquid**	EMIMCl	BASF	**Ionic liquid**	EMIMAc	BASF
				EMIMAc	BASF			
				AMIMCl	Solvionic			
				BMIMCl	Solvionic			
				HEAF	in-house synthesized			
Reaction vessel	50 ml beaker placed in silicon oil bath		**Reaction vessel**	50 ml beaker placed in silicon oil bath		**Reaction vessel**	250 ml round bottom vessel placed in silicon oil bath	
Equipment	Magnetic stirrer operated at 500 rpm		**Equipment**	Magnetic stirrer operated at 500 rpm		**Equipment**	Rotary evaporator operated at 150 rpm	
Particle size	≤0.15 mm		**Particle size**	≤2 mm		**Particle size**	≤2 mm	

In Part 4.1, cotton stalks, which were received from a local producer in Adıyaman, were milled to pass a 10 mesh (2 mm) screen. EMIMCl (≥ 98% purity) was purchased from Solvionic (Toulouse, France). Pretreatment of cotton stalks in EMIMCl was conducted in 50 ml glass vessels immersed in silicon oil placed on a digital magnetic stirrer equipped with a temperature sensor (RCT Basic Safety Control, IKA-Werke, Staufen, Germany) at open atmosphere under stirring at 500 rpm.

In Part 4.2, cotton stalks, which were received from a local producer in Adıyaman, were milled to pass a 10 mesh (2 mm) screen (except part 4.2.4 in which particle size effect was investigated). Ionic liquids, EMIMCl (≥ 95% purity) and EMIMAc (≥ 90% purity) were produced by BASF and obtained from Sigma-Aldrich. Ionic liquids, AMIMCl (≥ 98% purity) and BMIMCl (≥ 98% purity) were purchased from Solvionic (Toulouse, France). HEAF was synthesized according to previously reported procedure which is given in Appendix B with detail (Bicak, 2005). Pretreatment of cotton stalks in ionic liquids was conducted in 50 ml glass vessels immersed in silicon oil placed on a digital magnetic stirrer equipped with a temperature sensor (RCT Basic Safety Control, IKA-Werke, Staufen, Germany) at open atmosphere under stirring at 500 rpm.

In part 4.3, cotton stalks, which were received from a local producer in Adana, were milled to pass a 10 mesh (2 mm) screen. EMIMAc (≥ 90% purity) was produced by BASF and obtained from Sigma-Aldrich. Cotton stalks and EMIMAc were put together in a 250 ml round bottom vessel in which a glass rod was used to mix the slurry in order to ensure that EMIMAc wetted cotton stalks completely prior to the incubation. The round bottom flask was placed into the silicon oil bath of a rotary evaporator (RV 10 Digital, IKA-Werke, Staufen, Germany). The flask was rotated inside silicon oil bath at 150 rpm and under atmospheric pressure. This rotation did not provide any stirring effect for the cotton stalks incubated in EMIMAc; it was employed in order to obtain a homogeneous temperature distribution in the silicon oil bath.

Operation conditions used in Parts 4.5 and 4.6 were not included in Table 3.3 since investigations in the aforementioned parts involved comparison of the cotton stalks that were subjected to pretreatments under particular conditions. These particular conditions were described in the indicated parts.

The steps following the ionic liquid pretreatments and prior to the enzymatic hydrolysis were carried out according to the following procedure. After completion of pretreatment deionized water, which was at 10-fold higher mass than the mass of the ionic liquid, was added to terminate the reaction between cotton stalks and ionic liquid. Later on, this suspension was stirred for 30 minutes at room temperature under vigorous stirring and finally filtered through a filter paper in order to recover the pretreated cotton stalks. Pretreated cotton stalks were washed five times with water at the same amount, which was initially used for recovery of the pretreated biomass, to remove residual ionic liquid from pretreated cotton stalks since presence of ionic liquid would have an adverse effect on the subsequent enzymatic hydrolysis (Turner et al., 2003; Y. Wang et al., 2011). Lastly, samples were dried at 60°C for 16 hours and weighed to determine the solid recovery (%) obtained after pretreatment (Equation 3.1).

3.6.2 Alkaline pretreatment

For alkaline pretreatment (Part 4.4), 3 g of cotton stalks at a biomass loading of 10% (w/v) were incubated in NaOH at concentrations of 0.5%, 1% and 2% (w/v) in autoclave (Hiclave HVE-50, Hirayama, Saitama, Japan) at 121°C for 1 hour. At the end of the pretreatments, cotton stalks were washed with 300 ml of deionized water for three times and during the final wash, pH was adjusted to 4.8 via glacial acetic acid. Lastly, samples were dried at 60°C for 16 hours and weighed to determine the solid recovery (%) obtained after pretreatment (Equation 3.1).

All pretreatments were performed in duplicates. The solid recovery upon ionic liquid and alkaline pretreatments was determined according to the following equation:

$$SR\ (\%) = \frac{W_{PRT}}{W_{UT}} x100 \qquad (3.1)$$

where W_{PRT} is the weight of pretreated cotton stalks recovered after pretreatment (g) and W_{UT} is the weight of untreated cotton stalks subjected to pretreatment (g).

3.7 Enzymatic hydrolysis

Enzymatic hydrolysis of cotton stalks was carried under conditions described in Table 3.4. As it was the case in the previous part (3.6.1), enzymatic hydrolysis conditions were somewhat different in particular parts of the study since enzyme and substrate-related parameters were specifically investigated with the aim of increasing the product concentrations.

Table 3.4 Operation conditions used for enzymatic hydrolysis of cotton stalks

Parts	Substrate type	Substrate loading (w/v)	Enzyme type	Enzyme loading (v/v)	Reaction vessel	Equipment
4.1	EMIMCl pretreated cotton stalks	3%	Celluclast 1.5L	2	50 ml falcon	Water Bath
4.2	Ionic liquid pretreated cotton stalks	3%	Celluclast 1.5L and Cellic Ctec2	2	50 ml falcon	Water Bath
4.3	EMIMAc pretreated cotton stalks	3%	Cellic Ctec2	2	50 ml falcon	Shaking incubator at 150 rpm
4.4	Alkaline pretreated cotton stalks	3%	Cellic Ctec2	2	50 ml falcon	Shaking incubator at 150 rpm
4.5	EMIMAc and alkaline pretreated cotton stalks	3-15%	Cellic Ctec2	2	50 ml falcon	Shaking incubator at 150 rpm
4.6	EMIMAc pretreated cotton stalks	3% and 15%	Cellic Ctec2	2	50 ml falcon	Shaking incubator at 150 rpm

In Part 4.1, EMIMCl pretreated cotton stalks at a substrate loading of 3% (w/v) were enzymatically hydrolyzed via Celluclast 1.5L (Novozymes, Denmark) with 2% (v/v) loading for 72 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in water bath at 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

In Part 4.2, ionic liquid pretreated cotton stalks at a substrate loading of 3% (w/v) were enzymatically hydrolyzed with 2% (v/v) enzyme loading (except Part 4.2.9 in which enzyme-related effects were investigated) for 72 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in water bath at 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

In Part 4.3, EMIMAc pretreated cotton stalks at a substrate loading of 3% (w/v) were enzymatically hydrolyzed via Cellic Ctec2 (Novozymes, Denmark) with 2% (v/v) loading for 48 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 150 rpm and 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

In Part 4.4, alkaline pretreated cotton stalks at a substrate loading of 3% (w/v) were hydrolyzed via Cellic Ctec2 (Novozymes, Denmark) at 2% (v/v) loading for 48 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 150 rpm and 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

In Part 4.5, EMIMAc and alkaline pretreated cotton stalks at different substrate loadings were subjected to hydrolysis via Cellic Ctec2 (Novozymes, Denmark) with 2% (v/v) loading for 48 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 150 rpm and 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

In the last part (4.6), a comparison was made between EMIMAc pretreated cotton stalks at a substrate loading of 3% (w/v) and 15% (w/v) were subjected to hydrolysis via Cellic Ctec2 (Novozymes, Denmark) with 2% (v/v) loading for 48 hours. Cotton stalk samples immersed in 10 ml of 0.05 M citrate buffer at pH 4.8 were preincubated in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) at 150 rpm and 50°C for 20 minutes and later on, their hydrolysis was initiated by addition of the enzyme.

The enzymatic reactions were monitored for 24-72 hours by withdrawing 100 µl of samples at specific time intervals. The samples were incubated at 100°C for 5 minutes in order to stop the enzymatic reaction and finally, centrifuged at 10000 rpm for 5 minutes prior to analysis. The reducing sugars and glucose released during the enzymatic reaction were analyzed via DNS assay (Miller, 1959) (the details are given in Appendix D) and high performance liquid chromatography (HPLC), respectively. All assays were performed in duplicates.

The digestibility of cotton stalks was calculated on the basis of the reducing sugars released from the biomass subjected to hydrolysis via the following equation:

$$\text{Digestibility (\%)} = \frac{C_R}{C_S} \times 100 \qquad (3.2)$$

where C_R is the reducing sugar concentration in the enzymatic hydrolyzate (g/L) and C_S is the initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L).

Two glucose yields were defined for cotton stalks subjected to enzymatic hydrolysis. Glucose yield (%) was calculated on the basis of the theoretical maximum amount of glucose that could be obtained from the cellulosic portion of the pretreated cotton stalks via the following equation:

$$\text{Glucose yield (\%)} = \frac{C_G}{[C_S \times C_{PRT}\,(\%) \times 1.11]/100} \times 100 \qquad (3.3)$$

where C_S is the initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L), C_G is the glucose concentration obtained at the 48th hour of the hydrolysis and C_{PRT} (%) is the cellulose content of the pretreated cotton stalks. In literature, this yield was generally expressed as the percentage of the theoretical maximum glucose yield.

Overall glucose yield (%) was calculated on the basis of the theoretical maximum amount of glucose that could be obtained from the cellulosic portion of the untreated cotton stalks via the following equation:

$$\text{Overall glucose yield (\%)} = \frac{\text{SR (\%)} \times C_G}{C_S \times C_{UT}\ (\%) \times 1.11} \text{ x } 100 \qquad (3.4)$$

where SR (%) is the solid recovery after pretreatment, C_G is the glucose concentration obtained at the 48th hour of the hydrolysis (g/L), C_S is the initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L) and C_{UT} (%) is the cellulose content of the untreated cotton stalks. Conversion factor, 1.11 is included in both equations (3.3 and 3.4) owing to the water gain during conversion of cellulose to equivalent glucose.

3.8 Fermentation

The hydrolyzates obtained upon enzymatic hydrolysis of ionic liquid and alkali pretreated cotton stalks were utilized for ethanol production. pH of the hydrolyzates was adjusted to 6.2 via 10 M NaOH prior to fermentation. The hydrolyzates, which were inoculated with 10% (v/v) of a 24 hour precultivated *Saccharomyces cerevisiae* NRRL Y-132, were also supplemented with the following nutrients: 10 g/L yeast extract, 6 g/L urea, 3 g/L $Na_2HPO_4.7H_2O$, 3 g/L KH_2PO_4, 0.25 g/L $MgSO_4.7H_2O$, 0.08 g/L $CaCl_2.2H_2O$ (Table 3.1). Ethanol production was carried out in shaking incubator (Minitron, Infors AG, Bottmingen, Switzerland) for 24-96 hours at 30°C and 100 rpm. One ml samples were withdrawn at specific time intervals and centrifuged (4790xg, 10 minutes) at 4°C. The supernatants were monitored via HPLC for determination of glucose consumption and ethanol production during fermentation. Dry cell weight was determined directly from the absorbance of the samples withdrawn in which the absorbance was measured at 600 nm by a UV-visible spectrophotometer (Nicolet Evolution 100, Thermo Fisher Scientific Inc., USA) and converted to dry cell concentration (g/L) using a corresponding standard curve (Appendix C).

Similar to the glucose yields previously given in Part 3.6, two ethanol yields were defined for fermentation. Ethanol yield (%) was calculated on the basis of the theoretical maximum amount of ethanol that could be obtained from the glucose that was initially present in the fermentation media via the following equation:

$$\text{Ethanol yield (\%)} = \frac{C_E}{C_{G,i} x 0.51} \text{ x } 100 \qquad (3.5)$$

where C_E is the ethanol concentration obtained during fermentation (g/L), and $C_{G,i}$ is the initial concentration of glucose that is present in the fermentation media (g/L). 0.51 is the conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast. In literature, this yield was generally expressed as the percentage of the theoretical maximum ethanol yield.

Overall ethanol yield (%) was calculated on the basis of the theoretical maximum amount of ethanol that could be obtained from the cellulosic portion of the untreated cotton stalks via the following equation:

$$\text{Overall ethanol yield (\%)} = \frac{\text{SR (\%)} \times C_E}{[C_S \times C_{UT}\ (\%) \times 1.11 \times 0.51]/1.3} \times 100 \qquad (3.6)$$

where SR (%) is the solid recovery after pretreatment, C_E is the ethanol concentration obtained during fermentation (g/L), C_S is the initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L) and C_{UT} (%) is the cellulose content of the untreated cotton stalks. The correction factor, 1.3 is the dilution ratio for the hydrolyzate prior to being used as fermentation medium. Conversion factor, 1.11 is included in the equation owing to the water gain during conversion of cellulose to equivalent glucose. Furthermore, 0.51 is the conversion factor for glucose to ethanol based on the stoichiometric biochemistry of yeast (Equation 2-2).

3.9 The characterization of cotton stalks

Cotton stalks were screened via various characterization techniques at particular stages of the research with the aim of observing the structural changes in the biomass. These characterization techniques were described in the following sections.

3.9.1 Compositional analysis

Cotton stalks were examined in respect of the composition of the biomass prior to pretreatments and as well as following the pretreatments and enzymatic hydrolysis. The composition of the biomass was determined according to the National Renewable Energy Laboratory (NREL) laboratory analytical procedure (LAP) (Sluiter et al., 2008) in which two-step sulfuric acid hydrolysis was conducted to analyze the structural carbohydrates as cellulose and hemicellulose and also lignin as acid insoluble lignin (AIL) and acid soluble lignin (ASL) in cotton stalks. The following steps describe the procedure in detail.

1-Borosilicate glass filter crucibles were placed in an oven at 575±25°C for a minimum of four hours.
2-Cotton stalks were incubated in oven at 105°C for a minimum of four hours to attain a constant weight for the biomass.
3-0.3±0.01 g of cotton stalks and 3 ml of 72% (w/w) sulfuric acid were put together in a long test tube where a glass rod was used to ensure that cotton stalks were mixed with sulfuric acid completely. The tube was placed in a water bath where it was incubated at 30°C for one hour. During incubation, mixing was provided with the same glass rod at five to ten minutes of intervals without removing tube from the water bath.

4-Following the completion of the incubation in water bath, 84 ml of deionized water was added into the tube to dilute 72% (w/w) of sulfuric acid to 4% (w/w). The resulting solution was transferred into 100 ml of borosilicate glass bottle and autoclaved for 1 hour at 121°C.
5-Following the completion of the hydrolysis in autoclave, the bottle was removed and allowed to cool near to ambient temperature. After cooling, the hydrolyzate was vacuum filtered through the crucible, which was previously incubated at 575±25°C for a minimum of four hours and weighed prior to the filtration.
6-The filtrate, which would be further used for determination of structural carbohydrates (cellulose and hemicellulose) and also acid soluble lignin (AIL), was transferred into a 50 ml falcon. The residue obtained on the crucible was washed with deionized water gently without loss of any solid residue and incubated at 105°C for a minimum of four hours to attain a constant weight. This insoluble part represented the acid insoluble lignin portion of the cotton stalks. The percentage acid insoluble lignin (AIL) was determined according to the equation given below:

$$AIL\ (\%) = \frac{m_f - m_c}{m_{cs}} \times 100 \qquad (3.7)$$

where m_f is the weight of the crucible with acid insoluble lignin (g), m_c is the weight of the empty crucible (g) and m_{cs} is the initial weight of the dry cotton stalks (g).

7- One ml of filtrate was taken and used for determination of acid soluble lignin (ASL) in UV-visible spectrophotometer (Nicolet Evolution 100, Thermo Fisher Scientific Inc., USA). The measurement was conducted at 205 nm in a 1-cm light path quartz cuvette. 4% (w/w) of sulfuric acid was used as the reference blank and also for dilution of the hydrolyzate appropriately to attain an absorbance between 0.2-0.7. Acid soluble lignin (%) was determined according to the equation given below:

$$ASL\ (\%) = \frac{A}{a \times b} \times DF \times \frac{V}{m_{CS}} \times 29 \times 100 \qquad (3.8)$$

where A is the absorbance at 205 nm, DF is the dilution factor, V is the initial volume of 72% (w/w) of sulfuric acid solution (ml), m_{cs} is the initial weight of the dry cotton stalks (g), b is cell path length which is 1 cm and a is the absorptivity which is equal to 110 L/g.cm.

8-The rest of the filtrate (hydrolyzate) was used for determination of the structural carbohydrates present in cotton stalks via HPLC analysis. Prior to HPLC analysis, pH of the hydrolyzate was adjusted to 5-6 with calcium carbonate. pH adjustment of the hydrolyzate was carried out in a controlled manner in which pH was monitored to prevent any sudden increase in pH after addition of each loop of calcium carbonate. It was reported that an increase to a pH of above 9 would result with sugar loss (Sluiter et al., 2008). After adjusting pH of the samples properly, the sample was centrifuged at 6000 rpm for 5 minutes. The supernatant was decanted and filtered through a 0.2 µm filter before being monitored in HPLC. The cellulose (%) and hemicellulose (%) content of the cotton stalks were determined according to the equations 3.11 and 3.12, respectively.

3.9.2 High performance liquid chromatography (HPLC) analysis

In this study, HPLC analysis was conducted for the following reasons:

-To monitor the time courses of glucose production (g/L) during enzymatic hydrolysis and also, glucose consumption (g/L) and ethanol production (g/L) during fermentation.
-To determine the cellulose (%) and hemicellulose content (%) of the cotton stalks prior to pretreatments and as well as following the pretreatments and enzymatic hydrolysis.

The analysis was performed with Shimadzu LC-20A HPLC system (Kyoto, Japan) equipped with a BIORAD Aminex HPX-87H column (Hercules, CA, USA) operated at 55°C with a flow rate of 0.6 ml/min using 5 mM H_2SO_4 as the mobile phase. All samples were filtered through 0.2 µm filter before being analyzed, transferred to the HPLC vials and analyzed under the same operation conditions. Ultra-pure MilliQ deionized water, which had a conductivity of 18.2 MΩ.cm at room temperature, was utilized for dilution of the samples and also preparation of the standards. The calibration range of the standards; glucose, xylose and ethanol were 0.5-5 g/L. The standard calibration curves were obtained by plotting peak areas as a function of standard concentrations (g/L) and given in Appendix E. The concentrations of glucose (g/L), xylose (g/L) and ethanol (g/L) were determined considering the standard calibration curves and the area of the peaks obtained upon HPLC analysis of the samples. The concentrations of glucose (C_G), and ethanol (C_E) obtained either in enzymatic hydrolysis or fermentation were calculated using equations 3.9 and 3.10, respectively:

$$C_G = \frac{A_G}{S_G} \text{ x DF} \tag{3.9}$$

$$C_E = \frac{A_E}{S_E} \text{ x DF} \tag{3.10}$$

where A_G and A_E are the areas of the peaks obtained for glucose and ethanol present in the analyzed samples, respectively. S_G and S_E are the slopes (g^{-1}/L^{-1}) of the standard calibration curves obtained for glucose and ethanol, respectively (Appendix E). DF is the dilution factor.

The cellulose and hemicellulose content of the cotton stalks (%) were calculated using equations 3.11 and 3.12, respectively.

$$\text{Cellulose (\%)} = \frac{C_{G,C} \times V}{1.11 \times m_{CS}} \text{ x } 100 \tag{3.11}$$

$$\text{Hemicellulose (\%)} = \frac{C_{X,C} \times V}{1.14 \times m_{CS}} \text{ x } 100 \tag{3.12}$$

where $C_{G,C}$ and $C_{X,C}$ are glucose and xylose concentrations (g/L), respectively obtained upon HPLC analysis of the sample (the filtrate as described in Part 3.8.1) derived from two-step sulfuric acid hydrolysis of cotton stalks. V is the initial volume of 72% (w/w) of sulfuric acid solution (ml), m_{cs} is the initial weight of the dry cotton stalks (g) subjected to compositional analysis. The conversion factors, 1.11 and 1.14 are included in above equations due to the water gain during conversion of cellulose and hemicellulose to glucose and xylose, respectively.

The cellulose loss (%) and extracted lignin from the untreated cotton stalks (%) were calculated on the basis of the amount of cellulose and lignin present in the untreated cotton stalks and pretreated cotton stalks according to the equations given below:

$$\text{Cellulose loss (\%)} = \frac{C_{UT}(\%) - \frac{SR(\%) \times C_{PRT}(\%)}{100}}{C_{UT}(\%)} \times 100 \quad (3.13)$$

where C_{UT} (%) is the cellulose content of untreated cotton stalks, SR(%) is the solid recovery and C_{PRT} (%) is the cellulose content of the cotton stalk after pretreatment.

$$\text{Extracted lignin (\%)} = \frac{L_{UT}(\%) - \frac{SR(\%) \times L_{PRT}(\%)}{100}}{L_{UT}(\%)} \times 100 \quad (3.14)$$

where L_{UT} (%) is the lignin content of untreated cotton stalk, SR(%) is the solid recovery and L_{PRT} (%) is the lignin content of the cotton stalk after pretreatment (Haykir et al., 2013).

3.9.3 Scanning Electron Microscopy (SEM)

SEM images were obtained using a QUANTA 400F Field Emission SEM (Oregon, USA) operating at 20 kV. All samples were sputter coated with gold/palladium (Au/Pd) to provide conductivity prior to analysis. SEM images of all cotton stalk samples were taken at 2000X magnification.

3.9.4 X-ray Diffraction (XRD)

XRD analysis of the cotton stalk samples was conducted with Rigaku Ultima-IV Diffractometer (Japan) between 2Θ= 10-30° Bragg angles using Cu radiation at room temperature with a step size of 0.02° at a scanning speed of 1°/min.

3.9.5 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The infrared spectra of the samples were recorded with Bruker, Equinox 55 ATR-FTIR spectrometer (Massachusetts, USA) equipped with diamond-germanium ATR single reflection crystal. The samples were incubated in an oven at 60°C overnight prior to analysis and analyzed in powder form in the absorption band mode in the range of 4000-700 cm^{-1}.

3.10 Enzyme assays

The cellulase and xylanase assays conducted in order to define cellulase and xylanase activites of the enzymes, Celluclast 1.5L and Cellc Ctec2 are given in Appendix D.

CHAPTER 4

RESULTS AND DISCUSSION

Pretreatment of lignocellulosic biomass with ionic liquids as a novel and unique approach has been under intensive investigation for the last 10 years. From the time that Swatloski et al. (2002) demonstrated the dissolution of cellulose in BMIMCl (1-butyl-3-methyl imidazolium chloride), attempts were made to attain the most effective dissolution or pretreatment condition for conversion of the lignocellulosic biomass to value-added products. Though dissolution of cellulose and several types of lignocellulosic biomass in various ionic liquids has been of primary importance, recently reported studies have focused particularly on biomass pretreatment to enhance the enzymatic digestibility of the lignocellulosic feedstocks to fermentable sugars. Similarly in this study, cotton stalks were subjected to ionic liquid pretreatment to improve the enzymatic accessibility of the biomass with the aim of ethanol production. This novel pretreatment technique was assessed with respect to its effects on the structural changes derived in cotton stalks and especially, enzymatic accessibility of the biomass. To attain the most suitable condition that provided conversion of cotton stalks to ethanol effectively, several parameters were investigated. Not only pretreatment conditions were assessed, but also enzymatic hydrolysis and fermentation related parameters were evaluated.

The results were interpreted in six major parts as summarized in Figure 4.1. The first part covered preliminary studies that were employed to understand the interaction of cotton stalks with ionic liquids. Cotton stalks were subjected to EMIMCl pretreatment in which the variations in solid recovery and digestibility upon pretreatment and enzymatic hydrolysis were monitored with respect to the pretreatment conditions. EMIMCl was utilized due to its low cost and available amount compared to the more costly EMIMAc. Though EMIMCl was not effective to improve the digestibility of cotton stalks, the findings were useful to estimate appropriate ranges for the major parameters providing higher solid recovery and digestibility for cotton stalks.

In the second part (4.2), cotton stalks were subjected to EMIMAc pretreatment for production of cellulosic ethanol after ending up with the conclusion that EMIMAc introduced promising findings in respect of the enzymatic digestibility of cotton stalks to fermentable sugars. The common techniques that have been employed for characterization of the lignocellulosic biomass; SEM, XRD and ATR-FTIR were also employed to clarify the structural changes in the cotton stalks upon pretreatment. After selecting the most appropriate pretreatment and hydrolysis conditions that resulted with the highest glucose concentration, fermentation was carried out. The hydrolyzate, which was derived upon enzymatic hydrolysis of the EMIMAc pretreated cotton stalks, was utilized by the wild type yeast, *Saccharomyces cerevisae* NRRL Y-132 for ethanol production. High glucose and ethanol yields were obtained at each step, enzymatic hydrolysis and fermentation, respectively. However the yields, which were determined on the basis of the theoretical maximum amount of glucose and ethanol that could be obtained from cellulosic portion of the untreated cotton stalks, were low due to the considerable amount of cellulose lost during EMIMAc pretreatment, which was conducted at 10% of biomass loading with stirring. Accordingly, an alternative solution was offered in the following part; EMIMAc pretreatment was conducted at higher biomass loadings with the aim of decreasing the cellulose loss during pretreatment.

In the third part, the effect of higher biomass loadings on the structure and enzymatic accessibility of cotton stalks was examined. It was found that employment of EMIMAc pretreatment at higher biomass loadings without stirring was more advantageous compared to the pretreatment conducted at a biomass loading of 10% (w cotton stalks/w EMIMAc) with stirring. It was possible to enhance the enzymatic accessibility of the biomass only through disrupting the crystalline structure of the cotton stalks without resulting with noticeable changes in the composition of the biomass. By this way, much higher cellulose recoveries and higher glucose yields, considering the cellulosic content of the untreated cotton stalks, were obtained.

In the fourth part, cotton stalks were subjected to alkaline pretreatment which was recognized as a promising method that improved the biomass accessibility to enzymatic attack by means of removing lignin and hemicellulose. The changes in the structure and enzymatic digestibility of the cotton stalks with NaOH concentration was monitored in order to decide on the most suitable condition that provided the highest enzymatic digestibility of the biomass.

In the fifth part, a comparison was made between ionic liquid and alkaline pretreated cotton stalks regarding their digestibility at higher substrate loadings together with their conversion to ethanol. Besides, structural changes in the cotton stalks were also taken into account in order to present advantages of ionic liquid pretreatment over the conventional method, alkaline pretreatment.

In the last part of the study, ethanol production from EMIMAc pretreated cotton stalks was assessed with respect to the changes in biomass and substrate loadings in pretreatment and enzymatic hydrolysis, respectively. It was demonstrated that pretreatment conditions that were exploited for EMIMAc pretreatment were of vital importance for effective conversion of cotton stalks to cellulosic ethanol.

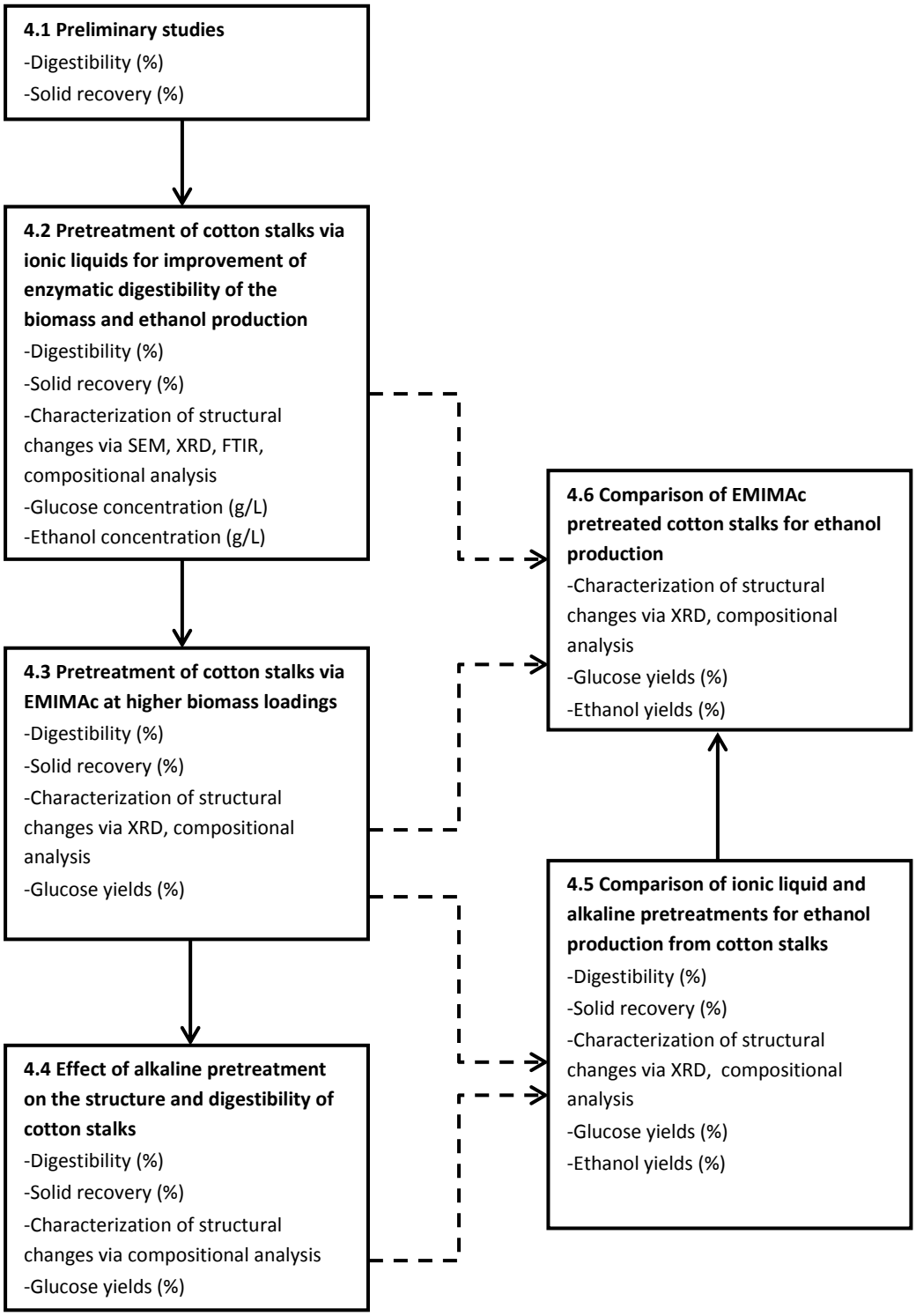


Figure 4.1 Major steps followed throughout the study

4.1 Preliminary studies

The preliminary experiments were conducted to understand the interaction of ionic liquids with cotton stalks with respect to their effects on the enzymatic digestibility and solid recovery. The variables evaluated are summarized in the Table 4.1. As indicated, the effects of pretreatment temperature, pretreatment period, biomass loading, antisolvent type and ionic liquid recycling were evaluated for EMIMCl pretreated cotton stalks having particle size of ≤0.15 mm prior to pretreatment. On the other hand, the effects of laccase supplementation during hydrolysis and particle size were investigated for EMIMAc pretreated cotton stalks. The major reason for investigating the effects of the variables in two different reaction media is the inadequate amount of EMIMAc which would not be sufficient to carry out the complete analysis. Instead, the less expensive ionic liquid, EMIMCl was utilized for the majority of the analysis.

Table 4.1 The parameters and their ranges investigated for the preliminary experiments

Ionic liquid	Parameters	Ranges
EMIMCl	Pretreatment temperature	80°C-120°C-150°C
	Pretreatment period	1h- 2h
	Biomass loading	5%-10% (w biomass/w IL)
	Antisolvent type	Water, ethanol, methanol, acetone
	EMIMCl recycling	-
	Particle size	≤0.15 mm
EMIMAc	Laccase supplementation	5% (v/v) laccase

4.1.1 Effect of pretreatment temperature on the digestibility of cotton stalks

Effect of pretreatment temperature on the digestibility of the cotton stalks were investigated at the following conditions: cotton stalks with particle size of <0.15 mm were incubated in EMIMCl at a biomass loading of 5% (w biomass/w IL) at 80°C, 120°C and 150°C for 2 hours. Solid recovery and digestibility attained at the 24^{th} hour of the enzymatic hydrolysis for the pretreated and untreated cotton stalks are shown in Table 4.2. As shown in Table 4.2, digestibility of the cotton stalks increased 2-fold with an increase in temperature from 120°C to 150°C. However, that increase in pretreatment temperature was shown to decrease the solid recovery dramatically; from 75% to 20%. The increase in pretreatment temperature from 80°C to 120°C did not have any noticeable impact on either solid recovery or digestibility of the cotton stalks.

Despite exhibiting superior properties compared to the conventional solvents, high viscosity of ionic liquids has been considered as a drawback during pretreatment. The most common approach to reduce the viscosity of the reaction mixture has been to increase the temperature of the slurry which would facilitate the diffusion of the IL into the biomass structure. The viscosities of the different ionic liquid-cellulose solutions, which were strongly linked to the cations and anions that ILs are made up of, have been investigated. In a previously reported study, the zero shear viscosity (the viscosity at rest) of cellulose-imidazolium based ionic liquid mixtures at 85°C was reported in which chloride based ILs were shown to have nearly 5-10 fold higher viscosity compared to acetate based ILs (Kosan et al., 2008). It has been shown that viscosity of the cellulose-IL mixtures was a strong function of the pretreatment temperature. The viscosities of the cellulose-IL solutions containing EMIMAc and BMIMCl were shown to decrease with an increase in temperature in which the viscosity of BMIMCl-cellulose solution was 5-6 fold higher than EMIMAc-cellulose solution (Sescousse et al., 2010). In another study, the viscosities of EMIMAc-cellulose solutions were reported to be directly related to the dissolution temperature in which higher dissolution temperatures accelerated the dissolution of cellulose in EMIMAc via the rapid decrease in viscosity of the solution (Cruz et al., 2012). According to the previous findings, pretreatment temperature should climb as high as 120°C with the aim of attaining reduced viscosity and thus more effective conditions for pretreatment. However, as indicated in the present study higher temperatures would have adverse effects on the solid recovery upon pretreatment. At elevated temperatures, the ionic liquid would behave more like a dissolution agent rather than a pretreatment agent which would result with a significant degradation of the polysaccharides, especially cellulosic portion of the untreated biomass (Sun et al., 2009, Vitz et al., 2009). Despite the fact that no compositional analysis has been carried out for the pretreated samples in order to confirm the degradation of carbohydrate-based materials, 20% of solid recovery obtained upon EMIMCl pretreatment at 150°C, which was critically low, obviously linked to the degradation of the biomass. But as a starting approach, 150°C has been considered as the most appropriate pretreatment temperature for EMIMCl since digestibility should be considered as a more vital parameter compared to solid recovery at this elementary level.

Table 4.2 The variation of solid recovery and digestibility of cotton stalks with respect to the pretreatment temperature.

Pretreatment temperature	Solid recovery(%)	Digestibility(%)
80°C	76	6
120°C	75	6
150°C	20	11
Untreated CS	100	3

4.1.2 Effect of pretreatment period on the digestibility of cotton stalks

Pretreatment period has been regarded as one of the key parameters which determined the function of the ionic liquid for biomass processing whether it was utilized as a dissolution agent or pretreatment agent. Obviously, the longer the pretreatment was carried out, the higher the amount of biomass that is dissolved in an ionic liquid. However, the pretreatment of the biomass in ionic liquids should be carried out in a controlled fashion such that minimal carbohydrate degradation would be observed. For this purpose, the effect of pretreatment period was assessed with the aim of enhancing the digestibility of cotton stalks while minimizing the loss of cellulosic portion of cotton stalks.

The effect of pretreatment period was investigated at three different temperatures as given in the previous part (80°C, 120°C and 150°C) regarding the combined effect of both pretreatment temperature and period on the digestibility and solid recovery for the cotton stalks. At each temperature, the variation of pretreatment period from 1 h to 2h was found to influence the solid recovery obtained upon pretreatment (Table 4.3). The solid recoveries were found to decrease with an increase in pretreatment period. The highest solid recovery was attained as 83% for the cotton stalks subjected to EMIMCl pretreatment at 80°C for 1 hour. While the solid recovery was found to increase nearly 2-fold (from 20% to 37%) with a decrease in pretreatment period from 2 h to 1 h at 150°C, the digestibility of cotton stalks increased nearly 3-fold. This result implied that longer pretreatment period not only lowered the solid recovery but also decreased the release of fermentable sugars due to its disruptive effect on the biomass. For the samples pretreated at 80°C and 120°C, the effect of pretreatment period on the enzymatic digestibility was insignificant in which digestibility ranged between 4 and 6%.

Table 4.3 The variation of solid recovery and digestibility of cotton stalks with respect to the pretreatment period.

Pretreatment temperature	**Pretreatment period**	**Solid recovery(%)**	**Digestibility(%)**
80°C	1 hr	83	4
	2 hr	76	6
120°C	1 hr	74	6
	2 hr	75	6
150°C	1 hr	37	27
	2 hr	20	11
Untreated		100	3

The drastic effect of pretreatment temperature on solid recovery could be also observed from the given results. As temperature increased from 120°C to 150°C, the solid recoveries were found to decrease nearly 2-fold. Although the pretreatment carried out at 150°C resulted with a considerable decrease in solid recovery, the digestibility of cotton stalks subjected to EMIMCl pretreatment for one hour was increased 9-fold compared to the digestibility of untreated samples. One could conclude that, 150°C and 1 hour of pretreatment temperature and period could be chosen to further continue with investigation of the parameters with respect to their effects on the solid recovery and digestibility of cotton stalks.

4.1.3 Effect of biomass loading on the digestibility of cotton stalks

In the early years of this technology, the reaction between cellulose and ionic liquids was carried out at lower loadings with the aim of dissolving the cellulose completely to produce regenerated cellulose (Swatloski et al., 2002; Fort et al., 2007; Fukaya et al., 2008). Generally, biomass loadings were reported to change from 1% to 5% (w cellulose/w solution). In the later years, the studies on cellulose dissolution were followed by ionic liquid pretreatment of the lignocellulosic biomass in which the reaction between the ILs and biomass were conducted at higher biomass loading up to 10%. (S.H. Lee et al., 2009, Arora et al., 2010, Nguyen et al., 2010, Samayam and Schall, 2010, Shill et al., 2011, Li et al., 2011). Interestingly, a recently reported study showed that biomass loading as high as 50% (w biomass/w IL) could also work for an effective pretreatment in which enhanced hydrolysis yields were achieved for corn stover upon its pretreatment via EMIMAc at 50% of biomass loading (H.Wu et al., 2011).

As the pretreatment has been the focus of the present study, the effect of biomass loading was investigated at 5% and 10% (w biomass/w IL) for the cotton stalks having particle size of <0.15 mm that were subjected to EMIMCl pretreatment for 1 hour at 80°C, 120°C and 150°C.

Table 4.4 The variation of solid recovery and digestibility of cotton stalks with respect to the biomass loading

Pretreatment temperature (°C)	Biomass Loading (%)	Solid recovery (%)	Digestibility (%)
80	5	83	4
	10	86	6
120	5	74	6
	10	82	8
150	5	37	27
	10	78	25
Untreated		100	3

As shown in Table 4.4, the effect of biomass loading on solid recovery at each temperature showed the same trend: solid recoveries increased as the biomass loading increased from 5% to 10%. The impact of biomass loading on the solid recovery upon EMIMCl pretreatment was shown to be more pronounced at 150°C in which solid recovery increased 2-fold, from 37% to 78% with an increase of biomass loading from 5% to 10%. Biomass loading exhibited less effect on the digestibility of the cotton stalks upon EMIMCl pretreatment at 150°C than it did on solid recovery. Similarly, the digestibility of cotton stalks upon EMIMCl pretreatment at 80°C and 120°C was not enhanced with an increase in biomass loading. These results indicated that increasing the biomass loading from 5% to 10% only had noticeable effect on the solid recovery obtained after EMIMCl pretreatment at 150°C for 1 hour. In conclusion, EMIMCl readily served as a pretreatment agent at 10% of biomass loading, in which pretreated cotton stalks were almost 7-fold more digestible than untreated cotton stalks.

