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Production of Ethanol from Spruce at High Solids Concentrations - An Experimental Study on Process Development of Simultaneous Saccharification and Fermentation

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Production of Ethanol from Spruce at High Solids Concentrations

An Experimental Study on Process Development of Simultaneous Saccharification and Fermentation

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The faculty opponent is Prof. Liisa Viikari, Department of Food and Environmental Sciences, Helsinki University, Finland.

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Abstract

Replacing fossil fuels by biofuels such as ethanol is considered a promising alternative to reduce greenhouse gas (GHG) emissions and mitigate climate change. Biofuels produced from lignocellulosic biomass, so-called second generation biofuels, result in decreased GHG emissions and limit competition with food and animal feed production. Interest in producing ethanol from lignocellulosic biomass has therefore increased rapidly during recent years. Several pilot and demonstration plants for the biochemical conversion of lignocellulose to ethanol have been built, and the first commercial plants are planned to start large-scale production within the coming years. However, a great deal remains to be done in process development to increase the production efficiency and decrease production cost.

The work presented in this thesis focuses on the biochemical conversion of spruce to ethanol, using enzymatic hydrolysis and fermentation in simultaneous saccharification and fermentation (SSF). The main aim of this work was to achieve a high ethanol concentration after fermentation, in order to reduce the energy required in distillation, thus reducing the production cost. A final ethanol concentration of 65 g/L was achieved, which is well above the 4 wt% considered to be the limit for economically feasible distillation. Furthermore, these experimental studies on the production of ethanol from spruce have contributed to a better understanding of some of the fundamental steps in the production process.

Enzymatic hydrolysis and fermentation must be performed at higher solid substrate concentrations in order to increase the ethanol concentration after fermentation. In the first part of this work, it was shown that the decrease in ethanol yield in SSF with high solids concentration is a result of both increased mixing difficulty and increased inhibition of the yeast, and possibly the enzymes, due to increased levels of inhibitory substances. In the second part of the work, it was shown that the ethanol yield in high-solids SSF could be significantly increased by adding a prehydrolysis step prior to SSF. It was also shown that this positive

effect on ethanol production from spruce is a result of fibre degradation rather than decreased viscosity, as often suggested when using other lignocellulosic materials such as straw and grass. The addition of a prehydrolysis step prior to SSF shifts the process from being fermentation-limited to being hydrolysis-limited. Prehydrolysis thus enhances fermentation, rather than the overall performance of hydrolysis.

The initial dry matter content in SSF was increased from 5-10% water-insoluble solids (WIS) to 20% WIS. The process configuration in enzymatic hydrolysis and fermentation has been shown to significantly influence the overall ethanol yield. The highest ethanol concentration (65 g/L) with an overall ethanol yield of 72% was obtained in fed-batch SSF, where prehydrolysed steam-pretreated spruce was fed to the reactor over a period of time. Approximately a quarter of the cellulose was, however, not converted to glucose, and was thus not fermented to ethanol. There is thus further potential for improvements in the process, which could increase the ethanol concentration and yield.

Populärvetenskaplig sammanfattning

Intresset för förnybara bränslen har ökat stadigt under de senaste åren. Att byta ut fossila mot förnybara bränslen kan minska utsläppen av växthusgaser till atmosfären och därmed minska klimatpåverkan av dessa men även minska vårt beroende av olja. Biobränslen som t.ex. etanol, biodiesel eller biogas, räknas till förnybara bränslen eftersom de inte bidrar till en ackumulering av koldioxid i atmosfären då koldioxid som bildas vid deras förbränning tas upp vid bildning av ny biomassa som används för tillverkning av nya biobränslen. Det är viktigt att kunna framställa etanolen på ett effektivt sätt till en rimlig kostnad. Detta arbete har bidragit till att effektivisera processen för tillverkning av etanol från gran. Etanol till fordonsbränsle görs idag av vete, majs och sockerrör (så kallad biomassa). Dessa innehåller i huvudsak druvsocker i form av stärkelse eller socker. Så står etanolframställningen i konkurrens till matproduktion.

Även cellulosa, som utgör en stor del av biomassan i träd, halm och gräs, består i huvudsak av druvsockermolekyler. För att minska konkurrensen av biobränsleframställning med matproduktion och för att minska produktionskostnaden, har man på senare år därför börjat titta på framställning av etanol från cellulosa. Då används enzymer (biologiska katalysatorer) som kan bryta ner cellulosa till enskilda druvsockermolekyler i en så kallad hydrolys. Dessa kan sedan jäsas till etanol av vanlig bagerijäst. När man utför hydrolys och jäsning samtidigt, är det möjligt att få ut mer etanol ur råmaterialet. Efter jäsning koncentreras etanolen med hjälp av destillation. Jäsning av socker till etanol som öl och vin har gjorts sedan urminnes tider. I cellulosa finns sockret dock bundet i en struktur som är svår att bryta ner.

Som råmaterial i detta arbete har gran använts för framställning av etanol. En stor kostnad i framställningen av etanol är destillationen. Denna kostnad minskar avsevärt när etanolkoncentrationen är hög redan efter jäsningen. Därför är det viktigt att ha en hög koncentration av fast råmaterial redan under hydrolysen och jäsningen. Målet med mitt doktorandprojekt har varit att studera hur höga

koncentrationer av fast råmaterial påverkar tillverkningsprocessen. Arbetet har även syftat till att uppnå en hög etanolkoncentration efter jäsningen.

Processen för framställning av etanol från gran fungerar väl så länge det inte finns för hög halt av biomassa i reaktorn. Veden som har förbehandlats inför hydrolysen och jäsningen är en tjock massa, som snabbt blir svår att röra om och hantera när den inte späs ut med mycket vatten. Dessutom har det visat sig att inte lika mycket druvsocker och etanol kan produceras när processen körs vid högre koncentration av fast material. Detta har i mina studier visats bero på både omrörningssvårigheter men även ökade koncentrationer av ämnen som påverkar jästen när koncentrationen av biomassa ökas. Dessa ämnen finns redan i veden eller frigörs under produktionsprocessen.

Jag har visat att det är möjligt att uppnå höga koncentrationer av etanol även vid högre utgångskoncentrationer av fast råmaterial när man låter enzymerna bryta ner cellulosan redan innan man tillsätter jästen. Detta har gjorts tidigare för andra råmaterial, men man har förklarat den positiva effekten med att det blir lättare att röra om materialet när enzymerna har fått bryta ner det, vilket gör materialet mer flytande. I denna avhandling har jag dock kunnat visa att jästen även arbetar mer effektivt i kontakt med redan nedbrutet material. Resultaten antyder att det inte enbart är koncentrationen av fast material som påverkar hur mycket etanol jästen kan framställa, men även till vilken grad cellulosan är nedbruten. En etanolkoncentration på 4-5% brukar anses vara det som måste uppnås för att kunna producera etanol från lignocellulosa till en konkurrenskraftig kostnad. I optimeringen i denna avhandling har jag framställt etanol från gran i koncentrationer upp till 6,5%.

Under den senaste tiden har ett antal demonstrationsanläggningar för cellulosabaserad etanol byggts runt om i världen och de första anläggningar som producerar etanol från cellulosa i kommersiell skala förväntas sättas i drift inom detta eller nästa år. Forskning som den presenterad i denna doktorsavhandling är därför ytterst aktuell och central för att minska tillverkningskostnaden och göra cellulosabaserad etanol konkurrenskraftig med fossila bränslen.

Publications

This thesis is based on the following papers. The papers are appended in the end of the thesis, and will be referred to in the text by their roman numerals.

I. **Hoyer, K.**, Galbe, M. and Zacchi, G. (2009). Production of fuel ethanol from softwood by simultaneous saccharification and fermentation at high dry matter content. *Journal of Chemical Technology and Biotechnology* 84: 570-577.

II. **Hoyer, K.**, Galbe, M. and Zacchi, G. (2010). Effects of enzyme feeding strategy on ethanol yield in fed-batch simultaneous saccharification and fermentation of spruce at high dry matter. *Biotechnology for Biofuels* 3:14.

III. **Hoyer, K.**, Galbe, M. and Zacchi, G. (2013). The effect of prehydrolysis and improved mixing on high-solids batch simultaneous saccharification and fermentation of spruce to ethanol. *Process Biochemistry* 48: 289-293.

IV. **Hoyer, K.**, Galbe, M. and Zacchi, G. (2013). Influence of fiber degradation and concentration of fermentable sugars on simultaneous saccharification and fermentation of high-solids spruce slurry to ethanol. *Biotechnology for Biofuels* 6:145.

V. **Hoyer, K.**, Wallberg, O. and Galbe, M. (2013). Combined prehydrolysis and fed-batch simultaneous saccharification and fermentation for the production of ethanol from spruce at high solids concentrations. *Manuscript*.

In all the above studies, I did essentially all the work: I planned and carried out the experiments, evaluated the results and wrote the papers. The co-authors participated in the design of the studies, discussed the results and commented on the papers.

During the work described in **Papers I-III** and the experimental work presented in **Paper IV**, Prof. Guido Zacchi was my main supervisor and Dr. Mats Galbe was my assistant supervisor. During the writing of **Paper IV** and the entire work presented in **Paper V**, Dr. Mats Galbe was my main supervisor, and Dr. Ola Wallberg was my assistant supervisor.

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I have spent several years on this research, during which I had the privilege to work in a friendly and inspiring environment at the Department of Chemical Engineering. I would like to thank all those who have contributed to making my years as a PhD student such a wonderful experience and a pleasant time to remember.

Prof. Guido Zacchi, my main supervisor during the first years of my PhD studies; thank you for giving me the opportunity to dive into the world of ethanol fermentation. Thank you for many lively inspiring discussions, and for always making time for me when I needed help or encouragement.

Dr. Mats Galbe, my main supervisor during the last 18 months of my PhD studies, for agreeing to be my main supervisor, despite the fact that you were already more than busy. Thank you for all our inspiring discussions and for encouraging me to do things my way. You impress me with your wide knowledge, and still, you never hesitate to get your hands dirty helping out in the lab. I appreciate that you were always there to help out when things were not working or my fermentor was leaking.

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I would not have enjoyed my years as a PhD student as much as I did if it were not for the colleagues I have had during these years. Stefano, I could not have asked for a better roommate than you. Thank you for all the laughs we shared in the office. Thanks also to former and present members of the Ethanol Group. I have enjoyed working with you, and of course travelling the world for conferences. It wouldn't have been half as fun without all of you! Thank you to

all my colleagues at the Department of Chemical Engineering for creating such a pleasant working environment, and for interesting discussions during coffee breaks and lunches.

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I would like to thank my family for all their support during these years. My mother, Heike, the greatest believer in science that I know, for always being able to convince me that what I have been doing was important, even if it did not always feel like it. Thanks also to my brothers, Olaf and Nils, for many fun and interesting discussions, comparing beer and fuel ethanol production on many levels.

Last but not least, I would like to thank Kim for showing patience with me when I had to go to the lab at strange hours to take samples or feed the yeast. Thank you also, for always being there for me when I needed support. But most of all, thank you for coming to Sweden with me and for being here. I love you!

Maja and Rasmus, thank you for being patient with your crazy mother who spent almost an entire summer sitting on the terrace writing her PhD thesis. Thank you for distracting me with your spontaneous laughter when my work was intense and stressful, and thank you for just being you. You are the greatest kids I could have ever wished for!

Contents

Abbreviations

AFEX	Ammonia fibre explosion
ARP	Ammonia recycle percolation
ATP	Adenosine triphosphate
BG	β-glucosidase
BSA	Bovine serum albumin
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
CSTR	Continuously stirred tank reactor
CBP	Consolidated bioprocessing
EDP	Entner-Doudoroff pathway
EG	Endoglucanase
EMP	Embden-Meyerhof-Parnas pathway
GH	Glycoside hydrolase
GHG	Greenhouse gas
HHF	Hybrid hydrolysis and fermentation
HMF	Hydroxymethylfurfural
HPLC	High performance liquid chromatography
IEA	International Energy Agency
IL	Ionic liquid
iLUC	Indirect land-use change
IPCC	Intergovernmental Panel on Climate Change
LHW	Liquid hot water
PEG	Polyethylene glycol
PPP	Pentose phosphate pathway
PSSF	Prehydrolysis and simultaneous saccharification and fermentation
SHF	Separate saccharification and fermentation
SSF	Simultaneous saccharification and fermentation
SSCF	Simultaneous saccharification and co-fermentation
VHG	Very high gravity
WIS	Water-insoluble solids

1

Introduction

During recent decades, there has been a growing interest in renewable energy for several reasons. Concentrations of greenhouse gases (GHGs) (mainly carbon dioxide, methane and nitrous oxide) in the atmosphere, mostly from the combustion of fossil fuels, have increased enormously during the last century (IPCC, 2007). If these emissions continue to increase as today, their high concentrations in the atmosphere will seriously affect our climate. Most of the increase in GHG emission originates from human activities and to minimize climate change in the future, GHG emissions need to be reduced. One way of achieving this is by replacing fossil fuels with more GHG-neutral fuels. The security of energy supply is another driving force for the development of renewable energy. Most countries are importers of fossil fuels such as oil and gas, while only a few are exporters. Together with a widely fluctuating oil price, this motivates the development of a broader range of energy sources, including renewable energy. Furthermore, bioenergy may be produced more locally than fossil energy, which may lead to rural development and employment opportunities.

The International Energy Agency (IEA) was established in 1974 mainly to ensure energy security, by lessening our dependence on oil (IEA, 2013b). Energy security remains the main objective of the IEA, including among others the

United States and the European Union, and member states are obliged to maintain oil stocks so that they can react quickly to disturbances in oil supply. The IEA is also engaged in long-term energy security, and promotes efficiency, diversity and flexibility in the energy sector of its member states (IEA, 2013b). Bioenergy plays a vital role in this. Reduction of GHG emissions is a central issue in the energy policies of the European Union (EU, 2010). In 2009, the member states of the European Union made a commitment to reduce their consumption of primary energy by 20%, to reduce GHG emissions by 20%, and to include 20% renewable energy in their total energy consumption by 2020 (EU, 2009). This goal is commonly referred to as the 20-20-20 initiative.

The transport sector is responsible for a significant share of GHG emissions. In 2004, 23% of the world's energy-related CO_2 emissions originated from transport (IPCC, 2007). In the United States, 28% of the total GHG emissions in 2011 originated from the transport sector (EPA, 2013). The largest part of this was derived from road transport. This makes the transport sector the largest contributor to GHG emissions after electricity production. Furthermore, the transport sector is responsible for the greatest increase in GHG emissions between 1970 and 2004 (IPCC, 2007). Replacing the fossil fuels gasoline and diesel, which today are the predominantly used fuels in the transport sector, by biofuels potentially reduces GHG emissions.

1.1 Biofuels

Biofuels are derived from biomass, i.e. organic matter from plants or animals, such as wood, agricultural material, energy crops, municipal organic waste, manure and algae. Due to the relatively recent carbon fixation in biomass used for biofuel production, biofuels have the potential to contribute less to carbon dioxide emissions than fossil fuels. Biofuels can be liquid or gaseous, and include ethanol, biodiesel and biogas. From 2000 to 2011, the global annual biofuel production increased from 16 billion litres to 100 billion litres and today constitutes about 3% of the total road transport fuel globally (IEA, 2013a). Within the 20-20-20 target of the European Union, the proportion of renewable fuel sources in the transport sector is set to 10% of the final energy consumption in that sector (EU, 2010). There is significant technical potential in producing transportation fuels from biomass for GHG emission mitigation (IEA, 2013a). In the latest report from the Intergovernmental Panel on Climate Change (IPCC) on the mitigation of climate change in the transportation sector, second generation biofuels, together with electric and hybrid vehicles, have been identified as key mitigation technologies for commercialization before 2030 (IPCC, 2007). Nev-

ertheless, biofuels should be seen as only part of the solution, and a complement to more efficient vehicles and transport systems (IPCC, 2007; Börjesson et al., 2008; EPA, 2013).

The production of some conventional biofuels has been heavily criticized for causing deforestation (through direct or indirect land-use change (iLUC)) and for competing with food and animal feed production (IEA, 2013a). The debate on whether biofuels have the potential to increase sustainability in the energy sector has recently changed towards a discussion on which biofuels are sustainable and which are not (Börjesson, 2009; IEA, 2013a). International sustainability certification is considered vital to ensure that biofuels have positive environmental and social impacts. The way in which biofuels are assessed depends on the underlying motivation for their use. When the aim is to reduce GHG emissions, it is important that energy sources used during the production process are biological and not fossil, and that by-products are utilized efficiently. Furthermore, when cultivated crops are used as substrate, these should not be grown on carbon-rich soil, and nitrous oxide emissions should be kept to a minimum by efficient fertilization strategies. Several ethanol producers in the world today fulfil these demands, while others do not (Börjesson, 2009; IEA, 2013a). The combustion of ethanol from sugarcane in Brazil leads to a reduction in GHG emissions of about 85% compared with fossil fuels, while ethanol produced from corn in the USA only leads to a reduction of 20%, due to the extensive use of fossil coal and natural gas in its production (Börjesson, 2009). In an effort to ensure that future biofuels have the desired environmental effects, only biofuels that result in a certain reduction in GHG emissions (35% by 2017 and 50% after 2017) are included in the EU's 20-20-20 target (EU, 2010).

In contrast to fossil fuels, biofuels can be produced from various feedstocks and processes. This complicates general analyses such as sustainability studies. These are further complicated by the assumptions made in the various models used. Of course, different cultivation and production options can be used in calculations and simulations. Also, assumptions regarding new cropland for the cultivation of feedstock can have a highly increasing effect on the results of GHG emissions from the production (Börjesson, 2009). iLUC is the subject of much debate in connection with the production of biofuels. The conversion of rainforests, peatlands, savannas or grassland for the production of feedstock for biofuels can lead to more carbon dioxide being released to the atmosphere, as CO_2 that has been stored in the ground is released, and is not compensated by the cultivation of biofuels (Fargione et al., 2008; Searchinger et al., 2008). However, some arable land that is not currently used, could be used for biofuel production. In the EU, 10% of the total arable land area is currently lying fallow (Börjesson,

2009). In a long-term perspective, the agricultural sector may develop in many places, resulting in more efficient crop production. When discussing the risks of iLUC, the critical factors are thus the rate of expansion and the total volume of biofuels produced (Börjesson, 2009). Since agricultural land is limited in most countries, it is important to increase the yield of biofuel per hectare of land, and to increase the production of biofuels from waste products such as agricultural and forest residues. However, whether a material is considered waste or not, changes quickly once it can be converted into a valuable product. Certification of fuels produced from agricultural or forest residues is especially complex and difficult to implement on an industrial scale, since the path from raw material to fuel is not always straightforward (Balan et al., 2013).

1.2 Ethanol as a transportation fuel

Ethanol has many advantages as a transportation fuel. It can be blended with gasoline to reduce the use of gasoline, and increase the octane number, improving engine performance. Low-blending of ethanol with gasoline introduces oxygen into the fuel, resulting in more complete combustion and cleaner exhaust gases (Wyman, 1994). Ethanol can be used as a fuel alone, but usually 10-15% gasoline is blended in to improve cold start of the engine. Ethanol is used on a large scale in Brazil, the United States and some European countries, including Sweden, and is expected to be one of the dominating renewable fuels in the transport sector in the near future (IPCC, 2007). The ethanol currently available is produced from sugar from sugarcane, or starch from grains such as wheat and corn, and is referred to as first generation bioethanol. However, these raw materials are also used for food and feed production. Furthermore, first generation ethanol usually results in higher GHG emissions than second generation bioethanol, which is produced from lignocellulosic biomass such as wood, agricultural residues such as wheat straw, corn stover and sugarcane bagasse, and energy crops such as switch grass or *Salix*. Therefore, interest in second generation ethanol has been growing during recent years, and the latest IPCC report on the mitigation of climate change states that the global potential for biofuels will depend on the success of technologies utilizing cellulose biomass (IPCC, 2007). Various pilot and demonstration plants for the production of ethanol from lignocellulosic biomass have been built during recent years, and the first commercial plants are planned to start large-scale production within the next few years (Balan et al., 2013). The first planned commercial plants for the biochemical conversion of lignocellulosic biomass to ethanol are the DuPont Biofuels plant in the United States, where it is planned to produce ethanol from corn stover and switchgrass, and the Beta Renewables plant in Italy, where ethanol production

from giant cane (*Arundo donax*) and switchgrass is planned.

1.3 Softwood for ethanol production

More than half of the 40.8 million hectares of Sweden's total land area is covered by productive forest; 42% of it being Norway spruce (Swedish Forestry Agency, 2013b). From the net felling volume of 72.1 million cubic metres solid timber excluding bark (2011), about half is used as sawlogs and the other half as pulpwood. Only a small portion is used as fuelwood, mainly for heating purposes (Swedish Forestry Agency, 2013a). For fuel production, mainly sawdust, shavings, tops and branches, and recycled wood are used (Skogsindustrierna, 2012). Throughout the world, over 4 billion hectares are covered by forest; Russia, Brazil, Canada, the United States and China hosting more than half of the world's total forest area (Swedish Forestry Agency, 2013c). The total forest volume in Sweden has increased by over 80% since the 1920s (Swedish Forestry Agency, 2013b), and is believed to continue increasing (Skogsindustrierna, 2012), while forest volumes are decreasing slightly internationally (Swedish Forestry Agency, 2013c). However, the annual decrease in the world's total forest volume is well under 1%, and we are moving towards sustainable forest management without any losses of forest volume (Swedish Forestry Agency, 2013c).

Despite having only 1% of the world's forest area, Sweden is one of the main producers of pulp and paper in the world (Skogsindustrierna, 2012). There is thus a long tradition of forest industries, some of them producing more than one main product (Bioraffinaderiet Domsjö Fabriker, 2013). This means that Sweden has good potential to include biofuels such as ethanol or other alcohols in the product portfolio of a biorefinery, which by many has been identified as a promising option for the production of ethanol from lignocellulosic materials. In Norway, the Borregaard biorefinery has been producing ethanol, specialty cellulose and a variety of lignin-derived chemicals from softwood for over 100 years (Borregaard, 2013). The use of woody biomass for ethanol production has several advantages over agricultural residues. Wood can be harvested all year round, which reduces the need for storage, and has a higher density, lowering transportation cost (Zhu and Pan, 2010). Also, the low content of pentoses in softwood compared to agricultural residues simplifies the fermentation process, making it possible to produce ethanol using ordinary baker's yeast. Wood is, however, stronger and more recalcitrant, and thus more difficult to break down than agricultural materials.

1.4 Aim of this work and outline of this thesis

The aim of the research presented in this thesis was to increase the ethanol concentration after fermentation in simultaneous saccharification and fermentation (SSF) of steam-pretreated spruce by increasing the solid substrate loading. This thesis is based on the research presented in five papers. The study described in **Paper I** concerns the impact of high inhibitor concentrations and mixing on ethanol production in SSF with high solids concentrations. In the studies described in **Papers II-V**, the ability to achieve high ethanol concentrations at high solids loadings in SSF was studied using different process options: fed-batch (**Paper II**), prehydrolysis (**Papers III** and **IV**) and a combination of both (**Paper V**).

