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Bondesson, Pia-Maria

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Steam pretreatment was found to be a useful instrument to improve access of the main components of corn stover. This pretreatment resulted in high energy recovery. The choice of catalyst during steam pretreatment affected the overall energy recovery and product yield. Steam pretreatment with acetic acid or sulphuric acid improved the energy recovery compared with steam pretreatment with no catalyst or phosphoric acid. Phosphoric acid had toxic effects on ethanol and biogas production, while acetic acid was toxic only to ethanol production. The toxic effects on ethanol production were overcome by increasing the pH from 5.0 to 5.5. Process configuration also influenced the total energy recovery and product yield. This showed that not only the type of pretreatment, but also the process configuration, is important in an energy-driven biorefinery.

Acetic acid is a known inhibitor during ethanol production. Using the *S. cerevisiae* strain KE6-12b resulted in ethanol production from both glucose and xylose, despite the fact that acetic-acid-catalysed steam pretreatment was used. Fed-batch improved SSCF in terms of ethanol yield and final ethanol concentration. Increasing the water insoluble solids (WIS) concentration from 10% to 11.7% improved the ethanol concentration, but the higher amount of inhibitors had a negative effect on the ethanol yield. Increasing the yeast concentration improved the results with higher WIS, but improvements are still required to increase the ethanol yield and concentration.

Key words
Ethanol, biogas, lignocellulose, steam pretreatment, acid catalyst, acetic acid, phosphoric acid, sulphuric acid, xylose fermentation, co-fermentation, process design, SSCF

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Arriving at one goal is the starting point to another
— John Dewey

Abstract

In view of global climate change and the increasing energy demand there is a need for renewable energy resources. This thesis discusses an energy-driven biorefinery concept based on the agricultural residues corn stover and wheat straw. The work is divided into two main parts. The first part is concerned with the effects of steam pretreatment and choice of acid catalyst on ethanol and biogas production, as well as the overall energy yield. The second part focuses on the combination of acetic-acid-catalysed steam pretreatment and simultaneous saccharification and co-fermentation (SSCF) and the role of process configuration on SSCF.

Steam pretreatment was found to be a useful instrument to improve access of the main components of corn stover. This pretreatment resulted in high energy recovery. The choice of catalyst during steam pretreatment affected the overall energy recovery and product yield. Steam pretreatment with acetic acid or sulphuric acid improved the energy recovery compared with steam pretreatment with no catalyst or phosphoric acid. Phosphoric acid had toxic effects on ethanol and biogas production, while acetic acid was toxic only to ethanol production. The toxic effects on ethanol production were overcome by increasing the pH from 5.0 to 5.5. Process configuration also influenced the total energy recovery and product yield. This showed that not only the type of pretreatment, but also the process configuration, is important in an energy-driven biorefinery.

Acetic acid is a known inhibitor during ethanol production. Using the *S. cerevisiae* strain KE6-12b resulted in ethanol production from both glucose and xylose, despite the fact that acetic-acid-catalysed steam pretreatment was used. Fed-batch improved SSCF in terms of ethanol yield and final ethanol concentration. Increasing the water insoluble solids (WIS) concentration from 10% to 11.7% improved the ethanol concentration, but the higher amount of inhibitors had a negative effect on the ethanol yield. Increasing the yeast concentration improved the results with higher WIS, but improvements are still required to increase the ethanol yield and concentration.

Populärvetenskaplig sammanfattning på svenska

En väldigt stor del av energiinnehållet i majshalm, upp till 88%, har jag omvandlat till etanol, biogas och fast bränsle. Detta uppnåddes genom att behandla halmen med ättiksyra och högtrycksånga som ett första steg i processen.

År 2015 enades 195 länder om ett nytt klimatavtal i Paris. Ökningen av den globala medeltemperaturen ska vara maximalt 2°C jämfört med den temperatur som fanns innan industrialismen. Detta kräver att vi kritiskt utvärderar vilka energikällor och transportbränslen vi har idag och försöker hitta alternativ till dessa. I mitt arbete har jag studerat omvandlingen av majs- och vetehalm till energirika bränslen. Genom att undersöka det första steget, d.v.s. sönderdelningen av halm, kunde jag omvandla 88% av energiinnehållet i halm till energirika bränslen. Med ytterligare processutveckling kunde jag producera etanol från de två sockerarterna glukos och xylos.

Idag kommer majoriteten av allt bränsle som används inom energisektorn och transportsektorn från fossila bränslen och en synnerligen liten del från alternativa källor. Halm skulle dock kunna ersätta en del av de fossila bränslena för tillverkning av fordonsbränsle, värme och elektricitet. För att kunna omvandla halm till energirika bränslen krävs det ett förbehandlingssteg som sönderdelar halmen. Därefter kan den omvandlas till etanol, biogas och fast bränsle. I mitt arbete har jag bland annat studerat förbehandlingssteget och hur det har påverkat mängden produkter som har framställts. Detta gjordes för att utvinna så mycket energirika bränslen som möjligt från halmen.

Förbehandling av halm gjordes genom att använda enbart högtrycksånga eller högtrycksånga kombinerat med en katalysator i form av svavelsyra, ättiksyra eller fosforsyra. Tillsatsen av ättiksyra och svavelsyra resulterade i hög omvandling av halm till energirika produkter. Ättiksyran är sämre för etanolproduktionen eftersom den påverkar jästen, som behövs för att producera etanol, negativt men har mindre

miljöpåverkan än svavelsyra. På grund av den lägre miljöpåverkan och den höga energiomvandlingen är därför ättiksyra intressant för biobränsleproduktion. Idag är det vanligt att processa halm med enbart högtrycksånga, men genom att tillsätta en syra ökar effekten.

Etanol är väldigt intressant både som bränsle och som byggsten till andra kemikalier. Man bör eftersträva att utvinna så stor mängd etanol som möjligt ur halm. Orsakerna till detta är flera såsom minskad produktionskostnad och ökad mängd etanol tillgänglig som både bränsle och kemikaliebyggsten. Därför är det bra om förhållandet producerad etanol jämtemot mängd utnyttjad halm är så stor som möjligt.

Jag undersökte olika processalternativ för att producera etanol från vetehalm som förbehandlats med högtrycksånga och ättiksyra. För att öka mängden etanol utnyttjades de två sockerarterna glukos och xylos som är de vanligaste sockerarterna i halm. Glukos är lätt att omvandla till etanol och det räcker med vanlig bagerijäst för att få hög etanolproduktion. Xylos är däremot svårare att utnyttja och kräver en annorlunda typ av jäst, t.ex. en genmodifierad jäst. Ättiksyra, som påverkar bagerijäst negativt, har visat sig ha större negativ påverkan på genmodifierad jäst och därmed på etanolproduktionen från xylos. För att minska inverkan av ättiksyra har jag undersökt olika processalternativ och två genmodifierade jästtyper. Genom att öka pH i processen minskar den negativa inverkan från ättiksyra. Det kombinerade valet av jäst och process är också viktigt för att få fram etanol från både glukos och xylos. Genom att dela upp den förbehandlade halmen i vätska och fast material och tillsätta dessa i olika omgångar kunde jag öka mängden etanol som tillverkades jämfört med om allt hade tillsatts samtidigt.

I min avhandling diskuteras förbehandlingssteget och dess påverkan på produktionen av etanol, biogas och fast bränsle. Utöver det diskuteras olika processalternativ för att producera etanol från glukos och xylos när ättiksyra används som katalysator under förbehandlingssteget. Båda delarna är viktiga i designen av ett energiinriktat bioraffinaderi, där det är viktigt att utnyttja maximalt av energiinnehållet av råvaran. Att kunna producera energi och bränsle på ett hållbart sätt är viktigt och kommer att vara ännu viktigare i framtiden. Med denna avhandling har det tagits ytterligare ett steg mot produktionen av biobränsle.

List of publications

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **Bondesson P-M**, Galbe M, Zacchi G. (2013) Ethanol and biogas production after steam pretreatment of corn stover with or without the addition of sulphuric acid. Biotechnology for Biofuels 6:11

II. **Bondesson P-M**, Galbe M, Zacchi G. (2014) Comparision of energy potentials from combined ethanol and methane production using steam-pretreated corn stover impregnated with acetic acid. Biomass and Bioenergy 67:413–424

III. **Bondesson P-M**, Dupuy A, Galbe M, Zacchi G. (2014) Optimizing ethanol and methane production from steam-pretreated phosphoric-acid impregnated corn stover. Applied Biochemistry and Biotechnology 175(3):1371–1388

IV. **Bondesson P-M**, Galbe M. Process design of SSCF for ethanol production from steam-pretreated, acetic-acid-impregnated wheat straw. (*Accepted for publication in Biotechnology for Biofuels*)

My contributions to the studies

I. I planned the study and performed the experiments. I evaluated the results with my co-authors. I wrote the article, which was critically reviewed and commented by all authors. I handled the submission process.

II. I planned the study and performed the experiments. I evaluated the results with my co-authors. I wrote the article, which was critically reviewed and commented by all authors. I handled the submission process.

III. I planned the study and performed the biogas experiments. I evaluated the results with my co-authors. I wrote parts of the article, which was critically reviewed and commented by all authors. I handled the submission process.

IV. I planned the study and performed the experiments. I evaluated the results with my co-author. I wrote the article, which was critically reviewed and commented by all authors. I handled the submission process.

Other related publications

I have also contributed to the paper below. However, this publication is not included in the thesis

Gladis A, **Bondesson P-M**, Galbe M, Zacchi G. (2015) Influence of different SSF conditions on ethanol production from corn stover at high solids loadings. Energy Science & Engineering 3(5):481–489

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Abbreviations

AD	Anaerobic Digestion
AFEX	Ammonia Fibre Explosion
BMP	Biochemical Methane Potential
COP21	UN Climate Change Conference in Paris
DM	Dry Matter
FPU	Filter Paper Unit
HMF	5-Hydroxymethyl-2-Furaldehyde
IEA	International Energy Agency
LCF	Lignocellulosic Feedstock
LPMO	Lytic Polysaccharide Monooxygenases
PPP	Pentose Phosphate Pathway
SHCF	Separate Hydrolysis and co-Fermentation
SHF	Separate Hydrolysis and Fermentation
SSCF	Simultaneous Saccharification and co-Fermentation
SSF	Simultaneous Saccharification and Fermentation
WIS	Water Insoluble Solids
XDH	Xylitol Dehydrogenase
XI	Xylose Isomerase
XK	Xylulokinase
XR	Xylose Reductase

Contents

1

Introduction

The world's population is growing, and with it the demand for energy. Worldwide energy consumption has more than doubled in the past 30 years (IEA 2016). Most of the primary energy supply is still obtained from coal, oil and natural gas; oil being the major source in the transportation sector, accounting for 93% of the total (IEA 2016).

It has been projected that natural gas and oil production will level off as resources become depleted, while the demand and consumption will increase. This was expected to result in high oil and gas prices, and interest therefore turned to alternative energy sources. The production of biofuels has increased, but the proportion of biofuels has not increased (IEA 2016). The price of oil and gas has not increased as expected due to oil and gas production from extraction routes other than traditional ones and a lower increase in energy demand than expected in China and India. However, biofuels and other renewable energy sources are projected to be the fastest-growing fuels in the power sector (Newell et al. 2016).

1.1 Climate change, energy supply and political measures

The use of biofuels and other renewable energy sources is increasing, mainly as a result of political measures, the aim of which is to meet the challenges of climate change and increasing energy demand.

It is now accepted by the majority of researchers that climate change and global warming are largely the result of human activities (IPCC 2014). The longer the delay

before actions are taken to limit the effects of climate change, the greater the risk of severe and irreversible changes in the environment (IPCC 2014). Considerable efforts have thus been made worldwide to counteract climate change and slow down, or stop, global warming.

In 2015, 195 countries adopted the Paris Agreement[1] resulting from the UN Climate Change Conference in Paris (COP21), which is planned to come into effect in 2020. The goal of COP21 is to ensure that the average global increase in temperature does not exceed 2°C, preferably 1.5°C, compared to pre-industrial temperature levels. Each country sets their own goals, based on national conditions, and these will be followed up and updated every five years.

Apart from mitigating climate change, ensuring a secure energy supply will be a considerable challenge in the future. The demand for energy is growing, and most oil and natural gas resources are derived from politically unstable regions. Therefore, many countries and regions are eager to secure a local or regional energy supply, which has in turn led to political measures promoting the use of biofuels and other renewable energy sources.

The European Union (EU) has had the overall goal since 2007 of decreasing greenhouse gas emissions by 20% by 2020, compared with the emissions in 1990. Other goals have also been set by the EU, for example, to decrease energy utilization by 20%, to increase the proportion of renewable energy to 20% of the total energy consumption, and to increase the amount of renewable fuel in the transportation sector to 10% of the energy consumption in that sector[2]. National emission goals differ depending on national wealth, from decreasing, to being allowed to increase. In 2014, additional climate goals were set out for 2030 in a new EU framework, requiring greenhouse gas emissions to be reduced to 40% of the levels of 1990, and the proportion of renewable energy to be at least 27% of the total energy consumption in the EU[3].

The goals set in Sweden for 2020 are to reduce emissions by 40% compared with the levels of 1990; to have at least 50% renewable energy; to achieve a 20% improvement in energy efficiency compared with 1990; and that the proportion of renewable fuel in the transportation sector should be 10% of the energy consumption in that sector. In 2015, the Swedish Government presented an outlook on the possibility of achieving these goals in which it was stated that it is very likely that the goals will not only be fulfilled, but will be exceeded (Swedish Government 2015). The goals to be set after 2020 will be discussed in the EU during 2016 and 2017, resulting in a roadmap describing how

1. http://unfccc.int/files/meetings/paris_nov_2015/application/pdf/paris_agreement_english_.pdf, accessed 2016-09-30
2. http://ec.europa.eu/clima/policies/strategies/2020/index_en.htm, accessed 2016-09-30
3. http://ec.europa.eu/clima/policies/strategies/2030/index_en.htm, accessed 2016-09-30

the goals in EU's framework for 2030 are to be achieved[4]. The vision of the Swedish Government is that Sweden should have no net greenhouse gas emissions by the year 2050[5].