Up to this point, the effects of pretreatment temperature, pretreatment period and biomass loading were assessed with respect to changes in solid recovery and digestibility obtained upon EMIMCl pretreatment of cotton stalks. Based on the reported results, the most appropriate values of pretreatment temperature, pretreatment period and biomass loading were determined as 150°C, 1 hour and 10% (w biomass/w IL), respectively. Although, EMIMCl has been known as an effective ionic liquid for cellulose/lignocellulose processing (Kosan et al., 2008, Vitz et al.,2009, Zavrel et. al., 2009), it was believed that it would not suffice as a pretreatment agent due to the enzymatic digestibility which was obtained almost 25%. For this reason, other potential ILs would be under investigation in respect of their effects on the enzymatic digestibility of cotton stalks.

One important parameter that influenced the solid recovery and hence, digestibility of cotton stalks was believed to be the particle size of the biomass, since diffusion of ionic liquids through the interior parts of biomass and their capability to have an effective interaction with the biomass was related to the particle size of the biomass (Kilpaleinen et al., 2007, Sun et al., 2009, Bahcegul et al., 2012b). Though particle size reduction was expressed as a prerequisite to facilitate more effective pretreatment and enzymatic hydrolysis for the biomass, it would apparently increase the energy requirements in the process (Yang and Wyman, 2008, Zhu and Pan, 2010). As smaller sized particles were used, the easier the ionic liquids interacted with the interior structure of the cotton stalks. This might appear as an advantage but it would increase the degradation of the cellulosic portion of the cotton stalks and thereby decrease the solid recovery upon pretreatment. Regarding the specified issues above, sieve analysis was conducted to obtain the particle size distribution of the cotton stalks. The sample from each sieve was weighed, and particle size was expressed as a percentage of the total weight of the sample (Table 4.5). According to the analysis, the cotton stalks with a particle size of 0.3-1.0 mm constituted 63.5% as the largest fraction while those with a particle size of <0.15 mm constituted only 12.5% of the total weight of the sample. The analysis was further continued with the cotton stalks with a particle size of 0.3-1.0 mm since their utilization would provide higher enzymatic digestibility and solid recovery compared to those observed for the cotton stalks having particle size of <0.15 mm.

Table 4.5 Particle size distribution of cotton stalks (1 mesh is the number of openings in one inch of screen of sieve)

Mesh	mm	% weight
18	1	10,1
25	0,71	26,6
50	0,3	36,9
100	0,15	13,9
<100	0,15	12,5

4.1.4 Effect of IL type on the digestibility of cotton stalks

Cotton stalks with a particle size of 0.3-1.0 mm at biomass loading of 10% (w biomass/w IL) were incubated in 1-allyl-3-methylimidazolium chloride (AMIMCl), 1-ethyl-3-methylimidazolium chloride (EMIMCl) and 1-ethyl-3-methylimidazolium methyl phosphonate (EMIMMP) at 150°C for 1 hour and also in 1-ethyl-3-methylimidazolium acetate (EMIMAc) at 150°C for half an hour. The ionic liquids except EMIMAc were received from Solvionic (Toulouse, France) and EMIMAc was synthesized in-house. EMIMAc, which has been previously reported to exhibit superior solvation capability towards cellulose/lignocellulosic biomass compared to other typical ionic liquids, was employed for a shorter pretreatment period to prevent cellulose degradation. As expected, the lowest solid recovery was obtained for the cotton stalks subjected to EMIMAc pretreatment at a biomass loading of 10% and 150°C for 30 minutes (Table 4.6). Unlike solid recovery, cotton stalks that were subjected to EMIMAc pretreatment were more digestible than those subjected to the pretreatment via other ionic liquids. The highest digestibility was obtained as 68% at the 24^{th} hour of the enzymatic hydrolysis of EMIMAc pretreated cotton stalks which was almost 20-fold higher compared to the digestibility of untreated cotton stalks. Considering the effects of other ILs on the digestibility of cotton stalks, EMIMCl yielded better than AMIMCl and EMIMMP in which AMIMCl and EMIMMP pretreated cotton stalks resulted with digestibility of 16% and 11%, respectively.

Table 4.6 The variation of solid recovery and digestibility of cotton stalks with IL type

Ionic liquids	Pretreatment period	Solid recovery (%)	Digestibility (%)
EMIMCl	1 h	78	25
AMIMCl	1 h	82	16
EMIMMP	1 h	74	11
EMIMAc	30 min	69	68
Untreated		100	3

Despite the fact that commercial ionic liquids, AMIMCl, EMIMCl and EMIMEP were manufactured at higher purity compared to EMIMAc in which its synthesis was conducted in-house, the results obtained upon EMIMAc pretreatment were strongly encouraging. Similarly, EMIMAc was reported to exhibit superior properties towards lignocellulosic biomass compared to other typical ionic liquids (S.H. Lee et al., 2009, Bahcegul et al., 2012b, Haykir et al., 2013).

4.1.5 Effect of laccase treatment on the digestibility of EMIMAc pretreated cotton stalks

As an alternative and environmentally friendly approach for lignin removal, laccase treatment was reported to be carried out under energy efficient conditions and generate less toxic compounds compared to the conventional techniques (Moreno et al., 2012). Therefore, it was attempted to investigate the effect of laccase on the digestibility of EMIMAc pretreated cotton stalks. Laccase treatment was conducted for the cotton stalks that were subjected to EMIMAC pretreatment at a biomass laoding of 10% and 150°C for 30 minutes prior to enzymatic reaction via Celluclast 1.5L. The commercial laccase, Novozymes 51003 was used at an enzyme loading of 5% (v enzyme/v buffer) in 0.05 M citrate buffer (pH 4.8) at 60°C for 48 hours. As a control, untreated cotton stalks were also treated via laccase prior to enzymatic hydrolysis under the same conditions. The effect of laccase treatment on the solid recovery and digestibility of EMIMAc pretreated cotton stalks and untreated cotton stalks were given in Table 4.7.

Table 4.7 The effect of laccase treatment on the solid recovery and digestibility of EMIMAc pretreated cotton stalks and untreated cotton stalks

	Laccase treatment	Solid recovery (%)	Digestibility(%)
EMIMAc	No laccase treatment	69	68
EMIMAc	Laccase treatment	69	78
Untreated	No laccase treatment	100	3
Untreated	Laccase treatment	100	11

As shown, laccase treatment of EMIMAc pretreated cotton stalks prior to hydrolysis resulted with an increase in digestibility from 68% to 78%. For the untreated cotton stalks, digestibility increased almost 4-fold with laccase treatment.

A previously reported study showed that laccase treatment before and after hydrolysis affected the release of fermentable sugars from steam-exploded wheat straw in a different manner. The products formed upon laccase treatment, which was carried out before hydrolysis, inhibited the action of celluloytic enzymes and adversely affected the release of fermentable sugars upon hydrolysis. However, laccase treatment after hydrolysis not only increased the release of fermentable sugars but also enhanced the ethanol yields from steam-exploded straw (Jurado et al.,2009). Contrarily, another study demonstrated that laccase treatment before hydrolysis improved the enzymatic hydrolysis of steam exploded softwood (Palonen and Viikari, 2004). When compared to the improvements in digestibility of biomass via laccase treatment in the referred studies, increase in the digestibility of EMIMAc pretreated cotton stalks obtained in the present study was trivial. In fact, laccase treatment was mostly employed with the aim of detoxifying the soluble part of the product obtained upon pretreatment, which obviously contains the inhibitory compounds derived from lignin, in the cases that soluble part derived upon pretreatment was subjected to the hydrolysis with the insoluble parts (Chandel et al.,2007, Jurado et al.,2009).

Contrarily, only the insoluble part of the product upon EMIMAc pretreatment was subjected to hydrolysis in the current study since the soluble part was the recovered aqueous solution of EMIMAc. Futhermore, the insoluble part in other words, pretreated cotton stalks were intensively washed with water to prevent the inhibitory effect of any degradation product for the subsequent steps, enzymatic hydrolysis and fermentation. Therefore, laccase treatment was not essential for the current case.

4.1.6 Effect of antisolvent type and IL recycling on the digestibility of EMIMCl pretreated cotton stalks

Besides demonstrating peculiar features in biomass processing, ionic liquids have received attention due to the convenience in their recycling and recovery of the pretreated biomass. The selection of an appropriate antisolvent type has been regarded as a key parameter with respect to its effect on the recovery of the pretreated biomass upon pretreatment and the reuse of the ILs.

Employment of alternative types of antisolvents has been under research in order to extract the remaining biomass components from the aqueous ionic liquid solutions after recovery of the pretreated biomass. Researchers put an emphasis on the recovery of lignin residues from the IL solutions (Sun et al., 2009, Tan et al., 2009, Fu et al., 2010). Previous researches indicate that significant levels of lignin extraction were achieved via employment of organic solvents and alkaline solutions during the recycling of ionic liquids (Sun et al., 2009, Tan et al., 2009, Fu et al., 2010).

Though lignin extraction has been the focus of utilization of alternative solvents during recovery and recycling of ionic liquids in the previously reported studies, the antisolvent effect was monitored in order to enhance solid recovery upon pretreatment and facilitate EMIMCl recycling. The following antisolvents, water, ethanol, methanol, acetone/water mixture (1:1, v/v) were screened in the view of solid recovery and digestibility of cotton stalks after pretreatment. Cotton stalks were subjected to EMIMCl pretreatment (received from BASF) at 10% of biomass loading and 150°C for 1 hour and the indicated antisolvents were used for the recovery of cotton stalks subjected to EMIMCl pretreatment.

The solid recovery upon EMIMCl pretreatment and biomass digestibility obtained at the 24th hour of the enzymatic hydrolysis for EMIMCl pretreated cotton stalks are given in Table 4.8. As observed from the table, solid recovery and digestibility did not appear to be affected significantly from antisolvent type. The solid recovery achieved upon EMIMCl pretreatment was found to range from 88% to 92%. Similarly, the digestibility of EMIMCl pretreated cotton stalks was unaffected from the antisolvent type. These slight changes in the digestibility and solid recovery could be ignored due to the environmental issues since water is easier and safer to use when compared to other types of antisolvents distinguished under the name, volatile organic chemicals. Meanwhile, exploitation of water as antisolvent will obviously bring significant profits in terms of process costs.

Table 4.8 The effect of antisolvent type on the solid recovery and digestibility of EMIMCl pretreated cotton stalks

Antisolvent type	Cellulose recovery(%)	Digestibility (%)
Ethanol	89	8
Methanol	88	10
Water	92	9
Acetone:Water (1:1, v/v)	89	10

One interesting point was that EMIMCl received from BASF yielded higher solid recoveries and lower digestibility values compared to those obtained from EMIMCl received from Solvionics (Part 4.1.3). This implied that EMIMCl received from BASF was not as effective as the one received from Solvionics.

During EMIMCl recycling, the slurry containing pretreated cotton stalks, EMIMCl and antisolvent was filtered through coarse filter paper to recover the pretreated cotton stalks. The filtrate that consisted EMIMCl and the antisolvent was incubated in oven at 80°C until all the antisolvent was evaporated and a residue precipitated was obtained. Later, the recovered EMIMCl was filtered through blue ribbon (2 µm) filter paper for 2 times to make sure that all the precipitate was removed from the recycled ionic liquid. After using the recycled EMIMCl for pretreatment under the same operation conditions as conducted in the previous analysis, the same antisolvents were used to recover the pretreated cotton stalks.

As shown in Table 4.9, solid recovery upon pretreatment and digestibility of cotton stalks at the 24th hour of hydrolysis were found to remain the same or increase slightly due to the accumulation of the unrecovered biomass components. These results revealed that EMIMCl was capable of retaining its effectiveness as a pretreatment agent with respect to its effect on the solid recovery and the digestibility. Further investigations comprising the impact of ionic liquid recycling on the structural properties of biomass and the efficiency of ionic liquids with an increase in the number of recycling would be carried out in the following parts of the study.

Table 4.9 The effect of recycling and antisolvent type on the solid recovery and digestibility of pretreated cotton stalks with recycled EMIMCl.

	Non recycled EMIMCl	Recycled EMIMCl	Non recycled EMIMCl	Recycled EMIMCl
Antisolvent type	**Cellulose recovery (%)**	**Cellulose recovery (%)**	**Digestibility (%)**	**Digestibility(%)**
Ethanol	89	91	8	10
Methanol	88	90	10	11
Water	92	92	9	11
Acetone:Water (1:1, v/v)	89	92	10	11

In this part, the effects of parameters, pretreatment period, pretreatment temperature and biomass loading on the solid recovery and digestibility of EMIMCl pretreated cotton stalks were evaluated. It was shown that higher pretreatment period and lower biomass loading exhibited undesirable impacts on the solid recovery such that biomass degradation was observed under these conditions. Higher pretreatment temperatures were essential to attain higher biomass digestibility for EMIMCl pretreated cotton stalks since the diffusion of EMIMCl into the robust structure of cotton stalks was believed to be facilitated at higher pretreatment temperatures. Moreover, the effects of antisolvent type and recycling were assessed in terms of their effect on the digestibility. It was decided to exploit water as the most suitable antisolvent required for recovery of the pretreated biomass since digestibility of EMIMCl pretreated cotton stalks were not found to vary with antisolvent type. Besides, the findings obtained upon EMIMCl recycling supported this conclusion that water could be utilized in order to precipitate the pretreated cotton stalks in the further investigations.

According to our findings, the highest digestibility was achieved for the cotton stalks subjected to EMIMAc pretreatment at 10% of biomass loading and 150°C for 30 minutes in which 68% of biomass digestibility was attained at the 24^{th} hour of enzymatic hydrolysis. Unlike digestibility, solid recovery upon EMIMAc pretreatment was attained as the lowest (68%). Furthermore, laccase treatment prior to hydrolysis did not show any significant effect on the digestibility of EMIMAc pretreated cotton stalks.

EMIMAc as a pretreatment agent has received growing interest due to encouraging findings obtained upon EMIMAc pretreatment of various lignocellulosic biomass; enhancing the digestibility by disrupting the crystalline structure and extracting lignin (S.H. Lee et al., 2009, Arora et al., 2010, Li et al., 2010, Nguyen et al., 2010, Samayam and Schall, 2010, Li et al., 2011, Shill et al., 2011, Bahcegul et al., 2012b, Haykir et al., 2013). Even EMIMAc used in the recent study was synthesized in a laboratory; it yielded better results compared to other commercial ILs. In the following part, commercial EMIMAc received from BASF, which was expected to result better than the EMIMAc synthesized in-house, would be investigated not only in terms of its impact on the enzymatic accessibility of cotton stalks but also in terms of the structural changes in the cotton stalks.

In the next part, several variables would be investigated to enhance the enzymatic accessibility of EMIMAc pretreated cotton stalks. Furthermore, ethanol production would be performed from glucose that was present in the hydrolyzate of the EMIMAc pretreated cotton stalks. Prior to fermentation analysis, the wild type yeast *Saccharomyces cerevisiae* NRRL Y-132 would be examined in terms of microbial growth, glucose consumption, ethanol production and its tolerance towards ethanol in media containing pure glucose. During the ethanol production from the glucose derived upon hydrolysis of EMIMAc pretreated cotton stalks, the fermentation media would be investigated with respect to the effects of nitrogen sources and various salts with the aim of enhancing ethanol production. Results of these analyses would be given as supplementary information in Appendix C.

4.2 Pretreatment of cotton stalks via ionic liquids for improvement of enzymatic digestibility of the biomass and ethanol production

In this part, ionic liquid pretreatment was conducted with the aim of enhancing the digestibility of cotton stalks and conversion of the glucose derived upon enzymatic hydrolysis of ionic liquid pretreated cotton stalks to ethanol. For this purpose, the parameters given in the Table 4.8 were evaluated in terms of their effects on the structural features and digestibility of cotton stalks. First of all, the ionic liquids; 2-hydroxy ethyl ammonium formate (HEAF), 1-allyl-3-methyl imidazolium chloride (AMIMCl), 1-butyl-3-methyl imidazolium chloride (BMIMCl), 1-ethyl-3-methyl imidazolium chloride (EMIMCl) and 1-ethyl-3-methyl imidazolium acetate (EMIMAc) were screened with respect to their effect on the structure of cotton stalks as well as on the digestibility of pretreated cotton stalks during enzymatic hydrolysis. Even though an analysis regarding the effects of different ionic liquids was given in the previous chapter, this current analysis has been a more detailed one in which the results were interpreted in terms of characterization techniques in addition to the results obtained upon enzymatic hydrolysis. As expected, EMIMAc yielding the most prominent results was employed for the subsequent analysis as shown in Table 4.10 (next page).

Later on, the effects of particle size, pretreatment period, pretreatment temperature and biomass loading would be evaluated with respect to their effect on the digestibility of EMIMAc pretreated cotton stalks based on the reducing sugar concentration attained at the 72^{nd} hour of the hydrolysis. The effect of EMIMAC recycling on pretreatment efficiency was investigated regarding the extracted lignin from untreated cotton stalks upon pretreatment and also, enzymatic digestibility of the pretreated cotton stalks. EMIMAc recycling was also assessed through characterization techniques including scanning electron microscopy (SEM), X-ray diffraction (XRD) and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) to compare the structural modifications in the samples subjected to pretreatment via non-recycled and recycled EMIMAc.

Table 4.10 Summary of parameters investigated, their ranges and the related analysis

Substrate	Parameters	Ionic liquid	Ranges	Analysis conducted
Cotton stalks	Ionic liquid type	-	AMIMCl-BMIMCl-EMIMCl-EMIMAc-HEAF	Digestibility, solid recovery, glucose concentration, structural changes, lignin extraction
Avicel PH-101	-	EMIMAc	-	Digestibility, solid recovery, glucose concentration, structural changes
Cotton stalks	Particle size prior to pretreatment	EMIMAc	<0.3 mm, 0.3-1.0 mm, 1.0-2.0 mm, <2.0 mm	Digestibility, solid recovery, glucose concentration
	Temperature	EMIMAc	120°C-150°C	Digestibility, solid recovery, glucose concentration
	Biomass loading	EMIMAc	5%-10%-15% (w biomass/w IL)	Digestibility, solid recovery, glucose concentration
	Pretreatment period	EMIMAc	15 min.- 30 min.	Digestibility, solid recovery, glucose concentration
	Recycling	EMIMAc	Three times recycling	Digestibility, solid recovery, glucose concentration, structural changes, lignin extraction

4.2.1 Effect of ionic liquid type on the digestibility of cotton stalks

In this part, several ionic liquids were screened in order to determine their effects on the digestibility of cotton stalks. Among pretreated cotton stalks, the highest digestibility was attained for the cotton stalks subjected to EMIMAc pretreatment which was 65% at the 72nd hour of enzymatic hydrolysis, being almost 9-fold more digestible than untreated cotton stalks (Figure 4.2). The biomass digestibility for the cotton stalks subjected to HEAF, AMIMCl and EMIMCl pretreatment were obtained as 26%, 25% and 26%. Even previously being reported as ineffective for cellulose processing (Bicak, 2005; Pinkert et al., 2010), cotton stalks subjected to HEAF pretreatment possessed similar digestibility with those subjected to AMIMCl and EMIMC pretreatments. This result implied that cellulose dissolution was should not be regarded as a criterion for an IL to be promising pretreatment agent for pretreatment of biomass. Additionally, preparation of HEAF did not require intensive effort during its synthesis (as given in the previous chapter) and also the raw chemicals,

ethanolamine and formic acid, which were cheaper compared to alkylimidazolium cations and halogen counter anions, introduced more economical procedure for the synthesis of the ionic liquid. Unlike biomass digestibility, the lowest solid recovery was obtained upon EMIMAc pretreatment in which approximately 53% of cotton stalk was lost during pretreatment. Solid recoveries obtained at the end of pretreatments conducted via other ionic liquids were higher, which were between 54 – 67%. This outcome could be attributed to the effectiveness of EMIMAc towards deconstructing the lignocellulosic structure.

The results were also interpreted in terms of the glucose concentration attained at the 72nd hour of the enzymatic hydrolysis as shown in Figure 4.3. As expected, the highest glucose concentration was obtained as 17 g/L upon hydrolysis of the cotton stalks subjected to EMIMAc pretreatment at 10% of biomass loading and 150°C for 30 minutes. Again, HEAF pretreated cotton stalks resulted with similar glucose concentration with AMIMCl and EMIMCl pretreated cotton stalks in which almost 4 g/L of glucose was released at the 72nd hour of the hydrolysis.

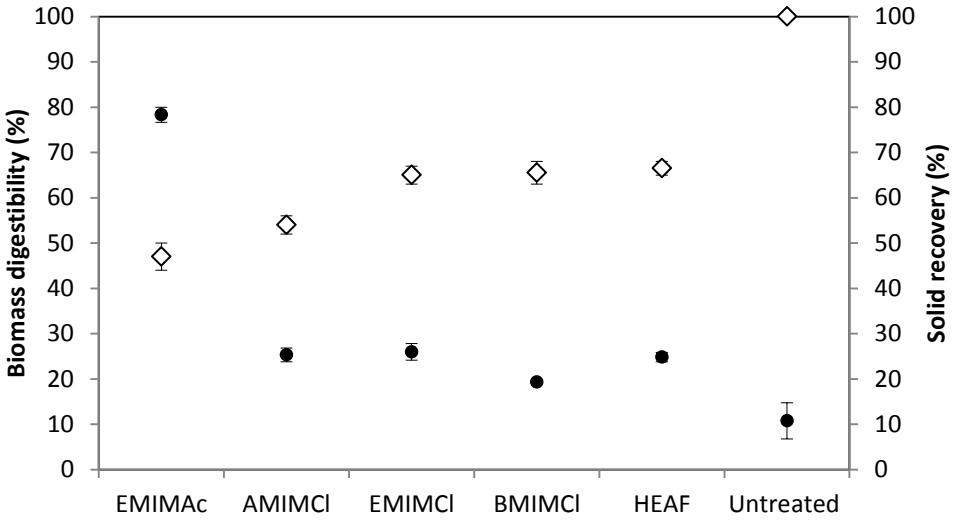


Figure 4.2 Effect of ionic liquid type on the digestibility of cotton stalks at the 72nd hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

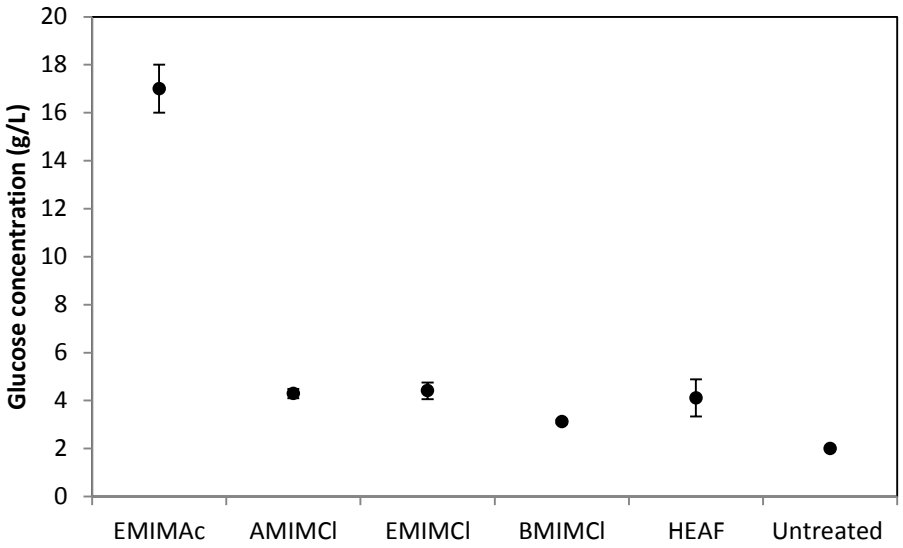


Figure 4.3 Effect of ionic liquid type on glucose concentration obtained at the 72nd hour of the enzymatic hydrolysis.

4.2.2 Effect of EMIMAc pretreatment on the digestibility and structure of cellulose and comparison of cellulose and cotton stalks with respect to their dissolution in the ionic liquid

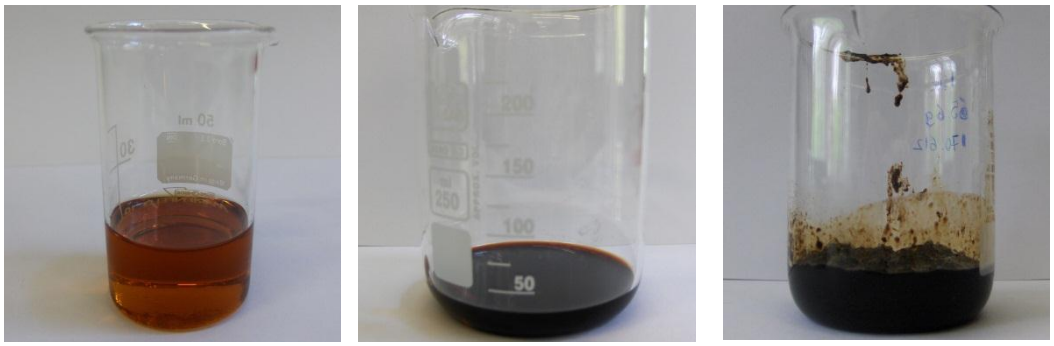
In this part, native cellulose, Avicel PH-101, was subjected to ionic liquid pretreatment with the aim of understanding the changes derived both in the structure and digestibility of the pure cellulose. This analysis was useful since a comparison was made between cellulose and lignocellulosic biomass with respect to their interaction with ionic liquids. For this purpose, Avicel PH-101 was subjected to EMIMAc pretreatment under the same conditions used for pretreatment of cotton stalks .

One distinctive outcome that could be derived after incubation of the substrates in EMIMAc was their dissolution. While Avicel PH-101 dissolved completely in EMIMAc (Figure 4.4b), cotton stalks dissolved partially in the ionic liquids under the same reaction conditions (Figure 4.4c). In addition to the reaction conditions conducted for pretreatment (pretreatment temperature, period, biomass loading…,etc) and physicochemical properties of ionic liquids (viscosity, anion-cation combination…,etc), dissolution of biomass in an ionic liquid also depended on substrate-related properties; composition, particle size, degree of polymerization…,etc (Olivier-Bourbigou et al., 2010). Avicel PH-101 as a commercial pure microcrystalline cellulose, possessing particles at an average particle size of 50 µm and lower DP (degree of polymerization) compared to the lignocellulosic biomass, was distinguishable from cotton stalks with respect to its manner of dissolution and even precipitation in EMIMAc.

To terminate the reaction between a cellulosic/lignocellulosic material and any ionic liquid, an antisolvent should be added to the reaction mixture in other words the slurry comprising the regenerated cellulose/pretreated biomass. As previously carried out, water has been selected as the most appropriate antisolvent for the recovery of the pretreated cotton stalks and EMIMCl recycling. Water with a 10-fold higher volume than the volume of the slurry was added to the reaction mixtures to terminate the reaction in which a precipitate was formed in both systems. However,

formation of gel like structure for the regenerated cellulose (Figure 4.5b, 4.5c, 4.5d) appeared as the most distinctive outcome considering the visual appearances of the pretreated material after antisolvent addition. Pretreated cotton stalks recovered as a precipitate after water addition has no visual similarity with the regenerated cellulose, yet (Figure 4.6).

After addition of water, regenerated cellulose and pretreated cotton stalks were recovered upon filtration of the aqueous mixtures containing EMIMAc, water and the precipitate through a coarse filter paper. The recovered precipitates were then washed 3-5 times with water to remove residual ionic liquid since presence of ionic liquid would have an adverse effect on the subsequent enzymatic hydrolysis (Turner et al., 2003). Finally, regenerated cellulose and pretreated cotton stalks were dried at 60°C for 16 hours.



(a) **(b)** **(c)**

Figure 4.4 EMIMAc before pretreatment (a), EMIMAc after cellulose dissolution (b), EMIMAc after pretreatment of cotton stalks

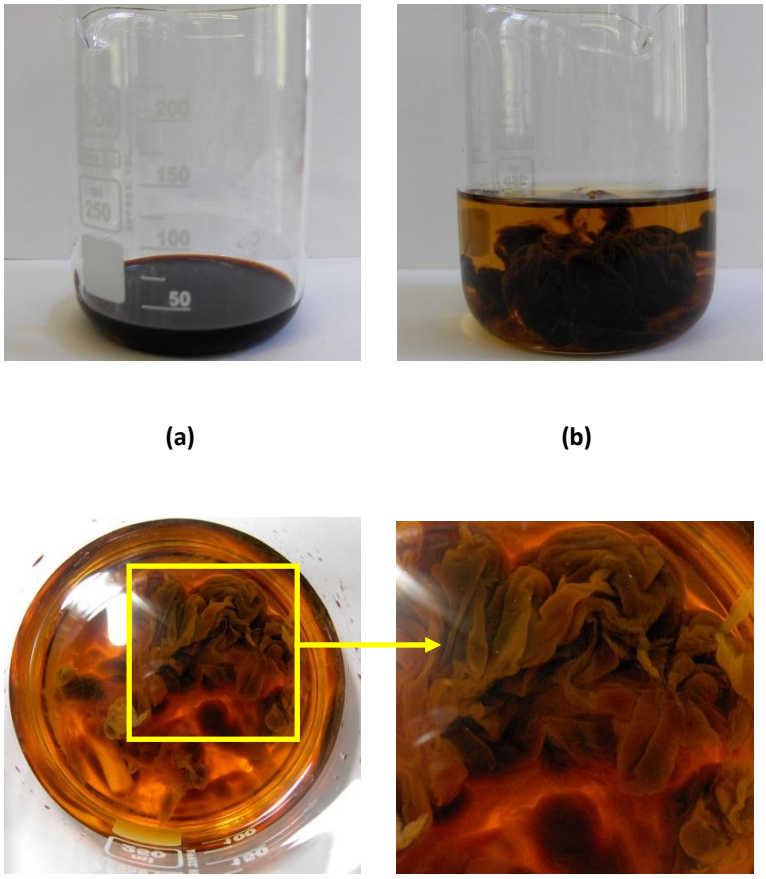
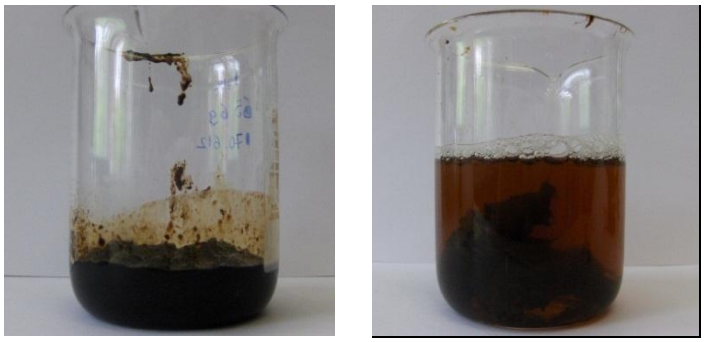


Figure 4.5 EMIMAc after cellulose dissolution and before water addition (a), precipitation of regenerated cellulose after antisolvent addition (b,c,d).



(a) **(b)**

Figure 4.6 EMIMAc after pretreatment of cotton stalks and before water addition (a), precipitation of pretreated cotton stalks after antisolvent addition (b).

(a) **(b)**

(c) **(d)**

Figure 4.7 Native Avicel PH-101 (a), regenerated Avicel PH-101 (b), untreated cotton stalks (c), EMIMAc pretreated cotton stalks (d).

As shown in Figure 4.7b, the regenerated cellulose, which comprised mainly coarse and very tough brown particles (Figure 4.7b), appeared to have an utterly different appearance compared to native cellulose which was composed of very fine particles (Figure 4.7a). Besides, cotton stalks which lost their fibrous-like appearance (Figure 4.7c) upon EMIMAc pretreatment possessed smooth and dark brown colored particles (Figure 4.7d). The color change derived for both samples upon pretreatment was an indicator of residual ionic liquid left in the biomass that could not be removed from the structure.

The results obtained upon hydrolysis of untreated and EMIMAc pretreated cotton stalks were discussed in the previous part and the results interpreted in terms of solid recovery upon pretreatment and also, digestibility and glucose concentrations attained at the 72nd hour of the hydrolysis. The same evaluations were conducted for the regenerated cellulose in addition to the changes in the crystalline structure of cellulose observed after EMIMAc pretreatment.

Considering the visual appearance of cellulose after EMIMAc pretreatment, the hydrolysis of those coarse and robust particles seemed be delicate compared to the hydrolysis of native cellulose consisting fine powders. Though the results derived from the enzymatic hydrolysis of cotton stalks demonstrated contrary to what has been predicted. As shown in Figure 4.8, the digestibility of cellulose subjected to EMIMAc pretreatment at 10% of loading and 150°C for 30 minutes was 95% which was found to be higher than the digestibility of native cellulose (70%). The digestibility of the samples was determined on the basis of the amount of glucose released at the 72nd hour of the hydrolysis since Avicel PH-101 was pure cellulose. Considering the cellulose recovery upon pretreatment via EMIMAc, almost half of the cellulose (49%) was recovered. The concentration of glucose that was released at the 72nd hour of the hydrolysis was found as 32 g/L for regenerated cellulose while it was 23 g/L for the native cellulose. Despite the digestibility of the samples were interpreted on the basis of the glucose released at the 72nd hour of the hydrolysis, enzymatic hydrolysis of regenerated and native cellulose was carried out for four days. Each day, the photo of each sample subjected to hydrolysis at a substrate loading of 3% (w/v) and 50°C in 0.05 M of citrate buffer (pH 4.8) was taken in order to monitor the time course of the hydrolysis (Figure 4.9). It could be clearly seen that at the end of the 4th day of the process, enzymatic hydrolysis of regenerated cellulose has been almost complete since there was trace amount of regenerated cellulose being unhydrolyzed.

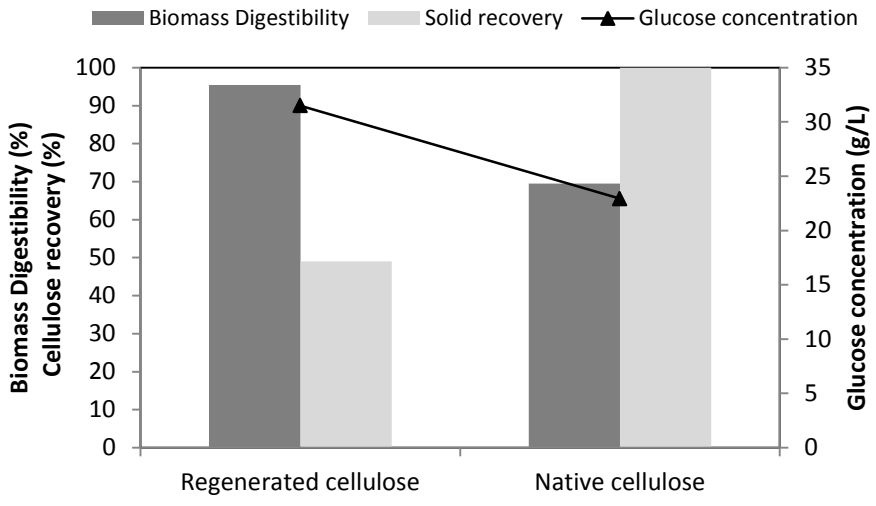
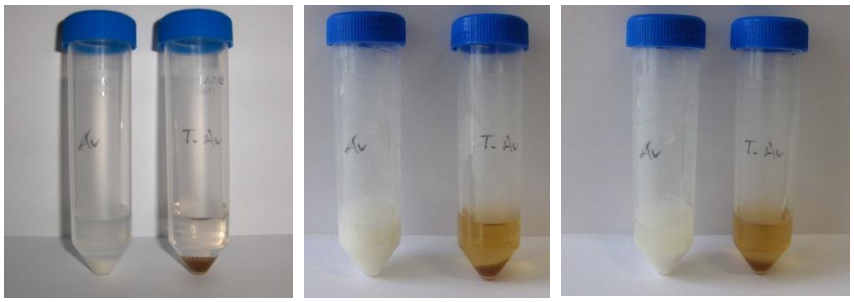
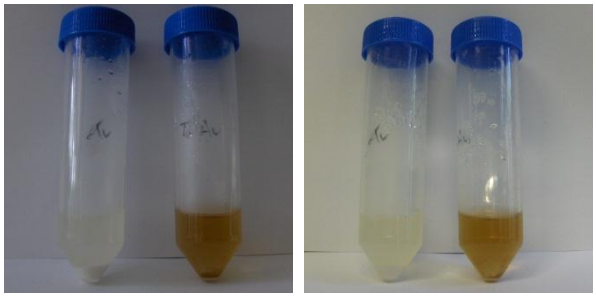


Figure 4.8 Effect of EMIMAc pretreatment on the cellulose recovery, digestibility and glucose concentration attained for Avicel PH-101 upon pretreatment and enzymatic hydrolysis.



(a) **(b)** **(c)**



(d) **(e)**

Figure 4.9 Enzymatic hydrolysis of untreated (falcon on the left hand-side) and EMIMAc pretreated Avicel PH-101 (falcon on the left hand-side) at t=0 h (a), t=24 h (b), t=48 h (c), t=72 h (d), t=96 h (e)

In addition to the findings obtained upon enzymatic hydrolysis, cellulose was assessed in terms of the changes that took place in its crystalline structure after EMIMAc pretreatment. For this purpose, XRD analysis was conducted for native and regenerated cellulose at Bragg angles between 2θ=10°-30°. As shown in Figure 4.10, two major peaks were detected at around 2θ=15° and 2θ=22.5° for native cellulose. Considering the XRD pattern of the regenerated cellulose, the location and intensity these peaks were seriously altered after the Avicel PH-101 was subjected to EMIMAc pretreatment. The larger peak at around 2θ=22.5° was broadened and shifted to lower Bragg angles in which this shift was previously reported as an indicator of modification of Cellulose I (Li et al., 2010; Reddy and Yang, 2009). In addition to these changes, the peak at around 2θ=15°, which was present in the XRD pattern of native cellulose, was absent in the XRD pattern of regenerated cellulose. Native cellulose which is designated as Cellulose I was shown to be less prone to enzymatic hydrolysis compared to cellulose II (Samayam et al., 2011, Kumar et al., 2010, Wada et al., 2010, Cheng et al., 2011). Modification of cellulose I in aqueous media was reported to result with its transformation to cellulose II which was more accessible to enzymatic attack (O'Sullivan, 1997). In addition to the modification in cellulose I, the presence of cellulose II in the structure could be verified from the peak appeared at around 2θ=12°. Similarly, that peak at around 2θ=12° was previously reported to predict the presence of cellulose II in the structure (Wada et al., 2010, Cheng et al., 2011).

Based on the reported changes above, one could conclude that the crystalline structure of the native cellulose, Avicel PH-101 was highly disrupted upon EMIMAc pretreatment. Obviously, the performance of the regenerated cellulose during enzymatic hydrolysis that resulted with enhanced digestibility compared to its native form should be linked to its greatly disrupted crystalline structure.

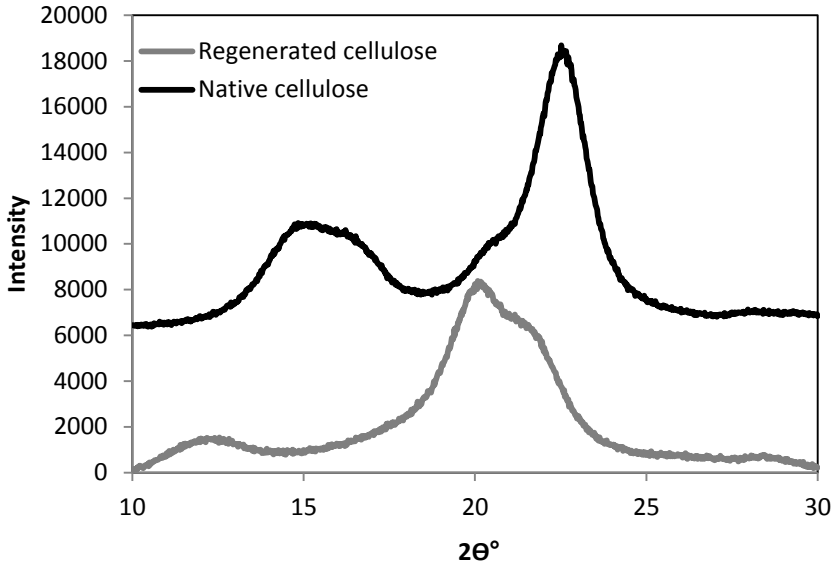


Figure 4.10 XRD patterns for native Avicel PH-101 and regenerated Avicel PH-101 obtained upon EMIMAc pretreatment.

