

# Biochemical conversion of biomass to biofuels

Pretreatment–Detoxification–Hydrolysis–  
Fermentation

**VENKATA PRABHAKAR SOUDHAM**



**Department of Chemistry**  
Umeå University, Sweden 2015

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Fermentation

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*This work is dedicated to my mother **Dhanalakshmi Addepalli/Soudham**, the strongest person I have ever known. Also, to my friend, mentor, and a great advisor **Dr. Tomas Brandberg**, without whom this degree would not have been possible.*

*“Every step was an effort of will”*



# **PREFACE**

This PhD dissertation serves as a partial fulfilment of the requirements for a PhD degree at the Department of Chemistry, Umeå University, Sweden. The PhD project was initiated in May 2010. First half of the PhD work was carried out under the supervision of Professor Leif Jönsson, Umeå University, and Dr. Björn Alriksson, SP Processum AB, Sweden. The second half of the PhD work was performed under the supervision of Professor Christer Larsson, Chalmers University of Technology, Sweden, Professor Jyri-Pekka Mikkola, Umeå University, Sweden & Åbo Akademi University, Finland, and Dr. Tomas Brandberg, Sweden.

## **Aims**

The aims of the research presented in this thesis concerns evaluating saccharification potential of different lignocellulose materials, investigating the potential of various solvents including less hazardous ionic liquids (ILs) for the use as lignocellulose pretreatment catalysts, and evaluating a battery of chemical detoxification methods to address the problems associated with toxic by-products that inhibit both cellulolytic enzymes and fermentation organisms.



## ABSTRACT

The use of lignocellulosic materials to replace fossil resources for the industrial production of fuels, chemicals, and materials is increasing. The carbohydrate composition of lignocellulose (i.e. cellulose and hemicellulose) is an abundant source of sugars. However, due to the feedstock recalcitrance, rigid and compact structure of plant cell walls, access to polysaccharides is hindered and release of fermentable sugars has become a bottle-neck. Thus, to overcome the recalcitrant barriers, thermochemical pretreatment with an acid catalyst is usually employed for the physical or chemical disruption of plant cell wall. After pretreatment, enzymatic hydrolysis is the preferred option to produce sugars that can be further converted into liquid fuels (e.g. ethanol) via fermentation by microbial biocatalysts.

However, during acid pretreatment, several inhibitory compounds namely furfural, 5-hydroxymethyl furfural, phenols, and aliphatic acids are released from the lignocellulose components. The presence of these compounds can greatly effect both enzymatic hydrolysis and microbial fermentation. For instance, when Avicel cellulose and acid treated spruce wood hydrolysate were mixed, 63% decrease in the enzymatic hydrolysis efficiency was observed compared to when Avicel was hydrolyzed in aqueous citrate buffer. In addition, the acid hydrolysates were essentially non-fermentable. Therefore, the associated problems of lignocellulose conversion can be addressed either by using feedstocks that are less recalcitrant or by developing efficient pretreatment techniques that do not cause formation of inhibitory by-products and simultaneously give high sugar yields.

A variety of lignocellulose materials including woody substrates (spruce, pine, and birch), agricultural residues (sugarcane bagasse and reed canary grass), bark (pine bark), and transgenic aspens were evaluated for their saccharification potential. Apparently, woody substrates were more recalcitrant than the rest of the species and bark was essentially amorphous. However, the saccharification efficiency of these substrates varied based on the pretreatment method used. For instance, untreated reed canary grass was more recalcitrant than woody materials whereas the acid treated reed canary grass gave a higher sugar yield (64%) than the woody substrates (max 34%). Genetic modification of plants was beneficial, since under similar pretreatment and enzymatic hydrolysis conditions, up to 28% higher sugar production was achieved from the transgenic plants compare to the wild type.

As an alternative to the commonly used acid catalysed pretreatments (prior to enzymatic hydrolysis) lignocellulose materials were treated with four ionic liquid solvents (ILs): two switchable ILs (SILs) -SO<sub>2</sub>DBUMEASIL and -CO<sub>2</sub>DBUMEASIL, and two other ILs [Amim][HCO<sub>2</sub>] and [AMMorp][OAc].

After enzymatic hydrolysis of IL treated substrates, a maximum amount of glucan to glucose conversion of between 75% and 97% and a maximum total sugar yields of between 71% and 94% were obtained. When using acid pretreatment these values varied between 13-77% for glucan to glucose conversion and 26-83% for total sugar yield. For woody substrates, the hemicellulose recovery (max 92%) was higher for the IL treated substrates than compared to acid treated samples. However, in case of reed canary grass and pine bark the hemicellulose recovery (90% and 88%, respectively) was significantly higher for the acid treated substrates than the IL treated samples.

To overcome the inhibitory problems associated with the lignocellulose hydrolysates, three chemical conditioning methods were used 1. detoxification with ferrous sulfate ( $\text{FeSO}_4$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) 2. application of reducing agents (sulfite, dithionite, or dithiothreitol) and 3. treatment with alkali:  $\text{Ca}(\text{OH})_2$ ,  $\text{NaOH}$ , and  $\text{NH}_4\text{OH}$ . The concentrations of inhibitory compounds were significantly lower after treatments with  $\text{FeSO}_4$  and  $\text{H}_2\text{O}_2$  or alkali. Using reducing agents did not cause any decrease in the concentration of inhibitors, but detoxification of spruce acid hydrolysates resulted in up to 54% improvement of the hydrolysis efficiency (in terms of sugar release) compared to untreated samples. On the other hand, application of detoxification procedures to the aqueous buffer resulted in up to 39% decrease in hydrolysis efficiency, thus confirming that the positive effect of detoxification was due to the chemical alteration of inhibitory compounds. In addition, the fermentability of detoxified hydrolysates were investigated using the yeast *Saccharomyces cerevisiae*. The detoxified hydrolysates were readily fermented to ethanol yielding a maximum ethanol concentration of 8.3 g/l while the undetoxified hydrolysates were basically non-fermentable.

**Keywords:** Lignocellulosic materials, Ionic liquids, Pretreatment, Inhibitors, Detoxification, Ferrous sulfate and hydrogen peroxide, reducing agents, alkaline treatments, Hydrolysis, Fermentation, Biofuels.

# LIST OF PUBLICATIONS

This thesis is based on the following research papers, referred to as **PAPER I–V** in the text.

**PAPER I: Soudham VP**, Gräsvik J, Alriksson B, Mikkola JP, Jönsson LJ (2013) Enzymatic hydrolysis of Norway spruce and sugarcane bagasse after treatment with 1-allyl-3-methylimidazolium formate. *Journal of Chemical Technology & Biotechnology* 88(12), 2209–2215.

**PAPER II: Soudham VP**, Raut DG, Anugwom I, Brandberg T, Larsson C, Mikkola JP (2015) Coupled enzymatic hydrolysis and ethanol fermentation: Ionic Liquid Pretreatment for Enhanced Yields. Manuscript – submitted to *Biotechnology for Biofuels*.

**PAPER III: Soudham VP**, Brandberg T, Mikkola JP, Larsson C (2014) Detoxification of acid pretreated spruce hydrolysates with ferrous sulfate and hydrogen peroxide improves enzymatic hydrolysis and fermentation. *Bioresource Technology* 166, 559–565.

**PAPER IV: Soudham VP**, Alriksson B, Jönsson LJ (2011) Reducing agents improve enzymatic hydrolysis of cellulosic substrates in the presence of pretreatment liquid. *Journal of Biotechnology* 155, 244–250

**PAPER V: Jönsson LJ**, Alriksson B, **Soudham VP** (2013) Methods for improvement of enzymatic hydrolysis of lignocellulosic material. WO 2013/000696 A1, PCT/EP2012/061021.

## Additional works by the author

**Soudham VP** and Jönsson LJ: Pretreatment and enzymatic hydrolysis of transgenic Aspens for the evaluation of their recalcitrance. Collaborating partners Bioimprove programme ([www.bioimprove.se](http://www.bioimprove.se)).

**Soudham VP**, Alriksson B, and Jönsson LJ: A comparative study of H<sub>2</sub>SO<sub>4</sub> and SO<sub>2</sub> impregnated steam pretreatments (pilot scale) of Norway spruce: chemical conditioning, enzymatic hydrolysis and fermentation.

**Soudham VP**, Rodriguez D, Rocha JM, Taherzadeh MJ, Martin C (2011) Acetosolv delignification of marabou (*Dichrostachys cinerea*) wood with and without acid prehydrolysis. *Forestry Studies in China* 13(1), 64–70.

## **Author contributions**

**PAPER I:** Conceived the idea together with co-authors, responsible for experimental design, performed all experimental work, conducted data analysis, and was main author of the manuscript.

**PAPER II:** Conceived the idea together with co-authors, responsible for experimental design, performed all experimental work, conducted data analysis, and was main author of the manuscript.

**PAPER III:** Responsible for the idea and experimental design, performed all experimental work, conducted data analysis, and was main author of the manuscript.

**PAPER IV:** Involved in experimental design, performed all experimental work, conducted data analysis, and was main author of the manuscript.

**PAPER V:** Conceived the idea together with co-authors, involved in experimental design, performed all experimental work, conducted data analysis, involved in writing.

# LIST OF NOTATIONS

[Amim][HCO <sub>2</sub> ]	1-allyl-3-methylimidazolium formate
[AMMorp][OAc]	N-allyl-N-methylmorpholinium acetate
AFEX	Ammonia fiber explosion
ARP	Ammonia recycle percolation
BGs	β-glucosidase
CAZy	Carbohydrate-Active enzymes
CBHs	Cellobiohydrolase
CBP	Consolidated bioprocessing
CF	Co-fermentation
CIL	Cellulose dissolving ionic liquid
CMC	Carboxymethyl cellulose
DBU	1,8-Diazabicycloundec-7-ene
DP	Degree of polymerisation
DTT	Dithiothreitol
EGs	Endoglucanase
GHG	Greenhouse gases
GRAS	Generally regarded as safe
HEC	2-hydroxyethyl cellulose
HMF	5-Hydroxymethyl furfural
IL	Ionic liquid
LHW	Liquid hot water
MEA	Monoethanolamine
OH·	Hydroxyl radical
OP's	Oxidation processes
PEG	Polyethylene glycol
PLA	Poly lactide
RCG	Reed canary grass
SAH	Spruce acid hydrolysate
SCF	Supercritical fluid
SF	Severity factor
SIL	Switchable ionic liquid
SSCF	Simultaneous saccharification and co-fermentation
SSH	Spruce slurry hydrolysate
SSF	Simultaneous saccharification and fermentation
WT	Wild type



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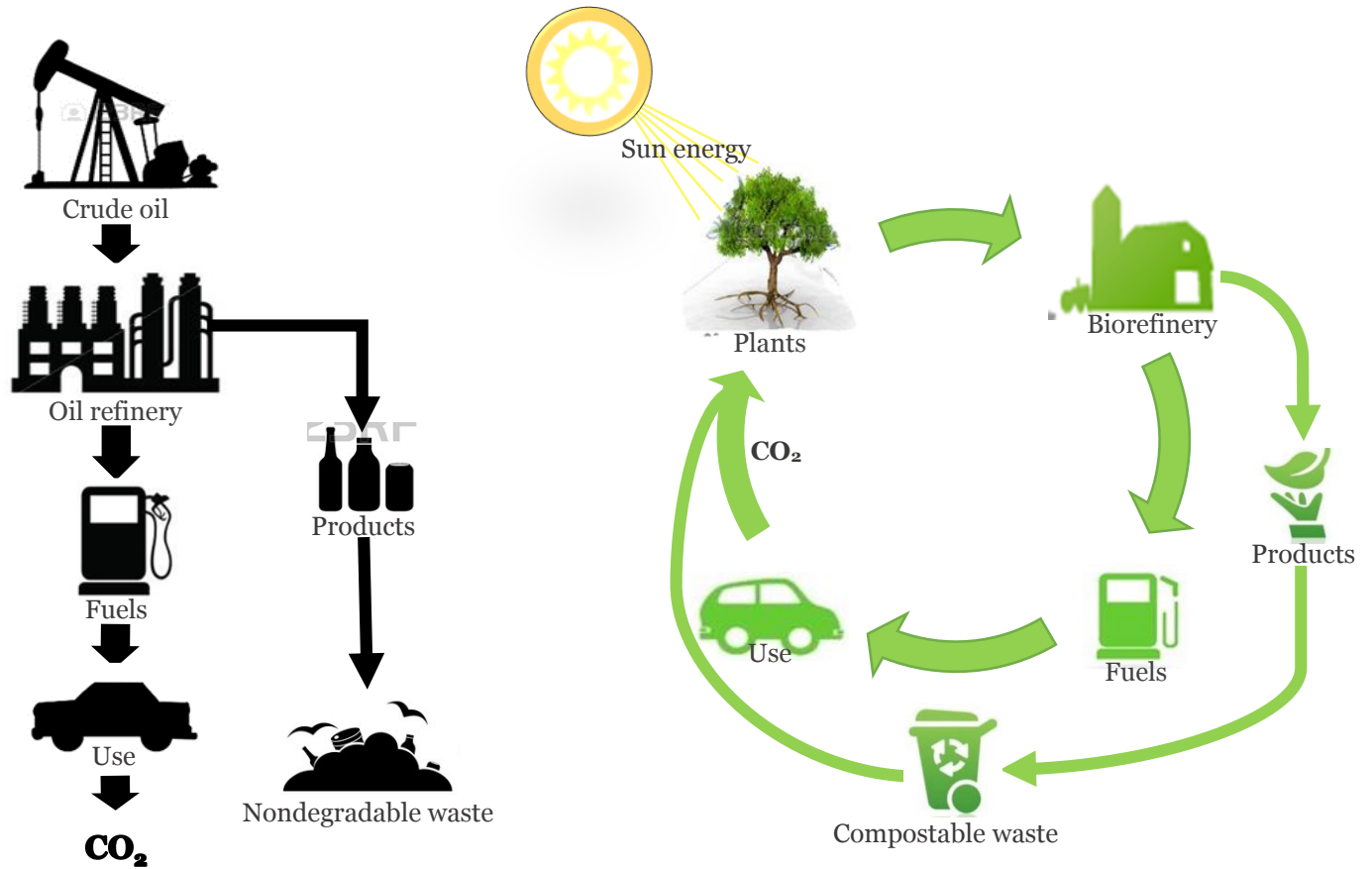
# **Biomass as a resource**

## **Introduction**

Fossil fuel refinery, based on resources such as coal, crude oil and natural gas, is essentially vital to sustain the life of modern world. A great fraction of worldwide energy carriers and a wide range of chemicals and material products comes from it (Cherubini 2010). In particular, the primary source of energy for transport sector is oil and over 90 wt% of organic chemicals are essentially derived from petroleum (Witcoff and Reuben 1996).

However, fossil reserves are of limited supply, cannot be replaced (takes millions of years to form, cycle time of  $> 10^7$  years) and are non-renewable (Clark and Deswarte 2008). Hence, their production follows a pattern called Hubbert Curve (Hubbert 1956), with output increasing, stabilizing and then falling to nothing over a period of time. Similarly, the global oil production was predicted to rise, peak and then fall off. Even if it is unclear as when, among other fossil reserves, various estimates presume that the oil would start to decline anywhere 20 years from now to more than a century in the future (Mousdale 2008). However, its demand continues to grow while the reserves dwindle. In 2014, oil consumption around the world was approximately 92 million barrels a day (EIA 2015) and by 2030 it is projected to be increase to about 116 million barrels (Cherubini 2010). Thus, we cannot forever pump and use an entire planet's worth of oil.

Nevertheless, advances in technology give some hope to replace the less available conventional oil with unconventional resources such as oil sands, ultra-heavy oils and shale oil etc. However, as understood by everybody, also they are finite resources and will thus eventually be depleted. In addition to this, these technologies for oil extraction and its use can be potentially harmful to the environment. For instance, greenhouse gases (GHG), such as carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ) and nitrous oxide ( $\text{N}_2\text{O}$ ), generated from the combustion of fossil fuels are allegedly perturbing the environment (IPCC 2014). Especially transportation accounts a significant share and has shown the highest growth rates in the GHG emissions (Szulczyk 2010). Furthermore, increasing world population, availability of energy per capita and the rise of emerging economies such as India and China will be driving forces of energy demand (Clark and Deswarte 2008). This would lead to rapid shrink in fossil reserves and increase in GHG emissions. In addition, the wastes associated with the conversion and consumption of fossil resources and their process auxiliaries are not environmentally compatible. The use of plastics, for example, gives rise to pollution that contaminates the environment for hundreds of years.



**Figure 1.** A comparative lifecycle description of current oil refinery and future biorefinery.

Therefore, considering the needs of world's development, population growth and with the resulting impact on environment, the reliance on short-term picture of a well-supplied fossil feedstock market must be redirected. Alternative solutions (feedstocks) that are renewable on a short timescale, able to alleviate climate change, and simultaneously reduce the consumption of fossil resources should be promoted instead (Cherubini 2010). For this, fundamentally, dematerialisation (use less resource) and transmaterialisation (replacement of current raw materials including energy) routes are necessary (Clark and Deswarte 2008). It is however recognized that there is not a single solution to these problems.

The shortfall in energy (i.e. heat and electricity) might be counterpoised by renewable and natural resources such as solar, wind, tides and traditionally used biomass. In contrast to non-renewables, these are in no danger of being over-exploited and can be considered as renewables (Pimentel et al. 2002). According to EU renewable energy statistics, Sweden is the leading country of using renewable sources of energy – In 2012, 51% of its total energy was coming from renewables, in which only the share of biomass was accounted for 60% (Eurostat 2014). Besides, the energy needs could in future also be fulfilled by technological developments such as unconventional nuclear fusion and fuel cells. It is important to note that, except biomass all other renewables would serve as source of energy, but biomass, besides fossil resources, are the only carbon rich material source available on our planet (Ragauskas *et al.* 2006) and can therefore be used to produce not only energy but also organic chemicals and materials.

Biomass feedstock based process auxiliaries, products and wastes can be designed to be rapidly degradable by bacteria or other biological means (biodegradable) and are thus environmentally compatible. For instance, unlike fossil derived plastics, polylactide (PLA) plastics derived from biomass are biodegradable. Furthermore, CO<sub>2</sub> that would generate from the use of biomass will again be consumed by plants during their growth (through photosynthesis), creating a closed loop of carbon cycle (Figure 1). Therefore, for organic chemicals and materials, a shift from fossil feedstock must mean a shift to biomass based feedstock.

Nevertheless, the main remaining question to be answered is what can substitute e.g. gasoline in all the internal combustion engine powered vehicles? Solvents, such as ethyl alcohol (ethanol: a simple alcohol found in beverages) and butyl alcohol (butanol), traditionally produced by means of fermentation processes in which microbial biocatalysts e.g. yeasts and bacteria convert simple sugars into alcohols, have properties similar to that of petroleum and can be used as alternatives (Wallner Scott and McConnell 2009; Szulczyk 2010). Fortunately, plants are the main and naturally

available abundant source of sugars and alcohol production (traditional wine and beer production from raw materials like fruits and grains by means of fermentation) is a technology evolved and with thousands of years accumulated knowledge (Mousdale 2008). Therefore, biomass as feedstock would offer another strong opportunity for the production of liquid fuels.

### **Ethanol as a transportation fuel**

Already back in 18<sup>th</sup> century ethanol was used as a fuel in combustion engines (Soni 2007). In 1908, Ford Model T (known as T-Ford) was demonstrated to run on either gasoline or pure alcohol (DiPardo 2000). However, by then, interest in using ethanol as a fuel was declined due to the available large quantities of low cost gasoline (DiPardo 2000). In 1970's, because of oil crisis, the focus was shifted to use of ethanol as alternative automobile fuel. For instance, in Brazil "Pró-Álcool" and in USA "Gasohol" programs were initiated and financed by the governments to phase out fossil derived automobile fuels (Berg 1999; Cortez *et al.* 2003). In 1980's, as a result of Pró-Álcool program, about 95% of new passenger cars in Brazil were powered by fuel ethanol (Cortez *et al.* 2003).

Nowadays, ethanol is used as an additive to gasoline to give the fuel clean burning and octane boosting properties (Walner Scott and McConnell 2009). In many countries it is blended with gasoline in various amounts for the use in flexible fuel vehicles (Goldemberg 2007). For instance, Sweden is the leading country in using highest number of E85 (fuel blend of 85% ethanol and 15% petrol) flexible-fuel vehicle in Europe and largest in ED95 (fuel blend of 95% ethanol and 5% ignition improver) bus fleet in the world (Bioethanol in Sweden 2015).

Most of the world's ethanol (>90%) is currently being produced from sugary or starchy feedstocks, sources often called as the "first generation" bioethanol raw materials (Mohr and Raman 2013). In 2013, world's top fuel ethanol producers were United States with 13.3 billion gallons (bg) and Brazil with 6.3 bg, together accounting for 84% of world production (RFA 2014). Major feedstocks used in US and in Brazil were corn and sugar cane, respectively. The advantages of first generation bioethanol is that the feedstocks has a high sugar content, conversion processes are fairly simple (Figure 2), and the technology is well established. Most of the Life Cycle Assessment (LCA) studies have shown a net reduction in GHG emissions when bioethanol is used to replace gasoline (Punter *et al.* 2004; Kim and Dale 2005; Dias de Oliveira *et al.* 2005; Farrell *et al.* 2006; Blottnitz von and Curran 2007).

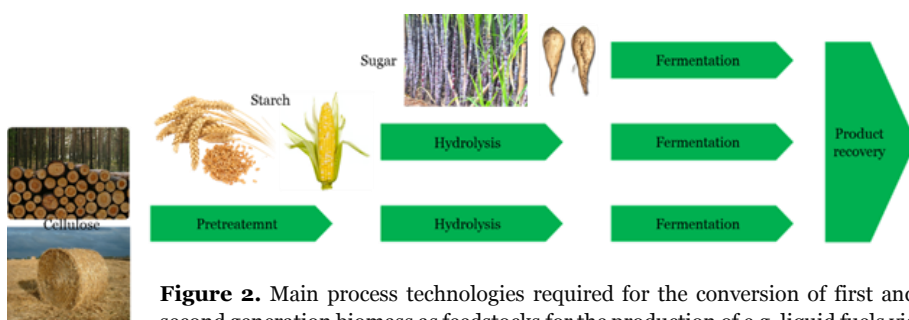
However, biomass conversion technologies and products indeed will have to compete with sophisticated existing and future petro refineries. This can be, to some extent, addressed by maximizing the value of biomass by

converting its major components into a variety of chemicals, materials, and energy. This integrated approach of biomass conversion corresponds to the biorefinery concept and is gaining increased attention around the world (Gravitis 2007; Clark and Deswarte 2008; Taylor G 2008). Similar to the oil-based refineries, biorefineries are expected to produce different industrial products (Cherubini 2010; Cheng and Wang 2013). For example, Novamont plant in Italy (feedstock: corn) <[www.novamont.com](http://www.novamont.com)>, Roquette site at Lestrem in France (feedstock: cereal grains) <[www.roquette.com](http://www.roquette.com)> produce a wide range of products, more than 600 carbohydrate derivatives. The aim of this bio-industry is to be competitive to oil refinery and lead to a progressive replacement of fossil derived products (Cherubini 2010). Nevertheless, due to large market demand of liquid fuels, biofuel generation will apparently be the backbone of bio-refineries.

Bio-refineries based on edible biomass as feedstock (e.g. sugar cane, sugar beet, corn, wheat, etc.) are identified as “1<sup>st</sup> generation” biorefineries and the fuels as 1<sup>st</sup> generation biofuels (Fernando *et al.* 2006; Clark and Deswarte 2008; Naik *et al.* 2010). As discussed earlier, food crops can indeed be used to produce energy, materials, and chemicals. Especially fuel ethanol is majorly produced from edible biomass. But, unfortunately, 1<sup>st</sup> generation biorefinery feedstocks are basically used for food and feed, which means that their utilization in this context would compete with food and feed industries for feedstock, fresh water, and fertile agricultural land (Rosillo-Calle 2012; Thompson *et al.* 2012; Zilberman *et al.* 2013). Therefore, the 1<sup>st</sup> generation biorefinery concept is of controversial and gives rise to ethical, environmental and political concerns (Thompson *et al.* 2012). In addition, feedstock is seasonal, availability is limited by soil fertility and the crop cultivation and conversion requires high energy input (Cherubini 2010). Thus, even though, plant based materials appear to be a gateway of moving from fossil resources, still, the use of edible biomass in a biorefinery concept would again raises some critical questions: can we produce and use enough plants while not compromising other essential needs? Do we have the technologies needed?