This thesis consists of three parts: a literature survey, a summary of my results and the detailed results appended in five research papers. Part one of this thesis is a literature survey that describes the structure of the lignocellulosic raw material (Chapter 2) and the production process of ethanol from softwood (Chapter 3) in detail. My work is then put into perspective in the second part that consists of Chapter 4-6 and focuses on enzymatic hydrolysis and fermentation at high solids concentrations. In Chapter 4, the challenges associated with high solids concentrations in enzymatic hydrolysis and SSF are discussed. In Chapter 5, the process configurations used in this work (i.e. prehydrolysis and fed-batch mode, as well as the combination of both) are discussed in detail. Chapter 6 summarizes the most important findings and suggests future work in this field. The detailed results of my work are presented in the third part, which consists of the five research papers making up this thesis.

2

Lignocellulosic biomass

2.1 Softwood structure and composition

Softwood contains three main polymers: cellulose, hemicellulose and lignin. These polymers are linked together in a complex structure so as to provide the tree with a transport system for water and nutrients, as well as mechanical strength (Sjöström, 1993).

2.1.1 The structure of wood cells

The wood cell wall consists of several layers: the middle lamella, the primary cell wall and the secondary cell wall. The middle lamella is located between the cells and is highly lignified, binding the different cells together (Sjöström, 1993). The primary cell wall, consisting mainly of cellulose and hemicellulose, is a thin layer (0.1-1.0 μm) around the middle lamella. Primary cell walls have a similar appearance in different cell types (Brett and Waldron, 1996). The cellulose chains are less ordered in the primary cell wall and are oriented in all directions within the plane of the cell wall (O'Sullivan, 1997). While some cells limit themselves to a middle lamella and a primary cell wall, others continue by forming a secondary cell wall, consisting of a thin outer and inner layer and a thicker

middle layer. The layers are made up of cellulose microfibrils, between which hemicellulose and lignin are located. It is the secondary cell wall that represents the most important characteristic feature of some cell types (Brett and Waldron, 1996). The middle layer in the secondary cell wall forms the main part of the cell wall with a thickness of 1 to 5 μm or 30-150 lamellae. The inner and outer layers are made up of few (3-4) lamellae, in which the microfibrils are arranged in a helix.

The carbohydrates that make up the building blocks of the cell walls are usually classified into monosaccharides, oligosaccharides and polysaccharides. D-glucose, D-mannose, D-galactose, D-xylose and L-arabinose are the main monosaccharides in the cell walls of wood; their proportions are given in Table 2.1. Oligosaccharides consist of up to ten monosaccharides linked together by glycosidic bonds, and the polysaccharides in wood cell walls consist of large numbers of monosaccharides linked together by glycosidic bonds.

2.1.2 Cellulose

Cellulose, the main polysaccharide in wood, is predominantly present in the secondary cell wall, constituting about 40-50% of the dry substance in most wood species (Fengel and Wegener, 1989; Sjöström, 1993). It consists of cellobiose units linked together by β-(1→4)-glycosidic bonds which form a linear homopolysaccharide, as illustrated in Figure 2.1. The degree of polymerization of cellulose ranges from 300 to 15 000 for different species. In softwood, it is about 8 000 (Fengel and Wegener, 1989). Chemical treatment such as wood pulping is known to decrease the degree of polymerization of cellulose, which also decreases with age in the living tree (Fengel and Wegener, 1989). Cellulose is insoluble in all common solvents including water, and has a strong tendency to form hydrogen bonds, both within the linear cellulose polymer (intramolecular linkages) and between polymer chains (intermolecular linkages) (Fengel and Wegener, 1989; Brett and Waldron, 1996). Whether the insolubility of cellulose in water is due to hydrogen bonding or its crystallinity, as believed by many, is, however, still being debated. Lindman et al. (2010) argue that the insolubility of cellulose in water is unlikely to be due to hydrogen bonding alone. Textbooks usually teach us that compounds capable of considerable hydrogen bonding are soluble in water simply because of the fact that they are able to form hydrogen bonds. This explains, for example, the solubility of compounds such as dextran or cellulose derivatives such as methyl cellulose and hydroxyethyl cellulose in water, as they have a high capacity for hydrogen bonding. Crystallinity is also unlikely to be the sole reason for the low solubility of cellulose in water since

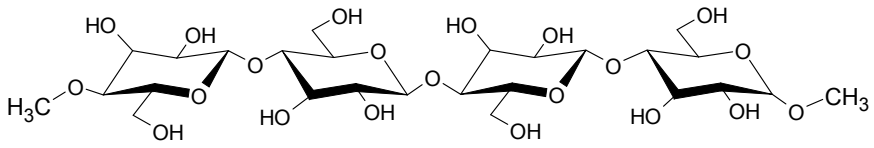

Figure 2.1: The structure of cellulose.

there is no major difference between the solubility of amorphous and crystalline cellulose in water. Lindman et al. (2010) suggested that the insolubility of cellulose in water may rather be due to its amphiphilic nature, as the cellulose chains are ordered in a structure resulting in hydrophobic regions that are responsible for the low solubility of cellulose in water.

Microfibrils are formed by 30-100 cellulose polymers joined together, where highly ordered, crystalline, regions alternate with less ordered, amorphous, regions (Sjöström, 1993). The crystallinity of native fibres has been reported to be at most 70% (O'Sullivan, 1997). Microfibrils make up fibrils, or lamellae, and finally cellulose fibres. The structure described above is called cellulose I, and is the only form commonly found in nature (Brett and Waldron, 1996). After thermal or chemical treatment of cellulose I, the internal crystal structure changes and cellulose II, III or IV are formed (Brett and Waldron, 1996; O'Sullivan, 1997).

2.1.3 Hemicellulose

Hemicelluloses are heteropolymers of the sugars D-glucose, D-mannose, D-galactose, D-xylose, L-arabinose and small amounts of L-rhamnose, D-glucoronic acid, 4-O-methyl-D-glucoronic acid and D-galactoronic acid. Most hemicelluloses have a degree of polymerization of only 200 and are highly branched (Sjöström, 1993). The composition and structure of hemicelluloses differ significantly between softwood and hardwood, as well as between different parts of the tree (Sjöström, 1993). Hemicellulose is bound to cellulose by hydrogen bonds, forming a network that provides the structural backbone of the plant cell wall (Mosier et al., 2005).

Between 20 and 30% of the dry substance in softwood is hemicellulose, mostly galactoglucomannans (Fengel and Wegener, 1989; Sjöström, 1993), that are shown in Figure 2.2. Galactoglucomannans are linear or slightly branched polymer chains consisting of (1→4)-linked β-D-glucopyranose and (1→4)-linked β-D-mannopyranose at a ratio of mannose to glucose units of about 3:1 (Fengel and Wegener, 1989). The hydroxyl groups in the units of the chain are partly substituted by galactose and O-acetyl groups (Sjöström, 1993).

Table 2.1: Composition of various lignocellulosic materials (as mass percentages)

	Glucan	Mannan	Xylan	Arabinan	Galactan	Lignin
Softwood						
Spruce[1]	45.0	12.6	5.0	1.0	1.8	33.4
Pine (Shannon et al., 2007)	44.9	11.5	6.2	1.9	3.0	26.6
Hardwood						
Poplar (Bura et al., 2009)	43.8	3.9	14.9	0.6	1.0	29.1
Agricultural residues						
Wheat straw (Linde et al., 2008)	32.6	0.0	20.1	3.3	0.8	26.5
Corn stover (Öhgren et al., 2007)	36.1	1.8	21.4	3.5	2.5	17.2
Sugar cane bagasse (Ferreira-Leitao et al., 2010)	41.4	3.4	22.5	1.3	1.3	23.6
Barley straw (Linde et al., 2006)	37.1	n.d.	21.4	3.1	0.0	19.5
Rice straw (Zhong et al., 2009)	34.7	-	15.1	2.2	-	19.1
Energy crops						
Switch grass (Xu et al., 2011)	32.0	-	17.9	1.9	1.7	21.4
Salix (Sassner et al., 2005)	41.5	3.0	15.0	1.8	2.1	25.2

[1] Average from **Papers I** and **II**

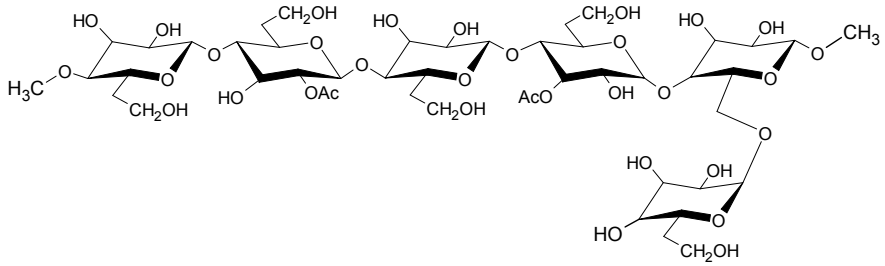

Figure 2.2: Representative structural unit of softwood galactoglucomannan.

In addition to galactoglucomannans, softwood contains arabinoglucoronoxylans, composed of a framework containing (1→4)-linked β-D-xylopyranose units, partly substituted by 4-O-methyl-α-D-glucoronic acid groups (Fengel and Wegener, 1989; Sjöström, 1993). In addition, arabinoglucoronoxylans contain α-L-arabinofuranose side chains, which are easily hydrolysed by acids (Sjöström, 1993).

2.1.4 Lignin

Lignin is the most abundant polymeric organic substance in the plant world after cellulose, and is responsible for the strength and water transport in plants (Sjöström, 1993). Although some fungal enzymes are able to degrade lignin biologically, in general, lignin serves as an efficient protection against degradation by most microorganisms (Vance et al., 1980; Lee, 1997). Lignins are aromatic polymers of phenylpropane units made up of coniferyl, sinapyl, p-coumaryl and p-hydroxy-cinnamyl alcohols, which are formed from D-glucose through complex reactions catalysed by enzymes. Lignin precursors are joined to lignin polymers by random polymerization to fill up the space between the preformed polysaccharides in the cell wall. The phenylpropane units are connected by ether bonds and carbon-to-carbon bonds to form lignin polymers that form hydrogen bonds and covalent bonds with practically all the constituents of hemicellulose (Fengel and Wegener, 1989; Lee, 1997) as well as some of the cellulose (Sjöström, 1993). The most common bonds between lignin and polysaccharides are ether, ester and glycosidic bonds. The glycosidic bonds between lignin and polysaccharides in the cell wall are easily cleaved with acid (Fengel and Wegener, 1989). Due to the complexity of isolating and investigating these compounds and bonds, lignin-polysaccharide binding is still the topic of much debate (Fengel and Wegener, 1989; Sjöström, 1993).

Softwoods contain about 30% lignin, while the lignin content in hardwood is

somewhat lower (Table 2.1). The highest lignin concentration can be found in the middle lamella. However, the greatest part of the lignin in softwood (about 70%) is found in the secondary cell wall, due to its thickness (Fengel and Wegener, 1989). Lignin can be divided into several classes, present at different amounts in different types of cell walls. Lignins of softwood, hardwood and grasses differ in their content of guainacyl, syringyl and p-hydroxy-phenyl units (Sjöström, 1993); softwood lignin consisting mainly of guaiacyl lignin, a large polymerization product of coniferyl alcohol (Fengel and Wegener, 1989; Ek et al., 2009). However, lignin structures also differ between the cell walls of cells of different origin in a single type of plant (Sjöström, 1993).

2.1.5 Residual components

Besides cellulose, hemicellulose and lignin, lignocellulosic biomass contains small amounts of extractives. These are composed of a variety of low-molecular-weight compounds such as fats, resin acids and phenolic compounds, which serve different functions, for example, as an energy source and in offering protection against microbiological attack (Sjöström, 1993). Extractives usually constitute less than 10% of the dry weight in wood, but concentrations are higher in certain parts of the tree, e.g. bark, which contains 20-40% extractives (Sjöström, 1993). Lignocellulosic biomass also contains small amounts of inorganic compounds, as well as minor quantities of other polysaccharides such as proteins, starch and pectic substances (Fengel and Wegener, 1989; Sjöström, 1993).

2.2 Composition of various lignocellulosic materials

The main lignocellulosic materials used for biological ethanol production differ in structure as well as composition. Lignocellulosic materials for ethanol production are usually divided into three categories: agricultural residues (e.g. wheat straw, sugarcane bagasse, corn stover), hardwoods and softwoods. The hemicellulose in agricultural residues and hardwoods is mainly composed of the pentose sugar xylose, while the hemicellulose in softwood contains mostly the hexose sugar mannose (see Table 2.1). This influences the ability to produce ethanol from the different materials. The maximal theoretical yield per kg of raw material is dependent on the amount of fermentable sugar in the raw material, and in agricultural residues and hardwood, a pentose-fermenting microorganism is needed to achieve high conversion of the entire amount of carbohydrates in the material to ethanol. In softwood, ordinary baker's yeast, which is only able to

ferment hexose sugars to ethanol, is sufficient to utilize most of the sugar. The content and distribution of the different polymers (cellulose, hemicellulose and lignin) and the structure in which they are connected, also influence the effectiveness of pretreatment and enzymatic hydrolysis of the materials. Woody biomass is, in general, physically larger and structurally stronger and denser than agricultural biomass. This, together with the higher lignin content in woody biomass, makes it more recalcitrant to microbial and enzymatic degradation (Zhu and Pan, 2010). While the general concept of producing ethanol from lignocellulosic material is the same for many lignocellulosic materials, the process should be fine tuned for each material, as it is not always possible to transfer the results from one kind of material to another (Galbe and Zacchi, 2007).

The composition of the raw materials used to produce ethanol is commonly reported as the amount of total sugars present in the material, as in Table 2.1. However, this provides no information on the way in which the monomeric sugars are distributed between the main polymers of the biomass.

3

Ethanol production from softwood

Ethanol can be produced from lignocellulosic material using either biochemical or thermochemical processes. Biochemical technology was used in the present work and involves four main process steps: pretreatment, hydrolysis, fermentation and product recovery (Figure 3.1). Pretreatment of the material is needed to increase the accessibility of the cellulose to the enzymes that break down the cellulose polymer into its monomeric glucose units in the subsequent hydrolysis step. Hydrolysis can also be performed using dilute or concentrated acids. The glucose molecules, together with monomeric sugar liberated from the hemicellulose, can then be fermented to ethanol using yeast or bacteria. After fermentation, the ethanol must be separated from the fermentation medium.

3.1 Pretreatment

Untreated lignocellulosic material can withstand enzymatic hydrolysis mainly due to the presence of lignin and hemicellulose, blocking the cellulose fibres. The crystalline nature of cellulose, the high degree of polymerization and the limited surface area available for enzyme attack, also limit the enzymatic hydrolysis of

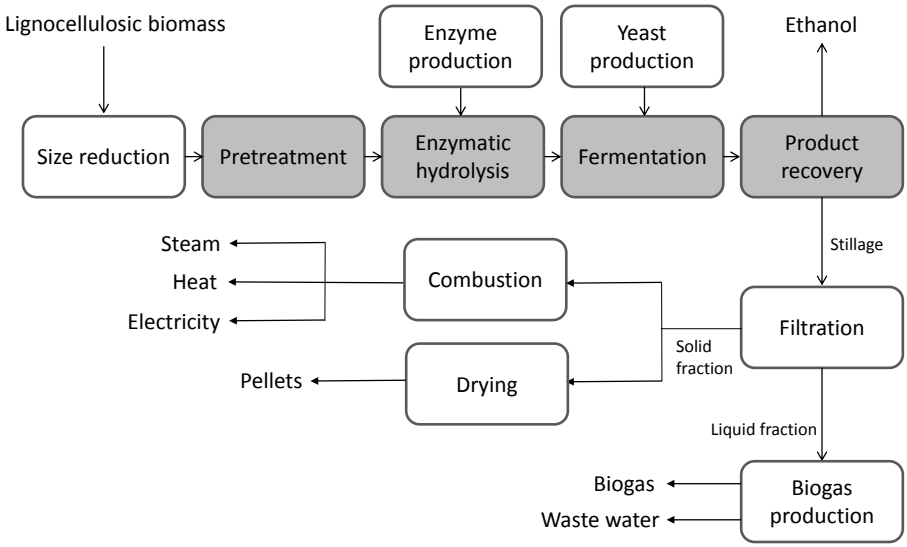

Figure 3.1: Example of a simplified process flowsheet for the production of ethanol from lignocellulosic biomass.

the material to monomeric sugars. To convert lignocellulosic material such as softwood to ethanol, the cellulose must thus be liberated and then broken down into monomeric sugar units, which can then be fermented to ethanol. Starch, which has been transformed to ethanol since ancient times by a well-known process, is a polysaccharide of glucose units linked together by α-(1→4)-glycosidic bonds instead of β-(1→4)-glycosidic bonds as in cellulose, and undergoes the transition from crystalline to amorphous when heated in water to 60-70°C, making the material more accessible to enzymes (Deguchi et al., 2006). Cellulose undergoes a similar transition but not until 320°C and 25 MPa (Deguchi et al., 2006). More advanced pretreatment of the material prior to enzymatic hydrolysis, is thus needed for lignocellulosic materials.

One of the main objectives of pretreatment is to increase the available surface area for enzymatic attack (Chandra et al., 2007; Alvira et al., 2010). The removal of hemicellulose increases the pore size and thus the accessibility of the substrate. Although the increase in exterior surface area with decreased particle size has a significant effect on the rate of hydrolysis by cellulase enzymes, it is the interior surface area that is affected by swelling of the fibres that is mainly responsible for enhancing cellulase hydrolysis rates (Chandra et al., 2007). If the pores are too small, enzymes may be prevented from entering, or may be trapped in the

pores. Furthermore, pretreatment influences the crystallinity of the substrate, although this phenomenon is not straightforward, and it has been observed that pretreatment that increased the digestibility of the substrate, also increased the degree of crystallinity of the cellulose fraction, in some cases (Alvira et al., 2010). This effect could possibly be explained by the fact that amorphous cellulose is hydrolysed first, resulting in the accumulation of the crystalline fraction in the cellulose fibres. The degree of polymerization is reduced during pretreatment, which influences the following enzymatic hydrolysis since different enzymes in the cellulase cocktail act on different parts of the polymer chain (see Section 3.2). It is, however, difficult to study the effect of a particular change resulting from pretreatment on enzymatic digestibility as pretreatment often leads to several structural changes simultaneously (Chang and Holtzapple, 2000). Lignin and hemicellulose remaining after pretreatment may reduce the efficiency of enzymatic hydrolysis by non-productive binding of enzyme to lignin, or by simply acting as a physical barrier between the enzymes and the substrate.

Several key properties are important in the choice of pretreatment method. It is important that the pretreatment is:

- **effective,** resulting in a highly digestible pretreated material with high sugar yields and high solids/sugar concentrations for a wide range of lignocellulosic materials. Recovery of lignin in a high value form and hemicellulose sugars in monomeric form to avoid the need for hemicellulases in the enzymatic hydrolysis. Effectiveness at low moisture content.
- **easy,** assuring minimum of treatment such as size reduction needed prior to pretreatment.
- **cheap,** enabling operation in minimal size reactors using minimum heat, power and chemicals.
- involving **minimum of unwanted side effects,** with minimum sugar loss and formation of degradation products inhibitory to the enzymes or microorganisms used downstream. No formation of waste compounds causing problems downstream.

There is no single method of pretreatment that meets all these demands, and the choice of method is a compromise, depending on the lignocellulosic material used. In the case of woody biomass, Zhu et al. (2010) pointed out that particular attention should be paid to the effectiveness as well as the energy consumption of the treatment process, especially regarding wood size reduction. According to them, size reduction is often neglected or overlooked in pretreatment studies,

but has a major impact on the energy demand and the cost of pretreatment of woody biomass. Jørgensen et al. (2007a) also emphasized that pretreatment methods must not only be optimized with regard to optimum convertibility of the material, but to ensure that they work in a full-scale process. This means that they should work with large particles, to reduce the energy needed for size reduction, they should work at high solids concentrations, to reduce water and energy usage, and it should be possible to integrate them with other processes when used in a biorefinery.

The nature of the substrate and the pretreatment method have significant impact on the subsequent process steps, which means that the choice of pretreatment method is crucial for the entire process. The various pretreatment options for lignocellulosic materials can be divided into biological, physical, chemical and physico-chemical methods.

3.1.1 Methods for pretreatment of lignocellulosic biomass

Biological pretreatment

Brown, white and soft-rot fungi

Some microorganisms such as brown, white and soft-rot fungi have the ability to degrade hemicellulose and lignin without affecting the cellulose, and can therefore be used to pretreat lignocellulose. This method of pretreatment has low environmental impact and works under mild conditions without the need of chemicals, and is therefore very cost-effective. However, the hydrolysis rates are very low, resulting in long pretreatment times (Galbe and Zacchi, 2007; Alvira et al., 2010) and the need of large amounts of space (Chandra et al., 2007). It is also difficult to maintain suitable growth conditions for the fungi, making it a complex process (Chandra et al., 2007). Furthermore, some of the material in hemicellulose, cellulose or lignin can be consumed by the microorganisms, resulting in a loss of yield (Galbe and Zacchi, 2007).

Physical pretreatment

The aim of physical pretreatment of biomass is to break down the biomass into smaller particles.

Mechanical communition

The surface area available for enzymatic attack can be increased and the cellulose crystallinity reduced by mechanical processes such as chipping, milling or grinding, in a way that is relatively insensitive to the physical and chemical char-

acteristics of the biomass. This, however, requires high amounts of energy, often more than the total energy content of the biomass (Galbe and Zacchi, 2007; Kumar et al., 2009), which results in this method not being economically feasible. Also, mechanical pretreatment alone does not remove the lignin from the material and combinations of different treatments, e.g. physical treatment in an extruder combined with heating and the addition of chemicals have been suggested as an interesting alternative (Galbe and Zacchi, 2007).

Chemical pretreatment

The main aim of chemical pretreatment of lignocellulosic biomass is the separation of hemicellulose and/or lignin from the cellulose, and in this way enhance enzymatic hydrolysis.

Alkali pretreatment

Adding alkali, mostly NaOH or $Ca(OH)_2$, to a lignocellulosic material results in lignin solubilisation, while only having a slight effect on the cellulose and hemicellulose. Alkali pretreatment is carried out at low temperatures and pressures, but pretreatment times are in the range of hours or days, rather than minutes. Some of the alkali is converted to salts that are incorporated into the biomass. The major effect of alkali pretreatment is the removal of lignin from the biomass, which is greatly improved by the addition of oxygen, especially in the case of highly lignified materials (Mosier et al., 2005). Pretreatment with alkali also causes fibre swelling, which increases the internal surface area (Galbe and Zacchi, 2007) and induces lignin structure disruption (Galbe and Zacchi, 2007; Alvira et al., 2010). Alkali pretreatment was shown to be more effective on agricultural residues than on more lignified wood materials (Chandra et al., 2007; Galbe and Zacchi, 2007; Alvira et al., 2010). Although pretreatment methods using alkali are common in the pulp and paper industry, alkali pretreatment is not considered an economically feasible pretreatment method for wood in ethanol production (Chandra et al., 2007).

