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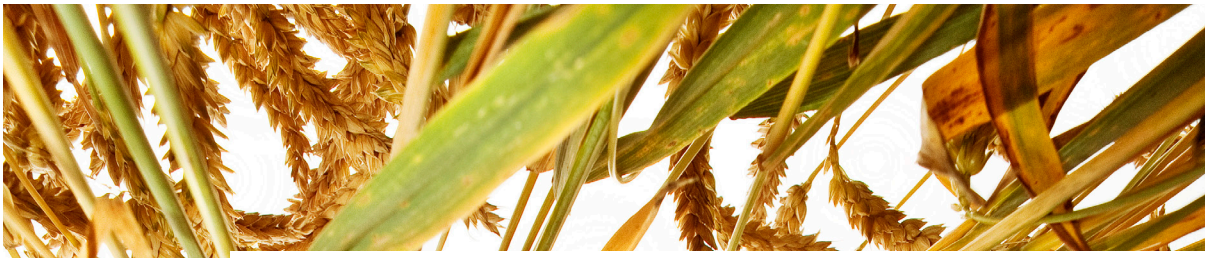
Erdei, Borbala

2013



Development of integrated cellulose- and starch-based ethanol production and process design for improved xylose conversion

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Doctoral Thesis
2013

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Academic thesis which by due permission of the Faculty of Engineering of Lund University will be publicly defended on 13 June at 1 pm in lecture hall K:B at the Center of Chemistry and Chemical Engineering, Getingevägen 60, Lund, for the degree of Doctor of Philosophy in Engineering. The Faculty opponent is Professor Johann Görgens, Department of Process Engineering, Stellenbosch University, South Africa.

Akademisk avhandling för avläggande av teknologie doktorsexamen vid tekniska fakulteten, Lunds universitet, som kommer att offentligen försvaras på engelska den 13 juni 2013, kl 13.00, i K:B auditorium, Kemicentrum, Getingevägen 60, Lund. Fakultetsopponent är Professor Johann Görgens, Department of Process Engineering, Stellenbosch University, Sydafrika.

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To my Grandfather†

Abstract

Transportation fuels from renewable resources such as ethanol are one of the alternatives to ensure energy security and decrease the net emission of carbon dioxide. First-generation ethanol production from sugar- and starch-based raw materials (1G) is today well established in many countries, and the focus of research has thus shifted to the development and demonstration of the production of second-generation bioethanol from lignocellulose (2G).

This thesis deals with the development of process configurations for bioethanol production from wheat straw integrated with wheat starch-based ethanol production. One part of the work focused on integration in simultaneous saccharification and fermentation (SSF) of steam-pretreated wheat straw (SPWS) with pre-saccharified, completely saccharified or fermented wheat meal using baker's yeast, *Saccharomyces cerevisiae*. Mixing wheat straw and pre-saccharified or saccharified wheat meal was shown to be beneficial for both 1G and 2G ethanol production. Not only the ethanol concentrations, but also the ethanol yields, increased when pre-saccharified wheat meal was mixed with SPWS. The highest ethanol yield achieved was higher than that obtained with SSF of either raw material alone. Ethanol yields above 80% of the theoretical (from the hexose sugars) and ethanol concentrations of about 6% (w/v) were achieved in batch SSF. Ethanol concentrations at such levels reduce the energy demand in distillation, thus lowering the production cost.

Since wheat straw contains a large amount of xylose, integrated process configurations were developed and investigated in an attempt to improve xylose utilization by a xylose-fermenting, genetically modified strain, *S. cerevisiae* TMB3400, in the second part of the work. The most promising configuration for co-fermentation of glucose and xylose was separate hydrolysis and co-fermentation of SPWS, as this allowed the glucose concentration to be controlled by the wheat-starch hydrolysate feed. An ethanol yield of 92% was achieved after fermentation based on glucose and xylose, and almost complete xylose consumption was achieved.

In the last part of the work, differences in the performance of two mutated strains of *S. cerevisiae* TMB3400 were revealed. It was shown that KE6-13i was more tolerant to inhibitors, while KE6-12 performed better in less inhibitory environments.

Populärvetenskaplig sammanfattning

Förbränning av fossila bränslen som bensin, kol, olja och naturgas orsakar att koldioxid ackumuleras i atmosfären, vilket är en av orsakerna till global uppvärmning. Transportsektorn står för största delen av Sveriges användning av bensin och olja. Oljan är importerad från andra länder, som gör oss väldigt beroende av omvärlden. Ett av de mest lovande alternativa bränsle som kan ersätta delar av dagens stora förbrukning av fordonsbränslen är bioetanol, som redan idag används i varierande andelar i bensin i Sverige. Bioetanol tillverkas idag huvudsakligen av socker- eller stärkelsebaserade råvaror, som sockerrör, sockerbetor eller spannmål, ofta betecknat som första-generationens bioetanol (1G). Emellertid utnyttjar man då resurser som delvis konkurrerar med matproduktion. Cellulosahaltiga biprodukter har därför stor potential att användas som råvara för framtidens andra-generationens bioetanol (2G).

Jordbruksbiprodukter, som vetehalm, innehåller sockerpolymerer, som kan brytas ner av enzymer till enskilda sockermolekyler (glukos) som kan jäsas till etanol med mikroorganismer, till exempel jäst. Omvandlingen av lignocellulosa till sockerarter är tyvärr svår. Man måste förbehandla materialet vid höga temperaturer, runt 170-220°C så att strukturen öppnas och för att enzymer ska nå cellulosafibrerna. Under förbehandlingen genereras dessutom produkter av nedbrutna sockerarter- inhibitorer- som förhindrar jästen att fungera optimalt.

För att nå hög etanolkoncentration efter jäsningen, behöver man öka mängden förbehandlat material i processen (mängden jäsbart socker är direkt relaterat till mängden råvara), vilket kan resultera i minskat utbyte. Målsättningen i denna studie har varit att höja utbytet och koncentrationen av etanol för att minska energibehovet under destillationen. Genom integration med 1G-etanol från vete kan man då sänka produktionskostnaden av 2G-etanol från vetehalm. Målet var också att utveckla och designa integrerade processer för att ta fram tekno-ekonomiska data för bästa driftsätt för den kombinerade processen, avseende både högsta etanolproduktion och optimalt utnyttjande av värdefulla biprodukter.

I avhandlingen visas att delvis eller helt försockrat vete inte bara ökar koncentrationen i processen, utan också att utbytet av etanol kan stiga. Detta beror på möjligheten att späda inhibitorkoncentrationerna till en lagom nivå där jästen känner en måttlig stress, vilket faktiskt kan medföra en ökad produktion av etanol. Fördelen med tillsättning av helt försockrad vätska är att man kan utvinna restprodukter från 1G processen och sälja dem som djurfoder.

För att öka etanolutbytet, kan man också utnyttja sockerarter från hemicellulosan, som utgör en stor del av jordbruksbiprodukter. Hemicellulosa innehåller dock mest sockerarter med fem kolatomer (pentoser) som inte är jäsbara med vanlig bagerijäst.

Nu finns det modifierad jäst, som har förmågan att jäsa glukos och pentos samtidigt, om det inte finns för mycket glukos, som oftast är den sockerart, som jästen föredrar. Olika metoder för tillsats av glukos genom att utnyttja försockrat vete och lämpliga integrerade processkonfigurationer för att förbättra omsättningen av pentoserna har också undersökts i denna studie.

Tudományos összefoglaló

A fosszilis üzemanyagok, mint a szén, a kőolaj és a földgáz elégetése során szén-dioxid szabadul fel, ami a légkörben felhalmozódva hozzájárul a globális felmelegedéshez, emellett a kőolaj import kiszolgáltatott helyzetet teremt az európai országok számára. A benzin és a gázolaj felhasználásában a transzportszektornak van a legjelentősebb szerepe, melyben az egyik legígéretesebb helyettesítő, azaz alternatív üzemanyag a bioetanol, amit üzemanyag adalékként már ma is használnak. A bioetanolt elsősorban cukor- és keményítő-tartalmú nyersanyagokból állítják elő, mint a cukornád, a cukorrépa vagy gabonafélék, ezt első-generációs (1G) bioetanolnak nevezzük. Azonban probléma, hogy élelmiszer vagy takarmány alapanyagokat etikus-e bioetanol gyártásra használni, ezért a cellulóz (természetes glükóz polimer) alapú melléktermékek előnyben részesülnek a bioetanol gyártás során. Az utóbbiakból előállított alkoholt nevezzük második-generációs (2G) bioetanolnak.

A lignocellulóz alapú mezőgazdasági melléktermékek, mint például a búzaszalma, cukorpolimereket tartalmaznak, amit bizonyos enzimek egyszerű cukormolekulákká, glükózzá bontanak le. A glükózt egyes mikroorganizmusok, mint például az élesztő, képesek alkohollá alakítani. A növényi lignocellulózt összetett struktúrája miatt viszont nehéz monomer cukorrá lebontani, ezért a nyersanyagot magas hőmérsékleten (170-220°C) elő kell kezelni, hogy a cellulózrostok hozzáférhetőek legyenek az enzimek számára. Az előkezelés során a lebontott cukrokból inhibítorok is keletkezhetnek, amik gátolják az élesztő működését. Ahhoz, hogy nagy etanol koncentrációt lehessen elérni, növelni kell az előkezelt anyag sűrűségét a folyamatban, ez azonban megnövekedett inhibítor koncentrációt jelent és csökkenti a hozamot.

Jelen tudományos munka célja az etanol hozam és koncentráció növelése volt az 1G és 2G etanol gyártási technológia integrálása által, ami a desztillálásban kisebb energiafelhasználáshoz vezet, ezáltal javítja a gyártás gazdaságosságát. A munka során az egyesített folyamatok tervezése és fejlesztése is cél volt a maximális hozam és az optimális végtermék koncentráció elérését szem előtt tartva. A részlegesen vagy teljesen elcukrosított búzaliszt hozzákeverése a búzaszalma alapú folyamathoz növelte az etanol koncentrációtés az etanol hozamot is. Ennek oka lehet, hogy a megfelelően alacsony inhibítor koncentráció valójában még növelheti is az etanol hozamot azáltal, hogy enyhe stresszt gyakorol az élesztőre. A teljesen elcukrosított búzaliszt hozzáadásának további előnye egy 1G melléktermék (DDGS) kinyerésének és értékesíthetőségének a lehetősége.

Az etanolhozam növelése céljából a hemicellulóz cukrokat is érdemes felhasználni, ami jelentős részét teszi ki a mezőgazdasági melléktermékeknek. A hemicellulóz viszont nagyrészt xilózt tartalmaz, amit a pékélesztő nem tud etanollá alakítani. Igaz, ma már van olyan genetikailag módosított változata, ami képes erre, de csak abban az esetben,

ha nincs túl sok glükóz jelen, amit az élesztő előnyben részesít. A glükóz rátáplálás megfelelő módja elősegítheti a xilóz felhasználását az integrált folyamatokban, ennek vizsgálata szintén e munka célja volt.

List of publications

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

I. **Erdei, B.**, Barta, Z., Sipos, B., Réczey, K., Galbe, M., and Zacchi, G. Ethanol production from mixtures of wheat straw and wheat meal. *Biotechnology for Biofuels*, 3: 16, 2010

II. **Erdei, B.**, Galbe, M., and Zacchi, G. Simultaneous saccharification and co-fermentation of whole wheat in integrated bioethanol production. (*Biomass and Bioenergy,* submitted)

III. **Erdei, B.**, Frankó, B., Galbe, M., and Zacchi, G. Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw. *Biotechnology for Biofuels*, 5: 12, 2012

IV. **Erdei, B.**, Frankó, B., Galbe, M., and Zacchi, G. Glucose and xylose co-fermentation of pretreated wheat straw using mutants of *S. cerevisiae* TMB3400. *Journal of Biotechnology*, 164:50-58, 2013

V. **Erdei, B.**, Hancz, D., Galbe, M., and Zacchi, G. SSF of steam-pretreated wheat straw with the addition of saccharified or fermented wheat meal in integrated ethanol processes. (*Biotechnology for Biofuels,* submitted)

My contributions to the studies

Paper I I planned the study and evaluated the results together with the co-authors. Zsolt Barta and Bálint Sipos performed the experimental work under my supervision. I wrote the major part of the paper.