4.2.3 Effect of ionic liquid pretreatment on the structure of cotton stalks

To examine the structural changes in the cotton stalks upon pretreatment via various ionic liquids, SEM images were obtained for untreated and ionic liquid pretreated samples. SEM was reported to be a useful characterization technique which has been capable of monitoring the structural modifications in the lignocellulosic biomass upon ionic liquid pretreatment (Sun et al., 2009, Li et al., 2010). For instance, Singh et al. (2009) showed the impact of EMIMAc pretreatment on structure of switchgrass samples by SEM analysis in which lignin extraction from the biomass via ionic liquid pretreatment could be clearly visualized. In another study, the structural changes of maple wood flour upon pretreatments via EMIMAc, BMIMAc and $BMIMMeSO_4$ were expressed through SEM analysis in which a comparison in their structural changes was related to the effectiveness of the ionic liquids (Doherty et al., 2010).

As shown in Figure 4.11, the intact structure exhibited by untreated cotton stalk (Figure 4.11a) was deconstructed upon EMIMAc (Figure 4.11b). The enhancement in the digestibility of cotton stalks upon EMIMAc pretreatment which was 9-fold higher compared to that of untreated cotton stalks were obviously related to the highly distrupted structure of the EMIMAc pretreated samples. SEM images of cotton stalks subjected to HEAF, AMIMCl and EMIMCl (Figure 4.11f, 4.11c, 4.11d) showed

that that these ioni liquids were only capable of resulting with inconsiderable changes in the structure of cotton stalks compared to the changes obtained EMIMAc. HEAF (Figure 6.2f) seemed as prominent as AMIMCl and EMIMCl with respect to its effect on the structural changes obtained upon pretreatment. Futhermore, BMIMCl pretreatment was found to show the least impact on the structure of cotton stalks in which the SEM image of the BMIMCl pretreated cotton stalks was almost identical to that of untreated samples. The changes in the structures of cotton stalks upon pretreatment visualized via SEM were also in accordance with the digestibility of pretreated cotton stalks, such that the digestibility of the HEAF, AMIMCl amd EMIMCl pretreated samples which exhibited alike morphologies to each other had identical biomass digestibility at the 72nd hour of the hydrolysis. Similarly, BMIMCl pretreatment which was ineffective to alter the structure of the cotton stalks resulted with the lowest biomass digestibility.

Besides monitoring the structural changes via SEM, the samples were also assessed with respect to the changes in their chemical compositions upon pretreatment and the extracted lignin (%) from the samples after pretreatment. The extracted lignin from untreated cotton stalks are calculated according to equation 3.14 in Part 3. As shown in Figure 4.12, extracted lignin upon ionic liquid pretreatment were found to change between 31%-46%. The highest extracted lignin upon pretreatment was achieved for the cotton stalks subjected to EMIMAc and AMIMCl pretreatment in which almost 46% of lignin extraction was achieved. HEAF, which was shown to yield promising results with respect to its effect on digestibility and morphology of cotton stalks, was able to extract 38% of lignin from the cotton stalks as similarly achieved upon EMIMCl pretreatment.

As demonstrated previously, BMIMCl which did not possess significant impact on the digestibility and the mosphology of the samples, identically yielded the least effect on the lignin removal (31%) for the cotton stalks.

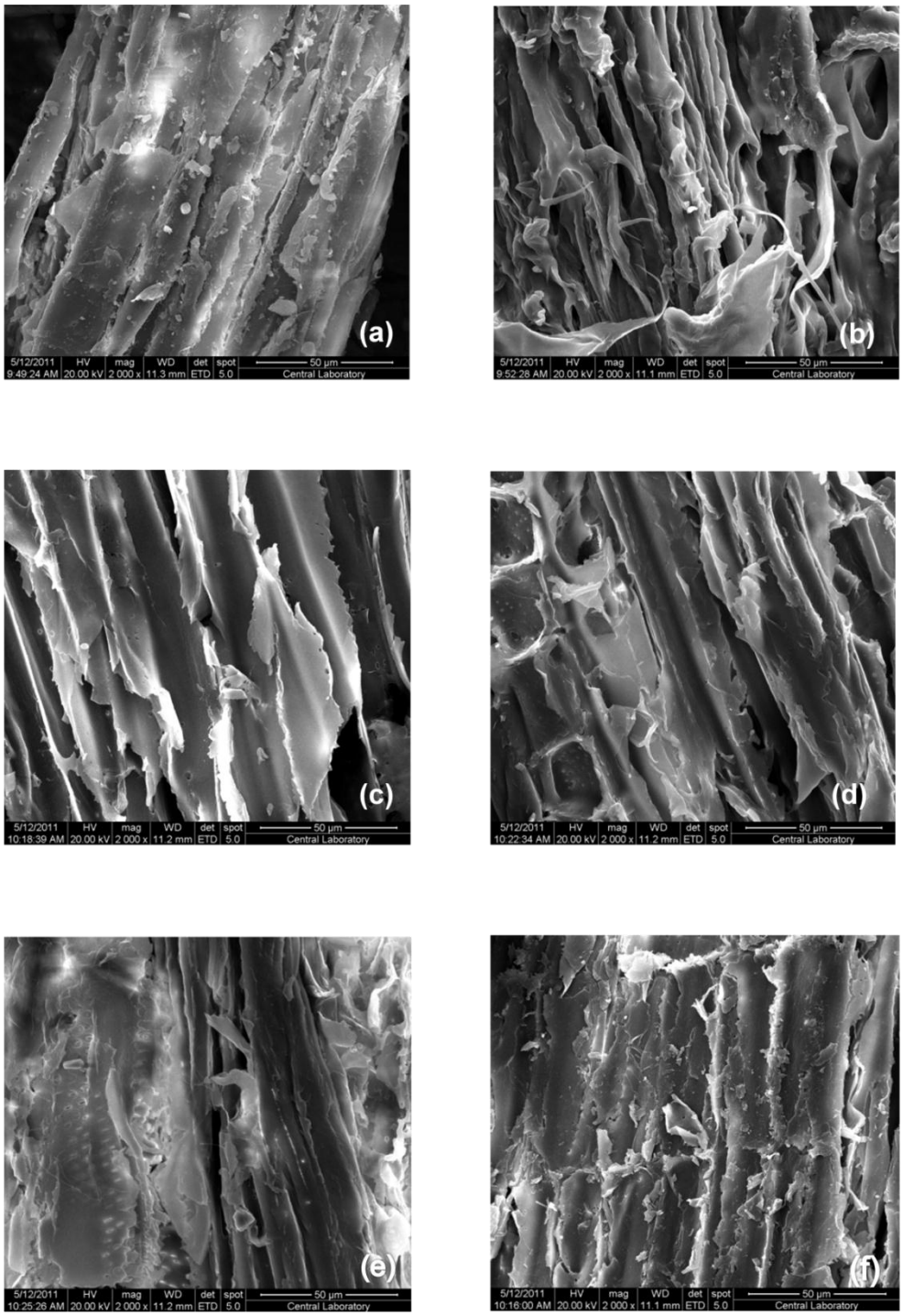


Figure 4.11. SEM images of untreated cotton stalk (a), pretreated cotton stalk samples via EMIMAc (b), AMIMCl (c), EMIMCl (d), BMIMCl (e) and HEAF (f) at magnification of 2000X.

Being shown to extract considerable amounts of lignin, the comparison of ionic liquid pretreatment with other methods would be beneficial for revealing the advantageous points of ionic liquid pretreatment for cotton stalks. For instance, alkaline pretreatment was shown to remove high amounts of lignin from cotton stalks such that alkaline pretreatment with 2% (w/v) of NaOH in an autoclave for 2 hours enabled lignin extraction of up to 65% (Silverstein et al., 2007). In another study, lignin removal, which was as high as 100%, was obtained upon alkaline pretreatment of cotton stalks with 4% (w/w) of NaOH in a high pressure reactor (Binod et al., 2012). Despite being highly effective in lignin removal, alkaline pretreatment required high temperatures and pressures and was not favorable compared to ionic liquid pretreatment which was conducted under milder conditions.

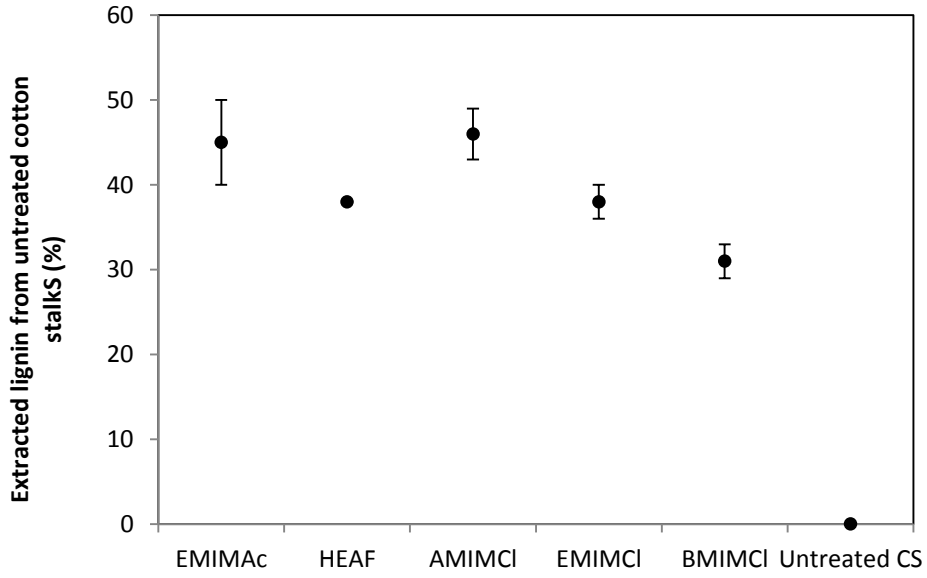


Figure 4.12 Effect of ionic liquid type on the extracted lignin from untreated cotton stalk (%).

Another vital parameter, which should be assessed to gather the changes in the biomass structure, was the crystalline structure of the biomass which has been reported to yield adverse effects on the digestibility of the biomass (Hendriks and Zeeman, 2009). For this reason, pretreated cotton stalks were evaluated in terms of their crystalline structure which was expressed according the changes in two main peaks at around 2θ=15° and 22°. As shown in Figure 4.13, the intensity of the peak at around 2θ=22° decreased for the cotton stalks subjected to AMIMCl, EMIMCl and EMIMAc pretreatment indicating that they possessed reduced crystallinity compared to untreated cotton stalks. In addition to the decrease in its intensity, the peak at around 2θ=22° shifted to lower Bragg angles particularly for EMIMAc pretreated cotton stalks. This change has been reported as the modification of cellulose I (Reddy and Yang, 2009, Li et al., 2010) which implied that EMIMAc pretreatment introduced the most drastic effect on the crystalline structure of cotton stalks. As expected, BMIMCl pretreatment had minimal impact on the crystallinity of cotton stalks in which the XRD pattern of the BMIMCl pretreated cotton stalks resembled to that of untreated samples.

Based on the SEM images, XRD patterns of cotton stalks and lignin extracted upon pretreatments, EMIMAc exhibited the most striking effect on the structure of cotton stalks. EMIMAc was capable of deconstructing the structure of cotton stalks through transforming its crystalline structure into amorphous form and extracting a noticeable amount of lignin. These results enhanced the accessibility of the cotton stalks to enzymatic hydrolysis in which EMIMAc pretreated cotton stalks were almost 9-fold and 3-fold more digestible compared to untreated samples and those pretreated via HEAF, EMIMCl and AMIMCl. For this reason, the following analysis would be carried out for EMIMAc pretreated cotton stalks.

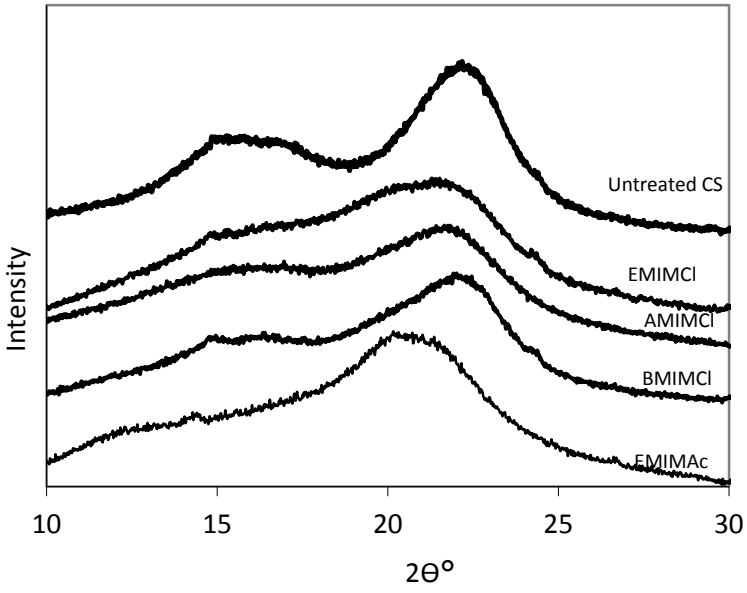


Figure 4.13 XRD patterns for untreated cotton stalk and cotton stalk samples pretreated via AMIMCl, BMIMCl, EMIMCl and EMIMAc.

4.2.4 Effect of particle size on the digestibility of EMIMAc pretreated cotton stalks

In this part, the digestibility of EMIMAc pretreated cotton stalks were monitored according to the changes in the particle size of cotton stalks prior to pretreatment. Cotton stalks having particle size of <0.3 mm, 0.3-1.0 mm, 1.0-2.0 mm and <2.0 mm prior to pretreatment were subjected to EMIMAc pretreatment at 10% of biomass loading and 150°C for 30 minutes. Cotton stalks having a particle size of <2.0 mm prior to pretreatment represented the entire amount of biomass obtained upon milling without any separation conducted. However, the other samples were obtained upon separating the milled cotton stalks via sieve analysis. As shown in Figure 4.14, the digestibility of EMIMAc pretreated cotton stalks at the 72nd hour of the hydrolysis ranged between 71%-78%. The highest digestibility was attained as 78% for the cotton stalks having a particle size of <2.0 mm. EMIMAc pretreated cotton stalks having the smallest particle size prior to pretreatment exhibited the lowest biomass digestibility. This was an expected finding which should be attributed to the degradation of the cellulose as similarly yielded for EMIMCl pretreated samples which had a particle size of <0.15 mm prior to pretreatment as reported in the previous chapter.

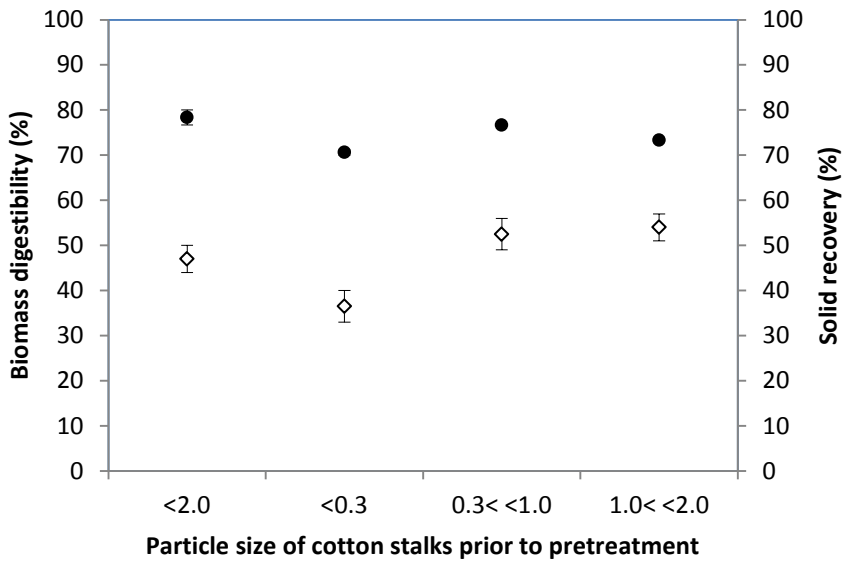


Figure 4.14 Effect of particle size of cotton stalks prior to pretreatment on the digestibility of cotton stalks at the 72nd hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

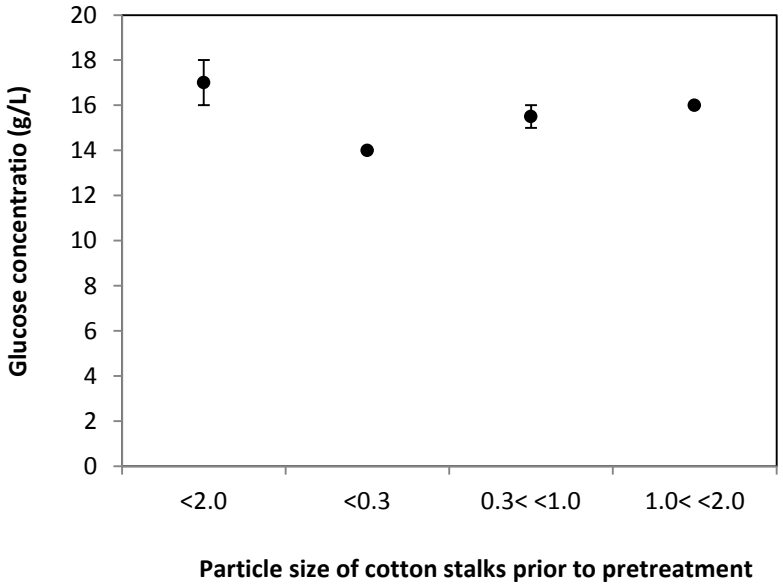


Figure 4.15 Effect of particle size of cotton stalks prior to pretreatment on glucose concentration obtained at the 72nd hour of the enzymatic hydrolysis.

Regarding the glucose released at the 72nd hour of the hydrolysis, the highest glucose concentration was attained as 17 g/L for the cotton stalks having particle size of <2.0 mm. The lowest glucose concentration was found as 14 g/L for the cotton stalks having the smallest particle size. Clearly, EMIMAc pretreatment resulted with cellulose degradation for the cotton stalks possessing the smallest particle size (<0.3 mm) since they yielded the lowest glucose concentration as 14 g/L at the 72nd hour of the hydrolysis.

After monitoring the variation of biomass digestibility and solid recovery against the particle size of cotton stalks prior to pretreatment, a decision should be made to continue with an appropriate particle size of cotton stalks for the subsequent analysis. Regarding the digestibility and glucose concentration attained in which the highest biomass digestibility (78%) and glucose concentration (17 g/L) were attained, the cotton stalks having a particle size of <2.0 mm prior to pretreatment should be utilized to further continue with analysis.

4.2.5 Effect of pretreatment temperature, pretreatment period and biomass loading on the digestibility of EMIMAc pretreated cotton stalks

As previously discussed in Part 4.1, pretreatment temperature, period and biomass loading were considered as crucial factors determining the accessibility of the biomass to enzymatic hydrolysis. In Part 4.1, the effects of these parameters were evaluated step by step to observe the changes in digestibility and solid recovery for the cotton stalks subjected to EMIMCl pretreatment. For convenience, these three parameters were evaluated all together for EMIMAc pretreated cotton stalks instead of conducting a step by step analysis. The parameters and their ranges are given in Table 4.11. Differing from the previous investigation conducted for EMIMCl pretreated cotton stalks, the pretreatment temperature was investigated above 100°C. Previously, high viscosity of ionic liquids and the heterogeneous structure of lignocellulosic biomass were reported to demonstrate limiting effects for the reaction between ionic liquids and biomass. Employment of the reaction at temperatures higher than 100°C has been useful for alleviating the mentioned limitations since selection of higher process temperatures introduced better penetration and pretreatment capabilities for ionic liquids towards lignocellulosic structures. Considering the superior solvation properties of EMIMAc towards any lignocellulosic biomass, the pretreatment period was kept at 30 minutes or shorter. The biomass loading was again investigated at 5% -10% (w cotton stalks/w EMIMAc).

Table 4.11 Summary of parameters investigated, their ranges for EMIMAc pretreatment of cotton stalks

Ionic liquid	Parameters	Ranges
EMIMAc	Pretreatment temperature	120°C-150°C
	Pretreatment period	15 min.-30 min.
	Biomass loading	5%-10% (w cotton stalks/w EMIMAc)

Before discussing the impact of pretreatment temperature, period and biomass loading on the solid recovery and biomass digestibility, pretreated cotton stalks were assessed according to their visual appearances as shown in Figure 6.4. A visual examination was conducted for the cotton stalks subjected to EMIMAc pretreatment at 120°C (Figure 4.16) and 150°C (Figure 4.16c) at 10% of biomass loading for 30 minutes. As clearly shown in the figure, the structure of cotton stalks pretreated at 120°C possessed much more similarity with that of untreated cotton stalks, mostly preserving their initial appearance. The appearances of samples pretreated at 150°C were utterly different from the untreated sample such that they lost the original fibrous-like structure of cotton stalks through deconstruction of the native structure at 150°C.

(a) **(b)** **(c)**

Figure 4.16 Visual appearances of untreated cotton stalks (b) and cotton stalks subjected to EMIMAc pretreatment at 120°C (b) and 150°C (c) at 10% of biomass loading for 30 minutes.

The effects of pretreatment temperature, period and biomass loading on solid recovery and biomass digestibility were clearly shown in Figure 4.17. The digestibility of EMIMAc pretreated cotton stalks at the 72nd hour of the hydrolysis ranged from 68% to 80%. Evidently, the increase in pretreatment temperature from 120°C to 150°C resulted with a slight increase in biomass digestibility. However, the solid recovery was found to decrease significantly from a range of 60-73% to a range of 49-68% with an increase in temperature from from 120°C to 150°C. Based on these findings, 120°C appeared to be more favorable as a pretreatment temperature compared to 150°C with respect to its effect on the solid recovery. The variation in pretreatment period and biomass digestibility was found to have no effect on the digestibility of the pretreated cotton stalks at 120°C . However as the pretreatment temperature increased to 150°C, the variation in biomass digestibility as a function of pretreatment period could be readily observed. The biomass digestibility was found to decrease from 80% to 76% with a decrease in pretreatment period from 30 minutes to 15 minutes for cotton stalks subjected to EMIMAc pretreatment at 150°C and 5% of biomass loading. Similarly, the biomass digestibility decreased from 78% to 68% with a decrease in pretreatment period from 30 minutes to 15 minutes for cotton stalks subjected to EMIMAc pretreatment at 150°C and 10% of biomass loading. The highest biomass digestibility was attained as 80% for the cotton stalks subjected to EMIMAc pretreatment at 150°C and 5% of biomass loading for 30 minutes.

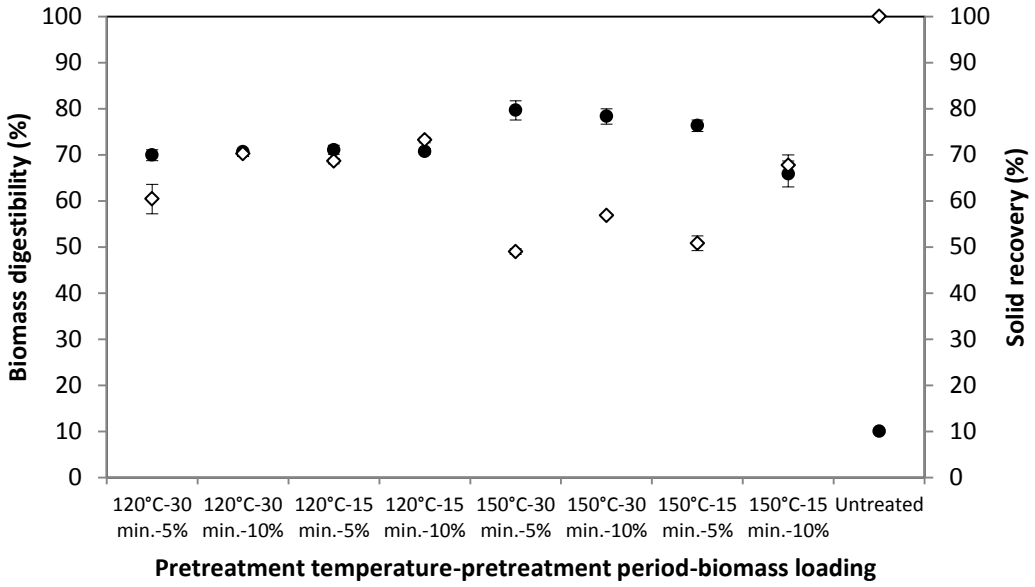


Figure 4.17 Effect of pretreatment temperature, pretreatment period and biomass loading on the digestibility of cotton stalks at the 72nd hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

As expected, solid recoveries upon EMIMAc pretreatment were found to change in a wider range compared to the variation of biomass digestibility with operation conditions during pretreatment. Solid recoveries upon EMIMAc pretreatment at 120°C yielded higher than those obtained upon EMIMAc pretreatment at 150°C. Despite enhancing the digestibility of the EMIMAc pretreated cotton stalks, elevated temperatures obviously caused significant loss of biomass components upon EMIMAc pretreatment. Furthermore, the effect of pretreatment period and biomass loading on solid recovery was more striking at 150°C compared to its effect on solid recovery at 120°C. Such that, the solid recovery was increased from 49% to 57% with an increase in biomass loading from 5% to 10% upon EMIMAc pretreatment for 30 minutes. For the cotton stalks subjected to EMIMAc pretreatment for 15 minutes, the solid recovery increased from 51% to 68% with an increase in biomass loading from 5% to 10%.

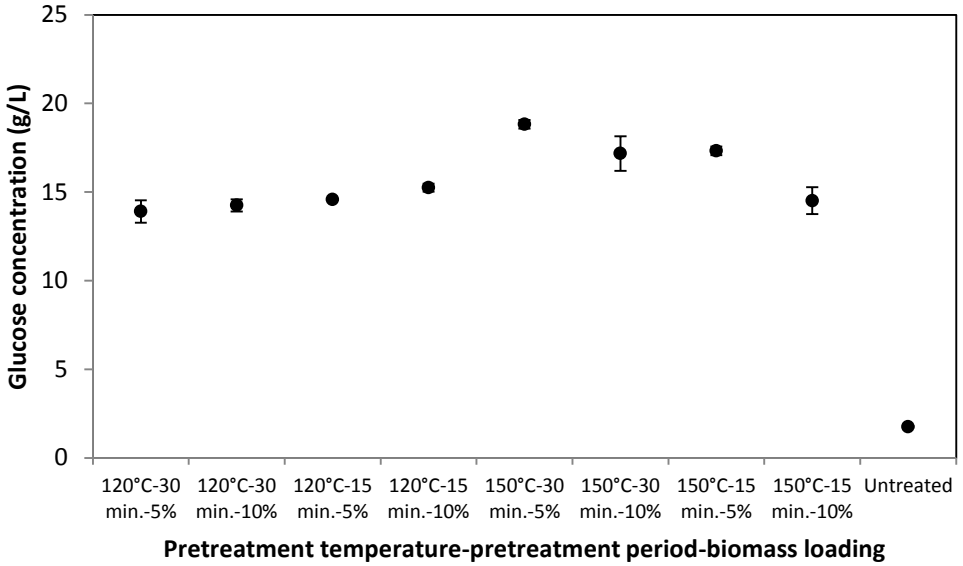


Figure 4.18 Effect of pretreatment temperature, pretreatment period and biomass loading on glucose concentration obtained at the 72nd hour of the enzymatic hydrolysis.

In addition to the analysis in terms of biomass digestibility based on the reducing sugar concentration attained at the 72nd hour of the hydrolysis and solid recovery, the parameters were assessed with respect to their effects on glucose concentration attained at the 72nd hour of the hydrolysis. As shown in Figure 4.18, process parameters yielded insignificant impacts on glucose concentrations for the cotton stalks subjected to EMIMAc pretreatment at 120°C in which such an observation was similarly obtained with respect to their effect on the biomass digestibility. As presumed, glucose concentration was found to change in a notable fashion for the cotton stalks subjected to EMIMAc pretreatment at 150°C. Such that, glucose concentration was found to decrease with an increase in biomass loading and decrease in pretreatment period. For instance, the glucose concentration was found to decrease from 19 g/L to 17 g/L with an increase in biomass loading from 5% to 10%. Despite the fact that the highest biomass digestibility and glucose concentration were attained for the cotton stalks subjected to EMIMAc pretreatment at 5% of biomass loading and 150° for 30 minutes, cotton stalks subjected to EMIMAc pretreatment at 10% of biomass loading and 150° for 30 minutes were selected in the continuation of the analysis. Utilization of higher biomass concentrations was regarded as important from the economical point of view since there would be an opportunity to pretreat a higher amount of biomass with the same amount of ionic liquid as it was the case in this study. Besides their powerful solvation characteristics towards lignocellulosic structures, ionic liquids do have high cost. Therefore, the subsequent analysis would be conducted with the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading and 150°C for 30 minutes of period which would be much more economically attractive.

4.2.6 Effect of EMIMAc recycling on the digestibility and structure of cotton stalks

In this part, the effect of EMIMAc recycling was examined in terms of the changes that took place in the structure and digestibility of cotton stalks. In Part 4.1, EMIMCl recycling was investigated with respect to its effect on digestibility together with assessment of the effect of antisolvent type on EMIMCl recycling. Water has been selected as the most appropriate solvent considering the environmental and economic aspects of the process and EMIMCl recycling was shown to yield insignificant effect on the solid recovery and biomass digestibility attained at the 24th hour of the hydrolysis. Recalling the procedure conducted during EMIMCl recycling, the insoluble products were removed from the recovered EMIMCl-antisolvent solutions as much as possible via their filtration simply through filter paper after evaporation of the antisolvents. Contrarily, the recovered aqueous EMIMAc solutions were directly utilized for the subsequent analysis without any purification in this part. Moreover, the results concerning the changes that took place in the structure of cotton stalks upon pretreatment via recycled EMIMAc were interpreted through SEM, XRD and ATR-FTIR analysis.
Initially, the structural changes for the cotton stalks pretreated via non-recycled and recycled EMIMAc were visualized by SEM analysis as shown in Figure 4.19. According to the images, EMIMAc recycling did not yield any significant result with respect to its effect on the deconstruction of the structure of cotton stalks. The SEM images of cotton stalks obtained upon pretreatment via recycled EMIMAc (Figure 4.19c) resembled to that obtained upon pretreatment via non-recycled EMIMAc (Figure 4.19b).

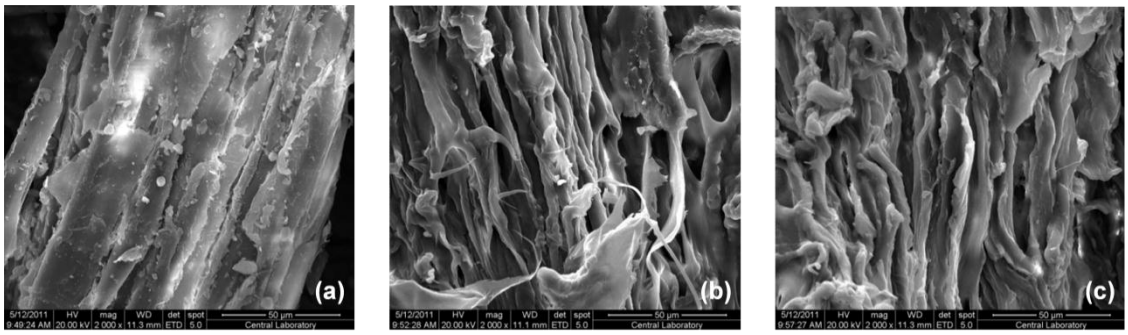


Figure 4.19 SEM images of untreated cotton stalks (a), pretreated cotton stalks via non-recycled EMIMAc (b) and recycled EMIMAc (c).

Furthermore, the cotton stalks were analyzed for the changes that resulted in their crystalline structure. The changes in peaks that were appeared around 2θ = 15° and 22° for untreated cotton stalks were considered to make a comparison between EMIMAc pretreated samples regarding their crystalline structure. According Figure 4.20, strong similarities were attained between the XRD patterns of two samples. This implied that recycled EMIMAc resulted with the same impact; reducing the crystalline structure of cotton stalks as caused by non-recycled EMIMAc.

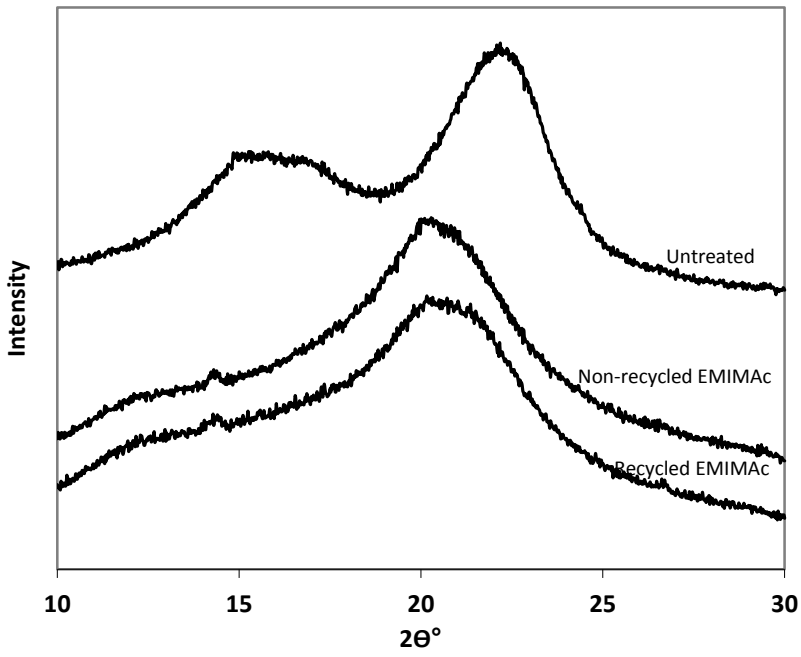


Figure 4.20 XRD patterns for untreated cotton stalk and cotton stalk samples pretreated via non-recycled EMIMAc and recycled EMIMAc.

The structural changes in the pretreated samples were also discussed according to the results obtained from ATR-FTIR analysis (Figure 4.21). This analysis was capable of demonstrating the structural changes in the cellulosic and lignin fractions of the cotton stalks upon pretreatment. The intensities of the bands as obtained from the work of Gupta and Lee (2010) at 1030 cm^{-1} (C-H in plane deformation in guaiacyl), 1241–1360 cm^{-1} (guaicyl ring breathing with C-O stretching), 1405–1430 cm^{-1} (aromatic skeletal vibration with C-H in plane deformation), 1500 cm^{-1}: (aromatic skeletal vibrations) were obviously decreased for both samples pretreated with recycled and non-recycled EMIMAc. The disappearence of these peaks or the decrease in their intensities can be attributed to the distruption of lignin bonds within cotton stalk pretreated via EMIMAc.

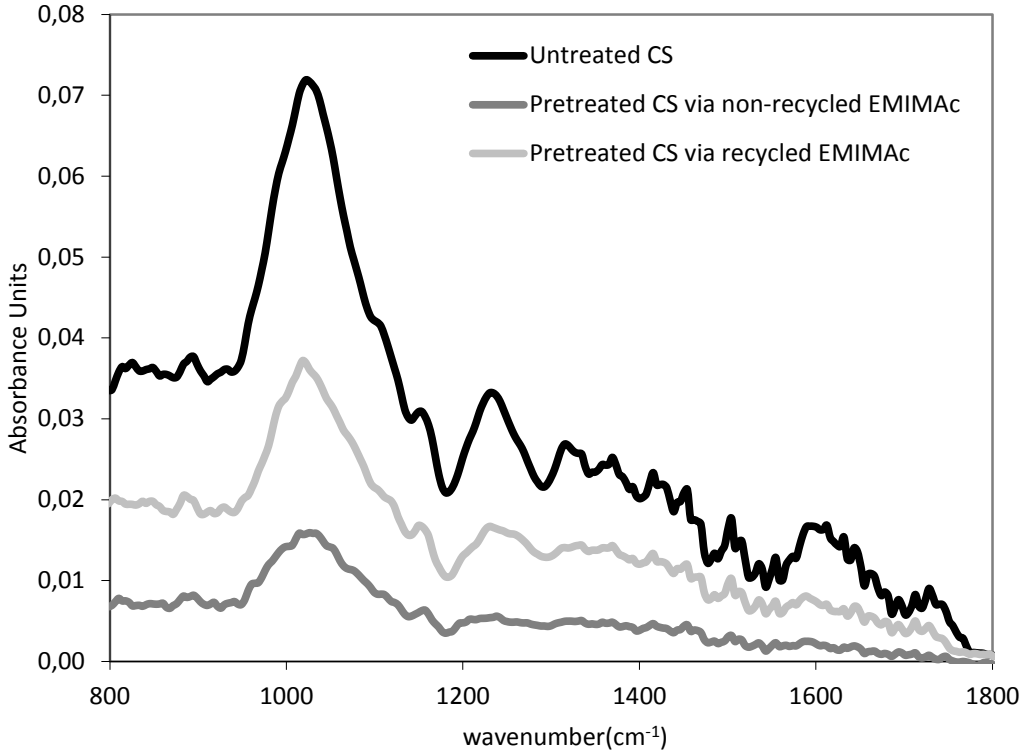


Figure 4.21 ATR-FTIR spectra for untreated cotton stalks and cotton stalks pretreated via non-recycled EMIMAc and recycled EMIMAc.

It has been also reported that the band at around 1430 cm^{-1} has been associated to the crystalline structure of cellulose (Bodirlau et al., 2010). Regarding the decrease in the band at around 1430 cm^{-1} indicated the reduction in the crystalline structure of the cotton stalks pretreated via non-recycled and recycled EMIMAc. When the patterns of pretreated cotton stalks were compared, the bands associated to the delignification of the samples were somewhat more intense for samples pretreated with recycled EMIMAc compared to those attained for cotton stalks pretreated with non-recycled EMIMAc. This finding was well correlated with the variation in the lignin extraction from cotton stalks upon EMIMAc pretreatment as shown in Figure 4.22. The extracted lignin from untreated cotton stalks are calculated according to equation 3.14 in Part 3. While 45% of lignin was extracted upon pretreatment via non-recycled EMIMAc, lower amounts of lignin was extracted from the cotton stalks upon pretreatment via recycled EMIMAc which was only 27% at the first recycle of EMIMAc.

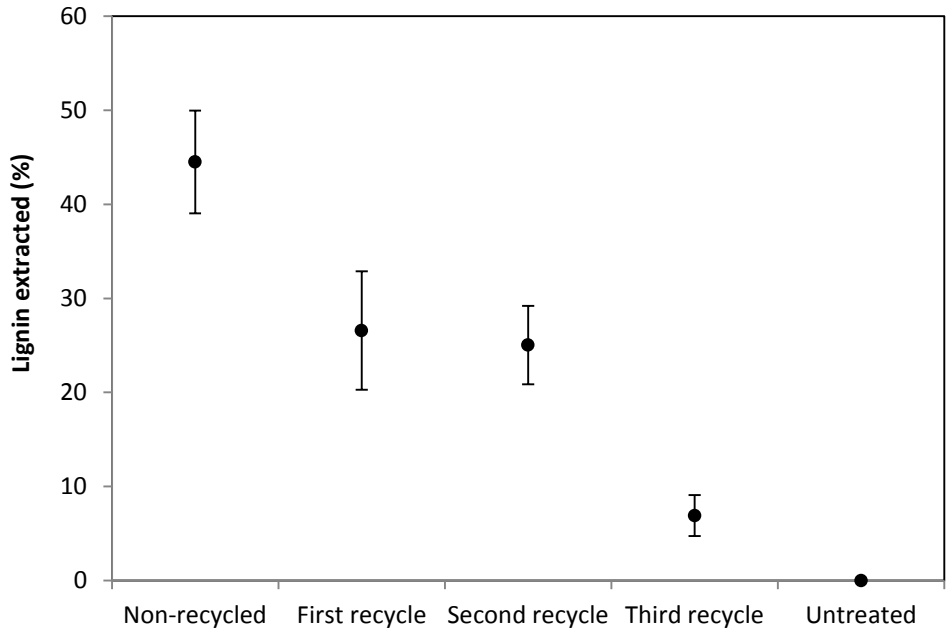


Figure 4.22 Effect of EMIMAc recycling on the extracted lignin from untreated cotton stalks.