Many different strategies must be applied if we are to achieve the goals set out in the Paris Agreement. Political steering is important in reducing future greenhouse gas emissions (IEA 2015), but this must be accompanied by technological development, including the implementation of low-carbon and carbon-neutral technologies (IPCC 2014). Replacing fossil fuels with biofuels is one step towards reducing greenhouse gas emissions, as well as offering a secure supply of sustainable energy in the future.

1.2 Aims and outline of this thesis

This thesis deals with an energy-driven biorefinery concept, i.e. a biorefinery where the biomass is utilized for the production of fuels, power and heat. The process investigated is based on the use of corn stover and wheat straw to produce ethanol and biogas for the transportation sector and for heat and power generation, and solid fuel for heat and power generation. The overall goal was to develop a process that converts as much as possible of the raw materials into useful energy.

The research was divided into two parts: a study to identify suitable pretreatment conditions, and a study of various simultaneous saccharification and co-fermentation (SSCF) strategies. The aim was to use cellulose for ethanol production, hemicellulose for biogas and/or ethanol production, and lignin as a solid fuel. It is important to utilize all the components of the biomass to maximize the overall energy yield. Pretreatment was therefore investigated as the first fractionation step, to separate cellulose, hemicellulose and lignin. The research on pretreatment focused on the impact of steam pretreatment using different acid catalysts on the subsequent process steps in relation to liquid, gaseous and solid products, as well as the overall energy recovery. The work on SSCF focused on the design of SSCF, when using acetic-acid-catalysed steam pretreated wheat straw, to produce ethanol from both glucose and xylose with modified yeast strains.

Chapter 2 presents a description of the biorefinery concept together with the structure of lignocellulosic biomass. The key processes in this work: pretreatment, ethanol production and biogas production, are also described in this chapter. In Chapter 3, the choice of catalyst in steam pretreatment and the influence on the downstream

4. http://www.regeringen.se/artiklar/2016/05/forhandlingar-om-hur-eus-klimatmal-till-2030-ska-nas/, accessed 2016-09-30
5. http://www.riksdagen.se/sv/dokument-lagar/dokument/kommittedirektiv/klimatfardplan-2050-strategi-for-hur-visionen_H2B153, accessed 2016-09-30

processes and on the product yields is discussed. Two different process configurations were investigated with respect to their product yields and overall energy recovery. The main difference between the two configurations is that in the second configuration the hemicellulose-rich liquid is separated from the cellulose- and lignin-rich solids after pretreatment. Chapter 4 focuses on ethanol production by the fermentation of both glucose and xylose through SSCF. Different SSCF configurations together with two recombinant strains of *S. cerevisiae* were investigated. In the final chapter, Chapter 5, the main findings are summarized together with suggestions for future research.

2

Biorefining

Biorefining is defined by the International Energy Agency (IEA) Bioenergy Task 42 as: "the sustainable processing of biomass into a spectrum of marketable biobased products and bioenergy"[6]. Biorefining is not a new concept. The production of vegetable oil, sugar, starch and vitamins in the food industry, as well as pulp and paper production, are examples of biorefineries that have been in operation for a considerable time. Today, the development of biorefineries has two strategic goals: an energy goal and an economic goal. The former aims at replacing fossil fuels with renewable domestic raw materials to ensure a secure supply of energy and reduce environmental impact, while the latter is to establish an economically viable biobased industry (Bozell & Petersen 2010).

The biorefinery concept is a wide one due to the large variety of raw materials, platforms (intermediates such as sugars, syngas and biogas), products (biofuels, food, feed, chemicals and materials) and conversion processes (biochemical, thermochemical, chemical and mechanical). However, regardless of the type of biorefinery, the ultimate goal is to utilize biomass efficiently and sustainably (de Jong & Jungmeier 2015). This requires optimization of biomass conversion and minimization of feedstock requirement as the availability of biomass is limited, while the range of energy and products needed is extensive.

A biorefinery is often compared to the traditional petrochemical refinery, in which raw oil is converted into various fuels and chemicals. However, there are many differences between a traditional petrochemical refinery and a biorefinery, although

6. http://www.iea-bioenergy.task42-biorefineries.com/en/ieabiorefinery/Factsheets.htm, accessed 2016-09-30

the basic concept is the same, i.e. to convert raw material into various products (de Jong & Jungmeier 2015). One important difference is the heterogeneity of the raw materials used in a biorefinery, which have a high content of oxygen, compared with the relatively homogeneous, low-oxygen oil used in a petrochemical refinery. The heterogeneity of the raw materials is reflected in the wide range of processes used to obtain the final products in a biorefinery. The difference in the composition of the components involved in the two kinds of refinery is also important. While a petrochemical refinery mainly utilizes well-defined, simple molecules such as ethylene, propylene, methane, benzene, toluene and xylene isomers, a biorefinery utilizes sugar monomers, such as glucose and xylose, as well as fatty acids, phenols and many other compounds.

2.1 Lignocellulosic feedstock biorefinery

The concept of a lignocellulosic feedstock (LCF) biorefinery implies refining lignocellulosic biomass into its basic macromolecules, which are then processed into various products and bioenergy (Kamm & Kamm 2007). LCF biorefineries have the potential to be successful because of the diversity and moderate costs of lignocellulosic biomass compared with traditional biorefinery feedstock such as wheat, maize and sugar cane. Furthermore, there is no competition with food and feed production. The product portfolio is also similar to that of an oil refinery; the products can replace those produced in petrochemical refineries, as well as providing new products for a future bio-based product market (de Jong & Jungmeier 2015, Kamm & Kamm 2007). However, a LCF biorefinery must be developed for the conversion of lignocellulosic biomass to valuable products to be technically and economically feasible (FitzPatrick et al. 2010). The key to a successful LCF biorefinery is to be able to efficiently separate the different fractions making up lignocellulosic biomass, namely, cellulose, hemicellulose and lignin. The most important step in an LCF biorefinery is, therefore, the pretreatment step, where fractionation takes place. An example of an energy-driven LCF biorefinery is shown in Figure 2.1. Pretreatment is used to separate hemicellulose from cellulose and lignin. The cellulose is used for ethanol production, while the lignin-rich residue is used as a solid fuel. Hemicellulose is used for biogas production.

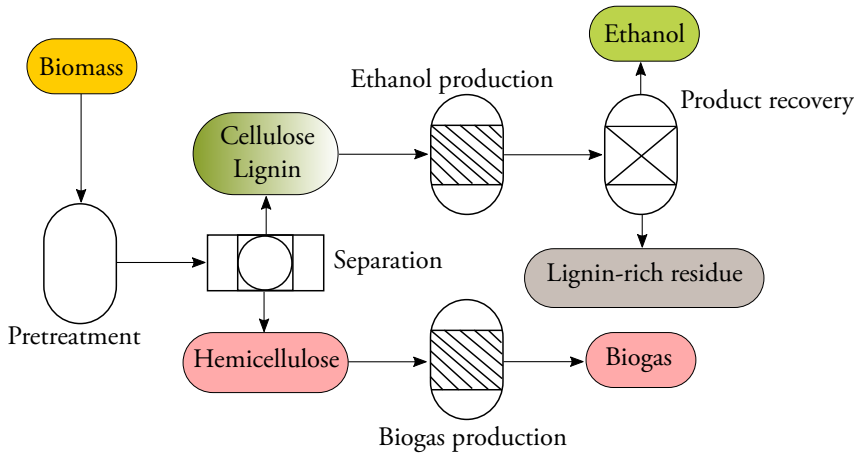

Figure 2.1: Simplified view of an energy-driven lignocellulosic feedstock biorefinery.

2.2 Lignocellulosic biomass

Many different raw materials can be described as lignocellulosic, including wood and forest residues from softwood and hardwood, agricultural residues and energy crops and grasses. The choice of biomass feedstock depends on the availability, yield per hectare and variation in quality, as well as market price and political decisions. The composition of the main macromolecules differs depending on the type of biomass, as can be seen from Table 2.1, which affects the choice of processes. There may also be differences in the composition of the same biomass due to differences in cultivation and harvesting conditions, as well as seasonal variations (Öhgren et al. 2005, Sander 1997).

2.2.1 Biomass composition

The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. These components are found in the plant cell wall, where they are highly interlinked (Figure 2.2). In addition to these three main components, lignocellulosic biomass contains small amounts of other components such as pectins, fats, resin acids, proteins and inorganic compounds (Sjöström 1993). Cellulose is an unbranched polysaccharide that affords structural strength to the cell wall. Cellulose consists of repeating units of cellobiose, which consists of two glucose molecules. These cellulose chains can be crystallized into bundles of chains, called microfibrils. The strength of the cell wall depends on the length, angle and crystallinity of the microfibrils (McFarlane et al. 2014).

Table 2.1: Composition of different kinds of lignocellulosic biomass (% of DM)

	Cellulose	Hemicellulose				Lignin	Reference
	Glucan[a]	Xylan	Galactan	Arabinan	Mannan		
Agricultural residue							
Corn stover	34.9	18.7	1.1	2.8	N.D.	14.1	1
Corn stover	38.0	22.8	1.5	3.9	0.7	20.4	2
Wheat straw	29.3	21.6	0.3	3.2	0.1	27.5[b]	3
Softwoods							
Pine	44.9	6.2	3.0	1.9	11.5	26.6	4
Spruce	44.9	5.2	2.2	2.0	12.0	31.1	5
Hardwoods							
Poplar	43.8	14.9	1.0	0.6	3.9	29.1	6
Willow	43.0	14.9	2.0	1.2	3.2	26.6	7
Energy crops/grasses							
Switchgrass	36.6	21.1	1.0	2.8	0.8	18.3	8
Giant reed	35.7	18.6	0.6	1.6	0.2	22.3	9

[1] Paper I-II, [2] Paper III, [3] Paper IV, [4] Ewanick et al. (2007), [5] Hoyer et al. (2010), [6] Bura et al. (2009), [7] Sassner et al. (2006), [8] Suryawati et al. (2009), [9] Scordia et al. (2011)

[a] Glucan can be found in both cellulose and hemicellulose, but the main fraction is found in cellulose.

[b] Including ash

N .D. Not detected

Hemicellulose comprises a heterogenic group of branched polysaccharides. These polysaccharides consist mainly of the sugars xylose, glucose, arabinose, galactose and mannose. Part of the backbone or sidechains is acetylated, or other chemical groups, such as ferulic acid esters and glucuronic acid, may be attached (Scheller & Ulvskov 2010). In most cell walls, one type of hemicellulose dominates, while the others are only present at small amounts. In straw the hemicellulose arabinoxylan dominates (Brigham et al. 1996), while in softwoods it is galactoglucomannan (Ademark et al. 1998). The main role of hemicellulose is to glue and tether the cellulose fibrils, providing strength and flexibility in the cell wall (Scheller & Ulvskov 2010, Viikari et al. 2012).

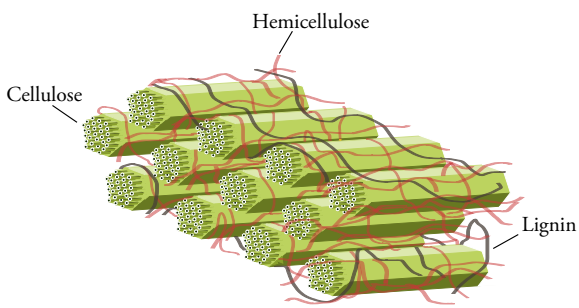

Figure 2.2: The structure of lignocellulose within the cell wall.

The last main component in lignocellulosic biomass is lignin. Lignin is an aromatic polymer made up of coniferyl, sinapyl and p-coumaryl alcohols. The structure is very complex, and it is difficult to isolate and investigate in its intact state. The structure of lignin is thus still the subject of debate (Albersheim et al. 2010). Lignin acts as a kind of glue in the cell wall contributing to the strength of the wall, and offering protection of the polysaccharides against microbial degradation. Lignin also serves as an internal water transport system, especially in trees. Water is transported vertically through lignin tubes in the tree, while the hydrophobic nature of lignin serves as a barrier to lateral water transport (Albersheim et al. 2010).

2.3 Pretreatment

Due to the complex and recalcitrant structure of lignocellulosic biomass, pretreatment is necessary to improve the utilization of the different macromolecules. The structure, size and chemical composition of the components in the biomass are changed by pretreatment making them more accessible (Mosier et al. 2005). Pretreatment is, therefore, the most crucial step in the process. It is also one of the most expensive steps in the conversion of biomass (Yang & Wyman 2008). Pretreatment has a considerable impact on all the other steps in the process, since the design and outcome of further process steps are dependent on the outcome of the pretreatment step (Galbe & Zacchi 2007, Yang & Wyman 2008). The choice of pretreatment method depends on the composition of the biomass and the choice of products. Some pretreatment methods solubilize hemicellulose, some solubilize lignin, while some only change the structure of the solids. The amount and kind of degradation products also differ between pretreatment methods and conditions. The choice of biomass is important as different kinds of biomass contain different amounts of sugars and lignin. Therefore, the combination of pretreatment method and raw material affects the overall process design. To evaluate the performance of pretreatment, it is important to investigate how the following process steps and the final product yields are affected, as well as the production cost (Galbe & Zacchi 2007).