Acid pretreatment

The dilute acid pretreatment process was previously considered to be one of the most promising options for commercialization in the near future, and has been successfully applied to various agricultural feedstocks, as well as short-rotation hardwoods and herbaceous energy crops (Wyman, 1994). In this process, the feedstock is mixed with about 0.5% sulphuric acid and heated to about 140-220°C for 5-20 minutes, which results in hydrolysis of most of the hemicellulose, leaving a cellulose residue with increased enzymatic digestibility. It has been commercially used in the production of furfural from lignocellulosic biomass,

where the xylose formed in the hydrolysis of the hemicellulose reacted further to produce furfural (Mosier et al., 2005). Using acids in pretreatment results in the need to neutralize the material before further processing, and places higher demands on the materials used in the process to avoid corrosion problems. Also, although it results in high sugar yields, acid hydrolysis also leads to further degradation of the sugars released to compounds known to inhibit fermentation. Using concentrated acid is less interesting on an industrial scale due to the high cost and problems associated with corrosion.

Organosolv

Numerous organic and aqueous solvents such as methanol, ethanol, acetone and ethylene glycol can be used to solubilise lignin to increase the enzymatic digestibility of lignocellulose. Solvents must be recycled when used on a large scale, due to their cost and because they might be inhibitory to the enzymes or the yeast used later in the process (Galbe and Zacchi, 2007; Alvira et al., 2010; Zhu and Pan, 2010). Zhu et al. (2010) also mentioned that the successful commercialization of organosolv pretreatment will depend on the development of high-value co-products from lignin and hemicellulose. Another drawback is the fact that some of these solvents are explosive and highly flammable, and thus difficult to handle.

Ozonolysis

Ozone is a powerful oxidant able to delignify lignocellulose at room temperature and moderate pressures. Pretreatment with ozone does not lead to the formation of any inhibitory compounds, but the large amounts of ozone needed and the cost associated with this make the process economically unviable (Alvira et al., 2010).

Ionic liquids (ILs)

A pretreatment method that has received much attention recently is the solubilisation of cellulose in ionic liquids (ILs). ILs are salts that are in their liquid state at low temperatures. They typically consist of a large organic cation and a small inorganic anion. Examples of ILs studied for the pretreatment of lignocellulosic biomass are 1-butyl-3-methylimidazodium chloride (Swatloski et al., 2002; Heinze et al., 2005; Weerachanchai and Lee, 2013), 1-ethyl-3-methylimidazolium acetate (Yong-Chang et al., 2013), 1-butyl-3-methylimidazolium acesulfamate (Xu et al., 2013), benzyldimethyl (tetradecyl) ammonium chloride (Heinze et al., 2005), 1-methyl-3-methoxylbenzylimidazolium chloride (Kilpeläinen et al., 2007) and 1-methyl-3-benzyl-imidazolium dicyanamide (Kilpeläinen et al., 2007). ILs are able to dissolve carbohydrates such as cellulose, hemicellulose and lignin

with minimal formation of degradation products. ILs have considerable potential in the pretreatment of lignocellulosic biomass prior to enzymatic hydrolysis and fermentation, but development is still at the research stage, and further economic optimization is needed to make ILs economically compatible with other pretreatment techniques (Alvira et al., 2010). The greatest challenge today is the recovery of the ILs after treatment, which is necessary due to their high cost. Possible effects on health and toxic effects on enzymes or yeast also require further investigation. Moreover, in order to dissolve biomass components in ILs with limited production of degradation products, pretreatment must be performed at low temperatures, which is a challenge on an industrial scale due to the high viscosity of ILs at low temperatures (Weerachanchai and Lee, 2013).

Ammonia fibre explosion (AFEX)

In ammonia fibre explosion (AFEX), biomass is treated with liquid anhydrous ammonia at 60-100°C. The pressure is initially kept high to maintain the ammonia in liquid form, and is then released causing rapid expansion, which causes swelling and physical disruption of the biomass fibres. AFEX decreases cellulose crystallinity and cleaves carbohydrate linkages, but it does not solubilise very much of the solid material. Although AFEX does not remove hemicellulose or lignin, it increases the enzymatic digestibility of the material, suggesting that ammonia affects lignin in a way that reduces unproductive binding of enzymes to lignin (Alvira et al., 2010). Because both glucan and xylan remain in the solid material after AFEX, hemicellulases are required together with cellulases in the subsequent enzymatic hydrolysis to break down all the carbohydrates to monomeric sugars. One of the main advantages of AFEX is the low formation of degradation products, but ammonia recovery remains a challenge that must be overcome before the technique can be commercially viable since up to 2 kg of ammonia is needed per kg of dry biomass (Galbe and Zacchi, 2007). However, the high sugar yields and minimal production of degradation products during pretreatment may justify the high costs, as the economics of biomass pretreatment is strongly influenced by sugar yields and inhibition during downstream processing. AFEX is a very effective method of pretreatment giving yields close to the theoretical in enzymatic hydrolysis at low enzyme loadings. It is more suited to herbaceous and agricultural residues, works moderately well on hardwoods but is not attractive for softwoods due to their high lignin content (Mosier et al., 2005; Chandra et al., 2007; Galbe and Zacchi, 2007; Alvira et al., 2010; Balat, 2011).

Another process using ammonia to pretreat lignocellulosic biomass is ammonia recycle percolation (ARP), in which aqueous ammonia solution (5-15%) is passed through a reactor filled with the lignocellulosic material at temperatures

around 140-210°C for longer time. The aqueous ammonia reacts with lignin, causing depolymerization of lignin and cleavage of lignin-carbohydrate bonds (Mosier et al., 2005). It also removes some hemicellulose and swells the cellulose fibres, making the cellulose more accessible to the enzymes (Mosier et al., 2005; Alvira et al., 2010). ARP leaves both the glucan and xylan in the solid material, which might be advantageous when using a fermenting organism that can utilize both glucose and xylose. As in AFEX, ARP is an efficient delignification method for agricultural residues and hardwoods, but is less effective for softwoods (Galbe and Zacchi, 2007).

Physicochemical pretreatment

Steam pretreatment

In steam pretreatment, high-pressure saturated steam is applied to the material for a few minutes, with or without the addition of a chemical catalyst. The pressure is then rapidly decreased by discharging the material into a flash vessel. Steam pretreatment is usually run at temperatures around 160-240°C corresponding to pressures between 6 and 34 bar. Hemicellulose is hydrolysed by acids released from the acetyl groups in the hemicellulose and in the case of acid-catalysed steam pretreatment from the acid catalyst added to the process. Water itself also acts as an acid at high temperatures. This method of pretreatment used to be called steam explosion due to the fact that the rapid decrease in pressure opens up the particulate structure of the biomass. It has, however, been shown that this only has a slight effect on the enzymatic digestibility of the cellulose (Mosier et al., 2005; Galbe and Zacchi, 2007; Jørgensen et al., 2007a; Kumar et al., 2009), and the major changes in lignocellulosic biomass caused by steam pretreatment are often attributed to the removal of hemicellulose due to acid hydrolysis of the hemicellulose (Hahn-Hägerdal et al., 2006; Galbe and Zacchi, 2007). Adding an acid catalyst such as SO_2 during steam pretreatment increases the recovery of hemicellulose sugars and improves the enzymatic hydrolysis of the solid fraction (Stenberg et al., 1998; Hahn-Hägerdal et al., 2006; Chandra et al., 2007; Galbe and Zacchi, 2007). It has also been shown to decrease the formation of inhibitors during pretreatment (Stenberg et al., 1998). Lignin is only removed to a limited extent in steam pretreatment but is redistributed on the fibre surface as a result of melting and depolymerization/repolymerization reactions (Kumar et al., 2009; Alvira et al., 2010).

The effectiveness of steam pretreatment is determined by the temperature and residence time, together with the particle size. The severity of steam pretreatment is defined using the so-called severity factor R_0.

$$R_0 = t \cdot e^{[T-100/14.75]}$$

where t is the residence time in minutes and T is the treatment temperature in °C. Higher severity results in increased hydrolysis of hemicellulose and increased enzymatic digestibility, but also promotes the formation of degradation products inhibitory to the enzymes and fermenting organism in the downstream processing. Maximum yields of sugars derived from hemicellulose in steam pretreatment are thus obtained at lower severities. Two-stage steam pretreatment has been suggested to combine high mannose and glucose yields (Söderström et al., 2003a,b). In this way, the first step can be performed at lower severity to hydrolyse the hemicellulose, and the solid fraction obtained can then be treated again at higher severity. Two-step steam pretreatment of softwood has been shown to result in a higher ethanol yield in the subsequent SSF step at lower levels of enzymes and water, but requires more capital and energy (Söderström et al., 2003a,b; Wingren et al., 2004). Wingren et al. (2004) have also shown that the overall ethanol production cost is very dependent on the way two-step steam pretreatment is performed, and it is thus important to perform economic analysis in order to be able to optimize process conditions. They also pointed out that a two-stage process is more complex than a one-stage process, making optimization more difficult.

A major drawback of steam pretreatment is the degradation of lignin and sugars derived from hemicellulose and cellulose to 5-hydroxymethylfurfural (HMF), furfural, aliphatic acids such as acetic, formic and levulinic acid, as well as phenolic compounds. Higher treatment severity results in greater hydrolysis of the hemicellulose, and thus increased accessibility of cellulose, but also increases the formation of degradation products.

Steam pretreatment is considered one of the most promising pretreatment methods on industrial scale (Alvira et al., 2010; Chandra et al., 2007). This is due to the fact that it results in high sugar recovery with low environmental impact, and the capital investment is lower than other alternatives. It also has good potential for energy optimization and can be used to produce relatively concentrated hemicellulosic sugar streams. The main drawbacks of steam pretreatment are the formation of degradation compounds and requirements on process equipment associated with the use of acids. Steam pretreatment has been tested in several pilot-scale facilities (US DOE Bioethanol Pilot Plant, 2000; Sekab, 2013; Iogen Corporation, 2013).

Various agricultural residues including wheat straw and corn stover, as well as hardwood residues from poplar and olive trees, have shown good results when pretreated with steam (Alvira et al., 2010). It is, however, not as efficient on softwoods due to the lack of acetyl groups in softwood hemicellulose (Alvira et al., 2010). Compared to other materials, where it can often be performed without the addition of a catalyst (so-called auto-hydrolysis), steam pretreatment of softwood, requires the addition of a catalyst such as SO_2 to achieve reasonable sugar yields (Kumar et al., 2009; Alvira et al., 2010).

Liquid hot water pretreatment (LHW)

Liquid hot water (LHW) pretreatment is run at 160-240°C for about 15 minutes. The pressure is chosen to maintain the water in a liquid state, in contrast to steam pretreatment. In LHW treatment, most of the hemicellulose, as well as half of the lignin and part of the cellulose, is dissolved. Since no acids are added, the need for neutralization after pretreatment is minimized, and it has been reported that both the residual cellulose and the liquid hydrolysate remaining after pretreatment can be fermented to ethanol with high yields (Mosier et al., 2005). LHW pretreatment generates organic acids through the cleaving of acetyl and uronic acid groups from the hemicellulose. These acids help catalyse the hydrolysis of mainly hemicellulose to monomeric sugars. These sugars are partially broken down into the inhibitors HMF and furfural. Inhibitor formation is, however, not as extensive as in steam pretreatment, especially when the pH is maintained between 4 and 7 to keep the hemicellulose sugars in the oligomeric form (Alvira et al., 2010). Liquid hot water pretreatment has shown good results for corn stover, sugarcane bagasse and wheat straw (Alvira et al., 2010) but is less efficient on softwood biomass. The reasons for this are not well understood (Mosier et al., 2005). Economically, LHW pretreatment is attractive due to the fact that no catalysts or chemicals are needed, but the water and energy demands are high, and the process has not been developed on commercial scale (Alvira et al., 2010). However, since the amount of water used in LHW treatment is much higher than in steam pretreatment, for example, the resulting material after pretreatment is much more diluted, which results in a higher energy demand and thus a higher cost of product recovery after fermentation (Galbe and Zacchi, 2007).

Wet oxidation

In wet oxidation, the biomass is treated at 170-200°C and 10-12 bar for 10-15 minutes with the addition of oxygen or air. Lignin and hemicellulose are solubilised resulting in increased digestibility of the remaining cellulose. Phenolic compounds from the lignin are degraded to carboxylic acids, while the formation of HMF and furfural is low during wet oxidation compared to steam pretreatment

and LHW pretreatment (Alvira et al., 2010). As the lignin is solubilised to a great extent, it is not possible to use it as a solid fuel. Also, the high costs of oxygen and catalyst are considered a challenge (Alvira et al., 2010). Wet oxidation is mainly suited to materials with a low lignin content, and does not work very efficiently on more lignified woody biomass (Galbe and Zacchi, 2007).

3.1.2 Assessment of pretreatment

Pretreatment can be assessed in different ways. Analysis of the sugar content in the solid and liquid fractions before and after pretreatment gives an indication of the degree of sugar recovery. To assess the digestibility of the cellulose (and sometimes the hemicellulose) after pretreatment, enzymatic hydrolysis or SSF is usually performed on the pretreated material. This can be done under various process conditions (substrate concentration, enzyme dosage, yeast dosage, temperature, stirring speed, retention time, etc.), using either washed or whole pretreated slurry, which complicates comparisons between different pretreatment methods carried out under different conditions. In an attempt to overcome this problem, pretreatment experiments have been performed on the same material using the same conditions, as well as calculations of yields and digestibility, for example, in the CAFI study (Wyman et al., 2005). This facilitates comparisons between different methods, but these are limited to the conditions defined in the project. For example, in the CAFI study, the initial glucan concentration was 1%, which is far from realistic in an industrial process.

A common way of assessing pretreatment with enzymatic hydrolysis is to use washed material at a solids concentration of about 2% water-insoluble solids (WIS) (about 1% cellulose). This provides a measure of the maximum digestibility of the pretreated substrate, but gives little information on the digestibility under more realistic process conditions. In a real full-scale process, it is assumed that the whole pretreated slurry would be used to avoid the need for further separation steps, and that the process would be run at higher solids loadings to minimize energy demands and the cost of product recovery after fermentation (Galbe and Zacchi, 2007). To assess pretreatment under more realistic process conditions, enzymatic hydrolysis or SSF should, therefore, be performed using the whole pretreated slurry at higher solids loadings (e.g. 10% WIS). To assess the effect of inhibitors in the pretreated material on the fermentation organism, fermentation tests are usually performed on the liquid fraction of the pretreated material. These tests can be performed on the liquid fraction obtained directly after pretreatment, or after dilution to that used in the final process. Performing SSF as an assessment of pretreatment, provides information about the pre-

treatment efficiency resulting from synergistic effects of enzymes and yeast. For example, Tengborg et al. (2001b) showed that the fermenting yeast in SSF detoxified the liquid for the enzymes by converting inhibitors to less toxic compounds. This gives a more complete, but also a more complex, picture of the production of ethanol from the material after pretreatment. To further complicate the assessment of pretreatment, different pretreatment methods and conditions affect the rest of the process steps, and all these process steps should, therefore, be optimized simultaneously under real process conditions (Galbe and Zacchi, 2007). Furthermore, most of the results from different pretreatment studies have been obtained using batch-operating equipment on a small scale, and the results may well be different on a larger scale.

3.1.3 Softwood pretreatment

Some of the pretreatment methods described above may be suitable for use in an industrial process. Pretreatment using ionic liquids is still on a research level and many fundamental issues need to be resolved before this can be considered a potential method in a real full-scale process. Methods that involve high costs in the form of energy demand, chemical recovery or slow hydrolysis rates (e.g. biological pretreatment, mechanical communition, organosolv and ozonolysis) are not considered interesting options in a full-scale process today. The most promising pretreatment methods for a full-scale process are the chemical and thermochemical options (Chandra et al., 2007; Alvira et al., 2010), which involve the addition of an acid or a base. Alkali, liquid hot water, ammonia fiber explosion and wet oxidation pretreatment work well on materials with a low lignin content, but have been found not to be suitable for more lignified materials such as softwood (see Section 3.1.1). There is thus a wide choice of pretreatment methods for low-lignin materials such as agricultural residues and herbaceous crops. Due to the high lignin content of wood, dilute acid pretreatment and steam pretreatment are the only feasible pretreatment methods in a full-scale softwood-to-ethanol process today. Steam pretreatment with the addition of an acid catalyst (H_2SO_4 or SO_2) is considered the most suitable option for obtaining high sugar yields from softwood (Hahn-Hägerdal et al., 2006; Chandra et al., 2007). This is also the pretreatment method closest to commercialization, being used in several pilot plants in Sweden, Denmark, France, Spain and Canada (Hahn-Hägerdal et al., 2006; Larsen et al., 2008; Iogen Corporation, 2013; Sekab, 2013; US DOE Bioethanol Pilot Plant, 2000). Steam pretreatment with the addition of SO_2 was used in the present work.

3.2 Hydrolysis

The hemicellulose in softwood is solubilised as a result of acid-facilitated pretreatment giving a slurry in which the solid part consists mainly of cellulose and residual lignin, and the liquid fraction contains mainly solubilised mannose with small amounts of xylose, galactose, arabinose and glucose (Table 3.1). The remaining cellulose can be further hydrolysed using acids or enzymes to form monomeric glucose.

Acid hydrolysis of cellulose is an old process developed in the 19^{th} and 20^{th} centuries. Dilute acid hydrolysis is carried out with an acid concentration in the range of 1-5% and requires high temperatures (160-230°C) and high pressures (about 10 atm), with retention times of seconds to minutes. It is efficient in hydrolysing the hemicellulose, but only results in low cellulose hydrolysis yields (Badger, 2002; Kumar et al., 2009; Balat, 2011). Concentrated acid hydrolysis, on the other hand, is able to hydrolyse cellulose almost completely with an acid concentration of about 10-30% (Wyman, 1994; Kumar et al., 2009). Concentrated acid hydrolysis can be performed at moderate temperatures (below 50°C) and atmospheric pressure, but requires longer retention times. In acid hydrolysis, part of the glucose is further broken down to HMF and xylose is broken down to form furfural. This constitutes a loss of sugar that could have been fermented to ethanol. In order to achieve a cost-competitive process, strong acids such as sulphuric acid should be used (Wyman, 1994). Sugar degradation is somewhat less in concentrated acid hydrolysis than in dilute acid hydrolysis (Balat, 2011), but the large quantities of acid needed make the recovery of a substantial amount of these acids necessary for economically feasible operation (Wyman, 1994; Balat, 2011). When using acid hydrolysis in the production of ethanol from lignocellulosic biomass, pretreatment and hydrolysis can be carried out in a single step.

Compared to acid hydrolysis, enzymatic hydrolysis is highly specific and is carried out under milder conditions (about 50°C and pH 5). Other advantages of enzymatic hydrolysis over acid hydrolysis are the fact that enzymes are biodegradable and environmentally benign (Wyman, 1994), and hydrolyse cellulose without the formation of by-products that inhibit enzyme or yeast activity (Kumar et al., 2009). One drawback of enzymatic hydrolysis is the fact that it requires much longer retention times than acid hydrolysis. In the work presented in this thesis, the pretreated spruce was hydrolysed using cellulolytic enzymes, and only enzymatic hydrolysis will be discussed below.

Table 3.1: Composition of steam-pretreated spruce used in the presented work

	Paper I (batch 1)	**Paper I** (batch 2)	**Paper II** (batch 1)	**Paper II** (batch 2)	**Paper III**	**Paper IV**	**Paper V** (batch 2)
Composition of washed fibers, expressed as wt% of dry matter							
Glucan	57.1	47.8	46.7	53.4	50.6	49.0	49.0
Mannan	2.3	5.3	1.9	0.7	0.0	0.0	0.0
Xylan	1.4	0.9	1.6	0.4	0.0	0.0	0.0
Galactan	0.6	0.3	1.2	0.1	0.0	0.0	0.0
Arabinan	0.4	0.0	1.2	0.0	0.0	0.0	0.0
Lignin	53.1	45.4	44.9	45.3	49.9	48.2	46.8
Total sugar concentrations in the prehydrolysate (g/L)							
Glucose	31.5	29.7	13.3	27.1	36.7	34.8	26.3
Mannose	30.0	27.9	22.1	28.7	27.8	28.6	21.7
Xylose	11.3	8.9	8.6	11.4	12.2	13.2	23.2
Galactose	4.7	4.4	3.6	4.5	5.6	6.6	8.4
Arabinose	2.4	0.0	0.0	0.0	3.5	0.0	0.0

3.2.1 Cellulases and their action

Microorganisms, including several bacteria and fungi, produce multiple enzymes, so-called enzyme systems, which degrade plant cell walls. Within the enzyme group of glycoside hydrolases (GHs), which hydrolyse the bonds between carbohydrates, cellulases are enzymes that cleave glycosidic β-1-4 bonds, such as the ones found in cellulose. The three most important activities in cellulase systems are described below.

- **Exoglucanases or cellobiohydrolases (EC 3.2.1.91)** act on the reducing and nonreducing ends of the cellulose chains respectively, producing either glucose (glucanohydrolases) or cellobiose (cellobiohydrolases). Exoglucanases are responsible for the solubilisation of solid cellulose, and play a minor role in changing the chemical properties of residual solid cellulose.
- **Endoglucanases (EC 3.2.1.4)** cleave cellulose chains at random internal amorphous sites, decreasing the degree of polymerization of cellulose, generating shorter oligomers and creating new reducing and nonreducing ends. Endoglucanases are thought to be mostly responsible for the chemical changes in solid cellulose, and contribute less to the solubilisation of cellulose.
- **β-glucosidases (EC 3.2.1.21)** complete hydrolysis by splitting cellobiose molecules into glucose monomers.

The different cellulases in a cellulase system act synergistically, making their collective activity higher than the sum of the individual activities. Synergism between endo- and exoglucanases is quantitatively the most important for the hydrolysis of crystalline cellulose, and is the most widely studied form of enzyme synergism. In endo-exo synergism, endoglucanases cleave cellulose chains, creating new substrate for exoglucanases. Eriksson et al. (2002b) showed that a significant contribution to the synergy between exoglucanase Cel7A (formerly CBHI) and endoglucanase Cel7B (formerly EGI) is due to the cellulases removing obstacles from the cellulose chain for each other, and in this way liberating unproductively bound enzymes. Zhang and Lynd (2004) suggested that the degree of polymerization plays an important role in determining whether the degree of synergism for a substrate is large or small, but found it difficult to draw any conclusions about the crystallinity index. This could be partly due to the difficulty in attributing certain changes in a substrate to the crystallinity index alone, since most forms of pretreatment also change other properties, such as the available surface area. Exoglucanases that attack the reducing ends of the cellulose

chain also exhibit synergism with those attacking the nonreducing ends (Himmel et al., 1996). β-glucosidases remove cellobiose units liberated by exoglucanases, decreasing end product inhibition of the exoglucanases.