Recently, the scientific community began to recognize the opportunities offered by nonedible biomass like lignocellulose materials such as forestry wood, agriculture residues (e.g. corn stover, straw, sugar cane bagasse, and grasses), and municipal solid wastes (Kamm and Kamm 2004; Cheng and Zhu 2009). Similar to 1<sup>st</sup> generation biorefinery feedstocks, lignocellulose materials also contain high amount of sugars, but in the form of polysaccharides, and can be used in the context of biorefinery (Fernando *et al.* 2006; Menon and Rao 2012). These substrates are abundant, geographically widely distributed, inexpensive, do not compete with food, freshwater and fertile land (Cherubini 2010; Menon and Rao 2012). Therefore, the limitations of 1<sup>st</sup> generation biorefineries are expected to be overcome by utilizing

lignocellulose biomass as feedstocks and the concept of its conversion into valuable products is referred to as “2<sup>nd</sup> generation” biorefinery (Friedl 2011). However, not many commercial second generation biorefineries (dedicated to liquid fuels production) exist at present, but extensive development work is being carried out in some European countries. For example, Borregaard <www.borregaard.com> (Norway, feedstock: Nordic wood), The Icelandic Biomass Company (Iceland, feedstock: hay, lupine straw and barley straw), and SP Processum AB <www.processum.se> (Sweden, an integrated cluster company of different industries including Aditya Birla Domsjoe mill as the core operations; feedstock: forestry wood) are companies dedicated to converting lignocellulose materials into different chemicals, materials and energy in a biorefinery context. In fact, among other, the SP Processum AB cluster is a good example of industrial symbiosis – one industry uses the waste of another as a raw material (Clark and Deswarte 2008).



**Figure 2.** Main process technologies required for the conversion of first and second generation biomass as feedstocks for the production of e.g. liquid fuels via biochemical pathways.

Unfortunately, large scale industrial fuel production from lignocellulose biomass via state-of-the-art biochemical conversion technique is still long way from meeting the commercial requirements, technological advances and commercialization have not occurred as quickly as expected (National Research Council 2011). This is because of the feedstock’s perishable characteristics, known as biomass recalcitrance, see section: structural features of lignocellulose materials. Unlike sugary and starchy substrates, due to its recalcitrance, release of fermentable sugars has become a bottle-neck in the conversion of lignocellulose biomass. Therefore, to overcome the lignocellulose recalcitrance, an additional feedstock treatment is required.

Already, many process options have been investigated worldwide for the conversion of lignocellulose materials. Today the preferred one is to thermochemically pretreat the biomass material and, subsequently, enzymatically hydrolyze the pretreated material to fermentable sugars that can then be converted to e.g. fuel ethanol. A simplified flow diagram of the lignocellulose conversion process (e.g. ethanol production) and its comparison to the 1<sup>st</sup> generation feedstocks is given in figure 2.

Thermochemical treatment (pretreatment) alters the chemical composition and physical structure of lignocellulose substrates and facilitates the enzymatic hydrolysis of polysaccharides. These steps are the most expensive stages of lignocellulose conversion.

However, a major issue for lignocellulose as a raw material for the industrial product manufacture is variability (chemical composition of lignocelluloses greatly vary within and in between species) and the conversion yield. A well-functioning system therefore requires the pairing of appropriate feedstock and conversion technologies (Robbins *et al.* 2012). In an ideal case, use of lignocellulose that are less recalcitrant and has high growth rate is beneficial. Nevertheless, a wide range of native lignocelluloses are highly recalcitrant and require a harsh pretreatment. The drawback is that during harsh thermochemical treatment, along with sugars, a variety of undesired by-products are produced from the lignocellulose components (see section: By-products of lignocellulose pretreatment). These compounds have been reported to be highly inhibitory to the downstream enzymatic hydrolysis and microbial fermentation processes. Thus, along with underlying great opportunities, there are number of challenges to be overcome for the use of lignocelluloses in a biorefinery context. Even though, technological advances and the efficiency gives reasons for optimism, still efforts are needed to improve the process for better. Developing an environmentally friendly, low-energy consuming and a cost-effective infrastructure is essential. The use of clean technologies and application of green chemistry principles throughout the lignocellulose conversion process is required in order to minimize the environmental footprints of lignocellulose biorefinery products and to ensure its sustainability (Clark *et al.* 2006).

This thesis is aimed to address some of the critical problems associated with the biochemical conversion of lignocellulose biomass through the integration of green chemistry.

Specifically

- Identifying less recalcitrance biomass which requires no pretreatment or a mild pretreatment and can be readily hydrolyzed to fermentable sugars.
- Developing optimized lignocellulose pretreatment techniques with the aim of overcoming the lignocellulose recalcitrance, regardless type of biomass used.
- Establishing a battery of chemical detoxification methods to overcome the problems associated with the byproducts formed from the thermochemical pretreatment of lignocelluloses.

## Lignocellulose biomass: an abundant renewable resource

Worldwide annual production of lignocellulose biomass has been estimated to approach 10–50 billion dry tons (Sánchez and Cardona 2008; Zhao *et al.* 2012a), this being the most abundant available renewable carbon source. The major constituents of lignocellulose materials are cellulose, hemicelluloses and lignin (Harmsen *et al.* 2010; Gilbert 2010). These are the main processing projects of biorefinery for the production of fuels as well as a variety of commodity chemicals and material products. However, lignocellulose biomass include a variety of materials, but in this work they are actually referred to as woody substrates and agriculture residues.

In lignocellulose, both cellulose and hemicelluloses are carbohydrate sugar polymers while lignin is highly branched complex aromatic polymer (Alonso *et al.* 2012; Sorek *et al.* 2014). Different species of plants have significant differences in the proportions of the main compositions (Pereira *et al.* 2008; Menon and Rao 2012). Also, the ratios between these components vary in the same plant species depending on age, stage of growth, and other conditions (Pauly and Keegstra 2010). Table 1 summarizes the chemical composition of different lignocellulose species used in this study.

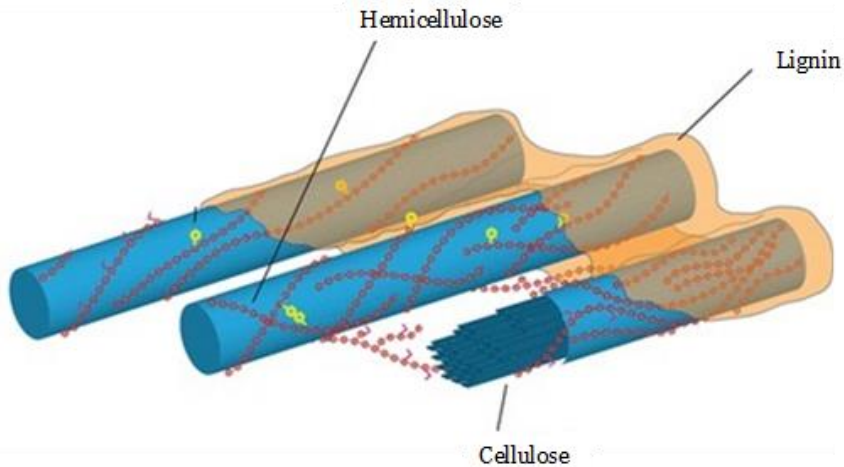
**Table 1** Chemical composition of the different lignocellulose materials

Lignocellulose	Component % (dry wt)							Source
	Arabinan	Galactan	Glucan	Xylan	Mannan	<sup>a</sup> Lignin	<sup>b</sup> Extractives	
Spruce	0.9	1.5	39.0	4.6	11.0	27.4	NA	PAPER I
Spruce	1.9	1.6	41.0	4.7	9.7	28.3	2.8	PAPER II
Pine	2.0	2.7	40.3	5.2	9.2	27.5	4.0	PAPER II
Birch	0.9	0.6	38.0	17.5	1.6	23.7	3.3	PAPER II
Aspen	0.4	0.5	45.6	16.4	1.4	20.2	NA	Wang <i>et al.</i> , 2012
Bagasse	1.6	0.5	32	18	0.2	25.1	NA	PAPER I
Reed canary grass	2.6	1.2	36.8	16.4	0.8	23.1	3.5	PAPER II
Pine bark	12.2	3.0	16.7	1.7	1.5	40.3	19.4	PAPER II

NA: not available; a: total lignin i.e. acid soluble and insoluble; b: ethanol extractives

Generally, cellulose is the dominant structural polysaccharide of lignocelluloses followed by hemicelluloses and lignin (Saha 2003). Woody biomass has a relatively high content of cellulose and lignin, whereas grasses have a higher content of hemicellulose and less lignin (Zhao *et al.* 2012a). Apart from these three major components, plant cell walls also contain a minor amounts of other substances such as pectins, proteins, extractives, several inorganic compounds, and ashes (Sjöström 1993), but they do not have significant impact in forming the lignocellulose structure (Raven *et al.* 1992). It is generally believed that the presence of lignin, hemicelluloses,

pectin, etc., and their spatial interlinks with cellulose have constructed rigid and compact structure (Figure 3) (physical barriers) of plant cell wall which is recalcitrant for the microbial degradation (Zhao *et al.* 2012a,b).



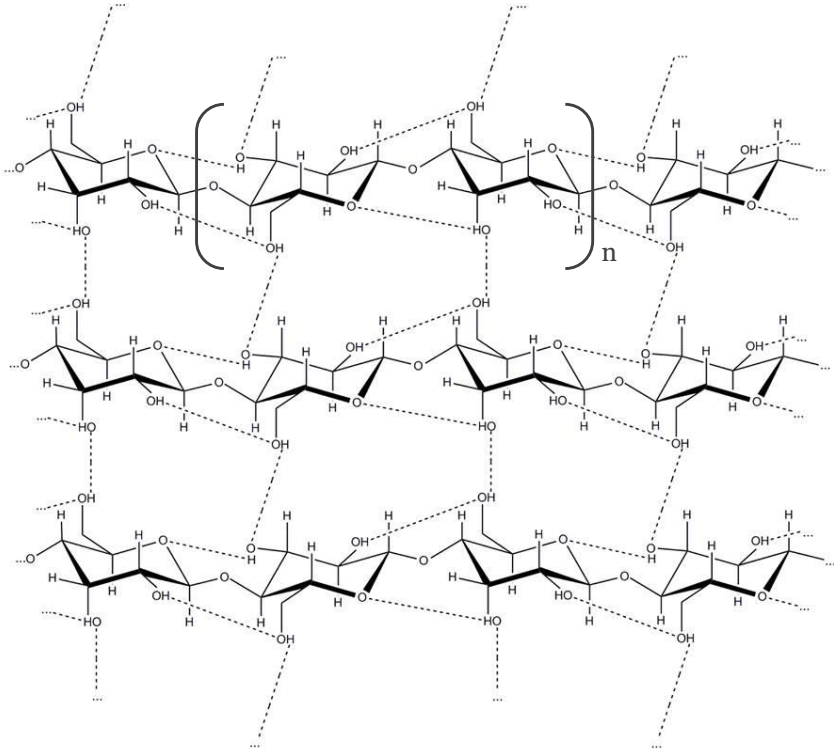
**Figure 3.** Arrangement of the major structural components in plant cell walls (Source: Sorek *et al.* 2014, reprinted with permission)

## Structural features of lignocellulose materials

### Cellulose

Cellulose, the major structural component of plant cell wall, is a high molecular weight linear condensation polysaccharide consisting of several repeated cellobiose (an oligomer of two anhydrous glucose units joined together with  $\beta$  (1 $\rightarrow$ 4) glycosidic bond) units (Figure 4; Klemm *et al.* 1998). It was previously expressed that the cellulose polymer(s)  $(C_6H_{10}O_5)_n$  typically contains between 100 and 20,000  $\beta$  (1 $\rightarrow$ 4) linked D-glucose molecules (Morohoshi 1991; Delmer and Amor 1995; O'Sullivan 1997; Zhang and Lynd 2004; Taherzadeh and Karimi 2007a,b; Mohnen *et al.* 2008), which represents its degree of polymerisation (DP). In lignocellulose, about 36 cellulose polymers are parallel linked together with hydrogen bonds and van der Waal's forces (Zhao *et al.* 2012a), forming the so called elementary fibrils which results in a crystalline structure with straight, stable supra-molecular fibers of great tensile strength and low accessibility (Percival Zhang *et al.* 2006). The elementary fibrils are again attached to other plant cell wall components i.e. hemicelluloses, pectin and covered with lignin (Figure 3). This compact form of cellulose bundles is referred to as cellulose microfibrils (Ha *et al.* 1998) and provide mechanical strength and chemical stability to the plants (Harmsen *et al.* 2010). Several of cellulose microfibrils are often associated together in the form of macrofibrils (Delmer and Amor 1995). Cellulose is generally insoluble in water and common organic solvents

(highly-ordered, crystalline, and less degradable), but it also has some soluble (amorphous, non-crystalline, easily degradable) regions in which the molecules are less-ordered (Zhang and Lynd 2004; Taherzadeh and Karimi 2008). However, many properties of cellulose typically depends on its DP (Harmsen *et al.* 2010).



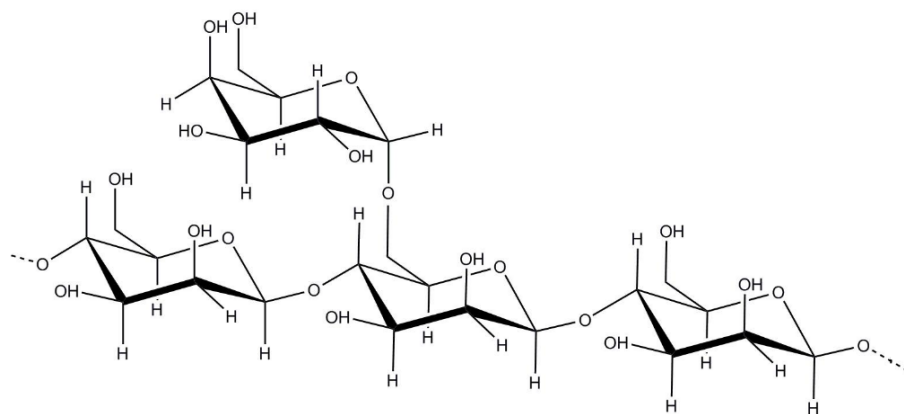
**Figure 4.** Structure of cellulose. In parenthesis, cellobiose, a disaccharide of two glucose residues linked through  $\beta$  (1 $\rightarrow$ 4) glycosidic bonds, the fundamental building blocks and the repeating structural unit of cellulose.

### Hemicellulose

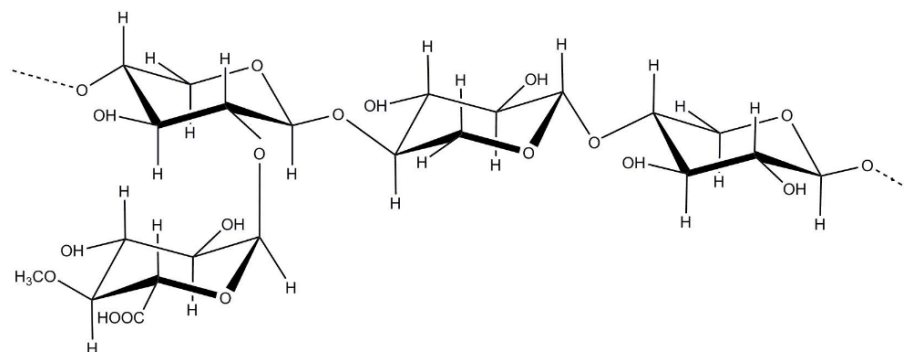
After cellulose, hemicellulose (a collective term of polysaccharides) is the second major carbohydrate constituent of lignocelluloses (Saha 2003). Hemicelluloses are a diverse group of short-chain linear and branched heterogeneous sugar polymers, typically made up of five different sugars – L-arabinose, D-galactose, D-glucose, D-mannose, and D-xylose (Zhao *et al.* 2012a; Figure 5). In these, L-arabinose and D-xylose are pentose ( $C_5$ ) sugars while the rest are hexose ( $C_6$ ) sugars. The most common type of sugar polymer of hemicellulose family is xylan. Further, hemicelluloses may also contain small amounts of other sugars such as  $\alpha$ -L-rhamnose and  $\alpha$ -L-fucose, organic acids such as acetic, 4-O-methyl glucuronic, galacturonic, and ferulic acid and

the acetyl groups can be partially substituted for the hydroxyl groups of sugars (Girio *et al.* 2010; Mussatto and Teixeira 2010; Martins *et al.* 2011; Xu *et al.* 2013).

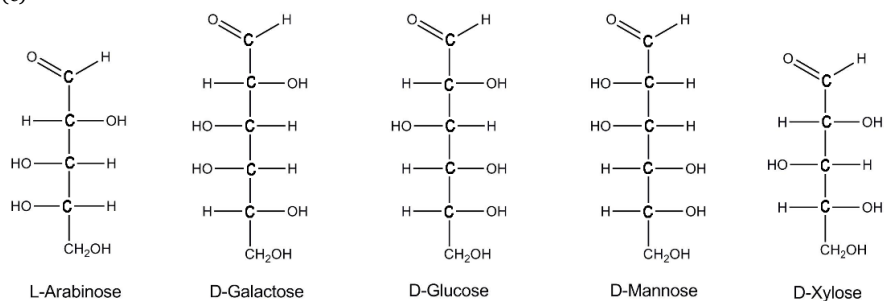
(a)



(b)



(c)



**Figure 5.** Structure of hemicelluloses a) Galactomannan b) Glucuronoxylan and c) sugar monosaccharides

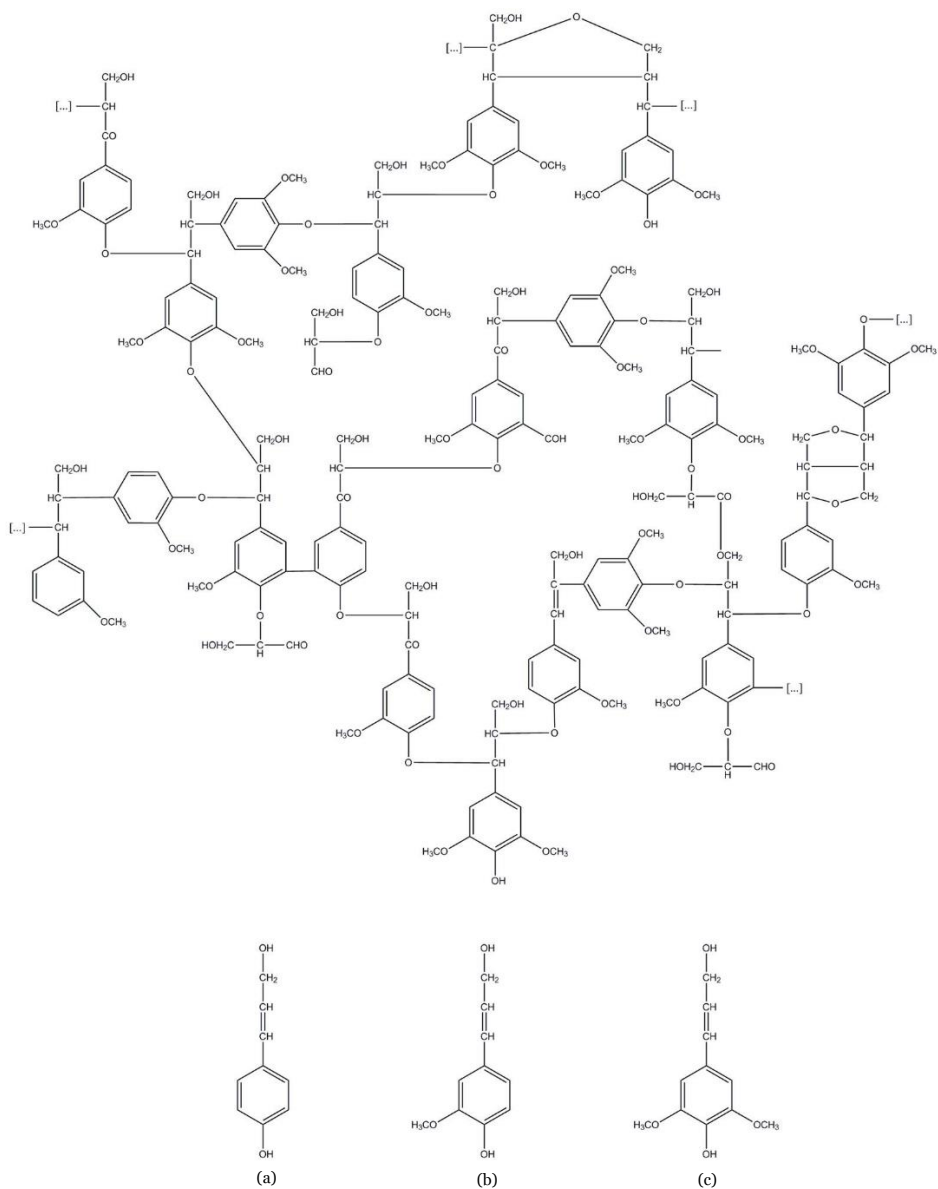
However, the backbone of hemicellulose is mainly composed of 1, 4-linked  $\beta$ -D-hexosyl residues and it may contain pentoses, hexoses, and/or uronic acids (Zhao *et al.* 2012a). Unlike cellulose, hemicellulose composition and structure (e.g. type of glycosidic linkages and side-chain composition) varies depending on their source (Scheller HV and Ulvskov 2010; Chundawat *et al.* 2011; Table 1). Hemicellulose(s) chain (-side) composition can be either homopolymer (consist of single sugar repeated unit e.g., xylans, mannans, and glucans) or heteropolymer (mixture of different sugars e.g. arabinogalactan, arabinoxylans, arabinoglucuronoxylans, galactoglucomannans, glucomannans, glucuronoxylans, and xyloglucans) (Girio *et al.* 2010; Figure 5). For instance, hemicellulose of hardwoods consist of O-acetyl-4-O-methylglucuronoxylans, in case of soft woods of O-acetylgalactoglucomannan and, in agricultural residues, the main hemicellulose is arabinoxylan (Peng *et al.* 2011; Table 1). Unlike cellulose, hemicelluloses lack crystallinity – to a large extent because of their short-chain branched structure in combination with the presence of acetyl groups attached to the polymer chains (Mussatto *et al.* 2010). Its DP consists between 70 and 200 thus being an amorphous polymer and easily degradable (Ragauskas *et al.* 2006; Harmsen *et al.* 2010; Zhao *et al.* 2012a).

## Lignin

After carbohydrates, lignin is another major component of lignocellulose biomass and it is, by far, nature's dominant source of aromatic polymer (Ragauskas *et al.* 2014; Calvo-Flores and Dobado 2010). Lignin is an amorphous and highly branched irregular complex polymer, predominantly constituting of three phenylpropane units as the major building blocks: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which give rise to a random sequence of p-hydroxyphenyl (H-lignin), guaicyl (G-lignin), and syringyl (S-lignin) subunits in the polymer, respectively (Ralph *et al.* 2004; Guo *et al.* 2014; Figure 6). The composition of H, G, and S-lignin's vary depending on source of lignocellulose (Guo *et al.* 2014). It has been identified that softwood lignins are mostly composed of G units with the remaining being H units, hardwood contain G and S monolignols with trace amounts of H units, and herbaceous plants contain significant amounts of all three G, S, and H lignins but in different ratios (Buranov and Mazza 2008; Chundawat *et al.* 2011).

In plant cells, lignin works as a strengthening agent: binding substance of cells, fibers, and vessels - gluing the cellulose fibers together and filling the space in between cellulose, hemicelluloses and other components of cell wall (Sticklen 2008). It would also affects the transport of water, nutrients and metabolites (Sorek *et al.* 2014). Thus, plays an important role in plant cell development, building the complex structure of lignocellulose, protecting the

plants from the pathogen and insect attacks (Mussatto and Teixeira 2010). Even though, some fungi and bacteria are capable of degrading lignin still it can persist for a long time (Bugg *et al.* 2011).



**Figure 6.** Schematic representation of lignin and the three major monolignol precursors a) p-coumaryl alcohol b) coniferyl alcohol and c) sinapyl alcohol.

## Other constituents

Lignocellulose cell wall also contain some other substances such as pectin – a component composed of acidic sugar (usually galacturonic acid) containing backbones with neutral sugar containing side chains (Scheller HV and Ulvskov 2010; Xiao *et al.* 2013), extractives (e.g. terpenoids, steroids, fats, waxes, and phenolic constituents) (Sjöström 1993), proteins, and ashes.

Pectins are highly branched and complex heterogeneous polysaccharides (Caffall and Mohnen 2009; Scheller and Ulvskov 2010) – composed of different subclasses: homogalacturonan, rhamnogalacturonan, and xylogalacturonan (Mohnen 2008). They functions in cell adhesion and wall hydration, and their crosslinking influences wall porosity and plant morphogenesis (Xiao *et al.* 2013).

The roles of extractives are diverse, some are involved in plant protection, some are precursors of certain chemicals, and for many the role has not been completely understood (Alriksson 2009; Rowell *et al.* 2012).

## Chemical interaction between cellulose, hemicellulose and lignin

As described earlier, in lignocellulose, cellulose acts as a core (skeleton) of the structure, hemicelluloses are arranged between the cellulose micro- and microfibrils, whereby both cellulose and hemicelluloses are embedded in lignin (Figure 3). Between these three components, mainly four interpolymer (in between different components) and intrapolymer (within individual components) linkages are identified: ether, ester, carbon-carbon, and hydrogen bonds (Faulon *et al.* 1994). A summary of the bonding position is given in table 2.