Many different pretreatment methods are available, and can be divided into biological, physical, chemical, and a combination of physical and chemical, i.e. physicochemical pretreatment (Alvira et al. 2010, Galbe & Zacchi 2007, Sun & Cheng 2002). Biological pretreatment involves the use of microorganisms, mainly brown, white and soft-rot fungi, which degrade mainly lignin and hemicellulose (Alvira et al. 2010, Hatakka 1983, Sun & Cheng 2002). Physical pretreatment often involves size reduction (comminution) and extrusion. The lignocellulosic structure is broken down through cutting/grinding and defibrillation, resulting in increased surface area and opening of the fibre structure (Duque et al. 2013, Karunanithy et al.

2012). In chemical pretreatment, the chemicals used solubilize either hemicellulose or lignin from the lignocellulosic structure. The pH is often important in such methods (Yang & Wyman 2008). Physicochemical pretreatment methods include ammonia fibre explosion (AFEX), wet oxidation and steam pretreatment with a catalyst (Alvira et al. 2010, Elander et al. 2009, Holtzapple et al. 1991). Steam pretreatment without a catalyst is difficult to categorize in any of those categories, but is often regarded as a kind of physicochemical pretreatment.

2.3.1 Steam pretreatment

One of the most studied and used pretreatment methods for lignocellulosic biomass is steam pretreatment (Galbe & Zacchi 2007, Kravanja et al. 2012). Steam pretreatment is sometimes called steam explosion as it was believed that an "explosion" caused the cellulose fibres to split open, making them more accessible to enzyme degradation and other forms of hydrolysis. However, it has been shown that the "explosion" itself may not be the main mechanism in improving enzymatic digestibility, but the most important mechanism behind steam pretreatment is a mechanism similar to acid hydrolysis (Brownell et al. 1986, Muzamal et al. 2015).

During steam pretreatment, the material is subjected to high-pressure saturated steam at a temperature between 160 and 240°C for a period of several seconds to some minutes. Water can act as an acid at high temperatures since high temperature causes self-ionization, which promotes autohydrolysis. The water cleaves acetyl groups from the hemicellulose, which form acetic acid. The free acetic acid further catalyses the hydrolysis of hemicellulose into soluble oligomeric and monomeric sugars (Schultz et al. 1986). Mechanical effects occur together with the chemical effect of hydrolysis. Mechanical effects result from synergistic effects of vapour expansion, the rapid pressure release and the collision of material on vessel walls, which cause fibre separation (Muzamal et al. 2015). Autohydrolysis and fibre separation cause partial hydrolysis and solubilization of the hemicellulose, as well as redistribution and, to some extent, solubilization of lignin (Figure 2.3) (Alvira et al. 2010). The result of steam pretreatment is a liquid fraction containing mainly hemicellulose in monomeric (single sugar molecules) and oligomeric (chains of a few to several sugar molecules) forms, and a solid fraction containing mainly cellulose and redistributed lignin. The redistributed lignin is formed through melting and depolymerization/repolymerization (Donaldson et al. 1988, Li et al. 2007, Shevchenko et al. 1999).

The original concept of steam pretreatment using only steam is not sufficient to degrade some kinds of lignocellulosic biomass. It is much more difficult to initiate the autohydrolysis of softwood due to the small amounts of organic acids attached to hemicellulose (Galbe & Zacchi 2012, Jørgensen et al. 2007a, Kumar et al. 2009). The

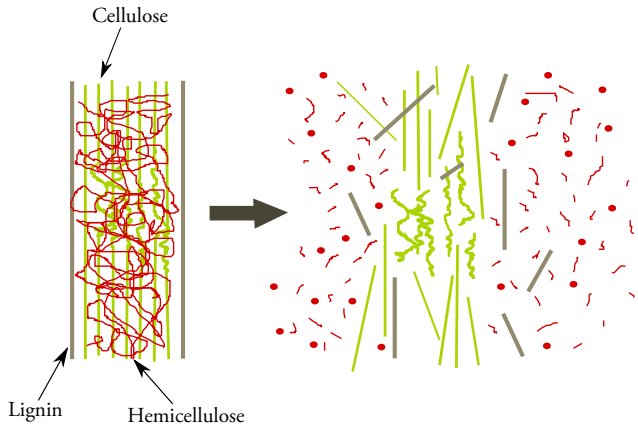

Figure 2.3: Illustration of the degradation of biomass using steam pretreatment. (Adapted from Mosier et al. (2005))

efficiency of hemicellulose hydrolysis can be improved by adding a catalyst, for example, an acid (Clark & Mackie 1987, Schwald et al. 1989, Stenberg et al. 1998). This is also true for other kinds of lignocellulosic biomass. An acid catalyst not only improves the hydrolysis of hemicellulose, it can also reduce the time and temperature required, thus lowering the amount of degradation products formed (Ballesteros et al. 2006, Bura et al. 2003).

Steam pretreatment, with or without an acid catalyst, has several advantages and disadvantages. According to Alvira et al. (2010), the advantages compared with other pretreatment methods are potential for less environmental impact with less hazardous process chemicals, lower capital cost, higher energy efficiency and high sugar recovery. Steam pretreatment also has the advantage that it can be used for many types of biomass, by choosing an appropriate catalyst. Steam pretreatment has also been shown to work on a large scale, and has been implemented in several pilot/demonstration/full-scale plants producing ethanol from lignocellulosic biomass. Examples of such plants are: DOE's pilot plant in Golden, Colorado (USA)[7]; SP Processum's Biorefinery demo plant in Örnsköldsvik (Sweden)[8]; Inbicon's demonstration plant in Kalundborg (Denmark) (Larsen et al. 2012); Iogen's demonstration plant in Ottawa (Canada)[9]; Beta Renewable's commercial plant in Crescentino (Italy)[10]; POET-DSM's commercial plant in Emmetsburg, Iowa (USA)[11] and Abengoa's commercial plant in Hugoton, Kansas (USA)[12].

7. http://www.nrel.gov/docs/fy00osti/28397.pdf , accessed 2016-09-30
8. http://www.sekab.com/biorefinery/demo-plant/ , accessed 2016-09-30
9. http://www.iogen.ca/cellulosic_ethanol/index.html , accessed 2016-09-30
10. http://www.betarenewables.com/crescentino/project , accessed 2016-09-30
11. http://www.poetdsm.com/liberty , accessed 2016-09-30
12. http://www.abengoabioenergy.com/web/en/2g_hugoton_project/ , accessed 2016-09-30

The disadvantages of steam pretreatment with an acid catalyst include the cost of the acid and acid removal, and the demands placed on non-corrosive equipment (Alvira et al. 2010, Yang & Wyman 2008). The main disadvantage of steam pretreatment, regardless of whether an acid catalyst is used or not, is the formation of degradation products, mainly from hemicellulose and lignin, which may be toxic or inhibitory to the microorganisms in the following process steps. However, inhibitor formation is a common problem in many pretreatment methods and is not unique to steam pretreatment.

Inhibitors

The formation of inhibitory compounds increases with increasing severity of steam pretreatment. Time, temperature and acid concentration all affect the severity of pretreatment (Abatzoglou et al. 1992, Chum et al. 1990). Inhibitors may be found naturally in biomass (for example, acetyl groups and extractives), or may be formed by the degradation of cellulose, hemicellulose and lignin (Figure 2.4).

The major inhibitors are furan derivatives, weak acids and phenolic compounds. Furan derivatives are formed by the degradation of sugar molecules. The main compounds, furfural and 5-hydroxymethyl-2-furaldehyde (HMF), are formed by the degradation of pentoses (5-carbon sugars) and hexoses (6-carbon sugars), respectively. Weak acids such as formic and levulinic acid are formed by the further degradation of furfural and HMF, while acetic acid is formed by the release of acetyl groups attached to hemicellulose. Phenolic compounds are formed during the degradation of lignin. All these compounds are potentially inhibitory, or toxic, to some degree, to some or all of the microorganisms used in a biorefinery (Almeida et al. 2007, Horváth et al. 2001, Larsson et al. 1999, 2000, Palmqvist et al. 1999b, Palmqvist & Hahn-Hägerdal 2000).

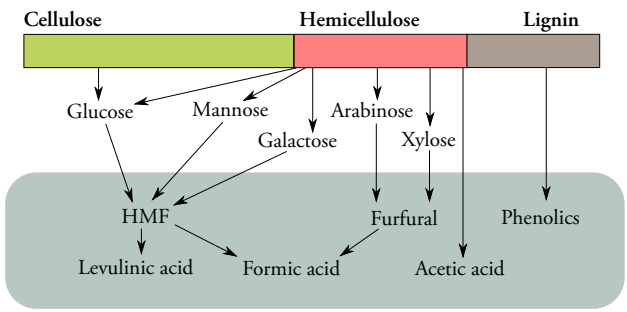

Figure 2.4: Common inhibitors formed by the degradation of cellulose, hemicellulose and lignin.

2.4 Ethanol production

Ethanol is a chemical of considerable interest in biorefining. Ethanol can be used as a transportation fuel by itself, or blended with gasoline, to replace fossil fuels in the transportation sector. Ethanol is also one of the potential top 10 chemical building blocks proposed for the future derivation of chemicals from biomass (Bozell & Petersen 2010).

Ethanol can be produced from lignocellulosic biomass through thermochemical or biochemical processes. Thermochemical processes comprise pretreatment followed by gasification into syngas. After purification, this can be used for ethanol production using catalytic synthesis or fermentation (Dwivedi et al. 2009). Ethanol production using biochemical processes is part of the so-called "sugar platform", where sugar molecules are the important intermediate products obtained from biomass. The production of ethanol using biochemical processes includes four main steps:

i. pretreatment,
ii. hydrolysis,
iii. fermentation and
iv. product recovery.

Hydrolysis involves breaking down the cellulose and hemicellulose into monomeric sugar building blocks, and can be performed with acids or enzymatically. In pure acid hydrolysis, no pretreatment is needed. The liberated sugar molecules are converted into ethanol by fermentation with yeast or bacteria. In the last step, product recovery, ethanol is separated from the rest of the medium, normally by distillation or a combination of distillation and evaporation.

2.4.1 Enzymatic hydrolysis

Enzymatic hydrolysis involves the conversion of polysaccharides into monomeric sugars using enzymes. Pretreatment of the lignocellulosic biomass is necessary before enzymatic hydrolysis can be performed effectively. Enzymatic hydrolysis is a complex process due to the recalcitrant nature of lignocellulosic biomass (Viikari et al. 2012). Enzymatic hydrolysis was long considered one of the most costly steps in the conversion of lignocellulosic biomass into ethanol. However, advances in enzyme technology have reduced the cost (Viikari et al. 2012).

Cellulose consists of β-1,4 linked glucose units, which are ordered into microfibrils through hydrogen bonds and van der Waals interactions. The fibrils are tightly packed and consist of ordered, crystalline, non-soluble regions and disordered, amorphous

regions. The crystalline regions pose a greater challenge to cellulose-degrading enzymes. Hemicellulose, on the other hand, is much easier to degrade, but requires several types of enzymes. Xylans, which are common in agricultural residues such as straw, consist of β-1,4 linked xylose units in the backbone. The backbone and branched structure have a high degree of acetyl esterification and, in the case of straw, arabinose substitutions. However, complex branching and acetylation patterns make some of the structures recalcitrant (Horn et al. 2012).

Several enzymes are needed to degrade the polysaccharides into sugar units. The most commonly used enzyme cocktails are derived from the fungus *Trichoderma reesei*. In the classical view of cellulose degradation, cellulose is degraded into glucose units synergistically by three groups of enzymes endo-1,4-β-glucanases, exo-1,4-β-glucanases and β-glucosidases (Horn et al. 2012, Persson et al. 1991, Van Dyk & Pletschke 2012). Endo-1,4-β-glucanases cleave cellulose bonds randomly in the cellulose chain, preferably in the amorphous regions. Cleavage results in new ends in the chain that are exposed to the exo-1,4-β-glucanases. Exo-1,4-β-glucanases generate cellobiose (cellobiohydrolases) or glucose units (glucanohydrolases) from the reducing or non-reducing end of the cellulose chain. Endo- and exoglucanases have different preferences regarding cellulose structure (crystalline, amorphous), and a commercial cocktail often contains many different enzymes of these types (Horn et al. 2012). The third group, β-glucosidases, degrades the resulting cellobiose units from the two other enzyme groups into glucose units.

A fourth group of cellulose-degrading enzymes is included in modern enzyme cocktails. These are lytic polysaccharide monooxygenases (LPMOs) and act by oxidizing polysaccharides, both cellulose and hemicellulose, into aldonic acids such as gluconic acid. LPMOs have flat substrate binding sites, which improves the degradation of cellulose, since LPMOs can attach to flat crystalline surfaces, resulting in new entry sites for exoglucanases (Cannella & Jørgensen 2014, Horn et al. 2012).

The hydrolysis of hemicellulose requires more types of enzymes due to the greater variation in its structure. Enzymes can be divided into those needed for cleavage of the backbone and those needed to remove substituents (Moreira & Filho 2016, Van Dyk & Pletschke 2012). The hemicellulose fraction can be removed and disrupted by pretreatment, especially pretreatment with acidic catalysts. However, since pretreatment can result in degradation products, milder pretreatment methods are preferred. Milder pretreatment results in a higher amount of oligomeric sugar that requires enzymes for further hydrolysis into monomeric sugars. Furthermore, some hemicellulose is not solubilized during pretreatment and can interfere with cellulose hydrolysis (Hu et al. 2011, Öhgren et al. 2007a). Therefore, hemicellulases may also be important in ethanol production and as a component in commercial enzyme cocktails.