Paper II I did essentially all the experimental work, evaluated the results and wrote the paper.

Paper III I planned the study and performed the experimental work together with Balázs Frankó. I evaluated the results and wrote the paper.

Paper IV I planned the study and evaluated the results together with the co-authors. Balázs Frankó performed the experimental work under my supervision. I wrote the major part of the paper.

Paper V I planned the study and Dóra Hancz performed the experimental work under my supervision. I evaluated the results together with the co-authors and I wrote the paper.

Abbreviations

AFEX	Ammonia fibre explosion
AMG	Amyloglucosidase
CBH	Cellobiohydrolases
DDGS	Distiller's dried grains with solubles
DM	Dry matter
EG	Endoglucanases
FWM	Fermented wheat meal
GHG	Greenhouse gas
HMF	5-hydroxymethylfurfural
LCA	Life cycle assessment
LWM	Liquefied wheat meal
PWM	Pre-saccharified wheat meal
SHCF	Separate hydrolysis and co-fermentation
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
SSCF	Simultaneous saccharification and co-fermentation
SPWS	Steam-pretreated wheat straw
SWM	Saccharified wheat meal
WIS	Water-insoluble solids
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase
1G	First generation
2G	Second generation

Contents

1 Introduction

The world's population has doubled during the last fifty years hence looking ahead we face major challenges in satisfying needs for food and energy. The extensive use of oil, coal and natural gas has made today's economy highly dependent on fossil fuels. Economic growth will increase the demand for personal mobility, and the transport sector in emerging economies will require greater oil supplies. Since crude oil production at existing oil fields is declining (IEA 2010), oil companies are being forced to turn to more costly sources of oil. According to the International Energy Agency, oil prices will remain high, or increase, in the next twenty years (IEA 2011). Renewable energy systems must, therefore, be implemented globally to satisfy the increasing energy demand and ensure national energy security.

Another important reason for replacing today's fossil-fuel-based energy systems with biomass-based systems is to reduce the accumulation of carbon dioxide (CO_2) in the atmosphere. CO_2 is a greenhouse gas (GHG) and is responsible for the greatest proportion of the greenhouse effect after water vapour (IPCC 2007). Burning fossil fuels releases CO_2 that has been stored for millions of years in deep reserves. An increase in GHGs traps more heat in the Earth's atmosphere leading to an increase in the average temperature and ultimately, climate change. Climate change has been intensively debated during the past 20 years, and several international and national agreements have been signed to reduce its environmental impact. The first agreement, which legally established binding obligations for developed countries to mitigate their GHG emissions, was the Kyoto Protocol. It was adopted in Kyoto in 1997, and implemented in 2005. One of the targets was to achieve an average 5% reduction in GHG emissions during the period 2008-2012, compared to the level in 1990 (UNFCCC 2013). In 2009, the European Union (EU) pledged a 20% unilateral reduction target for 2020, compared to 1990 levels. It is hoped that this target may increase to 30% through the cooperation of other developed countries (WRI 2013). Sweden has set a target of reducing its GHG emission by 40% by 2020, compared with 1990 levels, and plans to have a transportation sector with no net emission of GHGs into the atmosphere by 2050 (Energimyndigheten 2011).

GHG emissions can be most efficiently reduced in the transport sector, which accounted for over 60% of oil consumption worldwide in 2010 (IEA 2012). In the USA, transportation contributed about one third of the total GHG emission, and consumed most of its imported oil (Ross Morrow et al. 2010). Renewable

transportation fuels produced from biomass (biofuels) are therefore desirable alternatives to fossil fuels. Plants use CO_2 and water for photosynthesis, i.e. for glucose and oxygen production. This creates a carbon sink, as the CO_2 released by the combustion of other plant material is used during growth. This is called the carbon cycle, and in the ideal case this will be neutral, if the same amount of CO_2 is sequestered as is released during the combustion of biofuel products. However, all the environmental effects of producing and using biofuels require careful consideration. Life cycle assessment (LCA) has become the main method used for both qualitative and quantitative evaluation of the environmental impact of biofuels (de Vries et al. 2010; Fazio and Monti 2011). However, the variation in the results obtained in LCA studies depends on the quality of the input data (Börjesson and Tufvesson 2011). It is, therefore, difficult to compare biofuel chains due to the variations in conditions and parameters; for instance, different local agricultural practices and growth rates can lead to significant uncertainties (Chiaramonti and Recchia 2010). A certain biofuel can thus be a good alternative or a poor alternative, in terms of GHG emission, depending on the raw material used, and the production process and location (Börjesson 2009; Kendall and Chang 2009). Consequently, the impact of the whole production chain of biofuels based on LCA must be carefully considered when developing certification systems for biofuels.

Ethanol produced from biomass, also called bioethanol, has a considerable potential to replace oil to some degree, as it is a liquid fuel that can be easily integrated into the existing infrastructure for fuel distribution. Ethanol as a fuel is not a discovery of the 21st century. The engine of the first flexi-fuel automobile designed by Henry Ford (1863-1947) was capable of running on ethanol or gasoline, or a combination of the two. Today's flexi-fuel vehicles can use ethanol blends up to 100% depending on the climate. Today, ethanol is mostly used as an additive to gasoline up to a maximum of 20%, and can be used in most modern spark-ignition engines without the need for any modification. In Sweden, gasoline contains 5% ethanol, and there is an established distribution network for E85 (85% denatured ethanol fuel and 15% gasoline), which has accelerated the large-scale adoption of flexi-fuel vehicles since 2005 (Pacini and Silveira 2011). Although the domestic production of bioethanol is increasing, most is imported, mainly cheap ethanol originating from sugarcane, in Brazil (Dahlbacka 2009).

1.1 Ethanol from biomass

In order to produce ethanol from biomass a source of sugar is required. Since sugar can be produced from three types of feedstock: i) sucrose-containing sugar crops (e.g. sugarcane or sugar beet), ii) starchy cereals (e.g. wheat, corn and barley), or iii)

lignocellulosic biomass (e.g. wood, straw or herbaceous grasses) (Balat et al. 2008), so can bioethanol.

Today, bioethanol is produced commercially from sugar- or starch-based raw materials, and is often referred to as "first-generation (1G) bioethanol". Ethanol from sucrose is simply produced by fermentation after the extraction of sugars from the raw material. In a starch-based process, the starch must first be liquefied and saccharified to produce sugars that can be fermented to ethanol.

Data on ethanol production from the most significant producers in the world are collected in Table 1.1. The largest ethanol producer today, the USA, more than doubled its production based on corn between 2007 and 2011, as the number of ethanol plants increased from 110 in 2007 to over 200 in 2011 (RFA 2012).

Brazil is the second largest producer, and its ethanol industry has been based on sugarcane since 1976. In Europe, Germany and France produce the largest quantities of bioethanol, mainly from starch-rich raw materials such as wheat and barley.

Table 1.1 World fuel ethanol production from 2007 to 2011 (billions of litres). US (Renewable Fuel Association 2012)

	2007	**2008**	**2009**	**2010**	**2011**
USA	24.60	34.07	40.12	50.08	52.61
Brazil	19.00	24.50	24.90	26.20	21.09
European Union	2.16	2.78	3.93	4.57	4.42
China	1.84	1.90	2.05	2.05	2.10
Canada	0.80	0.90	1.10	1.35	1.75
Australia	0.10	0.10	0.21	0.25	0.33

Although 1G ethanol production is well established and understood, questions arise regarding not only sustainability, but also the ethics of using food resources for fuel production. The influence of biofuel production on food commodity prices is widely debated. Assessments are very uncertain (Sims et al. 2010), ranging from zero up to as high as 75% (Chakrabortty 2008) of the total increase in food price. In a systematic investigation, Baffes and Haniotis argue that the effect has not been as large as originally thought (Baffes and Haniotis 2010).

Future expansion of the bioethanol industry must, in any case, be based on production from lignocellulosic resources. Therefore, research has been shifted to the development of second-generation (2G) bioethanol. However, 2G ethanol is still not economically feasible due to low biomass utilization and high production cost. Lignocellulosic materials contain carbohydrates in the form of cellulose and hemicellulose, which can be converted into ethanol through acid or enzymatic processes (Galbe and Zacchi 2002). Conversion with the help of biodegradable enzymes as catalysts is preferred over acids, which may have a toxic effect. However, the enzymes are not able to degrade biomass on a reasonable timescale, due to the natural recalcitrance of lignocelluloses. To enhance the accessibility of the cellulose fibres to the enzymes, a pretreatment step is necessary. Pretreatment is considered to be the key for efficient utilization of lignocellulose (Galbe and Zacchi 2012), as it has a considerable impact on all the other steps in the process, such as hydrolysis, fermentation and product recovery by distillation.

Distillation has a large impact on the overall energy demand, which is highly dependent on the ethanol concentration in the feed (Galbe et al. 2011). A minimum of 4-5% (w/w) ethanol is required in a lignocellulosic process. To reach such a high ethanol concentration, a high water-insoluble solids (WIS) content is required in the hydrolysis and fermentation steps; however, this usually results in a lower yield due to inhibition caused by degradation products or poor mass transfer (Hoyer et al. 2009; Jorgensen et al. 2007).

One promising option to improve the ethanol concentration is to integrate 1G and 2G ethanol production, i.e., to use the whole crop. This could be beneficial for both processes as it is difficult to reach high ethanol concentrations in the 2G processes, while starch-based ethanol production often requires dilution of the sugar. With a higher ethanol concentration, the cost of distillation in the 2G process would be reduced due to the reduction in the energy required for distillation. In return, the surplus energy, which could be obtained by the combustion of lignin, could provide both 1G and 2G plants with heat and electricity. The two processes can be integrated in many ways, and the investigation of plausible process alternatives was one of the main subjects of the research presented in this thesis.

Another alternative to improve ethanol yield is to also utilize the hemicellulose fraction of herbaceous (and hardwood) crops, which contains mostly xylose (C5 sugar). Most microorganisms can ferment C6 sugars such as glucose easily, but not xylose. Modified microorganisms exist today that can ferment both C5 and C6 sugars simultaneously; but the process configuration must be appropriately designed to achieve efficient utilization of both sugars. The improvement of glucose and xylose co-fermentation with applications in some integrated scenarios has also been addressed in this work.

1.2 Scope and outline of this thesis

The work described in this thesis is focused mainly on the development of process configurations for the integration of 2G bioethanol production from wheat straw with 1G wheat starch-based ethanol production. The main objective of the work was to improve the ethanol yield and to reach a sufficiently high ethanol concentration following fermentation to reduce the energy requirements in downstream processes, thus improving the economy of the process. Studies were also carried out to develop and investigate combined processes with respect to optimal use of the co-products.

Another aspect of the present work was to find ways of increasing ethanol production from wheat straw by improving xylose conversion in different process configurations. To achieve enhanced glucose and xylose co-fermentation, integrated process configurations with suitable glucose feeding requirements were developed in separate hydrolysis and co-fermentation (SHCF) and simultaneous saccharification and co-fermentation (SSCF). The glucose and xylose co-fermentation performance of recently developed xylose-fermenting microorganisms in batch and fed-batch SHF was also investigated.