**Table 2** Overview of main linkages exist within and in between the three major components of lignocellulose (Harmsen *et al.* 2010).

Linkage	Intrapolymer	Interpolymer
Ether bond	Lignin, (hemi)cellulose	Cellulose-Lignin, Hemicellulose-lignin
Carbon-carbon bond	Lignin	NI
Hydrogen bond	Cellulose	Cellulose-hemicellulose Hemicellulose-Lignin Cellulose-Lignin
Ester bond	Hemicellulose	Hemicellulose-lignin

NI: not identified

Cellulose polymers are made up of two main linkages i.e. ether and hydrogen bonds. Glycosidic bonds of cellulose can be considered as ether

bonds since they connect two carbon atoms with an oxygen molecule (Solomon 1988). Hydrogen bonds form between two hydroxyl groups of different cellulose polymer chains. In fact, hydrogen bonds in cellulose fibers are considered to be responsible for various properties of native cellulose including its crystallinity (Poletto *et al.* 2014). Like in cellulose, the ether type of bonds are the main linkages in hemicelluloses. Instead, the hydrogen bonds are absent and significant amounts of ester (carboxyl) groups are present (Harmsen 2010). The intra-polymer linkages of lignin monomer units contain ether (70%) and carbon-carbon (30%) bonds (Harmsen 2010). They appear within or in between allylic and aryl carbon atoms (Adler 1977).

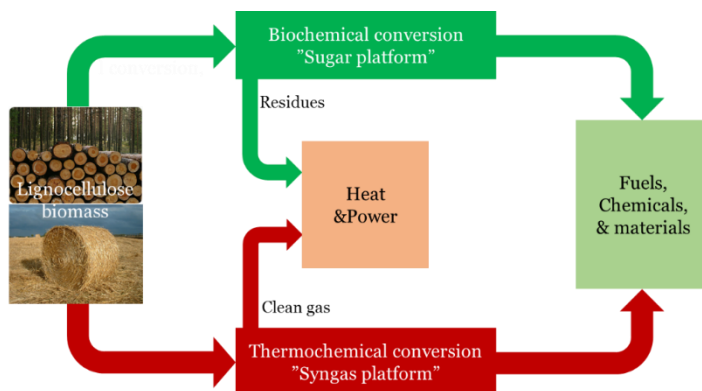
In inter-polymer linkages, hydrogen bonds were identified between cellulose, hemicellulose and lignin (Faulon *et al.* 1994). Lignin and polysaccharide complexes are primarily composed of ether and ester bridges (Ilyama *et al.* 1990) and lignin is connected to hemicellulose via ester bonds. Even though, ether groups are known to be present in between lignin and the polysaccharides, still it is not yet conclusively defined whether they exist between lignin and hemicellulose or lignin and cellulose (Harmsen 2010).

# Biomass conversion to biofuels

Technologies of biomass conversion in a biorefinery concept has majorly been described in two distinct pathways: thermochemical and biochemical processes (Demirbas 2007; Kamm *et al.* 2005; Menon and Rao 2012; Nanda *et al.* 2014; Figure 7).

Thermochemical conversion, also known as the syngas platform, involves controlled heating or oxidation of biomass (Demirbas 2004; Goyal *et al.* 2008; Tanger *et al.* 2013) into synthetic gas (syngas - intermediate product), which can be upgraded to valuable products. Biochemical conversion involves production of fermentable sugars and their conversion into liquid fuels (e.g. ethanol, butanol) or gaseous compounds (methane) by use of specific microbial population (Cuellar and Straathof 2014). This is known as the sugar platform since sugars are the key intermediates of the process.

Energy analysis calculations have shown that both thermochemical and biochemical technologies are competitive in their energy conversion efficiencies (Mousdale 2008; Foust *et al.* 2009). Also, it has been shown that the overall economics of these two processes are similar (Foust *et al.* 2009). Nevertheless, the comparative life cycle assessment suggest that the biochemical conversion would have better performance regarding greenhouse gas emissions and energy balance (Mu *et al.* 2010). However, there are inherent limitations in each of these processes and a careful pairing of technologies is required for an effective biomass conversion (Tanger *et al.* 2013). Indeed, there should be a role for both in advanced biofuel deployment. For fuel alcohols, biochemical conversion routes appear to be well suited, whereas for hydrocarbon fuels, the chosen production technologies tend to favour the thermochemical conversion routes (Foust *et al.* 2009).



**Figure 7.** Overview of biomass conversion pathways

## **Thermochemical**

Combustion, gasification, and pyrolysis are methods that are referred to as thermochemical conversion technologies of biomass, which can be used to produce heat, electricity, or gaseous or liquid intermediates for upgrading to liquid fuels or chemical (Figure 7; Butler *et al.* 2011; Wang *et al.* 2011; Brar *et al.* 2012; Bridgwater 2012; Solantausta *et al.* 2012; Tanger *et al.* 2013).

However, the compounds produced by thermochemical conversion of biomass and their relative amounts typically depend on process conditions i.e. temperature, pressure, feed rate, time of heating, particle size of biomass, and any quenching processes that are applied (Probestein and Hicks 2006). Before industry will proceed with their commercialization, thermochemical processes will have to overcome a number of technical and non-technical barriers such as feedstock moisture, high energy input, cleaning of intermediate products (e.g. syngas and bio-oil), relatively expensive catalytic conversions (e.g. Fischer Tropsch), ash content (inorganic ash materials have a very low melting point and they may solidify and attach to thermal conversion surfaces which would lead to slagging and fouling problems), particulates, char, and many other. Most importantly, due to severe thermal conditions, the efficient utilization of biomass major components would be in risk.

## **Biochemical**

Biochemical conversion is a major and an efficient pathway for the production of biomass derived fuels chemicals and materials (Figure 7). It mainly involves hydrolysis of lignocellulose polysaccharides (i.e. cellulose and hemicellulose) into simple sugars and their further conversion into e.g. fuel ethanol by fermentation organisms (Balat 2011). But, due to the compact rigid structure and recalcitrant nature to biodegradation - known as biomass recalcitrance, release of fermentable sugars has become paramount for industrialization of lignocelluloses as feedstock (Zhao *et al.* 2012a). Lignocellulose recalcitrance blocks decomposition of its structural carbohydrates from the microbial and animal kingdoms (Himmel *et al.* 2007). This property of woody biomass creates technical barriers to the cost effective transformation of lignocellulose biomass to sugars and then further to fuels.

In the past 80 years, several technologies have been developed with a clear objective of making the process cost-competitive to allow this conversion process to occur (Himmel *et al.* 2007). The major process steps required for biochemical conversion of lignocellulose biomass to the product of choice i.e. ethanol is presented in figure 2 – the focus of this thesis.

Unlike sugar extraction and ethanol production from 1<sup>st</sup> generation feedstocks, lignocelluloses as raw material would require three necessary process steps (Figure 2): pretreatment, hydrolysis of cellulose, and fermentation of lignocellulose derived sugars. Degradation of cellulose to glucose by means of enzymatic process is regarded as the most attractive way (Galbe and Zacchi 2007). However, due to structural characteristics of biomass, pretreatment is essential for enzyme catalysed cellulose conversion. Without a pretreatment, enzymatic hydrolysis of cellulose is ineffective as native cellulose is well protected by hemicellulose and lignin (Alvira *et al.* 2010; Yang *et al.* 2011; Zhao *et al.* 2012b). Therefore, after initial processing (e.g. size reduction) of lignocelluloses, fermentable sugars are usually produced in a coupled two step approach:

1. A pretreatment process is used to disrupt the physical and chemical barriers of lignocellulose and make the cellulose polymers more accessible for the enzymatic degradation. In this step, depending on method and the process conditions used, hydrolysis of hemicellulose as well as separation of lignin may occur.

2. Cellulose is hydrolysed using cellulase enzymes that are produced on site or acquired from an enzyme manufacturers. Even though glucose production and its conversion is the major route of biochemical pathway, still to facilitate an economically feasible process, it is important that all components of the raw material mainly hemicellulose and lignin should be efficiently utilized.

## **Pretreatment**

According to Zhao *et al.* (2012a) the factors affecting the accessibility of biomass cellulose can be divided into direct and indirect factors. The direct factors considered to be accessible surface area, and the indirect factors referred to biomass structure-relevant characteristic (pore size and volume, particle size, and specific surface area), chemical compositions (lignin, hemicelluloses, and other), and cellulose structure-relevant factors (cellulose crystallinity and degree of polymerization) (Zhao *et al.* 2012a). Pretreatment is actually the process, with an objective of removing the recalcitrant barriers of biomass by altering its indirect factors and improving direct factors thus enhancing the cellulose accessibility to enzymes that would degrade carbohydrate polymers into simple sugars (Mosier *et al.* 2005; García *et al.* 2011). Also, a partial or complete removal of hemicelluloses and/or lignin would help in improving the hydrolysis of cellulose.

In past several decades, a large number of pre-treatment techniques (some presented in Table 4 have been investigated and are comprehensively reviewed by Hsu (1996), Chandra *et al.* (2007), Mosier *et al.* (2005), Kilpeläinen *et al.* (2007), Galbe and Zacchi (2007), Carvalheiro *et al.* (2008),

Taherzadeh and Karimi (2008), Yang and Wyman (2008), Hendriks and Zeeman (2009), Kumar *et al.* (2009), Alvira *et al.* (2010), Harmsen *et al.* (2010), Mäki-Arvela *et al.* (2010), Pedersen and Meyer (2010), Brodeur *et al.* (2011), and Zhang B and Shahbazi (2011). In literature, lignocellulose pretreatment technologies are majorly classified into: physical & mechanical, chemical, physico-chemical, and biological. Some of the most commonly employed pretreatment techniques of lignocelluloses are described in following sections and their comprehensive comparison is presented in table 4.

Besides being considered as crucial, production of fermentable sugars based on pretreatment and enzymatic hydrolysis processes represents the main economic costs in lignocellulose bio-conversion (Gnansounou and Dauriat 2010; Klein-Marcuschamer *et al.* 2012). Therefore, pretreatment research has been mainly focused on developing methods that basically supports subsequent enzymatic hydrolysis which should result in higher sugar yields with lower enzyme dosage and shorter reaction times (Alvira *et al.* 2010).

### **Mechanical & Physical**

Among many, some treatments employed and categorised in mechanical & physical treatments include comminution, extrusion, torrefaction, and irradiation (microwaves, electron beam, ultrasound, and gamma ray). Feedstock size reduction by means of chipping, milling or grinding is often needed to make the material handling easier. If lignocellulose suspension is treated with enough energy (e.g. irradiation) the hydrogen bonds of substrate would break down which is expected to result in improving subsequent polysaccharides hydrolysis (Harmsen *et al.* 2010). The aim of these treatments include increasing the accessible surface area and pores size, decreasing cellulose crystallinity and DP, and densification of feedstock. If mechanical or physical treatments applied individually, the subsequent substrate enzymatic hydrolysis is inefficient and often not satisfactory (Agbor *et al.* 2011). Therefore, physical & mechanical treatments are used in the feedstock preparation prior to any chemical or physico-chemical pretreatment. Generally, energy required for these processes is relatively high and typically depends on the biomass characteristics and the final particle size required. Beyond a limit (e.g. size reduction via milling, grinding, and/or cutting) these methods become economically unfeasible (Hendriks and Zeeman 2009).

### **Chemical & Physico-chemical**

A variety of pretreatments are being developed using chemical reactions to disrupt the crystalline structure of lignocellulose and to partially or

completely hydrolyze lignin and/or (hemi) cellulose fractions. Hemicelluloses can be readily hydrolyzed e.g. under mild acidic or alkaline conditions, but cellulose is more resistant and requires a rigorous treatment. Pretreatments that involve chemical reactions can be classified as according to the following categories:

**Acid catalysed:** Pretreatment of biomass at low pH:

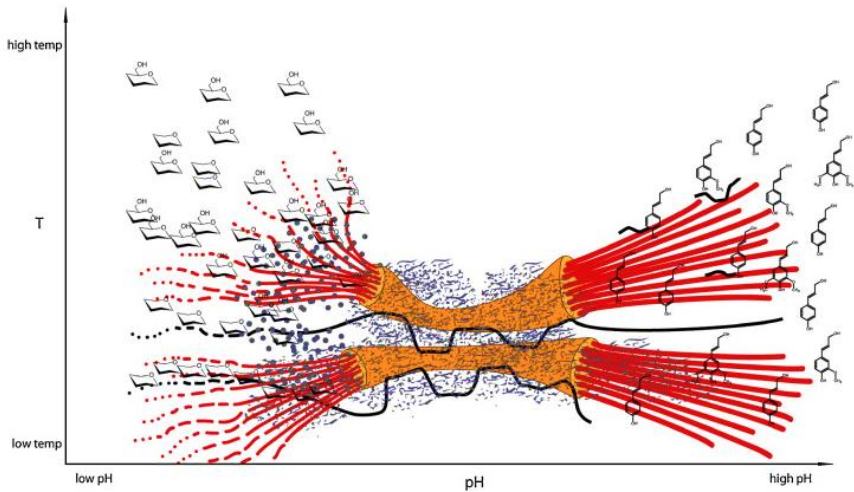
Acid pretreatment also referred to as acid hydrolysis is one of the most effective and traditionally used method to treat lignocellulose biomass. Inorganic (mineral) acids e.g. H<sub>2</sub>SO<sub>4</sub> (Shuai *et al.* 2010; Digman *et al.* 2010; Thomsen *et al.* 2009; Wyman *et al.* 2009), HCl (Wang *et al.* 2010), H<sub>3</sub>PO<sub>4</sub> (Marzialetti *et al.* 2008), and HNO<sub>3</sub> (Himmel *et al.* 1997) are generally used as catalysts, but, organic acids such as fumaric acid or maleic acid can be used as alternatives (Kootstra *et al.* 2009). However, most commonly used acid is H<sub>2</sub>SO<sub>4</sub>, which has been commercially used to pretreat a wide variety of biomass types and is a powerful agents for cellulose hydrolysis (Sun and Cheng 2002; Brodeur *et al.* 2011)

*Dilute acid hydrolysis:* Acid catalyst, 0.2-5 wt%, is mixed with the raw material and the suspension is treated under high pressure at 160-220°C for a short period of time up to few minutes. Hydrolysis of hemicelluloses and amorphous cellulose then occur, releasing monomer sugars and soluble oligomers into the hydrolysate and leaving most of the cellulose and lignin in the solid phase. Removal of hemicellulose leads to improved porosity and accessible surface area of cellulose. Due to the treatment, lignin is partially effected and depolymerised. The solubilized lignin may redeposit on cellulose fibers (Figure 8).

**Non-catalysed:** Pretreatment of biomass at neutral conditions:

Biomass pretreatment with e.g. steam (often referred to as steam explosion) and liquid hot water (LHW) at high temperature (160 – 260°C) and pressure, also referred to as hydrothermolysis, hydrothermal pretreatment, aqueous fractionation, solvolysis, autohydrolysis, or aquasolv, could remove most of the hemicelluloses (Sun and Cheng 2002; Mosier *et al.* 2005; Harmsen *et al.* 2010). As a result, cellulose in solid phase becomes more accessible to further degradation. It should be noted that the acids released from biomass components (e.g. acetic acid is released from O-acetyl groups in the polysaccharides) would lower the pH of suspension and influence the treatment efficiency (Chen *et al.* 2010). The advantage is that these treatments could reduce the need of chemicals and corrosion problems are limited (Petersen *et al.* 2009; Xiao *et al.* 2011). However, hemicelluloses being hydrolysed and dissolved in water usually do not result in complete conversion into monomer sugars. Therefore, hydrolysis (with acid or

enzymes) of liquid fraction is required to convert soluble oligosaccharides. Treatment of biomass at neutral conditions (e.g. steam explosion) are recognized as cost-effective processes for agricultural residues and hardwoods, but less efficient for softwoods (Clark and Mackie 1987). Performance of these treatments can be improved by using an acid catalyst such as H<sub>2</sub>SO<sub>4</sub> or SO<sub>2</sub> (Sun and Cheng 2002). But, similar to dilute acid treatments, due to severe treatment conditions (high temperature) sugar, lignin degradation products may form and lignin is partially depolymerised (Xiao *et al.* 2011).



**Figure 8.** Schematic representation of lignocellulose biomass affected by the pretreatment temperature and the pH. Orange and red tubes indicate cellulose fibrils and microfibrils, respectively; black curved lines illustrate hemicellulose; gray ‘veil and dots’ indicates lignin (source: Pedersen and Meyer 2010, reprinted with permission).

**Alkaline catalyzed:** Pretreatment of biomass at high pH:

Alkaline pretreatments of lignocellulose, soaking of the material in an alkali such as aqueous ammonia (Kim *et al.* 2009), calcium (lime) (Sierra *et al.* 2009), or sodium hydroxide (Wang *et al.* 2010) and then heating it for a certain time will increase the internal surface area due to swelling, decrease the DP and crystallinity, breaks the structural linkages between lignin and polysaccharides, and disrupts the lignin structure (Fan *et al.* 1987). The mechanism of alkaline treatments was reported to be the hydrolysis of intermolecular ester bonds (Sun and Cheng 2002), but they also remove acetyl and various uronic acid substitutions of hemicellulose (Chang and Holtzapple 2000) which makes carbohydrates more accessible. Alkaline treatments mainly involve delignification of the material, whereupon a major fraction of the lignin is solubilized. Some of the hemicelluloses are also

hydrolysed by the treatments, but a mostly recovered as oligomers. Thus, leaving the biomass enriched with cellulose, Chang and Holtzapple (2000) showed that biomass digestibility is enhanced with increasing lignin removal.

Alkaline treatments were reported to be effective especially for low lignin containing substrates such as hardwood and agriculture residues. For lignin rich softwood species a small or no effect was observed (Sun and Cheng 2002). But, a catalyst e.g. oxygen addition to the reaction mixture significantly improves the delignification of especially highly lignified materials such as soft woods (Chang and Holtzapple 2000). However, during the treatments with calcium or sodium hydroxide, salts are formed which need to be removed (González *et al.* 1986). Due to the mild conditions, degradation of sugars to by-products is limited and also prevents lignin condensation on cellulose (Figure 8).

***AFEX and ARP:*** Other forms of alkaline treatment techniques include ammonia fiber explosion (AFEX) and ammonia recycle percolation (ARP) processes. In AFEX, biomass is treated with liquid ammonia (typically 1-2 kg ammonia/kg dry biomass) at moderate temperature (<100°C) and high pressure (> 3 MPa) for about 10-60 min (Teymouri *et al.* 2005). After treatment, the pressure is quickly reduced. This reduces the lignin content, removes only a small amount of hemicellulose, de-crystallizes and increases digestibility of cellulose. The hemicelluloses degraded into oligomer sugars and are deacetylated, which is a probable reason for the hemicellulose not becoming soluble (Galbe and Zacchi 2007). In ARP, biomass is treated with aqueous ammonia (10-15 wt%) in a flow-through column reactor. The liquid flows through the biomass packed reactor column under pressure and elevated temperatures (150-170°C) (Kim *et al.* 2003; Kim and Lee 2005). After treatment, the hemi(cellulose) rich solids are separated from the liquid fraction and the ammonia, lignin and other dissolved sugars present in liquid fraction are recovered. Like the other alkaline treatments, both AFEX and ARP were also reported to be inefficient for high lignin containing materials. However, the cost of ammonia and its recovery drives the treatment feasibility (Harmsen *et al.* 2010).

## **Other**

**Organosolv processes:** Organic or aqueous-organic solvent mixtures such as alcohols (e.g. ethanol, methanol, acetone, and ethylene glycol) and aliphatic acids (e.g. acetic, formic, salicylic, and oxalic) with or without addition of an acid catalyst (catalyst include inorganic (e.g. HCl, H<sub>2</sub>SO<sub>4</sub>) or organic (HCOOH) acids) are used to break the linkages between lignin and carbohydrate polymers of lignocellulose (Sarkanen 1980; Chum *et al.* 1988; Aziz and Sarkanen 1989; Ligeró *et al.* 2007, 2009; Zhao *et al.* 2009; Soudham *et al.* 2011). These methods are reported to be effective for the separation of

lignocellulose main components, lignin is solubilized (extensively removed), hemicellulose hydrolysis occur and cellulose rich pulp is obtained, which leads to an improved accessibility of the cellulose fibers. The problems associated with cellulase enzymes absorption to lignin is minimized as lignin is being removed before hydrolysis step (Harmsen *et al.* 2010). In addition, lignin dissolved and recovered at a high quality might facilitate lignin applications to produce higher-value products.

However, the pulp must be thoroughly washed before hydrolysis, as the solvent may act as inhibitor to the enzymes and subsequent fermentation process. Also, solvent removal and recovery is necessary to make the process economically viable and to reduce the environmental impact (Sun and Cheng 2002; Harmsen *et al.* 2010).

**Oxidative processes:** Involves delignification and structural disruption of lignocellulose by treatment with an oxidizing agent such as hydrogen peroxide (Gould 1985; Azzam 1989; Andrade *et al.* 2014; Sua *et al.* 2014), ozone (Neely 1984; Silverstein *et al.* 2007; García-Cubero *et al.* 2009; Sugimoto *et al.* 2009), oxygen or air. Sometimes oxidation treatments are performed with the addition of an alkali catalyst (Mosier *et al.* 2005; Kumar *et al.* 2009). The mechanism is attributed to the high reactivity of oxidizing chemicals with the aromatic ring and its conversion into smaller molecules (Sun and Cheng 2002). Oxidants also break the side chain monomer units of lignin (Harmsen *et al.* 2010). Oxidation processes that perform with oxygen or air together with water at high temperature (150-350 °C) and pressure (5-20 MPa) (McGinnis *et al.* 1983; Jørgensen *et al.* 2007) are usually referred to as wet oxidation. However, during the treatments, lignin degradation products e.g. carboxylic acids are formed as well as hemicellulose degradation may occur.

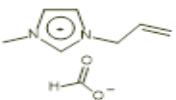
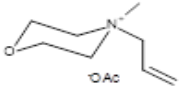
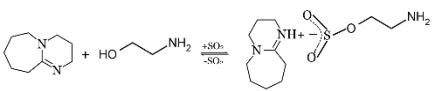
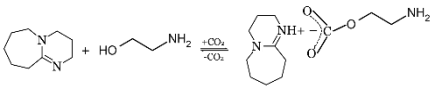
**CO<sub>2</sub> explosion:** Is similar to AFEX process. Biomass treatment with CO<sub>2</sub> at high temperatures and pressure and release of pressure by an explosive decompression disrupts the lignocellulose structure and consequently improves the cellulose accessible surface area (Hendriks and Zeeman 2009). Another way of using CO<sub>2</sub> is in its supercritical fluid form (SC-CO<sub>2</sub>), supercritical fluid is referred to a fluid that is in a gaseous form but at temperatures above critical it is compressed to a liquid like density (Alvira *et al.* 2010). It was believed that, in aqueous solution CO<sub>2</sub> forms carbonic acid which aids the hydrolysis of biomass (Puri and Mamers 1983; Brodeur *et al.* 2011). SC-CO<sub>2</sub> can remove lignin and its effect can be improved with the addition of a co-solvent such as ethanol. However, these treatments are considered to be not preferable for the lignocellulose materials since the sugar yields were low, efficiency is often not satisfactory, and are relatively expensive (Kim and Hong 2001). Nevertheless, CO<sub>2</sub> pretreatment is an

interesting approach because CO<sub>2</sub>, a GHG gas, will be utilized in the process – thus reducing its environmental footprint.

### Ionic liquids (ILs)

Another method for improving biomass digestibility is to use neoteric and more tractable emerging green solvents, ionic liquids (ILs). ILs are molten salts with a melting point near or below ambient temperature. They are exclusively composed of ions detained by coulombic forces (Tsuzuki *et al.* 2005; Binod *et al.* 2011; Fernandes *et al.* 2011). A vast variety of ILs are available, see e.g. reviews by Mäki-Arvela *et al.* (2010), Olivier-Bourbigou *et al.* (2010), Sun *et al.* (2011), Vancov *et al.* (2012), Liu *et al.* (2012); Costa Lopes *et al.* (2013), and Table 3 (ILs used in current study). However, they share a common feature which is that they usually consist of an organic cation and an organic or inorganic anion (Harmsen *et al.* 2010).