As shown in Figure 4.22, there has been a decrease in the amount of lignin extracted from cotton stalks upon recycling of EMIMAc from 45% to 7%. This finding should be regarded as an important one since it demonstrated the efficiency of EMIMAc recycling with respect to its impact on lignin extraction. Recycled EMIMAc was shown to be incapable of extracting lignin from the untreated cotton stalks as the number of recycling increased. Despite the fact that no analysis was conducted to analyze the recovered aqueous EMIMAc solution after each pretreatment, the inefficiency of EMIMAc to extract lignin from untreated cotton stalks could be related to the gradual accumulation of the residual biomass components and water through sequential recycling of EMIMAc based on the previously reported studies (S.H. Lee et al., 2009, Doherty et al., 2010, Nguyen et al., 2010).

Together with the reported findings, the variation in biomass digestibility should be also covered as a vital aspect of ionic liquid recycling. For this purpose, cotton stalks were enzymatically hydrolyzed for 72 hours upon each pretreatment conducted via recycled EMIMAc. As shown in Figure 4.23, the digestibility of EMIMAc pretreated cotton stalks attained at the 72nd hour of the hydrolysis varied in a narrow range, 63%-67%, indicating that EMIMAc recycling did not show any adverse effect on the digestibility of the pretreated samples. Unlike digestibility, solid recovery upon EMIMAc pretreatment increased from 47% to 67% with EMIMAc recycling. The variation of the solid recovery that demonstrated an ascending profile with EMIMAc recycling could be associated to the residual biomass components that could not be recovered in the former pretreatment. Similar to the variation of biomass digestibility with EMIMAc recycling, glucose released at the 72nd hour of the hydrolysis attained identical concentrations upon EMIMAc recycling (Figure4.24).

Albeit the presence of lignin in the biomass structure was reported to hinder biomass accessibility to enzymatic attack, the current findings showed that biomass digestibility was not affected from the inefficiency in delignification upon EMIMAc recycling. Even though pretreated cotton stalks were not examined in terms of their crystalline structure after each pretreatment, EMIMAc appeared to be successful in reducing the crystallinity of cotton stalk at each recycling resulting with identical digestibility in all enzymatic reactions. The crystalline structure of the biomass has been revealed as a more crucial parameter in determining the biomass digestibility compared to its lignin content as similarly reported in the previous studies (Rollin et al., 2011, H.Wu et al., 2011).

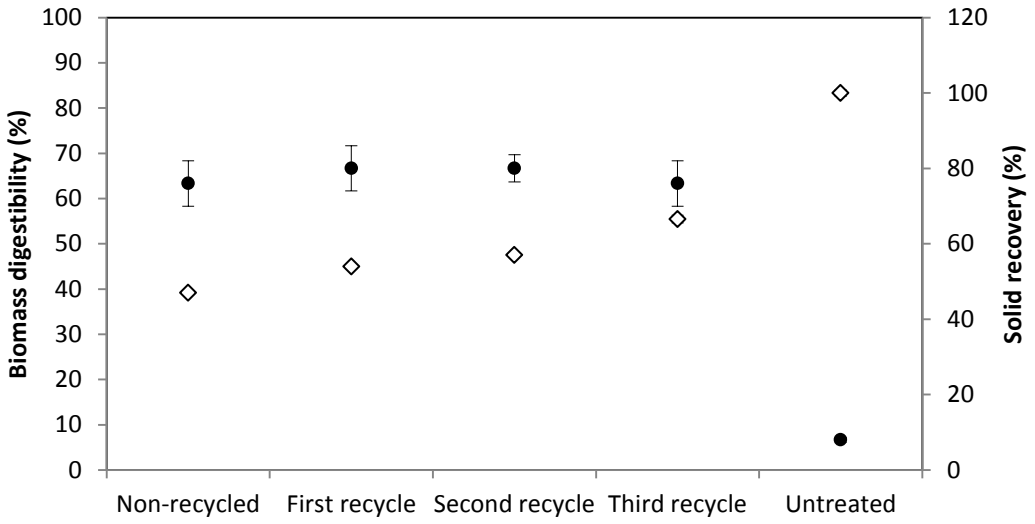


Figure 4.23 Effect of EMIMAc recycling on the digestibility of cotton stalks at the 72nd hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

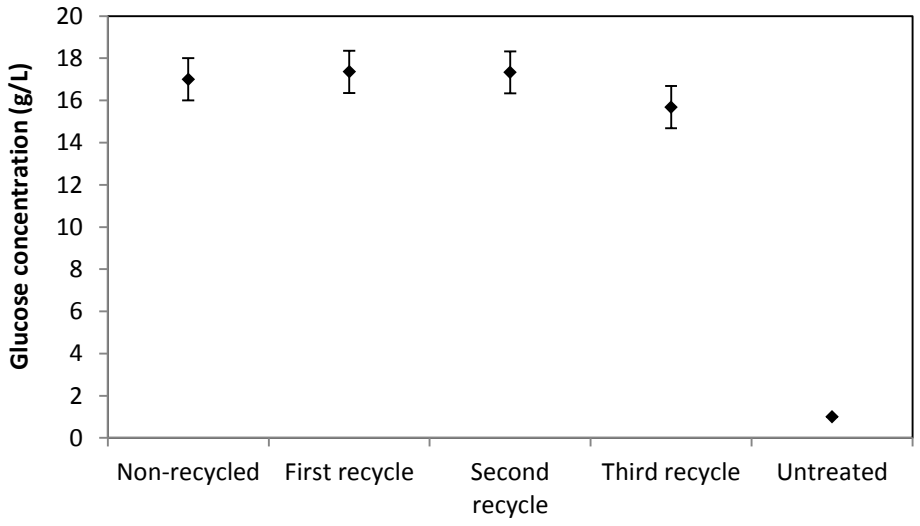


Figure 4.24 Effect of EMIMAc recycling on glucose concentration obtained at the 72nd hour of the enzymatic hydrolysis.

Up to this point, several typical ILs were assessed with respect to their effect on the solid recovery and digestibility attained upon pretreatment and enzymatic hydrolysis of cotton stalks, respectively. EMIMAc was selected as the most efficient pretreatment agent considering its impact on deconstruction of the structure of cotton stalks through significant levels in delignification and disruption of crystalline structure. Cotton stalks that were subjected to EMIMAc pretreatment were examined in terms of the effects of pretreatment temperature, pretreatment period, biomass loading, and particle size prior to pretreatment. The highest digestibility was attained as 78% at the 72^{nd} hour of the hydrolysis for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading and 150°C for 30 minutes. EMIMAc recycling was shown to be successful with respect to its effect on disrupting crystalline structure of cotton stalks and enhancing accessibility of cotton stalks to enzymatic hydrolysis in each recycle. EMIMAc pretreated cotton stalks were subjected to the hydrolysis at a substrate loading of 3% (w/v) and enzyme loading of 2% (v/v) (Celluclast 1.5L) for 72 hours; 17 g/L of glucose was derived from the pretreated biomass at the end of the enzymatic reaction.

In the following sections, it would be concentrated more on the enzymatic hydrolysis of cotton stalks. The steps considering the analysis that would be conducted given below in order to avoid any misunderstanding (Figure 4.25). Before going through ethanol production, a simple evaluation for enzymatic hydrolysis of EMIMAc pretreated cotton stalks should be employed to enhance the glucose yields as high as possible. For this purpose, the initial intention was to examine the variation of the cotton stalks digestibility with hydrolysis period. It not gave an idea about the time course of the enzymatic reaction but also put an emphasis on the initial hydrolysis rates. After deciding on the hydrolysis period that the reaction would be terminated, the cotton stalks would be investigated in terms of their compositions with the aim of determining the maximum amount of glucose that would be released at the end of the enzymatic hydrolysis. Later on, the effects of enzyme loading and enzyme type on the glucose released upon hydrolysis of EMIMAc pretreated cotton stalks would be examined at a fixed substrate loading, 3% (w/v) which would be a concise analysis. After deciding on the appropriate enzyme loading and enzyme type, glucose derived in the hydrolysis would be converted to ethanol and the effect of initial glucose concentration present in the fermentation medium on ethanol production would be investigated.

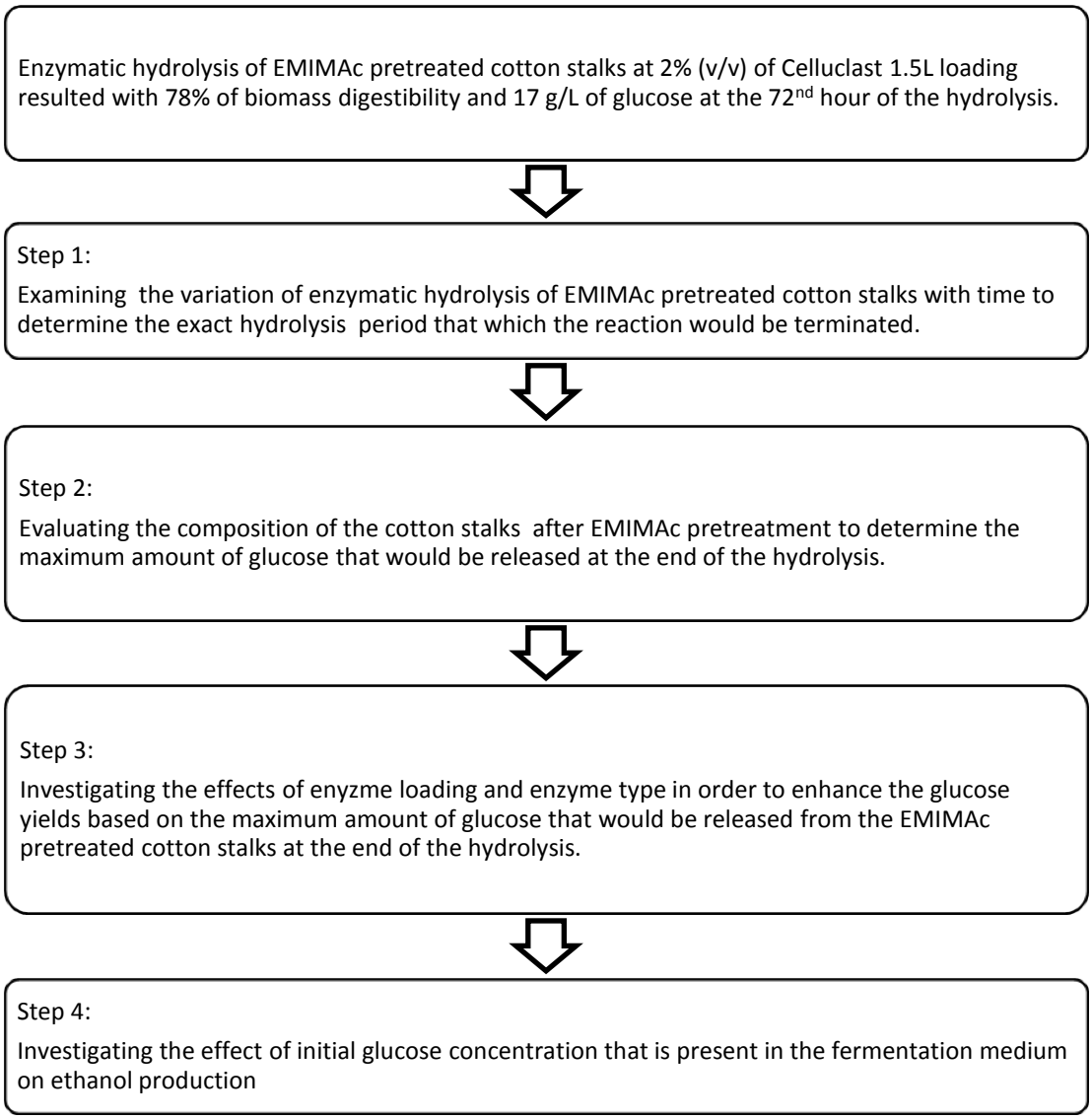


Figure 4.25 Steps regarding the analysis that would be conducted to optimize the enzymatic hydrolysis for EMIMAc pretreated cotton stalks with the aim of improving the amount of glucose that would be released upon enzymatic hydrolysis.

4.2.7 The variation of the enzymatic digestibility of untreated and EMIMAc pretreated cotton stalks with time

In this part, the intention was to evaluate the time course of hydrolysis for untreated cotton stalks and EMIMAc pretreated cotton stalks and determine the period that the reaction would be terminated. For this purpose, enzymatic hydrolysis of both samples was monitored for 72 hours of period and the samples were withdrawn more regularly in the first 24 hours of the reaction. As shown in Figure 4.26, the digestibility of EMIMAc pretreated cotton stalks reached a plateau after 24 hours of the reaction attaining 78% at the 48th hour of the reaction. After that, the digestibility decreased to 76% at the 72nd hour of the hydrolysis implying that 48 hours of hydrolysis period would be appropriate to terminate the enzymatic reaction and utilize the glucose released at that time further for fermentation.

Together with gathering information about the time course of hydrolysis, the figure also demonstrated a distinctive progress for hydrolysis of cotton stalks in the first 3 hours of the process. EMIMAc pretreated cotton stalk exhibited a much faster hydrolysis kinetics; almost 10 fold higher digestibility (56%) compared to that obtained upon hydrolysis of untreated cotton stalks (5%) over the same time interval. The enhancement of the initial rates of hydrolysis for cellulose and a variety of lignocellulosic biomass subjected to ionic liquid pretreatment was also discussed by the previously reported studies (Dadi et al., 2007, Arora et al., 2010, Li et al., 2010, Li et al., 2011).

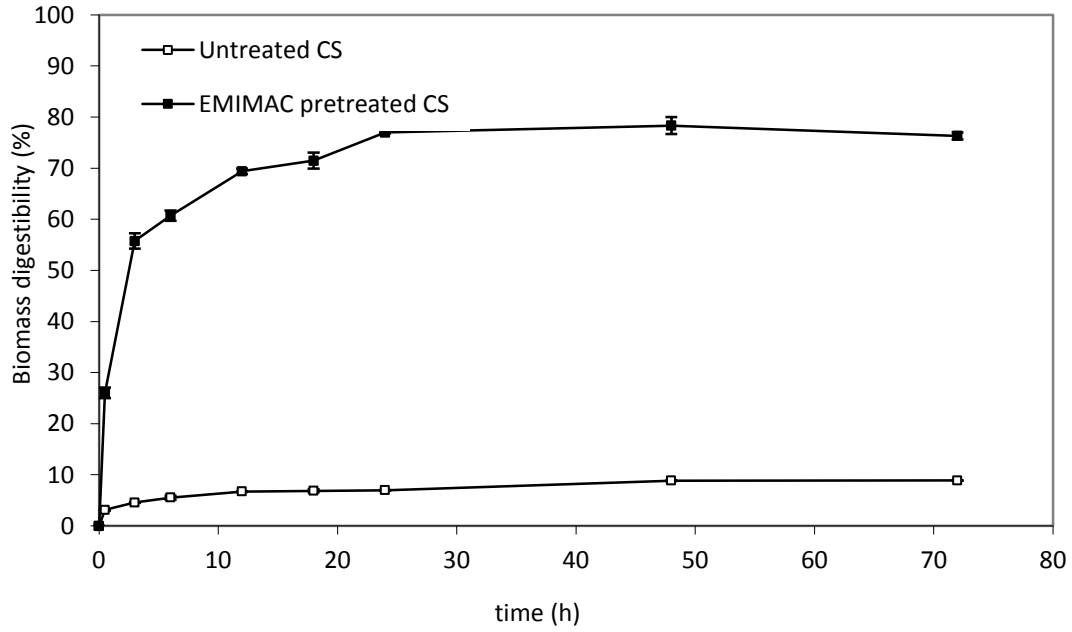


Figure 4.26 The variation of the digestibility of untreated and EMIMAc pretreated cotton stalks with time.

4.2.8 Compositional analysis of cotton stalks before and after EMIMAc pretreatment

Compositional analysis for the cotton stalks before and after EMIMAc pretreatment was crucial since it obviously enabled the determination of the glucose yields on the basis of the maximum amount of the glucose that would be released at the 48th hour of the enzymatic hydrolysis. For this purpose, a two-step sulfuric acid hydrolysis was conducted to analyze the structural carbohydrates and lignin in the cotton stalks according to the laboratory analytical procedure (LAP) provided by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008).

The compositions of the untreated and EMIMAc pretreated cotton stalk were given in Table 4.12. Upon EMIMAc pretreatment, the cellulose content of the cotton stalks increased from 40% to 60%. The cellulose enrichment for the biomass resulted with noticeable increase in the glucose concentration released at the 48th hour of the hydrolysis; from 1 g/L to 17 g/L. Based on the maximum amount of glucose that would be released from untreated and pretreated cotton stalks, glucose yields were found as 8% and 85%, respectively. Glucose yield (%) was determined according to equation 4.1 which is the ratio of the amount of glucose concentration that was derived at the 48th hour of the enzymatic hydrolysis (g/L) to the theoretical maximum amount of glucose that would be released from the biomass subjected to hydrolysis (g/L). Same equation is also given in a similar form in Part 3 as equation 3.3.

$$\text{Glucose yield}\,(\%) = \frac{C_G}{[C_S \times C_{cellulose}\,(\%) \times 1.11]/100} \times 100 \qquad (4.1)$$

where C_S is the initial concentration of the substrate in the hydrolysis buffer that is subjected to enzymatic hydrolysis (g/L), C_G is the glucose concentration obtained at the 48th hour of the hydrolysis and $C_{cellulose}$ (%) is the cellulose content of the cotton stalks (either untreated or pretreated) subjected to enzymatic hydrolysis.

Table 4.12 Compositional analysis of cotton stalks before and after EMIMAc pretreatment

	Solid recovery (%)	**Cellulose (%)**	**Hemicellulose (%)**	**Acid insoluble lignin (%)**	**Acid soluble lignin (%)**	**Total lignin (%)**
Untreated cotton stalks	100±0	40±1	16±0	23±1	3±1	25±2
EMIMAc pretreated cotton stalks	47±4	60±1	10±0	28±0	2±0	30±0

Though total lignin appeared to increase from 25% to 30%, the lignin extracted from untreated cotton stalks was found as 45% (referring Part 4.2.6). This slight increase did not make any sense when the following advantages were considered; cotton stalks were enriched in cellulose and their crystalline structure was disrupted upon EMIMAc pretreatment. There has been also a reduction in the hemicellulose content of the cotton stalks upon pretreatment from 18% to 10%. Even it might seem as a disadvantage considering the production of total fermentable sugars upon hydrolysis; this decrease could be ignored since the major component of interest was cellulose for this study.

4.2.9 Effect of enzyme type and loading on the enzymatic hydrolysis of cotton stalks

In this part, untreated and EMIMAc pretreated cotton stalks were subjected to ezymatic hydrolysis at a substrate loading of 3% (w/v) via commercial enzymes, Celluclast 1.5L and Cellic Ctec2 at enzyme loadings ranging from 1 to 4% (v enzyme/v buffer). The results were interpreted in terms of glucose concentration and also glucose yield (%) on the basis of the theoretical maximum amount of glucose that would be released from the biomass subjected to hydrolysis.

Before discussing the results obtained upon enzymatic hydrolysis, the commercial enzyme blends, Celluclast 1.5L and Cellic Ctec2 were assessed in terms of their protein content and activities of each enzyme present in the cocktail (Table 4.13). Cellic Ctec2 as a novel commercial enyzme preparation exhibited 3-fold higher cellulase activity (225 FPU/ml) compared to Celluclast 1.5L (75 FPU/ml). It also possessed 2-fold higher total amount of protein compared to Celluclast 1.5L. Cellic Ctec2 also demonstrated much higher β-glucosidase activity, which has been essential for hydrolysis of cellobiose molecules to glucose, compared to Celluclast 1.5L (Canella et al., 2012). Furthermore, it was found to possess higher xylanase activity for the hydrolysis of hemicellulose. Though utilization of xylose would not be considered for ethanol production, the presence of higher xylanase activity in the novel enzyme preparation would be beneficial for the enzymatic function of cellulases. It would facilitate cellulose hydrolysis and thus, release of glucose. The success of the novel enzyme preparation not only owes its success to the higher activities of cellulolytic enzymes but also to the synergistic effect between enzymes.

Table 4.13 Protein content and enzyme activities of Celluclast 1.5L and Cellic Ctec2 (a:taken from Canella et al., 2012).

	Protein content (mg/ml)	**Cellulase (FPU/ml)**	**β-glucosidase (U/ml)**	**Xylanase (U/ml)**
Celluclast 1.5L	60	75	15[a]	45
Cellic Ctec2	120	225	2731[a]	60

As shown in Figure 4.27 and 4.28, EMIMAc pretreated cotton stalks that were subjected to enzymatic hydrolysis at 2% of Cellic Ctec2 loading resulted with the highest glucose concentration and glucose yield as 19 g/L and 95%, respectively at the 48th hour of the hydrolysis. Regarding the cotton stalks that were hydrolyzed via Celluclast 1.5L, the highest glucose concentration and yield were achieved as 17 g/L and 83%, respectively for EMIMAc pretreated cotton stalks that were

enzymatically hydrolyzed at an enzyme loading of 2%. Increasing enzyme concentration from 2% to 4% for both enzyme blends did not make any sense; glucose concentrations and yields were either lowered slightly or did not change for EMIMAc pretreated cotton stalk samples. However, the highest glucose concentrations and yields for untreated cotton stalks were obtained for the samples that were hydrolyzed at an enzyme loading of 4% in which 2 g/L (14%) and 5 g/L (39%) of glucose concentrations were attained for the untreated cotton stalks subjected to hydrolysis via 4% of Celluclast 1.5L and Cellic Ctec2, respectively.

There have been a few studies employing Cellic Ctec2 and its former version, Cellic Ctec for enzymatic hydrolysis of lignocellulosic feedstocks. In the recent studies that featured the utilization of this novel product, it was attempted to lower the enzyme loadings and shorten the hydrolysis periods in order to develop an economically compatible process for cellulosic ethanol production (Alvira et al., 2011, Van Eylen et al. 2011, Xu et al., 2011, Zhang et al., 2011).

Glucose (19 g/L) derived from the hydrolysis of EMIMAc pretreated cotton stalks at 2% (v/v) of Cellic ctec2 would be utilized for ethanol production. That glucose would be concentrated to different glucose concentrations with the aim of investigating the effects of initial glucose concentration on ethanol production.

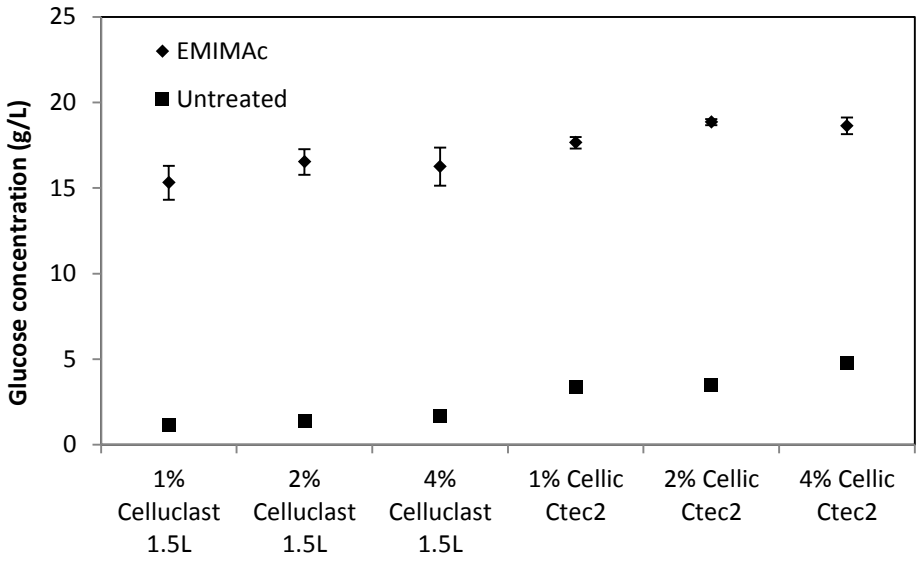


Figure 4.27 Effect of enzyme loading (% v/v) and type on the glucose concentration attained at the 48th hour of the enzymatic hydrolysis for untreated and EMIMAc pretreated cotton stalks.

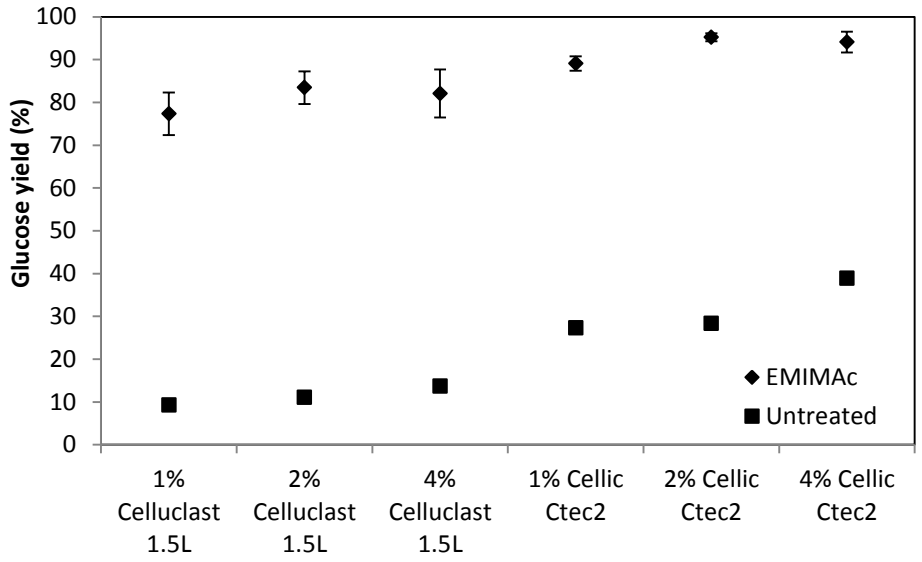


Figure 4.28 Effect of enzyme loading (% v/v) and type on the glucose yield attained at the 48th hour of the enzymatic hydrolysis for untreated and EMIMAc pretreated cotton stalks.

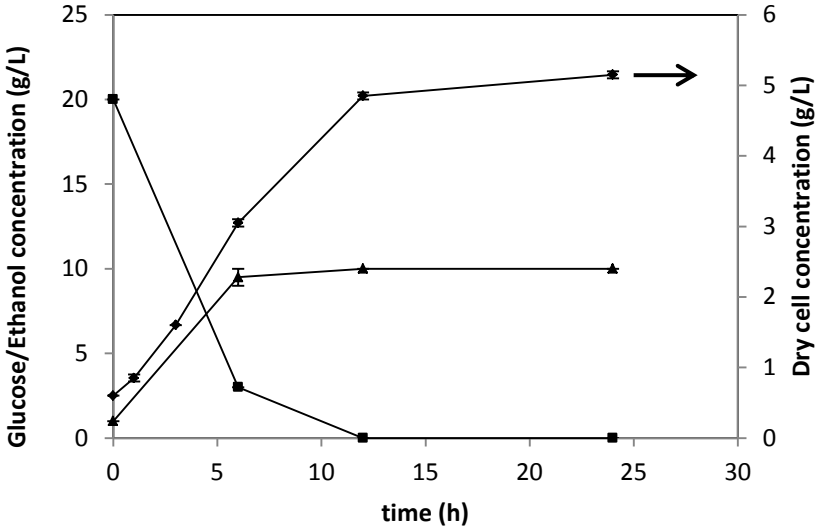
4.2.10 Conversion of cotton stalks to ethanol by the wild type yeast, *Saccharomyces cerevisiae* NRRL Y-132

In this section, the aim was to assess the conversion of glucose that was derived upon the enzymatic hydrolysis of EMIMAc pretreated cotton stalks, to ethanol at varying initial glucose concentrations of the fermentation medium. The effect of initial glucose concentrations was investigated at the following glucose concentrations, 20, 50 and 100 g/L. These concentrations were attained by concentrating the hydrolyzate, which contained almost 19 g/L of glucose originally, obtained upon hydrolysis. The wild type strain, *Saccharomyces cerevisiae* NRRL Y-132 was monitored in terms of time course of cell growth, glucose consumption and ethanol production (Figure 4.29, 4.30 and 4.31). To make a comparison, exactly the same media containing pure glucose at the same initial concentrations were also provided as controls. The results were also expressed by the following fermentation parameters, ethanol titre (v/v), ethanol yield based on the theoretical maximum amount of ethanol that can be produced from the initial amount of glucose present in the fermentation medium (%), yield coefficient for ethanol, $Y_{P/S}$ (g ethanol/g glucose), yield coefficient for dry cell, $Y_{X/S}$ (g cell/g glucose) (Table 4.14).

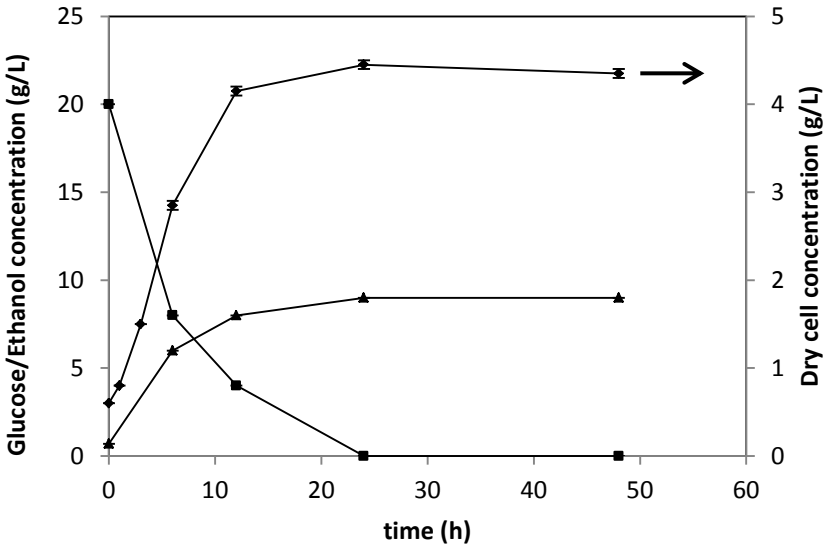
Prior to this investigation, an optimization regarding the effect of medium components of the hydrolyzate medium on ethanol production was performed. Besides, the ethanol tolerance of *Saccharomyces cerevisiae* NRRL Y-132 and the other growth related parameters were investigated. The relevant information for the aforementioned investigations were not introduced in this part, instead they were given in Appendix C as a supplementary data since the major aim for this part was just to understand the effect of the initial glucose concentration present in the hydrolyzate medium on ethanol production.

Table 4.14 The variation of fermentation parameters with initial glucose concentration present in the fermentation media and type of medium.

	Initial glucose concentration (g/L)					
	20		**50**		**100**	
	control	**hydrolyzate**	**control**	**hydrolyzate**	**control**	**hydrolyzate**
Ethanol concentration (g/L)	9	10	26	26	51	51
Ethanol titre (% v/v)	1.2	1.3	3.3	3.3	6.5	6.5
Ethanol yield (%)	88	98	100	100	100	100
Yield coefficient for ethanol, $Y_{P/S}$ (g ethanol/g glucose)	0.45	0.50	0.51	0.51	0.51	0.51
Yield coefficient for dry cell, $Y_{X/S}$ (g dry cell/g glucose)	0.19	0.23	0.16	0.10	0.09	0.03



(a)



(b)

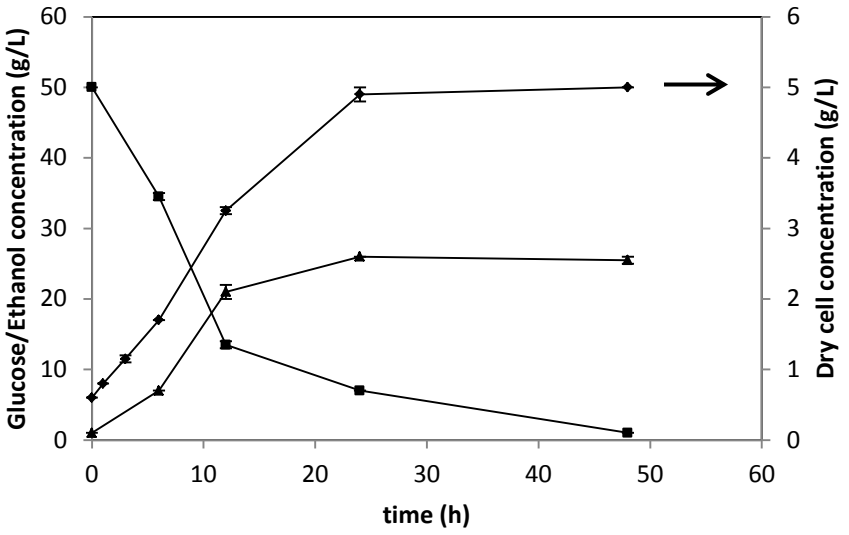
Figure 4.29 Time course of cell growth (♦), glucose consumption (■) and ethanol production (▲) for the hydrolyzate (a) and control (b) containing initial glucose concentration of 20 g/L.

As shown in Figure 4.29a, the glucose was quickly consumed in the first 12 hours of the fermentation and converted to 10 g/L of ethanol for the hydrolyzate medium containing 20 g/L of glucose concentration initially whereas it took 24 hours for the wild-type yeast to consume glucose and produce 9 g/L of ethanol in the medium containing pure glucose initially (Figure 4.29b). Considering the variation of yeast growth with time, higher dry cell concentration (5 g/L) was obtained for the hydrolyzate medium when compared to that obtained for the medium containing pure glucose (4.4 g/L). For both media, it was found that the wild type yeast reached the stationary phase of the growth almost within 24 hours of the fermentation.

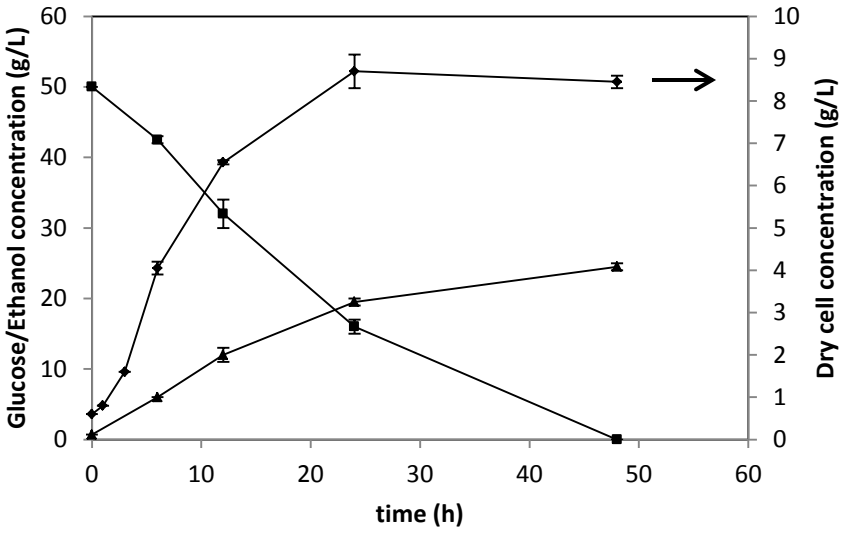
Fermentation of the medium containing 50 g/L of pure glucose (Figure 4.30b) and glucose present in the hydrolyzate initially (Figure 4.30a) resulted with 26 g/L of ethanol concentration corresponding to 3.3% (v/v) ethanol titer. It was observed that glucose was consumed totally within 48 hours for both media. Although yeast performed very well in both media resulting with 100% of the theoretical maximum ethanol yield, dry cell yield over glucose consumption, $Y_{X/S}$ (g dry cell/g glucose) was attained lower in the hydrolyzate medium compared to that obtained in the medium containing pure glucose at the same initial concentration (Table 4.14). This result could be attributed to the composition of the hydrolyzate which was concentrated from 19 g/L (the resultant glucose concentration obtained upon enzymatic hydrolysis of EMIMAc pretreated cotton stalks) to 50 g/L prior to fermentation. Though it was aimed to achieve a specific glucose concentration by concentrating the hydrolyzate, concentrations of the components in the rest of the hydrolyzate such as citric acid, sodium citrate, residual EMIMAc, typical degradation compounds (acetic acid, hydroxymethyl furfural and furfural) were increased and increased concentrations of these components might present toxic effects for the growth of the yeast.

The inhibitors, acetic acid, HMF (hydroxymethylfurfural) and also furfural were reported to result from sugar degradation owing to severe operation conditions conducted during pretreatment such as acidic environments and high temperatures (Palmqvist and Hahn-Hagerdal, 2000). Regarding the current pretreatment temperature and period (150°C and 30 minutes), formation of inhibitors during pretreatment were likely. Another potential inhibitor to cell growth would be the residual EMIMAc that remained on the pretreated cotton stalks even after the pretreated biomass was washed. According to a previously reported study, growth of *Saccharomyces cerevisiae* was found to decline significantly in the presence of EMIMAc at concentration of 30-35 mM. The analysis concerning the effects of various cations on fermentation media, showed that the cation, $EMIM^+$ was the major inhibitor for the yeast growth and ethanol production (Ouellet et al., 2011).

According to the HPLC analysis carried out for the samples withdrawn during fermentation, the peaks representing acetate, HMF and furfural peaks were not detected in the chromatograms. Excessive washing of the pretreated biomass (at least 5 times with water having 10-fold higher mass than the mass of the ionic liquid) most probably resulted with the removal of these inhibitor compounds. Obviously, increased concentrations of the salts, citric acid and sodium citrate might cause a decline in the yeast growth. It was also predicted that the adverse effect of these compounds on the cell growth would be more noticeable for the fermentation medium that was more concentrated to higher glucose concentration such that the similar effects would be observed for the hydrolyzate medium containing 100 g/L of glucose initially.



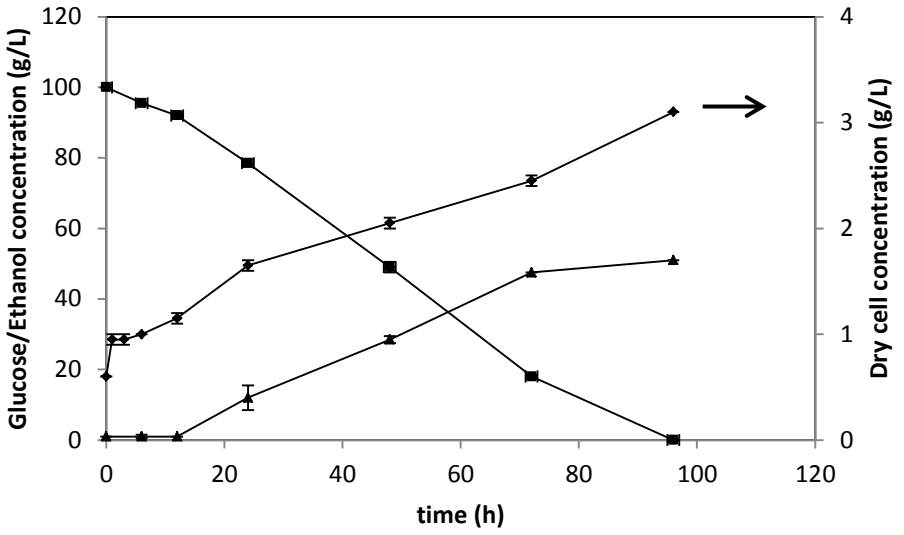
(a)



(b)

Figure 4.30 Time course of cell growth (♦), glucose consumption (■) and ethanol production (▲) for the hydrolyzate (a) and control (b) containing initial glucose concentration of 50 g/L.