In Chapter 1, the current studies are put in perspective. Chapter 2 describes the structure of starch and how ethanol can be produced from starch-based raw materials, such as wheat grain. A description of the structures of the constituents of lignocellulosic biomass, and the composition of different feedstocks used for ethanol production, particularly wheat straw, is provided in Chapter 3. This chapter also presents an overview of the technological routes used for the production of ethanol from lignocellulose, as well as the current state of commercialization. Chapter 4 explains in detail how ethanol can be produced from lignocellulose-based raw materials by the enzymatic conversion process. Different alternatives for the integration of starch- and cellulose-based processes investigated and developed in this work are presented in Chapter 5. In this chapter the results presented in Paper I and the results related to integration in Papers II and V are discussed. Chapter 6 deals with glucose and xylose co-fermentation. This chapter summarizes the results given in Papers III and IV and the results related to glucose and xylose co-fermentation in Papers II and V. The most important findings and some suggestions for future work are summarized in Chapter 7.

2 Ethanol from starch

2.1 Cereal crops as raw materials

Various starch-containing materials, such as corn and wheat, are traditional raw materials in today's ethanol production. Starch is the most abundant constituent of cereal kernels, and varies with the type of grain. Grain starch consists primarily of amylose and amylopectin. Amylose is the straight-chained polymer of D-glucose units, which are linked by β-1,4 glucosidic linkages with only a few side chains (Figure 2.1), giving it its helical characteristics. In contrast, amylopectin also has branches connected with β-1,6 linkages (Kelsall and Lyons 2003). The ratio of amylose to amylopectin is specific to each starch source, and affects its gelatinization properties (Power 2003) (as discussed in Section 2.2). Corn and wheat have similar starch contents, and the amylose to amylopectin ratios are also similar.

Wheat, derived from the wild *Triticum*, a grass native to the Middle East, is today the fifth major cereal plant cultivated in the world (Geohive 2012). It is the main cereal used in bread making because of its high value as a food commodity; hence the use of wheat for industrial purposes has been fairly limited until recently. Today, it is one of the main raw materials used for ethanol production in Europe. In Sweden, ethanol is produced from wheat, rye and barley in a dry-mill plant in Norrköping (the Agroetanol Plant), where 210 000 m^3 ethanol are produced from 550 000 tons of grain annually. (Agroetanol 2013). Dry-milled wheat grain obtained from Agroetanol was used as the raw material in the studies described in Papers I-III and V. The wheat meal consisted of 72.7% starch and 24.3% starch-free fibre, based on dry weight. The composition of the starch-free fibre fraction was as follows: 17.5% glucan, 14.4% xylan, 8.5% arabinan, 1.6% galactan and 18.2% lignin.

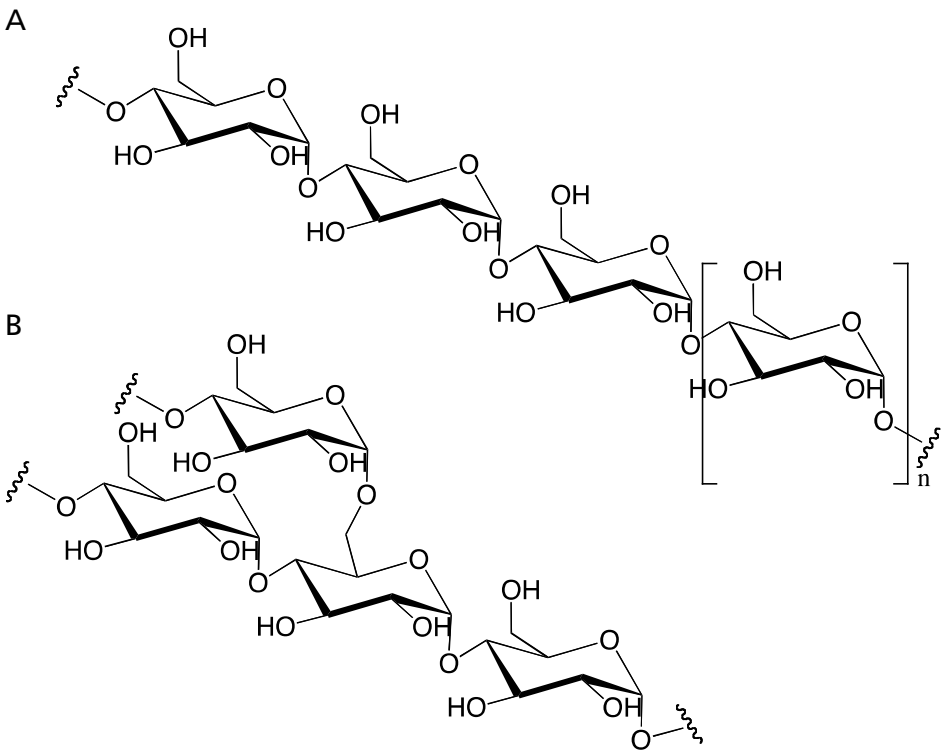

Figure 2.1 Structure of amylose (A) and amylopectin (B), the constituents of starch. In amylose, the glucose units are bound by α-1,4 linkages, while in amylopectin the branches are connected by α-1,6 linkages.

2.2 Conversion of starch to ethanol

The technologies currently used as pretreatment for the production of ethanol from cereals are dry milling or wet milling. A simplified representation of the two processes is shown in Figure 2.2.

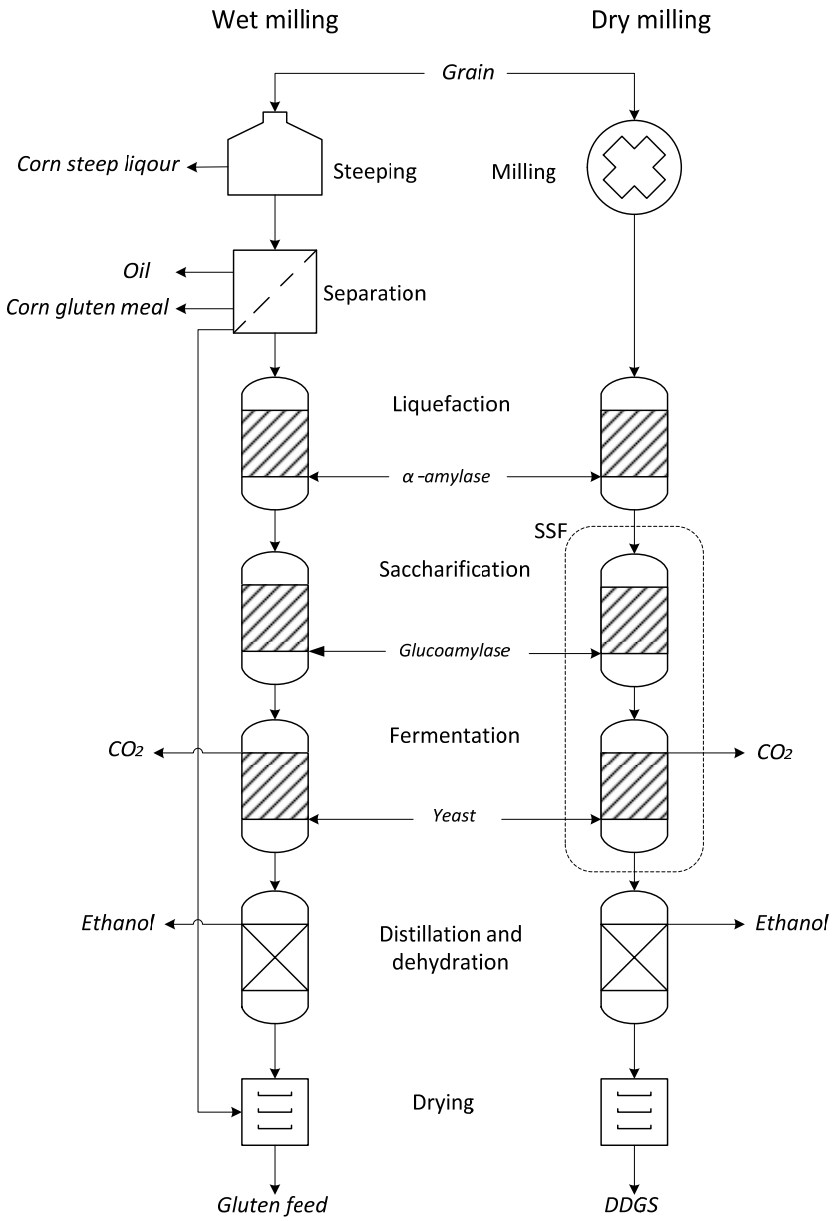

Figure 2.2 Flow sheet for the production of ethanol from starch-containing materials by wet- and dry-milling. Saccharification and fermentation can be performed simultaneously in SSF. DDGS denotes distiller's dried grains with solubles.

Wet milling, which is used for corn, is the process in which the components are separated after hydration of the starch-containing grain. The major components separated are: the germ, corn gluten meal, corn gluten feed and starch. Starch is the primary product of the process and can be converted into various products, including ethanol (Elander and Putsche 1996). The first step of the process is steeping at about 49-53 °C. The protein-rich steep liquor is an excellent fermentation nutrient source, and is used as an additive in the production of organic acids, amino acids, enzymes and ethanol (Destexhe et al. 2004). The germ, which typically contains up to 50% oil, is further processed to extract corn oil, the most valuable product of the process, and the gluten is used as animal feed. Corn gluten feed is the high-fibre, low-protein fraction in the process, and can be used as a source of energy, or as a source of protein and fibre for cattle feed, while corn gluten meal is the high-protein, low-fibre fraction which can be used to generate energy, or as a source of protein, vitamins and minerals for poultry (Ramirez et al. 2008). The grain is milled very gently to ensure high quality of the starch and high purity of the co-products. Since wet mill plants are multi-product biorefineries, they are more expensive to build than single-purpose dry-mill facilities. Therefore, the dry-mill process is more common in the processing of corn today. Other grains, such as wheat or barley, are only processed by dry-milling. Dry milling provides higher ethanol yields than wet milling (Entrix 2010).

In the dry-milling process, the whole grain is ground a fine particle size to facilitate penetration of the water during cooking, which has several purposes, including sterilization, solubilization of sugars and the release of the bound sugars and dextrins (long sugar chains). The conversion of starch into sugars is followed by fermentation and the distillation of the ethanol, which are similar in both the wet- and dry-milling process.

The purpose of cooking and saccharification is to hydrolyse the starch into fermentable monomer sugars, since starch cannot be directly metabolized by yeast. This is accomplished by the endo-enzyme α-amylase followed by the action of the exo-enzyme glucoamylase (amyloglucosidase). During liquefaction, the actual enzyme attack is preceded by the gelatinization phase, during which the starch granules lose their crystalline structure and become available to the α-amylase while being cooked at 90-120 °C. During gelatinization, the mash reaches its maximum viscosity. When thermostable α-amylases start breaking down the starch polymer into soluble dextrins, by randomly hydrolysing α-1,4 bonds, the viscosity decreases rapidly. The dextrinized mash is then cooled to 55-70 °C, and after the pH has been readjusted, glucoamylase is added. Glucoamylase converts the liquefied mixture of dextrins into individual glucose molecules in the saccharification step. The shorter the length of the chains, the less work remains for the exo-enzyme glucoamylase, which releases single glucose molecules by hydrolysing the α-1,4 linkages, beginning at the non-reducing end of the dextrin chain (Kelsall and Lyons 2003).

After saccharification, the mash is cooled to 32 °C and the glucose is converted to ethanol by yeast, for instance, *Saccharomyces cerevisiae*.