**Table 3** Ionic liquid solvents used in present study.

IL Name	Abbreviation & Structure	Reference
1-allyl-3-methylimidazolium formate	[Amim][HCO <sub>2</sub> ] 	PAPER I, II
N-allyl-N-methylmorpholinium acetate	[AMMorp][OAc] 	PAPER II
DBUH+ [MEA- Sulfonate] <sup>-</sup>	SO <sub>2</sub> DBU- MEA SIL 	PAPER II
DBUH+ [MEA- Carbonate] <sup>-</sup>	CO <sub>2</sub> DBU- MEA SIL 	PAPER II

ILs frequently exhibit very interesting properties such as chemical inertness, low volatility, good thermal stability, negligible vapour pressure, and solvation abilities etc. (Binod *et al.* 2011), which renders them important alternatives for the traditional lignocellulose treatment solvents (Marsh *et al.* 2002). ILs can function as selective solvents of lignin or (hemi) cellulose (Zavrel *et al.* 2009; Hossain and Aldous 2012; Casas *et al.* 2012; Isik *et al.* 2014; Glas *et al.* 2014). This property, separation and/or dissolution of lignocellulose components under ambient conditions, allows improved cellulose accessibility with no use of acid or alkaline solutions, thus avoiding

the formation of lignocellulose degradation products (Yang and Wyman 2008). Nevertheless, not all ILs has the ability to dissolve lignocellulose components and their efficiency can considerably vary (Pinkert *et al.* 2009). For instance, among the ILs used in current study, switchable ILs (SIL's) SO<sub>2</sub> DBU-MEASIL and CO<sub>2</sub> DBU-MEASIL are efficient in optimal fractionation and selective removal of lignin (Anugwom *et al.* 2014a, b). In addition, these solvents are capable of dissolving not only lignin but also pectin and hemicellulose the other constituents of lignocellulose (Anugwom *et al.* 2014a). Unlike SILs, conventional cellulose dissolving ILs (CIL's) [Amim][HCO<sub>2</sub>] and [AMMorp][OAc] do not remove lignin, but they disrupt the crystalline structure of lignocellulose.

In 1934, Charles Graenacher proposed a concept for dissolving cellulose in molten organic salts, N-alkyl or N-arylpiperidinium chlorides in the presence of nitrogen-containing bases (Graenacher 1934). However, by then, the invention had a little attention (Zhao *et al.* 2012; Binod *et al.* 2011). In the early 20<sup>th</sup> century Robin Rogers's research team at the University of Alabama used 1-butyl-3-methylimidazolium chloride IL for the dissolution of cellulose (Swatloski *et al.* 2002). Since then, ILs have attracted rising interest for the treatment of biomass. The feasibility of introducing different structural functionalities to the anionic or cationic part has made it possible to design a variety and new ILs (Marsh *et al.* 2002; Olivier-Bourbigou *et al.* 2010; Costa Lopes *et al.* 2013). The combination of anion and cation however profoundly affects the ILs physical and chemical properties such as melting points, viscosity, density, conductivity, polarity, hydrophobicity, and hydrolysis stability (Khupse and Kumar 2010). Therefore, ILs are tuneable for a more specific and targeted properties for certain applications. For instance, in table 3, the synthesized ILs [Amim][HCO<sub>2</sub>] and [AMMorp][OAc] were targeting cellulose dissolution, while SILs SO<sub>2</sub> DBU-MEASIL and CO<sub>2</sub> DBU-MEASIL were prepared to fibrillate lignocellulose and selectively remove lignin from lignocellulose biomass (Anugwom *et al.* 2014a,b). In fact, considerable efforts have been made to design enzyme-compatible ionic liquids that can dissolve carbohydrates (Zhao *et al.* 2008) – simultaneous pretreatment and saccharification of lignocellulose polysaccharides in a single pot. However, cellulose-dissolving capability is usually driven by the anion of an IL such as chloride, formate, acetate or alkyl phosphonate; these ions are capable of breaking the intermolecular hydrogen bonds within the cellulosic structures as they are strong hydrogen bond acceptors (Moultrop *et al.* 2005; Pinkert *et al.* 2009; Isik *et al.* 2014). Cations with cyclic structures such as the imidazolium-based ILs showed the best results (Kuhlmann *et al.* 2007; Isik *et al.* 2014)-suggesting that cations with a flatter molecular structure may support dissolution. Ionic liquids compete with lignocellulosic components for hydrogen bonding (Moultrop *et al.* 2005) and, as a result, the complex

network of non-covalent interactions between the biomass components cellulose, hemicellulose, and lignin is effectively disrupted (Yang and Wyman 2008; Lee *et al.* 2014).

Due to their polarity and unique properties, IL treatment of biomass is an emerging technique to improve the saccharification efficiency of recalcitrant lignocelluloses under mild treatment conditions than earlier described conventional pretreatment processes. However, despite their potential, IL treatment of lignocelluloses faces several challenges due to the lack of experience (Binod *et al.* 2011). One important challenge, out of several challenges is recovery and reuse of ILs (as cost of several ILs is still high; however, this is often a question of non-existing scaled up production). Also, recovery of dissolved compounds (lignin and hemicelluloses) from the ILs after cellulose has been extracted can be tedious. In addition, since lignocellulosic materials are heterogeneous, a large number of ILs need to be evaluated in order to find a suitable solvent for a particular substrate. Furthermore, presence of water may decrease the solubility of cellulose through competitive hydrogen bonding processes. Thus, it needs to be emphasized that the use of ILs as solvents for the pretreatment of lignocelluloses is still at an exploratory stage (Zhu 2008) and as of yet there are no industrial applications employing ILs in biomass processing (although in other applications).

The dissolved lignocellulose components (e.g. cellulose) can be recovered from the IL-biomass solution by the addition of anti-solvent like water, methanol, ethanol, or acetone (Weerachanchai *et al.* 2014); the dissolved cellulose is coagulated and can be regenerated by centrifugation or filtration. The mechanism is that the ions of the IL are extracted into the aqueous phase by the hydrogen bonding, dipolar and coulombic forces (Zavrel *et al.* 2009; Crosthwaite *et al.* 2005) - water molecules forms hydrodynamic network around the ions and disrupts the direct interaction of IL ions with e.g. cellulose (Costa Lopes *et al.* 2013) and the intra- and inter-molecular hydrogen bonds are rebuilt which results in cellulose precipitates (Zavrel *et al.* 2009). The residual IL in the regenerated substrate can be easily removed by washing with water since these types of ILs have a very high affinity to water. Also, the low volatile nature of ILs permits distillation of the volatile substances (Seddon 1996; Weerachanchai and Lee 2014), making IL recovery feasible (Binod *et al.* 2011; Vancov *et al.* 2012). Therefore, it is suggested that IL-based treatment of lignocellulosic biomass may offer a unique and environmentally friendly approach.

### **Biological**

Biological pretreatment involves the use of lignocellulose degrading microorganisms, mainly white, brown and soft rot-fungi, to alter the material

(Narayanaswamy *et al.* 2013; Zhang *et al.* 2007). These bio-catalysts are capable of degrading hemicellulose and lignin but leaves the cellulose intact, thus increasing the feedstock digestibility (Sánchez C 2009; Nanda *et al.* 2014). The major advantages of biological pretreatments are low energy requirement, mild operation conditions, avoids usage of hazardous chemicals, no damaging waste products are generated, and are considered to be environmentally friendly (Galbe and Zacchi 2007; Mousdale 2008). However, the pretreatment rates are generally very low and longer times (several days) are required (Cardona and Sanchez 2007; Sun and Cheng 2002; Tengerdy and Szakacs 2003). Also, careful control of microorganism growth conditions, space required to perform the treatments (a large amounts of space is required), and degradation of polysaccharides rendered these treatments commercially less attractive (Agbor *et al.* 2011).

In terms of efficiency, neither physical-mechanical nor biological processes are competitive with the chemical and physicochemical treatments of biomass. Based on solvents used pretreatment methods pose different characteristics, for instance, acid catalysts are used for hydrolysis of hemicellulose and partially remove lignin while alkaline and organic solvents are used to remove lignin and partially hydrolyse hemicelluloses. On the other hand, ILs can be used as potential alternatives to replace the conventions solvents with similar functionalities. Each method has its own characteristics and a large impact in the overall lignocellulose conversion process. The goal of these methods, however, remains leaving the pulp rich in cellulose fraction. Critical challenges in designing a pretreatment strategy include achieving a high degree of lignin removal without destroying the fermentable sugar content, increasing the degradability of cellulose and hemicellulose, and retaining the structural and chemical features of the lignin for further potential use (Lee *et al.* 2009).

**Table 4** Advantages and disadvantages of some lignocellulose pretreatment methods (Source: Kumar *et al.* 2009; Alvira *et al.* 2010; Brodeur *et al.* 2011; Nanda *et al.* 2014).

<b>Pretreatment method</b>	<b>Advantages</b>	<b>Disadvantages</b>
Mechanical comminution	Reduces cellulose crystallinity	Power consumption is usually high
Alkali	Efficient removal of lignin Low inhibitor formation	High cost of alkali Alters lignin structure
Acid (dilute)	Solubilizes hemicellulose Low acid consumption Short processing time Acid recovery is not required	High pressure and temperature are needed Cellulose hydrolysis is not effective Formation of inhibitors Equipment corrosion

AFEX	Highly effective for agricultural residues Cellulose becomes more accessible Reduce the lignin content Low formation of inhibitors	High cost of ammonia Ammonia recycling is needed Alters lignin structure Less effective for the lignin rich materials e.g. softwoods
ARP	Removes most of the lignin Cellulose rich pulps are obtained after pretreatment Most suitable to herbaceous	High energy costs and liquid loading Less satisfactory for softwoods
CO <sub>2</sub> explosion	Accessible surface area is increased Cost effective Does not cause formation of byproducts	Lignin or hemicelluloses are unaffected
Green solvents (ILs)	Lignin and hemicellulose hydrolysis Ability to dissolve high loadings of different type of lignocelluloses Mild processing conditions	Potentially high solvent costs Need for solvent recovery and recycle Unknown eco-toxicology of many formulations
LHW	Separation of nearly pure hemicellulose from rest of feedstock No need for catalyst Hydrolysis of hemicellulose	High energy/water input Solid mass left over will need to be dealt with (cellulose/lignin)
Ozonolysis	Reduces lignin content Does not produce byproducts	Large amount of ozone is required Expensive
Organosolv	Hydrolyzes lignin and hemicelluloses	Solvents recovery and recycle is needed Requires high energy High cost
SCF	Low degradation of sugars Cost effective Increases cellulose accessible area	High pressure requirements Lignin and hemicelluloses are unaffected
Steam	Cost effective Lignin transformation Solubilization of hemicelluloses	Hemicellulose degradation Acid catalyst is needed to improve the process performance Formation of byproducts
Biological	Degrades lignin and hemicelluloses Low energy requirement	Hydrolysis is very slow

AFEX: Ammonia Fiber Explosion; ARP: Ammonia Recycle Percolation; IL: Ionic liquid; LHW: Liquid hot water; SCF: Supercritical fluid

To assess the direct effect of a pretreatment on lignocellulose materials and to compare various pretreatment techniques, the severity factor (SF) is often used: it incorporates the time period used in the pretreatment performed at a certain temperature (see the equation below) (Pedersen and Meyer 2010).

$$SF = \log (t \cdot \exp((T - T_{ref})/14.75))$$

In the equation, “t” is the holding time of treatment in minutes, “T” is treatment temperature, “Tref” is reference temperature i.e. 100°C, and 14.75 is an empirically determined constant.

Other factors used to assess the efficacy of a pretreatment technique include: enzymatic digestibility of pre-treated solids, sugars degradation, generation inhibitors, cost effective, sugar concentration, fermentation compatibility of hydrolysates, lignin recovery, and heat and power requirements.

However, the major barriers in existing pretreatment techniques include insufficient separation of lignin and cellulose - which reduces the efficiency of subsequent cellulose hydrolysis, by-products formation, high use of energy and/or chemicals, and waste production (Harmsen *et al.* 2010). Also, since lignocellulose materials are diverse and have different physico-chemical characteristics, it is necessary to screen and adopt a suitable pretreatment technique to each raw material based on their properties.

## **Hydrolysis**

Hydrolysis of lignocellulose polysaccharides can comply with either a concentrated acid or enzyme catalysts, a comparative summary is given in Table 5. However, combination of dilute acid and enzymatic hydrolysis is the most preferred approach to achieve high sugar yields.

### **Concentrated acid**

Biomass is treated with mineral acids (e.g. H<sub>2</sub>SO<sub>4</sub>, HCl) at relatively low temperatures (T < 50°C) (Kumar *et al.* 2009) and a high acid concentration 30–70 wt% (Goldstein *et al.* 1983; Taherzadeh and Karimi 2007a). Hydrolysis of (hemi) cellulose then occur, releasing the sugars into hydrolysate and leaving mostly lignin in the solid phase. Sugar yield from concentrated acid hydrolysis is usually significantly higher compare to dilute acid hydrolysis. Also, when dilute acid is used a subsequent enzymatic hydrolysis is needed, while this is not required in case of concentrated acid hydrolysis. In addition, concentrated acid hydrolysis is flexible in terms of feedstock choice which means that it can be principally applied to any kind of biomass.

Both dilute and concentrated acid hydrolysis, however, offers good performance in terms of sugars recovery. But, there are several drawbacks, the released sugars would further degrade into by-products (furanaldehydes, organic acids) and lignin degradation products would also form. Concentrated acids are highly corrosive, toxic and hazardous, and require corrosion resistant equipment (Sun and Cheng 2002). In addition, the spent acid must

be recovered in order to make the process economically viable (Sivers and Zacchi 1995; Galbe and Zacchi 2002 ) and the hydrolysate pH neutralization requires a high amount of alkali (Agbor *et al.* 2011) which would result in formation of solid waste. Therefore, considering environmental impacts, high investment and maintenance costs have greatly limited the commercial interests of concentrated acid hydrolysis process (Wyman *et al.* 1996).

**Table 5.** A comparison of traditional acid hydrolysis and the current enzymatic hydrolysis.

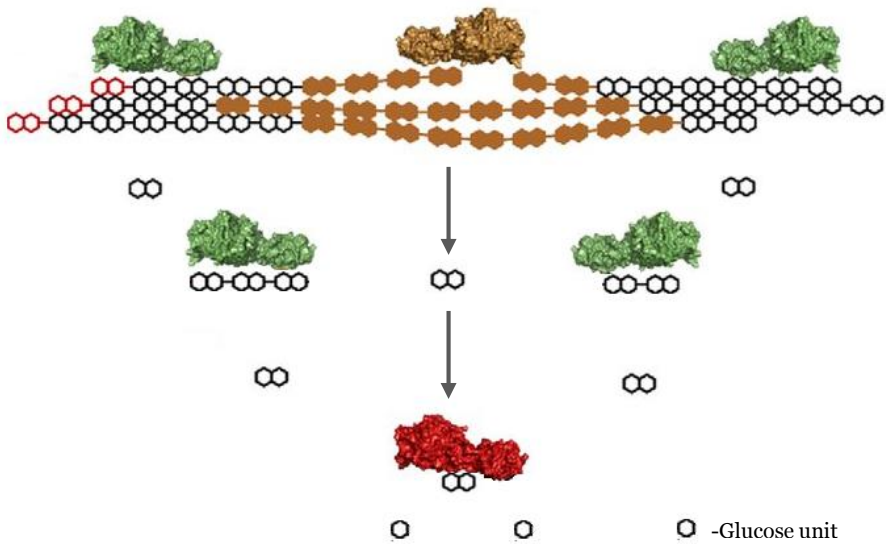
Hydrolysis process	Advantages	Disadvantages
Concentrated acid	Mild reaction conditions (T<50°C) High sugar yield Pretreatment is not required	Acid consumption is high Longer reaction time (2–6 h) Severe corrosion of equipment Acid recovery is needed
Enzymatic	Mild reaction conditions (~pH 5.5, T 50°C) High sugar yield No formation of inhibitors	Enzyme cost is high, at present Hydrolysis is usually performed for a long time, several days Pretreatment is required

## Enzymatic

Considering many advantages (Table 5), enzymatic hydrolysis is regarded as the most attractive way over concentrated acid hydrolysis. Due to the complex chemical structure of lignocellulose, multiple enzymes are often needed for the degradation of its carbohydrate polymers. For instance cellulose is hydrolysed by a mixture of cellulase enzymes and hemicellulose is hydrolysed by the action of different hemicellulases e.g. xylanases, mannanases, and other (Bhat 2000).

A widely accepted mechanism for the enzymatic hydrolysis of cellulose to glucose, a multi-step heterogeneous reaction divided into as primary hydrolysis and secondary hydrolysis, involves synergistic action of three distinct classes of enzymes: endoglucanase (EGs), exoglucanase also known as cellobiohydrolase (CBHs), and  $\beta$ -glucosidase (BGs) (Henrissat 1994; Knowles *et al.* 1987; Lynd *et al.* 2002; Teeri 1997; Wood and Garica-Campayo, 1990; Zhang and Lynd 2004; Percival Zhang *et al.* 2006; Binod *et al.* 2011; Yang *et al.* 2011). In primary hydrolysis, EGs breaks the low-crystallinity regions (randomly hydrolyses intramolecular  $\beta$ -1,4- glucosidic bonds) of cellulose fibre and forms new free chain-ends and CBHs further cleave the cellulose chains from free ends to release cellobiose units. This process takes place on the substrate solid surface and releases soluble sugars (DP up to 6) into the liquid phase (Binod *et al.* 2011). Secondary hydrolysis occurs in the liquid phase primarily involving the hydrolysis cellobiose units into glucose molecules by BGs (Percival Zhang *et al.* 2006; Binod *et al.* 2011). These

processes occur simultaneously (Woodward 1991) as shown in Figure 9. The synergetic endo-exo depolymerisation is the rate-limiting step for the whole cellulose hydrolysis process (Yang *et al.* 2011; Fox *et al.* 2012). During hydrolysis, the substrate characteristics vary, the combined actions of EGs and CBHs modify the cellulose surface characteristics over time (Eibinger *et al.* 2014), resulting in rapid changes in hydrolysis rates.



**Figure 9.** Enzymatic degradation of crystalline-ordered and amorphous-unordered (brown) cellulose by endoglucanase (EG brown), cellobiohydrolase (CBH green), and  $\beta$ -glucosidase (BG red). Cellulose reducing ends are shown in red. (Source: Eibinger *et al.* 2014, modified)

In addition, some proteins such as Swollenin (Saloheimo *et al.* 2002; Gourlay *et al.* 2013) have been identified as capable of non-hydrolytically loosening the packaging of cellulose fibril network, a process called amorphogenesis (Arantes and Saddler 2010). These proteins synergistically act together with the hydrolytic enzymes and increases the accessibility of individual cellulose chains to the cellulases. These helper proteins are therefore referred as amorphogenesis-inducing agents (Binod *et al.* 2011).

Cellulases distinguish themselves from most other classes of enzymes by being able to hydrolyze cellulose. According to the CAZy (Carbohydrate-Active enzymes) classification system cellulases are classified in glycosyl hydrolase families (Bhat 2000). Fungi such as *Aspergillus*, *Bacillus*, *Humicola*, *Phanerochaete* and *Trichoderma* are good source for producing cellulose catalytic enzymes (Sukumaran *et al.* 2005). Among all, *Trichoderma reesei* (*T. reesei*) is potent and widely used in the enzyme industry for producing a wide range of commercial enzymes including cellulases and

hemicellulases (Sheehan and Himmel 1999). *T. reesei* has also the advantage of being non-toxic and non-pathogenic which is important in large scale industrial processes (Nevalainen and Neethling 1998).

The main advantages of enzymatic hydrolysis include high sugar yield, only moderate temperatures are required for the reactions, does not create problems related to the equipment corrosion, and the formation of by-products is very low. But, the major bottleneck is that, at present, the enzymes cost is relatively high and the reaction rates are slow. In addition, there are also problems related to the enzyme - product inhibition.

However, there are several factors that are involved in influencing the enzymatic hydrolysis of cellulose. Specifically, type of pretreatment employed, modifications (chemical and structural) occurring in the feedstock during the pretreatment, particle size, amount and composition of lignin present in the pretreated biomass - lignin in the biomass critically affects the formation of soluble sugars during the enzymatic hydrolysis (cellulases tend to irreversibly bind to lignin through hydrophobic interactions that cause loss in enzyme activity), substrate concentration (a high concentration of substrate can hinder the mass transfer), type of enzymes employed, enzyme activity and loading, synergism, and hydrolysis conditions (pH, temperature and mixing) (Klyosov 1986; Gregg and Saddler 1996; Chang and Holtzapple 2000; Sun and Chen 2002; Jørgensen *et al.* 2007; Zhu *et al.* 2008; Kristensen *et al.* 2009; Alvira *et al.* 2010; Binod *et al.* 2011; Yang *et al.* 2011; Zhao *et al.* 2012b; Guo *et al.* 2014). Temperature has a profound effect on enzymatic conversion of lignocellulosic biomass, it has shown to influence cellulase adsorption - a positive relationship between adsorption and saccharification of cellulosic substrate was observed at temperatures below 60°C whereas the activities was decreased beyond 60°C, possibly because of the loss of enzyme configuration leading to denaturation of enzyme activity (Binod *et al.* 2011). Even though the individual impact of these factors on determining the efficiency of enzymatic hydrolysis has not been fully resolved, many of these factors are found to be interrelated during the saccharification process.

## **Fermentation**

The sugar rich lignocellulose hydrolysates, obtained from pretreatment and enzymatic hydrolysis can be fermented to ethanol using different types of microorganisms. The yeast *Saccharomyces cerevisiae* (*S. cerevisiae*, also known as Bakers' yeast) is the most frequently used and commercially dominant organism employed for this purpose (Badger 2002; Gray 2006). The others include *Zymomonas mobilis* (*Z. mobilis*) (Rogers *et al.* 1982), *Escherichia coli* (*E. coli*) (Alterthum and Ingram 1989) and thermophilic

bacteria such as *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum* (Ng *et al.* 1981; Lynd 1989).

The advantages of *S. cerevisiae* are that give high ethanol yields, exhibit relatively high ethanol and general inhibitors tolerance, as well as having GRAS (generally regarded as safe) status. A disadvantage is that they cannot utilize the abundant pentose sugars (Lin and Tanaka 2006; Nogue´ and Karhumaa 2014). This problem can be addressed by recombinant, genetically engineered, strains (Aristidou and Penttilä 2000; Jeffries 2006; Matsushika *et al.* 2009).

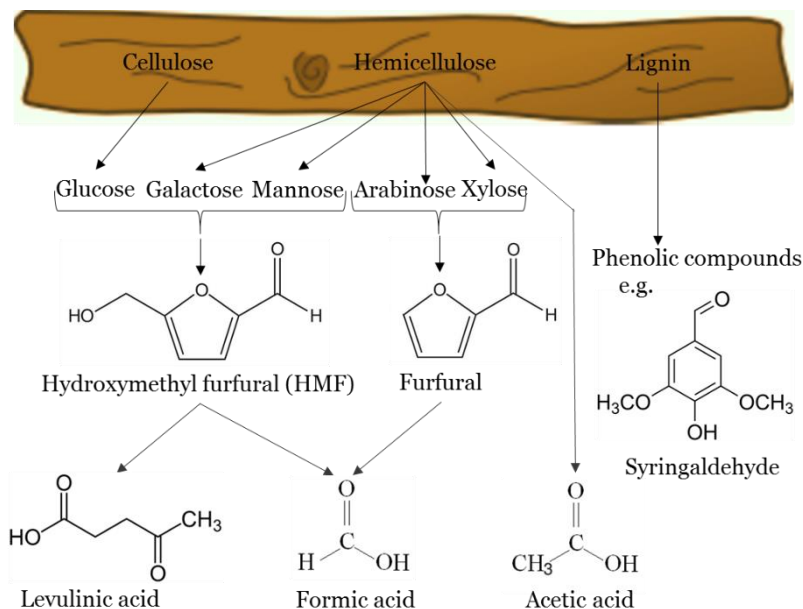
*Mode of fermentation:* Fermentations can be performed either in batch, fed-batch or continuous modes. In batch mode, a microorganism is inoculated to a specific volume of media and the fermentation is performed until the sugars are depleted. This is fairly simple, inexpensive, contain low risk of contamination and possibility to utilize the sugars efficiently. The disadvantages of batch cultivations is that when lignocellulose hydrolysates are used the cells are exposed directly to a high concentration of lignocellulose inhibitors (Nilsson *et al.* 2005). Also, they are labour intensive, time consuming (interception when fresh media should be added, cleaning, sterilisation, cell lag phase, cell growth and harvesting for each batch), and the overall productivity is low.

Fed-batch fermentations means that new medium is continuously added to the fermentation, and substrate concentration can be kept low. Furthermore, microorganisms sudden exposure to high concentrations of toxic lignocellulose hydrolysates can be avoided (Nilsson 1999). The disadvantage, in addition to those mentioned, is that the maximum working volume of the fermentation vessel is not utilized all the time.

Alternatively, in continuous mode, media is constantly added to the fermentation vessel and at the same rate the product (fermentation broth) is removed. Thus the fermentation volume is kept constant. In this way, unlike batch or fed-batch processes, ethanol is produced continuously. But, the main disadvantages are that the cells are constantly drained out from the reactor, contamination problems, and genetic instability. Continuous fermentations with flocculating yeast, or cell immobilization may help to improve the process. However, when configuring the fermentation process, several parameters must be considered: the ethanol yield and productivity should be high, and the equipment cost should be low (Palmqvist and Hahn-Hagerdal 2000a).