In addition to the enzymes that directly degrade the polysaccharides, other enzymes and proteins may be important in enzymatic hydrolysis. These are enzymes that contribute to wall loosening (swollenins and expansins), protein- and lignin-degrading enzymes, and enzymes degrading small molecules that inhibit the other enzymes (Banerjee et al. 2010).

2.4.2 Fermentation

Ethanol can be fermented into sugar by various kinds of yeast or bacteria. The sugar molecules utilized for ethanol production differ depending on the microorganism. One of the most commonly used yeast strains in ethanol production from sugar and starch is *Saccharomyces cerevisiae*, which is ordinary baker's yeast. *S. cerevisiae* is also important in ethanol production from lignocellulosic biomass. It is a robust yeast strain with a relatively high tolerance to pretreated lignocellulosic material, high ethanol tolerance and high glucose utilization with minimal by-product formation (Ghareib et al. 1988, Olsson & Hahn-Hägerdal 1993).

The drawback of native *S. cerevisiae* is that it can only ferment hexose sugars such as glucose and mannose, and not pentose sugars such as xylose. Agricultural residues usually contain mainly glucose and xylose, and the utilization of both sugars is therefore one way of increasing the amount of ethanol produced from lignocellulosic biomass. Various microorganisms can utilize xylose, such as *Clostridium saccharolyticum*, *Scheffersomyces stipitis* (formerly known as *Pichia stipitis*) and *Candida shehatae* (Olsson & Hahn-Hägerdal 1996). The drawback of these microorganisms is that they are not as robust as *S. cerevisiae* (Olsson & Hahn-Hägerdal 1993).

Since *S. cerevisiae* is considered the most robust ethanol-fermenting organism, much attention has been given to genetic and metabolic engineering of this yeast to make it pentose fermenting. Two main pathways can be used, which are usually referred to as the XR/XDH and the XI pathways (Van Maris et al. 2007). In the XR/XDH pathway, xylose is reduced to xylitol by the enzyme xylose reductase (XR) and the xylitol is oxidized to xylulose by the enzyme xylitol dehydrogenase (XDH) (Figure 2.5). In the XI pathway, xylose is catalysed to xylulose through isomerization by the enzyme xylose isomerase (XI). Xylulose is then phosphorylated to xylulose-5-P by the enzyme xylulokinase (XK). Xylulose-5-P is one of the intermediates in the pentose phosphate pathway (PPP), and can be metabolized into fructose-6-P or glyceraldehyde-3-P, which are intermediates in glycolysis.

The main metabolic pathway for the production of ethanol from glucose is glycolysis. Glucose is metabolized to two molecules of pyruvate, which are then reduced to ethanol and carbon dioxide when no oxygen is available. Under aerobic conditions, pyruvate

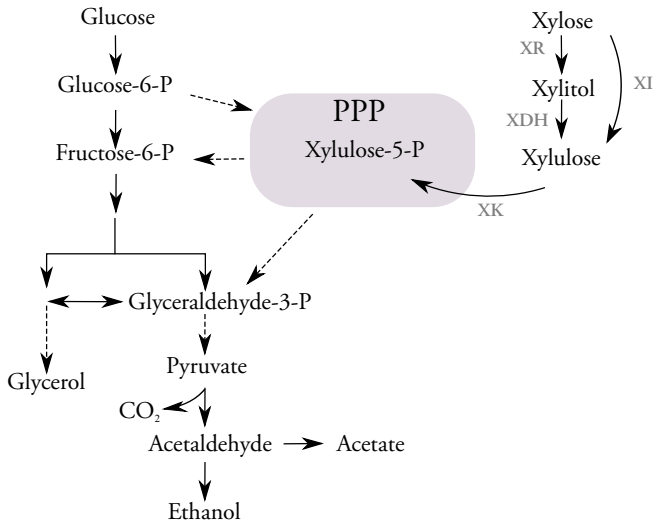

Figure 2.5: Scheme illustrating the production of ethanol by the utilization of glucose and xylose.

is instead converted to acetyl-CoA and subsequently oxidized to carbon dioxide in the following tricarboxylic acid cycle. The theoretical ethanol yield is 0.51 g/g consumed glucose, but some glucose is usually consumed to produce by-products such as glycerol and acetic acid, for maintenance, and to produce biomass.

The slurry of pretreated lignocellulosic biomass is a rather harsh environment for microorganisms. Native *S. cerevisiae* is known to have a relatively high tolerance to this environment, but genetically modified strains may be less tolerant. Xylose consumption seems to be more affected by inhibitors than glucose consumption (Casey et al. 2010, Hasunuma et al. 2011). Inhibition can be dealt with by a combination of strain robustness and process configuration (Almeida et al. 2007). The process configuration together with the choice of yeast strain and the presence of the inhibitor acetic acid will be further discussed in Chapter 4.

2.4.3 Combinations of enzymatic hydrolysis and fermentation

Different strategies can be used to combine enzymatic hydrolysis and fermentation of lignocellulosic biomass. Historically, two strategies have been used: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Tomás-Pejó et al. 2008). In SHF the two steps are performed sequentially in one vessel, or in separate reactor vessels, while in SSF hydrolysis and fermentation are carried out simultaneously in the same vessel. Both strategies have advantages and disadvantages. The main advantage of using SHF is the possibility of

performing both fermentation and hydrolysis under conditions that are optimal for the microorganism and the enzymes. The main disadvantage is end-product inhibition of the enzymes. If the liquid is separated from the solids after enzymatic hydrolysis, fermentation is only performed on the liquid. This facilitates fermentation, and yeast recycling is possible (Ask et al. 2012). SSF, on the other hand, has the advantage that the problem of end-product inhibition is alleviated, since when the glucose is released it is converted into ethanol. The disadvantage is that the conditions are determined by the most sensitive component, usually the microorganism. Enzyme cocktails often have optimal temperature ranges in the region of 45-50°C, while many microorganisms prefer temperatures no higher than 35°C (Cannella & Jørgensen 2014).

Historically, SSF has been considered superior to SHF in terms of the ethanol yield (Tomás-Pejó et al. 2008, Wingren et al. 2003). In the study by Wingren et al. (2003) it was shown that the capital cost of SSF is lower than SHF. However, improvements in enzyme cocktails have reduced end-product inhibition and the ethanol yield is sometimes better in SHF (Cannella & Jørgensen 2014). Modern enzyme cocktails contain LPMO enzymes, which require oxygen. During SSF oxygen is not available due to anaerobic conditions, and therefore the efficiency of LPMOs is low. LPMOs result in aldonic acids after the cleavage of cellulose. This will result in gluconic acid being formed instead of glucose as the other glucose units are released. Gluconic acid cannot be metabolized by *S. cerevisiae*, and SHF may therefore not be suitable if gluconic acid is generated at the expense of ethanol (Cannella & Jørgensen 2014).

As pentose fermentation has become more and more interesting for the production of ethanol, the strategies of separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF) have been introduced. These are similar to their forerunners, but employ a pentose-fermenting microorganism. The advantages and disadvantages are the same as for their predecessors, however, co-consumption can be affected by the glucose concentration, making SSCF more advantageous than SHCF (Meinander & Hahn-Hägerdal 1997).

Considerable progress has been made in developing strategies to optimize the ethanol yield and the ethanol conversion rate. Combining SHF and SSF by adding a liquefaction step or a pre-hydrolysis step at a higher temperature before lowering the temperature and adding the yeast, while the enzymes are still active in SSF is one option (Hoyer et al. 2013, Öhgren et al. 2007b, Palmqvist & Lidén 2014, Varga et al. 2004a). Using different feeding strategies for biomass, yeast and enzymes is another (Koppram & Olsson 2014, Olofsson et al. 2010). The choice of biomass, pretreatment, microorganism and enzymes determine which strategy should be used. The dependence of SSCF on process configuration will be discussed in more detail in Chapter 4.

2.5 Biogas production

Biogas contains 55-65% methane and 35-45% carbon dioxide (Balat & Balat 2009). Other components of biogas include small amounts of hydrogen sulphide, nitrogen, hydrogen, oxygen and ammonia. The composition of biogas depends on the raw material, and it is often used for heat and power generation. It can also be upgraded to methane by removal of the carbon dioxide and trace amounts of the other components. This methane-rich gas can then be used in the same way as natural gas, for example, as transportation fuel (Chandra et al. 2012, Weiland 2010).

2.5.1 Anaerobic digestion

Anaerobic digestion (AD) is the microbial decomposition of biomass into biogas in the absence of oxygen. As in ethanol production, lignocellulosic material should preferably be pretreated prior to AD. It is possible to degrade lignocellulosic biomass to biogas without pretreatment, but pretreatment increases the accessibility and shortens the residence time, while increasing the biogas yield (Ahring et al. 2015, Bauer et al. 2009, Chandra et al. 2012, Vivekanand et al. 2013). AD is a complex process, and is commonly divided into four steps:

i. hydrolysis,
ii. acidogenesis,
iii. acetogenesis and
iv. methanogenesis.

These steps are carried out by different groups of microorganisms which are partly dependent on each other and have different environmental requirements (Chandra et al. 2012, Weiland 2010). The first step, hydrolysis, is similar to enzymatic hydrolysis in ethanol production, but other macromolecules apart from polysaccharides, such as fats and proteins, are enzymatically hydrolysed into the monomeric compounds sugar, fatty acids and amino acids. It is more difficult to degrade lignin due to its complexity, and the process is slow and incomplete (Ahring et al. 2015, Chandra et al. 2012).

During the acidogenesis step, the compounds formed in the previous step are converted into organic acids, alcohols, hydrogen and carbon dioxide. The active microorganism is often the same as that producing the extracellular enzymes used in the previous step. Organic acids, apart from acetic acid, and alcohols are used as substrate in acetogenesis. In this step, the organic acids and alcohols are converted into acetate. In the fourth and final step, two groups of bacteria convert acetate or hydrogen and carbon dioxide into methane.

There is more knowledge about the basic function of AD than about the metabolism of the microorganisms employed and the interactions between them (Weiland 2010). Therefore, it is important to choose the source of microorganisms (active sludge) carefully. The active sludge can behave differently depending on the substrate. Active sludge from a biogas plant using a similar substrate should preferably be used. If this is not possible, a mixture of different sludges can be used to obtain a wide range of microorganisms (Angelidaki et al. 2009). The microorganisms are sensitive to inhibitors to different degrees. Since many different microorganisms are used, many compounds may be inhibitory. Some examples of potentially inhibitory compounds are calcium, magnesium and potassium ions, ammonia, sulphide, heavy metals and organic compounds such as lignin and long-chain fatty acids (Chen et al. 2008). Degradation products derived from steam pretreatment, such as phenolic compounds, furfural and HMF, can also be inhibitory, but the benefits of opening the structure of lignocellulosic biomass outweigh the effects of potential inhibitors (Monlau et al. 2014, Vivekanand et al. 2012).

3

Effect of pretreatment on combined ethanol, biogas and solid fuel production

Corn stover and wheat straw, which were the raw materials used in the studies described in this thesis, have the main components cellulose, hemicellulose (mainly xylan) and lignin. These three components can be used to generate three different kinds of energy products/fuels. **Papers I-III** discuss an energy-driven biorefinery concept based on corn stover, where cellulose is used for ethanol production, hemicellulose together with liquid by-products for the production of biogas, and lignin and other solid residues are used for solid fuel production. The aim is to achieve a high energy recovery, i.e. to convert the chemical energy in corn stover to useful energy. The pretreatment step is believed to be the most important step, having the greatest influence on the energy recovery of the whole process as it determines the kind and amounts of components available for each type of fuel (Galbe & Zacchi 2012, Kumar et al. 2009).

This chapter discusses steam pretreatment and its impact on enzymatic hydrolysis, SSF, AD and the total outcome in a combined ethanol, biogas and solid fuel biorefinery. In addition to pretreatment, the overall process design of the biorefinery is discussed by comparing two different process configurations (Figure 3.1). In Configuration I the whole material after pretreatment is subjected to SSF. After the ethanol is distilled off,

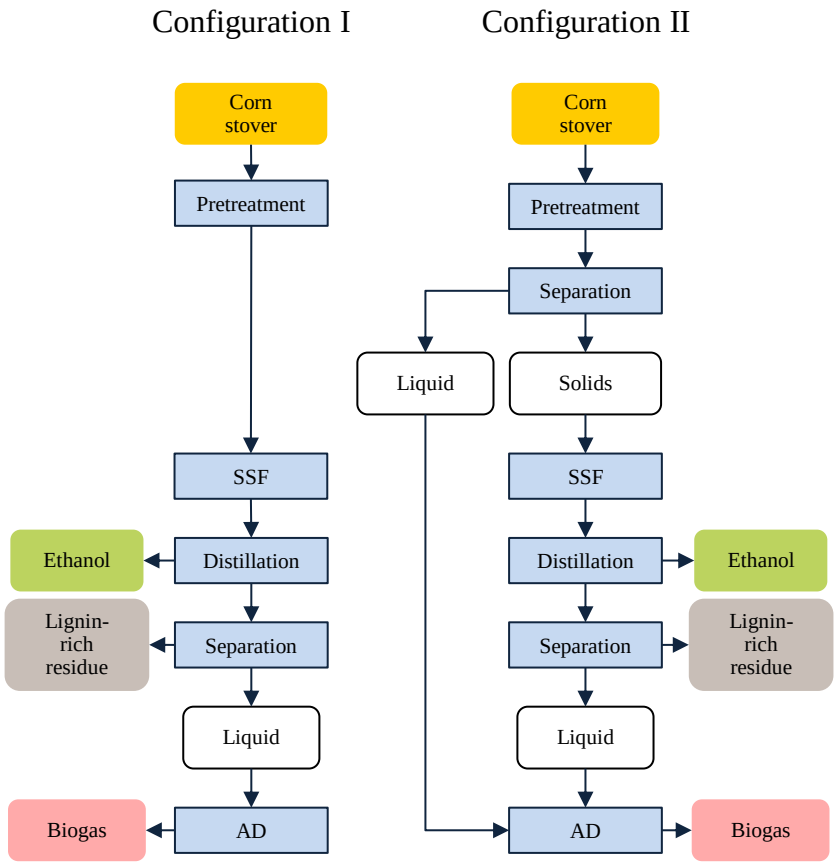

Figure 3.1: Flow charts describing the two process configurations studied. (Adapted from **Papers I-III**)

the remaining material is separated into a solid lignin-rich residue and a xylose-rich liquid. The liquid is then used in AD. In Configuration II, the solids are removed and washed directly after pretreatment. The xylose-rich liquid is then subjected to AD, while the washed solids are subjected to SSF. After distillation, the remaining material is separated and the remaining liquid is used in AD.