Fermentation requires 48-72 hours, and a final ethanol concentration of 10-12% is obtained (Bothast and Schlicher 2005). In many plants, however, saccharification is often performed in the same vessel as fermentation after a short pre-saccharification phase, where the released glucose is simultaneously converted to ethanol using SSF. In this configuration, the process time is reduced, the risk of infection is minimized, and end-product inhibition from enzymatic hydrolysis is eliminated (Bothast and Schlicher 2005; Destexhe et al. 2004). Either batch or continuous fermentation systems may be used, although batch processing is more common. Very high gravity fermentation systems are designed to minimize dilution by water, giving the potential of increasing the final ethanol concentration, thus reducing the energy required in the processes following fermentation (Bothast and Schlicher 2005; Jones and Ingledew 1994b).

After distillation of the ethanol, the stillage is centrifuged and separated into a solid and a liquid fraction. The liquid is then concentrated by evaporation and mixed with the solid fraction again. In the final step, the mixture is dried to give a valuable co-product, called distiller's dried grains with solubles (DDGS). DDGS is sold as animal feed as it contains compounds with high nutritional value (Galbe et al. 2011).

2.2.1 Enzymatic conversion of wheat meal

Dry-milled wheat grain, here denoted wheat meal, was used as the raw material in the current work when partially or completely saccharified wheat meal was integrated into the lignocellulosic process (described in Chapter 3). The first step of wheat meal processing was liquefaction. In this step, the wheat meal slurry, with a dry matter (DM) content adjusted to 35% (w/w), was supplemented with α-amylase. The slurry after liquefaction, denoted liquefied wheat meal (LWM) was used for integration with the lignocellulosic ethanol process (Paper II). Saccharification of the LWM was accomplished by adding amyloglucosidase to the slurry. In the study presented in Paper I, a short (2-hour) partial saccharification step was performed, resulting in a solution containing 53% of the total amount of glucose, corresponding to 68 dextrose equivalents, which is an indication of the total amount of reducing sugars, expressed as the D-glucose present in the solution. A value around 50-70 has been recommended as optimal in the starch-to-ethanol process (Destexhe et al. 2004; Montesinos and Navarro 2000). The material obtained was denoted pre-saccharified wheat meal (PWM), while in the studies presented in Papers III and V, the supernatant of completely saccharified wheat meal (SWM) was used together with the lignocellulose ethanol process. In the study presented in Paper V, fermentation of SWM was also

performed in the starch-based process, where the addition of fermented wheat meal (FWM) to the lignocellulose ethanol process was investigated.

2.2.2 Wheat mash fermentation

The wheat mash was fermented in two different ways. Batch SSF was performed on the entire PWM slurry (Paper I) by adjusting the WIS content to 2.8% (w/w) to restrict the ethanol concentration to 60 g/L, to ensure complete fermentation. SSF was performed at 36.5 °C, at pH 5, for 72 hours using baker's yeast, *S. cerevisiae*, at a concentration of 5 g/L. As nutrients, $(NH_4)_2HPO_4$, $MgSO_4 \cdot 7\ H_2O$ and yeast extract were used at concentrations of 0.5, 0.025 and 1.0 g/L, respectively.

In the second case, fed-batch fermentation was applied using the supernatant from SWM (Paper V), which was separated from the solids prior to fermentation. The water needed for dilution was first used to wash the filter cake to minimize sugar losses and increase the amount of sugars to be fermented. In another configuration, water was added to the fermentation step, resulting in a lower amount of added sugars. Fermentation was performed at 32 °C and pH 5, for 48 hours, after which the broth was centrifuged and the liquid fraction (FWM) was subjected to SSF in the integrated scenarios.

3 Ethanol from lignocellulose

3.1 Raw material

Lignocellulosic biomass includes a wide range of material such as agricultural and forestry residues, waste from the pulp and paper industry, paper and remaining fractions of municipal solid waste, and dedicated energy crops and grasses. All these materials represent a vast resource that can potentially be used for ethanol production; however, most of these materials are resistant to degradation, which makes it necessary to include process steps that facilitate further processing.

3.1.1 Structure and composition

The major fraction of plant matter is lignocellulose. It consists of three types of polymers: cellulose, hemicellulose and lignin, which are strongly interconnected and chemically bound by non-covalent forces and covalent cross-linkages (Pérez et al. 2002). Cellulose is a linear homopolysaccharide composed of β-D glucopyranose units, which are linked together by β-(1-4)-glycosidic bonds, forming cellobiose molecules connected in long chains (Figure 3.1). In nature the cellulose chains have a degree of polymerization, around 10 000 in wood, and as high as 15 000 in cotton (Fengel and Wegener 1989; O'Sullivan 1997). The cellulose crystallites are longer in straw than in wood, but not as long as in cotton cellulose. In addition, the degree of crystallinity of straw pulps appears to be lower than that of wood pulp (Liu and Sun 2010).

Several hundred cellulose chains associate through numerous intra- and inter-molecular hydrogen bonds and van der Waals forces, which are responsible for the formation of insoluble and rigid microfibrils. In the microfibrils the chains form highly ordered, crystalline domains, disrupted by more disordered amorphous regions (Béguin and Aubert 1994).

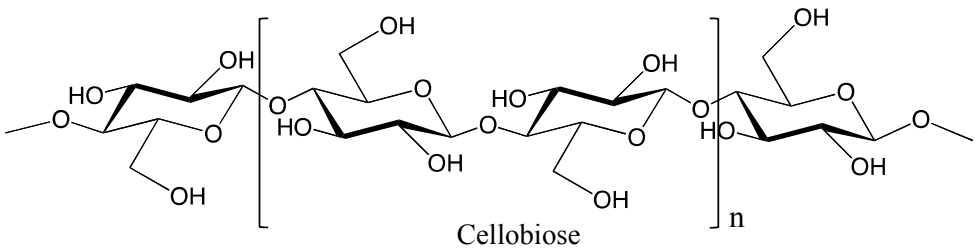

Figure 3.1 The structure of cellulose. Glucose molecules are connected by β-(1-4) bonds. The dimer cellobiose (in brackets) is a repeating unit in the homopolymer.

Hemicellulose is a complex carbohydrate polymer, and is the linking agent between cellulose and lignin. Hemicelluloses can also be referred to as "pentosans" or "polyoses", since they are composed of various sugar units such as D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose and glucuronic acids (Fengel and Wegener 1989). Sugars are linked together by β-1,4- and occasionally β-1,3-glycosidic bonds (Pérez et al. 2002). Subclasses of hemicellulose can be found depending on the plant species, stage of development and tissue type, and these are generally classified by the main sugar composing the backbone, e.g. xylans, mannans or glucans. Due to their highly branched structures (except for linear mannans), hemicelluloses can naturally become heavily hydrated, forming gels (Wyman et al. 2004). Hemicellulose makes up about 1/3 of hardwoods and herbaceous plants. Mannan-type hemicelluloses (glucomannans or galactogluco-mannans), or glucuronoxylans are the major components in softwood and hardwood, respectively, while arabino-glucuronoxylan, glucurono-arabinoxylan and arabinoxylan are the major components of cereal residues, such as wheat straw and grasses (Gírio et al. 2010).

After cellulose, lignin is the most abundant polymer in nature, providing structural support, impermeability and mechanical resistance of the cell wall. Regarding its structure, lignin is the most complex, three-dimensional, highly cross-linked amorphous heteropolymer, which consists of phenyl-propane units joined together by different types of linkages. The polymer consists of three main phenyl-propanoid units: p-hydroxyphenyl (H), guaiacyl propanol (G) and syringyl propanol (S), synthesized from the precursors coumaryl, coniferyl and sinapyl alcohol, see Figure 3.2 (Pérez et al. 2002; Ralph et al. 2004). The proportions of these building blocks depend on the origin; G being the main unit in softwood, G and S being most

common in hardwood (Sjöström 1993), while GSH lignins are present in wheat straw and grasses (Sun et al. 1997).

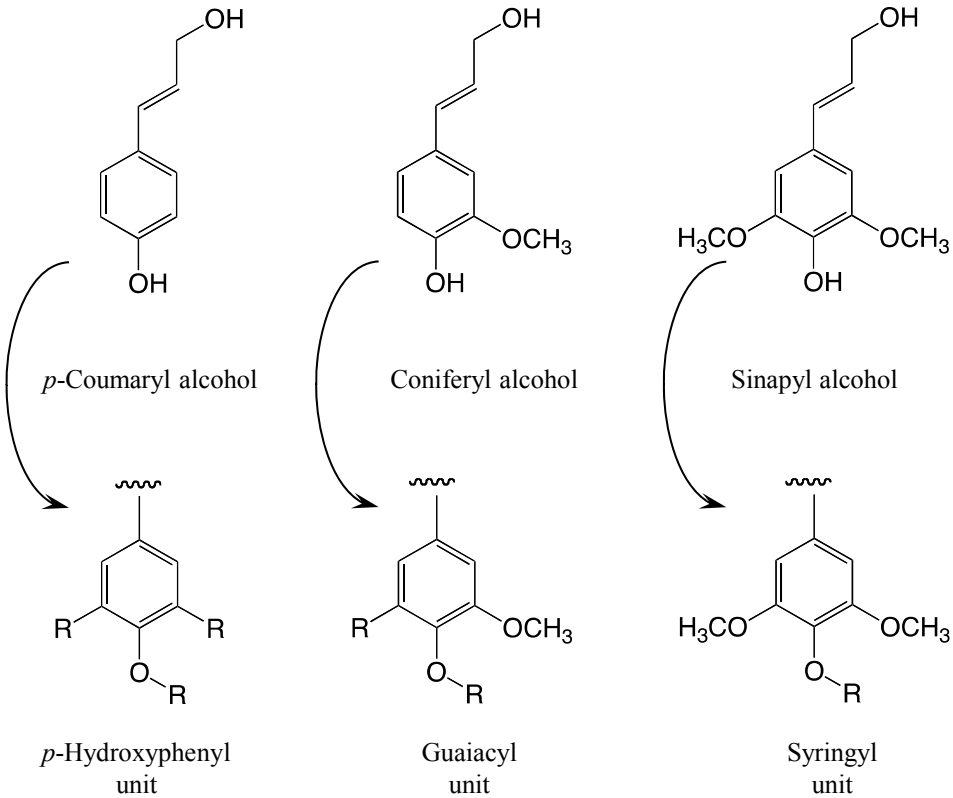

Figure 3.2 The three lignin units formed (p-hydroxyphenyl, guaiacyl and syringyl) from their precursors (p-coumaryl, conyferyl and synapyl alcohol).

Plants have developed advanced systems to support their own structure, to provide sufficient nutrition to every part of the plant, and to protect themselves from extreme weather conditions and, last but not least, from microbial degradation. There are compositional variations between species, as well as between the various cell wall layers. The plant cell wall is made up of several layers: the middle lamella, primary wall and the secondary wall (Figure 3.3). These layers differ in their structure as well as their composition (Sjöström 1993). The thin layer between the cells is the middle lamella, which holds the cells together (Fengel and Wegener 1989). At the early stage of growth it is composed of pectic substances, and it eventually becomes highly lignified. The primary wall is a thin layer that consists of a rigid skeleton of cellulose

microfibrils embedded in a matrix composed of hemicellulose and pectic compounds, and it is rich in lignin. The secondary wall is divided into three layers: thin outer and inner layers (S1 and S3) and a thick middle layer (S2), which contains the highest proportion of carbohydrates. The three layers differ with respect to the orientation of the microfibrils. The inner surface of the cell wall, the warty layer, is a thin membrane that contains warty material deposits (Sjöström 1993).