## Degradation products of lignocellulose pretreatment –inhibitors

Pretreatment of lignocellulose biomass may produce degradation products, mostly from sugars and lignin, such as furanldehydes, aliphatic acids and phenolic compounds (Figure 10). Presence of these compounds affects both saccharification and fermentation steps in the bio-conversion of lignocellulose.



**Figure 10.** Schematic representation of lignocellulose components degradation to inhibitory products (Source: Leif *et al.* 2013, modified).

### Sugar degradation products

Extensive degradation of (hemi)celluloses are responsible for the formation of furanldehydes, predominantly furfural and 5-Hydroxymethyl furfural (HMF) (Harmsen *et al.* 2010; Figure 10). Kinetic studies have shown that the biomass pretreatments (e.g. using acid catalyst) at high temperatures (>160°C) and a longer residence time have been reported to be significant for formation of furanldehydes (McKillip and Collin 2002) and their concentrations strongly increase with increasing temperature and reaction time. Pentose sugars dehydrate to furfural, while hexose sugars degrade to HMF (Saeman 1945).

## **Lignin degradation products**

Lignin degradation may result in formation of a variety aldehydic, aromatic, phenolic, and polyaromatic compounds. Among others, phenols are well known by-products of lignin, and may also form from sugars (Popoff and Theander 1976) and also some extractives are phenols (Rowell *et al.* 2005; Sjöström 1993). Phenolic compounds are however diverse (Clark and Mackie 1984) and a large number of different phenols e.g. vanillin, dihydroconiferyl alcohol, coniferyl aldehyde, vanillic acid, hydroquinone, catechol, acetoguaiacone, homovanillic acid, and 4-hydroxybenzoic acid are produced by the plants (Larsson *et al.* 1999b). The main factors influencing formation of phenols are pretreatment temperature, residence time, type of catalyst and catalyst concentrations.

## **Aliphatic acids**

Acetic, formic, and levulinic acids are the three common degradation products of lignocellulose polysaccharides. Acetic acid is formed from the acetyl groups, deacetylation, of hemicellulose. Formic acid is formed for the degradation of both furfural and HMF (Ulbricht *et al.* 1984), while levulinic acid is produced from the degradation product of HMF only (Dunlop 1948; Ulbricht *et al.* 1984).

Generation of pretreatment by-products however strongly dependents on type of raw material (due to the heterogeneity of biomass, plant cell walls vary in their ease of degradation), pretreatment method, conditions, and the catalyst. Thus, the composition of inhibitors in lignocellulosic hydrolysates will greatly vary. In further, lignocellulose hydrolysates are complex and contain other yet unidentified compounds which could also incorporate the inhibitory effect.

## **Inhibition by degradation products**

Phenolic compounds produced by plants were reported to be major inhibitors of cellulose hydrolysis, enzymatic hydrolysis of cellulose was inhibited in the presence of phenols at a concentrations  $\mu\text{M}$  to  $\text{mM}$  (Vohra *et al.* 1980; Martin and Akin 1988; Paul *et al.* 2003; Berlin *et al.* 2006; Pan 2008; Ximenes *et al.* 2010, 2011). In the presence of liquid fraction of thermochemically treated woody hydrolysates, enzymatic hydrolysis of cellulose was decreased by half due to the combination effect of inhibition, deactivation, and precipitation of the enzymes (Ximenes *et al.* 2010; Kim *et al.* 2011). Preincubation of cellulases together with vanillin and gallic, tannic, hydroxy-cinnamic, and 4-hydroxybenzoic acids caused 20–80% enzyme deactivation (Ximenes *et al.* 2011). Thus, phenols removal is expected to improve the enzyme activity and reduce the enzyme loading for a given level of cellulose conversion, which is

important for an economical viable cellulose conversion process (Kim *et al.* 2011).

Some phenols, importantly low molecular weight compounds, are highly inhibitory even at relatively low concentrations, while others require much higher concentrations to have an inhibitory effect (Ando *et al.* 1986; Larsson *et al.* 2000; Zhuang *et al.* 2009). They cause partition of fermentation organisms, loss of cell membrane integrity and reduces cell growth, sugar assimilation, and ethanol productivity (Larsson *et al.* 2000). Phenols will interfere with the cell membrane, which will influence cell function and change its protein-to-lipid ratio (Keweloh *et al.* 1990). In most cases, the mechanism of toxicity has not been elucidated. However, *S. cerevisiae* can convert some of the phenolic compounds to less inhibitory compounds e.g. during fermentation vanillin and coniferyl aldehyde are reduced to the corresponding alcohols, and coniferyl alcohol is converted to dihydroconiferyl alcohol (Larsson *et al.* 2000).

Furfural and HMF inhibits several glycolytic enzymes (Banerjee *et al.* 1981a; Modig *et al.* 2002), cell growth, respiration, and decrease the ethanol productivity of yeast (Chung and Lee 1984; Larsson *et al.* 1999a). However, under anaerobic conditions, yeast *S. cerevisiae* can convert furanaldehydes into alcohol (Diaz De Villegas *et al.* 1992; Taherzadeh *et al.* 1999a; Taherzadeh *et al.* 2000).

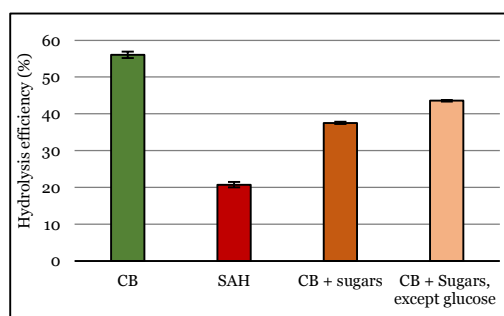
Larsson *et al.* (1999a) showed that concentrations of acetic acid, formic acid and levulinic acid over 100 mmol/L decrease the ethanol fermentation by *S. cerevisiae*. At low pH, acetic acid ( $pK_a=4.75$ ) is in its undissociated form and diffuses into the cells due to liposoluble properties (Pampulha and Loureiro-Dias 1989). In principle, moderate amounts of acetic acid may thus stimulate ethanol production, since stabilisation of pH requires input of ATP (adenine tri-phosphate, intracellular energy), which is produced by fermentation under anaerobic conditions (Taherzadeh *et al.* 1997). However, at high concentrations, acetic acid inhibits cell activity, which may lead to cell death (Verduyn *et al.* 1990).

Other compounds such as extractives (e.g. acidic resins, tanninic and terpene acids derives from lignocellulose), heavy metal ions (e.g. Cr, Cu, Fe, and Ni) and medium additive may also inhibits enzymes and fermenting organisms. But, these are less toxic (negligible) than that are discussed earlier. However, individual inhibitors may not have a strong effect, but in combination involving synergetic effect they can drastically hamper the hydrolysis and fermentation reactions (Mussatto and Roberto 2004; Chandel *et al.* 2012). However, as the composition of inhibitors differ from hydrolysate to hydrolysate their influence on enzymatic hydrolysis and fermentation performance will consequently vary.

In addition, as hydrolysis progresses, several other factors including end-product inhibition (Cellobiose inhibits cellulases (Mandels and reese 1963, 1965), BGs inhibited by glucose (Holtzapple et al.1990), and fermentation products (e.g. ethanol, butanol) inhibits to both hydrolytic enzymes (Qi *et al.* 2014)), low substrate reactivity, enzyme inactivation, and loss of enzyme/cells, will slow down hydrolysis rates (O'Dwyer *et al.* 2007) and may result in incomplete cellulose conversion. Likewise, fermentation reactions also suffer with product inhibition, cell loss, and other. These problems may be addressed, to some extent, by means of recovery of enzymes/fermentation organisms, immobilization to retain enzymes/cells in the reactor, and combined hydrolysis and fermentation steps to avoid product inhibition, which are greatly discussed in later sections.

However, the unavoidable problem is the inhibitory effect of lignocellulose hydrolysates on both hydrolysis and fermentation processes, which was elucidate as a part of current study, presented below.

Enzymatic hydrolysis efficiency of a model cellulosic substrate Avicel in pure citrate buffer media was 56%, while under same conditions replacing citrate buffer with spruce acid hydrolysate (SAH) media resulted in only 21% efficiency (Figure 11). Evidently, compounds present in acid hydrolysate were responsible for this inefficient, 63% lower, hydrolysis. It was evaluated that the monosaccharides present in SAH were responsible for 52% of the caused inhibition (only glucose only accounted 17% of it) while the rest 48% was due to the other compounds. Also, the incomplete conversion of cellulose even in pure citrate buffer was apparently due to the product inhibition.

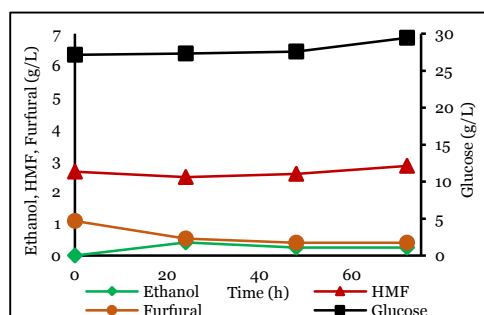


**Figure 11.** Efficiency of enzymatic hydrolysis (total glucose released as a percentage of total available) of Avicel cellulose (10 wt%) in either citrate buffer media or spruce acid hydrolysate (SAH) media. Hydrolysis in citrate buffer media was performed with or without addition of monosaccharides: arabinose, galactose, glucose, mannose, and xylose. The monosaccharide concentrations were equal to that of SAH. Hydrolysis experiments were performed with 2 wt%

enzyme load (1% each Celluclast 1.5L and Novozyme 188) and at 45°C for 96 h (Paper IV). SAH contained 22.7 g/l mannose, 18.4 g/l glucose, 11.3 g/l xylose, 5.6 g/l galactose, 3.6 g/l arabinose, 5.6 g/l acetic acid, 3.6 g/l 5-hydroxymethylfurfural (HMF), 2.1 g/l furfural, and 4.2 g/l phenols.

Qing *et al.* (2010) reported that the presence of xylose, xylan, and xylooligomers dramatically decreased cellulose conversion rates and yields. The inhibitory effect of xylooligomers (even at concentration of 1.67 mg/ml) higher than xylose or xylan (Qing *et al.* 2010). Presence of xylooligomers at a

concentration of 12.5 mg/ml severely inhibited enzymatic hydrolysis of Avicel, the initial hydrolysis rates and final glucose yields were inhibited by 82% and 38%, respectively (Qing *et al.* 2010). Supplementation of xylanases could reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose (Qing and Wyman 2011). Simple and oligomeric phenolics could also inhibit enzymatic cellulolysis by adsorbing onto cellulose and inactivating cellulases (Ximenes *et al.* 2011; Tejirian *et al.* 2011; Li *et al.* 2014). Lignocellulose derived aliphatic acids were also reported to be inhibitory to both cellulases and xylanases (Binod *et al.* 2011). However, the specific mechanism behind the decreased hydrolytic efficiency is complex and not fully studied.



**Figure 12.** Ethanol fermentation of spruce acid hydrolysate (SAH). Fermentations were performed with 1.6 g/L (cell dry wt) of *S. cerevisiae*, *Thermosacc* at 30°C (Paper III). SAH initially contained 30.1 g/l glucose, 5.3 g/l furan aldehydes (1.8 g/l furfural and 3.5 g/l HMF), 10.5 g/l aliphatic acids (6.1 g/l acetic, 3.5 g/l formic, 0.9 g/l levulinic), and 5.2 g/l total phenols.

In addition, ethanol fermentation of SAH by the yeast *S. cerevisiae* failed (Figure 12), apparently due to the toxicity of SAH. Glucose was essentially not consumed by the yeast. However, a fraction of furfural was utilized by *S. cerevisiae* and was probably converted into its respective alcohol.

As the inhibition problems of lignocellulose hydrolysates is apparent, in order to maximize enzymatic activity, ethanol productivity, and for an economically viable lignocellulose conversion process, removal of inhibitors is essential.

## Strategies to overcome inhibitory problems

Optimization of upstream processing i.e. use of pretreatment technique that do not result in formation of by-products or use of less severe pretreatment to minimise the by-product formation is one way to avoid the inhibitory problems of lignocellulose hydrolysates. However, with the current technology, a harsh pretreatment is required to overcome the obstacles associated with the biomass recalcitrance. Therefore, preventing the formation of inhibitors is difficult. Another approach is that separation of solid and liquid fractions of the pretreated material, proper wash of solids,

and hydrolysis of solids in an optimal environment would help to obtain high sugar yields. But, it would be economically expensive since more process units are required. In addition, fermentation of sugar rich pretreatment liquid hydrolysates would anyway need of addressing inhibitory problems. Hence, removal of inhibitory compounds present in lignocellulose hydrolysates is an important research area of research.

Over the years, different techniques categorised as physical, chemical, biological and in their combination were developed to lighten the toxic effect of lignocellulose hydrolysates. These include the use of chemical additives, microbial treatments, enzymatic treatments, heating and vaporization, liquid-liquid extraction, and liquid-solid extraction (Jönsson *et al.* 2013). However, many of these methods are effective only for a certain inhibitor or a class of inhibitors (Gracia *et al.* 2011). Also, some have been found to be more efficient than other, and some are more realistic in terms of industrial application (Cavka 2013). Among all, chemical detoxifications have been the most popular approaches to either remove or alter the structure of lignocellulose inhibitors (Jönsson *et al.* 2013).

### **Physical treatments**

Evaporation of acid willow hydrolysate (Palmqvist *et al.* 1996; Wilson *et al.* 1989), wood hydrolysates (Larsson *et al.* 1999), sugarcane bagasse hydrolysate (evaporated followed by activated charcoal treatment) (Rodrigues *et al.* 2001), and prehydrolysate corn stover (complex extraction with 30% trialkylamine-50% n-octanol-20% kerosene) (Zhu *et al.* 2011) were successfully demonstrated to eliminate volatile compounds (in variant extent) such as acetic acid, furfural and vanillin. However, the drawback is that this method is energy intensive and the non-volatile inhibitory compounds are unaffected – they remain in the hydrolysates (Chandel *et al.* 2011b).

Adsorptive techniques, using micro porous membranes (e.g. organic phase alamine 336) - have surface functional groups attached to their internal pores, were used for the removal of acetic acid from dilute sulphuric acid pretreated corn stover hydrolysate (Grzenia *et al.* 2008; Grzenia *et al.* 2010). In addition, formic and levulinic acids as well as HMF and furfural were also removed when Octanol and oetyl alcohol were used as organic phase solvents and Alamine 336 as the aliphatic amine extractant (Grzenia *et al.* 2010). However, these are complicated processes and might not be suitable in an industrial context.

### **Chemical detoxification of lignocellulose hydrolysates**

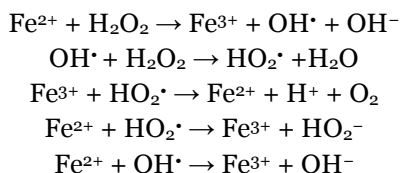
Chemical detoxification include a variety of techniques, discussed below.

## Treatment with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and ferrous sulphate (FeSO<sub>4</sub>)

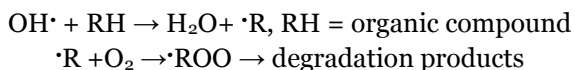
Recently, in our study, a novel method based on the use of FeSO<sub>4</sub> and/or H<sub>2</sub>O<sub>2</sub> has been explored for the detoxification of lignocellulose hydrolysates (Paper III).

Oxidative reactions that involve ferrous ions (Fe<sup>2+</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are generally referred to as Fenton reagents. These are environmentally acceptable and the most effective oxidation processes (OP's) commonly employed to remove organic pollutants of aqueous solutions (Amilcar *et al.* 2012; Zheng *et al.* 2013; Ebrahiem *et al.* 2013). In acidic environment, H<sub>2</sub>O<sub>2</sub> and Fe<sup>2+</sup> have very powerful oxidizing properties (Fenton 1894). The reaction of Fe<sup>2+</sup> with H<sub>2</sub>O<sub>2</sub> leads to the formation of highly reactive hydroxyl radicals (OH·) (Haber and Weiss 1934; Barbusinski 2009) and, consequently, this ion system is an important chemical source of OH·. These OH· are strong reagents that could oxidize and destroy a wide range of organic compounds including aliphatic, aromatic, and phenolics (Siedlecka and Stepnowski 2005; Amilcar *et al.* 2012; Benatti and Granhen 2012) as well as inorganic compounds, non-selectively (Yavuz *et al.* 2007). A suggested mechanism is that the OH· adds to the carbon double bonds and initiates a polymerisation reaction (Baxendale *et al.* 1946). Furthermore, Walling (1975) presented evidence of OH· involvement in the oxidation of various organic compounds.

The classic interpretation of Fenton reagent involves a simple redox reaction in which Fe<sup>2+</sup> is oxidized to Fe<sup>3+</sup> and H<sub>2</sub>O<sub>2</sub> is reduced to hydroxide ion, OH<sup>-</sup>, and hydroxyl radicals, OH·, (Amilcar *et al.* 2012), yielding only water and oxygen as by-products. The free radical mechanism proposed consists of the following reaction steps (Barb *et al.* 1951; Kremer *et al.* 1999).



The hydroxyl radicals then readily reacts with organic compounds and yields versatile oxidation products (Benatti and Granhen 2012), as shown in reactions below.



The detoxified hydrolysates were investigated in terms of glucose released by addition of hydrolytic enzymes and fermentability by the yeast *Saccharomyces cerevisiae*. Furthermore, a quantification of potentially inhibitory compounds was made from the detoxified and un-detoxified hydrolysates to evaluate the efficiency of the aforementioned treatments in this respect (Table 6).

Previously, it was observed that FeSO<sub>4</sub>, at lower concentrations, can enhance the pretreatment efficiency of cellulose-rich material (Zhao *et al.* 2011; Monavari *et al.* 2011), possibly by facilitating the interaction between cellulose and cellulase, thereby reducing overall enzyme requirement for cellulose hydrolysis.

### **Alkaline treatments:**

For a long time, alkaline detoxifications (treatments with the combination of high pH and temperature) have been considered to be promising methods to deal with the inhibitory problems of lignocellulose hydrolysates (Leonard and Hajny 1945; Van Zyl *et al.* 1998; Larsson *et al.* 1999b; Martinez *et al.* 2001; Persson *et al.* 2002; Chandel *et al.* 2007a).

Alkali such as NH<sub>4</sub>(OH), Ca(OH)<sub>2</sub>, and NaOH were demonstrated to degrade inhibitory compounds such as furfural, HMF, and phenols, but the degradation was typically depended on conditions (pH, temperature, and treatment time) used for the treatments (Table 6; Nilvebrant *et al.* 2003; Sárvári Horváth *et al.* 2005; Alriksson *et al.* 2005, 2006; Chandel *et al.* 2011a, b; 2009; Martinez *et al.* 2000; Ranatunga *et al.* 2000). In some cases, total phenol concentrations were higher in treated hydrolysates compared to the original ones (Sárvári Horváth *et al.* 2005; Alriksson *et al.* 2006). Compared to other, detoxification with Ca(OH)<sub>2</sub> (overliming) is considered to be one of the best methods and also an economical choice (Larsson *et al.* 1999b; Ranatunga *et al.* 2000; Martinez *et al.* 2001; Cantarella *et al.* 2004).

However, a major problem associated with the alkaline detoxification is that, along with inhibitors, sugars also degraded by the treatments - under harsh conditions the sugar loss was extensive (Martinez *et al.* 2000; Millati *et al.* 2002; Nilvebrant *et al.* 2003; Sárvári Horváth *et al.* 2005; Alriksson *et al.* 2005, 2006; Chandel *et al.* 2009, 2011a, 2011b) which could lead to a reduced end-product yield. Sugar degradation during the alkaline treatments e.g. by Ca(OH)<sub>2</sub> is due to stabilisation of enolate intermediates by the calcium ions (Jönsson *et al.* 2013). Another problem of overliming is that the addition of Ca(OH)<sub>2</sub> to the acid hydrolysates results in the formation of gypsum (CaSO<sub>4</sub>) (Alriksson *et al.* 2005; Martinez *et al.* 2001), but gypsum can be used as plaster of paris having many commercial applications (Chandel *et al.* 2011b).

Alternatively, NH<sub>4</sub>(OH) and NaOH can be used as they do not give rise to formation of gypsum (Alriksson *et al.* 2005, 2006).

**Table 6** The effect of different chemical detoxifications used for the removal of inhibitory compounds present in spruce acid hydrolysates.

Detoxification method	Conditions used	Effect on inhibitors and <sup>a</sup> sugars	Reference
H <sub>2</sub> O <sub>2</sub> /FeSO <sub>4</sub>	pH 3.8, 60°C, 2 h	Furfural, decrease: 50% HMF, decrease: 37% Phenols, decrease: 24% Formic acid, increase: 82%	PAPER III
Na <sub>2</sub> SO <sub>3</sub> Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub> C <sub>4</sub> H <sub>10</sub> O <sub>2</sub> S <sub>2</sub>	pH 5.5, 23°C, 10 min	No effect	Alriksson <i>et al.</i> 2006; PAPER IV
Ca(OH) <sub>2</sub>	pH 11, 30°C, 3 h	Furfural, decrease: 93% HMF, decrease: 92% Phenols, increase: 3% Sugars, decrease: 5% Formic acid, increase: 115%	Soudham <i>et al.</i> unpublished
NaOH	pH 9, 80°C, 3 h	Furfural, decrease: 71% HMF, decrease: 70% Phenols, increase: 10% Formic acid, increase: 85% Sugars, decrease: 19%	Soudham <i>et al.</i> unpublished
NH <sub>4</sub> OH	pH 9, 55°C, 3 h	Furfural, decrease: 17% HMF, decrease: 19% Formic acid, decrease: 8% Phenols, decrease: 13% Sugars, decrease: ~11%	Soudham <i>et al.</i> unpublished

<sup>a</sup>Total sugars

Sárvári Horváth *et al.* (2005) and Alriksson *et al.* (2006) investigated different conditions (combination of different pH and temperature) and proposed that the optimal conditions, based on removal of inhibitors, sugar degradation and product (ethanol) yield, for the detoxification of lignocellulose hydrolysates using NH<sub>4</sub>(OH), NaOH, and Ca(OH)<sub>2</sub> were pH 9 and 55°C, pH 9 and 80°C, and pH 11 and 30°C, respectively, and the treatment time was 3h for all of them. These treatments were later used (paper V) for the

detoxification of acid treated spruce hydrolysates to improve the enzymatic degradation of cellulose substrates.

Even though, alkaline detoxification is a well-established technique for the treatment of lignocellulose hydrolysates, still, its mechanism is not yet clearly explained. The effect of  $\text{Ca(OH)}_2$  treatment was proposed to be due to the precipitation of inhibitory compounds (Van Zyl *et al.* 1988). But, Persson *et al.* (2002) studied the chemistry of precipitants and the chemical composition of hydrolysates treated with not only  $\text{Ca(OH)}_2$  but also other alkali and reported that the positive effect was due to the chemical conversion of toxic substances rather than the precipitated compounds.

### **Use of reducing agents**

Addition of reducing agents such as sodium sulphite, sodium dithionite, dithiothreitol (DTT), and sodium borohydride is another technique used for the detoxification of lignocellulose hydrolysates (Paper IV; Alriksson *et al.* 2011; Cavka A and Jönsson 2013). These chemicals are commonly used in pulp and paper industry. Sodium sulfite has long been used in alkaline sulfite pulping (Thompson *et al.* 2013) and dithionite is used for bleaching purpose (Suess 2010). In aqueous environment and under the presence of oxygen, dithionite forms bisulfite and bisulfate molecules (Tao *et al.* 2008; Cavka 2013). In some pulp mills, sodium borohydride is used for the on-site production of sodium dithionite (Suess 2010). DTT is another reagent used to reduce the disulfide bonds of proteins – DTT prevents formation of intramolecular and intermolecular disulfide bonds between the cysteine residues of proteins.

Detoxification with reducing agents appeared to have no effect on degrading the inhibitory compounds of lignocellulose hydrolysates (Table 6; Paper IV). However, Cavka *et al.* (2011) studied the mechanism of reducing agents on lignocellulose hydrolysates and reported that the inhibitory compounds - more specifically phenols were sulfonated by the reducing agents, which made them unreactive (Cavka *et al.* 2011; Jönsson *et al.* 2013).

### **Other techniques**

**Liquid-solid extraction:** Activated charcoal was used to adsorb toxic compounds, phenolics, from the lignocellulose hydrolysates without removing any dissolved sugars (Mussatto and Roberto 2004; Chandel *et al.* 2007a; Canilha *et al.* 2008). However, its efficiency depends on several parameters such as the amount of activated charcoal, hydrolysate volume, pH, temperature, and contact time (Prakasham *et al.* 2009; Chandel *et al.* 2011).

Also, lignin, residues of lignocellulose processing, can be used for the same task (Björklund *et al.* 2002).