3.1 Steam pretreatment and choice of catalyst

Steam pretreatment is one of the most studied and used methods of pretreating lignocellulosic biomass (Kravanja et al. 2012). The outcome of pretreatment sets the stage for all the following process steps; therefore, pretreatment is crucial (Galbe & Zacchi 2012). Steam pretreatment can be run with steam only or with an acidic catalyst.

Using steam only has the advantage that no chemicals are required, but the disadvantages are that a higher pretreatment temperature and/or residence time are necessary. Different acid catalysts can be used during steam pretreatment. Sulphuric acid is the most common and the most investigated acid catalyst, and has been shown to result in higher hemicellulose recovery and increased ethanol formation than with no catalyst (Ballesteros et al. 2006, Varga et al. 2004b). However, requirements on the equipment, due to the corrosive nature of sulphuric acid, together with environmental concerns, make its use less desirable. Acetic acid and phosphoric acid have been less frequently investigated as a catalyst in steam pretreatment. One advantage of acetic acid is that it is easy to handle in the waste stream as it can be easily converted to biogas, while still having a positive effect on accessibility compared with using no catalyst. Disadvantages are that acetic acid is a known inhibitor of ethanol fermentation (Casey et al. 2010, Palmqvist et al. 1999b), and that it is a weaker acid than sulphuric acid and, therefore, a higher acid concentration, a higher pretreatment temperature or a longer pretreatment time is required to ensure efficient pretreatment. Phosphoric acid has the advantage of being a nutrient source for the microorganisms (Boonsombuti et al. 2015), but it is considered expensive, and technical grade phosphoric acid can be very corrosive due to impurities (Geddes et al. 2010).

Steam pretreatment of different kinds of lignocellulosic biomass using sulphuric acid, phosphoric acid or no catalyst has been investigated previously, usually in connection with ethanol production or enzymatic hydrolysis, but also in some cases with biogas production (Bauer et al. 2009, Geddes et al. 2010, Linde et al. 2007, Varga et al. 2004b, Vivekanand et al. 2013). The effect of steam pretreatment and the choice of catalyst on enzymatic hydrolysis, SSF, AD and the total energy recovery has not been as thoroughly investigated, and was the aim of the studies described in **Papers I-III**. In the study presented in **Paper I**, steam pretreatment together with sulphuric acid or no catalyst were investigated. **Papers II** and **III** describe the use of acetic acid and phosphoric acid, respectively, in steam pretreatment. Corn stover was used as the raw material in all three studies.

3.1.1 Effect of acid catalysts on steam pretreatment and enzymatic hydrolysis

Enzymatic hydrolysis has been used to investigate pretreatment (Lloyd & Wyman 2005). High glucose and xylose yields are desirable, and they can be used as a measure of the accessibility of the pretreated material to enzymes. Glucose and xylose are the predominant sugars in corn stover; thus even though other sugars are present in hemicellulose, xylose is the hemicellulose sugar considered in this thesis. It can be difficult to achieve both high glucose and xylose yield as xylose (hemicellulose) prevents the cellulolytic enzymes from reaching the cellulose. Adding hemicellulases improves the total enzymatic accessibility, but in many studies where pretreatment has been investigated, only cellulases have been used (Hu et al. 2011, Kim et al. 2011, Linde et al. 2007, Varga et al. 2004b). The accessibility to cellulose increases with pretreatment severity, but the hemicellulose is then often converted not only to xylose, but also further degraded to inhibitors such as furfural and formic acid, which lower the xylose yield.

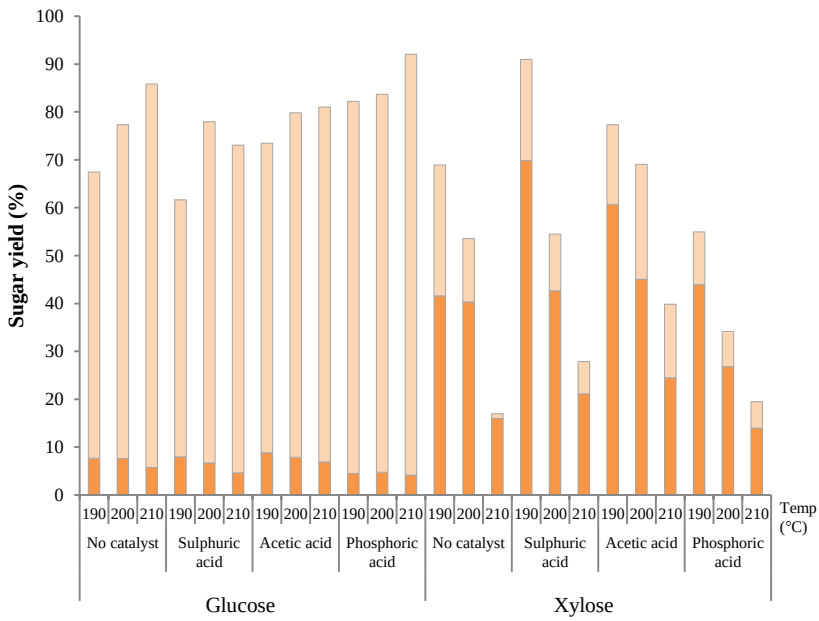

Figure 3.2: Glucose and xylose yields as % of the theoretical accessible glucose and xylose content in corn stover from steam pretreatment (orange) and enzymatic hydrolysis (pale orange). The pretreatment time was 10 minutes in all cases. Three pretreatment temperatures were studied: 190, 200 and 210°C. Enzymatic hydrolysis was performed at 5% WIS, 7.5 FPU enzyme mixture/g WIS, 40°C, 96 h with no acid catalyst, 0.2% sulphuric acid and 1% acetic acid. 10% WIS, 10 FPU enzyme mixture/g WIS, 45°C, 96 h were used with 0.4% phosphoric acid. (Adapted from **Papers I-III**)

It was found in this work that using acetic acid in steam pretreatment resulted in a higher recovery of xylose than the other acids and no catalyst at high temperatures (200 and 210°C) (Figure 3.2). Glucose recovery was not affected to the same extent by the different catalysts; therefore, adding an acid catalyst had little effect on glucose recovery. However, the sum of glucose and xylose recovery indicates that no catalyst and phosphoric acid (at 0.4%) degrade the lignocellulosic structure more than the others due to low xylose recoveries although the glucose recoveries were high.

3.1.2 The impact of the acid catalyst on SSF

The impact of the catalyst on SSF differed depending on the configuration (Figure 3.1). Using washed pretreated solids in SSF can provide an indication of how the solids affect the yeast and the enzymes. In addition, insight can also be gained into cellulosic enzymatic effectivity without glucose and cellobiose end-product inhibition. Whole slurry can be used as the substrate to evaluate the combined effect of the inhibitors in the liquid and the solid fractions from pretreatment on the yeast.

The results of the study on SSF with washed solids (Configuration II) indicated that pretreatment with no catalyst or together with phosphoric acid (which resulted in the highest glucose yields after enzymatic hydrolysis), did not provide any increase in the final ethanol yield (Figure 3.3a). The choice of catalyst had a clearer effect when using whole slurry (Figure 3.3b). Neither material steam pretreated with acetic acid nor high-temperature phosphoric-acid-pretreated material was easily fermented into ethanol. This was most evident after acetic acid pretreatment. The lack of ethanol production was alleviated by increasing the pH in the reactor from 5.0 to 5.5.

Acetic acid is known to be inhibitory to *S. cerevisiae* as it diffuses through the cell plasma membrane and dissociates inside the cell, resulting in a decrease in intracellular pH. To maintain the intracellular pH, the free protons generated must be transported from the cell, which requires ATP, which is formed at the expense of biomass (Palmqvist & Hahn-Hägerdal 2000). At low concentrations of weak acids, the ethanol yield is increased due to the requirement of ATP production. However, at high acid concentrations, the ethanol production is also affected (Graves et al. 2006). This inhibitory effect has been reported for other weak acids such as formic and levulinic acid, which are also inhibitors that can be formed during pretreatment (Larsson et al. 1999). However, only the undissociated form of weak acids diffuses through the plasma membrane. Therefore, the amount of undissociated acids can be decreased by increasing the pH, which explains the increase in ethanol yield from acetic-acid-pretreated material at pH 5.5.

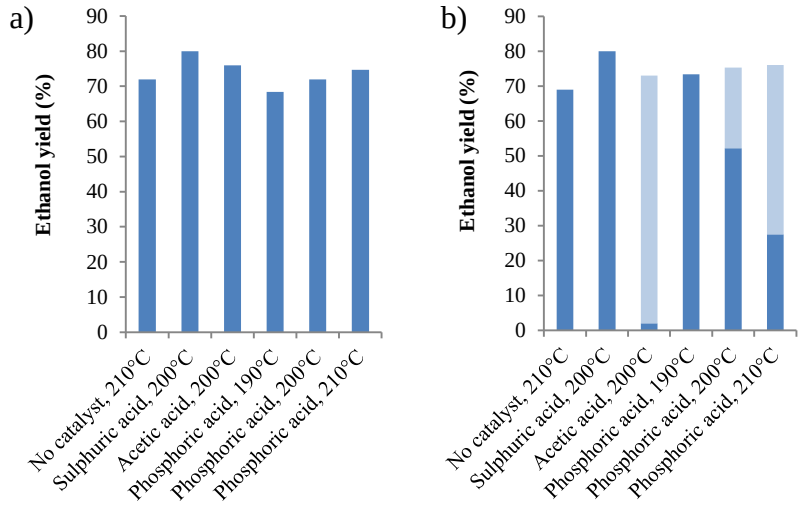

Figure 3.3: Ethanol yield, as % of the theoretical, based on the glucose content in corn stover. a) SSF of washed solids. b) SSF of whole slurry. The SSF conditions were the same in all cases: 10% WIS, 10 FPU enzyme mixture/g WIS, 3 g dry yeast/L, 35°C and 96 h. Dark blue shading pH 5. Pale blue shading shows the extra ethanol production obtained when the pH was increased to 5.5. (Adapted from **Papers I-III**)

Although the weak acids can explain the low ethanol production with acetic acid pretreatment at pH 5, it cannot explain the lower ethanol production following phosphoric acid pretreatment. Neither the concentration of acetic acid nor formic acid is high in comparison with the acid levels in the other pretreatment studies (Table 3.1). The concentration of furfural, another known inhibitor, was however, higher than in the other pretreatment studies. Furfural and HMF have been shown to affect the growth rate of the microorganism, as well as the ethanol production (Heer & Sauer 2008, Horváth et al. 2001, Larsson et al. 1999). *S. cerevisiae* can detoxify furfural and HMF by converting them into their less toxic alcohols, but this detoxification results in a lag phase that is partly explained by the competition for the same enzyme as is used for the production of ethanol in glycolysis (Palmqvist et al. 1999a, Taherzadeh et al. 1999). When using acetic acid pretreatment the yeast was not able to detoxify furfural at pH 5, but in the presence of phosphoric acid it was possible, although the concentration of furfural was almost twice as high (Figure 3.4). However, the detoxification rate was slower at pH 5 than at pH 5.5. By changing the duration of SSF, the importance of slow detoxification could have been reduced.

The phenolic compounds released from lignin during pretreatment are also considered to be inhibitory. Phenolics are a heterogenic group of inhibitors and the mechanism of inhibition is not completely known. Some toxicity data for phenolics and information on the dependency of toxicity on the structure are however available (Larsson et al.

Table 3.1: Concentration (g/L) of inhibitors after pretreatment with different acid catalysts. (Adapted from **Papers I-III**)

	Acetic acid	Formic acid	HMF	Furfural
No catalyst, 210°C	5.3	2.7	0.2	1.0
Sulphuric acid, 200°C	5.9	0.8	0.5	2.9
Acetic acid, 200°C	7.8[a]	2.1	0.7	2.7
Phosphoric acid, 190°C	1.3	0.8	0.2	2.6
Phosphoric acid, 200°C	2.4	1.3	0.4	4.6
Phosphoric acid, 210°C	2.9	1.5	0.7	5.2

[a] Including the acetic acid added in pretreatment.

2000). It has been suggested that phenolics can be converted to less toxic compounds in the same manner as furfural and HMF (Heer & Sauer 2008). However, the structure of the phenolics was outside the scope of the present work.

All the inhibitors (weak acids, furfuraldehydes and phenolics) cause synergistic effects (Ding et al. 2011, Palmqvist et al. 1999b). The effect of the weak acids can be reduced by increasing the pH. When this is done, the detoxification of the solution, by the conversion of furfural into alcohol, becomes apparent when using acetic acid. This may also be the case for phosphoric acid. However, higher pH might also reduce the toxic effect of some of the phenolic compounds. It could be concluded from this work that inhibitors are formed during pretreatment with phosphoric acid, creating a harsh environment for the yeast in the fermentation of glucose to ethanol. Increasing the pH improves the environment despite the fact that the amount of weak acids is relatively low from the beginning.