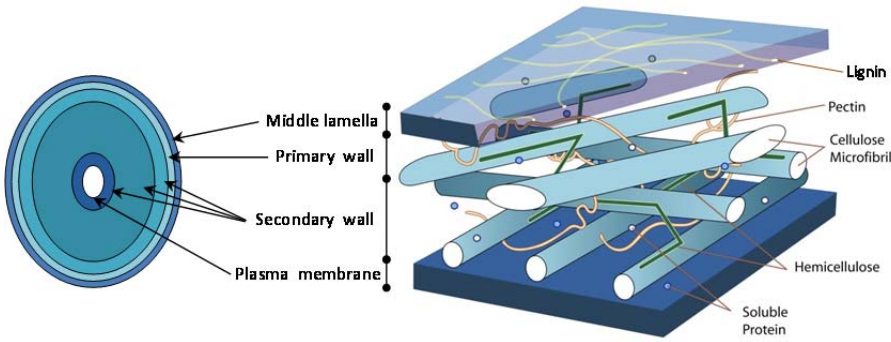

Figure 3.3 Schematic illustration of the cell wall structure. (The illustration on the right was adopted from Wikipedia: Public domain.)

3.2 Agricultural residues

Cereal crops cultivated for food or ethanol production produce enormous amounts of residual lignocellulosic materials that have different applications. Agricultural residues such as straw, stover and bagasse can comprise up to 50% of the agricultural production. Sugarcane bagasse and straw have recently been used to generate heat and power by burning in boilers, but agricultural residues have huge potential to serve as raw materials for the production of liquid, gaseous or solid fuels.

3.2.1 Wheat straw

Among agricultural residues, wheat straw is one of the most ideal feedstocks for fuel production due to its abundance, renewability and low lignin content. Around $7 \cdot 10^8$ tons of wheat was produced worldwide in 2011, according to the Food and Agricultural Organization of the United Nations. Considering that the residue-to-crop ratio for wheat is approximately 1.3:1.0 (based on weight) (Milbrandt 2005), $9.1 \cdot 10^8$ tons of wheat straw are generated annually. The amount of straw that can be collected and used in industrial processes is, however, significantly lower than that produced. Soil conservation requires about 50% of the straw to be left on the field (Mabee and Saddler 2010). Furthermore, some cereal straw is normally used by farmers for livestock feed. Considering all these factors, and combining them with the year-to-year variation in crop yield, it has been suggested that between 15% and 40% of the total residue produced could be available for fuel production (Bowyer and Stockmann 2001).

Wheat straw has been investigated in a number of studies regarding its structure (Hansen et al. 2011; Liu and Sun 2010; Merali et al. 2013; Sun et al. 1997), biodegradability (Singh et al. 2011) and suitability for fuel production (Jørgensen 2009; Linde et al. 2008; Nidetzky et al. 1993; Olofsson et al. 2010a; Saha et al. 2005). Wheat straw was used as the lignocellulosic raw material in all the studies described in this thesis. The composition of the raw material used in these studies is given in Table 3.1, and compared with that obtained in other studies, as well as other lignocellulosic raw materials used for the production of ethanol.

Table 3.1 Composition of wheat straw and other lignocellulosic materials expressed as percentage of dry matter

	Glucan	Xylan	Arabinan	Mannan	Galactan	Lignin[a]	Reference
Agricultural residues							
Wheat straw	38.8	22.2	4.7	1.7	-	18.5	Paper I
	38.8	22.2	1.4	1.7	2.7	18.5	Paper II
	34.2	25.2	3.3	0.5	-	21.9	Paper III
	31.6	23.4	4.7	-	-	26.2	Paper IV
	31.6	22.0	4.0	-	-	21.4	Paper V
	32.6	20.1	3.3	-	0.8	26.5	1
	37.3	18.2	n. r.	n. r.	n. r.	n. r.	2
	48.6	27.7[b]	n. r.	n. r.	n. r.	8.2	3
Barley straw	36.8	17.2	5.3	-	2.2	14.3	4
Corn stover	36.8	22.2	5.5	n. r.	2.9	23.1	5
Sugarcane bagasse	43.0	26.0	1.5	n. r.	0.4	24.6[c]	6
Softwood							
Spruce	49.9	5.3	1.7	12.3	2.3	28.7	7
Hardwood							
Poplar	39.8	14.8	1.2	2.4	-	29.1	8
Energy crops							
Willow	43.0	14.9	1.2	3.2	2.0	26.6	9

References: 1 (Linde et al. 2008), 2 (Nidetzky et al. 1993), 3 (Saha et al. 2005), 4 (Linde et al. 2007), 5 (Ohgren et al. 2005), 6 (Rudolf et al. 2008), 7 (Söderström et al. 2003), 8 (Esteghlalian et al. 1997), 9 (Sassner et al. 2006)

n. r. – not reported

[a] Including acid-soluble and acid-insoluble lignin

[b] All hemicellulose sugars

[c] Including ash

3.3 Conversion of lignocellulose to ethanol

The generation of fermentable sugars from lignocellulosic material requires harsher treatment than for starch-based materials, because of the recalcitrant structure of lignocellulosic biomass (Galbe and Zacchi 2012). The first step in the conversion of biomass to ethanol is size reduction followed by hydrolysis of the carbohydrates. Figure 3.4 shows the three principal methods of hydrolysis: concentrated-acid hydrolysis, dilute-acid hydrolysis and enzymatic hydrolysis.

Acid hydrolysis is the oldest known technique for the hydrolysis of biomass. The most commonly investigated acid is sulphuric acid, which has been found to be the most effective on both cellulose and hemicellulose. Concentrated-acid processes typically involve acid concentrations of 60-90%, and are operated at low temperatures and moderate pressures (Wang and Sun 2010). Under these conditions concentrated sulphuric acid hydrolyses cellulose and hemicellulose with little sugar degradation, resulting in high ethanol yields (Wyman 1994). However, the need to recover large quantities of acid and the need for expensive corrosion-resistant materials constitute major disadvantages (Taherzadeh and Karimi 2007).

Although dilute-acid hydrolysis has the advantage of low acid consumption (0.5-4% (w/w)), but it must be operated at higher temperatures (160-230 °C), which results in sugar degradation and corrosion problems. Low sugar yields, in the range of 50-60% of the theoretical, make ethanol production using this technology very inefficient (Wyman 1994), and high amounts of sugar degradation by-products inhibit the fermenting organisms. Sugar degradation can be reduced by applying two-stage hydrolysis. In the initial step, hemicellulose sugars can be solubilized using milder conditions (150-190 °C), and the remaining cellulose is subsequently hydrolysed under more severe conditions (190-230 °C). Although higher recovery of hemicelluloses can be achieved in this way, the glucose yields are still rather low (Nguyen et al. 1999).

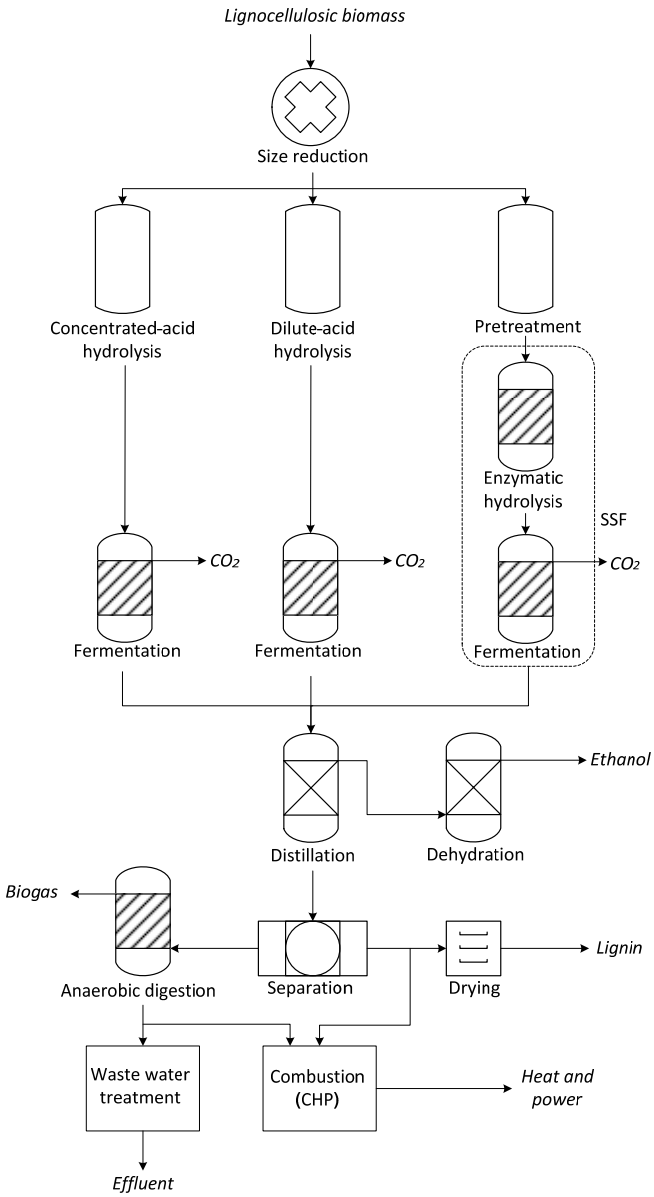

Figure 3.4 Flow sheet for the production of ethanol from lignocellulosic biomass, using concentrated-acid hydrolysis, dilute-acid hydrolysis or pretreatment followed by enzymatic hydrolysis, or SSF. Some alternative downstream-processing options are also included.

The process based on enzymatic hydrolysis and fermentation, or simultaneous saccharification and fermentation (SSF), is currently the most attractive way to convert biomass into ethanol in an energy-efficient way. Milder conditions result in higher yields due to less sugar degradation products, and lower production cost because less expensive construction materials can be used (Galbe et al. 2011).

Some of the most important factors in achieving an economically feasible process are efficient utilization of the raw material through high yields, i.e., conversion of all the sugars through the process steps and high productivity. It is also crucial to achieve a high ethanol concentration at the end of fermentation, so as to minimize the energy demand in the downstream processes, for instance, distillation and evaporation. Finally, if some compounds cannot be converted into ethanol or recovered as lignin, they should be utilized in alternative downstream process steps, such as anaerobic digestion. In anaerobic digestion, substances such as organic acids and residual sugars can be converted into biogas at wastewater treatment plants. Whether this would be useful as an alternative for C5 sugar utilization must be decided when the process is being designed.

The conversion routes described above are those in use in today's pilot, demonstration, and full-scale plants for the production of ethanol from lignocellulosic feedstock.

3.4 The outlook for ethanol production from cellulosic materials

Considerable effort is currently being devoted to the commercialization of the production of biofuels from lignocellulosic raw materials. Some commercial biorefineries already produce ethanol from lignocellulosic feedstock, for example, the Borregaard plant in Norway (Table 3.2). By implementing the biorefinery concept, they produce 15 800 tons of ethanol from 500 000 tons of wood annually, together with their main products, specialty cellulose and lignin (Borregaard 2013). In Sweden, the Domsjö pulp and paper plant produces specialty cellulose, while the fermentable sugars from hemicellulose are converted to ethanol. Concrete additives are also produced from lignin (Domsjö 2013).

A number of conversion processes have been demonstrated in smaller-scale plants using various raw materials such as wood, straw and dedicated energy crops (see Table 3.2). The Abengoa Bioenergy plant in Salamanca, Spain, which is one of the largest demonstration facilities in the world, using 25000 tons of straw annually, is an example of those, applying biochemical conversion, where the pretreatment step is followed by enzymatic hydrolysis and fermentation. Gasification followed by catalytic

conversion of syngas (Enerkem, Westbury, Canada) and the combination of these two, gasification followed by fermentation of the synthesis gas into ethanol (Coskata, Madison, PA, USA) are also represented. Despite the disadvantages of concentrated-acid conversion, this is still applied in some of the pilot plants in the USA, such as the BlueFire Ethanol Plant in Irvine, CA, due to the high sugar yield (about 90%) (BlueFire 2013). In Sweden, SEKAB E-Technology has demonstrated a dilute-acid continuous process in a pilot ethanol plant (SEKAB 2013).