**Liquid-liquid extraction:** Extraction of lignocellulose hydrolysates with ethyl acetate (Wilson *et al.* 1989; Cruz *et al.* 1999) or diethyl ether (Cruz *et al.* 1999) could eliminate acetic acid, furfural, and phenolics from the wood hydrolysates. Pasha *et al.* (2007) detoxified lignocellulose acid hydrolysates with calcium hydroxide in combination with ethyl acetate, subsequently, upon fermentation of detoxified hydrolysates the glucose consumption rates were significantly increased - giving an improved overall ethanol production (Pasha *et al.* 2007).

**Use of surfactants:** Surfactants are amphiphilic compounds that contain a hydrophilic head and a hydrophobic tail. It has been shown that some surfactants (e.g. fatty acid esters of sorbitan polyethoxylates (tween80, tween20 (Wu and Ju 1998)) and polyethylene glycol (PEG) (Börjesson *et al.* 2007) have a positive effect on enzymatic hydrolysis. Addition of surfactants to the hydrolytic system especially to the lignin-containing substrates significantly improved the hydrolysis efficiency, allowed either a faster reaction rate or lower enzyme loading (Helle *et al.* 1993). The primary mechanism behind the increased hydrolysis efficiency is due to the hydrophobic interaction between lignin surfaces and surfactants rather effecting inhibitory compounds (Kristensen *et al.* 2007; Börjesson *et al.* 2007). In addition, surfactants may increase the enzyme stability and prevent enzyme denaturation, could disrupt the structure of substrate and promote available reaction sites, and/or could positively affect enzyme-substrate interactions (Kaar and Holtzapple 1998; Eriksson *et al.* 2002; Binod *et al.* 2011).

**Ion exchange resins:** Treatment of lignocellulose hydrolysates with ion (anion or cation) exchange resins, or a resin without charged groups (Nilvebrant *et al.* 2001), have been shown to remove lignin-derived inhibitors, aliphatic acid and furan-aldehydes (Larsson *et al.* 1999b; Nilvebrant *et al.* 2001; Sárvári Horváth *et al.* 2004; Villarreal *et al.* 2006; Chandel *et al.* 2007a), especially they are most efficient when the hydrolysate is adjusted to a high pH (10) (Nilvebrant *et al.* 2001; Wilson and Tekere 2009; Ranjan *et al.* 2011). Although, ion exchange resins effectively remove lignocellulose inhibitors there are still several drawbacks: ion exchange resins are not cost efficient (Lee *et al.* 1999), leads to loss of fermentable sugars (Villarreal *et al.* 2006), and pH (10) adjustment requires significant amount of alkaline solvents which reflects its limited feasibility in commercial industrial purposes.

## Biological detoxification

Several biological methods including treatment with enzymes such as laccases or use of the natural or targeted genetic engineered micro-organism are proposed to overcome the inhibitory effects of pre-treated lignocellulose materials.

Micro-organisms, such as some species of yeasts (Schneider 1996; Hou-Rui *et al.* 2009; Fonseca *et al.* 2011), fungi (Nichols *et al.* 2008; López *et al.* 2004), and bacteria (Okuda *et al.* 2008; López *et al.* 2004) can naturally detoxify inhibitory compounds, specifically furanladehydes, aliphatic acids, and aromatic compounds (Boopathy *et al.* 1993; Gutiérrez *et al.* 2002, 2006; Koenig and Andreesen 1989; Wang *et al.* 1994). However, the selectivity of degradation varies depending on microorganism used. *T. reesei* was shown to degrade furanladehydes, acetic acid, and benzoic acid derivatives of a willow hydrolysate (Palmqvist *et al.* 1997). *Desulfovibrio furfuralis* could use furfural, furfuryl alcohol and 2-furoic acid as carbon and energy sources (Folkerts *et al.* 1989) and degrades furfural to acetate, CO<sub>2</sub> and/or methane (Boopathy and Daniels 1991). *E. coli* strains was found to convert furfural into furfuryl alcohol (Gutiérrez *et al.* 2002; 2006). However, conversion rates of these organisms were low and treatments would require several days. During this period they also consumed sugars from hydrolysates, and e.g. *T. reesei* consumed 35% of fermentable sugars (Larsson *et al.* 1999b).

Other approaches such as use of large inoculum (Chung and Lee 1985), modification of strains through genetic engineering to be more tolerant towards inhibitors e.g. phenolics (Larsson *et al.* 2001a,b), furan aldehydes (Pettersson *et al.* 2006; Gorsich *et al.* 2006), and aliphatic acids (Hasunuma *et al.* 2011a,b)), strain adaption (Keller *et al.* 1998; Martín *et al.* 2007), cell immobilization (Inoles *et al.* 1983; Nagashima *et al.* 1984), and encapsulation (Talebnia and Taherzadeh 2006; Westman *et al.* 2012a,b) have been proposed to improve the fermentability of lignocellulose hydrolysates. These solutions are however less attractive and they do not address the inhibitory problems during enzymatic hydrolysis.

Enzymatic, laccases and peroxidases - particularly produced from basidiomycetous (white-rot fungi) treatment is another approach to detoxify lignocellulose hydrolysates. Advantages of using these enzymes is that the detoxification can be achieved faster than what possible by microbial cultures. Laccases are multi-copper enzymes capable of oxidizing phenols and aromatic amines (Couto and Toca-Herrera 2007; Blevé *et al.* 2008). Treatment of willow acid hydrolysate with laccase and lignin peroxidase (Jönsson *et al.* 1998), spruce acid hydrolysate with the phenoloxidase/laccase (Larsson *et al.* 1999b), and sugarcane bagasse acid hydrolysates with laccase (Chandel *et al.* 2007a; Martin *et al.* 2002) selectively removed phenolic monomers and

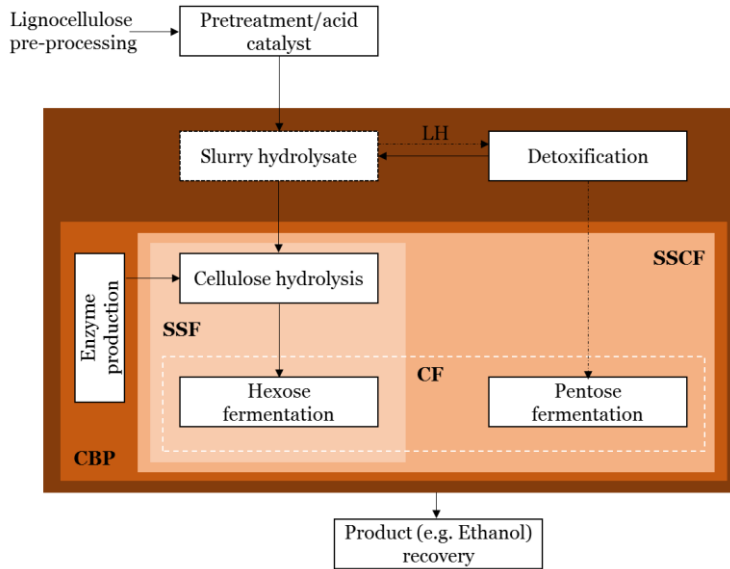
phenolic acids without affecting furans, acetic acid and aromatic compounds. The detoxifying mechanism was suggested to be oxidative polymerization of low-molecular weight phenolic compounds (Jönsson *et al.* 1998). However, the major disadvantage is that the production cost of these enzymes is high and commercial enzymes are still expensive (Parawira and Tekere 2011; Couto and TocaHerrera 2007).

So far, detoxification studies have had a major focus on improving fermentability of lignocellulose hydrolysates i.e. addressing the inhibitory effect on fermenting microorganism, while strategies to decrease the inhibition of enzymes have received relatively little attention (Jönsson *et al.* 2013).

## **Process configuration of enzymatic hydrolysis and fermentation**

Due to inhibitory compounds released during the pretreatment of lignocelluloses and the difficulties associated with the fermentation of pentose and hexose sugars, the overall lignocellulose biochemical conversion usually contain several solid–liquid separation, washing and other process units (Figure 13; Brethauer and studder 2014). Therefore, process complexity remains a major challenge and the simplification of process scheme by integration of as many unit operations as possible is necessary to reduce processing costs. Generally, there are four process configurations considered for the production of cellulosic ethanol: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (Hamelinck *et al.* 2005; Galbe and Zacchi 2007). In addition, these processes can be complemented with an integrated detoxification method (Figure 13).

SHF involves separation of cellulose-rich solids from the hemicellulose hydrolysate, solids washing, cellulose hydrolysis (cellulose is hydrolysed alone before fermentation), and separate fermentation of hydrolysates. The advantages of SHF processes is that both hydrolysis and fermentations can be performed at their optimal conditions: Optimum temperature for enzymatic hydrolysis ~50°C and for fermentation close to 30°C. However, the drawback is that the enzymatic hydrolysis suffers from end product inhibition. In addition, separate hydrolysis and fermentation means using separate process units.



**Figure 13.** Process configuration for the ethanol production from lignocellulosic biomass. Possibilities of process integration are shown inside the boxes: CF, co-fermentation; SSF, simultaneous saccharification and fermentation; SSCF, simultaneous saccharification and co-fermentation; CBP, consolidated bioprocessing. LH: liquid hydrolysate.

In SSF, cellulose saccharification and fermentation is performed simultaneously. SSCF is performed just as SSF, except in SSCF, fermentation of hemi (cellulose) hydrolysates performed in a single vessel. Hence, the risk of enzyme product inhibition can be avoided by SS(C)F since glucose generated from hydrolysis is immediately consumed by the organisms. Moreover, some of the compounds that are inhibitory to the enzymes are converted to less inhibitory compounds by the fermentation organisms (Almeida *et al.* 2007). Thus, SS(C)F is usually a more efficient alternative than SHF. The risk of SS(C)F is that the optimal conditions cannot be used and has to be a compromise, which means that neither hydrolysis nor fermentations are performed under their respective optimal conditions. In addition, the fermentation product i.e. ethanol is inhibitory to cellulolytic enzymes, but not to the same extent as glucose (Chen and Jin 2006; Holtzapple *et al.* 1990).

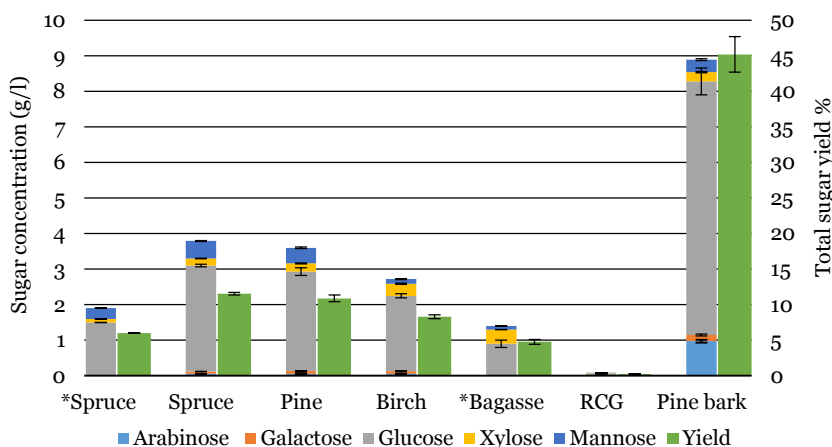
CBP involves simultaneous enzyme production, cellulose saccharification and fermentation of hemi (cellulose) hydrolysates in a single vessel using a genetically engineered superior biocatalyst that is capable of producing all the enzymes required for the hydrolysing lignocellulose polysaccharides and is also capable of fermenting resulting sugars (Lynd *et al.* 2005; Geddes *et al.* 2011). However, so far no commercially viable CBP organism has been reported (Brethauer and studder 2014).

# Evaluation of biomass recalcitrance

Recalcitrance, a major limitation for the bioconversion of biomass to fermentable sugars, was evaluated with respect to saccharification potential of different lignocelluloses. Untreated or acid treated substrates were enzymatically hydrolysed and substrate recalcitrance was assessed based on sugar release.

## Native lignocellulose materials

Figure 14 shows the enzymatic hydrolysis efficiency of different lignocelluloses (untreated) used in this study (Paper I, II). Sugar release from the hydrolysis was varied depending on type of biomass, enzyme preparation and the conditions used. When native spruce was hydrolysed with CTech2 enzyme preparation at 50°C and pH 5.8 the sugar release was improved 1.9 folds compared to results with the enzyme mix of Celluclast 1.5L and Novozyme 188 at 45°C and pH 4.8 (Figure 14), indicating the necessity of an efficient enzyme mix and optimum condition for the biomass hydrolysis.

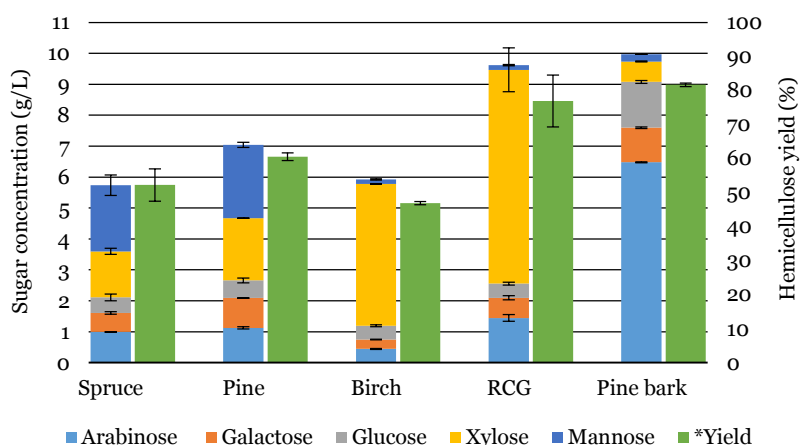


**Figure 14.** Sugar concentrations and the total sugar yields (g sugars released as a percentage of g total available) obtained from 48 h enzymatic hydrolysis of dried, milled native lignocellulose materials. Solids, 5 wt %, were hydrolysed in 50 mM citrate buffer with an enzyme load of 2 wt %. Experiments were performed either (\*) with the enzyme mix of Celluclast 1.5L and Novozyme 188 (1 wt% each) at 45°C, pH 4.8 (or) with CTech2 enzyme preparation at 50°C, pH 5.8. RCG: Reed canary grass; Source: Paper I, II.

The difference in sugar yields of three woody substrates was very small, i.e. it varied from 8–12%. The soft wood substrates i.e. spruce and pine appeared to have similar enzyme selectivity in terms of sugar release while the hard wood substrate birch had it slightly lower. Sugar production from the agriculture residues was lower compared to the other substrates. Only 5%

sugars were released from the bagasse, which was less than what was obtained from spruce wood (Figure 14). Surprisingly, even with highly efficient CTech2 enzyme preparation, no (< 0.1 g/l) sugars were released from the hydrolysis of untreated reed canary grass, showing it as a highly recalcitrance substrate. On the other hand, high amounts of sugars 8.9 g/l (45% total sugar yield) with a glucan to glucose conversion of 77% were obtained from the untreated pine bark, suggesting that pine bark is an amorphous substrate that can be readily hydrolysed to fermentable sugars. However, for most of the substrates ~80% of the released sugars contained only glucose (Figure 14), indicating selective enzymatic degradation of cellulose.

Another, common, way of evaluating lignocellulose recalcitrance is based on sugar release from a combination of pretreatment and enzymatic hydrolysis. Therefore, lignocelluloses were treated with dilute sulphuric acid (T 205°C, t 10 min; SF 4.1) and the resulting solid residues were subsequently hydrolysed using cellulolytic enzymes. A summary of sugars released from the acid treatment (pre-hydrolysis) of different lignocelluloses is given in figure 15. Sugars present in the acid hydrolysates (AHs) were, in order of abundance (Table 1), mainly hemicellulose sugars (85 – 95% of the total released sugars were composed of arabinose, galactose, xylose and mannose) reflecting their respective polymeric composition of lignocelluloses.



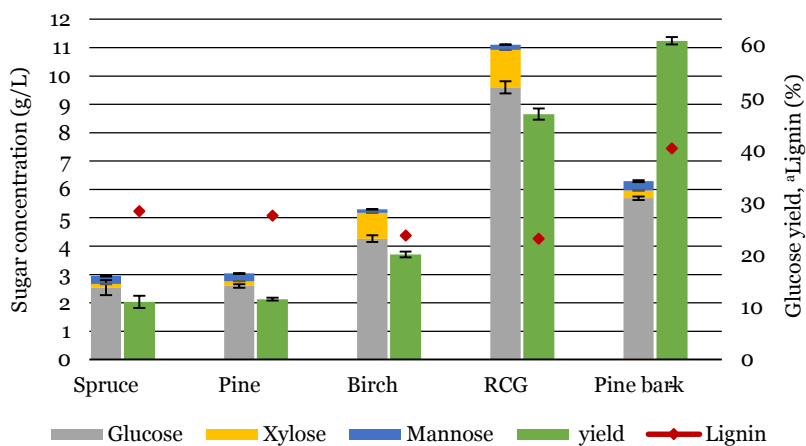
**Figure 15.** Sugar concentrations of different lignocellulose acid hydrolysates and their respective hemicellulose yields (g total hemicelluloses released as a percentage of g total available). Acid treatments were performed at 205°C for 10 min (SF 4.1) with 5 wt % solids and a H<sub>2</sub>SO<sub>4</sub> concentration of 1 wt %. \* Yields were calculated by excluding glucose as one of the hemicellulose sugars. RCG: Reed canary grass; Source: Paper II.

Acid hydrolysates of reed canary grass and birch wood contained high amount of xylose. While, arabinose was rich in pine bark hydrolysates. Soft

wood hydrolysates contained slightly higher concentrations of mannose than xylose and arabinose. There was also a small amount of other sugars present in the acid hydrolysates (Figure 15). Except for pine bark, glucan content of the substrates was essentially not affected by the acid treatment. For pine bark about 16% of glucose was released from the acid treatment, whereas only 2% was released for other substrates.

The main role of dilute acid pretreatment is to solubilize hemicelluloses and make the cellulose content more accessible to cellulolytic enzymes (Ungurean *et al.* 2011). Results of this study also confirm that the dilute acid pre-hydrolysis removes significant amounts of hemicelluloses; 82% of hemicelluloses were released from pine bark whereas 77% was released from reed canary grass. This, in turn, resulted in residues with higher glucan and lignin content. However, the applied acid treatment was slightly less efficient for woody substrates, only 52, 60 and 47% of hemicellulose was dissolved from spruce, pine and birch, respectively.

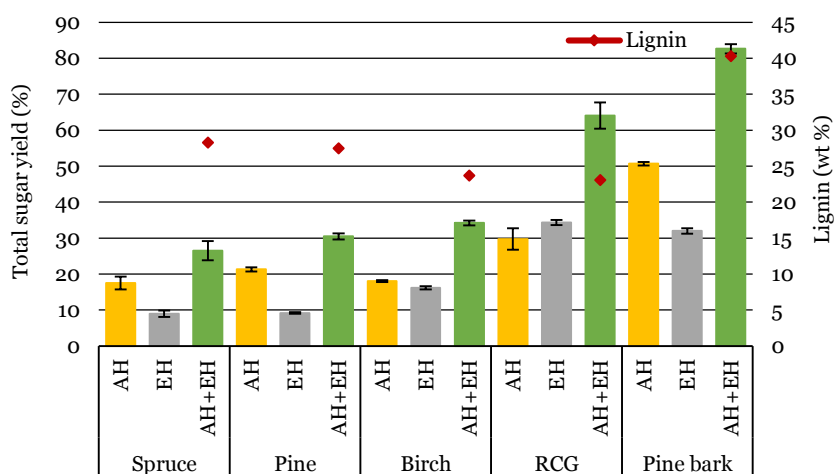
Sugars released from the enzymatic hydrolysis of acid treated substrates corresponded to 9, 9, 16, 34, and 32%, respectively, of the amount present in the starting material of spruce, pine, birch, reed canary grass and pine bark, respectively. The sugar composition of enzymatic hydrolysates contained 80–90 % glucose, a small amount of xylose and mannose, but no arabinose and galactose were present (Figure. 16).



**Figure 16.** Sugar concentrations and glucose yields (g glucose released as a percentage of g total available) obtained from 48 h enzymatic hydrolysis of acid treated (1 wt % H<sub>2</sub>SO<sub>4</sub>, 205°C for 10 min; SF 4.1) lignocellulose substrates. Hydrolysis reactions were performed at 50°C with an enzyme (CTech 2) load of 2 wt % and 50 mM citrate buffer (pH 5.8). a: lignin content (wt%) in the starting material, RCG: Reed canary grass; Source: Paper II.

Results from the enzymatic hydrolysis show that, compared to untreated substrates (Figure 14), dilute acid treated soft wood substrates (i.e. spruce and

pine, Figure 16), display no change or even lower efficiency in terms of sugar release. While the sugar production was improved 2 folds for birch wood. A high amount of sugars 11.1 g/l were released from the enzymatic hydrolysis of acid treated reed canary grass, and it should be noted that there was no sugars (<0.1 g/l) released from the enzymatic hydrolysis of untreated reed canary grass (Figure 14). Pine bark, which is non-recalcitrant, showed a lower sugar yield after enzymatic hydrolysis for acid treated material compared to its untreated. This is due to the fact that a high amount of sugars present in pine bark were removed by the acid treatment (Figure 15). However, the total sugar yield 83% obtained from the combined acid and enzymatic hydrolysis of pine bark was significantly higher than what was obtained from the untreated substrate (Figure 14, 16). However, the glucose yield 77% (concentration 7.1 g/l) from the enzymatic hydrolysis of untreated or from the combined acid and enzymatic hydrolysis of pine bark was equal. The only difference was the hemicellulose yield, 17% for untreated and 88% for combined acid and enzymatic treated pine bark.



**Figure 17.** Sugar yields (g sugars released as a percentage of g total available) from the combined acid treatment and enzymatic hydrolysis of lignocelluloses and their respective lignin content. AH: acid hydrolysate and EH: enzymatic hydrolysate. Acid treatments were performed at 205°C for 10 min (SF 4.1) with 5 wt % solids and a H<sub>2</sub>SO<sub>4</sub> concentration of 1 wt %. Enzymatic hydrolysis of acid pretreated substrates were performed at 50°C with an enzyme (CTech 2) load of 2 wt % and 50 mM citrate buffer (pH 5.8). RCG: Reed canary grass; Source: Paper II.

Similarly, total sugar yields from the combined acid and enzymatic hydrolysis of woody substrates were 26% for spruce, 30% for pine and 34% for birch (Figure 17). These values are higher than those obtained from the enzymatic hydrolysis of untreated substrates (Figure 14), indicating the advantage of employing a pretreatment prior to enzymatic hydrolysis. The effect of dilute acid treatment was especially significant for the reed canary

grass, which appeared as a highly recalcitrant material (Figure 17). About 64% of available sugars were released from the combined acid and enzymatic hydrolysis of reed canary grass.

Based on hydrolysis efficiency of acid treated substrates (Figure 16) and/or from the combined acid and enzymatic hydrolysis (Figure 17), it can be concluded that recalcitrance of lignocellulose substrates used in this study differ in the order of soft wood (spruce > pine) > hardwood (birch) > agriculture residues (reed canary grass) >> bark (pine bark).

Except for pine bark, there was a negative correlation between lignin content and the sugar (specifically glucose) release from the enzymatic hydrolysis of acid treated substrates. Low amounts of sugars were released from the substrates containing high lignin content. Apparently, acid treatment doesn't remove lignin thus leaving lignin rich pulp. Interestingly, even though the lignin content of pine bark was as high as 40 wt %, there was still a high amount of sugars released during enzymatic hydrolysis, regardless of whether it was untreated or acid treated. Apparently, the fluffy, porous structure of pine bark allows for unexpected efficient transport of enzymes and could, consequently, disintegrate it in a very thorough manner.

Even though, from the results of untreated substrates, reed canary grass appeared to be more recalcitrant than the other materials tested, after acid treatment, it was the second least recalcitrant material after pine bark. This may be due to reed canary grass contain less lignin than woody substrates. In addition, high amount of hemicelluloses were removed by the acid treatment, thus leaving cellulose more accessible to enzymatic hydrolysis. The accessibility of cellulose in plant cell walls has strongly influenced by the hemicellulose and lignin content (Chang *et al.* 2000; Studer *et al.* 2011). However, the improved release of hemicellulose sugars as a function of reduced lignin amount was not evident.

As demonstrated, woody materials are highly recalcitrant and will require sophisticated, probably harsh chemical treatment methods to separate their main components and facilitate hydrolysis.

Results suggesting that for untreated material, both lignin content and chemical composition might affect cellulose substrate-enzyme interactions during the enzymatic hydrolysis, whereas, for acid treated substrates, lignin composition is a major factor affecting the hydrolysis efficiency. However, the relationships between lignin content and saccharification of plant biomass is not well understood (Chen *et al.* 2007). This suggests that, beyond lignin, factors that influence on biomass recalcitrance to sugar release pointing to a critical need for deeper understanding of plant cell-wall structures.