Lastly, ethanol production was analyzed by concentrating the hydrolyzate almost 5-fold to obtain 100 g/L of initial glucose concentration in the fermentation medium. As seen in Figure 4.31, glucose was consumed within 96 hours of the fermentation and 51 g/L of ethanol, which corresponds to 6.5% (v/v) of ethanol titer, was produced by *Saccharomyces cerevisiae* in both fermentation media. As expected, dry cell concentrations attained for the hydrolyzate was lower compared to the medium containing pure glucose. According to Figure 4.31b, yeast was observed to reach the stationary phase of the growth within 24 hours in the medium possessing pure glucose whereas it has not yet reached the stationary phase in the hydrolyzate medium even at the 96^{th} hour of the process. Though, dry cell yield over glucose consumption, $Y_{X/S}$ (g dry cell/g glucose) was nearly 3 fold higher for the medium containing pure glucose compared to that achieved for the hydrolyzate medium, the wild-type yeast *Saccharomyces cerevisiae*, which resulted with at least 98% of the theoretical maximum ethanol yield, demonstrated a very satisfactory performance in the hydrolyzate media regardless of the initial glucose concentration.



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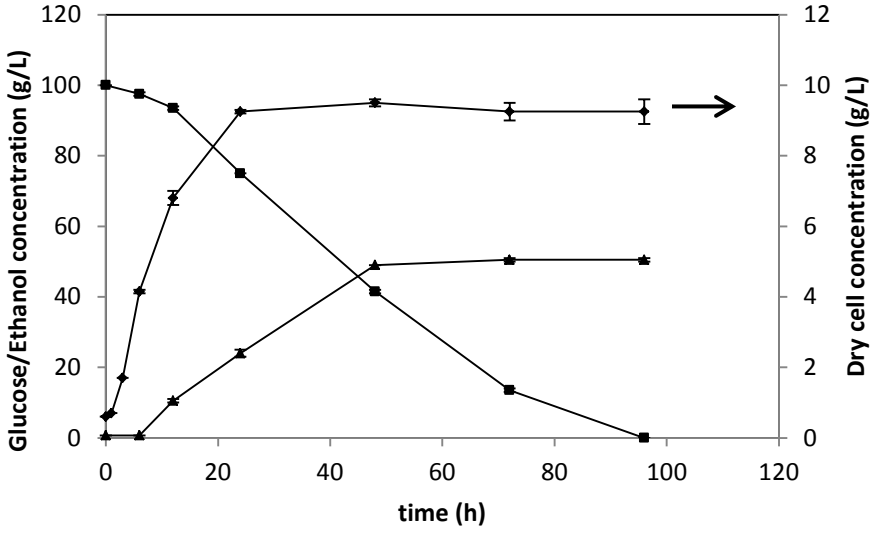


(b)

Figure 4.31 Time course of cell growth (♦), glucose consumption (■) and ethanol production (▲) for the hydrolyzate (a) and control (b) containing initial glucose concentration of 100 g/L.

4.2.11 Overall assessment of ethanol production from cotton stalks

In this study, the focus was to produce ethanol from cotton stalks. To achieve this target, pretreatment, enzymatic hydrolysis and fermentation were evaluated step by step and an optimization was performed at each step. Since pretreatment, which enhanced the digestibility of the lignocellulosic biomass, was considered as the most crucial step for cellulosic ethanol production, the major effort was given to conduct pretreatment at the most effective conditions. First of all, analyses were conducted to understand the interaction of cotton stalks with ionic liquids as discussed in the part 4.1, "Preliminary studies". The results were interpreted in terms of the solid recovery and digestibility obtained upon pretreatment and enzymatic hydrolysis, respectively. No structural analysis was performed and the amount of glucose released after the enzymatic reaction was not determined. In the following parts, much detailed analyses were performed; the structural changes gathered in the cotton stalks upon ionic liquid pretreatment were determined upon characterization of the pretreated biomass. Additionally, the glucose derived upon enzymatic hydrolysis of the pretreated cotton stalks was determined to decide on the best pretreatment condition giving the highest amount of glucose that would be utilized for ethanol production. EMIMAc as a pretreatment agent exhibited the most noticeable effect on the structure and digestibility of the cotton stalks. Following the pretreatment, enzymatic hydrolysis was investigated in terms of the effects of enzyme concentration and enzyme loading on the glucose concentration. Finally, ethanol production was carried out in the hydrolyzate media that possessed different glucose concentrations initially (obtained through concentrating the hydrolyzate) together with the performance of the wild type yeast, *Saccharomyces cerevisiae* NRRL Y-132 in the hydrolyzate media which was compared to that obtained in the media containing pure glucose.

Figure 4.32 presented an overall evaluation of the process; starting with untreated cotton stalks and ending up with the amount of ethanol produced. The mass balances were made on the basis of 100 g of untreated cotton stalks. The compositions of the solid and liquid products, percentage of the theoretical yields upon enzymatic hydrolysis and fermentation and the operation conditions were given in the flow chart. As seen in the flow chart, 100 g of untreated cotton stalks were found to possess almost 40 g of cellulose and 25 g of lignin. After EMIMAc pretreatment that was conducted at 10% of biomass loading and 150°C for 30 minutes, 47 g of cotton stalks were recovered as EMIMAc pretreated biomass. The rest of the biomass that remained in the recovered aqueous solution of EMIMAc could not be recovered. Pretreated cotton stalks were found to consist 28 g of cellulose and 14 g of lignin. Accordingly, 12 g of cellulose and 12 g of lignin could not be recovered after precipitation of the pretreated cotton stalks upon EMIMAc pretreatment. Later on, this 28 g of cellulose was enzymatically hydrolyzed to yield 30 g of glucose. The glucose yield was attained as 96% of the theoretical maximum yield (based on the maximum amount of glucose that could be produced from the cellulose present in the EMIMAc pretreated cotton stalks) at the 48^{th} hour of the hydrolysis. The solid product left after enzymatic hydrolysis (16 g), which was found to contain 88% of lignin (14 g), was regarded as an important by-product. Following the enzymatic hydrolysis, 30 g of glucose present in the hydrolyzate was converted to 15 g of ethanol by the wild type yeast, *Saccharomyces cerevisiae* NRRL Y-132 within 12 hours with 98% of the theoretical maximum yield.

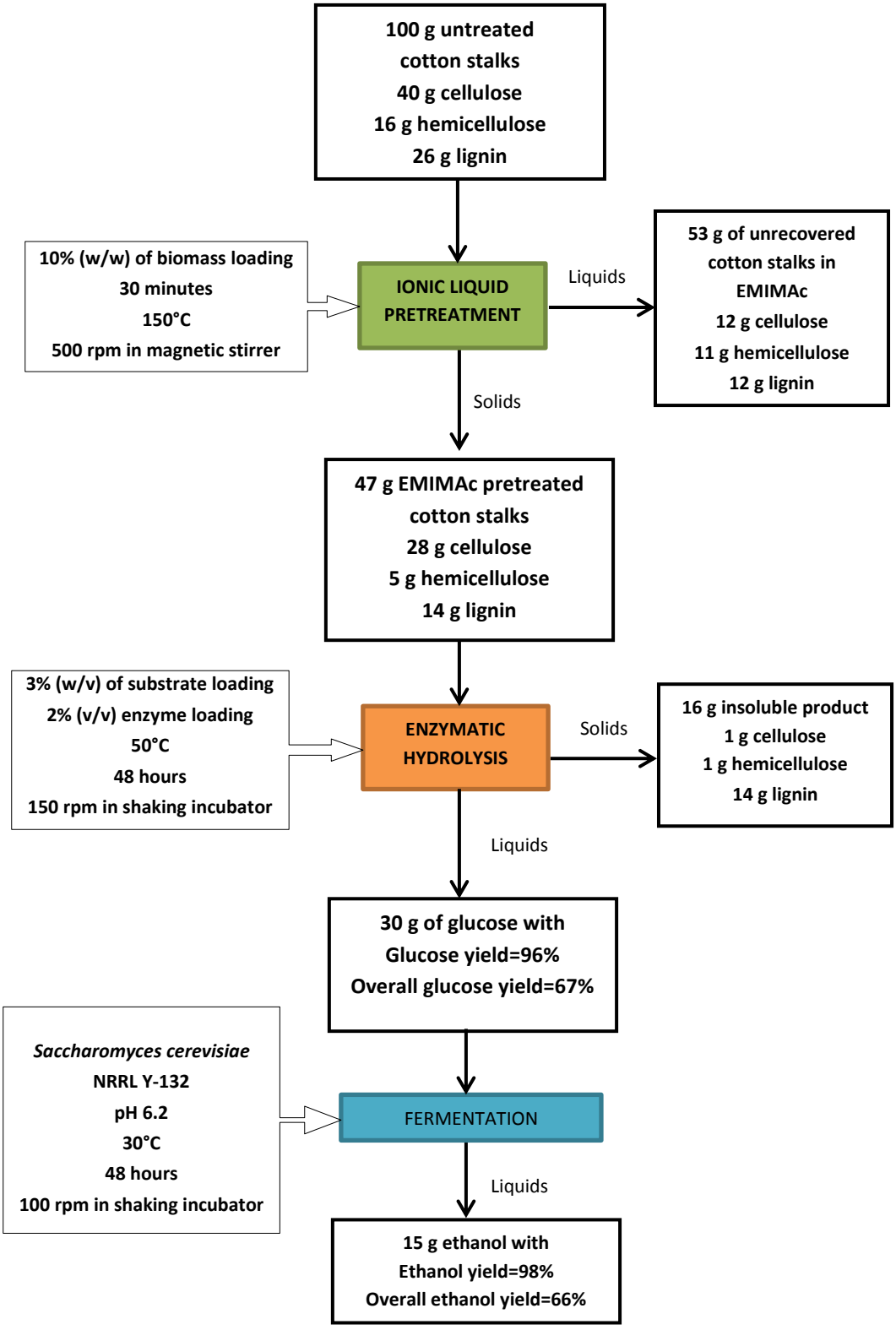


Figure 4.32 Flow chart for ethanol production from cotton stalks on the basis of 100 g untreated cotton stalks.

Glucose and ethanol yields, which were resulted as 96% and 98% of the theoretical maximum yields, respectively, appeared to be satisfactory when cellulose content of EMIMAc pretreated cotton stalks were considered. However, one should consider the maximum amount of glucose and ethanol that could be produced from the cellulosic portion of the untreated cotton stalks to discuss on the efficient utilization of cotton stalks with the aim of ethanol production. The glucose and ethanol yields were determined based on the cellulosic portion of the untreated cotton stalks and found as 67% and 66%, respectively. These low yields should be related to the significant amount of cellulose lost during EMIMAc pretreatment. As observed from the flowchart, almost 30% of cellulose present in untreated cotton stalks got degraded during pretreatment and thus could not be recovered within the precipitated portion after water addition.

Consequently in the following part, the efforts were made to increase the cellulose recovery and thus, enhance glucose and ethanol yields based on the cellulose content of the untreated cotton stalks. For this reason, EMIMAc pretreatment was conducted at higher biomass loadings without stirring with the aim of improving the cellulose recovery upon EMIMAc pretreatment.

Although the yields obtained regarding the efficient utilization of cellulosic portion of untreated cotton stalks to produce glucose and ethanol (67% for glucose and 66% for ethanol) were low, this part of the study should be considered as a starting point for ethanol production from lignocellulosic biomass that was subjected to ionic liquid pretreatment. The opportunities provided via EMIMAc and its utilization in pretreatment of cotton stalks to produce ethanol could be summarized one by one:

- EMIMAc, as a suitable pretreatment agent used in biomass processing, possessed relatively low volatility and thus, convenience in handling.
- The pretreatment period (30 minutes) conducted was shorter compared to most of the conventional methods and this 30 minutes of pretreatment period was sufficient to attain an efficient interaction between cotton stalks and EMIMAc.Even shorter periods during pretreatment can work to attain a structure that is prone to hydrolysis.
- Pretreated cotton stalks were conveniently recovered from the reaction mixture after the reaction was terminated by water addition. Following the solid recovery, EMIMAc was recycled by evaporation of the water and reused for further pretreatment without being purified.
- There was no need to adjust the pH of the pretreated cotton stalks prior to enzymatic hydrolysis. Pretreated cotton stalks were washed with sufficient amount of water to remove the residual EMIMAc and directly used in the enzymatic reaction.
- EMIMAc was capable of extracting 47% of the lignin present in the untreated cotton stalks and thus enhancing the cellulose content of the biomass from 40% to 60% prior to enzymatic hydrolysis.
- The conversion of pretreated cotton stalks to fermentable sugars was much faster compared to the hydrolysis of untreated cotton stalks which attributed to the efficiently disrupted crystalline structure of the cotton stalks upon EMIMAc pretreatment.
- The novel enzyme preparation Cellic Ctec2 at an enzyme loading of 2% (v/v) was able to convert 96% of the cellulose, which was present in EMIMAc pretreated cotton stalks at a substrate loading of 3% (w/v), to glucose at the 48^{th} hour of the hydrolysis. This finding was encouraging since enzymatic hydrolysis with this novel enzyme cocktail, which possessed 3-fold higher cellulase activity compared to Celluclast 1.5L, could provide the hydrolysis of cotton stalks at higher substrate loadings.
- *Saccharomyces cerevisiae* NRRL Y-132 metabolized almost all glucose present in the hydrolyzates prior to fermentation, resulting with over 95% of the theoretical maximum ethanol yield regardless of the initial glucose concentration of the fermentation media.

4.3 Pretreatment of cotton stalks via EMIMAc at higher biomass loadings

The impact of ionic liquid pretreatment on the structural features and enzymatic accessibility of the cotton stalks have been discussed in the previous part. Several ionic liquids were screened and among, EMIMAc was recognized as superior regarding its functionality in pretreatment of cotton stalks and thereby production of high concentrations of glucose (19 g/L) which enabled its comprehensive utilization for cellulosic ethanol production. However, almost half amount of the untreated cotton stalks was recovered after pretreatment; cotton stalks lost 30% and 47% of their cellulose and lignin, respectively as a consequence of superior solvation capability of EMIMAc towards lignocellulosic biomass. Cellulose loss, which was considered as a considerable amount, lowered glucose and hence ethanol yields that were based on the amount of cellulose present in untreated cotton stalks. To improve glucose and ethanol yields which were found as 67% and 66%, respectively; a solution was proposed regarding the study performed by Dordick and his co-workers (H.Wu et al.,2011). In the mentioned study, researchers conducted EMIMAc pretreatment at high corn stover loadings up to 50% (w corn stover/w slurry) with the aim of introducing an alternative way of pretreatment that was less expensive compared to the studies conducted at usual biomass loadings ranging from 5 to 10% (S.H. Lee et al., 2009, Arora et al., 2010, Nguyen et al., 2010, Samayam and Schall, 2010, Li et al., 2011, Shill et al., 2011), since there would be a chance to pretreat much amount of biomass in an ionic liquid. Dordick and his co-workers (2011) found that corn stovers that were pretreated at higher biomass loadings such as 33% (w corn stover/w slurry), were still possessing reduced crystallinity compared to the untreated samples. Accordingly, EMIMAc was found to be only capable of wetting the surface of the biomass at high biomass loadings and reducing the crystallinity of the biomass without changing its composition to a significant degree. Together with this finding, they concluded that crystallinity has been a more critical parameter compared to the extracted lignin with respect to their effect on the enzymatic accessibility of the biomass.

Based on these findings, cotton stalks loading increased from 10% to 50% (w cotton stalks/ w EMIMAc) without stirring considering that there would be less cellulose degradation under the conditions that EMIMAc acted as a pretreatment agent rather than a dissolution agent. By this way, effective utilization of the cellulosic portion of the cotton stalks to produce glucose and ethanol would be more likely.

In addition to the advantageous points stated above, less cellulose degradation and less amount of lignin extracted from the biomass would also facilitate the reuse of EMIMAc. In the case that more lignin was extracted from biomass or more cellulose degraded and remained in the recovered ionic liquid upon pretreatment; accumulation of the biomass components at each recycle of the ionic liquid would decrease the efficiency of EMIMAc as a pretreatment agent after a certain point. Accordingly, equilibrium would be attained with respect to the concentration of the residual biomass components and EMIMAc would be no more capable of disrupting the recalcitrant structure of cotton stalks to the same degree. Besides, employing higher biomass loadings or in other words lowering the amount of EMIMAc in pretreatment would obviously contribute to the attractiveness of this alternative solution in respect of the process costs.

4.3.1 Effect of biomass loading on the structure of cotton stalks

In this part, pretreated cotton stalks were examined in terms of their crystalline structures and composition since increased biomass loadings during EMIMAc pretreatment ended up with various effects on the structural features of the cotton stalks compared to the changes obtained upon EMIMAc pretreatment at 10% of biomass loading (w cotton stalks/w EMIMAc). Pretreatment was carried out in a round bottom vessel that was placed in an oil bath of a rotary evaporator and no stirring effect was introduced. In fact, it was not possible to stir the slurry during the incubation since EMIMAc just resulted with a wetting effect for the particles at high biomass loadings. However prior to the reaction, the slurry was mixed with a glass rod to ensure that EMIMAc wetted the cotton stalks completely.

The changes in the crystalline structure of the cotton stalks after EMIMAc pretreatment conducted at different biomass loadings were given in Figure 4.33. According to the XRD patterns of the pretreated samples, cotton stalks that were subjected to EMIMAc pretreatment up to 30% of biomass loading (w cotton stalks/w EMIMAc) were found to possess lower crystallinity compared to untreated cotton stalks; exhibiting similar patterns with the pretreated sample at 10% loading under stirring. XRD pattern of the samples that were subjected to EMIMAc pretreatment at biomass loadings of 40% and 50% were close to that obtained for the untreated cotton stalks as recognized from the two main peaks appeared slightly at around $2\theta=15°$ and 22°.

One major conclusion which could be derived from the XRD analysis is that EMIMAc was capable of disrupting the crystalline structure of the cotton stalks when pretreatment was carried out at 10% of biomass loading without stirring. As noticed, very similar XRD patterns were obtained for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading with and without stirring. Based on this finding, stirring conducted during pretreatment was not considered as a crucial parameter with respect to its effect on the crystallinity of the biomass. One might also realize that shifting of the peaks at around $2\theta=22°$ to lower Bragg angles, which was observed for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading with and without stirring, could not be obtained for the cotton stalks that were subjected to EMIMAc pretreatment at biomass loadings of 20% and 30%. This result indicated that the highest level of reduction in the crystallinity was attained for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading; since shifting of the peak that was observed at round $2\theta=22°$ to lower Bragg angles was directly linked to the modification of the cellulose I (Li et al., 2010, Reddy and Yang, 2009).

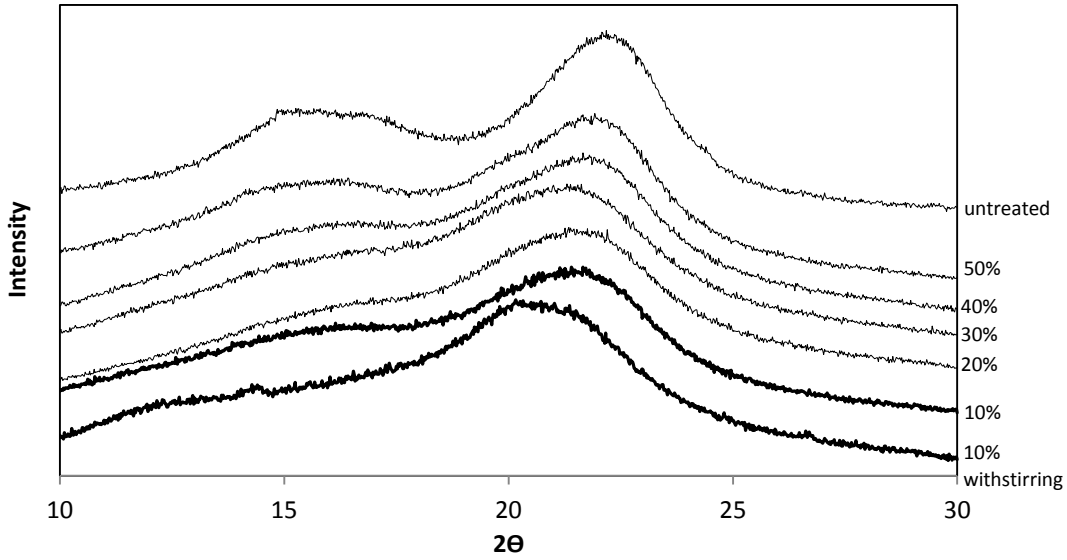


Figure 4.33 Effect of biomass loading (% w cotton stalks/w EMIMAc) during EMIMAc pretreatment on crystallinity of the cotton stalks.

In addition to the XRD analysis, the pretreated cotton stalks were examined in terms of their composition before and after EMIMAc pretreatment in order to determine the amount of cellulose loss (%) and extracted lignin from the untreated cotton stalks (%). For this reason, compositional analysis based on the procedure given by National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008) was followed. The cellulose loss (%) and extracted lignin from the untreated cotton stalks (%) were determined according to the equations, 3.13 and 3.14 given in Part 3.

Figures 4.34 and 4.35 demonstrated the variations of cellulose loss and extracted lignin with biomass loading during EMIMAc pretreatment. The most noticeable finding was obtained for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading; cellulose loss decreased from 30% to 6% when the pretreatment was employed without stirring. Though stirring had been considered as a necessity for an effective ionic liquid pretreatment in the previous experiments; this current result, which was in accordance with the conclusion derived from the XRD analysis, indicated that stirring was not essential for the effectiveness of the reaction between biomass and ionic liquid. Additionally, cellulose loss was found to decrease from 6% to 2% with an increase in the biomass loading from 10% to 50% for the samples pretreated via EMIMAc without stirring. Similarly, the variation in the amount of lignin extracted from the untreated cotton stalks was found as remarkable for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading; extracted lignin decreased from 47% to 17% when the pretreatment was conducted without stirring. The extracted lignin from the untreated cotton stalks was found to change between 0-17% and decrease as the biomass loading increased for the samples pretreated via EMIMAc without stirring.

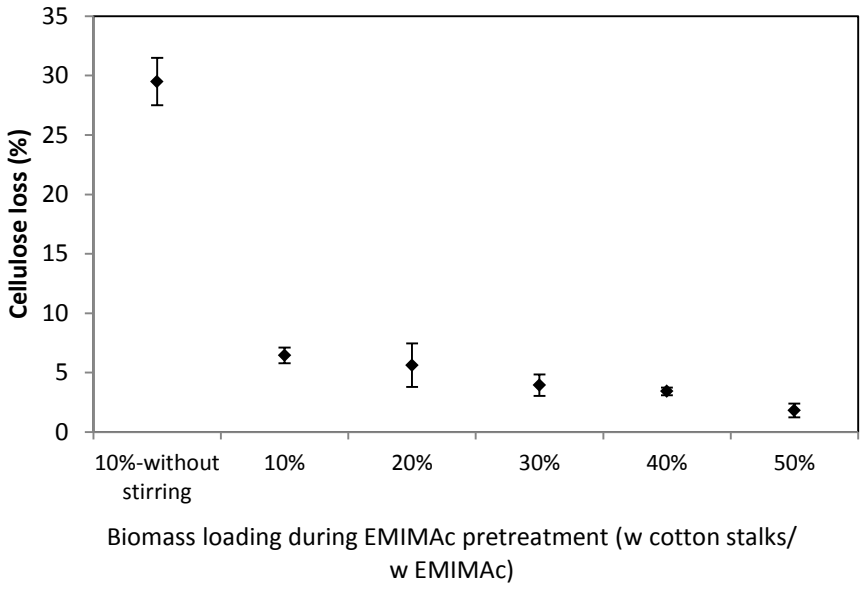


Figure 4.34 Effect of biomass loading (% w/w) conducted during EMIMAc pretreatment on cellulose loss (%).

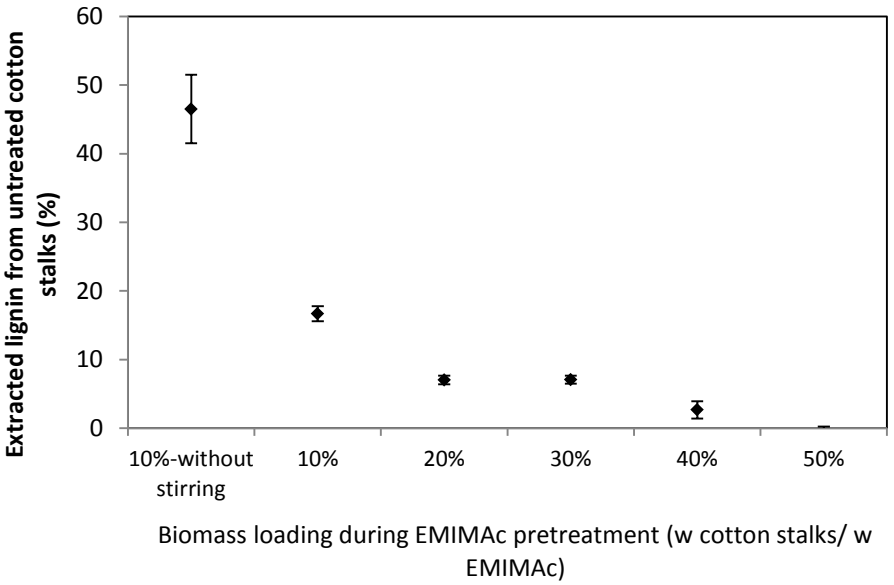


Figure 4.35 Effect of biomass loading (% w/w) conducted during EMIMAc pretreatment on extracted lignin from untreated cotton stalks (%).

Although the remarkable reductions achieved for cellulose loss at high biomass loadings were interpreted as a positive aspect that would provide effective conversion of the cotton stalks to glucose and ethanol, the decrease in the extracted lignin appeared to be a disadvantage considering the previously reported studies. Lignin extraction has been regarded as one of the major duties of ionic liquid pretreatment exploited for biomass processing (Sun et al., 2009, Tan et al., 2009, Fu et al., 2010). However, extraction of significant amounts of lignin from the biomass has been no longer considered as a crucial factor that determined the enzymatic accessibility of the biomass when compared to the leading role of the crystallinity as demonstrated by Dordick and his co-workers (H.Wu et al., 2011). Likewise in this study, the results derived upon enzymatic hydrolysis of the EMIMAc pretreated cotton stalks implied that crystallinity of the cotton stalks appeared as a more vital parameter compared to the impact of extracted lignin on the enzymatic accessibility of the pretreated cotton stalks.

4.3.2 Effect of biomass loading on the digestibility of the cotton stalks

The variations of the solid recovery and the digestibility of the cotton stalks with biomass loading during EMIMAc pretreatment were shown in Figure 4.36. As readily observed from the figure, the solid recovery upon EMIMAc pretreatment increased with an increase in biomass loading. This finding was expected since the major target was to perform EMIMAc pretreatment in a controlled fashion by means of preventing the excessive dissolution of the biomass in the ionic liquid. The solid recovery increased from 47% to 70% by excluding the stirring during EMIMAc pretreatment at 10% biomass loading. This result was very well in accordance with the sharp decreases obtained for cellulose degradation (from 30% to 6%) and extracted lignin (from 47% to 17%) with elimination of the stirring as discussed in the previous part.

Unlike the solid recovery, the digestibility of the cotton stalks was found to decrease with the increase in biomass loading during EMIMAc pretreatment. The digestibility of EMIMAc pretreated cotton stalks at the 48^{th} hour of the enzymatic hydrolysis decreased from 75% to 68% with elimination of the stirring for the cotton stalks subjected to EMIMAc pretreatment at 10% of biomass loading. Digestibility was found as 65% at the 48^{th} hour of the hydolysis for the cotton stalks subjected to EMIMAc pretreatment at 30% of biomass loading without stirring in which the extracted lignin from the cotton stalks was only 7% at the described conditions (Figure 4.35). While digestibility of the EMIMAc pretreated cotton stalks at the 48^{th} hour of the hydrolysis was observed to change between 65-75%, the extracted lignin from untreated cotton stalks ranged in a much wider range, 7-47% (Figure 4.35). Briefly, the incapability of EMIMAc to extract lignin at biomass loadings during pretreatment had minor effect on the digestibility of the cotton stalks. The reduction in the crystallinity was considered as a more effective parameter to enhance the digestibility of the biomass as similarly reported in a previous study (H.Wu et al., 2011).

In addition to the biomass digestibility, effect of biomass loading was also investigated with respect to its effect on the glucose yields calculated on the basis of both cellulose content of the untreated and EMIMAc pretreated cotton stalks (Figure 4.37). The glucose yield that is based on the glucose content of the EMIMAc pretreated cotton stalks (Glucose yield) demonstrated how effective the enzyme, Cellic Ctec2 converted the pretreated cotton stalks, which differed significantly in terms of their lignin content, to glucose. On the other hand, demonstrating the efficiency of the enzymatic reaction in terms of the glucose yield on the basis of the cellulose content of the untreated cotton stalks (Overall glucose yield) was more crucial compared to the former since the amount of cellulose that was recovered upon EMIMAc pretreatment was considered in its determination.

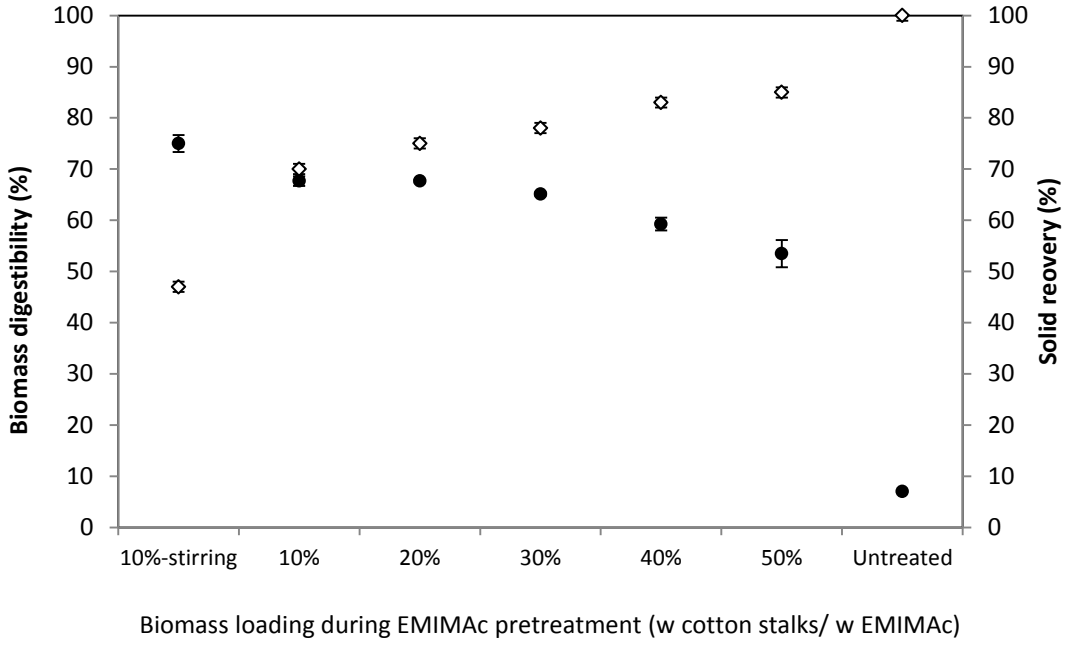


Figure 4.36 Effect of biomass loading on the digestibility of cotton stalks at the 48th hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

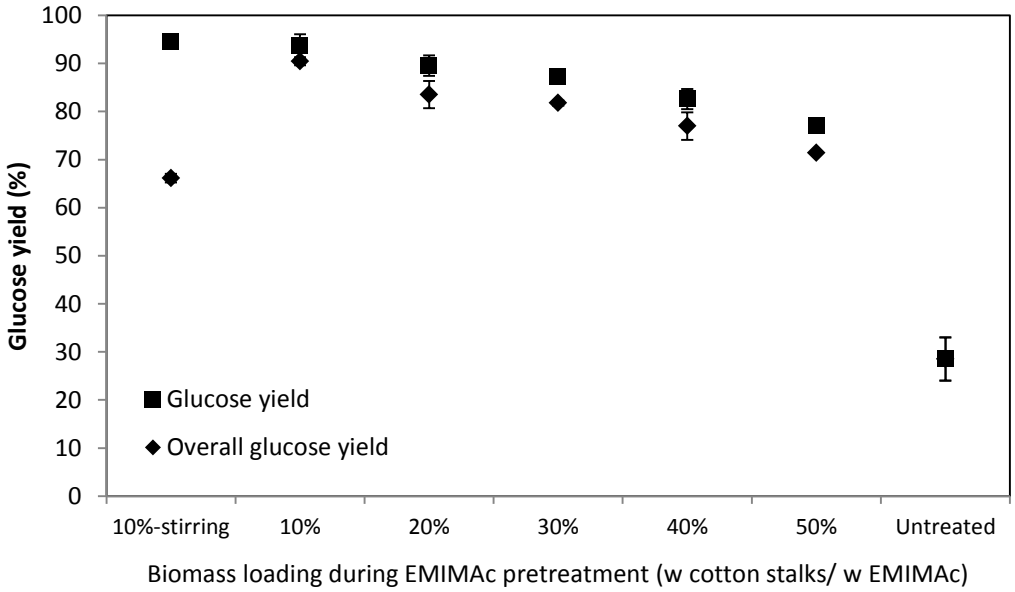


Figure 4.37 Effect of biomass loading on the glucose yields that were obtained on the basis of the theoretical maximum amount of glucose that can be obtained from the cellulosic portion of the EMIMAc pretreated cotton stalks, glucose yield and untreated cotton stalks, overall glucose yield.

According to the Figure 4.37, the highest glucose yield was obtained as 95% for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading under stirring. However, that sample exhibited the lowest glucose yield based on the cellulose content of the untreated cotton stalks which was 66%. This opposing trend was linked to the lower amount of cellulose recovered upon EMIMAc pretreatment at 10% of biomass loading under stirring. Briefly, under those conditions EMIMAc resulted with degradation of almost 30% of cellulose present in the untreated cotton stalks and only 66% of the cellulosic portion of the untreated cotton stalks was converted to glucose upon enzymatic hydrolysis. On the other hand, EMIMAc extracted considerable amount of lignin (the biomass was enriched in cellulose), disrupted the crystalline structure at the highest level under those conditions.

Elimination of stirring for the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading enhanced overall glucose yield from 66% to 90% since cellulose degradation during the pretreatment decreased from 30% to 6%. However, the exclusion of stirring during the process did not affect the glucose yield which was found as 94%. As seen in Figure 4.37, difference between glucose yields that were determined on the basis of cellulose content of different substrates were much less for the cotton stalks that were subjected to EMIMAc without stirring since much less cellulose degradation was attained under those conditions. Glucose yields were found to range between 82-90% for the cotton stalks that were subjected to EMIMAc pretreatment at biomass loadings between 10-30% without stirring. But much lower glucose yields were obtained for the cotton stalks that were subjected to EMIMAc pretreatment at biomass loadings at 40% and 50% which was in accordance with the results derived upon XRD analysis. The XRD patterns obtained for the cotton stalks that were pretreated via EMIMAc at biomass loadings, 40% and 50% were similar to that obtained for the untreated cotton stalks (Figure 4.33).

To decide on the most appropriate condition that would be selected to further continue with ethanol production, results should be interpreted by considering all major findings obtained; structural changes and yields obtained upon pretreatment and hydrolysis, respectively. The results were summarized in Table 4.15. According to the findings given in the table, utilization of the cotton stalks, which were subjected to EMIMAc pretreatment at a biomass loading of 30%, for ethanol production would be reasonable since they appeared as the sample pretreated at the highest biomass loading in which reduced crystallinity and also satisfying biomass digestibility and glucose yields were obtained. Utilization of the cotton stalks that were pretreated via EMIMAc at 30% of biomass loading would also provide significant benefits for the process costs since there would be an opportunity to pretreat 3-fold higher amount of biomass with EMIMAc without stirring when compared to the former pretreatment conditions conducted (10%-stirring) for ethanol production from EMIMAc pretreated cotton stalks.

Table 4.15 Results that summarize the effect of biomass loading on the structural changes obtained upon EMIMAc pretreatment and enzymatic accessibility of the pretreated cotton stalks

Biomass loading (%)	Crystalline structure	Cellulose loss (%)	Extracted lignin (%)	Biomass digestibility (%)	Glucose yield (%)	Overall glucose yield (%)
10% with stirring	The most amorphous	30	47	75	95	66
10%	Amorphous	6	17	68	94	90
20%	Amorphous	6	7	68	90	84
30%	Amorphous	4	7	65	87	82
40%	Less amorphous	3	3	59	83	77
50%	Less amorphous	2	-	53	77	71
Untreated	Crystalline	-	-	7	29	29

In the following part, alkaline pretreatment would be investigated with respect to its effect on the structural changes in the cotton stalks and also their enzymatic accessibility. Alkaline pretreatment has been considered as a promising approach for lignin removal from the biomass (Sun and Cheng, 2002, Hendriks and Zeeman, 2009, Alvira et al., 2010). Since cotton stalks possess substantial amounts of lignin (26-30%), alkaline pretreatment of cotton stalks was found to enhance the conversion of cotton stalks to fermentable sugars (Silverstein et al., 2007). The study reported by Silverstein et al. (2007) was followed and offered as an alternative to ionic liquid pretreatment for conversion of the cotton stalks to ethanol. These two promising methods were compared with respect to their effects on the structural changes obtained upon pretreatment and particularly digestibility of cotton stalks at high substrate loadings in order to improve glucose and ethanol concentrations.

4.4 Effect of alkaline pretreatment on the structure and digestibility of cotton stalks

In this part, alkaline pretreatment was investigated as an alternative approach to ionic liquid pretreatment at various NaOH concentrations. Pretreatment was carried out according to procedure of Silverstein et al. (2007) since it was one of the most cited articles in the field of biorefinery and the procedure was conducted particularly for cotton stalks. The pretreatment was found to be effective towards lignin removal; 65% of lignin was extracted from cotton stalks upon its pretreatment with 2% (w/v) of NaOH at 121°C for 90 minutes and additionally, pretreated samples exhibited higher

digestibility compared to those pretreated via, sulfuric acid, hydrogen peroxide and ozone pretreatments, major chemical pretreatment techniques for lignocellulosic biomass. In addition to its superior capability in lignin removal, alkaline pretreatment resulted with removal of hemicellulose which was almost 35% for the cotton stalks subjected to alkaline pretreatment at the conditions given above. Furthermore, Kaur et al. (2012) performed alkaline pretreatment with the focus on ethanol production from cotton stalks and found out that the highest lignin reduction for cotton stalks at a biomass loading of 10% (w/v) was obtained upon pretreatment conducted with 4% of NaOH for 90 minutes in an autoclave at 121°C. In another study, almost all lignin was removed upon pretreatment of cotton stalks at a biomass loading of 5% with 4% of NaOH at 180°C for 45 minutes (Binod et al., 2012).

The structural changes in the cotton stalks and digestibility of cotton stalks were examined at NaOH concentrations of 0.5%, 1% and 2% (w/v). Cotton stalks at a biomass loading of 10% (w/v) were incubated with NaOH solutions in autoclave at 121°C for 1 hour. At the end of the pretreatment, cotton stalks were washed with deionized water for three times and during the final wash, pH was adjusted to 4.8 via acetic acid.

To understand the structural changes in the biomass, cotton stalks were examined in terms of the compositional changes derived upon alkaline pretreatment. For this purpose, a compositional analysis was conducted for each alkaline pretreated sample for determination of the amount of lignin and hemicellulose removed upon pretreatment from the cotton stalks. The results were given in Figure 4.38 and 4.39. As seen in Figure 4.38, a linear relationship was found to exist between the extracted lignin from untreated cotton stalks (%) and percentages of NaOH (w/v). The highest lignin extraction was attained as 52% for the cotton stalks that were subjected to alkaline pretreatment at 2% of NaOH. For the cotton stalks that were pretreated via 0.5% of NaOH solution at 121°C for 1 hour, only 23% of lignin was extracted from the untreated cotton stalks. Similarly, the extracted hemicellulose from the untreated cotton stalks varied linearly with NaOH concentration (Figure 4.39). However, the variation in NaOH concentration was found to have a less considerable effect on the hemicellulose extracted than its effect on lignin reduction. The highest and lowest hemicellulose extraction were obtained as 49% and 61% for the cotton stalks that were subjected to alkaline pretreatment at 0.5% and 2% of NaOH, respectively. To sum up, pretreatment of cotton stalks by 0.5% NaOH was found to be sufficient for extraction of almost 50% of hemicellulose from untreated cotton stalks, however 2% NaOH was required for extraction for 52% of lignin from the biomass since the decomposition of lignin was much more difficult compared to hemicellulose removal.