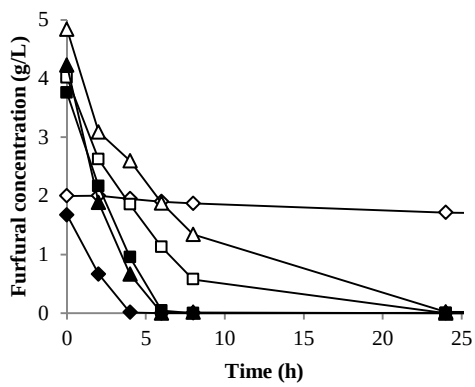

Figure 3.4: Furfural concentration during the first 24 hours of SSF at pH 5 (open symbols) and pH 5.5 (filled symbols) following acetic acid pretreatment at 200°C (diamonds), phosphoric acid pretreatment at 200°C (squares) and phosphoric acid pretreatment at 210°C (triangles).

In summary, the choice of acid catalyst affected the outcome of ethanol production. Steam together with no catalyst, sulphuric acid or phosphoric acid at the lowest temperature (190°C) resulted in a less toxic environment for the yeast at pH 5. Sulphuric acid appeared to be the most promising in terms of the final ethanol yield, although the difference was not remarkable. The use of acetic acid and phosphoric acid at the higher temperatures of 200 and 210°C was not suitable at pH 5, but the outcome was good when the pH was increased to 5.5.

3.1.3 Effect of the acid catalyst on anaerobic digestion

When studying the biogas production using AD, the biochemical methane potential (BMP) is often measured. This provides a measure of the practical methane potential of the substrate in a batch process with a high amount of active sludge, as this methodology only includes digestible compounds. The drawback of this method is that the long-term effects of inhibitory compounds in a continuous process cannot be studied. The high amount of active sludge dilutes the substrate and possible toxic compounds, while increasing the amount of nutrients in the mixture. Nutrient limitation and toxicity are therefore often not detected in BMP measurements. These can only be detected when the substrate is studied in a continuous process. BMP was measured in the first three studies (**Papers I-III**), providing information on the effects of the catalyst, but not in a long-term continuous process. In the third study (**Paper III**), the digestion of the cellulose standard was lower than in the other studies (**Paper I-II**). The BMP results from the third study are therefore normalized to the others.

In general, low xylose recovery during enzymatic hydrolysis and the choice of process configuration (Figure 3.1) affected the BMP most. The results of enzymatic hydrolysis implied that steam alone or together with phosphoric acid degraded the lignocellulosic structure more than the other acid catalysts, resulting in low xylose recovery. This was also reflected in the BMP results following these pretreatments (Figure 3.5). Harsher pretreatment conditions result in a lower amount of xylose as a considerable fraction is degraded into furfural and formic acid. These compounds can also be converted into biogas, but some of the degraded compounds are polymerized into solid pseudo-lignins during steam pretreatment (Brownell & Saddler 1984, Vivekanand et al. 2013), and are therefore not available for biogas production.

The choice of process configuration also affects the BMP. Two processes were evaluated, which will be discussed in more detail in Section 3.2. This resulted in three substrates for which the BMP could be measured. Configuration I resulted in a distilled liquid residue from the SSF of whole slurry. Configuration II resulted in the pretreatment liquid and a distilled liquid residue from the SSF of washed solids.

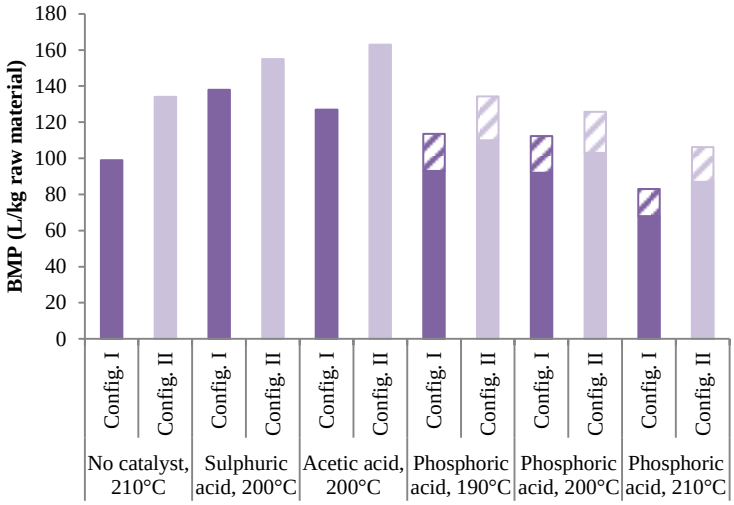

Figure 3.5: BMP obtained from pretreated corn stover and added acetic acid when using the different pretreatment catalysts and using Configuration I and Configuration II. The BMP results from the steam pretreatment with phosphoric acid were compensated for the lower digestion of the cellulose standard compared to the other BMP studies (purple/white stripes). (Adapted from **Papers I-III**)

Configuration II yielded a higher BMP than Configuration I. One possible explanation of this could be that the pretreatment liquid contains some glucose which would otherwise have been consumed by the yeast in SSF. Another possible explanation is that the washed material is more easily hydrolysed during SSF, and therefore results in more liquefied hemicellulose being available for biogas production. A third possible explanation is that some of the volatile compounds in the slurry is removed during distillation and therefore not used as substrate during AD.

Pretreatment with phosphoric acid led to toxic compounds in the pretreatment liquid, which were evident during SSF of the slurry (as discussed above). Toxic effects were also seen during AD of pretreatment liquid after phosphoric acid pretreatment at 200 and 210°C (Figure 3.6). Under these conditions, no methane was produced until 7-10 days after the active sludge was added, whereas methane production was observed after 1-3 days in the other cases. After the lag phase, the values of BMP were in the same range as for the other substrates. It has been shown that phenolic compounds, furfural and HMF can be inhibitory to the microorganisms used in AD (Monlau et al. 2014, Vivekanand et al. 2012). When using pretreatment liquid as the substrate in AD, the liquid is not detoxified by the yeast during SSF, as is the case in SSF of whole slurry. Since the pretreatment liquid was found to be toxic to *S. cerevisiae* it is not surprising that it was also toxic to the microorganisms in AD. The

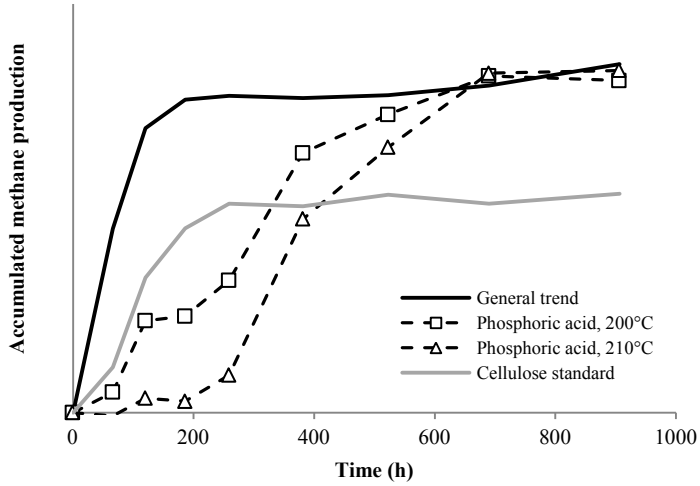

Figure 3.6: Methane production over time for the different substrates investigated. Cellulose standard; liquid from phosphoric acid pretreatment at 200°C; liquid from phosphoric acid pretreatment at 210°C; general trend for all but the two substrates mentioned above. (Adapted from **Papers I-III**)

liquid is detoxified by converting the compounds into non-toxic (or less toxic) compounds and then converted into methane and carbon dioxide. The lag phase is due to the period required by the microorganism for detoxification.

The impact of the acid catalyst is most obvious when using phosphoric acid, where a toxic effect can be seen after pretreatment. In general, high severity in pretreatment is undesirable as the amount of substrate that can be digested to biogas is reduced due to a lower amount of hemicellulose sugars being available for fermentation due to the production of more degradation compounds. The highest BMP obtained when using Configuration II was in combination with acetic acid pretreatment, while the highest BMP when using Configuration I was observed when using sulphuric acid pretreatment. One reason why the BMP was higher when using Configuration I after sulphuric acid pretreatment may be that part of the acetic acid is removed during distillation. Since acetic-acid-catalysed steam pretreatment resulted in a higher amount of acetic acid, the effect of acetic acid removal will be greater. The choice of process configuration has a considerable effect on AD. Pretreatment liquid together with the liquid residue after SSF of washed solids, gave a higher BMP than using the liquid residue after SSF of the whole slurry.

3.2 Steam pretreatment and process configuration in biorefinery concept

The choice of pretreatment catalyst affects the different process steps in a biorefinery. The process configuration affects how the process steps can be combined, which in turn affects the final outcome in terms of the product yield and concentrations. The impact of the pretreatment step may differ depending on how the process is designed.

Two process configurations were investigated (Figure 3.1). The first comprised only one separation step, while the second comprised two. The major difference between the two configurations is that the liquid, which is the most toxic part of the pretreated material, was included in the SSF substrate in Configuration I, and in the substrate for AD in Configuration II; in addition, extra water was added in Configuration II to remove the pretreatment liquid from the solids and to dilute the solids in SSF. The substrates used for SSF and AD differ depending on which configuration is used. In Configuration I, the material detoxified by the yeast during SSF is used in AD, which can have a positive effect. Furthermore, all the glucose will have been consumed in SSF. However, considering Configuration II, a less toxic stream as well as the possibility to run at higher WIS are advantageous during SSF, as this can result in a higher ethanol yield and concentration. This also means that volatile compounds can be used as substrate during AD, and therefore not be removed by distillation, which results in higher biogas production. From a process and capital cost perspective, extra process steps and additional water are costly (Wingren et al. 2003). This must be taken into consideration when comparing the product yield and concentration of different process alternatives.

3.2.1 The impact of the acid catalyst on process configuration

Process design, in terms of interconnectivity between the different process steps, is important. The total product energy yield can be increased by removing the inhibitory pretreatment liquid from the SSF step, and instead feeding it to the AD step (Figure 3.7). This is mainly due to increased biogas production and in some cases due to higher ethanol production. The difference was greater when pretreatment was performed with no catalyst, and with phosphoric acid at higher temperatures. Therefore, Configuration II may not be suitable for substrates subjected to low-severity pretreatment, as the process cost may be too high in relation to the increase in product yield. Decisions must, however, be based on techno-economic studies, which were outside the scope of this thesis.

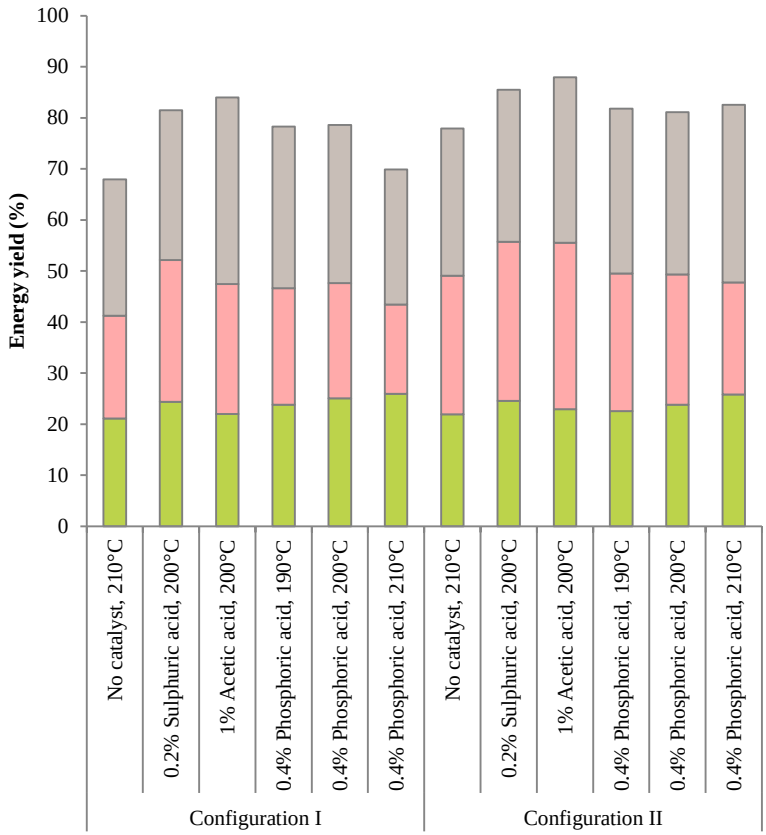

Figure 3.7: Energy yield as % of the energy in ethanol (green), methane (pink) and solid fuel (grey), compared to the energy content of corn stover and added acetic acid. The energy required to run the process is not considered in the energy yield. (Adapted from **Papers I-III**, where the BMP data from **Paper III** were normalized so as the same cellulose standard consumption was obtained as in the other two studies.)

Configuration II resulted in a higher energy recovery than Configuration I, regardless of the choice of catalyst. Upon comparing the catalysts, it was found that acetic acid and sulphuric acid resulted in somewhat higher product recovery, in terms of energy content, than no catalyst or phosphoric acid. The pretreatment with acetic acid and sulphuric acid improved the overall energy recovery, as well as the production of ethanol and methane from the SSF and AD steps, respectively. Pretreatment with acetic acid yielded the highest recovery of the energy content in corn stover (88%) due to greater solid material recovery, but the difference in total energy yield compared to sulphuric acid pretreatment was not sufficient to conclude that acetic acid pretreatment was superior.