## Transgenic Aspens

Several studies have proved that the genetic manipulation tools can be used to make the lignocelluloses less recalcitrant to both acid treatment and enzymatic digestion (Biswal *et al.* 2014; Lee *et al.* 2009; Chen *et al.* 2007). Chen *et al.* (2007) suggested that genetically modification or reduction of lignin can bypass the need for acid treatment and thereby facilitate bioprocess of biomass. Transgenic lines of down-regulated in lignin biosynthetic enzymes yielded nearly twice as much sugar from plant cell wall as wild-type plants (Chen *et al.* 2007). Also, large differences were observed in the enzymatic saccharification efficiencies, 67–79% for lines compare to 43% of wild type, of acid treated substrates (Chen *et al.* 2007).

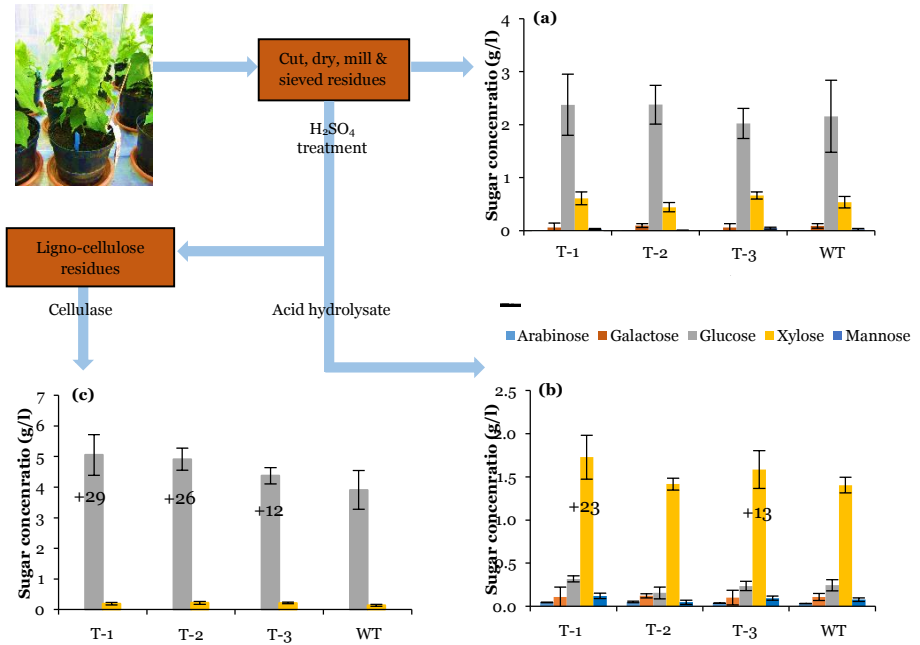
Therefore, as part of this study (collaborative work with the project BioImprove ([www.bioimprove.se](http://www.bioimprove.se)) - an initiative of Umeå Plant Science Centre (UPSC; [www.upsc.se](http://www.upsc.se))). About 210 individual transgenic aspens (representing a total of 14 constructs with 3 lines each and 5 plants for each line (selected by SweTree Technologies (STT; [www.swetree.com](http://www.swetree.com))), on the basis of growth characteristics height, width and lignin content) were evaluated for their saccharification potential.

Transgenic aspens were tested for total sugar release through enzymatic hydrolysis alone as well as through combined acid treatment (1 wt% H<sub>2</sub>SO<sub>4</sub>, 121°C and 40 min: SF 2.2) and enzymatic hydrolysis screening methods. The results were compared with that of selected wild type (WT) aspen plants. Total sugars released from the plants were varied among the constructs and the lines.

Figure 18 shows the saccharification potential of few selected transgenic (T1-3) and WT aspen plants. Without a pretreatment, no differences in the sugar production was observed from the enzymatic hydrolysis of transgenic and WT plants (Figure 18a). However, acid treatment resulted in the release of up to 23% more xylose from the transgenic plants than the wild type (Figure 18b). Also, 12-29% more glucose was released from the subsequent enzymatic hydrolysis of acid treated transgenic plants than the wild type (Figure 18a). Apparently, the increased glucose production after acid treatment (Figure 18C) is mainly attributed to improved cellulose digestibility of transgenic aspens. Overall, combined sugar release of pentoses (arabinose, xylose), hexoses (galactose, glucose, and mannose) and all monosaccharides after acid treatment and enzymatic saccharification were increased by up to 25%, 29% and 28%, respectively.

Along with acid treatment, a substrate delignification technique, acetosolv (Soudham *et al.* 2011; treatment of biomass 5 wt% with acetic acid 90 wt% and 0.2 wt% HCl at 121°C for 60 min: SF 2.4), was also used as a tool for

evaluating the recalcitrance of transgenic aspens. However, the pulp obtained after the acetosolv delignification showed poor enzymatic digestibility even compare to the untreated aspens (data not shown). This is probably due to the acetylation of cellulose during the acetic acid treatment (Zhao *et al.* 2012) and also lignin condensation on cellulose fibers.



**Figure 18.** Sugars released from the aspen plants subjected to (a) enzymatic hydrolysis without acid treatment (b) acid treatment and (c) enzymatic hydrolysis of the substrates that were first subjected to acid treatment. T (1-3) denote transgenic plants and WT are wild type plants. Acid pretreatments were performed with 2 wt % solids and 1 wt % H<sub>2</sub>SO<sub>4</sub> at 121°C for 40 min (SF 2.2). Enzymatic hydrolysis reactions were performed at 45°C with 50 mM citrate buffer (pH 4.8) and an enzyme load of 1 wt % (equal amount of Celluclast 1.5L and Novozyme 188 enzymes). Values are means of five biological replicates. Percentage differences are shown for transgenic plants significantly differing from wild type (WT) (post analysis-of-variance (ANOVA) *t*-test)  $P \leq 1\%$ .

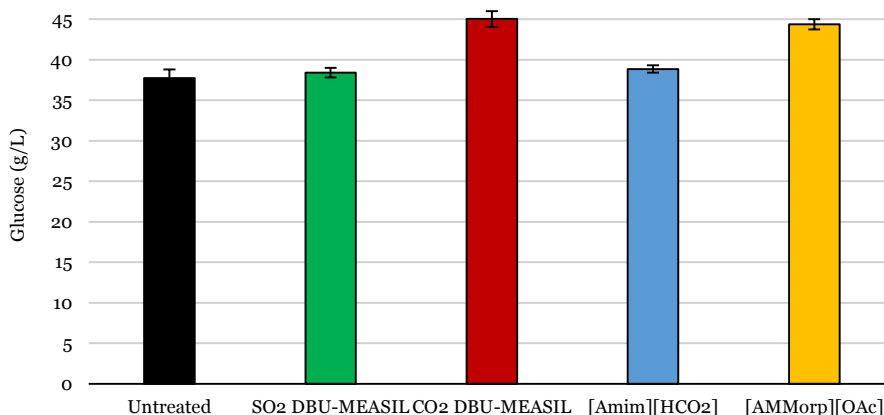
The study results confirms the advantage of using genetic manipulation tools to alter the lignocellulose recalcitrance, thus improving the accessibility of carbohydrate polysaccharides to the degrading enzymes. However, commercial utilisation of transgenic plants is far from reality. Also, considering recalcitrant wood materials, a chemical pretreatment might not be compatible with the positive effects of lignin modification on pretreatment efficiency and an increase enzymatic processing is apparently less obvious.

# Ionic liquid mediated pretreatment

Selective extraction of lignin under mild conditions and without chemical conversion, while reducing the crystallinity of the cellulose, represents an ideal strategy to achieve improved biomass bioconversion. As an alternative to the traditional chemical pretreatment techniques, a set of initially identified ILs (Table 3) which could hydrolyse high concentrations of lignin or dissolve cellulose and result in a substantial decrease in the cellulose crystallinity were used for the pretreatment of different lignocelluloses. The saccharification efficiency of IL treated substrates was then compared with the sulphuric acid treated and untreated substrates.

## Model cellulose substrate

In preliminary evaluation, a commercial microcrystalline cellulose Avicel was treated (at 120°C for 90 min, SF 2.5) with the mentioned four IL solvents and the regenerated substrates were subsequently enzymatically hydrolysed.

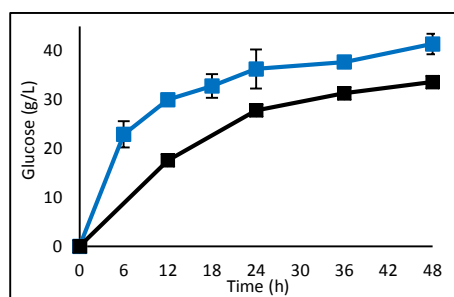


**Figure 19.** Glucose production from 48 h enzymatic hydrolysis of non-treated and IL treated Avicel cellulose. IL treatments of 5 wt% Avicel cellulose were performed at 120°C for 90 min (SF 2.5) and the enzymatic hydrolysis reactions were performed at 50°C with an enzyme (CTech 2) load of 2 wt % in 50 mM citrate buffer (pH 5.8). Source: Paper II.

After 48 h enzymatic hydrolysis, the glucose production was 45.0 ( $\pm 1.0$ ) and 44.4 ( $\pm 0.6$ ) g/l for the substrates treated with CO<sub>2</sub> DBU MEASIL and [AMMorp][OAc], respectively (Figure 19). Whereas the glucose production from the untreated control was 37.7 ( $\pm 1.1$ ) g/L. No significant differences in terms of glucose production was observed between untreated and the Avicel treated with either SO<sub>2</sub> DBU MEASIL (38.4  $\pm 0.58$  g/L) or [Amim][HCO<sub>2</sub>] (38.9  $\pm 0.5$  g/L). Also, the glucose production rates (GPR, from the first 4 h enzymatic hydrolysis) were higher in case of ILs CO<sub>2</sub> DBU MEASIL and

[AMMorp][OAc] compared to that of untreated Avicel ( $5.2$  vs  $3.6$   $\text{g}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ). Evidently, this indicates that the treatment with both  $-\text{CO}_2$  DBU MEASIL and [AMMorp][OAc] ILs did influence the cellulose crystallinity and improved hydrolysis efficiency. Treating Avicel cellulose alone with  $-\text{SO}_2$  DBU MEASIL appear to have no effect at all.

On the other hand, in as separate study, treatment with [Amim][HCO<sub>2</sub>] resulted in significantly improved glucose levels and GPR upon longer processing times (48 h at  $45^\circ\text{C}$ , SF 1.8) (Figure 20). About  $41.1\pm 2.1$  g/L of glucose was produced from the [Amim][HCO<sub>2</sub>] treated Avicel, while the glucose produced from untreated or water treated (at  $45^\circ\text{C}$  for 48 h) Avicel was only  $33.6\pm 0.7$  g/L. However, the glucose from the untreated sample could be enhanced to 42 g/L, after 120 h hydrolysis with an additional enzyme load.



**Figure 20.** Glucose production from 48 h enzymatic hydrolysis of untreated and [Amim][HCO<sub>2</sub>] treated Avicel cellulose. Avicel (5 wt%) was treated with [Amim][HCO<sub>2</sub>] at  $45^\circ\text{C}$  for 48 h (SF 1.8) and the regenerated substrates were enzymatically hydrolysed at  $45^\circ\text{C}$  with 50 mM citrate buffer (pH 4.8) and an enzyme load of 2 wt % (1 wt% of each Celluclast 1.5L and Novozyme 188). Source: Paper I.

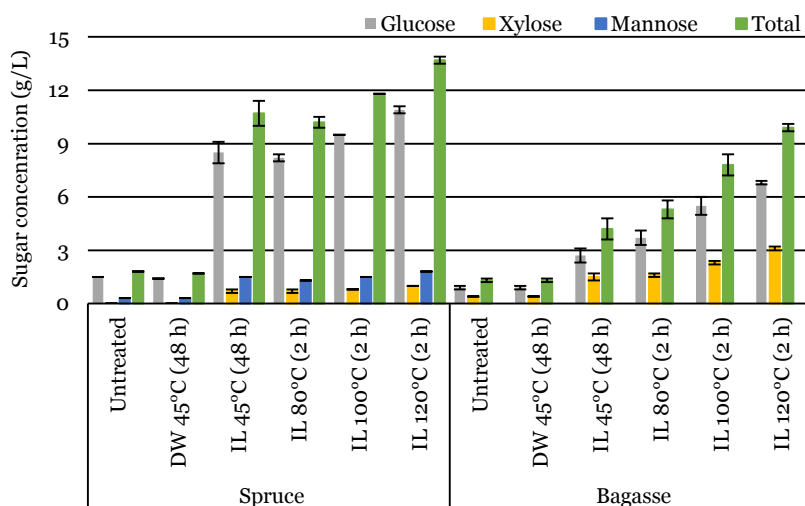
The use of enzymes in IL media was also studied to see whether the enzymes remain stable in IL environment or not. Thus, exploring the opportunities offered by IL solvents for the simultaneous pretreatment and saccharification of cellulose substrates. Avicel was incubated (at  $45^\circ\text{C}$  for 48 h) together with cellulase enzymes and the IL [Amim][HCO<sub>2</sub>]. Upon hydrolysis no cellulose to glucose conversion was detected, probably enzymes were denaturation by the IL.

However, from the investigation of ILs treatments of model cellulose substrate, it can be concluded that the  $\text{SO}_2$  DBU MEASIL- doesn't influence the cellulose crystallinity while the  $\text{CO}_2$  DBU MEASIL was able to attack the crystalline matrix of cellulose. In turn, both [AMMorp][OAc] and [Amim][HCO<sub>2</sub>] could attack the cellulose crystalline matrix, but the performance of [AMMorp][OAc] was clearly superior to that of [Amim][HCO<sub>2</sub>].

## Biomass substrates

Since the IL treatments clearly improved the saccharification efficiency of cellulose substrates, the four ILs were applied in the treatment of different lignocellulose substrates. In a preliminary study, spruce wood and sugar cane bagasse were treated with [Amim][HCO<sub>2</sub>] under mild conditions (SF 1.5–2.7) and the regenerated substrates were subsequently hydrolysed (Paper I). Figure 21 shows the sugars released (by enzymatic hydrolysis) from untreated, water treated, and IL treated spruce wood and sugar cane bagasse, at various conditions.

After 48 h enzymatic hydrolysis of spruce, the [Amim][HCO<sub>2</sub>] treated wood released 8.2 ( $\pm 0.2$ ) to 10.9 ( $\pm 0.2$ ) g/L of glucose and 10.7 ( $\pm 0.7$ ) to 13.7 ( $\pm 0.2$ ) of total sugars (Figure 21). This glucose release corresponded to 39–50% of the theoretical yield. In case of water treated and untreated spruce, 1.4–1.5 g/L sugars were released (corresponding only 6–7% of the theoretical yield). Thus, the glucose production was notably better from [Amim][HCO<sub>2</sub>] treated spruce substrates. Also, it should be noted that after treating spruce wood with [Amim][HCO<sub>2</sub>] at 45°C for 48 h or at 80°C for 2 h, similar sugar yields were obtained (apparently similar degree of treatment severities).

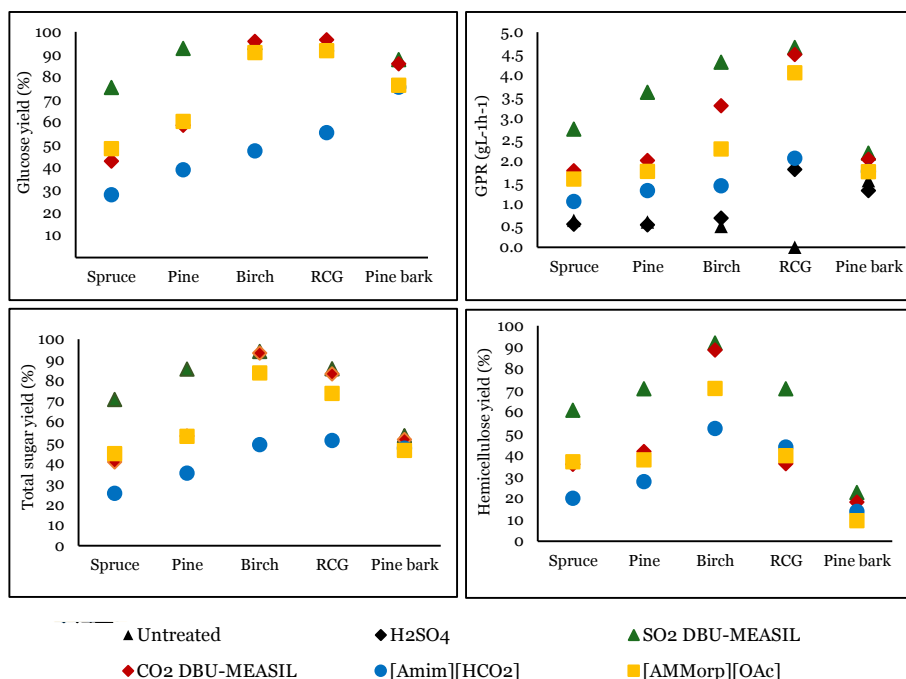


**Figure 21.** Sugars released from the enzymatic hydrolysis of non-treated, water treated and [Amim][HCO<sub>2</sub>] treated spruce wood and sugar cane bagasse at various condition. Enzymatic hydrolysis was performed at 45°C with citrate buffer (pH 4.8) and an enzyme load of 2 wt % (1 wt% of each Celluclast 1.5L and Novozyme 188). Source: Paper I.

Also in case of sugar cane bagasse, [Amim][HCO<sub>2</sub>] treatment enhanced the enzymatic hydrolysis (Figure 21). After 48 h hydrolysis, about 2.7 ( $\pm 0.4$ ) to 6.8 ( $\pm 0.1$ ) g/L glucose and 4.2 ( $\pm 0.6$ ) to 9.9 ( $\pm 0.2$ ) g/L total sugars were released. The glucose release corresponding to 15 – 38% of the theoretical

glucose yield. In case of water treated and untreated bagasse, the obtained glucose concentrations were equal i.e. 0.9 g/L (corresponds to 5% of the theoretical yield). The release of hemicelluloses from the enzymatic hydrolysis of IL treated substrates (Figure 21) indicates that the IL treatments facilitates not only hydrolysis of crystalline cellulose but also hydrolysis of non-crystalline hemicelluloses.

In general, increasing degree of severity upon IL treatment resulted in better hydrolysis yields. Thus, at the study was extended to even more severe conditions (Paper II).



**Figure 22.** Sugar yields (g sugars released as a percentage of g total available) and the glucose production rates (GPRs) obtained from the enzymatic hydrolysis of IL treated, untreated and acid treated lignocelluloses. Enzymatic hydrolysis reactions were performed at 50°C with an enzyme (CTech 2) load of 2 wt % in 50 mM citrate buffer (pH 5.8). GPRs are from the 4 h hydrolysis and the yields are from 48 h hydrolysis. Source: Paper II.

Consequently, the IL/SIL treatment was applied to all target species, spruce, pine, birch, RCG and pine bark and the efficiency of ionic liquids SO<sub>2</sub> DBU-MEASIL, CO<sub>2</sub> DBU-MEASIL, [Amim][HCO<sub>2</sub>] and [AMMorp][OAc] were compared. The following conditions were chosen for the IL treatments: 120°C for 90 min (SF 2.5), 160°C for 90 min (SF 3.7), and 180°C for 60 min (SF 4.1). Thereafter, the enzymatic digestibility of IL treated substrates were evaluated and the amount of sugars released were compared with that of acid treated (205°C and 10 min, SF 4.1) and untreated substrates. Sugars released

from the enzymatic hydrolysis of IL treated substrates are presented in paper II (Appendix A.1-A.5) and the best results are summarised in Figure 22.

IL treatments resulted in significant enhancement in sugar yields. At maximum, glucose yields ranging from 75–97 % and total sugar yield 71–94% were obtained, depending on the lignocellulosic species in question. A high amount of hemicelluloses (maximum 23% for pine bark – 92% for birch wood) were also recovered from the IL treated substrates (Figure 22). However, the total sugar recovery was higher (83%) for the acid treated pine bark compared to the (53%) IL treated. There is a simple explanation for this: the hemicelluloses were dissolved in IL solvents and were decanted. Nonetheless, hemicellulose recovery from the IL treated woody substrates were higher than the acid treated substrates (Figure 15, 22).

Regardless of the type of biomass used, SO<sub>2</sub> DBU-MEASIL- performed best. In particular when softwood substrates were used, the performance was superior. In case of birch and RCG, both SILs worked well. (Figure 22). The efficiency of IL [AMMorp][OAc] was same in magnitude of order as CO<sub>2</sub> DBU-MEASIL.

However, in terms of glucose production rates, SO<sub>2</sub> DBU-MEASIL- was the best, followed by CO<sub>2</sub> DBU-MEASIL. This is because SO<sub>2</sub> DBU-MEASIL is more powerful lignin removing solvent than CO<sub>2</sub> DBU-MEASIL. About 95% lignin was removed from birch wood when it was treated with SO<sub>2</sub> DBU-MEASIL at 160°C for 2 h, whereas this corresponded to only 50% for CO<sub>2</sub> DBU-MEASIL (Anugwom *et al.* 2014b). As already discussed, lignin content of the substrates is one of the major obstacles in the hydrolysis process. Compared to SILs, the glucose production rates of [AMMorp][OAc] treated lignocelluloses were lower, but [AMMorp][OAc] doesn't remove lignin. Apparently, the IL [Amim][HCO<sub>2</sub>] is not the best pretreatment solvent for lignocelluloses, since it does not remove lignin and it is less efficient in disrupting cellulose crystallinity (Figure 19).

In general, increasing pretreatment severity was beneficial. However, IL treatments at 160°C for 90 min and 180°C for 60 min gave similar results and shorter treatment times can be used if higher temperatures are employed. Also, for less recalcitrant substrates, a mild CO<sub>2</sub> DBU-MEASIL pretreatment is enough to obtain high sugar yields whereas for amorphous pine bark, the IL pretreatments did not help (Figure 22).

In summary, pretreatments with IL solvents have significantly improved the enzymatic hydrolysis of recalcitrant lignocellulose materials. Lignin removing SILs are superior to common ILs and the high lignin removal capacity appears to be crucial for enhancing the subsequent enzymatic hydrolysis. However, the recovery challenges of solvated hemicelluloses and lignin, also, the

(current) cost of many ILs are the major drawbacks. Further, the technology is not yet mature for industrial applications. Still today, acid pretreatment technology works albeit working with acid hydrolysates gives rise to its own challenges.

# Chemical conditioning of acid pretreated spruce wood hydrolysates

Softwood hydrolysates such as acid pretreated spruce wood was considered to be suitable for the investigations concerning the chemical detoxification. This is because, as previously reported, compared to other, spruce wood is highly recalcitrant substrate and would require a harsh chemical pretreatment to enhance its susceptibility by cellulase enzymes. Hence, the hydrolysates contain significant amount of by-products that are in sufficient concentrations to inhibit both enzymes and fermentation organisms. Acid catalysts such as sulfur dioxide gas and sulfite/hydrogen sulfite are commonly considered for pretreatment of lignocellulosic substrates (Galbe and Zacchi 2007; Wang *et al.* 2009). Thus, the pretreatment of softwood was performed at a high temperature (>200°C) with the addition of sulfur dioxide.

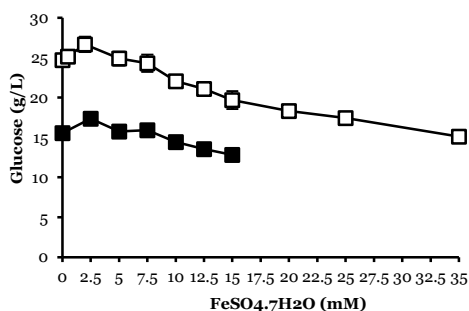
The effect of various chemical detoxifications proposed in this study were evaluated with either spruce acid hydrolysate (SAH, liquid fraction of the acid pretreated spruce wood) or spruce slurry hydrolysate (SSH). In the enzymatic hydrolysis experiments, Avicel was used as cellulose substrate in case of SAH was used as media. Thus, the experiments with SAH differ to the SSH, and any improvements observed in the hydrolysis experiments with SAH must therefore related to the detoxification effect on SAH rather than on solids since Avicel was never exposed to detoxification. The experiments with SSH indicate the effect of detoxification on realistic lignocellulose hydrolysates, SSH contain both solids (notably cellulose and lignin) and liquid fraction i.e. SAH.

## Detoxification with H<sub>2</sub>O<sub>2</sub> and FeSO<sub>4</sub>

The effect of in-situ addition of ferrous sulfate upon enzymatic hydrolysis of cellulose was evaluated by adding 0-35 mM FeSO<sub>4</sub> to the hydrolytic system i.e. SAH containing Avicel cellulose or SSL or 50 mM citrate buffer containing Avicel cellulose.

Results suggested that the glucose production from the enzymatic hydrolysis was effected by the addition of ferrous sulfate (Figure 23). An addition of 0-7.5 mM ferrous sulfate resulted in only a minor impact on the hydrolysis efficiency, but higher dosage resulted in decreased levels of glucose. Inclusion of 35 mM FeSO<sub>4</sub> resulted in 39% lower glucose release compared to the control (Figure 23). Apparently, an excess of Fe<sup>2+</sup> ions has an inhibitory effect on enzymatic degradation of cellulose.