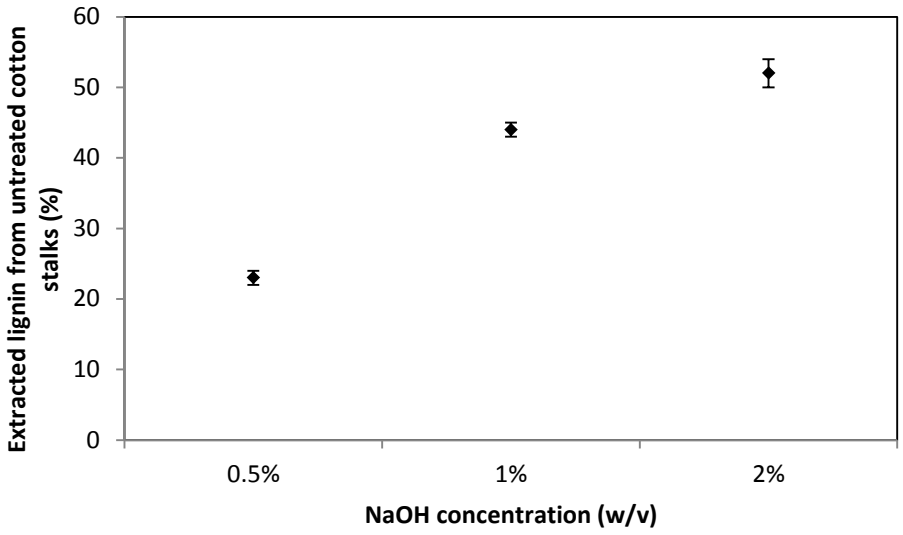


Figure 4.38. Effect of NaOH concentration (% w/w) during alkaline pretreatment on extracted lignin from untreated cotton stalks

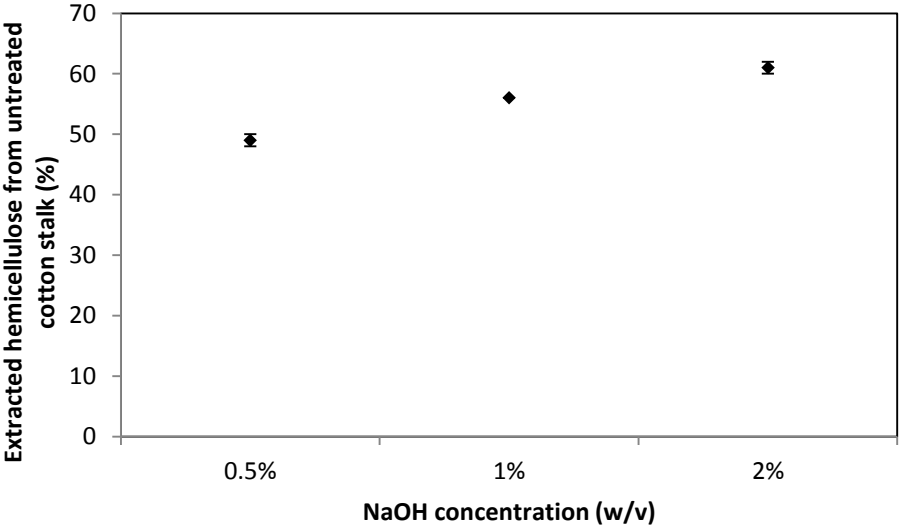


Figure 4.39 Effect of NaOH concentration (% w/w) during alkaline pretreatment on extracted hemicellulose from untreated cotton stalks

Besides its effect on the structure of pretreated cotton stalks, the variation in NaOH concentration was examined with respect to its effect on the digestibility of cotton stalks. Alkaline pretreated cotton stalks were subjected to enzymatic hydrolysis at the same conditions conducted for EMIMAc pretreated cotton stalks; biomass at a substrate loading of 3% (w/v) was enzymatically hydrolyzed with 2% (v/v) of Cellic Ctec2 for 48 hours. The digestibility of alkaline pretreated cotton stalks that is based on the reducing sugar concentration (g/L) attained at the 48^{th} hour of the hydrolysis was given in Figure 4.40. As shown in the figure, the digestibility of the alkaline pretreated cotton stalks increased from 54% to 69% with an increase in the concentration of NaOH from 0.5% to 2%. As expected, the solid recovery upon alkaline pretreatment decreased from 73% to 55% with an increase in NaOH concentration from 0.5% to 2%.

Pretreated cotton stalks were also investigated in terms of the glucose yields that were determined on the basis of cellulose content of alkaline pretreated cotton stalks (Glucose yield) and untreated cotton stalks (Overall glucose yield). As seen in Figure 4.40, glucose yield was the highest for the cotton stalks that were subjected to alkaline pretreatment at 1% (w/v) of NaOH concentration. However overall glucose yield was the highest (69%) for the cotton stalks that were subjected to alkaline pretreatment at 2% NaOH (w/v). The overall glucose yield was considered as more critical for determination of the condition that would be further utilized for ethanol production since it includes the cellulose recovered upon pretreatment. As discussed part in the previous part, the higher the cellulosic portion of the biomass was recovered upon pretreatment, the higher the amount of glucose obtained from the biomass in its native structure upon hydrolysis for its conversion to ethanol. For this reason, the alkaline pretreated cotton stalks at 2% of NaOH concentration would be utilized with the aim of conducting a comparison with EMIMAc pretreated cotton stalks for production of ethanol.

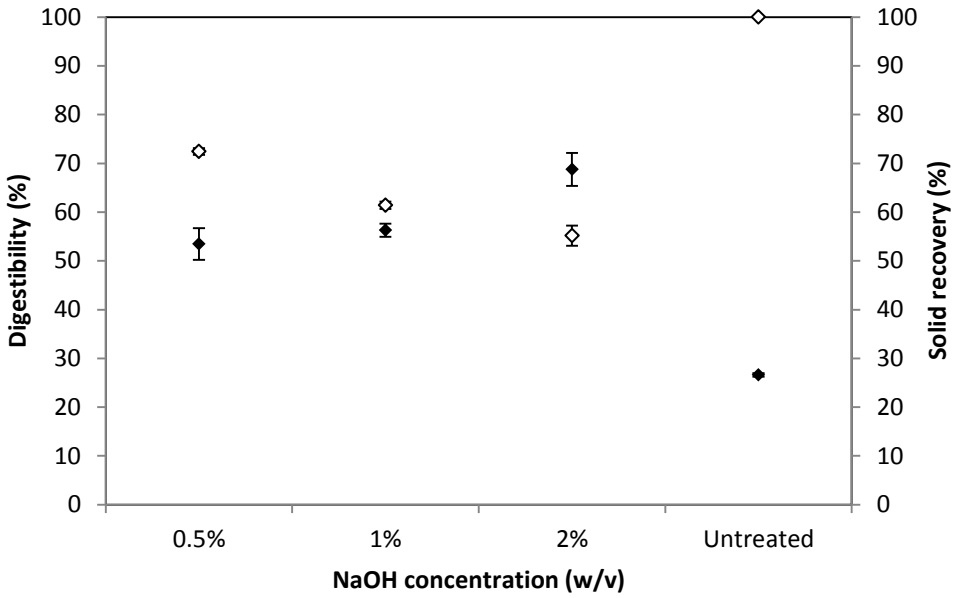


Figure 4.40 Effect of NaOH concentration (% w/v) during alkaline pretreatment on the digestibility of cotton stalks at the 48th hour of the hydrolysis (●) and solid recovery obtained upon pretreatment (◊).

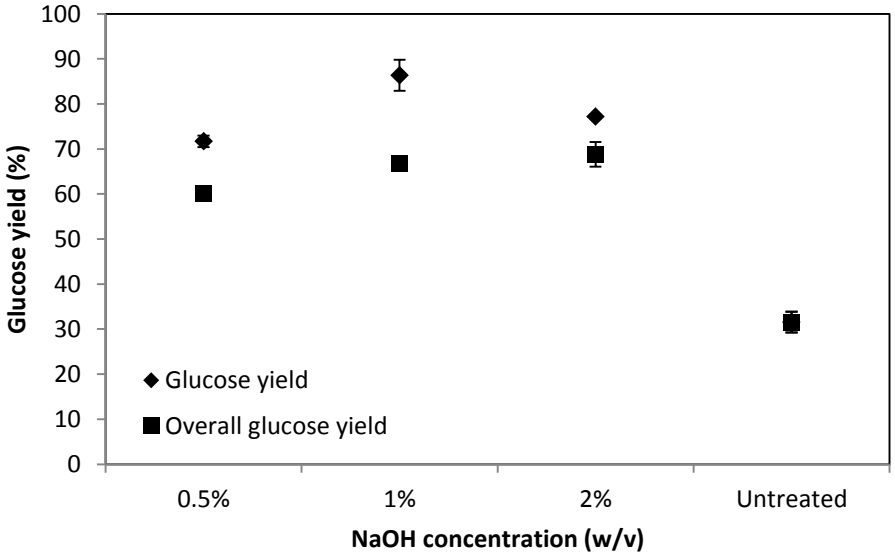


Figure 4.41 Effect of NaOH concentration (% w/v) on the glucose yields that was obtained on the basis of the theoretical maximum amount of glucose that can be obtained from the cellulosic portion of the alkaline pretreated cotton stalks, Glucose yield and untreated cotton stalks, Overall glucose yield.

4.5 Comparison of ionic liquid and alkaline pretreatments for ethanol production from cotton stalks

In this part, pretreatment techniques, ionic liquid and alkaline pretreatment were assessed in respect of their effects on the enzymatic digestibility of cotton stalks at high substrate loadings and ethanol production.

Alkaline pretreatment has been accepted as an encouraging approach for a variety of lignocellulosic biomass possessing particularly high contents of lignin and also hemicellulose (Silverstein et al., 2007, L.Wu et al., 2011a, L.Wu et al., 2011b). However, utilization of additional chemicals for pH adjustment for the hydrolyzate prior to its exploitation in enzymatic hydrolysis and fermentation and also disposal of the alkaline reagent after the process would obviously bring economic and environmental drawbacks. At this point ionic liquid pretreatment could be offered as an alternative that would bring much less environmental impact compared to alkaline pretreatment. Up to this point, these two techniques were examined independently in respect of their impact on the structural features and enzymatic accessibility of the biomass. To understand how one might introduce advantages/disadvantages over the other with respect to the efficient conversion of the cotton stalks to glucose and ethanol together with the following aspects; process economy and environmental impacts, a comparison should be performed. This comparison was essential since it would light the way to the conclusion of the whole study by questioning the feasibility of the ionic liquid technology in biomass processing for production of biofuels and other bio-based products. For this reason, starting out with their effect on the structural changes (changes in crystalline structure and composition) obtained upon pretreatment; these techniques would be compared with respect to their effect on the enzymatic accessibility of the pretreated cotton stalks at high substrate loadings, lastly conversion of the glucose that was derived from the hydrolysis conducted at the highest substrate loading to ethanol. Finally, an overall mass balance would be provided in order to enable a better consideration at each three major step of the process.

The operation conditions employed for the pretreatment of cotton stalks were given in Table 4.16. According to the table, ionic liquid pretreatment was carried out under more moderate conditions compared to alkaline pretreatment; almost 3-fold higher amount of biomass loading and shorter pretreatment period were used for incubation of cotton stalks with EMIMAc under atmospheric pressure. For alkaline pretreatment, cotton stalks having the same particle size but at a lower biomass loading were incubated in 2% (w/v) of NaOH solution in an autoclave that operated at 15 psi for 60 minutes. Accordingly, these two pretreatment techniques appeared to generate diverse impacts on the structure and enzymatic digestibility of cotton stalks.

Table 4.16 Operation conditions used for ionic liquid and alkaline pretreatment of cotton stalks

Pretreatment method	Chemical	Particle size of the biomass (mm)	Pressure	Biomass loading	Temperature (°C)	Period (min)	pH adjustment after pretreatment
Ionic liquid pretreatment	Pure EMIMAc	≤2	At atmospheric pressure	30% (w cotton stalks/w EMIMAc)	150	30	No pH adjustment
Alkaline pretreatment	2% (w/v) NaOH solution	≤2	In an autoclave at 15 psi	10% (w cotton stalks/v NaOH solution	121	60	pH of the pretreated biomass was adjusted to 4.8 via glacial acetic acid

4.5.1 Comparison of ionic liquid and alkaline pretreatments with respect to their effects on the structure of cotton stalks

In this part, cotton stalks were examined in terms of the changes in their composition and crystalline structure that took place after pretreatment. There have been a variety of pretreatment techniques that led to different changes in the composition of the cotton stalks and the efforts were mainly put on removal of the lignin from biomass and disruption of its crystalline structure (Silverstein et al., 2007, Bahcegul et al., 2012a, Binod et al., 2012, Kaur et al., 2012, Haykir et al., 2013). Table 4.15 showed the composition of the cotton stalks prior and after pretreatments. In addition to the composition of the biomass, the table showed the percentages of extracted lignin, hemicellulose and cellulose loss from the cotton stalks upon ionic liquid and alkaline pretreatments. According to the data given in the table, 51% of lignin was extracted from the cotton stalks that were subjected to alkaline pretreatment whereas EMIMAc pretreatment was able to extract only 7% of lignin from the cotton stalks at the conditions given in Table 4.17. This was an expected result since alkaline reagents have been recognized as very effective in extracting lignin from the biomass. Besides, the lower amount of lignin extracted from the cotton stalks via EMIMAc pretreatment was linked to the interaction between EMIMAc and cotton stalks at 30% of biomass loading which was not effective compared to the EMIMAc pretreatment conducted at lower biomass loadings. When the previously reported findings were recalled, EMIMAc was able to extract nearly 45% of lignin from the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading under stirring (Haykir et al., 2013). Alkaline pretreatment was also shown to be effective in the extraction of considerable amount of hemicellulose from the cotton stalks in which 61% of hemicellulose was extracted from the cotton stalks. The capability of EMIMAc to extract hemicellulose was much lower; only 11% of hemicellulose was removed from the cotton stalks that were subjected to EMIMAc pretreatment at 30% biomass loading and 150°C for 30 minutes. When solid recoveries were compared, much lower solid recovery was obtained upon alkaline pretreatment (55%) compared to ionic liquid

pretreatment (78%) and the solids that were unrecovered upon alkaline pretreatment apparently accounted for the considerable amount of lignin and hemicellulose extracted during the pretreatment. Cellulose content of the cotton stalks subjected to alkaline pretreatment increased from 37% to almost 60% owing to the considerable amounts of lignin and hemicellulose removed from the biomass. However, the cellulose content of the biomass was found to increase slightly; from 37% to 45% for EMIMAc pretreated cotton stalks. Furthermore, the cellulose loss was found somewhat higher for the cotton stalks that were subjected to alkaline pretreatment which was 11%, whereas only 4% of cellulose was lost during EMIMAc pretreatment.

Table 4.17 Compositional analysis for untreated cotton stalks and cotton stalks subjected to ionic liquid and alkaline pretreatment.

	Solid recovery (%)	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**	**Extracted lignin (%)**	**Extracted Hemicellulose (%)**	**Cellulose loss (%)**
Untreated	100	37	18	26	-	-	-
Ionic liquid	78	45	22	32	7	11	4
Alkaline	55	60	13	23	52	61	11

The comparison related to the effects of these two leading pretreatment methods on the structural properties of the cotton stalks, was further investigated with respect to their effects on the crystalline structure of the biomass. Crystalline structure of the biomass has been regarded as a key feature for a biomass that hindered the accessibility of the biomass to enzymes (Hendriks and Zeeman, 2009). For this reason, disruption of the crystalline structure of the biomass has been regarded as vital as extraction of lignin and hemicellulose from the biomass during pretreatment. In the current study, the changes in the crystalline structure of the cotton stalks were mainly interpreted according to the changes observed in the major peaks at round 2θ=15° and 22° as shown in the XRD pattern of untreated cotton stalks. As shown in Figure 6.32, disruption in the crystalline structure of the cotton stalks through EMIMAc pretreatment could be monitored from the disappearance of the peak at around 15° and the decrease of the intensity of the peak at 22° in addition to its shift to lower Bragg angles. These changes obtained upon EMIMAc pretreatment were described as the indicators of a biomass structure, which possessed reduced crystallinity and more enzymatic accessibility compared to its native structure (H.Wu et al., 2011).

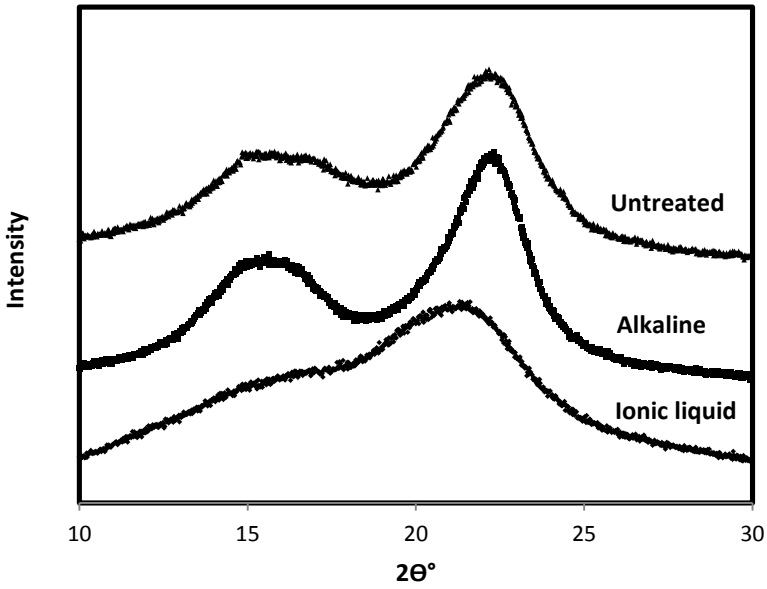


Figure 4.42 XRD patterns of untreated cotton stalks and cotton stalks that were subjected to ionic liquid and alkaline pretreatment

Unlike ionic liquid pretreated cotton stalks, the cotton stalks that were subjected to alkaline pretreatment exhibited an utterly different diffraction pattern in which the peaks at around 2θ=15° and 22° were found to retain their positions together with possessing higher intensities compared to untreated cotton stalks. Such a finding, which inferred to an increase in the crystalline structure of cotton stalks upon alkaline pretreatment, was also supported by the previously reported studies (L.Wu et al., 2011a, L.Wu et al., 2011b). Alkaline pretreatment of sweet sorghum bagasse via NaOH solution with a concentration of lower than 5M (corresponding to 20% (w/v) of NaOH) with the aim of delignification, resulted with an increase in the crystalline structure of the biomass and it was associated to the removal of amorphous portions (lignin and hemicellulose) of the biomass during pretreatment. However, conducting pretreatment at 5 M NaOH eventually had a positive effect on the crystallinity of bagasse; reductions in the intensity of the peaks observed for untreated bagasse and presence of the peak at around 2θ=12° were evidences of a structure possessing reduced crystallinity (L.Wu et al., 2011a). According to another study, crystallinity of the sugarcane bagasse was found to decrease as a result of its pretreatment via 5M of NaOH solution (Wada et al., 2010). Based on these findings, more concentrated NaOH solutions would be required to yield reduction in the crystalline structure of the biomass that was subjected to alkaline pretreatment.

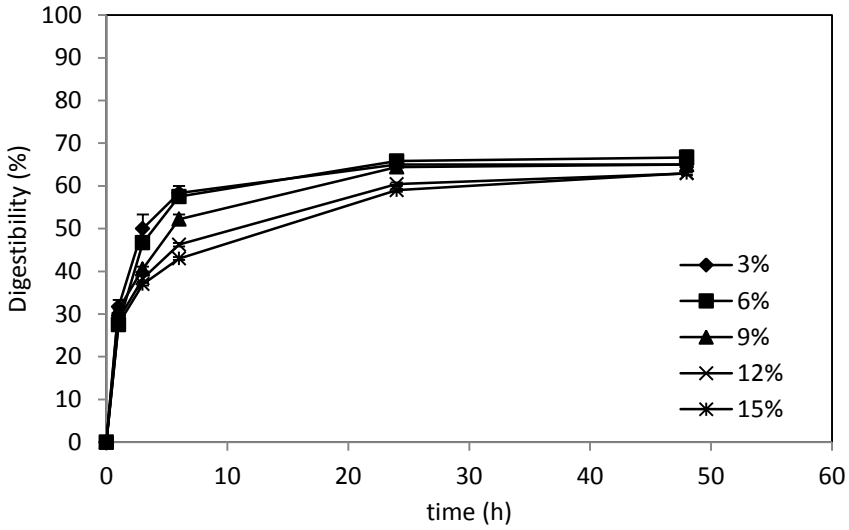
To sum up, two different pretreatment techniques generated various effects on the structure of the cotton stalks. While alkaline pretreatment was effective in extraction of high portions of lignin and hemicellulose from the biomass, EMIMAc pretreatment resulted with a disruption of the crystalline structure of the cotton stalks. In the following part, alkaline and ionic liquid pretreated cotton stalks would be examined in terms of enzymatic digestibility and glucose yields attained upon enzymatic hydrolysis conducted at different substrate loadings. This following analysis would enable us to conclude which one of the structural changes would prevail over the other regarding their impact on the enzymatic hydrolysis of the biomass.

4.5.2 Effect of substrate loading on the enzymatic digestibility of cotton stalks

In this part, alkaline and ionic liquid pretreated cotton stalks were subjected to enzymatic hydrolysis at substrate loadings that ranged from 3% to 15% (w/v). It was aimed to monitor the changes in the enzymatic hydrolysis of two pretreated biomass with substrate loading. The substrate loading conducted during enzymatic hydrolysis has been important since it would determine the concentration of glucose that would be further utilized for ethanol production. According to the previously reported studies, performing the hydrolysis at high substrate loadings would bring benefits in terms of process costs owing to the production of high percentages of ethanol titers (v ethanol/v fermentation medium) upon fermentation. Higher ethanol concentrations obtained upon fermentation would obviously facilitate the product recovery during distillation and reduce the process costs since less energy would be utilized to attain the final ethanol concentration during downstream processing (Wingren et al., 2003, Jørgensen et al., 2007). Though being less energy intensive in respect of product recovery, utilization of high substrate loadings during hydrolysis might generate mass transfer limitations and also bring problems due to the formation of inhibitors at high concentrations (Kristensen et al.,2009). Owing to those problems, digestibility of the biomass to fermentable sugars would be more difficult at higher substrate loadings (Jørgensen et al., 2007, Rosgaard et al., 2007, Hodge et al., 2008; Lu et al., 2010). The variations in the digestibility of ionic liquid and alkaline pretreated cotton stalks were given in Figure 4.43.

As seen in the Figure 4.43, ionic liquid pretreated cotton stalks got more easily digested when compared to alkaline pretreated cotton stalks. For EMIMAc pretreated cotton stalks, almost 24 hours of hydrolysis was found to be sufficient to reach the highest digestibility. However, a longer hydrolysis period was required for alkaline pretreated cotton stalks; the highest digestibility was attained at the 48^{th} hour of the hydrolysis for the samples hydrolyzed at 6% to 15% of substrate loading. These findings could be also supported with comparison of the initial hydrolysis rates. One could easily observe the hydrolysis of EMIMAc pretreated cotton stalks was much faster in the first hour of the reaction when compared to the hydrolysis of alkaline pretreated cotton stalks in the same period of time.

The main reason for getting much higher initial hydrolysis rates for ionic liquid pretreated cotton stalks compared to alkaline pretreated samples was obviously related to the structural changes derived for the biomass upon EMIMAc pretreatment. Recalling the major findings derived in the previous part; crystalline structure of the cotton stalks was disrupted through EMIMAc pretreatment, whereas such an effect could not be gathered upon alkaline pretreatment. The strong connection between the reduction in the crystalline structure of the biomass and enhanced initial hydrolysis rates was also supported by an increasing number of studies (Chang and Holtzapple, 2000, Laureano-Perez et al., 2005, Dadi et al., 2007, Hall et al., 2010).



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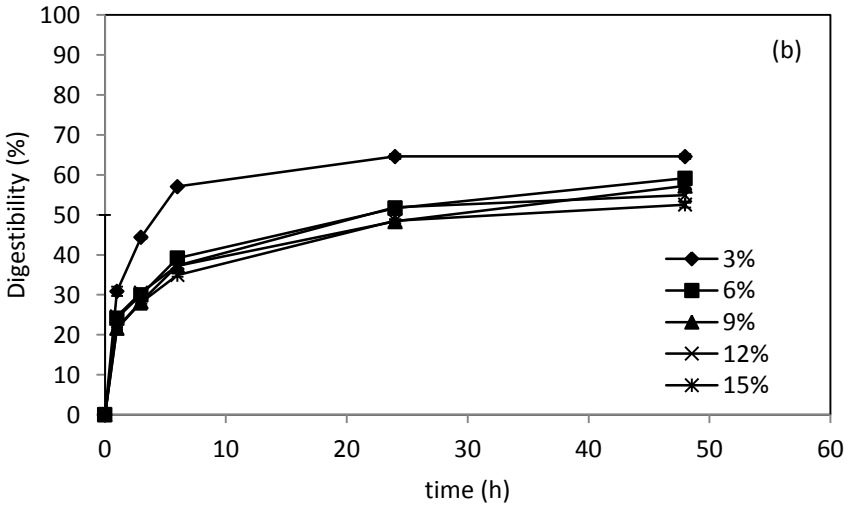


(b)

Figure 4.43 Effect of substrate loading on the digestibility of cotton stalks subjected to ionic liquid (a) and alkaline pretreatment (b)

Another critical result that could be derived from the profiles given in Figure 4.43 was that the digestibility of the cotton stalks decreased with an increase in substrate loadings for both substrates. This effect was observed more clearly for the alkaline pretreated cotton stalks; the digestibility of alkaline pretreated cotton stalks decreased from 68% to 53% at the 48th hour of the hydrolysis with an increase in the substrate loading from 3% to 15% (w/v). Such variations were similarly observed in the previously reported studies. For instance, conversion of cellulose and hemicellulose, which was present in steam pretreated wheat straw, was found to decrease with an increase in the substrate loading from 2% to 40% (w/w) (Jørgensen et al., 2007). The major causes for the decrease in sugar yields with increase in substrate loading were basically described as mass transfer limitations owing to the highly viscous hydrolysis media, pristine nature of the biomass and increased concentrations of inhibition products such as glucose and phenolics (Jørgensen et al., 2007, Hodge et al., 2008, Kristensen et al., 2009). In a previously reported study, the adverse effect of increased substrate loadings up to 20% (w/w) on conversion of cellulose to glucose was related to the presence of inhibitor compounds and also linked to the mass transfer problems at solid loadings higher than 20% (w/w) (Hodge et al., 2008). Unlike alkaline pretreated cotton stalks, the digestibility of the ionic liquid pretreated cotton stalks at the 48th hour of the hydrolysis was found to change in a narrow range; between 63-67% which implied that they were much less affected from the variation in the substrate loading.

The effect of substrate loading on the enzymatic hydrolysis of pretreated cotton stalks was also expressed in terms of the glucose yields which were determined on the basis of the cellulose content of the pretreated (Figure 4.44a) and untreated cotton stalks (Figure 4.44b). At first glance, one could easily realize that glucose yield was not affected by the variation in the substrate loading for the ionic liquid pretreated cotton stalks (Figure 6.34a); enzyme was able to convert almost 90% of the cellulose present in the EMIMAc pretreated cotton stalks to glucose. However that was not the case for the alkaline pretreated cotton stalks; glucose yield that was based on the cellulose content of the pretreated biomass was found to decrease from 75% to 61% with an increase in the substrate loading from 3% to 15%. For the untreated cotton stalks, glucose yield was found to range between 15-19%.

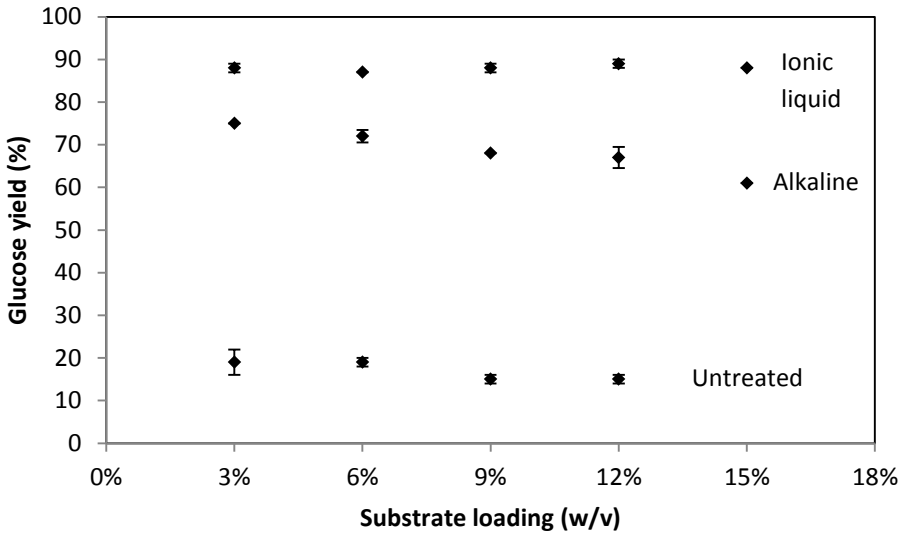
Even though ionic liquid pretreated cotton stalks possessed less amount of cellulose (45%) compared to alkaline pretreated biomass (60%); higher glucose concentration and thus, much higher glucose yield was obtained for EMIMAc pretreated cotton stalks. This result should be also regarded as an important one since it inferred the strong correlation between the crystallinity of a biomass and its accessibility to enzymatic attack. Therefore, the crystalline structure of the biomass should be regarded as a more decisive parameter compared to the variation in its composition with respect to their effect on the enzymatic accessibility of the biomass. A recently reported study was also supportive to this conclusion in which the crystalline structure of biomass was shown to hold a primary role for the enzymatic hydrolysis of a biomass (H.Wu et al., 2011).

In addition to its effect on the glucose yield, the effect of substrate loading was also expressed in terms of the overall glucose yield that was based on the cellulose content of the untreated cotton stalks. Overall glucose yield was regarded as more crucial compared to the former one since it considered the amount cellulose recovered upon pretreatment and therefore showed how efficient the cellulosic portion of the untreated biomass was converted to glucose. According to the results in Figure 4.44b, the profiles for the overall glucose yield derived for ionic liquid, alkaline pretreated and untreated cotton stalks were similar to the profiles obtained for glucose yield. While overall glucose yield was found to range between 82-85% for the ionic liquid cotton stalks; it decreased from 67% to 54% with an increase in substrate loading from 3% to 15% for the alkaline pretreated cotton stalks. For untreated cotton stalks the same glucose yields were achieved (glucose yield was equal to overall glucose yield) since no pretreatment was conducted and cellulose recovery was 100% for untreated cotton stalks. The major conclusion that could be derived from Figure 4.44b was that the difference between the overall glucose yields for ionic liquid and alkaline pretreated cotton stalks

became larger with an increase in substrate loading. During hydrolysis conducted at a substrate loading of 15%, 84% of overall glucose yield was obtained for ionic liquid pretreated cotton stalks while it was found much lower; 54% for the alkaline pretreated cotton stalks at the 48th hour of the hydrolysis. This substantial difference in overallglucose yields attained for the pretreated cotton stalks subjected to hydrolysis at 15% of substrate loading was important since it would obviously influence the ethanol yields that would be obtained upon fermentation.

Together with the findings related to the digestibility of the cotton stalks and overall glucose yield, which considered how efficient the cellulosic portion of the biomass was converted to glucose upon pretreatment and hydrolysis, indicated that the enzymatic accessibility of the biomass was strongly correlated to the modifications in the biomass structure. According to the current findings, transformation of the crystalline structure of the biomass to an amorphous form has been of primary importance compared to the other changes observed in the biomass structure such as extraction of lignin and hemicellulose. The reduction in the crystalline structure of the cotton stalks not only enhanced the conversion of the cellulosic portion of the biomass to glucose regardless of the substrate loading but also facilitated the completion of the enzymatic reaction.

The current investigation was followed by ethanol production; the glucose, which released from ionic liquid and alkaline pretreated cotton stalks that were subjected to enzymatic hydrolysis at 15% of substrate loading, was utilized in fermentation. Recalling the previous fermentation experiments (Part 4.2.10), the hydrolyzate media was concentrated to attain specific initial glucose concentrations with the focus on monitoring the effect of initial glucose concentration present in the fermentation media on ethanol production. In the following analysis, ethanol production was investigated in respect of how efficient the cellulosic portion of the untreated cotton stalks was converted to ethanol for ionic liquid and alkaline pretreated cotton stalks.



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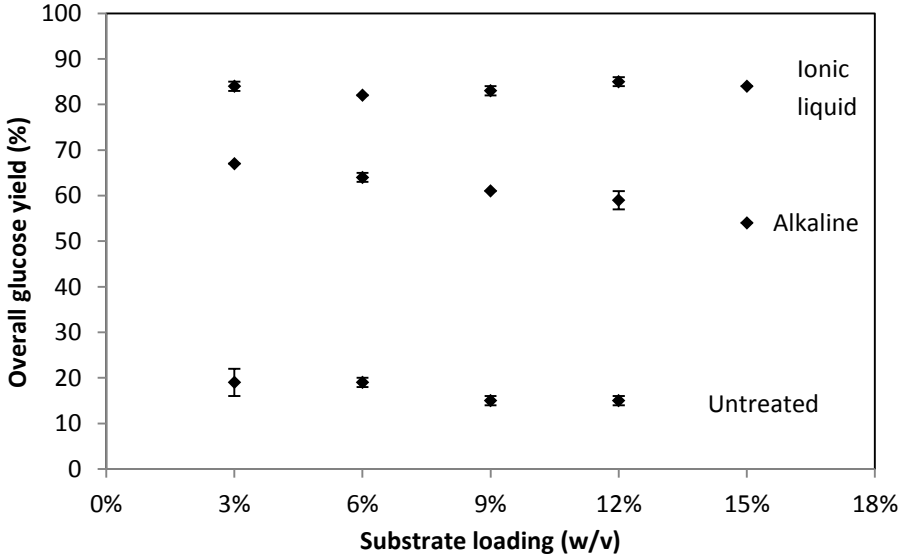


(b)

Figure 4.44 Effect of substrate loading on the glucose yield that was obtained on the basis of the theoretical maximum amount of glucose that can be obtained from the cellulosic portion of the cotton stalks, Glucose yield (a) and untreated cotton stalks, Overall glucose yield (b).

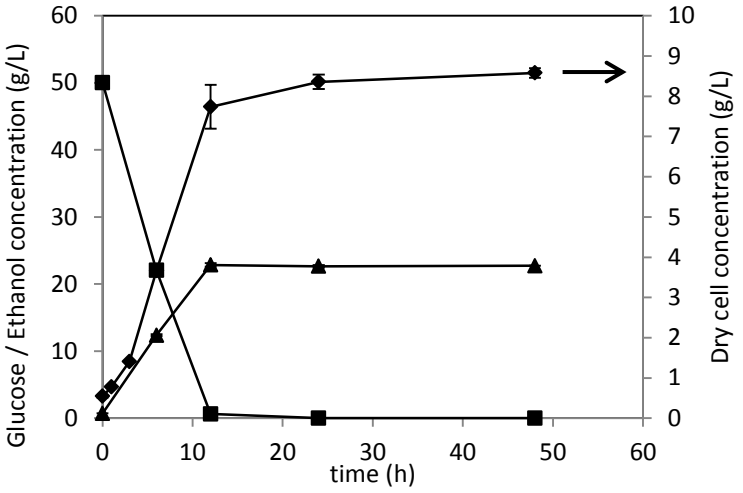
4.5.3 Ethanol production from ionic liquid and alkaline pretreated cotton stalks

In this part, the glucose derived upon enzymatic hydrolysis of ionic liquid and alkaline pretreated cotton stalks at 15% (w/v) of substrate loading was utilized for ethanol production. As discussed previously (Part 4.2.10), *S. cerevisiae* NRRL Y-132 was able to metabolize high concentrations of glucose that was initially present in the hydolyzate media and shown to result with high conversions of glucose to ethanol.

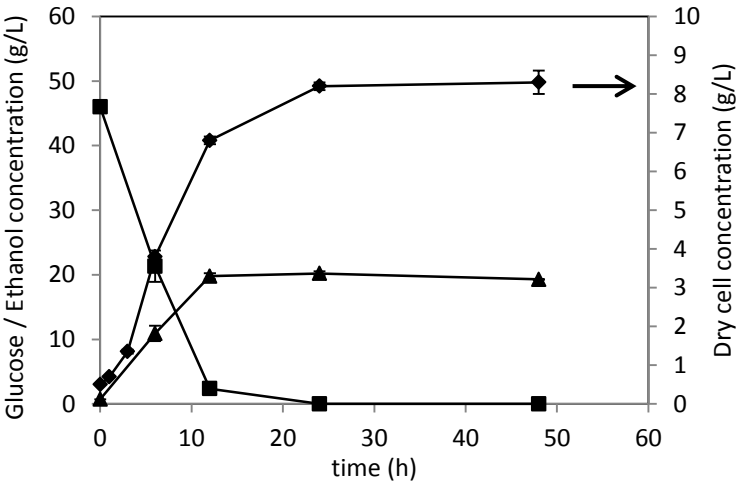
Though results were interpreted through glucose yields in the previous parts, glucose concentrations attained at the 48th hour of the hydrolysis for both substrates had to be known in order to have an idea about the initial glucose concentration of both fermentation media and monitor the time course of glucose consumption. The enzymatic hydrolysis of ionic liquid and alkaline pretreated samples at 15% of substrate loading resulted with 66 g/L and 61 g/L of glucose, respectively at the 48th hour of the enzymatic reaction. Before starting out fermentation, the hydrolyzates were diluted 1.3 fold after the precultivated strain and other essential nutrients were added to the hydrolyzates. Therefore, the initial glucose concentrations of the fermentation media were 50 g/L and 46 g/L for ionic liquid and alkaline pretreated cotton stalks prior to fermentation.

The Figure 4.45 presented the time courses for cell growth, glucose consumption and ethanol production for ionic liquid (Figure 4.45a) and alkaline pretreated cotton stalks (Figure 4.45b). According to the profiles, *S. cerevisiae* was found to consume glucose completely within 12 hours and reached the stationary phase of the growth at the 24th hour of the fermentation for both hydrolyzates. The highest ethanol concentrations were found as 23 g/L and 20 g/L for ionic liquid and alkaline pretreated cotton stalks, respectively at the 12th hour of the fermentation. Besides, dry cell concentrations were also close to each other; 8.4 g/L and 8 g/L of dry cell concentrations were obtained at the 24th hour of the fermentation for ionic liquid and alkaline pretreated cotton stalks, respectively.

Table 4.18 showed ethanol concentrations (g/L), titers (% v ethanol/v fermentation medium) and yields (%) that were obtained upon fermentation of the glucose which was derived upon hydrolysis of ionic liquid and alkaline pretreated cotton stalks. Ethanol yields given in the table were determined in the same way as the glucose yields were expressed in the previous parts of the study. Accordingly, ethanol yield was determined on the basis of the maximum amount of ethanol that could be produced from the glucose initially present in the fermentation medium and overall ethanol yield was determined on the basis of the maximum amount of ethanol that could be produced from the cellulose present in the untreated cotton stalks (Equations, 3-5 and 3-6). The latter one, which took the cellulose recovery upon pretreatment into consideration, indicated how effective the cellulosic portion of the untreated biomass was converted to ethanol. Regarding the findings in the table, the maximum ethanol titer was attained as 3% (v/v) upon fermentation of the glucose derived from the hydrolysis of EMIMAc pretreated cotton stalks. Moreover, close ethanol yields were obtained for ionic liquid and alkaline pretreated cotton stalks considering the conversion of the glucose present initially in the hydrolyzates to ethanol.