Most carbohydrate hydrolases contain a carbohydrate-binding module (CBM), which brings the catalytic module in closer contact with the substrate and ensures correct orientation. The presence of CBMs is especially important in the hydrolysis of an insoluble substrate such as cellulose (Suurnäkki et al., 2000; Zhang and Lynd, 2004). These CBMs have a distinct specificity to certain regions of the polysaccharide chain, bringing enzymes closer to their target, thus enhancing enzymatic hydrolysis compared to that without a CBM (Lynd et al., 2002). Besides bringing the active site of cellulases close to the cellulosic substrate, some CBMs have shown the ability to disrupt the crystalline structure of cellulose non-catalytically, enhancing the degradative capacity of the catalytic module (Boraston et al., 2004; Arantes and Saddler, 2010; Gourlay et al., 2012). Mandels and Reese introduced the C_1-C_x concept for the enzymatic hydrolysis of cellulose over 50 years ago (Mandels and T, 1964). In this concept, the catalytic breakdown of cellulose by cellulolytic enzymes (the C_x factor) is preceded by the C_1 factor which opens up the crystalline structure of cellulose. Due to the difficulty in measuring the effectiveness of the C_1 factor, this was not studied in detail for many years. In 1985, the swelling factor C_1 was connected with the reaction of amorphogenesis, where crystalline cellulose is opened up, increasing the amount of cellulose directly accessible to the enzymes (Coughlan, 1985). During amorphogenesis, the structure of cellulose is opened up, while the microfibrils remain molecularly almost unchanged, and no significant amount of soluble sugars is released (Arantes and Saddler, 2010; Gourlay et al., 2013). This results in a decrease in particle size and a corresponding increase in cellulose accessibility due to increased surface area (Jäger et al., 2011). Some CBMs have been shown to result in amorphogenesis of crystalline cellulose by weakening and splitting hydrogen bonds. It has been suggested that CBMs adsorb onto cellulose defects such as micro-cracks and then penetrate the cellulose structure as a result of mechanical action (Arantes and Saddler, 2010). This opens up the crystalline structure of cellulose and enables water molecules to enter the microfibrillar structure of cellulose. Plant-derived proteins called expansins also have the ability to open up the cellulose structure through non-catalytic action (Arantes and Saddler, 2010). Expansin-like proteins produced by fungi and bacteria, such as swollenin produced by *Trichoderma reesei*, have shown similar effects on various cellulose substrates (Jäger et al., 2011) and steam pretreated corn stover (Gourlay et al., 2013). In enzymatic hydrolysis of steam-pretreated corn stover, swollenin has been shown to affect both cellulose and hemicellu-

lose. Although the exact mechanism of amorphogenesis by different proteins is not yet known, the fact that most of these swelling agents possess a (potential) CBM suggests that this binding module plays a significant role in the non-catalytic amorphogenesis activity (Arantes and Saddler, 2010).

Free fungal enzymes are promising candidates for the deconstruction of lignocellulose as they can secrete proteins at high titres (more than 100 g/L), and their enzyme cocktails are efficient in biomass deconstruction (Chundawat et al., 2011). The fungi most studied with regard to industrial cellulose-degrading enzymes is *T. reesei*, which produces at least two exoglucanases (Cel7A (formerly CBHI) and Cel6A (formerly CBHII)), five endoglucanases (Cel7B (formerly EGI), Cel5A (formerly EGII), Cel12A (formerly EGIII), Cel61A (formerly EGIV) and Cel45A (formerly EGV)), two β-glucosidases (Cel3A (formerly BGLI) and Cel1A (formerly BGLII)), as well as various hemicellulases. Cellobiohydrolase activity is essential for cellulose hydrolysis, and Cel7A and Cel6A represent 60% and 20%, respectively, of the total cellulase protein produced by *T. reesei*. Both cellobiohydrolases are slow in reducing the degree of polymerization in cellulose (Lynd et al., 2002). The endoglucanases, which represent less than 20% of the total cellulase protein in T. reesei, are thought to be mainly responsible for the reduction of the degree of polymerization in cellulose. The main product of Cel7A and Cel6A, cellobiose, inhibits the activity of both cellobiohydrolases and endoglucanases, and a high β-glucosidase activity is therefore required to remove cellobiose, and thus increase hydrolysis efficiency. *T. reesei* produces β-glucosidases but these remain bound to the cell wall and are thus not effective in the industrial hydrolysis of cellulose. Only 0.5% of the protein mixture secreted by *T. reesei* is β-glucosidase (Merino and Cherry, 2007), and cellulase mixtures used for cellulose saccharification on an industrial scale are most often supplemented with β-glucosidase from *Aspergillus* (Lynd et al., 2002; Zhang et al., 2006).

3.2.2 Factors influencing enzymatic hydrolysis of lignocellulose

Enzymatic hydrolysis is affected by substrate properties as well as process parameters. In the hydrolysis of pretreated lignocellulose, the initial hydrolysis rate is usually high, but declines after a certain time.

For successful enzymatic hydrolysis of lignocellulose, the cellulases must be able to access the glycosidic bonds in cellulose, and the total available surface area of the substrate is thus an important factor. Exactly what constitutes the "available" surface area is, however, still under debate (Lynd et al., 2002). Although many pores in the cellulose structure are large enough to accommodate

a cellulolytic enzyme, synergism between more than one enzyme may be necessary to hydrolyse the cellulose effectively, and the pore volume may not be able accommodate more than one enzyme. The degree of polymerization and crystallinity also affect the hydrolysis rate (Lynd et al., 2002). It is often difficult to determine the extent to which the physical and chemical properties of cellulose influence enzymatic hydrolysis rates, partly due to the variation in size and shape of individual particles. Furthermore, pretreatment often influences more than one property. It is, for example, not uncommon to see an increase in the enzymatic digestibility of cellulose following different methods of pretreatment, one of which increases the degree of crystallinity while the other reduced the degree of crystallinity.

The amount of lignin remaining in the solid material is a major obstacle in enzymatic hydrolysis, as it prevents cellulases from accessing the cellulose (Mooney et al., 1998; Merino and Cherry, 2007), and binds unproductively to the cellulolytic enzymes (Berlin et al., 2005b, 2006; Jørgensen et al., 2007a; Várnai et al., 2010; Rahikainen et al., 2011; Sipos et al., 2011). Enzymatic activity is lost as a result of unproductive binding of enzymes to lignin, especially at higher temperatures such as those commonly used in enzymatic hydrolysis (Rahikainen et al., 2011). Lignocellulosic materials differ in carbohydrate composition and lignin content, and the influence of lignin on enzymatic hydrolysis is thus more pronounced in lignin-rich materials. In some cases, it can be advantageous to delignify the pretreated biomass before enzymatic hydrolysis. Residual lignin in steam-pretreated spruce has been identified as the main reason for reduced yield in enzymatic hydrolysis, while it has been found to be possible to hydrolyse delignified spruce almost completely (Várnai et al., 2010). Although delignification is possible on lab scale, it involves extra costs arising from the addition of an extra process step, as well as the risk of loss of sugar. It may still be an interesting process option if the lignin can be recovered as a valuable by-product (Jørgensen et al., 2007a). Some methods of pretreatment remove a significant part of the lignin present in the biomass. The unproductive binding of enzymes to lignin can be reduced and hydrolysis yields increased, especially in highly lignified substrates, by the addition of surfactants (Eriksson et al., 2002a; Kristensen et al., 2007), polymers such as polyethylene glycol (PEG) (Börjesson et al., 2007; Kristensen et al., 2007; Sipos et al., 2011) or proteins like bovine serum albumin (BSA) (Yang and Wyman, 2006; Kristensen et al., 2007; Brethauer et al., 2011; Wang et al., 2013). Not only the lignin content but also the chemical structure of the lignin in the substrate affects the increase in hydrolysis efficiency resulting from additives (Berlin et al., 2006; Kristensen et al., 2007; Rahikainen et al., 2011; Sipos et al., 2011). Unproductive binding of enzymes to lignin is affected

not only by the affinity of the lignins to the enzymes used, but also the available surface area (Rahikainen et al., 2011). Softwood lignins have shown stronger inhibition on enzymatic hydrolysis than other lignocellulosic materials (Nakagame et al., 2010). The presence of a CBM on cellulolytic enzymes also has a significant impact on unproductive binding of the enzymes to lignin (Palonen et al., 2004). Reduction of unproductive binding of enzymes to lignin not only improves enzymatic hydrolysis, but also increases the possibility of enzyme recycling (Sipos et al., 2011). Enzymatic hydrolysis rates and yields can differ significantly between hydrolysis of the whole pretreated slurry and the washed slurry due to the presence of compounds inhibiting enzymatic hydrolysis. Inhibition of enzymatic hydrolysis is discussed in more detail in Section 4.1.1.

The choice of pretreatment method has a significant impact on the subsequent enzymatic hydrolysis. Enzymatic hydrolysis of pretreated lignocellulosic biomass usually focuses on hydrolysis of the cellulose fraction. However, at low degrees of severity, less hemicellulose is solubilised during pretreatment, and hemicellulases may also be needed, as well as cellulases, to completely break down the lignocellulose structure. The addition of hemicellulases, mostly xylanases and mannanases, has been shown to increase both hemicellulose and cellulose hydrolysis (Berlin et al., 2005a; Várnai et al., 2010, 2011). In materials containing substantial amount of lignin, for example steam pretreated spruce, residual lignin affects the enzymatic hydrolysis more than residual hemicellulose and the effect of hemicellulase addition on enzymatic hydrolysis yield may be less pronounced (Várnai et al., 2010, 2011).

The optimal temperature for *T. reesei* cellulases is about 50°C, but decreases with increased retention time (Eklund et al., 1990; Tengborg et al., 2001a). This is probably due to increased enzyme deactivation at higher temperatures over longer time (Eklund et al., 1990; Jørgensen et al., 2007a). Longer retention times are, however, often needed in order to limit enzyme dosage and reduce production costs without suffering a reduction in enzymatic hydrolysis yield. The optimal temperature for enzymatic hydrolysis of lignocellulosic biomass is also dependent on the pH (Tengborg et al., 2001a). Part of the enzymatic hydrolysis is often performed simultaneously with fermentation. The fermentation organism used then determines the pH and temperature of the process; these are often not the same as the optimum pH and temperature for the enzymes.

It is desirable to perform enzymatic hydrolysis and fermentation at higher solids concentrations in order to obtain a more concentrated ethanol solution after fermentation. However, high substrate concentrations are associated with problems due to product inhibition of the cellulolytic enzymes, higher lignin concentrations and mass transfer limitations. Enzymatic hydrolysis and fermenta-

tion at high solids concentrations is discussed in more detail in Chapter 4. Increased shear rate, due to increased agitation of the material, has been shown to result in increased enzyme deactivation (Reese and Ryu, 1980). Increased agitation can, however, also have a positive effect on cellulose hydrolysis yields. The effect of agitation and mixing on the enzymatic hydrolysis of cellulose is discussed in more detail in Section 4.2.

3.2.3 Improving cellulase action

Extensive research has been carried out to improve the performance of *T. reesei* enzymes used in the production of fermentable sugars from lignocellulosic material since the 1950s. This involves screening for new enzyme-producing microorganisms, the modification of enzyme-producing fungi to increase the productivity of the desired enzymes and to make the fungi produce enzymes not usually produced. Research is also carried out on improvements to existing enzymes by rational design or directed evolution (Zhang et al., 2006; Banerjee et al., 2010; Chundawat et al., 2011) and the addition of new accessory enzymes to the enzyme cocktails (Chundawat et al., 2011).

The development of enzymes for the cost-effective production of ethanol from lignocellulosic biomass is a challenge due to the high enzyme loadings required and the low value of the final product, ethanol (Merino and Cherry, 2007). The cost of enzyme production can be reduced by increasing the productivity of the fungal strains producing the enzymes and reducing the cost of the carbon and nitrogen sources in the fermentation stage. Expressing all the enzymes needed in the final product in one organism also has the potential to reduce production costs.

In screening for new enzymes, the main advantage of directed evolution is that it requires no knowledge of enzyme structure and mechanism. However, it is less suited for the improvement of enzyme activity, but is widely used for the improvement of thermal stability (Chundawat et al., 2011). Furthermore, it is not sure whether it is always possible to obtain an enzyme with maximal effectiveness through a natural selection process, and sometimes rational design through protein engineering may yield more effective enzymes (Banerjee et al., 2010). Besides the lack of detailed knowledge of hydrolysis mechanisms for many enzymes involved in the hydrolysis of lignocellulose, other aspects such as protein secretion, inhibition, synergism and thermal stability, need to be considered. It is a complex matter to engineer enzymes through rational design with maintaining these properties simultaneously. The discovery and production of new enzymes leads to thousands of cellulases, hemicellulases, ligninases, lyases,

pectinases and esterases whose function and relative activities are unknown and need to be studied. According to Banerjee et al. (2010), it is currently not gene discovery in enzyme structures that is the limiting factor in the search for new and better enzymes but rather the capacity to evaluate the activities encoded by these genes. Unfortunately, there is no reliable way to predict enzyme performance on the basis of amino acid sequences (Banerjee et al., 2010), and new cellulases need to be tested, preferably on real industrial substrates since results obtained on one substrate can not always be transferred to another one (Zhang et al., 2006). Some enzymes that attack certain bonds in a synthetic substrate are unable to approach the same bonds in a real lignocellulosic substrate (Banerjee et al., 2010). In contrast, enzymes that show no effect on pure cellulose may have a positive effect on real lignocellulosic biomass, for example because they enhance cellulose degradation by attacking the lignin or hemicellulose fraction in lignocellulose (Merino and Cherry, 2007). Although today's commercial enzyme mixes are often optimized for a specific substrate, better enzyme mixes for differently pretreated materials could be developed using the material used in the process also for the optimization of the enzyme mix (Zhang et al., 2006; Merino and Cherry, 2007; Banerjee et al., 2010).

Since many enzyme cocktails are sold on a protein mass basis, research is often focused on increased specific activity per unit mass of protein, either by increasing the specific activity of certain enzymes, or by finding accessory enzymes that result in increased synergy (Banerjee et al., 2010). Therefore, the specific activity of enzymes needed for the deconstruction of lignocellulose needs to be increased, but also enzymes not participating must be identified and eliminated from the protein secretion palette of the host organism. Accessory enzymes, their function and need as well as synergistic effects between different enzymes are other aspects that are being investigated (Zhang et al., 2012). One example of the alteration of cellulase cocktails where the addition of new accessory enzymes has significantly increased the specific activity of the enzyme mixture is the discovery of copper-dependent proteins (AA9, formerly GH61) which are able to cleave cellulose chains by oxidation (Merino and Cherry, 2007; Harris et al., 2010; Wilson, 2012). These can more than double the total specific activity of an enzyme mix when less than 5% AA9 protein is added, despite the fact that AA9 shows no significant activity when used alone (Merino and Cherry, 2007; Harris et al., 2010).

Today, most commercial enzyme preparations used for bioenergy production have fairly similar activities and compositions due to the fact that they have been optimized for acid-pretreated corn stover (Merino and Cherry, 2007; Banerjee et al., 2010). One way to tailor enzyme cocktails for different feedstocks in the

future could be the use of a core mixture of cellulases that is supplemented with different accessory enzymes for different feedstocks (Banerjee et al., 2010). On-site (or near-site) production of enzymes is also being studied as a future option for enzyme production (Merino and Cherry, 2007). This could reduce production costs, but is a challenge due to the complexity of enzyme production. Another alternative is the on-site production of a basic mixture of enzymes, which is supplemented with commercial accessory enzymes or vice versa.

Other enzyme improvements targeted by the research community include modification of CBMs to improve interactions with cellulose, tolerance to end products and other inhibitors, and thermal and pH stability to enable operation at higher temperatures (Jørgensen et al., 2007a; Banerjee et al., 2010; Zhang et al., 2012) or more efficient conversion at low temperatures when enzymatic hydrolysis is performed simultaneously with the fermentation (Merino and Cherry, 2007).

3.3 Fermentation

During the production of ethanol from lignocellulosic biomass, two fermenting microorganisms are usually needed: one to produce the cellulolytic enzymes that liberate monomeric sugars from the lignocellulosic biomass, and one to ferment these sugars to ethanol. This section describes the fermentation of the monomeric sugars in the lignocellulose hydrolysate to ethanol.

3.3.1 Microorganisms fermenting sugar to ethanol

In order to achieve cost-effective conversion of biomass to ethanol, the microorganism used to ferment the monomeric sugars in the hydrolysate to ethanol must give a high ethanol yield and productivity, as well as being resistant to hydrolysates and high ethanol concentrations. In an industrial process, it is also important that the fermenting organism can utilize a broad range of substrates. Other desirable traits include high specific growth rate, low nutrition requirements, high salt tolerance, thermal tolerance and high shear tolerance. To meet these requirements, both improved fermenting microorganisms and better designs of fermentation processes are needed.

There is no single microorganism in nature that can fulfil all these requirements. The yeast *Saccharomyces cerevisiae* has been widely used for ethanol fermentation for centuries. Under anaerobic conditions, it ferments glucose to ethanol, and is known for its superior tolerance to ethanol. Furthermore, it is popular due to its GRAS status (generally regarded as safe by the US Food and

Drug Administration), and has been used successfully in industrial scale fermentation. The main disadvantage of *S. cerevisiae* is its narrow substrate utilization range. *S. cerevisiae* only ferments certain hexose sugars, but can not utilize the pentose sugars in the substrate efficiently as it lacks both a xylose-assimilation pathway and adequate levels of key pentose phosphate pathway (PPP) enzymes (Picataggio and Zhang, 1996).

Zymomonas mobilis is the only microorganism that metabolizes glucose anaerobically using the Entner-Doudoroff pathway (EDP), which yields half as much ATP per mole of glucose as the Embden-Meyerhof-Parnas (EMP) pathway used by *S. cerevisiae*. *Z. mobilis* thus produces less biomass, and more carbon is transferred to the fermentation products. It also has high ethanol tolerance, yields 5-10% more ethanol per gram glucose than e.g. *S. cerevisiae*, has a higher specific ethanol productivity than S. cerevisiae, and has simple nutritional needs (Lin and Tanaka, 2006). It has, therefore, attracted a great deal of attention from researchers studying ethanol production. However, *Z. mobilis* can only be used with a limited number biomass sources, and the more robust *S. cerevisiae* is still preferred in industry (Lin and Tanaka, 2006). Furthermore, *Z. mobilis* cannot utilize pentoses such as xylose (Olsson and Hahn-Hägerdal, 1996).

Escherichia coli has been widely used industrially, mainly for the production of recombinant protein. It is able to ferment a wide spectrum of sugars to ethanol and has low nutrient requirements, but can only be used in a narrow pH range (6.0-8.0), and is less hardy than yeast cultures (Lin and Tanaka, 2006; Balat, 2011). *E. coli* also suffers from adverse public perception due to the risk of bacterial infection.

Various anaerobic thermophilic bacteria are able to convert lignocellulosic biomass to ethanol, but the process has been shown to be very slow with a poor ethanol yield, partly due to the production of high levels of by-products such as acetic and lactic acid (Lin and Tanaka, 2006). Also, anaerobic bacteria have a very low ethanol tolerance (<2%, v/v) (Balat, 2011).

3.3.2 The yeast *Saccharomyces cerevisiae*

S. cerevisiae was used as the fermenting organism in the present work. It is able to grow under both aerobic and anaerobic conditions, at temperatures up to about 40°C and pH 3.5-6, with optimal conditions just above 30°C and pH 5.0-5.2. The metabolism of S. cerevisiae can be divided into respiration and fermentation. In the presence of oxygen, energy is created by respiration. Fermentation takes place under anaerobic conditions. Sugar uptake occurs by facilitated diffusion and *S. cerevisiae* has an advanced system for sugar uptake including at

least 20 transport proteins (Russel, 2003). Some transporter proteins are expressed regardless of sugar concentration, while others are induced at either high or low glucose concentration (Özcan and Johnston, 1995). *S. cerevisiae* is able to ferment the monosaccharides glucose, fructose, mannose and galactose, the disaccharides maltose and sucrose, and the trisaccharides raffinose and maltotriose, depending on the strain (Russel, 2003). Sucrose is the first sugar to disappear from the fermentation medium, followed by glucose. The most important catabolic pathway in *S. cerevisiae* is glycolysis, where glucose is converted to fructose-6-phosphate and then to pyruvate via the glycolytic pathway, also called the EMP pathway (Russel, 2003). In the presence of oxygen and low sugar concentrations, the yeast uses almost all the sugar for the production of energy and to create new cell mass. However, even under aerobic conditions, part of the glucose take the fermentative pathway and are converted to ethanol. This is called the Crabtree effect and occurs mostly at high external glucose concentrations, e.g. during cell cultivation, where it usually is considered a yield loss. Under anaerobic conditions, pyruvate enters the fermentative pathway giving ethanol and carbon dioxide as the main products. Other products include acetic acid and acetaldehyde. The most simplified expression for the fermentation of glucose and mannose to ethanol is:

$$C_6H_{12}O_6 + 2\ P_i + 2\ ADP \rightarrow 2\ C_2H_5OH + 2\ CO_2 + 2\ ATP$$

The maximum theoretical yield of ethanol from glucose in *S. cerevisiae* is 0.51 g ethanol/g glucose (2 moles ethanol/mole glucose). The same yield is obtained from mannose. The above equation does not take into account the fact that, even under anaerobic conditions, some sugar will be used for cell maintenance and growth, and the production of by-products such as glycerol and lactic and succinic acid (Russel, 2003). Together with glucose and fructose, mannose is a rapidly fermentable sugar by *S. cerevisiae* (Klein et al., 1998). It is taken up by the yeast and converted to fructose-6-phosphate and then follows the same path as glucose in the metabolic pathway (Figure 3.2).

The most common bacterial contamination in yeast fermentation is lactobacilli. These are very tolerant to ethanol and produce lactic and acetic acid. Bacterial contamination means a loss of fermentable sugar and thus a loss of ethanol. For every mole of lactic acid produced, one mole of ethanol is lost. Contamination by lactobacilli is often observed in SSF of washed fibres (e.g. **Paper II**) (Figure 3.3). The inhibitors present in the prehydrolysate, i.e. the liquid fraction of the biomass after pretreatment, thus also fulfil a positive function, by

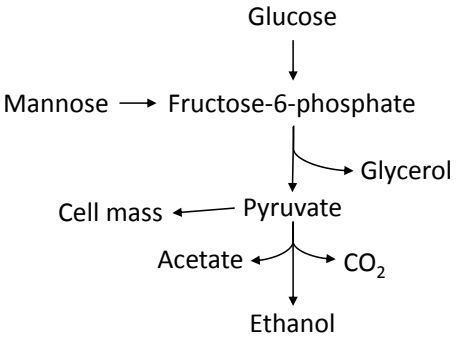

Figure 3.2: Simplified illustration of the glycolytic pathway (Embden-Meyerhof-Parnas pathway) in *S. cerevisiae*.

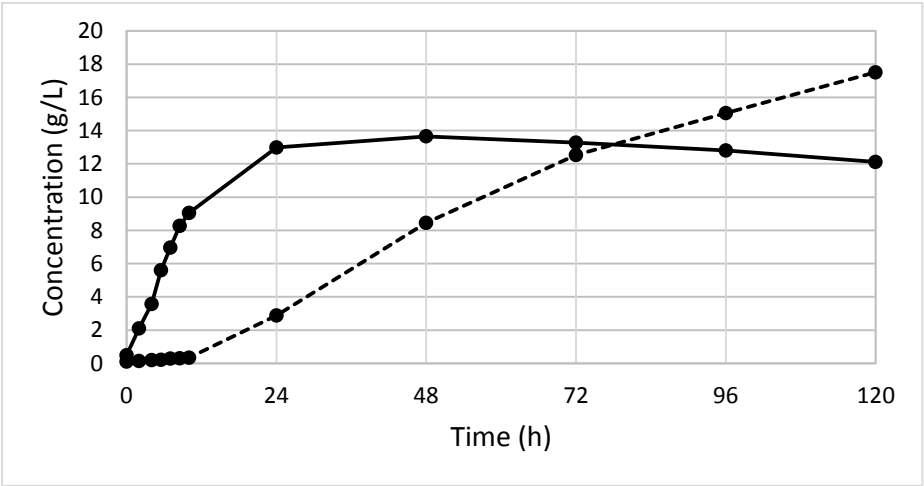

Figure 3.3: Concentrations of ethanol (solid line) and lactic acid (dashed line) in SSF with washed steam pretreated spruce with 14% WIS (**Paper II**). Production of lactic acid is a result of contamination by lactic acid bacteria.

repressing the growth of unwanted microorganisms. Reducing contamination is important on industrial scale, and it is thus desirable to perform fermentation on the unwashed fibres. Also, excluding a washing step simplifies the process. Most of the research presented in this thesis was, therefore, performed on whole pretreated spruce material.