Enzymatic hydrolysis of Avicel in citrate buffer, i.e. in the absence of lignocellulose inhibitors, indicated similar results and the effects corresponded well with the hydrolysates. Furthermore, increasing  $\text{FeSO}_4$  concentrations resulted in decreasing pH (as low as 3.7 with 35 mM  $\text{FeSO}_4$ ), but pH controlled experiments yielded similar results, i.e. there was a severe inhibition at high  $\text{FeSO}_4$  concentration. Consequently, this indicates that the pH was not the decisive factor behind reduced hydrolysis efficiency.



**Figure 23.** Effect of  $\text{FeSO}_4$  addition on glucose release during (□) 96 h enzymatic hydrolysis of Avicel (10 wt%) in spruce acid hydrolysate and (■) 24 h enzymatic hydrolysis of spruce slurry hydrolysate with 10 wt% suspended solids. Hydrolysis experiments were performed with an enzyme load of 2 wt% (1 wt% of each Celluclast 1.5L and Novozyme 188) at 45°C and pH 5.2. Source: Paper III.

$\text{Fe}^{2+}$  ions don't adsorb to on cellulose and, the observed negative effect should not be related to cellulose adsorption (Tejirian and Xu 2010). Rather, its impact is linked to interaction between  $\text{FeSO}_4$  and the hydrolytic enzymes. Due to its redox activity,  $\text{FeSO}_4$  could affect folding and stability of the dominating enzymes (Tejirian and Xu 2010). According to Tejirian and Xu (2010), at concentration of  $\text{FeSO}_4$  higher than 10 mM,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  ions act as strong inhibitors of cellulases which was also evident in this study. Besides, at moderate amounts,  $\text{Fe}^{2+}$  can be used to promote the interaction between cellulose substrate and enzyme active sites (Zhao *et al.* 2011), thus benefiting hydrolysis efficiency at low concentrations.

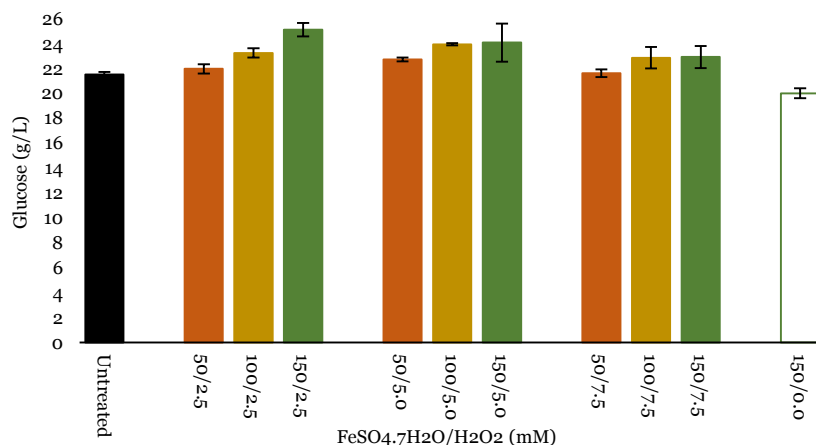
Detoxification effect of  $\text{H}_2\text{O}_2$  and  $\text{FeSO}_4$ , alone or in combination, on acid treated spruce hydrolysates was evaluated. Spruce hydrolysates were treated with various concentrations of  $\text{H}_2\text{O}_2$  and  $\text{FeSO}_4$  (Paper III) and the chemical composition of hydrolysates were determined before and after the treatments. In general, treatment with  $\text{H}_2\text{O}_2$  with or without  $\text{FeSO}_4$  influenced the chemical composition of hydrolysates. Formic acid, furfural, HMF, and phenols were all affected to a varying degree (Paper III). Precipitants (in dark brown color) were found in the hydrolysates treated with  $\text{H}_2\text{O}_2$  together with  $\text{FeSO}_4$ , while, no precipitate was observed in the hydrolysates treated with  $\text{H}_2\text{O}_2$  only. Treatment with  $\text{FeSO}_4$  alone had no impact on hydrolysates.

The chemical analysis showed that acetic acid, levulinic acid, and glucose concentration of hydrolysates were essentially unaffected by the treatments. Nevertheless, the concentration of formic acid was increased by up to 82 (w/v)%, while furfural, HMF and phenols were degraded to a large extent

(Paper III). The increased concentrations of formic acid resulted from the degradation of both furfural and HMF. Increased concentration of H<sub>2</sub>O<sub>2</sub> favour the degradation of furanlaldehydes, high amounts were degraded by the treatments with 150 mM H<sub>2</sub>O<sub>2</sub> and 7.5 mM FeSO<sub>4</sub>. However, treatments with H<sub>2</sub>O<sub>2</sub> and an increasing concentration of FeSO<sub>4</sub>, did not result in increased formic acid concentrations indicating that other reaction pathways were also active such as formic acid degradation to CO<sub>2</sub> (Sinnaraprasat and Fongsatitkul 2011).

Removal of phenols followed a similar trend as furanlaldehydes. However, increasing concentrations of FeSO<sub>4</sub> was not beneficial for degradation of phenols. FeSO<sub>4</sub> alone, without addition of H<sub>2</sub>O<sub>2</sub>, did not help, while the treatment with only 150 mM H<sub>2</sub>O<sub>2</sub> could remove the inhibitors almost to the same extent as obtained together with FeSO<sub>4</sub>.

By this methodology, enzymatic degradation of Avicel was improved in the presence of SAH treated with H<sub>2</sub>O<sub>2</sub> and FeSO<sub>4</sub>. After 72 hydrolysis, a maximum of 25.1 (±0.3) g/l glucose was produced from the hydrolysates treated with 150 mM H<sub>2</sub>O<sub>2</sub> and 2.5 mM FeSO<sub>4</sub>, which is about 17% higher compared to 21.5 (±0.2) g/l glucose produced from the untreated hydrolysates. A correlation between the extent of phenol degradation and improved enzymatic decomposition was observed, whereas the correlation was less apparent in case of furanlaldehydes. However, detoxification of spruce slurry hydrolysates had no impact on enzymatic hydrolysis.

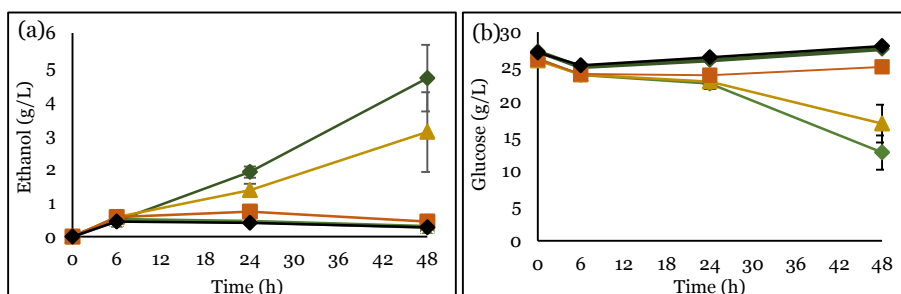


**Figure 24.** Glucose released from 72 h enzymatic hydrolysis of Avicel (10 wt%) in spruce acid hydrolysates untreated and treated with H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> (T 60°C; t 2h; pH 3.8). Hydrolysis experiments were performed with an enzyme load of 2 wt% (1 wt% of each Celluclast 1.5L and Novozyme 188) at 45°C and pH 5.2. Source: Paper III.

Treatment with H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> of Avicel cellulose in citrate buffer, i.e. in the absence of inhibitors, resulted in severe inhibition of enzymatic hydrolysis.

The glucose production decreased from  $48.2 \pm 1.9$  g/l for untreated to  $22.3 \pm 0.9$  g/l for 150/2.5 H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> treated samples. Evidently, improved glucose production in case of spruce hydrolysates was likely the result of interactions between hydroxyl radicals and inhibitory compounds. In addition, as reported by Tejirian and Xu (2010), oxidation of Fe<sup>2+</sup> by H<sub>2</sub>O<sub>2</sub> mitigated its inhibitory effect on cellulases. Thus, H<sub>2</sub>O<sub>2</sub> by itself offers no beneficial effect (Figure 24), whereas the combination of H<sub>2</sub>O<sub>2</sub> and FeSO<sub>4</sub> gives rise to a better performance.

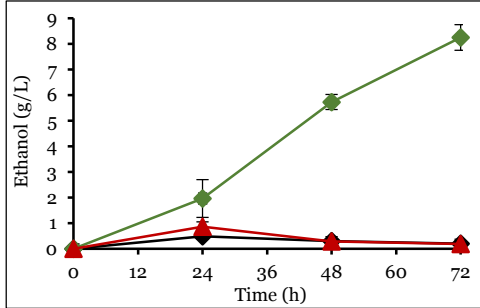
Based on degradation of inhibitors, treatments of spruce hydrolysates with 150/2.5, 150/5.0, and 150/7.5 mM of H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> as well as only 150 mM H<sub>2</sub>O<sub>2</sub> were tested for their fermentability with the yeast *S.cerevisiae*. After 48 h fermentations, hydrolysates treated with 150/2.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> produced  $4.7 \pm 1.0$  g/l ethanol, while the hydrolysates treated with 150/5.0 and 150/7.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> produced only  $3.1 \pm 1.2$  and  $0.4 \pm 0.1$  g/l, respectively. Fermentations of hydrolysates treated with 150/7.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> were not successful, which could be due to the high amount of FeSO<sub>4</sub>. FeSO<sub>4</sub> at concentrations above 5 mM has been reported to be toxic and severely inhibits microbial cell growth (Lee *et al.* 2012; Lin *et al.* 2011).



**Figure 25.** (a) Ethanol production and (b) glucose consumption from the fermentations of spruce acid hydrolysates treated (T 60°C; t 2h; pH 3.8) with H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub>·7H<sub>2</sub>O at concentrations of (◇) 150/0 mM (◆) 150/2.5 mM (▲) 150/5.0 mM (■) 150/7.5 and (◇) untreated, respectively. Fermentations were performed with 1.6 g/L (cell dry wt) of *S. cerevisiae*, *Thermosacc* at 30°C. Source: Paper III.

Also, the untreated hydrolysates were essentially non-fermentable. Even though, a comparable amount of inhibitors especially phenolic compounds were degraded by the treatment with 150 mM H<sub>2</sub>O<sub>2</sub>, the hydrolysates were still non-fermentable, which is in line with the results observed in hydrolysis experiments (Figure 24). In fermentations where yeast was active producing ethanol (Figure 25), there was also a consumption of furfural and HMF, which is consistent with results reported by Delgenes *et al.* (1996). Similar results were also observed from the fermentations of spruce slurry hydrolysates, spruce slurry treated with 150/2.5 and 150/5.05 mM of H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> were readily fermented to ethanol while the untreated ones were non-fermentable.

To elucidate the influence of detoxification and removal of inhibitors, specifically HMF and furfural, on the fermentability of hydrolysates, experiments were performed by re-adding the furanldehydes to their original concentrations in previously detoxified hydrolysate.



**Figure 26.** Ethanol production from the spruce acid hydrolysate: non-treated (♦), detoxified with 150/2.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub>·7H<sub>2</sub>O (◆), and detoxified with 150/2.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub>·7H<sub>2</sub>O and HMF and furfural added to the original concentrations before detoxification (▲). Fermentations were performed with 1.6 g/L (cell dry wt) of *S. cerevisiae*, *Thermosacc* at 30°C. Source: Paper III.

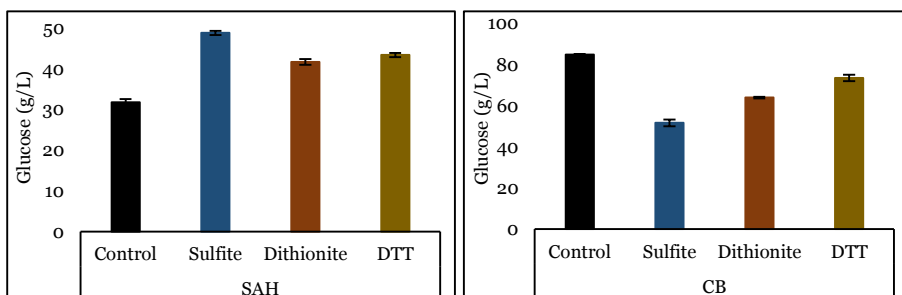
Two mixtures were detoxified with 150/2.5 mM H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub>. After detoxification, furfural (0.9 g/l) and HMF (1.1 g/l) (corresponding to the amounts degraded as a result of the detoxification) were added thus restoring the original concentrations. The detoxified hydrolysates, in which the inhibitors were not restored, were successfully fermented and 8.3±0.49 g/l ethanol was produced in 72 h. Fermentations of detoxified hydrolysates where furans were restored to the original level failed (Figure 26).

In summary, detoxification with H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> is presented as an efficient tool to deal with the inhibitory problems of toxic lignocellulose hydrolysates on not only fermenting organisms but also cellulolytic enzymes.

## Application of reducing agents

Sulfur oxyanions sulphite, dithionite and DTT (dithiothreitol: known as Cleland's reagent) were used to detoxify the spruce acid hydrolysates.

In the presence of spruce acid hydrolysate, inclusion of 15 mM reductants had a positive effect on the enzymatic hydrolysis of Avicel cellulose (Figure 27). The glucose production, after 120 h hydrolysis, reached  $48.7 \pm 0.5$ ,  $41.6 \pm 0.7$  and  $43.3 \pm 0.5$  for the hydrolysates with sulphite, dithionite and DTT, respectively. Thus, about 31–54% more glucose was produced compared to the control hydrolysate with no reducing agents added. Avicel hydrolysis in citrate buffer negatively affected by the presence of reducing agents (Figure 27). However, sulfite had large negative effect in citrate buffer, while it gave the highest final glucose concentration in the presence of hydrolysate (Figure 27). The results also showed that the reducing agents must be added before the enzymes are added to the hydrolytic system, otherwise part of the effect was lost.



**Figure 27.** Glucose produced from 120 h enzymatic hydrolysis of Avicel cellulose in either spruce acid hydrolysate (SAH) media or citrate buffer (CB) media, with and without addition of reducing agents. Hydrolysis experiments were performed with an enzyme load of 2 wt% (1 wt% of each Celluclast 1.5L and Novozyme 188) at 45°C and pH 5.2. Source: Paper IV.

Further, experiments were performed with first step hydrolysates from two-step dilute sulfuric acid pretreated spruce wood (Nilvebrant *et al.* 2003), thus investigating the effect of reducing agents with various hydrolysates. Compared to control, addition of 15 mM dithionite to the hydrolysis mixture has improved glucose production to about 26% (Paper IV).

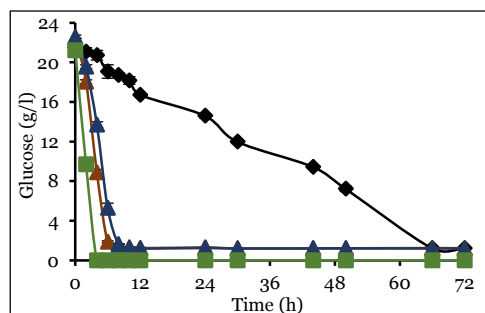
When dithionite (15 mM) was added to spruce slurry hydrolysate, only 13% better glucose production could be obtained which was lower compared to Avicel hydrolysis with added dithionite (Figure 27).

Along with Avicel, a variety of cellulose substrates namely, carboxymethyl cellulose (CMC),  $\alpha$ -cellulose, 2-hydroxyethyl cellulose (HEC), and solids (washed) from the spruce slurry hydrolysate were tested as cellulosic substrates in the presence of citrate buffer or acid hydrolysate with or without

15 mM of dithionite. In all cases glucose production was improved to a variable extent in hydrolysate, while citrate buffer experiments resulted in lower glucose concentrations. However, addition of reductant to citrate buffer containing solids of spruce slurry alleviated the negative effects of the reducing agents and improved the glucose production about 7%. Even though the solids of slurry hydrolysate were washed there was still some low-molecular mass compounds formed from the pretreatment process in the solid fraction, possibly by hydrophobic interactions, and these were present in the liquid phase when the material was suspended in citrate buffer. It is known that lignin can adsorb inhibitory compounds such as phenols and furan aldehydes (Björklund *et al.* 2002) and about 0.48 g/l phenols were detected in the citrate buffer contained solids of spruce slurry. As discussed in earlier chapters phenolic compounds severely inhibits cellulolytic enzymes.

However, experiments carried out to address the question concerning the contribution of phenolic compounds to the inhibitory effect, hydrolysate was treated with the phenol oxidase laccase. The laccase treatment resulted in a 45% decrease in the content of total phenolics, but no positive effect on enzymatic hydrolysis was detected. However, pretreatment liquid is expected to contain a wide variety of phenolic compounds and more detailed analyses are needed to clarify the role of phenolic compounds in the inhibition of enzymatic hydrolysis of cellulose.

In addition to the hydrolysis, spruce hydrolysates were anaerobically fermented with the yeast *S. cerevisiae* with or without addition of a reducing agent (Figure 28). As control a nutrient solution with the same glucose concentration as the hydrolysate was used.



**Figure 28.** Glucose consumption during the ethanol fermentation of spruce acid hydrolysates with no addition (◆), addition of reducing agents 15 mM sulphite (▲) and 15 mM Dithionite (▲), and reference fermentation of pure glucose solution (■). Fermentations were performed with 2.0 g/L (cell dry wt) of *S. cerevisiae* at 30°C and with the supplementation of nutrients. The data represent mean values of two experiments

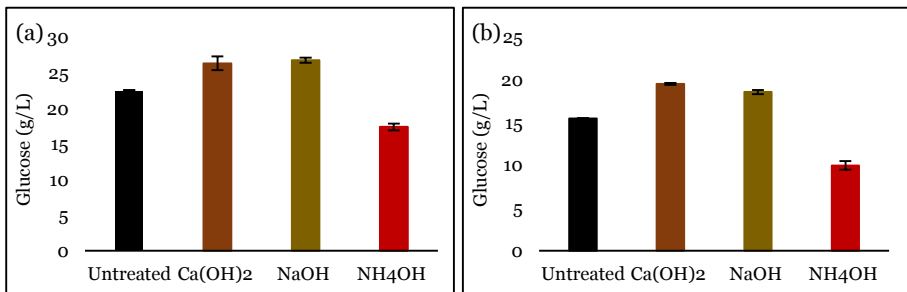
During fermentations, glucose in hydrolysates was as readily consumed as in the control with glucose media when a reducing agent sulfite or dithionite was added (Figure 28). While without addition of reducing agent the glucose consumption was very slow and lasted for 72 h. These results are consistent with that of reported in Alriksson *et al.* (2011).

In conclusion, the study indicates that the addition of sulphur oxyanions are useful in many ways: apparently for pretreatment, for improving enzymatic hydrolysis, and for improving the fermentability of inhibitory lignocellulosic hydrolysates.

## Alkaline detoxification

Three different types of alkali:  $\text{Ca}(\text{OH})_2$ ,  $\text{NaOH}$ , and  $\text{NH}_4\text{OH}$  were used as conditioning agents to alleviate the inhibition problems associated with the spruce hydrolysates. The conditions used for the treatments are presented in Table 6.

Enzymatic hydrolysis of spruce slurry hydrolysates detoxified  $\text{Ca}(\text{OH})_2$  or  $\text{NaOH}$  increased the glucose production with 17 and 19%, respectively (Figure 29a). Similarly, hydrolysis of Avicel cellulose in the presence of  $\text{Ca}(\text{OH})_2$  or  $\text{NaOH}$  treated acid hydrolysates gave better results compare to the hydrolysis of Avicel in un-detoxified acid hydrolysate (Figure 29b), the glucose production was improved 26 and 20% for  $\text{Ca}(\text{OH})_2$  and  $\text{NaOH}$ , respectively. The positive effect of alkaline treatments can be attributed to the degradation of inhibitory compounds (Table 6; Sárvári Horváth *et al.* 2005; Alriksson *et al.* 2006).



**Figure 29.** Glucose production from 72 h enzymatic hydrolysis of alkali-treated and untreated (a) spruce slurry hydrolysates (b) Avicel in spruce acid hydrolysates. Conditions used for the alkaline treatments were:  $\text{Ca}(\text{OH})_2$  - pH 11, 30°C, 3 h;  $\text{NaOH}$  - pH 9, 80°C, 3 h; and  $\text{NH}_4\text{OH}$  - pH 9, 55°C, 3 h. Hydrolysis experiments were performed with an enzyme load of 2 wt% (1 wt% of each Celluclast 1.5L and Novozyme 188) at 45°C and pH 5.2. Source: Paper V.

Conditioning with  $\text{NH}_4\text{OH}$  did, however, not improve the situation. In fact, compared to non-detoxified hydrolysates, significantly less glucose (about 23 – 36%) was released from the enzymatic hydrolysis when hydrolysates were treated with  $\text{NH}_4\text{OH}$  (Figure 29). The negative effect of ammonium hydroxide was apparently due to its high ionic strength (~0.83 M), a high amount of

ammonium hydroxide was used for the conditioning (Soudham *et al.* unpublished).

Addition of  $\text{NH}_4\text{Cl}$  (~0.83 M) to the hydrolysate media (containing citrate buffer and Avicel) potentially inhibited the enzymatic hydrolysis, resulted in about 37% less glucose compare to the reference without added  $\text{NH}_4\text{Cl}$ . Salt ions either competes or changes the configuration of the proteins, which results in decreasing hydrophobic interactions between the protein and the solid surface (Can *et al.* 2006).

Even though both  $\text{Ca}(\text{OH})_2$  and  $\text{NaOH}$  treatments gave better results, when considering sugar loss,  $\text{Ca}(\text{OH})_2$  was better conditioning agent than  $\text{NaOH}$  (Table 6). A high sugar degradation from the  $\text{NaOH}$  (19%) and  $\text{NH}_4\text{OH}$  (10%) treatments may be due to the high temperatures (80 and 55°C) used (Sárvári Horváth *et al.* 2005).

The results suggest that detoxification with a variety of alkaline reagents can be used to alleviate the inhibitory effect of compounds present in lignocellulose hydrolysates and subsequently improve the enzymatic saccharification and fermentability.

## Conclusions

Enzymatic hydrolysis of untreated lignocelluloses revealed that the agriculture residues e.g. reed canary grass were more recalcitrant than woody substrates. However, hydrolysis efficiency of acid treated agriculture residues were higher than the woody substrates. Nonetheless, among investigated, pine bark was found to be an amorphous substrate.

Genetic modification of plants proved to be beneficial for increasing the yields of monosaccharides. The saccharification efficiency of transgenic aspens was significantly higher than the wild type thus altering the plants to be more suitable as feedstock for the production of biofuel.

Ionic liquid treatments resulted in significant improvements of enzymatic hydrolysis efficiency. Lignin hydrolysing SO<sub>2</sub> DBU-MEASIL and CO<sub>2</sub> DBU-MEASIL were the most effective pretreatment solvents. However, for recalcitrant softwood substrates SO<sub>2</sub> DBU-MEASIL was more efficient than CO<sub>2</sub> DBU-MEASIL.

Problems associated with the conversion of toxic lignocellulose hydrolysates into biofuels were addressed by different strategies that are already in use at industries, but for other purposes. Detoxification of acid pretreated spruce wood hydrolysates with either H<sub>2</sub>O<sub>2</sub>/FeSO<sub>4</sub> or reducing agents or alkaline solutions significantly improved subsequent enzymatic hydrolysis and the hydrolysates were readily fermented to ethanol. Non-detoxified hydrolysates, on the other hand, suffered from low sugar production and were non-fermentable.

## Future perspective

Evaluation of lignocellulose recalcitrance will have significant role in the efficient conversion of lignocellulose materials. However, there are no standard tools for this purpose. Use of ionic liquids for the evaluation of biomass recalcitrance is an interesting option.

Results suggest that also many other factors beyond lignin influence on biomass recalcitrance to sugar release pointing to a critical need for deeper understanding of plant cell-wall structures. Lignocelluloses, with exceptionally high or low sugar release, identified in this study can be used for further investigations which would permit drawing conclusions on factors impacting lignocellulose recalcitrance.

The use of ILs as solvents for the pretreatment of lignocelluloses is promising. However, challenges that remain and have to be addressed includes recovery of lignocellulose components dissolved in IL and recovery and reuse of ILs. New types of ILs can be synthesised with more specific and targeted properties for certain applications.

Both detoxification and inhibition mechanisms are complex processes involving several factors. Observations in this work suggest that a varying degree of reduction and/or chemical transformation of inhibitory compounds would improve both enzymatic hydrolysis and microbial fermentation of the toxic lignocellulose hydrolysates. Thus, the use of chemicals that can convert lignocellulose inhibitors can be further explored.

The efficient detoxification concerning both sugar release and fermentability caused by  $H_2O_2/FeSO_4$  deserves further studies to find the optimal conditions.

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