(a)



(b)

Figure 4.45 Time courses for cell growth (♦), glucose consumption (■) and ethanol production (▲) during the fermentation of the hydrolyzates obtained upon enzymatic hydrolysis (15% of substrate loading) of cotton stalks subjected to ionic liquid pretreatment (a) and alkaline pretreatment (b).

Table 4.18 Fermentation parameters obtained for ethanol production from the hydrolyzates derived upon enzymatic hydrolysis (15% of substrate loading) of cotton stalks subjected to ionic liquid pretreatment and alkaline pretreatment.

	Initial glucose concentration (g/L)	Ethanol concentration (g/L)	Ethanol titre (% v/v)	Ethanol yield (%)	Overall ethanol yield (%)
Ionic liquid	50	23	3.0	90	77
Alkaline	46	20	2.5	86	46

Unlike ethanol yield, overall ethanol yield differed considerably with respect to the pretreatment method; 77% and 46% of overall ethanol yield were obtained for ionic liquid and alkaline pretreated cotton stalks, respectively. This was not surprising since the remarkable difference in overall glucose yield observed for ionic liquid and alkaline pretreated cotton stalks (Figure 4.44b) was a forecaster of this current finding; overall glucose yield for ionic liquid and alkaline pretreated cotton stalks were 84% and 54%, respectively.

Albeit more efficient conversion to glucose and ethanol were expected for alkaline pretreated cotton stalks owing to the higher cellulose content of the biomass upon pretreatment (60%) compared to that obtained upon ionic liquid pretreated cotton stalks (45%), the results were completely contradictory to what has been predicted. Ionic liquid pretreated cotton stalks resulted with much higher yields upon enzymatic hydrolysis and fermentation compared to alkaline pretreated cotton stalks which mainly attributed to the reduced crystalline structure obtained upon EMIMAc pretreatment.

Figure 4.46 represented the overall mass balances for each pretreatment type on the basis of 100 g of untreated cotton stalks by specifying the composition of the insoluble products at each major step together with the glucose and ethanol concentrations and corresponding yields. Untreated cotton stalks were found to possess 37 g of cellulose initially. The cotton stalks that were subjected to ionic liquid pretreatment were found to lose 1 g of cellulose whereas it was 4 g of cellulose upon alkaline pretreatment (Figure 4.46a). The cellulose that could not be recovered upon each pretreatment was in inconsiderable amounts. The most noticeable variation in the composition of the biomass was observed upon alkaline pretreatment in which 13 g and 11 g of lignin and hemicellulose were extracted from the cotton stalks, respectively (Figure 4.46b). However, EMIMAc pretreatment was able to remove 1 g of lignin and 1 g of hemicellulose from the biomass. Following the enzymatic hydrolysis, which was conducted at a substrate loading of 15% (w/v), 35 g of glucose was released from EMIMAc pretreated cotton stalks with a glucose yield of 88% whereas it was 22 g of glucose (61% of glucose yield), which was derived upon hydrolysis of alkaline pretreated biomass. The water insoluble product obtained upon hydrolysis of EMIMAc pretreated cotton stalks was found to be composed of 81% (w/w) of lignin and 19% (w/w) of residual polysaccharides that were not digested within 48 hours of hydrolysis. The lignin content of the water insoluble product obtained after hydrolysis of alkaline pretreated biomass was much lower; it consisted only 50% of lignin. Based on these recent findings related to the composition of the water insoluble product derived upon hydrolysis, one could conclude that the incapability of EMIMAc to extract lignin upon pretreatment

was favorable; almost 96% of the lignin that was initially present in untreated cotton stalks was preserved till the end of the hydrolysis. By this way, it was possible to fractionate the biomass into its major components; lignin as water insoluble product and cellulose and hemicellulose mainly in the form of monosaccharides as water soluble products. Together with hydrolyzate, utilization of the lignin rich product should be encouraged and assessed in a multiproduct perspective within the context of biorefinery.

When alkaline pretreatment was evaluated, it was observed that 50% of the lignin that was initially present in the untreated cotton stalks was found to remain in NaOH solution (2% w/v) at the end of alkaline pretreatment. Though it may seem advantageous regarding the adverse effects of lignin on the function of celluloytic enzymes during hydrolysis (Palonen et al., 2004, Yang and Wyman, 2006), extraction of lignin from the alkaline solution with the aim of treating lignin as a by-product after pretreatment would be problematic. Since this attempt would require the employment of further steps and chemical reagents which would generate adverse effects in respect of process costs and environment.

In addition to the discussions that were made above, delignification during alkaline pretreatment would result with formation of phenolics that would subsequently generate inhibitory effects on enzymatic hydrolysis and fermentation (Palmqvist and Hahn-Hagerdal, 2000). However it would not be the case for EMIMAc pretreatment which was conducted at 30% of biomass loading without stirring. In fact, the incapability of EMIMAc to extract lignin from the cotton stalks at the described conditions would also be advantageous regarding the recycling of ionic liquids. In the cases that ionic liquids were shown to extract significant amounts of lignin, continuous reuse of the ionic liquid resulted with accumulation of the extracted lignin (S.H. Lee et. al., 2009, Shill et al., 2011). Accumulation of the biomass components in the recovered solution with continuous reuse was found to decrease the efficiency of ionic liquids as pretreatment agents and even the glucose yields obtained upon enzymatic hydrolysis (Li et al., 2010; Nguyen et al., 2010).

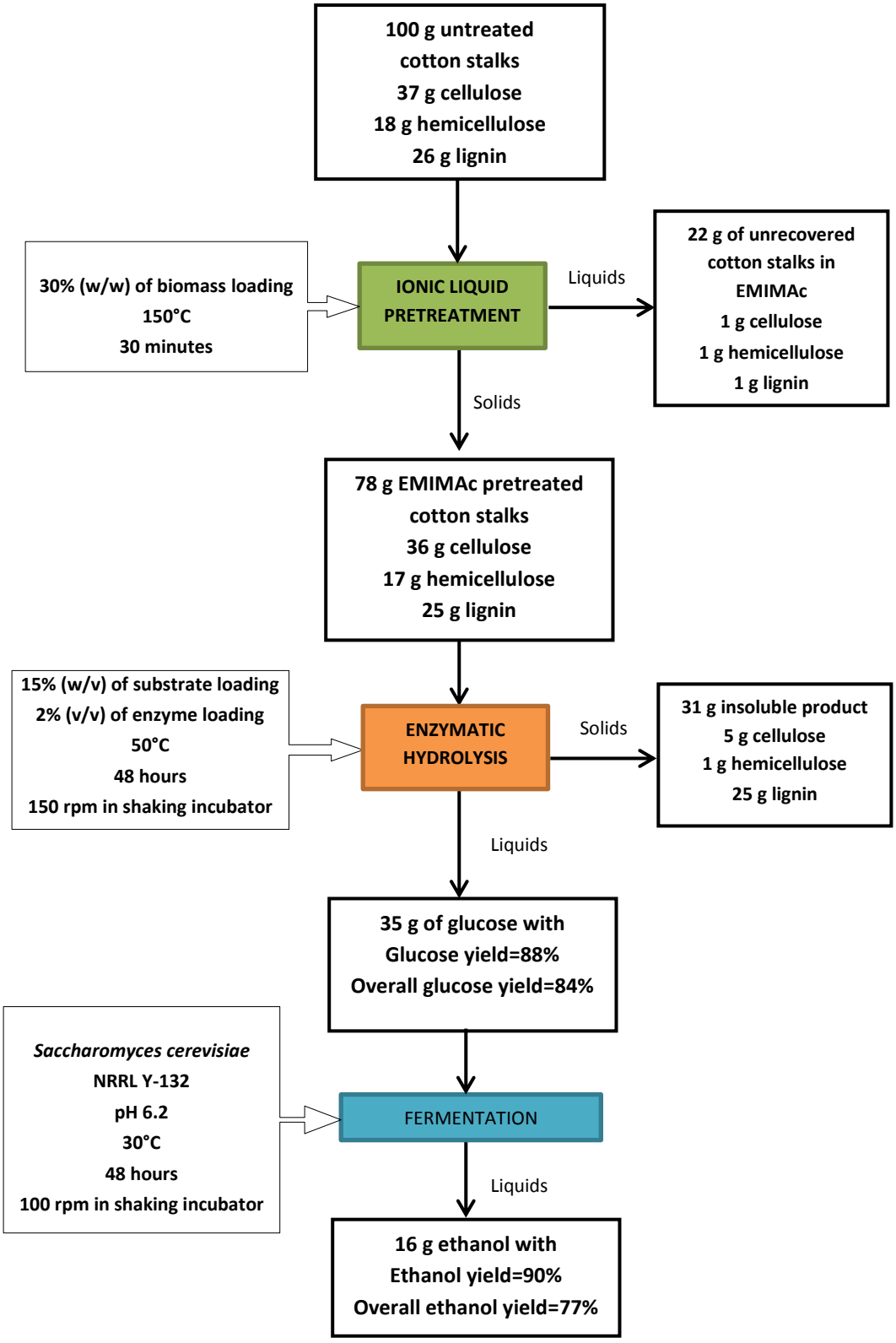


Figure 4.46a The flowchart for ethanol production from EMIMAc pretreated cotton stalks.

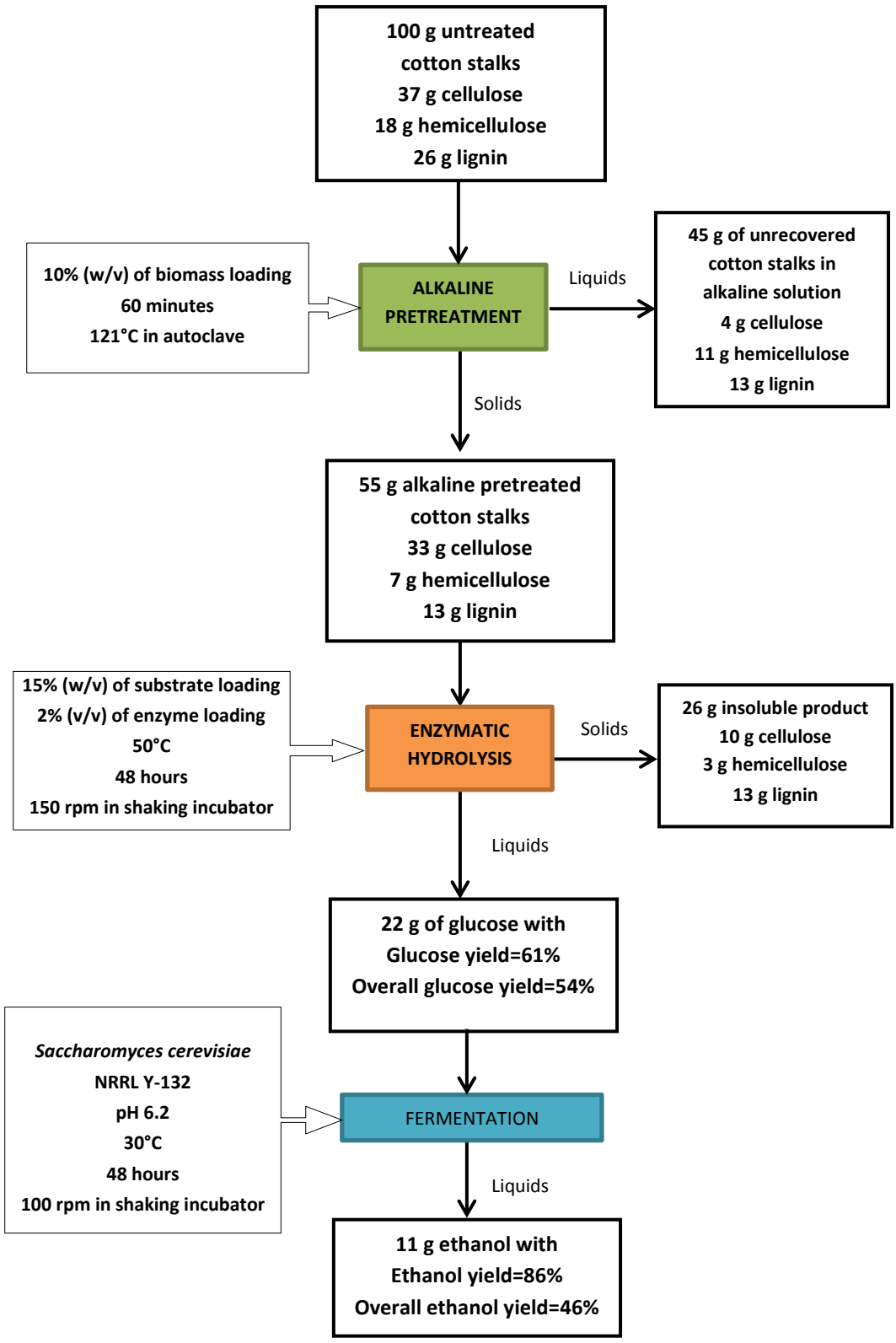


Figure 4.46b. The flowchart for ethanol production from alkaline pretreated cotton stalks

4.6 Comparison of EMIMAc pretreated cotton stalks for ethanol production

This section, as a brief review of ethanol production from EMIMAc pretreated cotton stalks, presents a comparison of two different cases that were carried out for an effective biomass processing to yield cellulosic ethanol. Operation conditions, compositional changes, enzymatic accessibility of the biomass and yields obtained upon hydrolysis and fermentation were all included in the comparison to conclude which one of the cases prevailed over the other in respect of effective utilization of cellulosic portion present in the untreated cotton stalks to produce ethanol. Case I represented ethanol production from the cotton stalks that were subjected to EMIMAc at 10% of biomass loading (w cotton stalks/w EMIMAc) and 150°C for 30 minutes under stirring. These pretreated cotton stalks were enzymatically hydrolyzed at a substrate loading of 3% (w/v) via 2% (v/v) of Cellic ctec2 for 48 hours. On the other hand, Case II represented ethanol production from the biomass that was subjected to EMIMAc pretreatment at 30% of biomass loading (w cotton stalks/w EMIMAc) and 150°C for 30 minutes without stirring. This time, cotton stalks were enzymatically hydrolyzed at a substrate loading of 15% (w/v) via 2% (v/v) of Cellic ctec2 for 48 hours.

Table 4.19 showed the details and the major findings derived upon each process. According to the table, the most noticeable dissimilarity was the compositional changes obtained upon EMIMAc pretreatment. In Case I, cotton stalks were found to lose almost 7-fold higher amount of cellulose, hemicellulose and lignin compared to those extracted in Case II. This substantial difference was obviously linked to the pretreatment conditions in which more moderate conditions were conducted in Case II compared to Case I. Apparently, the lower biomass loading utilized in Case I enabled lignin and hemicellulose extraction just like the alkaline pretreatment did, but it resulted with a considerable amount of cellulose loss. This cellulose loss, which was almost 30%, was adequate information to explore an alternative way that would enhance cellulose recovery. Since effective conversion of the cellulosic portion of untreated cotton stalks was of primary interest in this study, Case II was offered as an alternative to increase the cellulose recovery upon EMIMAc pretreatment. For this purpose, a comprehensive investigation was performed in respect of the influence of biomass loading on the structural changes and digestibility of the biomass (Part 4.3). That was followed by enzymatic hydrolysis conducted at higher substrate loadings than 3% (w/v) with the aim of increasing glucose concentration and hence, ethanol concentration at end of the process (Part 4.5). According to the previous investigations, the most appropriate conditions for biomass loading in EMIMAc pretreatment and substrate loading in enzymatic hydrolysis were selected as 30% (w cotton stalks/w EMIMAc) and 15% (w cotton stalks/v buffer), respectively. The cotton stalks were found to lose a minor amount of cellulose (4%) and they were still possessing reduced crystalline structure upon EMIMAc pretreatment at 30% of biomass loading.

Table 4.19 The comparison of two different cases of EMIMAc pretreatment for ethanol production from cotton stalks (a:Cellulose loss (%), b:Hemicellulose extracted (%), c:Lignin extracted (%), d: Glucose yield (%), e: Overall glucose yield (%), f:Ethanol yield (%), g: Overall ethanol yield (%)).

	Conditions for pretreatment		**Conditions for enzymatic hydrolysis**		**Conditions for fermentation**		**a**	**b**	**c**	**d**	**e**	**f**	**g**
Case I	Biomass loading (w CS / w EMIMAc)	10%	Substrate loading (w CS/v buffer)	3%	m.o.	*S. cerevisiae*	30	71	47	96	66	98	66
			Enzyme loading (v enzyme /v buffer)	2%	Temperature (°C)	30							
	Temperature(°C)	150	Temperature(°C)	50	pH	6.2							
	Period (min)	30	pH	4.8	Period (h)	48							
	Stirring rate (rpm)	500	Period (h)	48	Stirring rate (rpm)	100							
Case II	Biomass loading (w CS / w EMIMAc)	30%	Substrate loading (w CS/v buffer)	15%	m.o.	*S. cerevisiae*	4	11	7	88	84	90	76
			Enzyme loading (v enzyme/v buffer)	2%	Temperature (°C)	30							
	Temperature(°C)	150	Temperature(°C)	50	pH	6.2							
	Period (min)	30	pH	4.8	Period (h)	48							
	Stirring rate (rpm)	-	Period (h)	48	Stirring rate (rpm)	100							

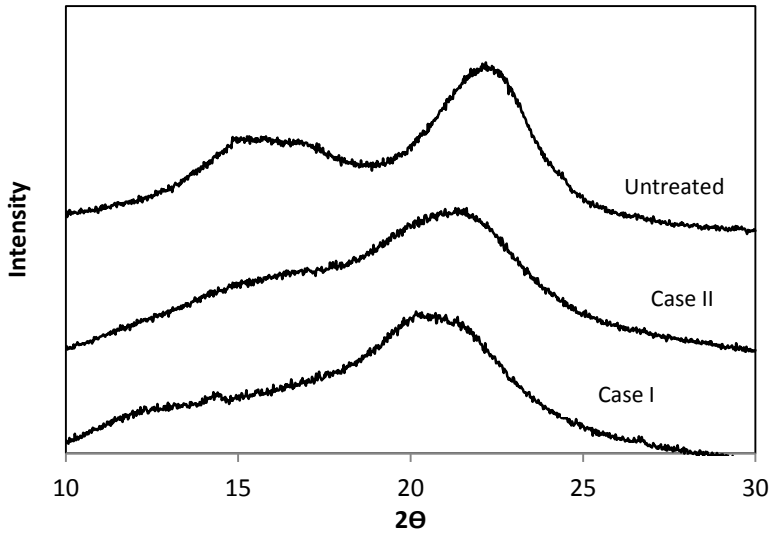


Figure 4.47 XRD patterns for untreated cotton stalks and cotton stalks subjected to EMIMAc pretreatment according to the conditions in Case I and II.

Additionally, EMIMAc pretreatment conducted at a 3-fold higher biomass loading compared to that conducted in the former case was more economically encouraging since less amount of EMIMAc was consumed for pretreatment of the same biomass amount. It was important to take the recent conclusion one step further and utilize the cotton stalks, which were subjected to EMIMAc pretreatment at 30% of biomass loading, for enzymatic hydrolysis at 15% of substrate loading with the aim of increasing glucose concentration. The increase in the cellulose recovery upon EMIMAc pretreatment at 30% of biomass loading resulted with a substantial increase in glucose yield that was based on the cellulosic content of the untreated cotton stalks (overall glucose yield); from 66% to 84% though the enzymatic hydrolysis was performed at 15% of substrate loading. This result eventually put an emphasis on the cellulose recovery upon pretreatment since the cellulose recovery and the conversion of the cellulosic portion of the biomass in its native state to glucose were strongly correlated to each other regardless of the substrate loading during enzymatic hydrolysis. Unlike overall glucose yield, the glucose yield that was based on the cellulosic portion of the EMIMAc pretreated cotton stalks was found to decrease somewhat; from 96% to 88% as we move from Case I to Case II. This slight decrease was particularly associated to the structural dissimilarities between the pretreated biomass. Pretreated cotton stalks in Case II were found to have higher levels of biomass crystallinity (Figure 4.47) and possess more lignin and hemicellulose compared to the pretreated biomass in Case I. The structural variations observed for the cotton stalks in Case II evidently decreased the extent of the hydrolysis owing to the fact that crystallinity of cellulose and strong association of cellulose with the other components were considered as the major complications for enzymatic accessibility of the cellulosic portion of the biomass.

Following the enzymatic hydrolysis, ethanol production was performed under the specified conditions in Table 4.17. Fermentation was conducted under exactly the same conditions excluding the initial glucose concentrations of the hydrolyzates utilized as the fermentation media for ethanol production. As the enzymatic reaction in Case II was carried out at a much higher substrate loading, the glucose concentration of the hydrolyzate was obviously much higher. The glucose concentrations obtained at the 48^{th} hour of the hydrolysis for each pretreated biomass were not indicated in the table since the hydrolyzates derived upon enzymatic reactions were diluted with the addition of

precultivated strain and other nutrient supplementations. Therefore, the concentrations of the glucose that was initially present in the fermentation media were different from those obtained at the end of hydrolysis. According to the table, ethanol yield was not observed to be affected considerably from the variation in structure-related factors and operation conditions. Ethanol yield, which was defined on the basis of the maximum amount of ethanol that could be produced from the glucose initially present in the fermentation medium, were 98% and 90% for the cases I and II, respectively. However, that was not the case for overall ethanol yield which was defined on the basis of the maximum amount of ethanol that could be produced from cellulose present in untreated cotton stalks. Owing to the fact that the cellulose recovery upon pretreatment was taken into account in the definition of the overall ethanol yield; the conversion of the cellulosic portion of the cotton stalks to ethanol was much higher in Case II; it was found as 76%. However, the cotton stalks that were subjected to EMIMAc pretreatment at 10% of biomass loading was resulted with 66% of ethanol yield due to 30% of cellulose lost upon pretreatment.

The recent findings showed that Case II was much favorable with respect to its effect on the effective conversion of the cotton stalks to ethanol compared to Case I. Case II not only provided high glucose and ethanol yields, but also introduced more economically viable conditions since it provided employment of EMIMAc in lower amounts. Increased biomass loadings conducted during pretreatment was thought to be beneficial considering the reuse of the ionic liquid. The recycling and reuse of ionic liquids were regarded as crucial aspects in the field of biomass processing owing to the very high costs of these unique pretreatment agents. The conditions in order to enhance the extent of the ionic liquid reuse and convenient ways to recycle the ionic liquid while recovering the biomass components effectively were recently recognized as key features for implementation of ionic liquid technology to the larger scales (Stark, 2011, Tadesse and Luque, 2011). According to Table 4.17, the vast majority of biomass components were recovered upon EMIMAc pretreatment in Case II when compared to Case I. This dissimilarity was attributed to the fact that EMIMAc was in charge of reducing the crystallinity without intending to remove large fractions of lignin and hemicellulose from the biomass. As lower amounts of residual biomass components were gathered in the recovered EMIMAc, there would be much less accumulation of the biomass components upon multiple recycles of the ionic liquid which would increase the extent of EMIMAc reuse. Recalling Figure 4.4 that demonstrated the variation of extracted lignin with EMIMAc recycling conducted under conditions described in Case I, the capability of EMIMAc to extract lignin was found to get lower as it got reused for three times. In fact lignin extraction was not the primary consideration according to our target; the figure has been useful to express how EMIMAc lost its effectiveness as a dissolution agent (not a pretreatment agent) with recycling. Though digestibility of the cotton stalks were found to remain unaffected from the recycling of EMIMAc (Figure 4.24), the reported studies revealed that the continuous accumulation of biomass components with the reuse of ionic liquid would eventually lower the enzymatic digestibility of the biomass after a certain point (Li et al., 2010, Nguyen et al., 2010), since the ionic liquid would not be able to function properly due to the high concentrations of the residual components. To sum up, Case I would bring complexities in respect of ionic liquid reuse owing to the continuous increase in the concentrations of unrecovered biomass components when compared to Case II.

One major conclusion that could be derived from the findings given in Table 4.17 was that EMIMAc pretreatment conducted in Case I was totally a biomass deconstruction while Case II was more likely a biomass fractionation. In other words, EMIMAc pretreatment was carried out in a more controlled fashion in Case II compared to the former case. Together with high glucose yields (84% considering the cellulosic portion of the untreated cotton stalks) derived in Case II, the insoluble residue left after the enzymatic reaction, which constituted 80% of lignin, was found to possess 93% of the lignin present in untreated cotton stalks. So in Case II, we were able to fractionate the cotton stalks into its major components upon pretreatment and enzymatic hydrolysis; most of the cellulose and hemicellulose in the form of water soluble product (glucose and xylose in the hydrolyzate) and also lignin in the form of insoluble product. However in Case I, we were not able to control the recovery of cellulose and hemicellulose upon EMIMAc pretreatment at 10% of biomass loading.

According to the discussions made above, Case II appeared as a more appropriate approach for conversion of cotton stalks to ethanol effectively. To make a final conclusion, the major advantages related to the ethanol production from the cotton stalks subjected to EMIMAc pretreatment, that was based on the conditions in Case II, were summarized below:

1) Cotton stalks were converted to glucose and ethanol more effectively. Higher glucose (84%) and ethanol yields (76%) (considering the cellulose content of untreated cotton stalks) were obtained upon enzymatic hydrolysis and fermentation, respectively.

2) Reduction in biomass crystallinity was satisfactory; there was no need to make any considerable compositional changes to improve the enzymatic accessibility of the biomass.

3) EMIMAc was employed for pretreatment of 3-fold higher amount of biomass indicating that it was possible to use much lower amounts of ionic liquid to pretreat a specific amount of biomass which was more economically attractive.

4) Since lignin removal during EMIMAc pretreatment was lower, majority of the lignin (93%) that was present in untreated cotton stalks was found to be preserved until the end of the hydrolysis as an insoluble product. Eventually, I was able to fractionate the biomass into its major components with promisingly high yields both in liquid state (cellulose and hemicellulose) and solid state (lignin).

CHAPTER 5

CONCLUSIONS

This study aims efficient conversion of cotton stalks to cellulosic ethanol through ionic liquid pretreatment and enhanced enzymatic hydrolysis. Accordingly, a variety of investigations were performed in order to attain the most appropriate operation conditions for ionic liquid pretreatment, enzymatic hydrolysis and fermentation with the goal of enhancing the yields and product concentrations. The major conclusions based on the investigations performed in this study are listed in the following bullet points.

- Preliminary studies comprise the investigations about effects of general pretreatment conditions on enzymatic digestibility (%) and solid recovery (%) of EMIMCl pretreated cotton stalks. Higher pretreatment temperatures (≥120°C) were found to favorable for the enzymatic digestibility of the biomass due to the lower viscosity of the reaction medium at elevated temperatures. On the other hand, higher pretreatment periods (>1 hour) and lower biomass loadings (<10% w CS/w IL) were found to decrease the solid recovery obtained upon pretreatment and hence, enzymatic digestibility of the pretreated biomass owing to the biomass degradation at the stated conditions. Based on these findings, pretreatment temperature, period and biomass loadings were selected as 150°C, 30 minutes and 10% (w CS/w IL), respectively for the subsequent analyses.

- Ionic liquids, AMIMCl, BMIMCl, EMIMCl, EMIMAc and HEAF were screened with respect to their effects on biomass structure and enzymatic digestibility of the biomass. Among, EMIMAc resulted with the highest biomass digestibility (65%) which was 9-fold higher than the digestibility of the untreated cotton stalks. In accordance with its enzymatic accessibility, EMIMAc pretreated cotton stalks exhibited superior structural variations compared to their native form. SEM images revealed an entirely deconstructed structure for EMIMAc pretreated cotton stalks, whereas less pronounced changes were gathered in the morphology of the other pretreated biomass samples. According to XRD analysis, the highest reduction in crystallinity was observed for EMIMAc pretreated cotton stalks. EMIMAc was also found to be capable of extracting 45% of the lignin present in the untreated cotton stalks.

- EMIMAc reuse did not exhibited any adverse effect on the enzymatic digestibility and as well as on the capability of EMIMAc to transform the crystalline structure of cotton stalks into an amorphous form. Though, EMIMAc was found to extract less lignin with an increase in its reuse. Accordingly, crystallinity of the biomass appeared as a more decisive factor compared to its lignin content with respect to their effects on the enzymatic digestibility of the biomass.

- Cotton stalks, which were subjected to EMIMAc pretreatment at 10% (w CS/w EMIMAc) of biomass loading and 150°C for 30 minutes, were enzymatically hydrolyzed at a substrate loading of 3% (w/v) with Cellic Ctec2 at a loading of 2% (v/v). Accordingly, 19 g/L of glucose, which corresponded to a glucose yield of 95% based on the cellulose content of the EMIMAc pretreated cotton stalks, was obtained at the 48th hour of the hydrolysis.

- The glucose obtained upon enzymatic hydrolysis of EMIMAc pretreated cotton stalks was fermented by the wild type yeast, *Saccharomyces cerevisiae* Y-132. The yeast metabolized all glucose present in the hydrolyzates and resulted with over 95% of ethanol yield regardless of the initial glucose concentration of the fermentation media. The highest ethanol concentration was obtained as 51 g/L at the 96th hour of the fermentation in the hydrolyzate medium containing 100 g/L of glucose, 10 g/L yeast extract, 6 g/L urea, 3 g/L $Na_2HPO_4.7H_2O$, 3 g/L KH_2PO_4, 0.25 g/L $MgSO_4.7H_2O$, 0.08 g/L $CaCl_2.2H_2O$.

- EMIMAc pretreated cotton stalks resulted with satisfactory glucose and ethanol yields based on the cellulose content of the pretreated cotton stalks and glucose concentration of the hydrolyzate prior to fermentation, respectively. However, glucose and ethanol yields based on the cellulose content of the untreated cotton stalks were found as 67% and 66%, respectively. These results conflicted with the major target of this study since cellulosic portion of the cotton stalks were not converted to glucose and ethanol effectively.

- Though EMIMAc was shown to enhance the enzymatic digestibility of the cotton stalks, superior solvation capability of EMIMAc was found to cause cellulose degradation. The compositional analysis conducted for cotton stalks before and after EMIMAc pretreatment revealed that almost 30% of cellulose could not be recovered upon precipitation of the pretreated biomass. Accordingly, this cellulose loss accounted for the low overall glucose and ethanol yields. In order to alleviate cellulose degradation during EMIMAc pretreatment and thus, provide efficient conversion of the cellulosic portion of cotton stalks to glucose and ethanol, EMIMAc pretreatment was conducted at higher biomass loadings (≥10% w CS/w EMIMAc) under no stirring.

- Cotton stalks, which were subjected to EMIMAc pretreatment at 10-50% (w CS/w EMIMAc) of biomass loading and 150°C for 30 minutes under no stirring, exhibited identical XRD profiles with the cotton stalks pretreated via EMIMAc at 10% (w CS/w EMIMAc) of biomass loading under stirring. Though, EMIMAc was not able to extract high fractions of lignin; the glucose yields based on the cellulose content of the EMIMAc pretreated cotton stalks were comparable (83-94%) to the glucose yield of the former EMIMAc pretreated cotton stalks (95%). Likewise, crystallinity was considered as a more critical parameter for the accessibility of the biomass to enzymatic hydrolysis based on the aforementioned findings. More to the point, negligible amounts of cellulose loss ranging between 2-6% were obtained and thus, higher overall glucose yields based on the cellulose content of the untreated cotton stalks, which ranged between 71-90%, were achieved.

- Cotton stalks pretreated via EMIMAc at 30% (w CS/w EMIMAc) of biomass loading and 150°C for 30 minutes under no stirring were selected for ethanol production owing to the very satisfactory overall glucose (82%) obtained and the positive aspects of employing higher biomass loadings under no stirring during pretreatment with respect to process costs.

- In order to introduce the significant advantages of ionic liquid pretreatment with respect to ethanol production from cotton stalks, a comparison was performed between EMIMAc and alkaline pretreated cotton stalks. Enzymatic hydrolysis was conducted at higher substrate loadings (3-15% w/v) for both pretreated biomass with the aim of achieving higher glucose and thus, ethanol concentrations upon hydrolysis and fermentation, respectively. The results showed that EMIMAc pretreatment (at 30% of biomass loading under no stirring) provided enhanced enzymatic hydrolysis for the cotton stalks even at the highest substrate loadings, 15% (w/v). Despite being capable of removing larger fractions of lignin from cotton stalks (52%), alkaline pretreatment was not as efficient as EMIMAc pretreatment in

cellulose conversion. Glucose yields of alkaline pretreated cotton stalks were observed to decrease with an increase in substrate loading. Prevailing effect of biomass crystallinity over lignin content of the biomass could clarify the considerable difference between the glucose yields derived upon hydrolysis of alkaline and EMIMAc pretreated cotton stalks.

- Similar to the enzymatic hydrolysis, EMIMAc pretreated cotton stalks were shown to be superior compared to alkaline pretreated samples with respect to ethanol production. The hydrolyzates obtained upon hydrolysis of EMIMAc and alkaline pretreated cotton stalks at 15% (w/v) of substrate loading were fermented and resulted with 77% and 46% of overall ethanol yields, respectively.

- The insoluble product obtained upon enzymatic hydrolysis of EMIMAc pretreated cotton stalks consisted 83% of lignin, whereas lignin accounted for only 50% of the insoluble product derived upon hydrolysis of alkaline pretreated cotton stalks. The incapability of EMIMAc (at 30% of biomass loading) to extract lignin was considered as an advantage since lignin, which has been a substantial by-product, was conveniently obtained without being depolymerized. This finding could be also beneficial for the lifetime of ionic liquids. Since accumulation of high amounts of lignin in ionic liquids was avoided, the efficiency for sequential uses of ionic liquids for biomass pretreatment would be enhanced.

- In general, the discussions put an emphasis on the reduction of the crystalline structure of cotton stalks upon EMIMAc pretreatment. This impact not only provided identical biomass digestibility upon EMIMAc reuse for multiple times, but also resulted with enhanced enzymatic accessibility for the biomass subjected to EMIMAc pretreatment at high biomass loadings. Therefore, the modifications in the crystalline structure of the biomass was regarded as more crucial compared to compositional changes in the biomass with respect to their effects on the enzymatic accessibility of the biomass.

- Moreover, EMIMAc pretreatment at 30% of biomass loading provided fractionation of the cotton stalks into its major components. Such that, cellulose and hemicellulose in the form of soluble products (glucose and xylose) and lignin in the form of an insoluble product were obtained upon enzymatic hydrolysis at 15% (w/v) of substrate loading with encouragingly high yields. However, EMIMAc pretreatment conducted at 10% of biomass loading under stirring was not only energy intensive but also resulted with large fractions of unrecovered cellulose and lignin. Therefore, biomass fractionation could not be the case under these conditions.

- High viscosity of EMIMAc was not a problem anymore for pretreatments conducted at high biomass loadings. EMIMAc was only capable of wetting the surface of the cotton stalks particles during pretreatment. Slurry formation was not observed and thus, stirring was not essential to relieve the challenges created by the high viscosity of EMIMAc as it was observed during pretreatment at 10% of biomass loading.

Consequently, utilization of high biomass loadings during EMIMAc pretreatment appeared as a promising tactic for implementation of this technology to industrial scales for production of cellulosic ethanol. But evidently, we need better solutions to alleviate the contribution of the ionic liquids to the process costs. Though reduction of ionic liquid costs does not appear to be possible for now, different strategies can be developed. In this context, minimization of water utilization which has been essential to remove residual ionic liquid from pretreated biomass prior to enzymatic hydrolysis and furthermore, enhancements in ionic liquid recovery are considered as cost effective approaches. As future work,

- The compatibility of cellulases and ionic liquids can be investigated with the aim of performing enzymatic hydrolysis in the presence of ionic liquids. Though inhibitory effects of imidazolium based ionic liquids on cellulose degrading enzymes were reported, cellulases were shown to retain their activity up to certain limits of ionic liquid concentrations in the hydrolysis medium. This strategy will obviously bring benefits in respect of process costs since the washing step conducted after pretreatment will be discarded and both steps will be performed in the same vessel. However, one must consider the recovery of the ionic liquid from the reaction medium for its subsequent uses and extraction of glucose for its conversion to ethanol. Utilization of biphasic systems (salting out agents such as K_3PO_4 and PEG) was shown to be promising for separation of hydrophilic ionic liquids from aqueous systems in the previously reported studies. This system can be adapted to suggested approach for attaining enhanced recovery for ionic liquids and extraction of glucose from the reaction.

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APPENDIX A

LIST OF CHEMICALS AND EQUIPMENTS USED IN THE STUDY

Table A.1 List of chemicals used in this study and their suppliers.

Chemical	Supplier
1-allyl-3-methyl imidazolium chloride	Solvionics (Toulouse, France)
1-butyl-3-methyl imidazolium chloride	Solvionics (Toulouse, France)
1-ethyl-3-methyl imidazolium chloride	Solvionics (Toulouse, France)
1-ethyl-3-methyl imidazolium chloride	Sigma-Aldrich (St. Louis, MO, USA)
1-ethyl-3-methyl imidazolium acetate	Sigma-Aldrich (St. Louis, MO, USA)
Cellic Ctec2	Novozymes (Bagsværd, *Denmark)*
Celluclast 1.5L	Novozymes (Bagsværd, *Denmark)*
Novozym 51003	Novozymes (Bagsværd, *Denmark)*
3,5-dinitrosalicyclic acid	Sigma-Aldrich (St. Louis, MO, USA)
Acetic acid	Merck (Darmstadt, Germany)
Agar granulated	Merck (Darmstadt, Germany)
Calcium carbonate	Merck (Darmstadt, Germany)
Calcium chloride dihydrate	Merck (Darmstadt, Germany)
Citric acid mono-hydrate	Merck (Darmstadt, Germany)
D-glucose	Merck (Darmstadt, Germany)
D-xylose	Merck (Darmstadt, Germany)
Disodium hydrogen phosphate heptahydrate	Merck (Darmstadt, Germany)
Ethanol	Sigma-Aldrich (St. Louis, MO, USA)
Ethanolamine	Sigma-Aldrich (St. Louis, MO, USA)
Formic acid	Merck (Darmstadt, Germany)
Magnesium sulfate heptahydrate	Merck (Darmstadt, Germany)
Peptone	Merck (Darmstadt, Germany)
Phenol	Sigma-Aldrich (St. Louis, MO, USA)
Potassium dihydrogen phosphate	Merck (Darmstadt, Germany)
Potassium sodium tartrate	Merck (Darmstadt, Germany)
Sodium hydroxide	Merck (Darmstadt, Germany)
Sodium sulfate	Sigma-Aldrich (St. Louis, MO, USA)
Sulfuric acid	Merck (Darmstadt, Germany)
Tri-sodium citrate dihydrate	Merck (Darmstadt, Germany)
Urea	Merck (Darmstadt, Germany)
Xylan from birchwood	Sigma-Aldrich (St. Louis, MO, USA)
Yeast extract	Merck (Darmstadt, Germany)

Table A.2 List of laboratory equipments used in this study, their models and suppliers.

Equipment	Model and Supplier
Shaking incubator	Minitron, Infors AG (Bottmingen, Switzerland)
Incubator	Nüve EN 055 (Ankara, Turkey)
Incubator	Nüve FN 400 (Ankara, Turkey)
Water bath	Grant SUB-6 (Essex, UK)
Digital magnetic stirrer with a temperature sensor	RCT Basic Safety Control, IKA Werke, (Staufen, Germany)
Autoclave	Hiclave HVE-50, Hirayama (Saitama, Japan)
Rotary evaporator	RV 10 Digital, IKA Werke (Staufen, Germany)
UV-Visible Spectrophotometer	Nicolet Evolution 100, Thermo Fisher Scientific Inc., (USA)
High Performance Liquid Chromatography	Shimadzu LC-20A HPLC system (Kyoto, Japan)
Carbohydrate column for HPLC analysis	Biorad Aminex HPX-87H (Hercules, CA, USA)
Scanning electron microscopy (SEM)	Quanta 400F Field Emission SEM (Oregon, USA)
X-ray diffraction (XRD)	Rigaku Ultima-IV Diffractometer (Japan)
Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)	Bruker Equinox 55 ATR-FTIR equipped with diamond-germanium ATR single reflection crystal (Massachusetts, USA)
Bench top centrifuge	Hettich Rotina 380 R (Tuttlingen Germany)
Micro centrifuge	Sigma 1-15 Microfuge (Postfach, Germany)
pH meter	Sartorius Basic pH Meter PB-11 (Goettingen, Germany)
Ice flaker	Scotsman AF 80 (Milan, Italy)
Magnetic stirrer with heating	Heidolph MR 3001 (Schwabach,Germany)
Vacuum pump	Gast Model doa-p104-aa (Michigan, USA)
Scale	Sartorius bp221s (Goettingen, Germany)
Scale	Sartorius CP323S (Goettingen, Germany)

APPENDIX B

B.1 Preparation of 0.05 M, pH 4.8 Citrate Buffer

Prepare 0.1 M stock solutions of citric acid mono-hydrate and tri-sodium citrate dehydrate. Add 25 ml of citric acid mono-hydrate solution to 25 ml of tri-sodium citrate dehydrate in order to obtain 0.05 M citrate buffer. Then, check the pH of the solution and adjust to 4.8 with 10 M NaOH if necessary. Store the buffer at 4°C before use.