The viability of the yeast used in fermentation is often measured by counting the number of yeast cells in a defined volume of yeast slurry. However, this only

provides a measure of the number of living yeast cells, and does not give any information on the expected performance of the yeast. Unfortunately, there is no accurate, simple inexpensive and quick method of testing yeast vitality (i.e. the ability of the yeast to ferment quickly and efficiently rather than just being alive). Therefore, yeast performance if often assessed using reference fermentation. In the fermentation of sugars in pretreated lignocellulosic material, this can be fermentation of the liquid hydrolysate. An example of this is presented in **Paper III**.

3.3.3 Research in fermentation of lignocellulosic hydrolysates

The challenge in fermenting the sugars in lignocellulosic hydrolysates to ethanol can be divided into two parts. First, lignocellulosic hydrolysates constitute a difficult medium for yeast due to the presence of inhibitors and the high osmotic pressure arising from the high sugar and solids concentrations, which reduce yeast performance. Second, it is important to utilize as much of the substrate as possible, but it is difficult to convert pentose sugars such as xylose to ethanol using *S. cerevisiae*.

Inhibitory compounds

When hemicellulose is degraded, for example during acid-facilitated pretreatment such as steam-pretreatment, xylose, mannose, galactose, glucose and acetic acid are liberated. Part of the cellulose may also be broken down to glucose. At high temperatures and pressures, some of these compounds are further broken down into compounds inhibitory to *S. cerevisiae*, including furans, weak acids and phenols.

Some hemicellulose in lignocellulosic biomass is highly acetylated, and acetic acid is liberated when this hemicellulose is broken down during pretreatment. Softwood hemicellulose is less acetylated than the hemicellulose in most agricultural residues, and prehydrolysates thus contain less acetic acid. Xylose can be degraded to furfural while hexose sugars are degraded to HMF. HMF may be further broken down to levulinic and formic acid. Furfural can also be broken down to formic acid. Furthermore, several phenolic compounds such as vanillin, guaiacol, coniferyl and syringaldehyde are released as a result of lignin degradation (Jönsson et al., 1998; Larsson et al., 1999b; Kothari and Lee, 2011). The effect of these compounds on fermentation of lignocellulosic biomass is discussed in detail in Section 4.1.2.

Fermentation with high concentrations of insoluble solids

Increased solids concentration in the production of ethanol from lignocellulosic material results in increased sugar concentrations after enzymatic hydrolysis, as well as increased ethanol concentrations. The increase in osmotic pressure as a result of high sugar concentration, and the high ethanol concentration constitute stress factors for the yeast. These stress factors can be more or less pronounced depending on the kind of lignocellulosic material being used. Mutturi and Lidén (2013) reported that the loss of fermentation capacity due to high temperature (39°C) was more pronounced in steam-pretreated spruce than in similarly treated giant cane (*Arundo donax*). These stress factors can also act synergistically (Russel, 2003). Yeast produces glycerol to protect itself from hyperosmotic conditions and heat shocks, and to maintain redox balances. Glycerol is the most abundant product of anaerobic yeast fermentation after ethanol and carbon dioxide. In the production of ethanol from lignocellulosic biomass, glycerol production is especially pronounced at the high solids concentrations that should result in high sugar concentrations during fermentation. This is further discussed in Section 5.3.

Pentose fermentation

One of the greatest challenges in the fermentation of lignocellulosic hydrolysates to ethanol is the utilization of all the sugars. The cost of the feedstock often represents a considerable proportion of the ethanol production cost using lignocellulosic biomass, and it is therefore important to use a microorganism that can also ferment the pentose fraction (Picataggio and Zhang, 1996). In agricultural residues, for example, the pentoses, mostly xylose, often constitute about 30% of the sugar in the material. The theoretical ethanol yield can thus be increased significantly when utilizing all sugars in contrast to only utilizing the hexose sugars, as illustrated in Table 3.2. The greater the amount of sugar that can be fermented, the higher the final concentration of ethanol, and the lower the cost of product recovery, as discussed below. The few naturally occurring microorganisms that are able to ferment all the sugars in lignocellulosic biomass to ethanol generally grow slowly, resulting in marginal ethanol yields and productivity (Olsson and Hahn-Hägerdal, 1996; Picataggio and Zhang, 1996). *S. cerevisiae* provides reasonable ethanol yields from the fermentation of xylose, but the process is slow (Gong et al., 1981). Therefore, attempts have been made to improve the ability of this, and similar microorganisms, to ferment pentoses using metabolic engineering (Olsson and Hahn-Hägerdal, 1996; Kumar et al., 2009). However, microorganisms that can ferment xylose to ethanol in laboratory media, may not necessarily ferment lignocellulosic hydrolysates efficiently

on a large scale and the genetic modification of organisms to increase xylose fermentation is often a matter of trial and error (Olsson and Hahn-Hägerdal, 1996).

Table 3.2: Theoretical ethanol yields from different lignocellulosic materials using *S. cerevisiae* (L/dry metric ton)

	From hexoses	From pentoses
Spruce[1]	421	60
Corn stover (calculated using Öhgren et al. (2007))	290	220
Salix (calculated using Sassner et al. (2005))	335	148

[1]Calculated from the average sugar contents reported in **Papers I** and **II**

3.4 Product recovery

The recovery of ethanol after fermentation is usually performed by distillation or a combination of distillation and evaporation. To allow blending of ethanol with gasoline, the water content must be reduced to less than 1% to avoid alcohol/water separation (Willkie et al., 2000). This is achieved by dehydration or drying, e.g. by molecular sieving. The residue after distillation contains residual lignin, untreated cellulose and hemicellulose, as well as enzymes and yeast (Kumar et al., 2009).

Distillation and evaporation are the process steps with the highest energy demand in the production of ethanol from softwood (Galbe et al., 2007; Wingren et al., 2008). Steam pretreatment also requires a great deal of energy in the form of high-pressure steam, but also produces significant amounts of secondary steam that can be used to replace some of the primary steam needed in distillation and evaporation (Wingren et al., 2008). Process intensification and heat integration in distillation and evaporation can potentially reduce the energy demand and thus the production cost (Galbe et al., 2007; Wingren et al., 2008). Significant energy savings can also be achieved in distillation by increasing the ethanol concentration in the feed (see Figure 3.4). In order to achieve a high ethanol concentration after fermentation, high substrate concentrations must be used. This was the main objective of the research presented in this thesis. The enzymatic hydrolysis and fermentation of high solids lignocellulosic material is discussed in more detail in Chapters 4 and 5.

Only part of the lignocellulosic raw material consists of carbohydrates that can be converted to ethanol. A significant fraction of the softwood raw material

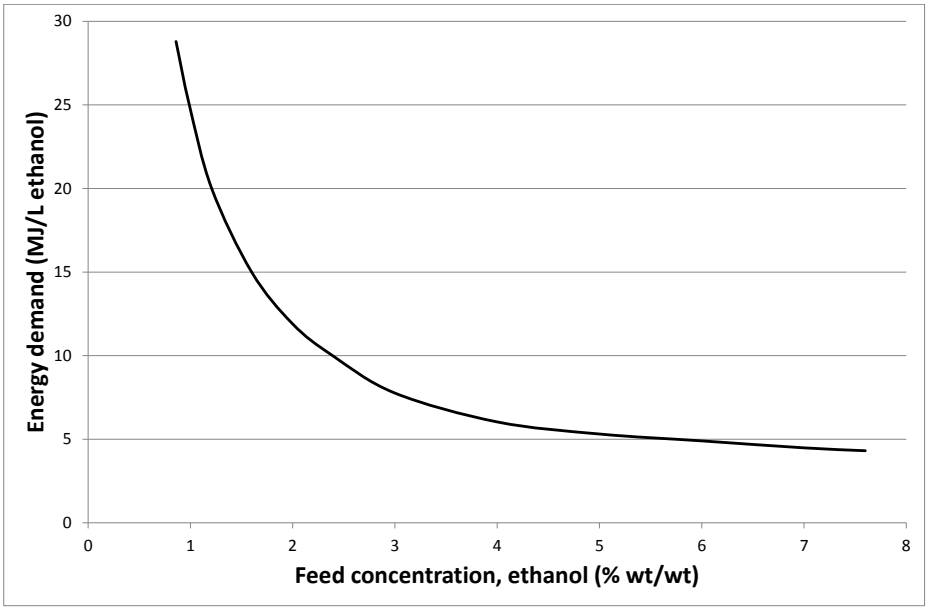

Figure 3.4: Energy demand in distillation. Adapted from Galbe et al. (2007).

ends up in the solid fraction after product recovery (Wingren et al., 2008). This can be used as solid burning fuel, either directly, after drying and pelletizing, or for the production of heat, steam or electricity. The liquid fraction after distillation can be converted to biogas by anaerobic digestion. In a biorefinery with multiple products besides ethanol, anaerobic digestion of the hemicellulose-rich liquid, after pretreatment, to biogas is also an option. This would also eliminate the need for fermentation of xylose to ethanol.

4

High solids concentrations in enzymatic hydrolysis and fermentation

The aim of the research presented in this thesis was to increase the ethanol concentration after the fermentation step in the production of ethanol from spruce. Increased ethanol concentration in the distillation feed significantly decreases the energy demand in product recovery and thus the production cost. Higher substrate and product concentrations could also potentially result in savings in capital cost since smaller reactors would be needed. The final ethanol concentrations obtained in SSF in the studies described in this thesis are summarized in Figure 4.1. In this, and the following chapter, the methods used to increase the final ethanol concentration after fermentation to 65 g/L, using different configurations of SSF, are discussed in detail.

When discussing enzymatic hydrolysis and SSF at high solids concentration, it is important to bear in mind that the meaning of "high solids concentration" has changed in the relative few years during which this research was performed. When the laboratory study described in **Paper I** was performed, it was common

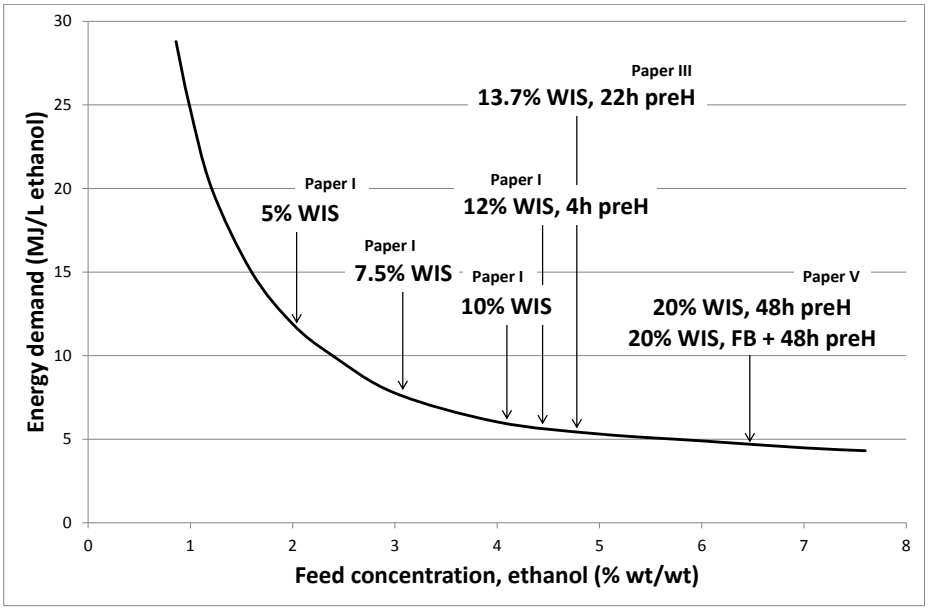

Figure 4.1: The final ethanol concentrations achieved in SSF in the studies presented in this thesis, indicated in the figure published by Galbe et al. (2007) (Figure 3.4 in Section 3.4) (preH = prehydrolysis, FB = fed-batch).

practice to run SSF at 5% WIS, and 10% WIS was considered high. Today, 10% WIS is considered standard, and research focusing on high solids concentrations, is now usually performed at WIS concentrations above 10%.

Enzymatic hydrolysis and fermentation of steam-pretreated spruce at 10% WIS results in final ethanol concentrations slightly above 40 g/L when the sugar in the raw material is almost completely converted to ethanol. When using other lignocellulosic materials that contain more pentose sugars in the hemicellulose fraction, initial WIS concentrations as high as 20% WIS may be required to reach a final ethanol concentration after fermentation of 4% (Larsen et al., 2008; Mutturi and Lidén, 2013). It is thus necessary to increase the concentrations of fermentable sugars to achieve final ethanol concentrations above 4-5%. The most obvious way of increasing the concentrations of fermentable sugars is by increasing the concentration of the carbohydrate substrate, i.e. the solids concentration in the enzymatic hydrolysis and fermentation. However, increasing the concentration of solids has been found to result in a decrease in overall yield in enzymatic hydrolysis (Tengborg et al., 2001a; Mais et al., 2002; Jørgensen et al., 2007b; Kristensen et al., 2009b; Manzanares et al., 2011; Gupta et al., 2012;

Palmqvist and Lidén, 2012) and SSF (Varga et al., 2004; Jørgensen et al., 2007b; Manzanares et al., 2011; **Paper I**). The cost of the raw material constitutes the highest single cost item in the production of ethanol from lignocellulosic biomass, and the overall ethanol yield is the single most important parameter in reducing the production cost (Sassner et al., 2008). Therefore, it is important to increase the substrate concentration and final ethanol concentration without any significant loss of overall ethanol yield. The decrease in yield at high solids concentrations is usually attributed to stirring and mixing difficulties due to the high viscosity of pretreated lignocellulosic slurries, and increased inhibition of the enzymes and yeast due to increased concentrations of inhibitors. End-product inhibition is another factor contributing to decreased hydrolysis yields at high substrate concentrations. When the present research was started, the reason for the decrease in yield in SSF had, however, not been studied in detail. The study presented in **Paper I** showed that stirring and inhibition both have a significant impact on ethanol yield.

4.1 Inhibition of cellulolytic enzymes and yeast

During the hydrolysis of lignocellulosic biomass, various degradation products are formed, as illustrated in Figure 4.2. Some of these compounds may be inhibitory to the enzymes or yeast used in the production of ethanol. Increasing the solids concentration will lead to an increase in the concentrations of these inhibitors. Also, in the industrial production of ethanol from lignocellulosic biomass the process streams will probably be recycled, leading to the accumulation of inhibitors. It is therefore important to understand the mechanisms of inhibition of enzymatic hydrolysis and fermentation in the biomass-to-ethanol process. Inhibitor concentrations in the prehydrolysate after steam pretreatment of the material used in the studies described in this thesis are presented in Table 4.1.

Table 4.1: Inhibitor concentrations (g/L) in the liquid fraction of the pretreated spruce slurries used in this work

	Furfural	HMF	Acetic acid	Formic acid	Levulinic acid
Paper I (batch 1)	1.5	3.1	7.1	n.a.	n.a.
Paper II (batch 1)	0.9	1.9	4.9	n.a.	n.a.
Paper II (batch 2)	1.2	2.7	5.9	n.a.	n.a.
Paper III	2.3	2.8	5.8	n.a.	1.7
Paper IV	2.1	1.8	5.0	1.6	1.7
Paper V (batch 2)	3.0	1.6	8.7	1.6	1.7

n.a. = not analysed

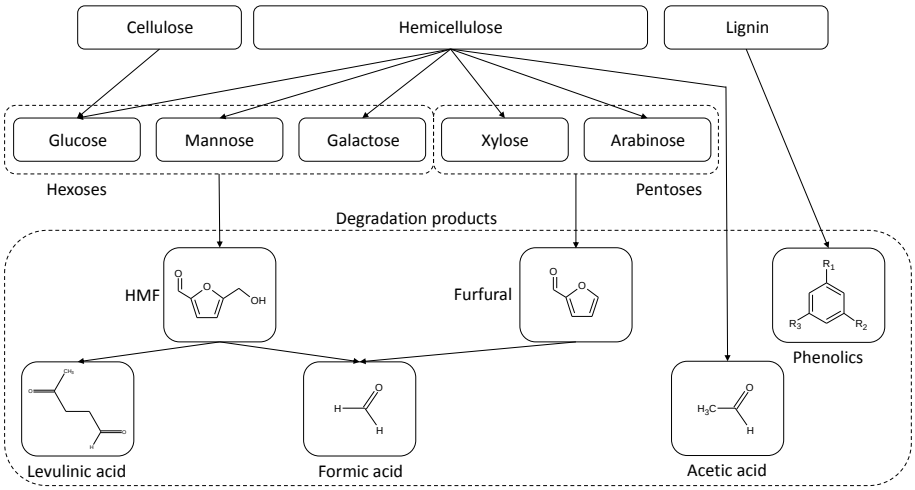

Figure 4.2: Main degradation products formed during the hydrolysis of lignocellulosic biomass.

Determination of the inhibiting effect of compounds present in the prehydrolysate of pretreated lignocellulosic biomass is not straightforward. Inhibition can be determined for single components or mixtures of components. Prehydrolysates, however, are complex mixtures and the complete composition is usually not known. Furthermore, enzymatic hydrolysis can be evaluated in different ways. In this work, yield from enzymatic hydrolysis was assessed as the amount of glucose as a function of total glucose available in the substrate. However, this provides no information on the degradation of the cellulose to shorter polymer chains. The results from inhibition studies carried out with different methods may differ, depending on which enzymatic activity in the cellulase system is inhibited. It is important to bear this in mind when comparing different studies. The fact that it is not possible to completely separate enzymatic hydrolysis and fermentation in SSF further complicates this.

4.1.1 Inhibition of cellulolytic enzymes

The inhibition of cellulolytic enzymes by degradation products has not been as thoroughly studied as the inhibition of *S. cerevisiae*, and divergent and conflicting results have been reported in the literature. It is, however, clear that prehydrolysate from steam-pretreated lignocellulosic biomass such as spruce (Tengborg et al., 2001b), willow (Palmqvist et al., 1996), poplar (Panagiotou and Olsson, 2007) and corn stover (Kothari and Lee, 2011) has an inhibitory effect on

enzymatic hydrolysis. Studies on the effect of single inhibiting compounds on enzymatic hydrolysis have shown that lignin derivatives and sugars have the greatest impact on hydrolysis, followed by organic acids and furans, and finally ethanol (Hodge et al., 2008; Jing et al., 2009; Kothari and Lee, 2011). The concentrations at which these compounds are usually present in lignocellulosic prehydrolysate is roughly the reverse. It is therefore important to consider not only the specific inhibition of a certain substance, but also its concentration in the medium.

Lignin and lignin derivatives, including various aromatic and polyaromatic compounds, inhibit both hydrolysis and fermentation. Lignin can sterically block the cellulose, and cellulases can bind unproductively to lignin, reducing hydrolysis efficiency, as discussed in Section 3.2.2. Enzymatic hydrolysis of cellulose is also inhibited by water-soluble lignin at low concentrations, although the individual compounds responsible for this have not been identified (Kothari and Lee, 2011; Kim, 2012). The lignin derivatives vanillin and syringaldehyde, often found in lignocellulosic prehydrolysates, do not inhibit cellulases significantly at the concentrations usually present (less than 1 g/L) (Jing et al., 2009; Kothari and Lee, 2011). Concentrations of 4-5 g/L are required before inhibition becomes noticeable. However, lignocellulosic prehydrolysates, contain a large number of unknown phenolic substances originating from lignin. The inhibition caused by lignin thus may originate from some of these unknown lignin derivatives. Lignins are a heterogeneous group of polyaromatic compounds, and their inhibitory effects differ depending on the physical and chemical properties of the lignin (Kim, 2012). Water-soluble lignin inhibits especially β-glucosidase activity (Mes-Hartree and Saddler, 1983; Kim et al., 2011). Therefore, increased levels of β-glucosidase will be required in enzymatic hydrolysis with higher solids loadings (Merino and Cherry, 2007).

It is well known that cellulolytic enzymes are end-product inhibited. Cellulases are thus inhibited by cellobiose and glucose, and hemicellulases by e.g. xylose. Pentose sugars, however, also inhibit cellulases, and hexose sugars such as glucose inhibit hemicellulases, but to a lesser extent (Kothari and Lee, 2011). Mannose, xylose and galactose have no significant inhibitory effect on β-glucosidase, but inhibit mainly cellobiohydrolases (Xiao et al., 2004). Glucose inhibits β-glucosidases as well as endoglucanases and cellobiohydrolases (Beltrame et al., 1984; Xiao et al., 2004). Although cellobiose is a more potent inhibitor of cellulases than glucose (Holtzapple et al., 1990), because of high concentrations of glucose, Hodge et al. (2008) identified glucose as the single component with the greatest impact on the enzymatic hydrolysis of dilute-acid-pretreated corn stover because of its high concentration.

Acetic acid is known to inhibit *S. cerevisiae* but has little effect on the enzymatic hydrolysis of cellulose at concentrations up to 25 g/L (Cantarella et al., 2004; Hodge et al., 2008; Jing et al., 2009; Kim et al., 2011). Formic acid has a stronger inhibitory effect on cellulases than acetic acid (Cantarella et al., 2004; Kothari and Lee, 2011), but is present in lignocellulosic hydrolysate at lower concentrations (Kothari and Lee, 2011) (Table 4.1). Minor inhibition of cellulases by levulinic acid has been observed for concentrations up to 29 g/L (Cantarella et al., 2004; Panagiotou and Olsson, 2007).

The inhibition of cellulolytic enzymes by lignocellulosic prehydrolysate was first suspected to be a result of increased levels of furfural and HMF, but this has proven not to be the case for the concentrations usually found in lignocellulosic prehydrolysates (up to about 5 g/L) (Mes-Hartree and Saddler, 1983; Tengborg et al., 2001b; Hodge et al., 2008; Jing et al., 2009; Kothari and Lee, 2011). The concentrations of furfural and HMF in the prehydrolysates used in the present work were well below 4-5 g/L (Table 4.1).

Although different researchers studying the effects of single or several inhibitors on the enzymatic hydrolysis of lignocellulose have come to different conclusions, the general conclusion is that the reduction in hydrolysis yield observed in the enzymatic hydrolysis of the whole pretreated lignocellulosic biomass can not be completely explained by the inhibitors identified and listed above (Palmqvist et al., 1996; Tengborg et al., 2001b; Panagiotou and Olsson, 2007; Kothari and Lee, 2011). This suggests that there are other, unidentified substances in lignocellulosic prehydrolysates, that have inhibitory effects. Lignin is a heterogeneous group of polyaromatic compounds, many of which have not been identified. Some of the substances often classified as "unidentified" may thus be lignins.