Several companies are planning to scale up their operations for commercial production, for example, Abengoa Bioenergy, Enerkem and Coskata. However, the Beta Renewables plant (a joint venture between Mossi & Ghisolfi Chemtex division and a capital investment company, TPG) in Crescentino, Italy started operations in 2012, and has become the first commercial-scale cellulosic ethanol plant. The plant is projected to produce 60 000 tons of ethanol per year (initially 40 000 tons), based on Arundo donax, a perennial cane, and wheat straw. Pretreatment will be followed by enzymatic hydrolysis and fermentation and, after ethanol recovery the residual material will be burnt in the boiler to generate steam and power for use in the plant.

Although the first commercial plant is now in operation, the expected rapid expansion of 2G ethanol has not yet taken place. Until experience is gained from large-scale production, it is difficult to predict the production cost of 2G ethanol (Galbe et al. 2007; Sims et al. 2010). Further research is needed to develop efficient and economically feasible processes, and to improve existing ones.

Table 3.2 Demonstration and commercial facilities for ethanol production from cellulosic materials (IEA 2013)

Country, location	Company	Feedstock	Output t EtOH/a
Operational			
Sarpsborg, Norway	Borregaard Industries AB[a]	Spruce	15800
Oulu, Finland	Chemopolis Ltd[b]	Straw, wood	5000
Kalundborg, Denmark	Inbicon (Dong Energy)[b]	Wheat straw	4300
Straubing, Germany	Sued-Chemie AG[b]	Wheat straw	1000
Babilafuente, Spain	Abengoa Bioenergy[b]	Straw	4000
Ottawa, Canada	Iogen Corp.[b]	Straw	1600
Westbury, Canada	Enerkem[b]	Wood	4000
Rome, NY, USA	Mascoma Corp.[b]	Wood, switchgrass	500
Madison, PA, USA	Coskata[b]	Wood	120
Vonore, TN, USA	Du-pont[b]	Corn stover	750
Jennings, LA, USA	BP Biofuel[b]	Bagasse, wood waste, switchgrass	4200
Crescentino, Italy	Beta Renewables[a]	Arundo donax	60000
Under construction			
Edmonton, Canada	Enerkem Aberta Biofuels LP[a]	MSW	30000
Emmetsburg, USA	POET-DSM Advanced Biofuels[a]	Corn cob	75000
Blairtown, USA	Fiberright LLC[a]	MSW	18000
Hugoton, USA	Abengoa Bioenergy[a]	Corn stover, wheat straw, switchgrass	75000
Vero Beach, USA	INEOS Bio[a]	Agricultural residue	24000

[a]Commercial plant

[b]Demonstration plant

MSW: Municipal solid waste

4 Enzymatic conversion of biomass to ethanol

As previously discussed, lignocellulosic materials are very resistant to enzymatic attack, due to their recalcitrant structure, and they must therefore be pretreated prior to hydrolysis to increase their digestibility. Various configurations of the enzymatic route were investigated in this work, and detailed descriptions of the process steps are provided in this chapter.

4.1 Pretreatment

Lignocellulosic biomass must be pretreated prior to enzymatic hydrolysis so that the enzymes can efficiently convert cellulose to monomer sugars. The rigid structure of the recalcitrant biomass must be modified to break down the lignin structure, remove the hemicelluloses and disrupt the crystalline structure of the cellulose to increase enzyme accessibility (Mosier et al. 2005). An ideal method of pretreatment should result in high yields of all sugars, avoid degradation or carbohydrate losses and the formation of inhibitory compounds, and result in high digestibility of the cellulose, and should, of course, be cost effective (Galbe et al. 2007; Sun and Cheng 2002).

Various types of pretreatment have been thoroughly investigated over the years. The purpose of physical pretreatment such as mechanical comminution is size reduction, which is an effective way to reduce cellulose crystallinity (Sun and Cheng 2002; Tomás-Pejó et al. 2011). Chemical methods, such as alkali (Carvalheiro et al. 2008), organosolv (Park et al. 2010) and ozonolysis (García-Cubero et al. 2009), as well as the physicochemical method ammonia fibre explosion (AFEX) (Holtzapple et al. 1991), have been shown to be effective for lignin solubilization. Acid pretreatment using sulphuric (Nguyen et al. 2000), nitric, hydrochloric or phosphoric acid (Mosier et al. 2005), and physicochemical pretreatment such as steam explosion (Balat 2011), wet oxidation and dilute-acid pretreatment, mainly act on the hemicellulose fraction (Tomás-Pejó et al. 2011).

As pretreatment is necessary prior to enzymatic hydrolysis, and since it affects all the other steps in the conversion process, it is important to choose the pretreatment

method carefully, depending on the process configuration and the raw material to be used. The most suitable methods of pretreatment of agricultural residues are alkaline or acid pretreatment, steam explosion or AFEX. Pretreatment at high pH, using alkaline solutions such as sodium, potassium or ammonium hydroxide while heating, disrupts the lignin structure and makes the carbohydrates more accessible to the enzymes. This kind of pretreatment is generally more effective on materials with low lignin contents (Chang et al. 1997). Alkali pretreatment combined with acid hydrolysis has also been evaluated (Govumoni et al. 2013). AFEX, another alkaline pretreatment method using high-pressure liquid ammonia, is operated at moderate temperature (below 100 °C) and high pressure (above 3 MPa), which is released very quickly causing explosion of the material (Holtzapple et al. 1991). In spite of the minimal removal of hemicellulose or lignin with AFEX, high enzymatic digestibility has been reported at low enzyme loadings compared with other pretreatment methods (Wyman et al. 2005). Furthermore, this kind of pretreatment does not require small particle sizes and no inhibitors are produced (Sun and Cheng 2002). However, the major problem associated with this kind of pretreatment is the high cost of ammonia and ammonia recovery, which makes this method economically unfeasible. Furthermore, handling large amounts of ammonia is hazardous (Holtzapple et al. 1991).

Steam pretreatment is one of the most promising methods of pretreatment and also the most widely used in the pilot and demonstration-scale facilities presented in Table 3.2. High-pressure saturated steam (6-33 bar) is used to increase the temperature to 160-240 °C in the reactor for a period of time ranging from seconds to several minutes, after which the pressure is suddenly released (Galbe and Zacchi 2002; Tomás-Pejó et al. 2011). This form of pretreatment can be performed with or without the addition of an acid. Agricultural residues contain sufficient amounts of organic acids (mainly acetic acid), to act as catalysts for hemicellulose hydrolysis, so-called autohydrolysis. However, the addition of acid catalysts such as sulphuric acid (Sassner et al. 2008b; Söderström et al. 2003) or sulphur dioxide (Ohgren et al. 2005) results in better hemicellulose removal and improved degradability of the cellulose in subsequent enzymatic hydrolysis (Galbe et al. 2011). Steam pretreatment with the addition of acids has been investigated and optimized on various agricultural residues, such as corn stover (Ohgren et al. 2005), barley straw (Linde et al. 2006) and sugarcane bagasse (Carrasco et al. 2010; Martin et al. 2002). Other studies have shown that impregnation of wheat straw with small amounts of H_2SO_4 before steam pretreatment results in improved sugar yields (Ballesteros et al. 2006; Linde et al. 2008).

In the present studies, wheat straw was impregnated with a dilute sulphuric acid solution and then pretreated in a steam pretreatment unit, comprising a batch steam gun and a 10-L reactor (Palmqvist et al. 1996a), using saturated steam at 190 °C for 10 minutes. The conditions for pretreatment were optimized previously by Linde et

al. (2008). The wheat straw slurry obtained after pretreatment comprises two fractions: a liquid fraction rich in monomeric and oligomeric sugars, arising mainly from hemicellulose, and a solid fraction consisting of digestible cellulose and lignin. The fraction of solid matter, i.e., the WIS, was determined and the composition of the solid and liquid fractions is given in Table 4.1.

One major drawback of steam explosion (as in other acid pretreatment methods) is the generation of toxic compounds derived from sugar degradation. These inhibitors will be present in the liquid fraction and can affect the following hydrolysis and fermentation steps.

Table 4.1 Composition of the steam-pretreated wheat straw

Paper	**I**	**II**		**III**		**IV**	**V**
Batch		1	2	1	2		
				% of slurry			
WIS	7.6	8.7	9.5	8.2	13.5	12.4	11.7
Solid fraction				**% of WIS**			
Glucan	67.6	62.5	63.4	62.2	58.6	51.4	59.3
Xylan	0.7	4.0	6.4	5.8	6.3	5.6	6.7
Galactan	-	-	1.5	-	-	-	-
Arabinan	0.4	1.1	-	0.6	0.4	-	-
Mannan	-	1.4	3.4	-	-	-	-
Lignin							
Acid-insoluble	23.1	22.5	25.1	21.9	26.9	29.2	28.3
Acid-soluble	5.1	3.5	3.3	1.6	0.8	2.0	0.7
Lignin ash	1.0	1.4	2.5	3.4	3.0	5.0	3.1
Liquid fraction				**g/L**			
Monomers							
Glucose	2.3	2.7	3.1	2.4	2.3	4.0	3.4
Xylose	22.0	23.9	25.6	22.0	24.8	30.0	24.5
Galactose	1.4	0.3	1.4	1.3	-	-	4.1
Arabinose	2.9	3.7	3.4	3.2	4.7	6.7	5.4
Mannose	1.1	-	1.3	0.8	-	-	1.1
Oligomers							
Glucose	0.9	1.5	2.1	2.6	7.5	3.9	5.0
Xylose	2.9	9.3	11.7	8.4	18.9	9.2	12.3
Galactose	-	-	2.8	2.3	-	-	1.3
Arabinose	-	1.0	1.2	0.7	1.1	-	0.3
Mannose	4.1	-	0.5	0.5	-	-	0.3
By-products							
Furfural	1.5	1.6	2.0	2.0	1.7	2.8	2.6
HMF	0.4	0.2	0.1	0.1	0.1	0.3	0.2
Acetic acid	1.7	2.3	2.9	2.9	2.4	3.6	2.4
Formic acid	nd	nd	nd	nd	nd	0.7	0.4

WIS: water-insoluble solids; nd: not determined; HMF: 5-hydroxymethylfurfural

4.1.1 Inhibitors

Sugar degradation products generated during steam pretreatment, illustrated in Figure 4.1, can be very inhibitory in the subsequent process steps, especially in fermentation. The most common degradation products are 5-hydroxymethylfurfural (HMF), furfural, organic acids, such as formic and levulinic acid, and phenolic compounds. Acetic acid, which is also a toxic organic acid, is formed by the hydrolysis of the acetyl groups of hemicellulose (Jonsson et al. 2013).

HMF and furfural are formed under severe conditions from hexose and pentose sugars, respectively (Dunlop 1948; Palmqvist and Hahn-Hägerdal 2000a; Ulbricht et al. 1984). These compounds have been shown to affect enzymatic hydrolysis (Tengborg et al. 2001) and microbial activity (Larsson et al. 1999; Sanchez and Bautista 1988; Taherzadeh et al. 2000).

Further degradation of furfural results in formic acid, while HMF gives both formic acid and levulinic acid (Ulbricht et al. 1984). Formic, levulinic and acetic acids are classified as weak acids because of their high pK_a value, which is the pH at which the concentrations of undissociated and dissociated forms of the acid are equal and the buffering capacity of the acid is therefore greatest. Despite its low pK_a value, formic acid is the most toxic, due to its small molecular size followed by levulinic and acetic acid. Smaller molecules such as formic acid are believed to diffuse more easily through the plasma membrane, while levulinic acid may be more inhibitory than acetic acid due to its greater lipophilicity. The toxicity of these acids is attributed to the undissociated form of the acids. Undissociated acids can enter the cell through the plasma membrane and dissociate inside the cell as a result of the neutral cytosolic pH (Pampulha and Loureiro-Dias 1989) which leads to a decrease in intracellular pH and cell death.