3.3 Final remarks

The choice of pretreatment conditions and catalyst is not straightforward. Not only is it dependent on the total combined yield of all the products, but also on the expected process economy, and which product is defined as the main product. If the results are evaluated based only on the total energy recovery, then acetic acid would be the best choice of catalyst. However, the use of sulphuric acid as a catalyst in pretreatment resulted in more ethanol than the use of acetic acid, and the total energy recovery was only slightly lower. Furthermore, a lower pH was possible in the fermenter for ethanol production when sulphuric acid was used; and a lower amount of acid was used during pretreatment, which means less chemicals and less risk of infections during SSF. If ethanol is considered more important than biogas, then sulphuric acid would be the better choice; whereas if biogas is considered more important, then acetic acid is the better choice. The long-term effect on biogas production was not evaluated in these experiments since they were performed batch-wise and not continuously. Sulphuric acid may be undesirable in a full-scale process as it is known to inhibit methane production by microorganisms (Harada et al. 1994). This acid is also considered more environmentally harmful than is acetic acid. The fact that no sulphur removal step is required may compensate for the lower production of ethanol. Taken together, the results indicate that acetic acid may be the preferable catalyst in pretreatment. However, higher amounts of acetic acid are required for impregnation than sulphuric acid for ethanol production, and the higher pH increases the risk of infections in SSF.

Phosphoric acid is not a realistic option today since clean phosphoric acid is expensive. Even if the price of phosphoric acid were to be lower in the future, no conclusive evidence was found that this would be better than any of the other acids. Steam, without the addition of catalyst, seems not to be an alternative, but using an acid increases the production cost. On the other hand, steam pretreatment without a catalyst requires a higher pretreatment temperature to reach a similar severity. Changing the way in which pretreatment is performed could be an option when using no catalyst. Two-step pretreatment is one option, where the first step is run at low severity and breaks down the hemicellulose. The second step is carried out at a higher severity to improve the accessibility to cellulose. However, this would require two pretreatment steps and higher energy requirements. Whether this is more preferable than adding chemicals depends on the final energy and product yields, as well as the total process cost. To determine whether pretreatment should be performed with or without an acid catalyst, not only experimental design, but also techno-economic evaluation is needed, together with considerations of the “best” mix of products.

The energy recovery in terms of the useful energy was good in both process configurations evaluated, which shows that steam pretreatment is a successful pretreatment method. Although the ethanol concentrations were low (the ethanol concentration should be about 5% by volume to be economically feasible, but never exceeded 3.5% by volume in these studies), the overall energy recovery is acceptable. Water was added to SSF in the configurations. The WIS content would be higher if no water was added, possibly increasing the ethanol concentration; however, in Configuration I, the inhibitor concentration would also be higher, which may reduce the ethanol yield. Other process configurations for SSF could be considered to reduce the effect of increasing inhibitor concentration, for example, fed-batch SSF.

4

Conversion of xylose to ethanol

In the previous chapter, the pretreatment and utilization of raw material to provide energy-rich products were discussed. Acetic acid showed promising results as a catalyst in steam pretreatment, and is an interesting option in an energy-based biorefinery. However, the ethanol recovery cost is expected to be high with the process configurations studied as the concentration of ethanol was low. It has been shown that the ethanol concentration entering the distillation step should be at least 5% by volume (Zacchi & Axelsson 1989). Two alternatives are being discussed to increase the ethanol concentration: increasing the WIS content (Hoyer et al. 2009, Jørgensen et al. 2007b, Koppram & Olsson 2014) or fermentation of the other available sugars (Koppram et al. 2013, Moreno et al. 2013, Olofsson et al. 2010, 2008). A combination of the two is also a possibility (Palmqvist & Lidén 2014).

Using a substrate with a higher WIS content could be an alternative in process Configuration II, by not adding water to the SSF step. Since the inhibitors in the pretreatment liquid are removed by the separation step in Configuration II, difficulties associated with a higher WIS content would only be caused by the higher amount of solids (Lu et al. 2010). In this scenario only glucose is available for fermentation and it is therefore possible to use native *S. cerevisiae* as it is known to have a relatively high tolerance to this environment. The WIS content was doubled and the same material and process configuration were used as in the previous studies with acetic-acid pretreated corn stover. The results are shown in Figure 4.1, where it can be seen that the ethanol concentration was almost doubled.

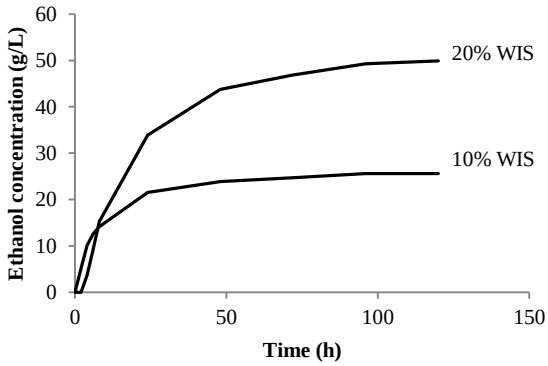

Figure 4.1: Ethanol concentration resulting from SSF of acetic-acid steam pretreated corn stover at 10% and 20% WIS when applying Configuration II.

However, the process configuration is highly dependent on the choice of products. It has previously been shown that the economic feasibility of such a plant, based on wheat straw, increases when ethanol is also produced from xylose in a configuration similar to Configuration II, with ethanol, biogas and solid fuels as products (Joelsson et al. 2016). The production of ethanol from xylose requires efficient xylose-fermenting microorganisms, many of which are not as tolerant to inhibitors as is native *S. cerevisiae*. An acetic-acid-catalysed steam pretreatment step will further increase the problems since high acetic acid concentrations can cause major problems even with native *S. cerevisiae*. A higher pH was found to be crucial to obtain ethanol from glucose, and this may be even more important during co-fermentation of xylose and glucose. The choice of SSCF configuration can also affect the co-fermentation, as will be discussed in more detail in this chapter. Therefore, it is important to identify the best configuration to be able to combine acetic acid pretreatment and glucose and xylose co-fermentation.

The configuration of different SSCF processes using acetic-acid pretreated wheat straw and corn stover with the recombinant *S. cerevisiae* strains KE6-12 and KE6-12b will be discussed in this chapter. The discussion is based on **Papers II** and **IV**, together with some experimental data that have not been published previously.

4.1 Acetic acid pretreatment and xylose fermentation

Combining acetic acid pretreatment and xylose fermentation leads to a number of challenges. In a study performed by Casey et al. (2010) it was shown that the ethanol production rate by *S. cerevisiae* 424A(LNH-ST) decreased in the presence of acetic acid. They also observed that the sugar consumption rate, especially that of xylose, decreased with increasing acetic acid concentration. The inhibitory effect of acetic acid was more severe on xylose fermentation than on glucose fermentation. Therefore, the effect of the pretreatment liquid on fermentation is an important aspect when investigating co-fermentation using a co-fermenting yeast strain.

Figure 4.2 shows the xylose consumption together with xylitol formation for three different liquid fermentation experiments with the *S. cerevisiae* strains KE6-12 and KE6-12b using pretreatment liquid from acetic-acid-catalysed steam pretreated corn stover and wheat straw. Corn stover was pretreated at 200°C and wheat straw at 190°C. KE6-12 did not consume xylose at a very high rate using corn stover as the substrate, while the xylose consumption was almost complete when using wheat straw. One possible explanation of this difference could be that the concentration of acetic acid was higher in the pretreatment liquid from corn stover (7.6 instead of 4.6 g/L), and acetic acid is known to affect the xylose consumption (Casey et al. 2010, Palmqvist & Lidén 2014). The amount of acetic acid formed by the disruption of hemicellulose differs depending on the type of raw material, the batch of raw material and pretreatment severity. In this work, corn stover has a higher content of acetyl groups than wheat straw leading to higher levels of acetic acid. In addition, the pretreatment temperature was 200°C for corn stover and 190°C for wheat straw.

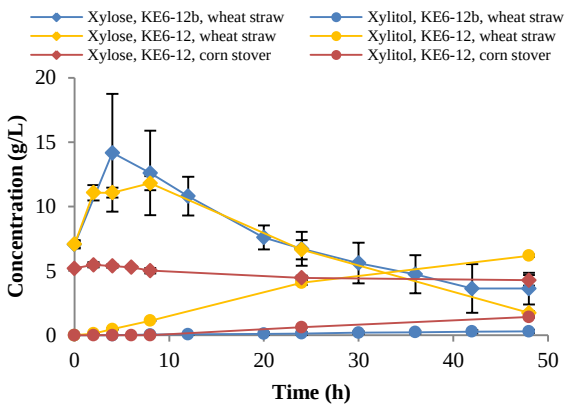

Figure 4.2: Concentration of xylose and xylitol during liquid fermentation experiments with the yeast strain KE6-12 using corn stover (adapted from **Paper II**), KE6-12 using wheat straw (not previously published) and KE6-12b using wheat straw (adapted from **Paper IV**).

However, according to a study by Palmqvist & Lidén (2014), the difference in the amount of xylose consumed at pH 5.5 at acetic acid concentrations of 4 and 8 g/L was negligible. The lack of xylose consumption could also be due to other inhibitory compounds. The difference between the two materials studied in this work was distinct, and may be due to a combination of material effects (formation of inhibitory compounds during pretreatment), acetic acid concentration and pretreatment severity.

KE6-12 produced large amounts of xylitol (Figure 4.2). The overproduction of xylitol is a result of co-factor imbalance between the enzymes converting xylose to xylulose via xylitol, which reduces the ethanol yield (Kötter & Ciriacy 1993, Matsushika & Sawayama 2008). KE6-12b showed no such overproduction of xylitol, and consumed xylose at the same rate as KE6-12. The cell concentration of KE6-12b added was also about half the cell concentration of KE6-12. Therefore, KE6-12b seems to be a better choice for co-fermentation.

4.2 SSCF and process configuration

Glucose and xylose share the same transport system (Kuyper et al. 2005, Meinander et al. 1999, Saloheimo et al. 2007). Since the affinity for xylose is many times lower, xylose transport is inhibited by glucose (Saloheimo et al. 2007). However, it has been shown that a low concentration of glucose is essential for an efficient xylose fermentation (Krahulec et al. 2010, Meinander et al. 1999). Overexpression of xylose transporters in yeast can improve the co-fermentation of xylose (Subtil & Boles 2012), but the choice of process configuration of SSCF can also improve co-fermentation. It has previously been shown that a fed-batch strategy for both material and enzymes can improve xylose consumption during SSCF (Olofsson et al. 2010, 2008). Using a fed-batch strategy for either the raw material, or enzymes, or both, keeps the glucose level low and is thus an efficient strategy for promoting xylose consumption.

The most common way of performing fed-batch SSF or SSCF is by feeding pretreated material, i.e. solids and liquid in a mixed slurry. Another strategy in fed-batch SSCF is to separate the liquid and the solids after the pretreatment step. The liquid stream has a higher concentration of xylose than glucose, since much of the hemicellulose fraction is hydrolysed during pretreatment. This will promote the consumption of xylose. After most of the xylose has been fermented, solids rich in cellulose (glucose) can be gradually fed to the reactor, changing the direction of the process towards cellulose hydrolysis and glucose fermentation. In the study presented in **Paper IV** three liquid and solid fed-batch strategies were investigated together with batch SSCF (Figure 4.3). After the initial addition of liquid and then solids, after 48 hours, (B), an extra pre-hydrolysis step

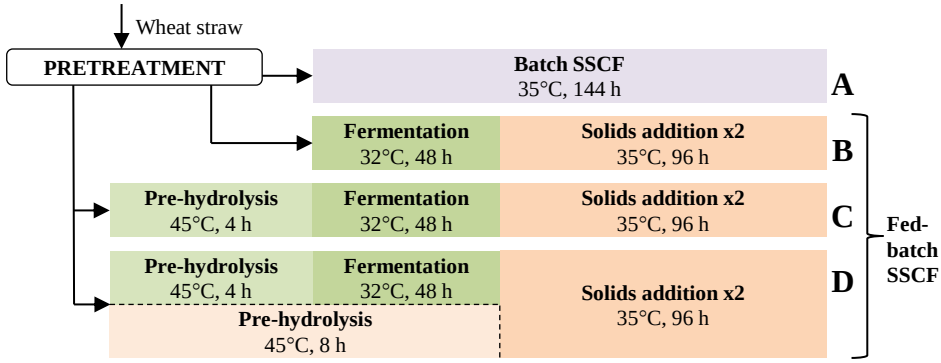

Figure 4.3: Overview of the four SSCF configurations investigated. A) Batch SSCF (base case). B) Fed-batch SSCF of the liquid, followed by the addition of solids at 48 and 50 hours. C) As in B, but with 4 hours' pre-hydrolysis of the liquid fraction. D) As in C, but with 8 hours' pre-hydrolysis of the solid fraction. (Adapted from **Paper IV**)

was investigated (C). Pre-hydrolysis of the liquid for 4 hours was conducted to increase the initial xylose concentration by the hydrolysis of oligomeric hemicellulose, when the yeast was added. In addition to the pre-hydrolysis of the liquid, also pre-hydrolysis of solids for 8 hours (D) was conducted to improve mixing and hydrolysis.

4.2.1 Batch and fed-batch

Co-fermentation in SSCF is dependent on the SSCF strategy as well as the microorganism used. Figure 4.4 gives the results obtained from the batch SSCF and the three fed-batch SSCF experiments at 10% WIS using the yeast strain KE6-12b. Batch SSCF with KE6-12b was successful, indicating that xylose transport was not substantially inhibited in KE6-12b. However, the ethanol yield was slightly improved when fed-batch strategies were used (Figure 4.5), indicating that some inhibition-related problems in batch SSCF are solved by applying a fed-batch procedure.