Insoluble solids have been reported not to affect enzymatic hydrolysis until a certain concentration is exceeded (Hodge et al., 2008). For steam-pretreated corn stover, this concentration was 25% WIS when applying enzymatic hydrolysis decreased to washed fibre.

4.1.2 Inhibition of yeast

The inhibition of *S. cerevisiae* by non-volatile compounds present in lignocellulosic prehydrolysate after steam pretreatment is more pronounced than that of cellulolytic enzymes (Palmqvist et al., 1996; Kothari and Lee, 2011). Substances that may have inhibitory effects on *S. cerevisiae* include aromatics (e.g. phenolic compounds), acids, furans, inorganic ions and fermentation products such as alcohols (Olsson and Hahn-Hägerdal, 1996; Jönsson et al., 2013). The con-

centrations present in lignocellulosic prehydrolysates are sufficient to have an inhibitory effect in fermentation (Palmqvist et al., 1996; Panagiotou and Olsson, 2007) and when concentrated five times, no ethanol was produced in the fermentations of lignocellulosic prehydrolysate performed by Palmqvist et al. (1996). Acetic acid and lignin degradation products appear to have the greatest inhibitory effect (Olsson and Hahn-Hägerdal, 1996), but the picture gained from the literature is not very clear. Apart from inhibiting ethanol production, the non-volatile compounds present in lignocellulosic prehydrolysate also inhibit cell growth, but this is outside the scope of this thesis.

Weak organic acids in dilute acid hydrolysates of spruce inhibit fermentation by *S. cerevisiae* at elevated concentrations (Delgenes et al., 1996; Larsson et al., 1999a). At concentrations up to 9 g/L, however, acetic acid increases the ethanol yield compared to reference fermentation (Taherzadeh et al., 1997; Larsson et al., 1999a; Palmqvist et al., 1999; Panagiotou and Olsson, 2007), simultaneously with decreases in glycerol and cell mass yields (Taherzadeh et al., 1997). Acetic acid concentrations vary depending on the lignocellulosic material, but are generally lower in softwood hydrolysates than, for example, in hydrolysates from agricultural residues, which contain more acetylated hemicellulose (Jönsson et al., 2013). In the material used in the present work, acetic acid concentrations were generally in the range of 5-9 g/L (Table 4.1). It is the undissociated form of acetic acid that inhibits *S. cerevisiae* (Taherzadeh et al., 1997; Jönsson et al., 2013). Therefore, the inhibition is pH dependent. At lower pH, more acetic acid is present in its undissociated form and is thus able to pass through the cell membrane. Among the weak organic acids present in lignocellulosic prehydrolysate, formic acid is the most inhibitory to *S. cerevisiae*, followed by levulinic and acetic acid (Larsson et al., 1999a; Panagiotou and Olsson, 2007; Jönsson et al., 2013).

Furfural and HMF present in the prehydrolysate also have a negative effect on ethanol productivity, already at low concentrations (1-5 g/L), but do not influence the final ethanol yield (Boyer et al., 1992; Delgenes et al., 1996; Larsson et al., 1999a; Palmqvist and Hahn-Hägerdal, 2000). *S. cerevisiae* is able to consume both furfural and HMF and in this way detoxifies the fermentation medium (Boyer1992). Furfural depletion is almost independent of the initial furfural concentration, but depends on the amount of yeast cells (Chung and Lee, 1985; Boyer et al., 1992). Increased concentrations of furfural and HMF in the fermentation medium result in cell death, but if the inoculum is sufficient, some cells will survive the initial furfural and HMF depletion phase and can continue fermentation when the furfural and HMF levels are reduced (Chung and Lee, 1985). Furfural and HMF are usually consumed during 24 hours and cause a

lag phase (Delgenes et al., 1996). HMF is a more potent inhibitor to *S. cerevisiae* than furfural at equal concentrations and is consumed more slowly by *S. cerevisiae* than furfural, causing a longer lag phase (Palmqvist and Hahn-Hägerdal, 2000).

As in enzymatic hydrolysis of lignocellulosic biomass, the inhibition observed in lignocellulosic hydrolysates is not uncommonly more pronounced than the inhibition in a model substrate containing equal concentrations of weak acids and furans (Palmqvist and Hahn-Hägerdal, 2000). This indicates that other, unspecified, compounds contribute significantly to yeast inhibition. These compounds may be lignin degradation products (Palmqvist and Hahn-Hägerdal, 2000). Vanillin, which constitutes a large fraction of the phenolic monomers in spruce hydrolysates (Palmqvist and Hahn-Hägerdal, 2000), syringaldehyde and p-hydroxybenzoic acid inhibit both cell growth and ethanol production by *S. cerevisiae* at concentrations below 1 g/L (Delgenes et al., 1996; Palmqvist and Hahn-Hägerdal, 2000).

Apart from inhibition, yeast may suffer from stress due to high temperature or high osmolarity in the fermentation medium (Thomas et al., 1994; Russel, 2003). The latter may occur when sugar or salt concentrations in the fermentation medium are high. In very high gravity (VHG) fermentation in the brewing and fuel ethanol industries, the insoluble solids are usually removed for practical reasons. Very little research has thus been carried out on the effect of insoluble material on ethanol fermentation. The addition of particulate, insoluble material, such as soy flour, alumina or sea sand, has been reported to have a stimulatory effect on high gravity fermentation of glucose to ethanol (Thomas et al., 1994). These particles were, however, only present at a solids concentration of 2%. The mechanism behind the effect of these materials on fermentation is, to the best of the author's knowledge, not known. In the present work (**Paper IV**), a significant difference was observed in overall ethanol yield when comparing SSF of the whole steam-pretreated spruce slurry with SSF of the liquid prehydrolysate (Figure 4.3). In SSF of the whole slurry, the glucose concentration increased throughout the entire SSF process, reaching a final concentration of 68 g/L, and only 3 g/L ethanol was produced, corresponding to an overall ethanol yield in SSF of 3.9%. The prehydrolysate, however, although it contained the same concentrations of inhibitors and soluble sugar as the whole slurry, gave an overall ethanol yield of 88.1%. SSF was thus fermentation-limited in the presence of the solid material. It is, however, not clear whether the negative effect of the solid material on ethanol fermentation originated from solid lignin or carbohydrates, or the fact that solids were present in general. Sufficient amounts of nutrients, especially nitrogen, during cultivation, have been shown to increase

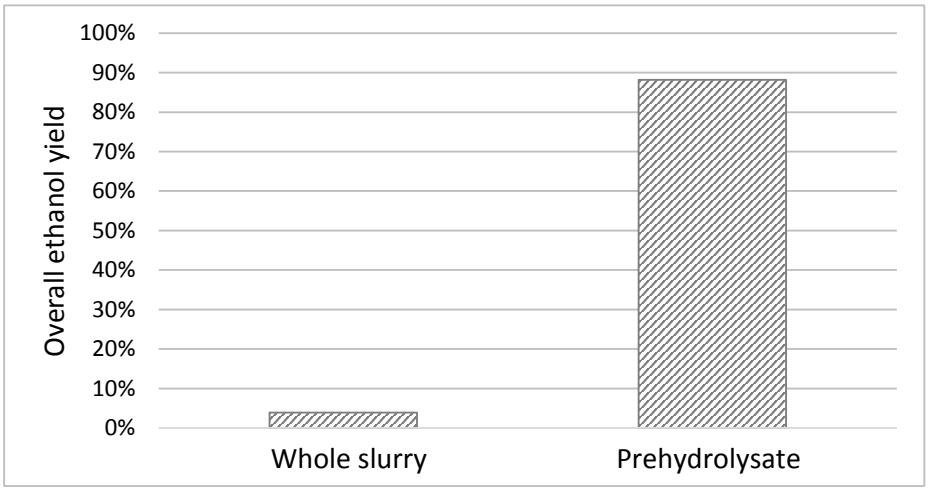

Figure 4.3: Overall ethanol yield (in % of theoretical) from SSF of steam-pretreated spruce slurry with 13.7% WIS, and SSF of prehydrolysate at a concentration corresponding to 13.7% WIS. From Paper IV.

the tolerance of yeast to stress (Jones and Ingledew, 1994).

Yeast can be engineered to be more inhibitor tolerant. Alternatively, prehydrolysates can be detoxified prior to fermentation to reduce yeast inhibition. However, detoxification adds to the production cost and alternatives involving process adaptation to mitigate yeast inhibition may be a cheaper and more robust alternative in industrial production of ethanol from lignocellulosic biomass. Yeast strains subjected to high inhibitor concentrations over an extended period develop some degree of inhibitor tolerance (Banerjee et al., 1981; Keller et al., 1998). Cultivating yeast for the production of ethanol from lignocellulosic biomass on prehydrolysate, thus reduces yeast inhibition in the fermentation process (Alkasrawi et al., 2006). Yeast is usually grown in multiple steps with a batch phase to produce a starting amount of yeast cells which is then fed with sugar slowly to avoid the Crabtree effect. All SSF and fermentation studies carried out in this work were performed with yeast cultivated on glucose solution, which was then fed with prehydrolysate supplemented with glucose. Similar to the adaptation of yeast to inhibitors in the prehydrolysate, yeast that has been subjected to a medium containing high concentrations of sugar and salts shows better fermentation performance in subsequent fermentation of sugar to ethanol in a hyperosmotic medium (Hirasawa and Tokoigawa, 2001).

Yeast inhibition can also be reduced by the appropriate choice of process con-

figurations. For example, fed-batch fermentation can be employed, in which the toxic material is fed to the reactor in smaller portions. Also, washing the pretreated slurry prior to enzymatic hydrolysis removes the inhibitors from the medium in both enzymatic hydrolysis and subsequent fermentation.

4.1.3 Inhibition in SSF

Combining enzymatic hydrolysis and fermentation in SSF, exposes the cellulolytic enzymes to high ethanol concentrations, which has an inhibitory effect on the cellulases (Ooshima et al., 1985; Holtzapple et al., 1990; Bezerra and Dias, 2005; Chen and Jin, 2006). An ethanol concentration of 60 g/L ethanol has, for example, been shown to reduce cellulose hydrolysis by 30% (Jing et al., 2009). Yeast has no effect on enzymatic hydrolysis of cellulose (Chen and Jin, 2006).

Another problem encountered in SSF is the difficulty in differentiating between enzyme and yeast inhibition. In the present work (**Paper I**), it was clearly shown that the ethanol yield was reduced when the concentration of the prehydrolysate was increased, at the same WIS concentration. In the experiments with higher concentrations of prehydrolysate, an increase in the concentration of residual fermentable sugar was observed, indicating that enzymatic hydrolysis continued to produce monomeric sugar throughout the whole SSF process. However, is was not clear from the experiments whether the performance of enzymatic hydrolysis deteriorated due to the increased concentrations of inhibitors. Jørgensen et al. (2007b) analysed the residual material after prehydrolysis and SSF of wheat straw and observed that the enzymes were still negatively affected by high solids concentrations, possibly due to inhibition by pentose sugars or ethanol. It may, however, be difficult to estimate the WIS content after successful SSF, as the yeast cells will contribute to the dry weight.

4.2 Stirring limitations

4.2.1 Rheology of pretreated lignocellulose

Slurries of pretreated spruce (Wiman et al., 2011), red oak (Dasari and Berson, 2007) and corn stover (Knutsen and Liberatore, 2009; Roche et al., 2009a; Viamajala et al., 2009; Ehrhardt et al., 2010) are shear thinning fluids, exhibiting rapidly increasing viscosity and yield stress with increasing WIS content. Below a critical value of shear stress, these slurries act like plastic materials, and do not flow when the WIS concentration is high (Viamajala et al., 2009). There is also an upper limit on the shear rate, above which the slurries become New-

tonian or even shear thickening fluids (Stickel and Powell, 2005; Dasari and Berson, 2007). In dilute-acid-pretreated corn stover, Viamajala et al. (2009) observed an increase in viscosity at increased WIS concentrations up to about 25% WIS. Above this, the viscosity appeared to be independent of WIS concentration. Ehrhardt et al. (2010) investigated acid-pretreated corn stover up to 30% WIS but observed no such plateau in yield stress at high solids concentrations. It is, however, difficult to compare the values of yield stress of different materials, since the materials differ, for example, in particle size distribution and fibre length (Ehrhardt et al., 2010). Therefore, different lignocellulosic materials may behave differently when agitated. Steam-pretreated spruce slurry requires a much higher power input to achieve a certain stirring rate than *Arundo donax* at the same WIS concentration (Palmqvist and Lidén, 2012). Furthermore, while the power required to maintain a certain stirring rate decreased rapidly during the first 2-6 hours of enzymatic hydrolysis of *Arundo donax* and was independent of the initial WIS content (10-20% WIS was tested), this was not the case for spruce (Palmqvist and Lidén, 2012). The highest final WIS concentration in the experiments performed on *Arundo donax* by Palmqvist and Lidén (2012) was 16-17%, and the rapid decrease in viscosity can thus not be the result of a decrease in WIS concentration, but may be due to structural and/or compositional changes in the material during hydrolysis. Furthermore, steam-pretreated agricultural materials differ visually from softwood slurries. Pretreated agricultural materials such as *Arundo donax* contain rather large fibres and have the appearance of a slurry of suspended solids, while pretreated spruce has a more paste-like appearance. Zhao et al. (2013) also reported a paste-like appearance of pretreated sugarcane bagasse after hydrolysis. Liquefaction during prehydrolysis, often reported for other materials such as corn stover (Roche et al., 2009b), wheat straw (Jørgensen et al., 2007b), *Arundo donax* (Palmqvist and Lidén, 2012) and paper sludge (Elliston et al., 2013) was not observed with spruce in the present work (**Papers III** and **V**), although high ethanol yields were obtained in SSF and in prehydrolysis and SSF (and thus high glucose yields in enzymatic hydrolysis).

Research results on the dependence of yield stress on particle size are inconsistent. Higher yield stress has been reported for milled slurry than the original slurry in steam-pretreated spruce slurry, as well as other, non-lignocellulosic, materials (Wiman et al., 2011). The opposite has, however, been observed for other pretreated lignocellulosic materials such as red oak and corn stover (Dasari and Berson, 2007; Viamajala et al., 2009; Ehrhardt et al., 2010). In general, a narrower particle size distribution, as in milled compared to original slurry, results in higher yield stress and viscosity due to more efficient packing (Roche et al., 2009a; Wiman et al., 2011). However, the fibrous nature of the particles in

biomass slurries may have a greater impact on the rheological properties and overshadow this effect (Dasari and Berson, 2007; Viamajala et al., 2009).

The WIS concentration, particle size distribution and chemical composition of the fibres change simultaneously during enzymatic hydrolysis. As hydrolysis proceeds, the yield stress decreases (Roche et al., 2009a; Wiman et al., 2011). Yield stress is dependent not only on WIS concentration, but also other properties of the fibre in a pretreated lignocellulosic slurry. Yield stress values at similar WIS contents have been found to be slightly lower in hydrolysed samples than in the original material (Viamajala et al., 2009; Wiman et al., 2011). This may be due to weakening of the cellulose structure by enzymes (in contrast to weakening of cellulose by shear force, as discussed below). It has also been suggested that this could be explained by changes in particle diameter and/or shape, leading to changes in rheological properties (Wiman et al., 2011).

4.2.2 Effect of stirring on enzymatic hydrolysis and fermentation

Due to the increased viscosity at increased WIS concentration, mixing the material in the reactor becomes a problem and the risk to obtain stagnant zones increases. If this is solved by increased agitation, thus in a continuously stirred tank reactor (CSTR) increased impeller speed, the power consumption for the impeller increases (Palmqvist et al., 2011) resulting in increased process costs. Especially due to the long residence times in enzymatic hydrolysis and fermentation, high power inputs are not economically feasible in an industrial biomass-to-ethanol process.

Instead of increasing the stirring in the reactor, a higher enzyme dosage can be used to decrease the viscosity of the material. However, Palmqvist et al. (2011), showed that doubling the enzyme dosage in the enzymatic hydrolysis of steam-pretreated spruce with 10% WIS only reduced the energy required for stirring by 15-25%, depending on the impeller speed. An alternative is to introduce a prehydrolysis step prior to enzymatic hydrolysis and fermentation, which can be performed at higher, more optimal, temperatures for the cellulolytic enzymes, and thus, potentially require less enzymes for the same viscosity reduction. Prehydrolysis of the pretreated material prior to SSF is further discussed in Section 5.2.

No correlation was observed between cellulose conversion and mixing rate when a rotating drum was used for the enzymatic hydrolysis of pretreated wheat straw and corn stover at a dry matter content of 20-25%, between 2 and 20 rpm

(Jørgensen et al., 2007b; Roche et al., 2009a). These rotating drums, however, work at very low mixing rates, and conflicting results have been obtained using shake flasks and CSTRs. The conversion of cellulose to glucose in the enzymatic hydrolysis of steam-pretreated softwood and pure cellulose in shake flasks has been shown to increase with increased mixing (Ingesson et al., 2001; Mais et al., 2002). However, at 7.5% WIS, low mixing (25 rpm) with intermittent mixing at higher rates (150 rpm), was found to give the same hydrolysis results as continuous high mixing (Ingesson et al., 2001; Mais et al., 2002). A similar effect was seen at higher solids content (10% WIS), but with slightly lower conversion. Agitation has also been shown to enhance the enzymatic hydrolysis of Avicel (Sakata et al., 1985) and steam-pretreated softwood (Tengborg et al., 2001a). In the case of softwood, intermittent stirring at higher speed did not improve hydrolysis yields. Palmqvist et al. (2011) investigated a larger range of stirring speeds and observed more than a twofold increase in enzymatic hydrolysis yield from pretreated softwood when the impeller speed was increased from 25 rpm, where stagnant zones occurred in the fermentor, to 500 rpm, where complete fluid motion was observed. The increase was almost linear in the range of the impeller speeds tested, regardless of enzyme dosage (Palmqvist et al., 2011). A positive effect of agitation on ethanol yield in SSF was also observed in the present work (**Paper I**), where the overall ethanol yield increased from 84.6% to 95.8% when the stirrer speed was increased from 200 to 700 rpm. A slight increase in glucose production was also observed in SSF of steam-pretreated spruce with 13.7% WIS in a CSTR, compared with a rotating drum reactor (**Paper III**) (Figure 4.4). Due to the difficulty in collecting representative samples from the viscous slurry in these experiments, it was, however, not possible to determine whether this difference was significant or not. Considering the large difference in hydrolysis yield reported by Palmqvist et al. (2011), it is, however, clear that the difference between free-fall mixing in the rotating drum and agitation using an anchor stirrer in a CSTR, was not of the same magnitude as that observed by Palmqvist et al. (2011).

The increase in hydrolysis rate with increased impeller speed may be explained by increased shear forces. The average shear rate in the reactor is proportional to the impeller speed (Metzner and Otto, 1957). The increase in hydrolysis yield with increasing impeller speed could also be due to an increase in enzyme adsorption or a reduction of end-product inhibition. Adsorption of cellulases increases with increased agitation (Sakata et al., 1985; Kaya et al., 1994) due to decreased laminar boundary layers close to particles, which facilitates mass transport to the surfaces (Eriksson et al., 2005). This probably also facilitates the transport of hydrolysis products from the surface to the bulk, possibly re-

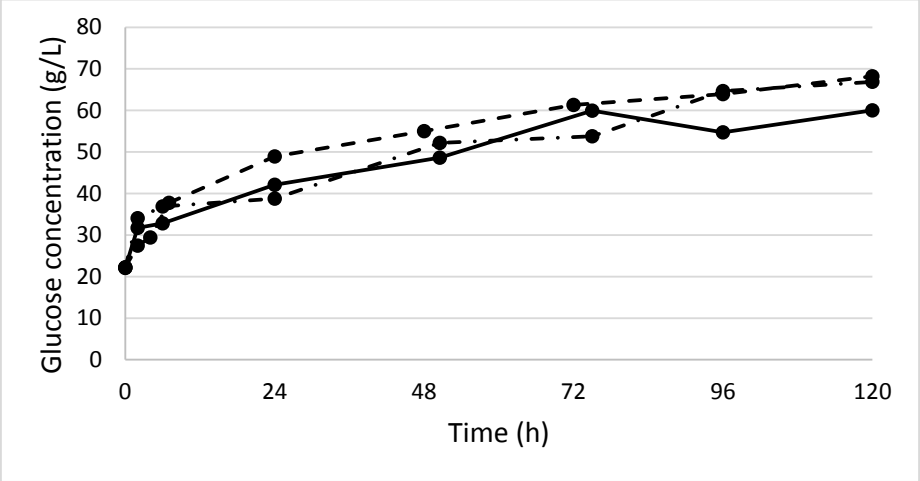

Figure 4.4: Glucose concentration in SSF of steam-pretreated spruce with 13.7% WIS using reactors with different methods of agitation. Rotating drum (solid line), CSTR 1 (dotted line) and CSTR 2 (dash-dotted line). The ethanol production was similar in all these SSF experiments and low (3-4 g/L final ethanol concentration). Adapted from **Paper III**.

ducing local end-product inhibition. However, at very high shear rates (stirring speeds of 3000-24000 rpm), Cao and Tan (2004) found that the enzymes denatured, resulting in a deterioration in hydrolysis. Denaturation may also occur at low shear rates when the enzymes are subjected to shear over a long time (Kaya et al., 1994).

During the enzymatic hydrolysis of steam-pretreated spruce, the impeller speed required to ensure thorough mixing of the material in a CSTR decreases. However, the positive effect of impeller speed on the yield from enzymatic hydrolysis is maintained throughout the entire hydrolysis stage and even increases with hydrolysis time (Palmqvist et al., 2011). According to Lenting and Warmoeskerken (2001), a certain level of shearing of the cellulose fibres is essential for optimal enzymatic hydrolysis. Cellulases primarily attack the amorphous regions in cellulose, and if they have a CBM their action can be very intense in a limited region. As a result of this, these areas become weakened and are more easily affected by shear forces (Lenting and Warmoeskerken, 2001). This could explain why the positive effect of shear forces becomes more pronounced as enzymatic hydrolysis progresses, as observed by Palmqvist et al. and why pre-shearing the material prior to enzymatic hydrolysis does not have any positive effect on the performance of hydrolysis (Palmqvist et al., 2011).

4.3 Yield calculations in enzymatic hydrolysis and SSF with high solids concentration

The yield from enzymatic hydrolysis is generally expressed as the amount of glucose released in relation to the initial amount of glucose present in the pretreated lignocellulosic material. The glucose content in the substrate can easily be calculated by analysing the material to determine its total content of sugars. It is common laboratory practice to take samples of the supernatant of the hydrolysis slurry and analyse their glucose content using high performance liquid chromatography (HPLC). Slurry volume and density are usually assumed to be constant during hydrolysis, and the density of the lignocellulosic slurry is assumed to be that of water, 1000 g/L. However, the saccharification of insoluble polymers to soluble sugar involves changes in volume, density and insoluble solids concentration. When performing enzymatic hydrolysis at high solids concentration when little (or no) free water is present in the material initially, simply measuring the glucose concentration in the supernatant, not taking changes in volume and density into account, results in an overestimation of the hydrolysis yield (Kristensen et al., 2009a; Zhu et al., 2011).