However, weak acids at low concentrations have also been shown to increase the ethanol yield in anaerobic fermentation at the expense of biomass formation (Pampulha and Loureiro-Dias 1989; Taherzadeh et al. 1997). This is thought to be the consequence of the cell's reaction, pumping out protons by the action of the plasma membrane ATPase to balance the intracellular pH (Verduyn et al. 1992; Verduyn et al. 1990; Viegas and Sa-Correia 1991).

Degradation of lignin results in phenolic compounds such as vanillin, 4-hydroxy-benzaldehyde, vanillic alcohol or acids such as syringic, sinapic or ferulic acid, which reduce the fermentation rate and biomass yield more than the ethanol yield (Klinke et al. 2003; Larsson et al. 2000). Vanillin and phenol can cause cell membrane damage, but apart from this, little is known about the inhibition mechanism of phenolics (Almeida 2009).

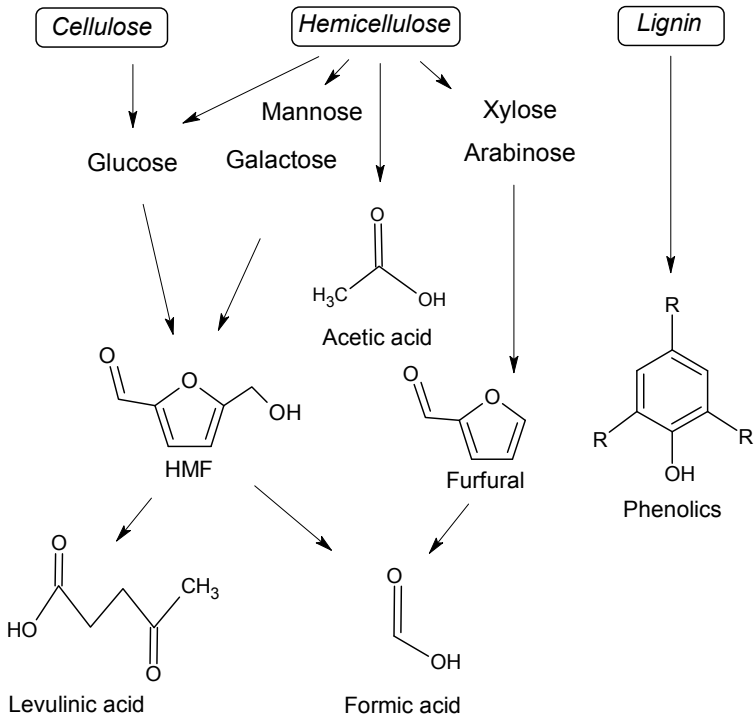

Figure 4.1 Inhibitory compounds formed during acid-catalysed hydrolysis of biomass. Adapted from (Palmqvist and Hahn-Hägerdal 2000a)

4.2 Enzymatic hydrolysis

Biomass is the most abundant carbon source in nature, providing energy to those species that can utilize it. A number of microorganisms can be found in nature that have developed efficient cellulose-degrading enzyme systems. *Trichoderma* and *Aspergillus* species are among the best characterized enzyme systems of aerobic filamentous fungi. These species are used for commercial cellulase enzyme production due to their high levels of secreted enzymes and intensive research on their development (Lynd et al. 2002).

4.2.1 Lignocellulose-degrading enzymes

Three major types of activities are represented in cellulase systems: endoglucanases (EGs), exoglucanases, including glucohydrolases and cellobiohydrolases (CBHs), and β-glucosidases. Endoglucanases act randomly on the amorphous region of the cellulose, attacking the β-1,4-glycosidic bonds, liberating glucose oligomers of various lengths and exposing new terminal ends. *T. reesei* produces five endoglucanases (EGI-V) which reduce the degree of polymerization of cellulose. Exoglucanases, on the other hand, bind to the end of the cellulose chain and act in a progressive manner, releasing either glucose units (glucohydrolases) or cellobiose units (cellobiohydrolases). *T reesei* produces two known cellobiohydrolases, CBHI and CBHII, which play a key role in the system. CBHI cleaves off cellobiose from the reducing end, while CBHII prefers the non-reducing end (Béguin and Aubert 1994; Lynd et al. 2002; Pérez et al. 2002). About 60% of the total protein produced for cellulose degradation is CBHI, and 20% is CBHII (Percival Zhang et al. 2006). To help enzyme binding on the cellulose surface, most cellulases have a carbohydrate-binding module, apart from the active site, which also facilitates hydrolysis by bringing the catalytic domain closer to the substrate. The synergistic action of endoglucanases and cellobiohydrolases is a very important feature of the cellulase system, resulting in a greater combined activity than the sum of the individual components. Cellulases are severely inhibited by their product, cellobiose, which confers an important role on β-glucosidase, which is not a cellulase but it hydrolyses the soluble cellobiose disaccharide into glucose units. *T. reesei* also produces two β-glucosidase enzymes, but a β-glucosidase preparation from an *Aspergillus* species is often added to facilitate biomass hydrolysis. β-glucosidase is also inhibited by its end-product, glucose, which may be a limiting factor in hydrolysis.

Hemicellulases are required for the hydrolysis of the complex structure of hemi-cellulose. Endo-1,4- β-D-xylanase and endo-1,4- β-D-mannase act by depolymerizing the hemicellulose backbone, while β-D-xylosidase, β-D-mannosidase and β-D-glucosidase hydrolyse small oligosaccharides into xylose, mannose or glucose, respectively, by cleaving the oligomer's β-1,4 bonds (Pérez et al. 2002).

Enzymatic hydrolysis is influenced by the structural characteristics of the biomass, the mode of enzyme operation, and also by the specific mixture of enzymes. Substrate-related factors such as the degree of polymerization, the accessible surface area of the cellulose, and the distribution of derived insoluble matter, such as hemicellulose and lignin, are important (Yang et al. 2011). Soluble matter in the hydrolysate has also been suggested to reduce the rate of enzymatic hydrolysis (Linde et al. 2008; Ohgren et al. 2007; Sipos et al. 2009). To overcome challenges of enzyme-related rate limiting factors, intensive research has been performed to maximize biomass degradation by understanding features of hydrolytic enzymes, by elucidating synergistic interaction of

the individual components (Yang et al. 2011), and by minimizing end-product inhibition and inactivation, while improving stability (Béguin and Aubert 1994).

Earlier techno-economical evaluations have shown that the cost of enzymes represents a considerable part of the total cost of ethanol production (Sassner et al. 2008a; Wingren et al. 2003). However, due to extensive research on ways of minimizing the amount of enzyme used by the design of more efficient enzymes and enzyme mixtures (Lynd et al. 2002; Sun and Cheng 2002; Yang et al. 2011), the cost of enzymes has been significantly decreased. The cost of enzymes is no longer a limiting factor in the commercial production of ethanol from cellulosic biomass (Viikari et al. 2012).

4.3 Fermentation

During fermentation, the sugars released during enzymatic hydrolysis are fermented into ethanol, together with the formation of carbon dioxide. Lignocellulose hydrolysates contain C6 and C5 sugars, lignin and lignin derivatives, and a number of inhibitory compounds, some present in the raw material and others arising from the degradation of sugars. The main sugars fermented in the hydrolysates from pretreated agricultural residues are glucose and xylose. In order for the production of ethanol to be economically feasible, the efficient and complete conversion of all sugars derived from hemicellulose and cellulose is highly desirable. The requirements for an ideal fermenting microorganism are, therefore, that it produces ethanol at a high productivity and high yield from all types of sugars, while at the same time tolerating high ethanol and inhibitor concentrations. Good temperature tolerance would also be beneficial in certain process configurations such as SSF. Tolerance to low pH is also desirable, as low pH reduces the risk of contamination.

Unfortunately, there is no single microorganism in nature that meets all these requirements. Among a number of reported bacteria, yeast and filamentous fungi that can ferment lignocellulosic hydrolysate, *Escherichia coli*, *Zymomonas mobilis*, *Saccharomyces cerevisiae*, *Pichia stipitis* and *Candida shehatae* are the most relevant for ethanol production (Balat 2011; Hahn-Hägerdal et al. 2007). Traditionally, *S. cerevisiae* and *Z. mobilis* have been used for ethanol production. These are capable of efficiently fermenting glucose to ethanol, but not xylose. *E. coli*, *P. stipitis* and *C. shehatae*, on the other hand, are naturally xylose-fermenting strains, but they have low ethanol and inhibitor tolerance, they perform best in a narrow, neutral pH range and, in the case of *E. coli* produce mixed products, which makes them unsuitable for industrial ethanol production (Gírio et al. 2010). The glucose-fermenting strain *Z. mobilis* has similar disadvantages apart from its ethanol tolerance, which is high enough to allow ethanol concentrations up to 12-13.5% (v/v), although significant

inhibition is seen at ethanol concentrations above 2% (v/v) (Osman and Ingram 1985).

4.3.1 Saccharomyces cerevisiae

Baker's yeast, *S. cerevisiae*, is the most commonly used microorganism in traditional sucrose and starch-based industrial ethanol fermentation. Due to its status as "generally recognized as safe" and its robustness, *S. cerevisiae* is also the preferred microorganism in the fermentation of lignocellulosic substrates. Wild type *S. cerevisiae* can efficiently ferment glucose, mannose and fructose, as well as the disaccharides, sucrose and maltose and, in addition to sugars, ethanol, glycerol and acetate can also be utilized for growth. Furthermore, as a facultative anaerobe, it is able to grow under both aerobic and anaerobic conditions (Walker 1998). The main product of this organism is ethanol, which is produced at a high yield (above 0.45 g/g glucose at optimal conditions, which corresponds to 88% of the theoretical), and high specific rate (up to 1.3 g/g cell mass/h) (Verduyn et al. 1990). The main metabolic pathway involved in fermentation to produce ethanol is glycolysis, in which one molecule of glucose is metabolized and two molecules of pyruvate are produced. If no oxygen is available, the pyruvate is further reduced to ethanol with the release of CO_2. The theoretical ethanol yield is 0.511, and that for CO_2 is 0.489, based on the mass of glucose metabolized. Since two ATPs are produced in glycolysis, which drives the biosynthesis of the cell, involving energy-requiring bioreactions, ethanol production is strongly related to cell growth in sugar fermentation, and thus yeast is produced as a by-product (Bai et al. 2008). However, during the fermentation of lignocellulose hydrolysates cell growth can be severely inhibited (Palmqvist and Hahn-Hägerdal 2000b), and in some cases cell death is seen (Tang et al. 2011). Other by-products include glycerol, the production of which is stimulated by higher osmotic pressure, and organic acids and higher alcohols, which are produced at much lower levels (Ingledew 1999).

S. cerevisiae has been shown to have relatively good tolerance to lignocellulose-derived inhibitors (Olsson and Hahn-Hägerdal 1993) and high osmotic pressure, and Casey and Ingledew reported no significant reduction in fermentation rate even over 10% (v/v) ethanol (Casey and Ingledew 1986). The main disadvantage of *S. cerevisiae,* i.e., its lack of ability to ferment xylose, can be overcome by metabolic engineering, in which substantial progress has been achieved (see Chapter 6).

4.4 Process configurations

The initial stages required in bioethanol production are pretreatment, cellulase production, hydrolysis of cellulose and other remaining hemicellulose (if present), fermentation of hexose sugars (glucose in the case of wheat straw) and the fermentation of pentose sugars, mainly xylose, with genetically engineered strains. Several process configurations can be used, based on the degree to which these particular steps are combined, as shown in Figure 4.2.