The fed-batch strategies were similar to each other in terms of ethanol yield. Adding a short pre-hydrolysis step of the liquid before liquid fermentation increased the initial amount of sugar, but did not change the final yield; neither did short pre-hydrolysis of the solids noticeably improve the yield. In experiments conducted by Palmqvist & Lidén (2014), 48 hours of pre-hydrolysis improved the glucan conversion but had a negative effect on the xylose consumption. Studies on the effects of longer pre-hydrolysis time than 8 hours may therefore be of interest, especially since most of the xylose in the liquid stream is already fermented.

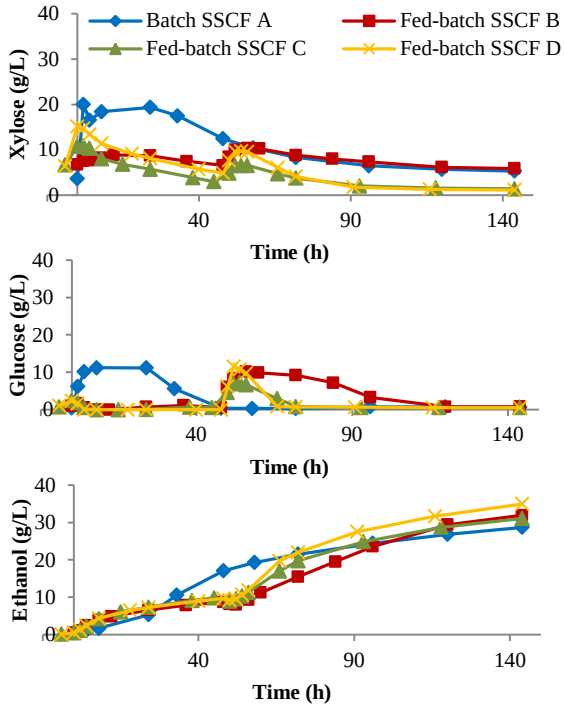

Figure 4.4: Average xylose, glucose and ethanol concentrations in different SSCF configurations at 10% WIS from two measurements. (Adapted from **Paper IV**)

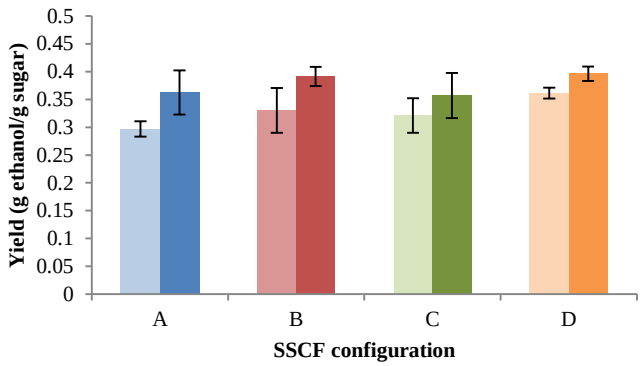

Figure 4.5: Overall (pale shading) and metabolic (dark shading) ethanol yield (g/g) based on available sugars and consumed sugars, respectively, for the four SSCF configurations defined in Figure 4.3. Average values of two measurements. The error bars represent the highest and lowest results. (Adapted from **Paper IV**)

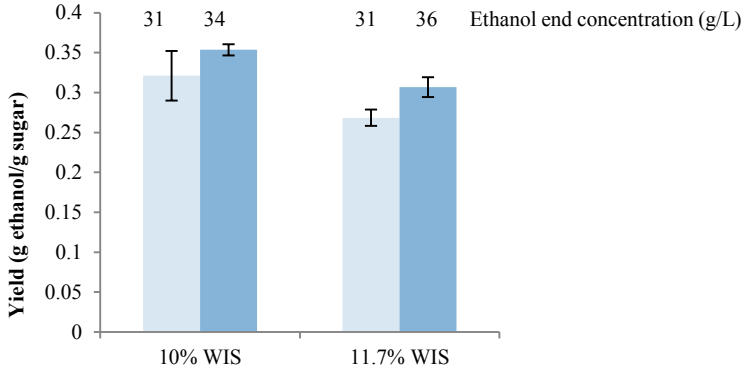

Figure 4.6: Overall ethanol yield for fed-batch SSCF at 10 and 11.7% WIS using yeast concentrations of 2.1 (pale blue) and 4.3 g/L (dark blue). Average values of two or three (11.7% WIS, 4.3 g/L) measurements. The error bars represent the highest and lowest results. The numbers above the bars are the average ethanol end concentration after SSCF. (Adapted from **Paper IV**)

4.2.2 Towards high WIS and co-fermentation

Both co-fermentation of glucose and xylose and a high WIS concentration are important in SSCF to obtain a high ethanol concentration. Increasing the WIS concentration has a negative effect on the overall ethanol yield when using native *S. cerevisiae*, mainly due to the higher inhibitor concentration (Jørgensen et al. 2007b, Varga et al. 2004a). Since the recombinant strains are often more sensitive, the combination of high WIS and co-fermentation is challenging.

The results of the experiments presented in **Paper IV** using SSCF with 10 and 11.7% WIS are shown in Figure 4.6. The small increase in WIS concentration clearly affected the ethanol yield. To overcome some of the difficulties associated with high WIS content, the yeast concentration was increased. The ethanol yield increased, showing that a higher yeast concentration was required to accommodate the higher WIS content.

Doubling the yeast concentration improved the ethanol yield and final ethanol concentration at higher WIS contents. The addition of yeast at concentrations above 4.3 g/L may be favourable since some of the xylose was not consumed (Figure 4.7). However, most of the unconsumed sugar is probably still available in the form of oligomers, since there is a difference between the overall and the metabolic yield. Some of the sugars may also be available as cellulose/hemicellulose, but this was not investigated. Therefore, the enzymatic conversion step (i.e. enzymatic hydrolysis) to glucose and xylose can be considered more important in improving SSCF. There are several possible ways of improving enzymatic conversion. The enzyme dose could be increased in the same way as the yeast; however, the cost of enzymes is a large contributor to the overall production cost. Laccase enzymes could be added to

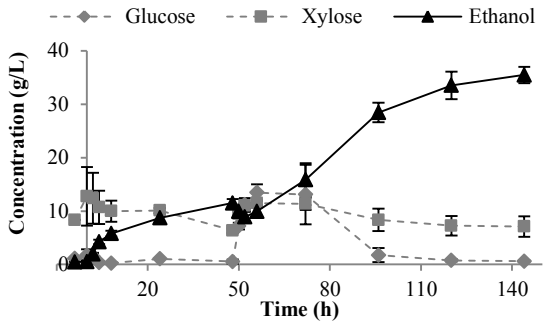

Figure 4.7: Glucose, xylose and ethanol concentration during fed-batch SSCF with KE6-12b, using acetic-acid-catalysed steam pretreated wheat straw with a WIS content of 11.7%. Average values of three measurements. The error bars represent the highest and lowest results. (Adapted from **Paper IV**)

detoxify the solids (Moreno et al. 2013), as they remove phenols which inhibit and deactivate enzymes (Ximenes et al. 2011, 2010). A prolonged pre-hydrolysis step could be added to obtain a solution closer to an SHCF solution (Palmqvist & Lidén 2014). All the mentioned options may increase the enzymatic conversion of the pretreated material, but the pretreatment step could also be improved to facilitate enzymatic conversion. In this context, two-step pretreatment (Söderström et al. 2003) is an interesting solution since the liquid fraction can be separated, while the solids are pretreated again under other, more severe conditions. This would improve the hydrolysis of cellulose as well as reduce the risk of inhibitor formation by hemicellulose degradation during pretreatment.

Improving the enzymatic hydrolysis during SSCF of acetic-acid-pretreated straw can be an efficient means of increasing the overall yields and final product concentrations. The risk of microbial infection by other microorganisms must also be considered as a higher pH increases the risk of infection. Process design and yeast design aimed at lowering the pH are also needed. Genetically modifying the yeast to be more tolerant to acetic acid (Mira et al. 2010) or making the yeast more tolerant through adaptation during cultivation (Sànchez i Nogué et al. 2013) could be possibilities.

4.3 Final remarks

Obtaining high ethanol yields from both glucose and xylose can be a considerable challenge. The process configuration is dependent not only on the kind of raw material, yeast and enzymes that are used, but also on which steam pretreatment catalyst is chosen. The combination of engineering solutions employing effective process configuration design with yeast strain evolution will help in the development of full-scale processes that are efficient in terms of conversion rates and yields. Acetic-acid-catalysed steam pretreatment of the raw material in combination with xylose-fermenting strains was found to be successful, although there is room for considerable improvement. The process configuration was appropriate for the selected yeast strains, but it was clear that the enzymatic hydrolysis step in SSCF was not fully optimized. Further studies are therefore required to optimize the design of SSCF when using steam pretreatment with acetic acid. Any new combination of raw materials, microorganisms and enzymes will require the optimization of process configuration design.

5

Concluding remarks

The aim of this work was to design a process to convert the energy in agricultural residues into useful energy in the form of the products ethanol, biogas and solid fuel. This is an important aspect of a biorefinery, where the raw material should be utilized in the best possible way. Attention was devoted to the pretreatment step and its impact on the different process steps, as well as on maximizing the total energy yield. Three different acid catalysts were investigated in combination with steam pretreatment, as well as steam pretreatment alone. New insights have been gained resulting in new possible pretreatment processes for future biofuel production facilities. Moreover, various process configurations for ethanol production from both glucose and xylose were investigated, such as acetic acid impregnation prior to the pretreatment step followed by utilization of a genetically modified *S. cerevisiae* strain. Since the presence of acetic acid during the fermentation of xylose to produce ethanol is known to be problematic, the findings presented in this thesis will be useful in the future development of xylose co-fermentation. The main findings of this work are summarized below.

Choice of pretreatment catalyst in combined ethanol, biogas and solid fuel production

- Using acetic acid or phosphoric acid in steam pretreatment yielded a more toxic material for ethanol production at pH 5. To overcome this toxicity, the pH was increased to reduce the amount of weak acids present in dissociated form. However, increasing the pH increases the risk of infection.
- Biogas production was affected more by the process configuration than by the choice of pretreatment catalyst. Pretreatment with high severity resulted in a lower BMP. Phosphoric acid pretreatment was the only pretreatment catalyst that led to visible toxic effects on biogas production. However, the long-term effect on biogas production could not be established in this case.
- Both process configuration and the choice of pretreatment catalyst affect the overall energy yield and the distribution between the products. The energy recovery was improved by separating the pretreatment stream into a solid and a liquid phase, and by using acetic acid or sulphuric acid as the catalyst.
- Steam pretreatment resulted in a high energy recovery in the products, and is therefore a good option for fractionation of the main components in agricultural residues for the production of energy-rich products in a biorefinery.

Production of ethanol from xylose

- The *S. cerevisiae* strain KE6-12b produced low amounts of xylitol and was able to produce ethanol from xylose, both when only pretreatment liquid was fermented and in SSCF, despite the fact that acetic acid was used as the pretreatment catalyst.
- Adopting a fed-batch strategy improved the ethanol production.
- The digestibility of the pretreated wheat straw, and therefore the enzymatic hydrolysis step, is one of the limiting factors during SSCF. This must be further improved by optimizing the pretreatment or enzymatic hydrolysis step to obtain a higher ethanol yield, higher ethanol concentration and shorter residence time.

The studies and the results presented in this thesis deal with a process configuration that could be part of an energy-driven biorefinery concept. Sustainable energy production is important, now and in the future, but it is also important to produce other compounds, such as plastics and polymers in a biorefinery to replace petrochemical refining. Ethanol is a potential building block for other chemicals and products, and methane can also be converted into other products. In this work, it was assumed that lignin would be used as a solid fuel to generate heat and power, but it is also valuable in the production of chemicals, high-value fuels and other products. The choice of products depends on the selling price, legislation and policies, and will therefore vary. Regardless of the products chosen now or in the future, appropriate process design and pretreatment methods are required for the fractionation of the different components in lignocellulosic biomass. From this perspective the work presented in this thesis is important.

Further research is needed to ensure that the lignin fraction is also utilized to its best. If lignin is to be used for products other than solid fuel, a detailed understanding of the structure of lignin and the changes that take place in it after pretreatment is needed. The changes in its structure may vary depending on the acid catalyst used in pretreatment, and it would be interesting to investigate this in the future. However, if lignin is used for the production of high-value polymers, it will no longer constitute a source of energy.

Another aspect, not covered in this work, is the production of carbon dioxide during both ethanol and biogas production, which is important from the environmental perspective. If we are to limit the average global increase in temperature, carbon emissions must be reduced. In fact, the net carbon emission should preferably be negative. One way of achieving this is to capture carbon dioxide and sequester it, or use it to produce other chemicals. The carbon dioxide produced during ethanol production is cleaner and more easily captured than that from combustion. Therefore, the utilization of carbon dioxide will be an important aspect of a future biorefinery and must be included in the overall process.

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Pia-Maria Bondesson began her studies in chemical engineering at Lund University in 2005, and received her master´s degree 2010. In 2011, she started as a Ph.D. student at the Department of Chemical Engineering, focusing on combined ethanol and biogas production from agricultural residues.

In view of global climate change and the increasing energy demand there is a need for renewable energy resources. This thesis discusses an energy-driven biorefinery concept based on the agricultural residues corn stover and wheat straw. The material is used to produce ethanol and biogas for the transportation sector and for heat and power generation, and solid fuel for heat and power generation. The overall goal was to develop a process that converts as much as possible of the raw materials into useful energy.

The work is divided into two main parts. The first part is concerned with the effects of steam pretreatment and choice of acid catalyst on ethanol and biogas production, as well as the overall energy yield. The second part focuses on the combination of acetic-acid-catalysed steam pretreatment and simultaneous saccharification and co-fermentation (SSCF) and the role of process configuration on SSCF.

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