The calculation of actual yields, taking volume and density changes into account, involves more complicated sampling, since the insoluble solids concentration and liquid density must also be measured in each sample. Kristensen et al. (2009a) observed an almost linear correlation between the actual yield and the yield based on initial volume and density, but the slope of the line obtained when plotting these yields against each other, may differ between substrates and most likely process conditions. The slope of this line can thus be used to determine the actual yield from the measured yield, for a specific substrate and process conditions. Zhu et al. (2011) proposed a similar method, in which the sugar concentrations measured in hydrolysis slurries are corrected using correction factors based on the change in volume resulting from the increase in sugar concentration in a sugar solution. To obtain accurate values of the yield from enzymatic hydrolysis of unwashed pretreated lignocellulosic slurry, it is necessary to take the initial density of the liquid into account when using this method. The method proposed by Zhu et al. has been shown to result in errors below 4% (Zhu et al., 2011).

In SSF, yield calculations at high solids concentrations are more complex. While water is incorporated into the glucose monomers during enzymatic hydrolysis, resulting in a decrease in volume, the fermentation of sugar to ethanol increases the liquid volume. The increase in liquid volume due to fermentation has a greater impact on the volume than the consumption of water during en-

zymatic hydrolysis when all the sugar released is fermented to ethanol (Zhang and Bao, 2012). When the change in liquid volume is not taken into account the yields from enzymatic hydrolysis are overestimated, while SSF yields are underestimated. Zhang and Bao (2012) suggested a modified method of calculating ethanol yields in SSF taking liquid volume change into account. This method is based on the measured ethanol concentration in the liquid phase, and assumes that all fermentable sugars are converted to ethanol.

In the studies included in this thesis, liquid volume change was not taken into account in yield calculations, and the reported yields are thus conservative, and the overall ethanol yields are the lowest possible. However, in research such as that presented in this thesis, the actual yields are of less importance, and rather the difference between the yields in different experiments is of interest.

5

Evaluation of configurations of SSF with high solids concentration

Chapter 3 describes the main steps in the production of ethanol from lignocellulose. These can be combined in different ways to achieve optimal conversion of the raw material to the final products. The most straightforward alternative is to perform all the process steps separately in sequence: pretreatment, enzymatic hydrolysis, fermentation and product recovery. This way to perform the enzymatic hydrolysis and fermentation is called separate hydrolysis and fermentation (SHF). When the hydrolysis of cellulose is carried out in the presence of a fermentative organism, the process is referred to as simultaneous saccharification and fermentation (SSF). When using a fermenting microorganism that can utilize both the hexose and pentose fraction of the sugars in the material, the process is often referred to as simultaneous saccharification and co-fermentation (SSCF). The SSF concept was first patented by Gauss et al. (1976). The term SSF is also used to describe solid state fermentation, and is somewhat misleading. It was not used by the authors of the original patent, but is today well established as the concept for simultaneous saccharification and fermentation in the

field of ethanol production from lignocellulosic biomass. The main advantage of SSF and SSCF over SHF is reduced end-product inhibition of the cellulolytic enzymes, since glucose is continuously removed from the medium and fermented to ethanol. Other advantages include reduced reactor volume and higher ethanol yields (Gauss et al., 1976; Mehmood et al., 2009; Zhao et al., 2013). However, in SSF, both enzymatic hydrolysis and fermentation are performed at the same temperature and pH. The cellulolytic enzymes, however, have an optimal temperature of about 50°C, while *S. cerevisiae* works best at about 30°C. Process conditions in SSF are thus a compromise. Also, reuse of the yeast is more difficult in SSF than in SHF due to difficulties in separating the yeast from the lignin after fermentation (Olofsson et al., 2008a). SSF can be broadened to include the production of the fermenting microorganism and the cellulolytic enzymes in so-called consolidated bioprocessing (CBP) (Lynd, 1996). However, such a system, in which the conversion of the lignocellulosic biomass to ethanol is performed in a single step, has not yet been tested on an industrial scale, and no single organism has been found that can convert lignocellulose to ethanol with acceptable yields (Garvey et al., 2013).

A number of strategies can be used to optimize SSF. The most common process designs after ordinary batch mode, are SSF in fed-batch mode, and the addition of an extra enzymatic hydrolysis step prior to SSF. The latter is commonly called prehydrolysis. Both of these process configurations have been used in the research included in this thesis. Fed-batch mode was studied in some detail in the study presented in **Paper II**, while the studies described in **Papers III-IV** focused on prehydrolysis, and fed-batch SSF was combined with an additional prehydrolysis step in the final study (**Paper V**). In this chapter, these configurations are discussed and linked to the studies presented in **Papers II-V**.

5.1 Fed-batch SSF

When SSF is performed at high WIS concentrations, the material is initially difficult to stir due to its high viscosity. This problem can be overcome by starting SSF with part of the substrate, at a lower solids concentration, and then adding the remaining substrate in fed-batch mode. Fed-batch SSF can be carried out in a number of ways. At low solids loadings, the prehydrolysate can be fed over a period of time in order to give the yeast time to adapt to the toxic environment. Also, when using a pentose-fermenting yeast, xylose uptake is suppressed by high glucose concentrations, making fed-batch SSF an alternative for ensuring low glucose concentrations during SSF (Olofsson et al., 2008b), and thus increasing the ethanol yield from xylose. The work presented in this thesis fo-

cuses on fed-batch SSF with the aim of increasing the solids loading in SSF, and "fed-batch" thus refers to the feeding of mainly the substrate.

There is no general increase in final ethanol yield using fed-batch operation, compared with batch operation. This has been reported for enzymatic hydrolysis of steam-pretreated barley straw with 5-15% DM (Rosgaard et al., 2007), for SSF of steam-pretreated spruce with a solids concentration of 10-14% WIS (Rudolf et al., 2005; **Paper II**) and dilute-acid-pretreated *Sorghum bicolor* at 5% DM (Mehmood et al., 2009). However, the amount of enzymes required can be reduced in fed-batch mode in some cases where higher enzyme loadings are needed with high solids concentrations (Ballesteros et al., 2002). On the other hand, high enzyme loading in SSF may have a negative effect on yeast performance due to increased sugar concentrations affecting the yeast, as shown by Zhao et al. (2013) for washed acid-pretreated sugarcane bagasse. They performed SSF at a temperature of 37-38°C, which may have been high enough to stress the yeast, which combined with high glucose concentrations, resulted in the cessation of ethanol production. When fresh yeast was added after 72 hours of SSF, the final ethanol yields did not differ between batch and fed-batch SSF using sugarcane bagasse with 20% WIS.

Increased glucose yields in fed-batch enzymatic hydrolysis have also been reported. Zhao et al. (2013) observed a slight increase in glucan conversion in fed-batch enzymatic hydrolysis of washed acid-pretreated sugarcane bagasse with 20% WIS, compared with batch hydrolysis. The difference in glucose yield increased at 30% WIS. Gupta et al. (2012) have also reported an increase in final glucose concentration, from 80.8 to 127.0 g/L, when performing enzymatic hydrolysis with 20% WIS of *Prosopis juliflora* wood delignified with sodium chlorite.

Fed-batch enzymatic hydrolysis or SSF is not commonly used to improve glucose or ethanol yields, but rather as a means of achieving reasonable glucose or ethanol yields at solids concentrations that result in decreased yields or stirring difficulties in batch mode. When comparing batch with fed-batch hydrolysis or SSF, the same solids concentrations are usually used to facilitate comparison. For example, fed-batch SSF with 15% WIS refers to a slurry containing 15% WIS, separated into one more dilute fraction that is used in the initial batch phase and a more concentrated fraction that is used as feed. Combining these fractions would result in a slurry with 15% WIS as in the case of the batch experiment. When fed-batch mode is used to facilitate stirring, the situation may be different. Firstly, in this case, it is not always possible to have a batch reference experiment, and secondly, the total solids concentration is not always clearly reported, since the main objective is not to achieve high yields using high solids concentrations,

but rather to achieve high concentrations of glucose or ethanol. A clear example of this is the enzymatic hydrolysis and SSF of paper sludge performed by Elliston et al. (2013). Already at 5% DM, paper sludge has a viscosity that makes stirring impossible in batch hydrolysis. However, using an initial concentration of 2.5% DM in enzymatic hydrolysis, and feeding additional portions of substrate and enzymes, followed by fed-batch SSF with substrate feeding, it was possible to stir the slurry and to reach a high final ethanol concentration of 47 g/L. The limiting factor in this process design (fed-batch prehydrolysis followed by fed-batch SSF), was the increase in viscosity in the reactor as non-degradable material accumulated, making stirring impossible. Using a different reactor equipped with a stronger motor, facilitating stirring at higher viscosities, allowed further substrate additions, resulting in an even higher ethanol concentration of 63 g/L. This could be further increased to 91.5 g/L when the material in the fermentor was allowed to fully degrade before new substrate was added (Elliston et al., 2013). Although a high final ethanol concentration was achieved in this process design, there is still scope for improvement of SSF, since ethanol yields were only 29 and 54% in the two last fed-batch designs. High glucose and ethanol concentrations have also been reported in fed-batch enzymatic hydrolysis and SSF of washed and detoxified pretreated corncob (SSF) (Zhang et al., 2010), washed and unwashed steam-pretreated corn stover (enzymatic hydrolysis) (Lu et al., 2010), steam-pretreated, delignified corn stover (enzymatic hydrolysis) (Yang et al., 2010), and alkali-pretreated washed empty palm fruit bunch fiber (SSF) (Park et al., 2013) using different fed-batch set-ups to maximize glucose or ethanol concentration.

5.1.1 Enzyme feeding strategy in fed-batch SSF

Although the final yield from hydrolysis or SSF is often not affected by the feeding of substrate in fed-batch enzymatic hydrolysis or SSF, increased hydrolysis productivity has been reported, especially in the initial phase. High initial hydrolysis rates are not surprising in fed-batch mode, since the whole enzyme dose is often added to the reactor during the batch phase. The initial enzyme concentration is then higher in relation to the substrate concentration in fed-batch compared to batch mode. In the study presented in **Paper II**, the initial ethanol production rate was in some cases the same in batch and fed-batch mode, and in some cases slightly higher in fed-batch SSF. The initial ethanol production rate was slightly higher in fed-batch compared with batch SSF even in some cases when part of the enzymes were fed together with the substrate. This may be due to a lower initial WIS concentration in fed-batch SSF. In all SSF experiments presented in **Paper II**, the initial soluble glucose in the reactor was consumed during the first 2 hours

of SSF and ethanol production rates thus probably reflect enzymatic hydrolysis rates.

When all enzymes are added at the start of hydrolysis, the substrate in the feed will have a shorter contact time with the enzymes compared with the substrate in the batch phase. This may decrease the yield from fed-batch compared to batch hydrolysis (Rosgaard et al., 2007). Also, the risk for unproductive binding of enzymes to lignin is probably higher in fed-batch hydrolysis, when the material is degraded and the relative lignin concentration increases. To prevent this, part of the enzymes can be added together with the substrate feed. It is thus important to consider the strategy of enzyme addition in fed-batch enzymatic hydrolysis and SSF. The impact of enzyme feeding strategy on the ethanol yield in SSF of steam-pretreated spruce was investigated in **Paper II**. The overall ethanol yield decreased from 77.4 to 68.9% in SSF of whole slurry of steam-pretreated spruce with 10% WIS when changing from batch to fed-batch mode, and adding all the enzymes at the beginning of fed-batch SSF (Figure 5.1). The reason for this may be the inhibition of enzymes by compounds in the prehydrolysate, which is supported by the fact that fed-batch SSF using washed slurry (14% WIS) and the addition of all enzymes at the beginning of the process resulted in a higher ethanol yield compared to batch SSF under the same conditions. In SSF with 10 and 14% WIS and prehydrolysate present during SSF, ethanol yields were higher when part of the enzymes were added together with the feed compared to the addition of all the enzymes at the start of the process. The opposite was observed for washed fibre at 14% WIS. This confirms the hypothesis that enzymes are inhibited by compounds in the prehydrolysate, probably soluble lignin. Zhao et al. (2013) recently reported no significant difference in glucose yield with different enzyme feeding strategies in the enzymatic hydrolysis of acid-pretreated sugarcane bagasse with 20% WIS. They investigated the same strategies as those described in **Paper II** (all enzymes added at the start and enzymes added together with the substrate throughout hydrolysis). However, they used washed (and delignified) fibre, so hydrolysis was not affected by the inhibition of enzymes due to soluble lignin, and the difference in contact time between the enzymes and the substrate was probably offset by the long retention times ($>$ 200 h).

It was also shown in this work (**Paper II**) that, when adding part of the enzymes together with the substrate feed, it is beneficial to mix the substrate and enzymes prior to addition to the reactor, rather than adding them separately. Visually, the feed material changed from dry crumbs to a more paste-like slurry during the 2-11 hours from the addition of the enzymes to the substrate feed (the same time as yeast addition in SSF) until the material was fed to the reactor. It is possible that mixing the substrate and enzymes resulted in the onset of enzy-

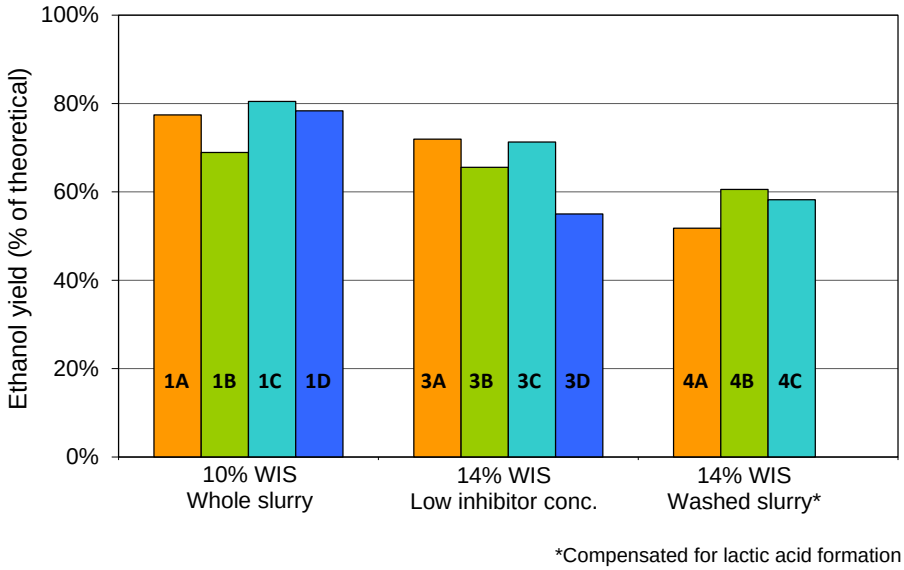

Figure 5.1: Overall ethanol yields (in % of theoretical) from batch and fed-batch SSF of steam-pretreated spruce with different enzyme feeding strategies: A: batch SSF, B: fed-batch SSF with all enzymes in batch, C: fed-batch SSF with enzymes fed mixed with the substrate, D: fed-batch SSF with enzymes fed separately from substrate. Adapted from **Paper II**. In SSF with 14% WIS and low inhibitor concentration, the concentration of prehydrolysate corresponded to 7.5% WIS.

matic hydrolysis, although the feed was kept at room temperature. Also, when the enzymes were mixed with the substrate prior to addition to the reactor, the enzymes were more evenly distributed over the substrate, and were in contact with the undegraded substrate in the feed, and not the partly degraded substrate already in the reactor, which may have influenced the enzymatic hydrolysis in SSF.

5.2 Prehydrolysis prior to SSF

In SSF with high solids concentrations, it has become common to perform an extra enzymatic hydrolysis step prior to SSF, in order to liquefy the material and facilitate stirring, as is a common procedure in the production of ethanol in first generation plants. This additional enzymatic hydrolysis step is often called prehydrolysis, but also other terms have been used, such as semi-simultaneous saccharification and fermentation (Elliston et al., 2013; Lu et al., 2013), non-

isothermal simultaneous saccharification and fermentation (Varga et al., 2004), hybrid hydrolysis and fermentation (HHF) (Merino and Cherry, 2007), or simply liquefaction (Jørgensen et al., 2007b; Georgieva et al., 2008). Practically, all of these refer to the same configuration of SSF. The term "nonisothermal SSF" is also used in SSF with varying temperature (Mutturi and Lidén, 2013). In some of the studies reported in the literature, the enzymes were added before the yeast, and sometimes prehydrolysis was performed at increased temperature, without further comments on this as a separate process step. In these studies, prehydrolysis is considered to be a practical step in the laboratory to facilitate mixing, and is often hardly mentioned. However, the addition of a prehydrolysis step may have significant impact on the ethanol yield, so it is important to differentiate between SSF with and without prehydrolysis. Combined prehydrolysis and SSF is referred to below as PSSF (prehydrolysis and simultaneous saccharification and fermentation). Prehydrolysis is commonly performed in the temperature range of 45-50°C, which are more optimal temperatures for the cellulolytic enzymes, than those in SSF, where the temperature is restricted to about 30-35°C by yeast viability. PSSF should not be confused with SHF. In SHF, the liquid and solid are separated after enzymatic hydrolysis, and only the liquid fraction is fermented. In SHF, therefore, no hydrolysis occurs during fermentation, and rather long retention times or high enzyme loadings are needed to reach a feasible glucose yield (Jørgensen et al., 2010). Hydrolysis yields are usually modest in SHF, even with long retention times. When using high concentrations of solid substrate, enzymatic hydrolysis may result in high glucose concentrations and severe end-product inhibition. Higher overall ethanol yields are thus possible in a shorter time in PSSF than in SHF when using high solids concentrations (Jørgensen et al., 2010).

At moderate solids concentrations, when the overall ethanol yield in SSF (and thus also the glucose yield in enzymatic hydrolysis) is not severely restricted by mixing problems, PSSF does not show any benefits with regard to ethanol yield compared to ordinary SSF. On the contrary, overall ethanol yields have been reported to decrease slightly when a prehydrolysis step was included prior to SSF in the case of steam-pretreated corn stover with 10-11.5% WIS (Öhgren et al., 2007), steam-pretreated barley straw with 7.5% WIS (Linde et al., 2007), washed LHW-pretreated olive tree prunings with 9% WIS (Manzanares et al., 2011) and washed, alkali-pretreated pressed palm fibre (a residue from palm oil extraction) (Boonsawang et al., 2012). This was also observed in SSF with steam-pretreated spruce with 7.5 and 10% WIS in the present work (**Paper I**), as shown in Figure 5.2. Mesa et al. (2011) observed a slightly higher ethanol yield in PSSF than in SSF using steam-pretreated, delignified and washed sugarcane

bagasse with 16% WIS. The retention time in PSSF in their experiments was, however, twice as long as the retention time in SSF (48 h instead of 24 h), and the prehydrolysis step probably had no positive impact on overall ethanol yield. In SSF with moderate solids concentrations, the ethanol yield is already high; overall ethanol yields of 85-95% were observed in the study described in **Paper I** using 7.5 and 10% WIS. The decrease in ethanol yield in PSSF, compared with SSF, at these solids loadings is often explained by possible deactivation of the cellulolytic enzymes due to the combination of high temperature during prehydrolysis and the long retention time. Although the use of a higher temperature in enzymatic hydrolysis increases the initial rate of hydrolysis, the temperature leading to the highest hydrolysis yield has been shown to be dependent on retention time (Eklund et al., 1990; Tengborg et al., 2001a). When prehydrolysis is added to SSF that already results in a high ethanol yield, the negative effect of deactivation of the enzymes may thus outweigh the positive effect of enhanced enzymatic hydrolysis during prehydrolysis at a higher temperature. Another negative effect of prehydrolysis on SSF is the high initial sugar concentration in the SSF step. High sugar concentrations in fermentation may increase the stress on the yeast and increase the production of cell mass or by-products such as glycerol. This may partially explain why a prehydrolysis step prior to SSF at moderate solids concentration resulted in a decrease in ethanol yield even when performed at the same temperature as SSF (Linde et al., 2007).

In the present work (**Papers I** and **III**), it was found that the addition of prehydrolysis prior to SSF at high solids concentrations significantly increased the overall ethanol yield and concentration, even when SSF was fermentation-limited (Figure 5.3). The final ethanol concentration from SSF of steam-pretreated spruce with 12% WIS increased from 21 to 45 g/L when a prehydrolysis step at 48°C was included (**Paper I**) while the final ethanol concentration increased from 3 to 48 g/L when a prehydrolysis step was added prior to SSF of steam-pretreated spruce with 13.7% WIS (**Paper III**). An increase in ethanol yield when using PSSF instead of SSF has also been reported for washed LHW-pretreated olive tree prunings with 23% WIS, although the low ethanol yield in SSF was due to poor fermentation (Manzanares et al., 2011).