B.2 Preparation of DNS reagent

Dissolve gently the following chemicals in 1 L of distilled water at 50°C in the following order. Stir the solution overnight in order to attain a completely clear solution. Filter the solution through a coarse filter paper and store it in a dark bottle at room temperature.
361.5 g Rochelle salt (sodium potassium tartrate)
10 g 3,5-dinitrosalicyclic acid
10 g NaOH
0.5 g Sodium sulfate
2 g Phenol

B.3 Preparation of the ionic liquid, 2-hydroxy ethyl ammonium formate (HEAF)

Preparation of the ionic liquid, 2-hydroxy ethyl ammonium formate (HEAF) is performed according to the previously reported procedure by Bicak (2005). The reactants, ethanol amine (2-Amino ethanol, ≥98%) and formic acid received from Sigma Aldrich are distilled prior to the synthesis of the ionic liquid with the aim of separating any impurity from the reactants. The experimental set-up consisted of a 2-necked flask placed onto ice bath equipped with a reflux condenser and a dropping funnel. Ethanol amine (119.8 g) is put into the 2-necked flask and formic acid (76 ml) is placed into the dropping funnel. The reaction between the reactants, which is an exothermic one, is conducted with drop wise addition of formic acid and under vigorous stirring of ethanolamine for 45 minutes. Stirring is provided for 24 hours at room temperature to ensure that a viscous and transparent liquid product is obtained. The product, 2-hydroxy ethyl ammonium formate (HEAF) can be stored at room temperature and used up to one month. Based on the thermogravimetric analysis (TGA) performed by Bicak (2005), the ionic liquid was found to decompose at temperature above 150°C. For that reason, pretreatment of cotton stalks via HEAF was conducted at 130°C in order to be on the safe side.

APPENDIX C

C.1 Preparation of YPD agar

1. Dissolve the given ingredients (Table C.1) in distilled water and sterilize the medium in an autoclavable bottle at 121°C for 20 minutes.
2. Allow the sterilized medium to cool to 55°C.
3. Transfer the liquid medium to the petri dishes and allow the medium to solidify.
4. Seal the agar plates with parafilm and store them at 4°C prior to inoculation.

Table C.1 Composition of YPD agar

Component	Concentration (g/L)
Yeast extract	10
Peptone	20
Glucose	20
Agar	20

C.2 Preparation of liquid YPD medium (Precultivation medium)

1. Dissolve the given ingredients (Table C.2) in distilled water and sterilize the medium in an autoclavable bottle at 121°C for 20 minutes.
2. Allow the sterilized medium to cool to room temperature before inoculation.
3. YPD medium should be prepared at most one day prior to inoculation.

Table C.2 Composition of liquid YPD medium

Component	Concentration (g/L)
Yeast extract	10
Peptone	20
Glucose	20

C3. Preparation of the fermentation medium

1. Dissolve the given ingredients (Table C.3) in distilled water and sterilize the solution in an autoclavable bottle at 121°C for 20 minutes.
2. Allow the sterilized medium to cool to room temperature.
3. Sterilize the enzymatic hydrolyzate by using sterilized filter having a pore size of 0.45 µm.
4. Inoculate the fermentation medium together with the filter sterilized hydrolyzate by 10% (v/v) of precultivated yeast.

Table C.3 Composition of the fermentation medium

Component	Concentration (g/L)
Yeast extract	10
Urea	6
$Na_2HPO_4.7H_2O$	3
KH_2PO_4	3
$MgSO_4.7H_2O$	0.25
$CaCl_2.2H_2O$	0.08

C.4 Strain storage and revival

The yeast cells in the late exponential phase are used for preparation of stock cultures. According to a previously reported protocol, 1 ml of late exponential culture is taken and put together with 1 ml solution of 30% (w/v) glycerol in a test tube with a screw cap. The glycerol solution and cells in the test tube are mixed and the tube is stored at -60°C. These frozen stock cultures (viable up to 5 years) can be revived by scrapping some of the cells from the surface, spreading them onto YPD agar plates and incubating them at 30°C for at least 2 days.

C.5 Precultivation of the yeast

During precultivation, yeast growth has been of primary importance. Accordingly, one loop of *yeast* from the *previously grown* agar plates is transferred into liquid YPD medium which constitutes no more than $1/5^{th}$ of the total volume of the erlenmeyer flask in order to attain effective aeration. The yeast in liquid medium is incubated at 30°C and 150 rpm for 24 hours prior to its transformation into the fermentation medium at 10% (v/v) of inoculation.

C.6 Calibration curve for *Saccharomyces cerevisiae* NRRL Y-132

The calibration for dry cell concentration of the yeast was given in the below figure.

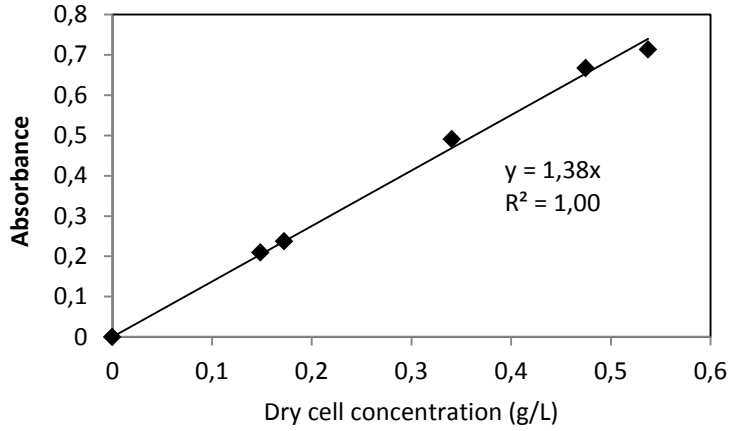


Figure C.1 Dry cell concentration versus absorbance measured at 600 nm.

C7. Effect of pH on growth of the yeast

Effect of pH on growth of the wild type yeast was investigated in liquid YPD medium. One loop of *yeast* from *previously grown* agar plates was incubated in liquid YPD medium at 30°C and 150 rpm for 48 hours. The initial pH of the fresh liquid YPD medium was 6.2 and it was adjusted to pH 4.8 via glacial acetic acid. The dry cell concentration was found to be higher for the medium at pH 6.2 according to the Figure C.2. For this reason, pH of the hydrolyzates derived upon enzymatic hydrolysis of the pretreated cotton stalks were adjusted to 6.2 via 10 M NaOH prior to fermentation. It was also observed that the wild type yeast, *Saccharomyces cerevisiae* NRRL Y-132 entered the late exponential phase of the growth almost after 24 hours and also, reached stationary phase of the growth after 32 hours. Conferring to the growth phases in Figure C.2, yeast cells were incubated for at most 24 hours in liquid YPD medium prior to the fermentation in order to be used as an inoculum for fermentation medium.

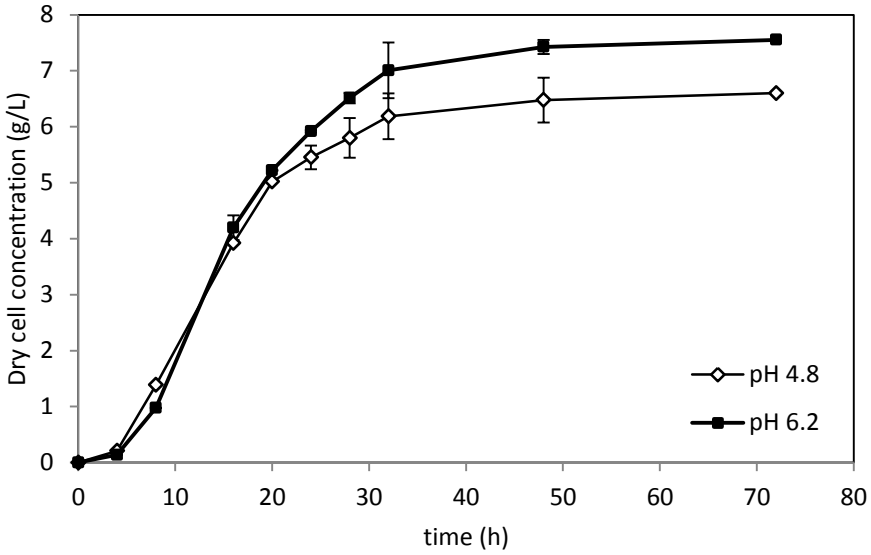


Figure C.2 Effect of pH on growth of *Saccharomyces cerevisiae* NRRL Y-132.

C8. Effect of initial glucose concentration on yeast growth and ethanol production in pure glucose media

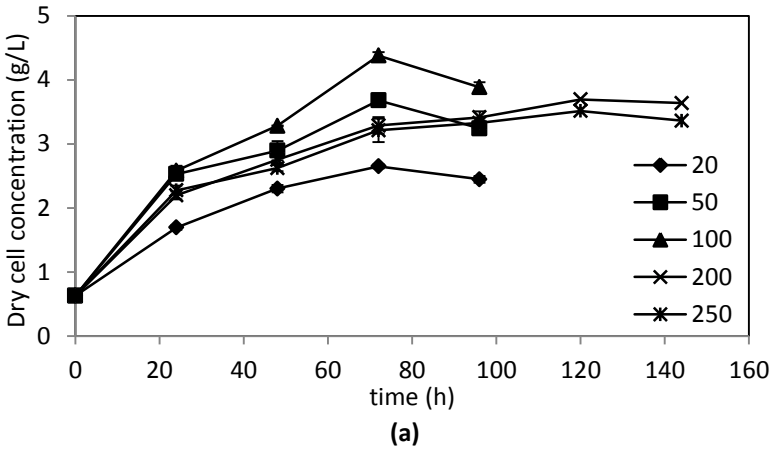
Prior to conducting any investigation in the hydrolyzate media derived upon enzymatic hydrolysis of EMIMAc pretreated cotton stalks, the initial glucose concentration in pure glucose media was assessed with respect to its effect on the growth of the wild type yeast, *S. cerevisiae* NRRL Y-132 and ethanol production. It was aimed to determine a range for the initial glucose concentration at which ethanol production from hydrolyzates would be investigated. For this purpose, analysis was carried out at glucose concentrations ranging from 20 to 200 g/L with the aim of monitoring the variations in the time courses of yeast growth, glucose consumption and ethanol production. The results were given in Figure C.3. As seen in the first figure (a) which demonstrated the time course of yeast growth, dry cell concentration increased with an increase in initial glucose concentration from 20 to 100 g/L. However a decrease was observed in dry cell concentrations when initial glucose concentration increased to 200 g/L and 250 g/L. Almost 3.3 g/L of dry cell concentration was obtained upon fermentation of 200 g/L and 250 g/L glucose whereas dry cell concentrations were almost 3.7 g/L and 4.4 g/L respectively for the media having 50 g/L and 100 g/L of glucose initially. Furthermore, the effect of initial glucose concentration was monitored with respect to the variation in glucose consumption of the yeast. As seen in the figure, 48-72 hours of time was enough for yeast to consume all glucose which was initially present at 20 to 100 g/L. However, yeast was found to be incapable of consuming glucose completely even after 144 hours of fermentation in the media containing 200 and 250 g/L of glucose initially. The variations in ethanol production were found to be in accordance with the time course of glucose consumption. The final ethanol concentration was attained almost within 72 hours of fermentation in the media having 20 to 100 g/L glucose initially. However, it took at least 120 hours to attain final concentration of ethanol in the media containing 200 g/L and 250 g/L glucose initially. Similar to the results obtained in dry cell concentration, ethanol production profiles for the media possessing 200 g/L and 250 g/L of glucose initially were close. They both appeared to reach the steady state conditions at the 120th hour of the fermentation at which the yeast was capable of producing almost 78 g/L of ethanol from both media.

Considering the results in Table C.4, *S. cerevisiae* NRRL Y-132 was able to convert at least 96% of the glucose to ethanol in the media containing 20 to 100 g/L glucose initially. However, 80% and 78% of the theoretical maximum ethanol yield were achieved, respectively upon fermentation in the pure glucose media having 200 g/L and 250 g/L of glucose initially.

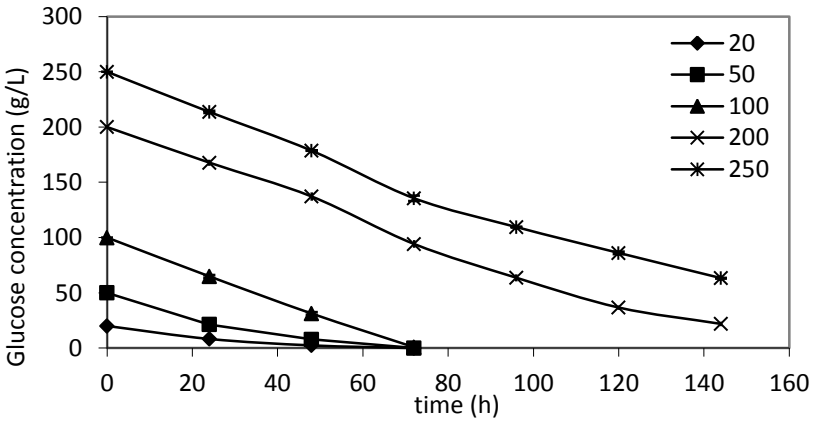
In this context, this effect was not investigated at glucose concentrations above 100 g/L for the hydrolyzate media obtained upon enzymatic hydrolysis of EMIMAc pretreated cotton stalks (Section 4.2.10).

Table C.4 Effect of initial glucose concentration on ethanol production in pure glucose media

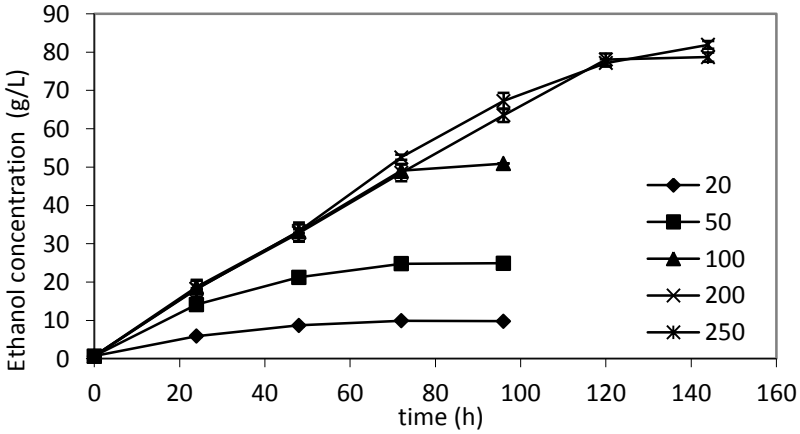
	Initial glucose concentration (g/L)				
	20	**50**	**100**	**200**	**250**
Ethanol concentration (g/L)	10	25	51	82	79
Ethanol titre (% v/v)	1.3	3.2	6.5	10.4	10.0
Ethanol yield (%)	98	96	100	80	78



(a)



(b)



(c)

Figure C.3 Effect of initial glucose concentration on the (a) yeast growth, (b) glucose consumption and (c) ethanol production in pure glucose media.

C.9 Effect of nutrients supplementation on the ethanol production from EMIMAc pretreated cotton stalks

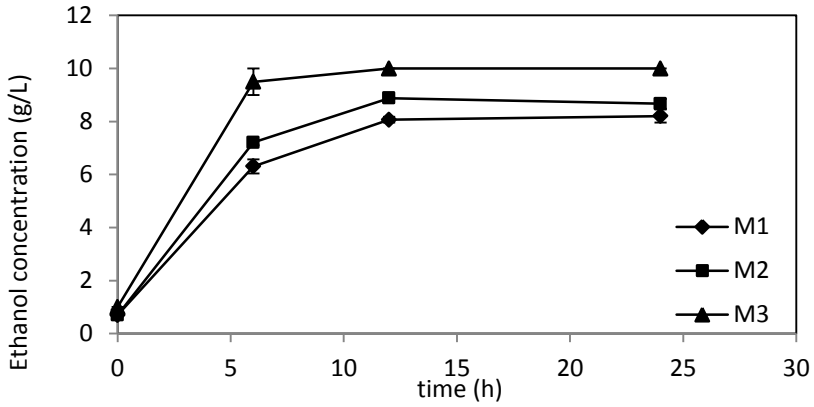
Three different media were assessed at each initial glucose concentration (20, 50 and 100 g/L) and their compositions were given in Table C.5. Experiments for each media were conducted under the same pH, temperature and stirring rate, 6.2, 30°C and 150 rpm, respectively. Initially, the medium was composed of only urea at concentration of 6 g/L and glucose derived from hydrolysis of EMIMAc pretreated cotton stalks. Later on, yeast extract at a concentration of 10 g/L was added to each medium. Finally, inorganic salts were added to the medium containing urea and yeast extract to investigate their effect on ethanol production. The major aim was to enhance ethanol yield by making modifications in the nutrient media. As described above, the analysis was carried out in a step wise manner based on the media given in Table C.5. The effect of initial glucose concentration on ethanol production, which was investigated in the final media (M3), was shown in Part 4.2.10 in more detail. Not only ethanol production was demonstrated, the variations in dry cell concentration and glucose consumption with initial glucose concentration were given in the aforementioned section. In the same section, ethanol production from the hydrolyzate supplemented with the components of the final medium was also compared to the medium containing pure glucose together with the same nutrients.

Table C.5 Composition of fermentation media

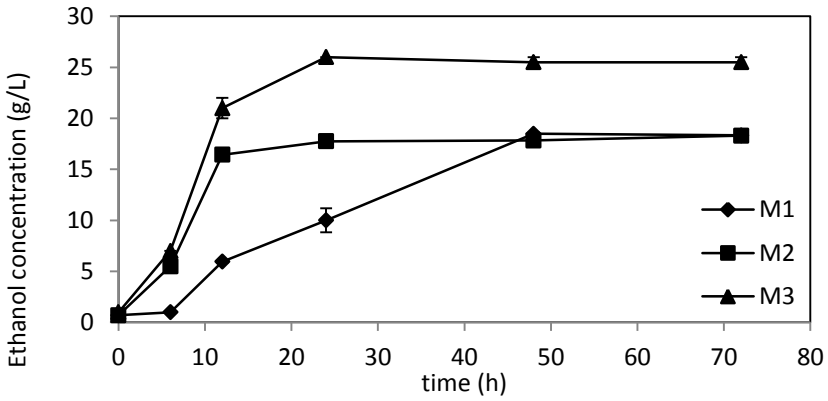
Medium 1 (M1)		**Medium 2 (M2)**		**Medium 3 (M3)**	
Component	**Concentration (g/L)**	**Component**	**Concentration (g/L)**	**Component**	**Concentration (g/L)**
Glucose	20-50-100	Glucose	20-50-100	Glucose	20-50-100
Urea	6	Urea	6	Urea	6
		Yeast extract	10	Yeast extract	10
				$Na_2HPO_4.7H_2O$	3
				KH_2PO_4	3
				$MgSO_4.7H_2O$	0.25
				$CaCl_2.2H_2O$	0.08

Figure C.4 showed the variation of ethanol production with nutrient media having the following initial glucose concentrations, 20, 50 and 100 g/L. According to the figure (Figure C.4), the highest ethanol production profile was attained in M3 at each initial glucose concentration. According to the figures, ethanol concentration reached the highest value within the first 12 hours for the media containing 20 g/L glucose initially whereas it took longer for the media having initial glucose concentrations, 50 and 100 g/L; at least 48 and 96 hours of time was required to reach the final ethanol concentration, respectively. Supplementation of M1 with yeast extract (M2) and then with inorganic salts (M3) not only enhanced the final ethanol concentrations but also decreased the time required to attain the highest ethanol concentration. Though, this effect was not much apparent for the media having 20 g/L glucose initially; M3 containing 50 and 100 g/L of glucose initially resulted with a different profile compared to M1 (Figures C4 (b) and C4 (c)). For instance, the highest ethanol concentration was attained as 26 g/L at the 24^{th} hour in M3 containing 50 g/L of glucose initially whereas 48 hours of time was required to reach the highest ethanol concentration in M1 which was

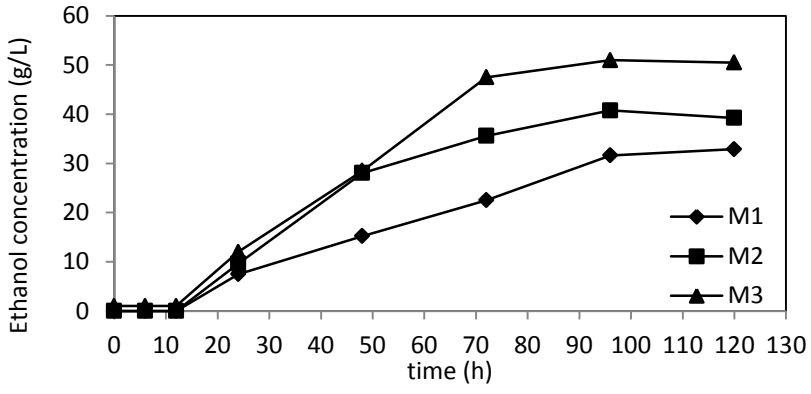
18 g/L. Similarly, the highest ethanol concentration was obtained as 51 g/L at the 72nd hour in M3 containing 50 g/L of glucose initially while it took at least 96 hours to attain the final ethanol concentration in M1 which was 33 g/L. The improvements derived upon supplementation of M1 with yeast extract and inorganic salts were also shown in Table C.6. According to the table, M3 at each initial glucose concentration resulted with at least 98% of the theoretical maximum ethanol yield however much lower yields were derived in M1 and M2. The highest ethanol titer was 6.5% (v/v) which was obtained upon fermentation of 100 g/L glucose in M3. Consequently, M3 was selected as the most appropriate medium and investigated in more detail together with its comparison with the medium containing pure glucose (Section 4.2.10).



(a)



(b)



(c)

Figure C.4 Effect of nutrient media on the time course of ethanol production from hydrolyzate having initial glucose concentrations of (a) 20 g/L, (b) 50 g/L and (c) 100 g/L.

Table C.6 Effect of nutrient media on ethanol production from hydrolyzate having initial glucose concentrations of 20, 50 and 100 g/L.

	Initial glucose concentration (g/L)								
	20			**50**			**100**		
Fermentation Media	**M1**	**M2**	**M3**	**M1**	**M2**	**M3**	**M1**	**M2**	**M3**
Ethanol concentration (g/L)	8	9	10	18	18	26	33	41	51
Ethanol titre (v/v %)	1.0	1.1	1.3	2.3	2.3	3.3	4.2	5.2	6.5
Ethanol yield (%)	80	87	98	69	69	100	65	80	100

APPENDIX D

D.1 Cellulase assay

Cellulase activities of the commercial enzymes, Celluclast 1.L and Cellic Ctec2 were determined on the basis of the procedure reported by National Renewable Energy Laboratory (NREL) laboratory analytical procedure (LAP) (Adney and Baker, 2008).The assay was conducted according to the following steps.

1- Prepare 0.05 g of Whatman No.1 filter paper strips having dimensions of 1 cm x 6 cm.

2- Put each rolled filter paper strips into the 13 mmx100mm long test tubes.

3- Add 1 ml of 0.05 M of sodium citrate buffer, pH 4.8 to the tubes ensuring that strips are completely immersed into the buffer.

4- Equilibrate the tubes to 50°C in a water bath.

5- Dilute the enzyme at least 100 fold with citrate buffer. In addition to this initial dilution ratio, DR_1, at least 4-5 more dilutions should be performed in order to obtain the enzyme dilution ratio that corresponds to the production of 2 mg of glucose/0.5 ml solution from filter paper strips.

6- Add 0.5 ml of diluted enzyme solutions to the tubes and incubate them at 50°C for 60 minutes.

7- At the end of 60 minutes, remove the tubes from the bath and add 3 ml of DNS to terminate the enzymatic reaction.

8- The controls used in the assay are:

- Reagent blank: 1.5 ml citrate buffer
- Enzyme control: 1 ml citrate buffer+0.5 ml enzyme solution
- Substrate control: filter paper strip+1.5 ml citrate buffer

Likewise, these controls should be incubated at 50°C along with the enzyme assay tubes for 60 minutes. Finally, add 3 ml of DNS to the control tubes at the end of the incubation.

9- Prepare 10 g/L of stock glucose solution and the following glucose standards by performing the appropriate dilutions, 1, 1.65, 2.5 and 3.35 g glucose/0.5 mL solution. Add 0.5 ml of each of the glucose standards to 1 ml of citrate buffer and similarly, incubate them all together at 50°C along with the enzyme assay tubes for 60 minutes. Finally, add 3 ml of DNS to the glucose standard tubes at the end of the incubation.

10- Boil all tubes (enzyme assay samples, controls, glucose standards) for 5 minutes. Allow the tubes to cool to room temperature. Make sure the pulps (deriving from filter paper strip) in the tubes to be settled. Unless, centrifuge the tubes and use the supernatants for the rest of the analysis.

11- Withdraw 0.2 ml of sample from each tube and dilute them with 2.5 ml of distilled water. Vortex each tube and measure their absorbance at 540 nm. Make sure that the absorbance of the samples does not exceed 0.9. If not, higher enzyme dilutions will be required.

12- Cellulase activity, which is named as one international filter paper unit (FPU), is defined as the amount of enzyme that produces 1 μmol of glucose per minute during the hydrolysis reaction (Ghose, 1987). Cellulase activity is calculated according to the following equations,

$$\text{Cellulase activity} = \frac{0.37}{[\text{enzyme dilution ratio releasing 2 mg glucose}]^{-1}} \quad \text{(D-1)}$$

$$DR = DR_1 \times \text{Subsequent dilution ratio} \quad \text{(D-2)}$$

In order to obtain the enzyme dilution ratio that corresponds to the production of 2 mg of glucose/0.5 ml solution upon the enzymatic hydrolysis of filter paper strips, the amount of glucose versus enzyme dilution ratio should be plotted as shown in Figure D.2.

13- Sample calculations and data given below demonstrate the findings obtained upon previously conducted cellulase assay for the commercial enzyme, Celluclast 1.5L.

Based on the slope of the glucose calibration curve (Figure D.1), glucose concentration, which corresponded to each enzyme dilution ratio, were calculated and given in Table D.2.

Table D.1 Glucose calibration data

glucose concentration (mg/0.5ml)	Absorbance measured at 540 nm
1	0.282
1.65	0.454
2.5	0.635
3.35	0.817

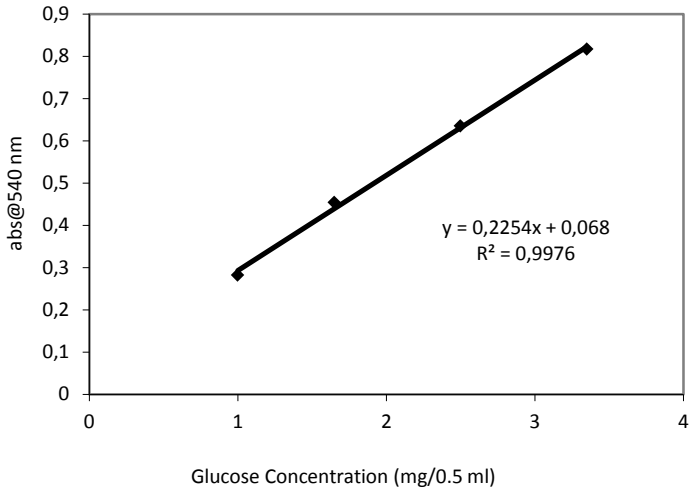


Figure D.1 Glucose calibration curve

Table D.2 Concentration of glucose, which was released upon enzymatic hydrolysis of filter paper strips, versus enzyme dilution ratio, DR.

DR	$[DR]^{-1}$	Glucose Concentration (mg/0.5ml solution)
100	0.01	3.10
200	0.005	2.01
300	0.00333	1.56
400	0.0025	1.30

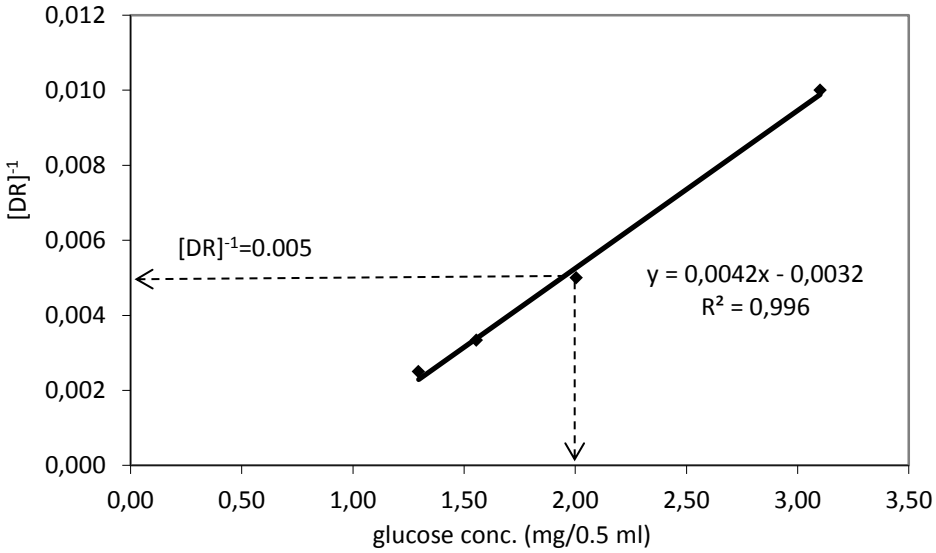


Figure D.2 Concentration of glucose, which was released upon enzymatic hydrolysis of filter paper strips, versus $[DR]^{-1}$.

Thus, cellulase activity for Celluclast 1.5 L was found as

$$\frac{0.37}{0.005} = 75 \text{ FPU/ml}$$

D.2 Xylanase assay

Xylanase activities of the commercial enzymes, Celluclast 1.L and Cellic Ctec2 were calculated on the basis of a previously reported procedure (Ghose, 1987). The assay was conducted according to the following steps.

1-Preparation of xylan substrate

I. Weigh 1 g of xylan from birchwood into a 100 ml of autoclavable bottle and add 80 ml 0.05 M, pH 4.8 citrate buffer of pH 4.8.
II. Place the bottle onto a magnetic stirrer with heater at 120°C. Loosen the cap of the bottle slightly and let the solution to boil for 5 minutes.
III. Turn off the heater, tighten the cap of the bottle and allow the solution to stir at room temperature overnight.
IV. Complete the solution to 100 ml with citrate buffer and allow the solution to stir for a while.
V. This slightly turbid solution can be stored at most 2 days at 4°C in order to be used as the xylan substrate for the xylanase assay.

2- Enzymatic reaction

I. Dilute the enzyme at least 200 fold with citrate buffer (DR_1).
II. Put 2 ml of xylan substrate into 50 ml falcon. Add 0.2 ml of diluted enzyme and vortex the reaction mixture in order to start the enzymatic reaction.
III. Withdraw at most 1 ml of samples (V_{sample}) at t=0 at specific time intervals from the reaction mixture for 5 minutes of reaction period and immediately add them into the tubes containing 1.5 ml of DNS reagent to terminate the enzymatic reaction.
IV. When the reaction is completed, boil the tubes for 5 minutes and allow them to cool to room temperature.
V. Measure the absorbance of the samples against a buffer blank at 540 nm. Make sure that the absorbance of the samples does not exceed 0.9. If not, higher enzyme dilutions will be required.
VI. Plot the absorbance versus time graph and determine the slope of the linear curve (m_1).
VII. Plot a calibration curve using xylose as a standard at the following concentrations, 60, 90, 120 and 150 µg/ml and determine its slope (m_2).
VIII. Xylanase activity (U/ml) is defined as the amount of enzyme that releases 1 µmol of xylose equivalent sugar per minute and determined according the following equation,

Xylanase activity (U/ml) =

$$DR_1 \times \frac{m_1}{m_2} \times \frac{1}{V_{sample}} \times \frac{V_{reaction\ mixture}}{V_{enzyme\ diluted}} \times \frac{1\ \mu mol\ xylose}{150 \mu g\ xylose} \qquad \text{(D-3)}$$

APPENDIX E

CALIBRATION CURVES OF THE STANDARDS USED IN HPLC ANALYSIS

E.1 Calibration curve for glucose

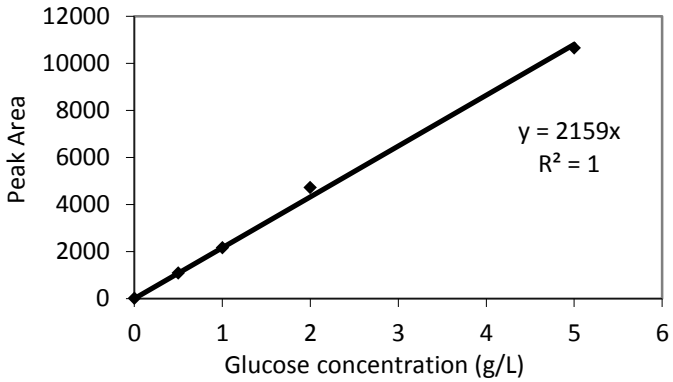


Figure E1. Calibration curve for glucose

E.2 Calibration curve for xylose

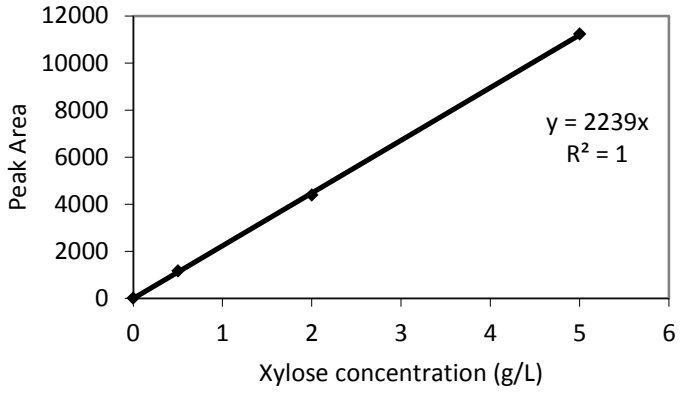


Figure E2. Calibration curve for xylose

E.3 Calibration curve for ethanol

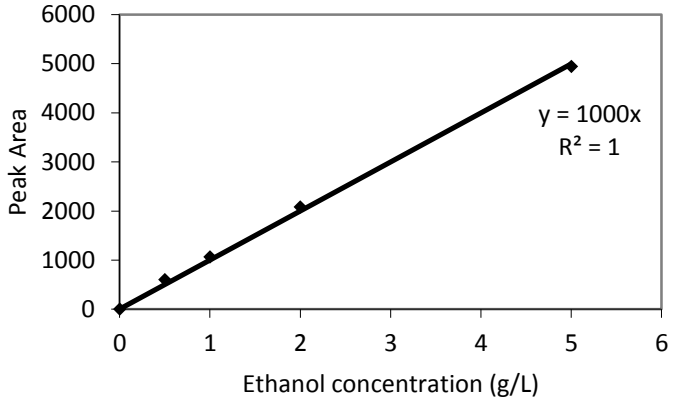


Figure E3. Calibration curve for ethanol

VITA

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B. Sc.	Hacettepe University, Chemical Engineering	2000-2004
High School	TED Ankara College	1993-2000

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Year	Place	Enrollment
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PUBLICATIONS

1. Peer-reviewed journal articles

i. Bahcegul, E., Apaydin, S., **Haykir, N.I.**, Tatli E., Bakir, U., 2012. Different ionic liquids favor different lignocellulosic biomass particle sizes during pretreatment to function efficiently. Green Chemistry. doi: 10.1039/C2GC35318K.

ii. **Haykir, N.I**., Bahcegul, E., Bicak, N., Bakir, U., 2012. Ionic liquid facilitated approach to cellulosic ethanol production: Improved biomass digestibility through ionic liquid pretreatment for ethanol production from cotton stalk. Accepted to 15th European Congress on Biotechnology, will be published in the special issue of New Biotechnology.

iii. **Haykir, N.I.**, Bahcegul, E., Bicak, N., Bakir, U., 2012. Pretreatment of cotton stalk with ionic liquids including 2-hydroxy ethyl ammonium formate to enhance biomass digestibility. Industrial Crops and Products. 41, 430-436.

iv. Bahcegul, E., Tatli, E., **Haykir, N.I.**, Apaydin, S., Bakir, U., 2011. Selecting the right blood glucose monitor for the determination of glucose during the enzymatic hydrolysis of corncob pretreated with different methods. Bioresource Technology. 102, 9646-9652.

v. **Haykir I**., 2009. A comparative study on lignocellulose pretreatments for bioethanol production from cotton stalk. 14th European Congress on Biotechnology, Special Issue of New Biotechnology. 25, 253-254.

vi. Çalik, P., Angardi, V., **Haykir, N.I.**, Boyaci, I.H.,2009. Glucose isomerase production on a xylan-based medium by *Bacillus thermoantarcticus*. Biochemical Engineering Journal. 43, 8-15.

2. International Conference Publications

i. **Haykir, N.I.**, Bahcegul, E., Bicak, N., Bakir, U., "Ionic liquid facilitated approach to cellulosic ethanol production: Improved biomass digestibility through ionic liquid pretreatment for ethanol production from cotton stalk", oral presentation in the 15th European Congress on Biotechnology, 23–26 September, Istanbul, Turkey, 2012.

i. **Haykır I.**, Bakır Bölükbaşı U., Bahçegül E., Apaydın S., "Ethanol production form cotton stalk via IL-mediated pretreatment and enhanced enzymatic hydrolysis", International symposium on alcoholic fuels (ISAF-XIX), 10-14 October, Verona, Italy, 2011.

ii. **Haykır I.**, Apaydın S., Bahcegül E., Tatlı E., Bıçak N., Bakır Bölükbaşı U.,"Pretreatment of cotton stalk with ionic liquids for enhanced enzymatic hydrolysis and ethanol production", 8th European Congress of Chemical Engineering, 25-29 September, Berlin, Germany, 2011.

iii. Apaydin S., Bahcegul E., **Haykir I.**, Tatli E., Bicak N., Bakir U., "Pretreatment of Lignocellulosic Agricultural Wastes with Ionic Liquids for the Production of Sugars via Improved Enzymatic Hydrolysis", 4th Annual Workshop of COST FP0602, 21-24 September, Izmir, Turkey, 2010.

iv. **Haykır I.**, Bakır U., "A comparative study on lignocellulose pretreatments for bioethanol production from cotton stalk", 18th European Biomass Conference and Exhibition, 3 - 7 May, Lyon, France, 2010.

v. **Haykır I.**, Bakır U., " A comparative study on lignocellulose pretreatments for bioethanol production from cotton stalk.", 14th European Congress on Biotechnology, 13–16 September, Barcelona, Spain, 2009.

3. National Conference Publications

i. **Haykır I.**, Bölükbaşı U., "Hasat sonrası tarlada kalan pamuk bitkisini bileşenlerine ayrıştırmak için iyonik sıvı ön işleminin etkilerinin araştırılması"**,** 9th National Chemical Engineering Congress, Ankara, 2010. (In Turkish)

ii. **Haykır I.**, Çalık P., Boyacı İ.H., "Investigation of Bioprocess Parameters for Glucose Isomerase Production by *Bacillus thermoantarcticus*", 1st National Catalysis Congress, Güzelyurt, Northern Cyprus, 2007.

References

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