As discussed above for fed-batch SSF, prehydrolysis is not always added to SSF to increase the ethanol yield, but in many cases simply to facilitate stirring in SSF of substrates with high solids concentrations. In these cases, the slurry may not be possible to stir, but after liquefaction during prehydrolysis, stirring is possible and SSF can be performed. It has been shown that unwashed steam-pretreated wheat straw can be converted to ethanol using PSSF at solids concentrations up to 40% DM (WIS concentration not reported), giving final ethanol concentrations

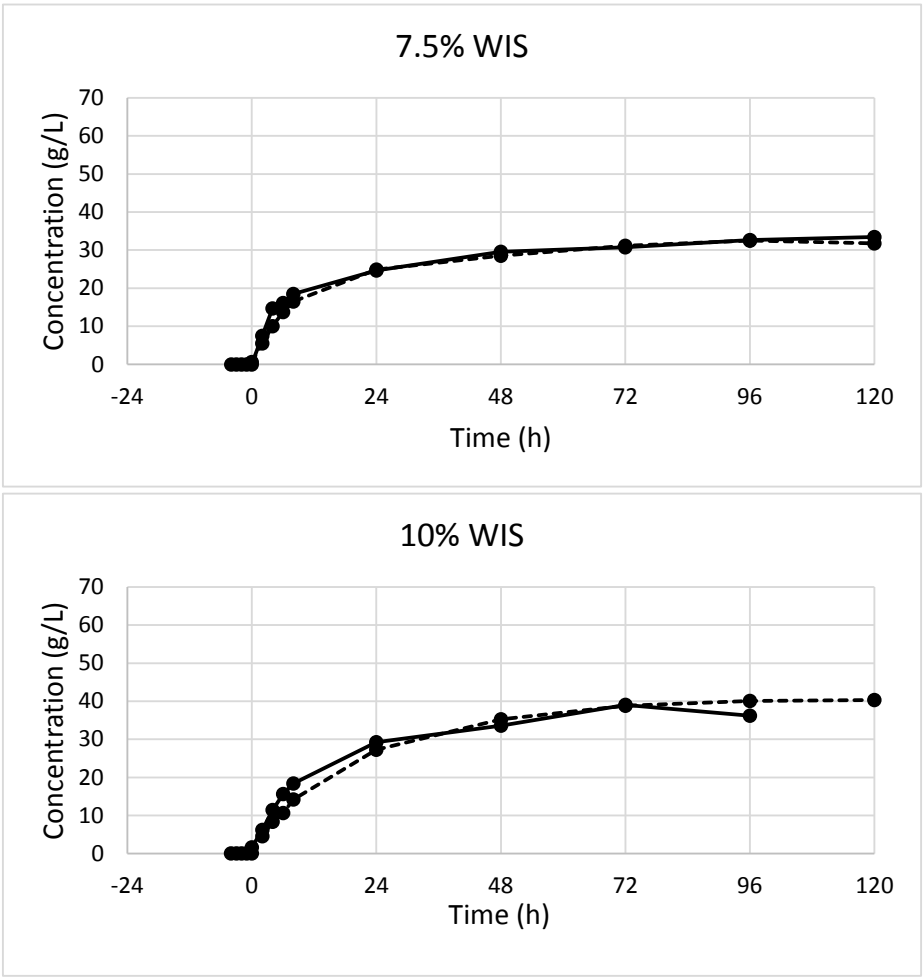

Figure 5.2: Ethanol concentrations in SSF of steam-pretreated spruce. Comparison of SSF without (dashed line) and with (solid line) 4 h prehydrolysis with 7.5 and 10% WIS. Adapted from **Paper I**.

of up to 48 g/L (Jørgensen et al., 2007b). Final ethanol concentrations of 60-70 g/kg slurry have been demonstrated for palm kernel press cake with 35% DM, however long retention times of 192-216 h were required (Jørgensen et al., 2010). As discussed above, different lignocellulosic materials behave differently in enzymatic hydrolysis. Agricultural materials such as *Arundo donax*, corn stover and wheat straw quickly lose their fibre network strength and become liquids during the first few hours of enzymatic hydrolysis, while steam-pretreated spruce

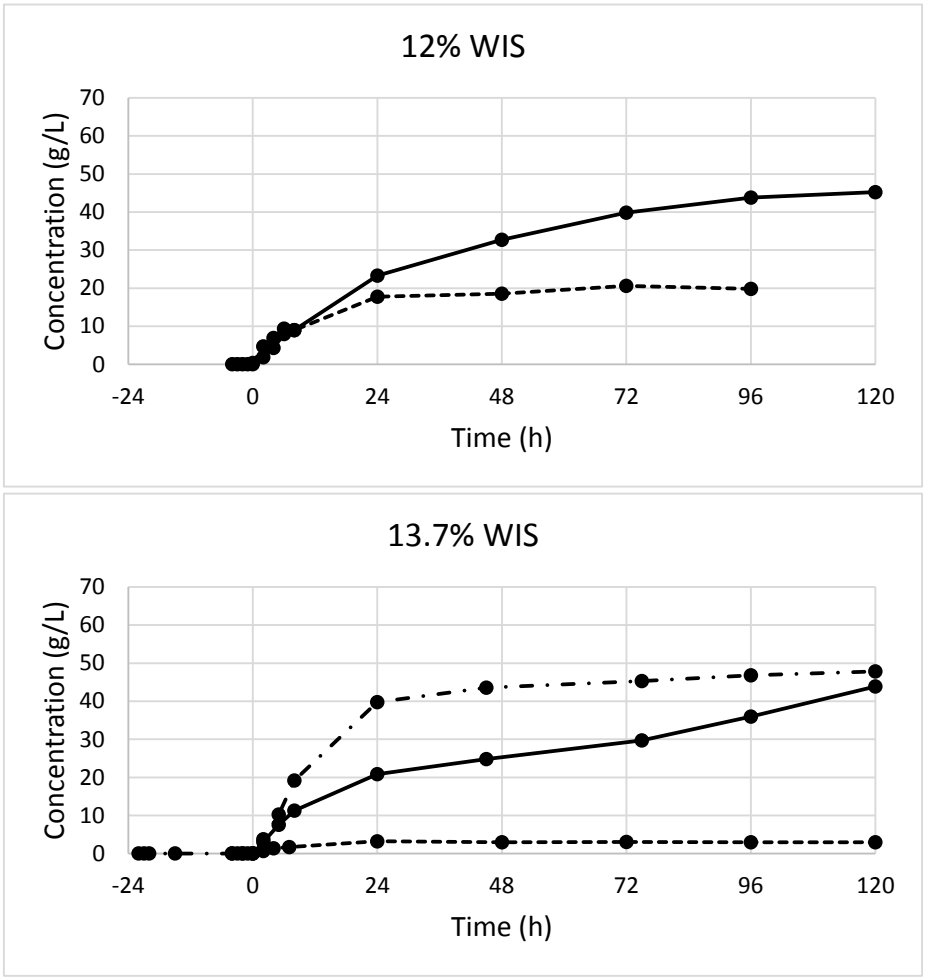

Figure 5.3: Ethanol concentrations in SSF of steam-pretreated spruce. Comparison of SSF without (dashed line) and with 4 (solid line) and 22 h (dash-dotted line) prehydrolysis with 12 and 13.7% WIS. Adapted from **Papers I** and **III**.

remains a thick paste. Although the viscosity has been reported to decrease less in the hydrolysis of spruce than in other materials such as *Arundo donax* (Palmqvist and Lidén, 2012), it was found in the present work (**Papers I, III** and **V**) that the addition of a prehydrolysis step prior to SSF of steam-pretreated spruce at high solids concentration significantly increased the ethanol yield. It was shown that it is possible to run PSSF with 20% WIS using the whole slurry of steam-pretreated spruce, with a final ethanol concentration of 64 g/L (**Paper V**).

The influence of different factors on the ethanol yield in SSF was studied in order to gain a better understanding of the influence of prehydrolysis on the overall ethanol yield (**Paper IV**). The degradation of the fibre network during enzymatic hydrolysis may have an impact on the subsequent SSF. Since no significant reduction in viscosity was observed during prehydrolysis, as has been reported for other lignocellulosic materials (see Section 4.2.1), this aspect was regarded in a broader sense as fibre degradation in general. Furthermore, PSSF using a substrate with 20% WIS resulted in significant ethanol production (**Paper V**), while SSF of a 13.7% WIS substrate led to total yeast inhibition (**Paper III**), although it is likely that the total WIS content in the PSSF experiment with 20% WIS was higher at the time of yeast addition than in SSF with 13.7% WIS. This indicates that not only the total concentration of WIS is the problem in SSF with 13.7% WIS, but also the chemical or physical structure of the substrate. The effects of the initial concentration of fermentable sugars and fibre degradation on the ethanol yield from the SSF step of PSSF were investigated in **Paper IV**.

5.2.1 Effect of initial concentration of fermentable sugars on ethanol yield in SSF

In SHF, the initial concentrations of fermentable sugars in fermentation are high, and increased glycerol production is observed with increasing sugar concentration (Zhao et al., 2013). In the present work (**Papers III** and **V**), increased sugar concentration after prehydrolysis resulted in increased glycerol production. Concentrations of glucose and mannose at yeast addition in PSSF were between 80 and 85 g/L (**Paper III**), resulting in a maximum glycerol concentration of 6 g/L after PSSF. Sugar concentrations were higher in a later study (116 g/L, **Paper V**), resulting in final glycerol concentrations as high as 11.4 g/L. Increased glycerol production indicates yeast stress, which will lead to a loss in ethanol yield, as discussed in Section 3.3.3. Hirasawa and Tokoigawa (2001) found increased fermentability in a hyperosmotic fermentation medium, compared to untreated yeast, when S. cerevisiae was subjected to high sugar concentrations for a short period of time (0.5-2 h). The amount of living yeast cells decreased, but this was compensated by a much higher fermentation ability of the surviving cells in the hyperosmotic medium. The increase in fermentation ability was believed to originate not only from the osmotic stress during cultivation, but also from the high concentration of fermentable sugar during fermentation to ethanol. High concentrations of fermentable sugars may thus have a boosting effect on fermentation. The increase in fermentation ability was only observed during the first hours of fermentation, after which the untreated and treated yeast showed the

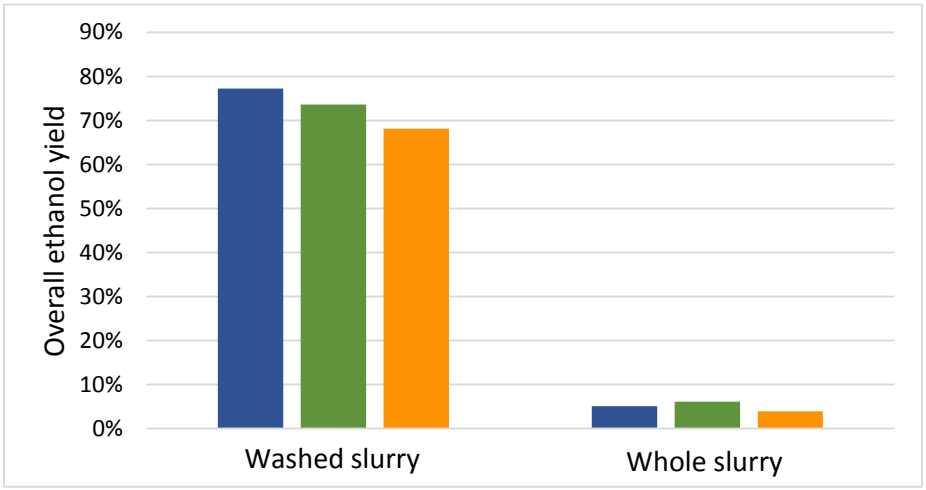

Figure 5.4: Overall ethanol yield (in % of theoretical) from SSF of steam-pretreated spruce with 13.7% WIS and varying initial concentrations of fermentable sugars: blue: low (36-38 g/L), green: moderate (61-76 g/L) and yellow: high (116-119 g/L). Adapted from **Paper IV**.

same fermentation ability. In SSF, fermentable sugars are continuously consumed by the yeast as they are produced by enzymatic hydrolysis, and their concentration is thus kept low. This results in less stress, but may also result in yeast starvation. The concentration of fermentable sugar in fermentation thus affects the yeast and ethanol production in different ways.

In the study presented in **Paper IV**, the initial concentration of fermentable sugar did not influence the overall ethanol yield from SSF significantly. In SSF of the washed spruce fibre after steam pretreatment, the yeast was able to ferment all the fermentable sugar to ethanol, regardless of the initial sugar concentration. A slight decrease in overall ethanol yield was observed at high sugar concentrations, probably due to the increased production of cell mass or by-products. This difference was, however, not sufficient to explain the difference between the overall ethanol yields in PSSF and SSF observed in **Papers I** and **III**. Glycerol was not detected during any of the SSF experiments described in Figure 5.4. SSF of the whole pretreated spruce slurry resulted in very low overall ethanol yields, regardless of the initial sugar concentration. The increase in final ethanol concentration when adding a prehydrolysis step to SSF is thus not likely to be the result of the increase in initial sugar concentration in fermentation in PSSF.

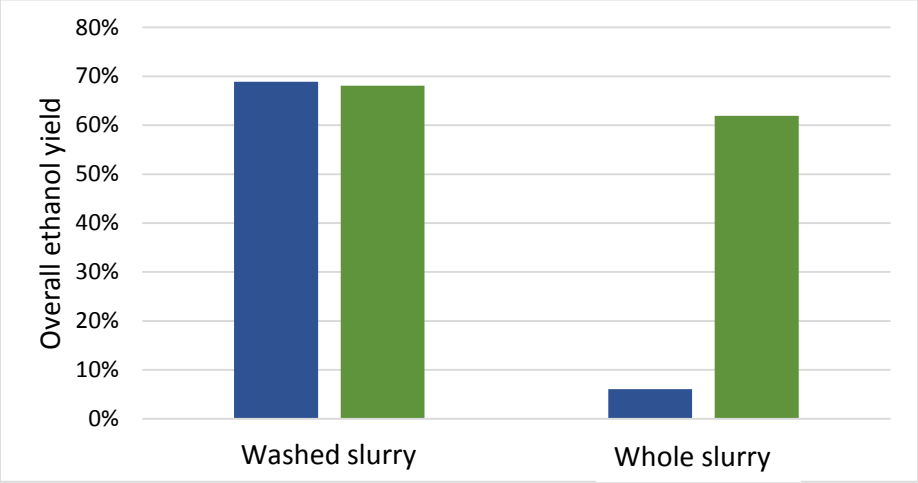

Figure 5.5: Overall ethanol yield (in % of theoretical) from SSF of steam-pretreated spruce with 13.7% WIS, with undegraded fibre (blue), and degraded fibre (green). Adapted from **Paper IV**.

5.2.2 Effect of initial degree of fibre degradation on ethanol yield in SSF

It was found to be possible to ferment the prehydrolysate of steam-pretreated spruce at a concentration corresponding to 13.7% WIS, giving an overall ethanol yield of 88.2% (**Paper IV**). SSF of washed pretreated spruce slurry also resulted in a high overall ethanol yield (77.2%). However, the yeast was not capable of fermenting glucose and mannose to ethanol when both prehydrolysate and fibre were present together (**Paper IV**). It is thus probable that a combination of the inhibitors present in the prehydrolysate and the presence of fibre prevented fermentation in SSF of the whole steam-pretreated slurry at high solids concentrations.

The yeast was able to ferment the sugar present in the liquid in the presence of fibre at a concentration of 13.7% WIS, regardless of whether the fibre was degraded or not, when using washed pretreated slurry (Figure 5.5). When using whole pretreated spruce slurry, the yeast was able to produce 47.8 g/L ethanol (overall ethanol yield of 62.1%) when the spruce fibre had been degraded by enzymes prior to SSF, while no significant ethanol production was observed in SSF with undegraded fibre. Fibre degradation thus appears to have a significant impact on ethanol production in the presence of prehydrolysate, which contains inhibitors.

The reason for the increase in ethanol production in PSSF compared with SSF at high solids concentrations is thus likely to be the degradation of the fibre prior to yeast addition. The term "fibre degradation" is used in **Paper IV** in a broad sense, and it remains to be investigated what exactly in the degradation of the pretreated fibre leads to increased fermentability. Apart from the reduction in viscosity during prehydrolysis, the cellulose is degraded, and the structure of the solid material in the pretreated slurry is altered. Furthermore, many compounds are liberated during hydrolysis. Acetic acid may be produced in enzymatic hydrolysis of agricultural residues rich in acetylated hemicellulose. This is generally not the case in softwood, which contains less acetylated hemicellulose. It has also been suggested that low water activity, which is a known stress factor in yeast fermentation, may be the reason for decreased fermentation in SSF with high solids concentration (Jørgensen et al., 2007b). We measured the water activity in steam-pretreated spruce slurry at different solids concentrations using a dew point hygrometer, and it was found that it decreased only slightly (from 1.000 to 0.987) when the WIS concentration was increased from 5 to 20%. These measurements are only preliminary, and the effect of water activity must be investigated in greater detail before the hypothesis of Jørgensen et al. (2007b) can be confirmed or rejected.

Batch SSF with steam-pretreated spruce at high solids concentrations is usually fermentation-limited. In SSF with 13.7% WIS, for example, only very little ethanol was produced, while the glucose concentration increased during the entire 120 h of the experiment (**Paper III**). Similar observations, of glucose accumulation, were made in all the experiments with insignificant ethanol production in the studies included in this thesis. When adding a prehydrolysis step of sufficient duration, all the glucose was consumed, but all the cellulose added to the reactor was not converted to glucose, indicating that enzymatic hydrolysis was limiting in PSSF (**Papers III** and **V**). In the studies on steam-pretreated spruce, presented in this thesis, the addition of prehydrolysis prior to SSF thus shifted the process from being fermentation-limited to being enzymatic-hydrolysis-limited.

5.3 Combinations of fed-batch SSF and PSSF

Prehydrolysis in PSSF can be performed in fed-batch mode to facilitate stirring of the material, allowing the solids concentration to be further increased (Varga et al., 2004; Elliston et al., 2013; Lu et al., 2013). Jørgensen et al. (2007b) reported a significant volume reduction in the prehydrolysis of steam-pretreated wheat straw, which would allow increased utilization of reactor space when performing prehydrolysis in PSSF in fed-batch mode.

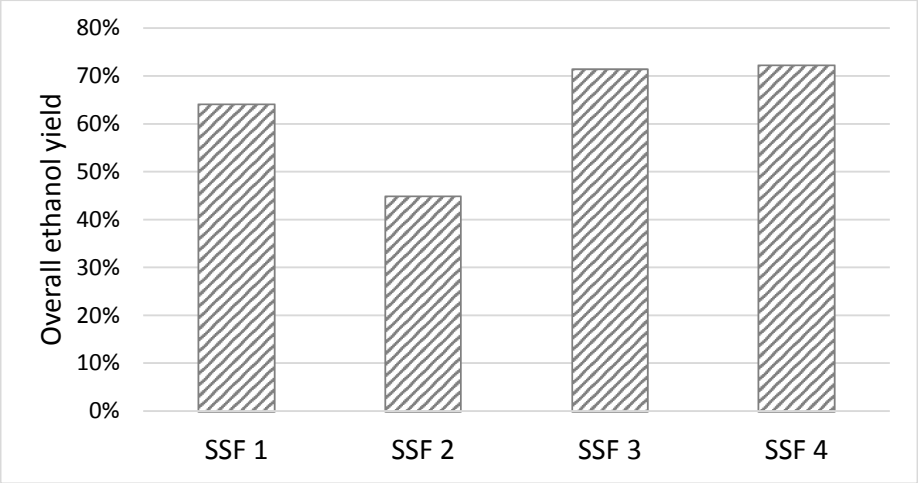

Figure 5.6: Overall ethanol yield (in % of theoretical) from PSSF and fed-batch SSF with prehydrolysed feed using steam-pretreated spruce with 20% WIS. SSF 1: PSSF with 48 h prehydrolysis, SSF 2: PSSF with 24 h prehydrolysis, SSF 3 and 4: fed-batch SSF with prehydrolysed feed and different feeding times. Adapted from **Paper V**.

In the present work (**Paper V**), fed-batch SSF and prehydrolysis were combined in another way. Steam-pretreated spruce slurry was prehydrolysed and then used as feed in fed-batch SSF. In batch PSSF of steam-pretreated spruce slurry with 20% WIS, the prehydrolysis time had a significant effect on the ethanol yield, as can be seen in Figure 5.6. Significant glycerol production was observed during PSSF with both 24 and 48 h prehydrolysis. The amount of glycerol produced in PSSF with 48 h prehydrolysis was almost twice that with 24 h prehydrolysis. Yeast produces glycerol to protect itself from hyperosmotic stress, which may originate from high concentrations of sugar. To ensure low concentrations of fermentable sugar during the entire SSF process fed-batch SSF was performed, using steam-pretreated spruce slurry that had been hydrolysed for 48 h at 48°C as feed over 8-11 h (**Paper V**). This increased the overall ethanol yield from 64% in batch PSSF to 72% (see Figure 5.6). Glycerol production was slightly lower in fed-batch SSF than in batch PSSF with 48 h prehydrolysis, but 8 g/L glycerol was produced, indicating that the yeast was also stressed when the concentrations of fermentable sugars were low.

As described in this chapter, it is clear that the choice of process configuration in enzymatic hydrolysis and fermentation has a significant impact on glucose and ethanol yields. SSF of steam-pretreated spruce with high solids concentrations appears to pose a challenge mainly to the fermenting organism. Sur-

prisingly, adding a prehydrolysis step facilitated fermentation and resulted in higher ethanol yields than in SSF without prehydrolysis. PSSF, on the other hand, was mainly hydrolysis-limited, and although ethanol yields up to 72% and final ethanol concentrations of 65 g/L were achieved with a combination of prehydrolysis and fed-batch SSF, enzymatic hydrolysis yields when using 20% WIS were still only 60-70%, showing that there is potential for further improvement.

6

Conclusions and future work

6.1 Conclusions

The aim of the present work was to increase the final ethanol concentration after fermentation of steam-pretreated spruce. This was done by evaluating and optimizing different configurations of SSF. The following main conclusions can be drawn from the results presented in this thesis.

- The overall ethanol yield in SSF decreases with increased solids concentration due to a combination of decreased mixing and increased inhibition.
- Fed-batch SSF with substrate feeding does not result in higher ethanol yield than batch SSF per se, but higher ethanol yields can be obtained if the enzymes are added in the right way.
- In fed-batch SSF with substrate feeding, enzyme feeding strategy affects the overall ethanol yield. When prehydrolysate is present, it is advantageous to feed part of the enzymes together with the substrate feed to minimize inhibition of the enzymes by compounds in the prehydrolysate, probably soluble lignin. When washed fibre is used, it is best to add all enzymes in

the batch phase to increase the contact time between enzymes and substrate.

- When feeding enzymes together with substrate in fed-batch SSF, enzymes should be mixed with the substrate feed instead of adding both separate.
- SSF of steam-pretreated spruce with 13.7% WIS did not result in any significant ethanol production in an ordinary CSTR or a rotating drum reactor designed to handle solid or semi-solid material. SSF was fermentation-limited.
- SSF of the washed solids or the liquid fraction of the pretreatment slurry with 13.7% WIS, separately, resulted in high ethanol yields, indicating that the yeast is able to ferment the sugars to ethanol in the presence of WIS or inhibitors separately.
- Prehydrolysis can significantly increase the ethanol yield in SSF, even when SSF is fermentation-limited. When adding a prehydrolysis step prior to SSF, the limitation shifts from being fermentation-limited in SSF to being hydrolysis-limited in PSSF.
- The positive effect of the addition of a prehydrolysis step in SSF of the whole slurry of steam-pretreated spruce at solids concentrations above 10% WIS appears to be a result of fibre degradation, rather than decreased viscosity or increased initial concentration of fermentable sugar.
- PSSF at high solids concentrations (20% WIS) resulted in increased glycerol production. This constitutes a yield loss in ethanol production, and indicates that the yeast is subjected to stress. Higher glucose concentrations at yeast addition resulted in higher glycerol production. A high concentration of fermentable sugar thus constitutes a stress factor for the yeast. However, this was not the only stress factor in PSSF with 20% WIS.
- A final ethanol concentration of 65 g/L with an overall ethanol yield of 72% was obtained in fed-batch SSF with 20% WIS, where prehydrolysed substrate was fed over a period of time ensuring low glucose concentrations throughout SSF. This is higher than the 4-5 wt% often stated as the limit for economically feasible distillation for the recovery of ethanol in the production of ethanol from lignocellulose. Combining fed-batch SSF and prehydrolysis in this way thus appears to be a promising alternative to achieve high ethanol concentration and yield in the production of ethanol from softwood.

6.2 Future work

SSF of whole steam-pretreated spruce slurry is limited by poor fermentation. The results presented in this thesis indicate that the yeast is negatively affected by the presence of solid material at high concentrations. The effects of solids on yeast fermentation are not well understood, and should be investigated in greater detail in SSF with high solids concentrations.

In PSSF of whole steam-pretreated spruce slurry at high solids concentrations, enzymatic hydrolysis may be the limiting step. It should be studied if this limitation is due to slow hydrolysis and thus could be overcome when performing enzymatic hydrolysis for longer time. If enzymatic hydrolysis cannot be improved by longer hydrolysis time, the inhibition of the enzymes, for example, by lignin, should be studied more extensively in order to improve the glucose yield and thus potentially the ethanol yield.

Even when performing successful PSSF with high solids concentrations, the high viscosity of the substrate poses a number of practical challenges in agitation, mixing, heating/cooling, as well as the control of pH and temperature. These must be understood well, especially when scaling up the process.

The aim of the present work was to optimize the SSF step in the production of ethanol from spruce. It is, however, important to consider the whole production process, and different process options need to be validated in the context of the entire process. The production of products other than ethanol obviously changes the optimal strategy of the process. Techno-economic evaluations are also needed to compare different process options.

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