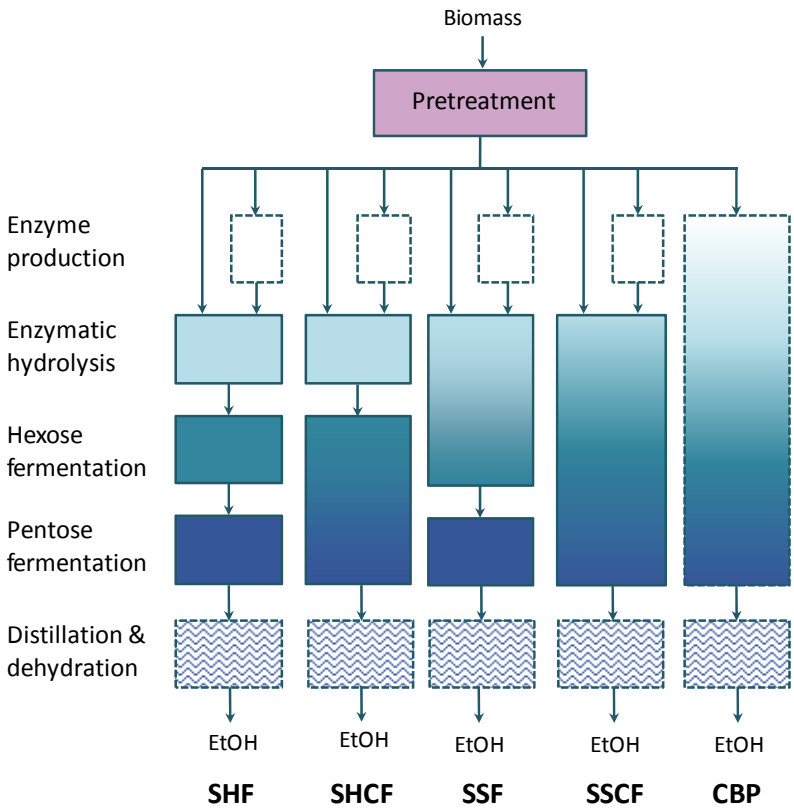

Figure 4.2 Alternative process configurations for ethanol production using enzymatic hydrolysis, and hexose and pentose fermentation. SHF: separate hydrolysis and fermentation, SHCF: separate hydrolysis and co-fermentation, SSF: simultaneous saccharification and fermentation, SSCF: simultaneous saccharification and co-fermentation. CBP: consolidated bioprocessing. Process steps with solid lines have been studied in this work.

4.4.1 SHF and SHCF

In separate hydrolysis and fermentation, SHF, the cellulose in the solid fraction is first hydrolysed by the cellulolytic enzymes, and the liquid fraction containing the sugars is then fermented to ethanol (Cardona and Sánchez 2007). There are two ways of performing SHF (Bacovsky et al. 2010). In the first alternative, the fermentation of C6 and C5 sugars is performed sequentially, i.e. the pretreated biomass slurry is subjected to hydrolysis, and then C6 sugar fermentation is carried out, followed by the removal of the ethanol. The residual stillage is then subjected to the fermentation of C5 sugars. Alternatively, the soluble C5 sugars can be removed after pretreatment, and fermented in parallel with the hydrolysed cellulose (C6) fraction. Finally, the ethanol from the separate fermentation of the sugars can be distilled together. In the second alternative, the C6 and C5 sugars are fermented in a single step using the same microorganism. This is called separate hydrolysis and co-fermentation, SHCF.

A major advantage of both SHF and SHCF is that each process step can be operated at its optimal temperature, i.e. around 50 °C for enzymatic hydrolysis and around 32 °C for fermentation, and the yeast can be easily separated and recycled after fermentation. However, sugar yields are usually lower due to the inhibition of hydrolysis by glucose and cellobiose. The advantages of glucose and xylose co-fermentation in the SHCF configuration will be thoroughly discussed in Chapter 6, based on the results presented in Papers III and IV.

4.4.2 SSF

The concept of SSF was introduced by Gauss et al. in 1976 to eliminate end-product inhibition of the enzymes. If fermentation is carried out in the same vessel simultaneously with hydrolysis, the sugars released by the enzymes are directly fermented to ethanol. This prevents the accumulation of glucose and cellobiose, eliminating end-product inhibition of hydrolysis (Gauss et al. 1976), and the enzyme loading can be reduced. SSF has been comprehensively reviewed by Olofsson et al. (Olofsson et al. 2008a). SSF has other advantages, such as the avoidance of sugar losses, since there is no need for sugar separation before fermentation, and the assimilation of degradation by-products that have inhibitory effects on the enzymes by the microorganisms, which may lead to improved enzymatic hydrolysis. The latter may explain the higher observed ethanol yields in SSF than in SHF (Söderström et al. 2005; Tengborg et al. 2001). Moreover, because of the higher ethanol yields achieved in the combined process, the investment costs for SSF can be 20% lower than those in SHF (Wingren et al. 2003), which is a very important factor in achieving economic feasibility in ethanol production from lignocellulosic material.

There are, however, also two major drawbacks of SSF. One is the operating temperature (35-37 °C), which is a compromise between the optimal temperatures for the separate steps. The development of thermo-tolerant strains that can ferment at elevated temperatures could substantially decrease the difference between the temperature optima of hydrolysis and fermentation, which may enhance the performance of SSF. The other drawback is that recycling of the yeast is very difficult due to the solid lignin residue present in SSF. This will result in a loss in yield when the yeast is produced on-site, while the loss of externally produced yeast would increase the operating cost.

4.4.3 Consolidated bioprocessing

Consolidated bioprocessing (previously also called direct microbial conversion) would be the ultimate all-in-one process. In this unified configuration, cellulase production, the hydrolysis of cellulose, and C6 and C5 sugar fermentation are performed simultaneously in a single vessel. The attractiveness of the process lies in its simplicity, which results in simplified operation, a reduction in the number of process steps and reduced cost of chemicals (Balat 2011; Olson et al. 2012). It would also offer the potential for lower production cost and higher efficiency. The smaller reactor volume would also lead to reduced capital investment. Unfortunately, low ethanol yield and the formation of significant amounts of by-products, together with the low ethanol tolerance of currently available microorganisms make this option economically unfeasible. If this configuration is to be realized, microorganisms must be developed that utilize cellulose and other fermentable compounds released from the pretreated biomass at a high conversion rate and which produce the desired product at high yield and titre (Lynd et al. 2005).

4.5 Product recovery

The recovery of the ethanol, i.e., solid-liquid separation and distillation, can have a considerable effect on the overall production cost (Galbe et al. 2011). The distillation step normally consists of stripper columns and rectification columns. The stripper columns separate the ethanol from the solid and non-volatile compounds, and concentrate the ethanol by removing most of the water. In the rectification columns, the ethanol is concentrated to a value close to the azeotropic point.

The ethanol concentration in the broth has a significant impact on the energy demand, at least up to an ethanol concentration of about 7-8% (w/w) ethanol in the distillation feed (Galbe et al. 2007; Zacchi and Axelsson 1989). The energy demand can be reduced by heat integration and by running the stripper and the rectifier in series, as shown by Galbe et al. (2007), using two stripper columns and a rectification column, which are heat integrated by operating at different pressures. Since the energy demand of the distillation step decreases as the concentration of ethanol in the feed increases, it is of great importance to obtain high ethanol concentrations in the distillation feed. In a starch-based process this stream normally has an ethanol concentration above 8% (w/w), which is sufficiently high to be economically feasible for 1G commercial ethanol production (see Chapter 1).

However, it is difficult to achieve ethanol concentrations above 5% (w/w) in the fermentation of lignocellulosic hydrolysates. High sugar concentrations require a high solids content, which also results in higher concentrations of inhibitors, which may result in lower yield. Two ways of increasing the ethanol concentration in lignocellulosic ethanol production are either to integrate the process with starch-based ethanol production or to increase the ethanol yield by co-fermentation of C6 and C5 sugars. The main objectives of this work were to find possible process configurations for the integration of the two processes (Chapter 5) and for facilitating the co-fermentation of glucose and xylose (Chapter 6).

5 Integration of the starch- and cellulose-based ethanol production processes

The integration of 2G ethanol production with existing 1G ethanol production offers a promising means of using the whole agricultural crop; in the current work, wheat. The wheat grain is the substrate used for 1G ethanol production and the straw for 2G ethanol production. Integration of the two processes can be beneficial for both processes. While the 2G plant would benefit from the reduction in energy demand and capital cost per ton fuel produced, the lignin residue would provide the integrated plant with heat and electricity.

It is generally difficult to reach high ethanol concentrations in the lignocellulosic process due to the high concentrations of inhibitory compounds resulting from high solids contents. At the same time, starch-based streams must be diluted somewhat to reduce the sugar concentration. Thus, both processes would benefit from co-processing by the integration of suitable streams. The energy required for distillation would be significantly reduced in the case of 2G production, reducing the overall production cost.

Not only a higher ethanol concentration, but also a higher ethanol yield, can be expected in an integrated plant. Dilution of the lignocellulosic stream helps reduce the inhibitor concentration, while the addition of some inhibitors to 1G fermentation can be beneficial in wheat meal fermentation, as low concentrations of weak acids have been shown to increase the ethanol yield (Pampulha and Loureiro-Dias 1989; Taherzadeh et al. 1997).

Among further benefits, a starch-derived hydrolysate could be used as a complex nutritional source, instead of chemicals, which would also reduce the cost of large-scale lignocellulosic process (Sassner et al. 2008a). It has been shown in previous studies that the use of chemicals could be reduced by supplementing the fermentation of lignocellulosic hydrolysate with wheat hydrolysate (Brandberg et al. 2007; Jones and Ingledew 1994a). In another study, corn hydrolysate was shown to be a potential substitute for yeast extract for SSF of lignocellulose residues (Tang et al, 2011).

Finally, some configurations could facilitate xylose fermentation, as a low glucose concentration is beneficial for efficient xylose utilization in the genetically modified strains of S. cerevisiae investigated in this work. The possible use of a starch-derived hydrolysate to provide a constant glucose feed in lignocellulosic fermentation motivated the investigation of some of the integration scenarios in this work.

5.1 Alternatives for process integration

Figure 5.1 shows a simplified scheme illustrating the possible alternatives for the integration of wheat starch-based ethanol production (1G) with ethanol production from wheat straw (2G). Due to similarities in the two processes, there are several suitable points for integration early in the process, e.g. after pretreatment of the raw materials, and not only in the downstream processes of distillation and evaporation.

The easiest point of integration would be to mix the liquid streams after fermentation, before distillation, to reap the benefits of a higher ethanol concentration, while using separate, dedicated equipment for each process up to the distillation step. In this way, the solid residues from each process can be utilized optimally. The protein-rich solid residue from the 1G process can be used to produce DDGS, which can be sold as animal feed, while the lignin residue from the 2G process can provide heat and power for both processes. However, the cost of the 2G equipment and the energy demand could be reduced by combining the material streams further upstream. The possible points of integration in the upstream processing are discussed below.

In the first study (Paper I), PWM was mixed with SPWS in SSF using baker's yeast. The effect of integration on the ethanol yield and concentration was investigated. Adding the entire PWM stream to SSF has the advantage of simplicity, as saccharification of both the wheat meal and SPWS can occur simultaneously in one vessel. This would, however, result in mixed residues after distillation, meaning that the valuable animal feed would be burnt with the lignin residue from the lignocellulosic process to produce heat and power. This is a major disadvantage of this configuration, as discussed in Papers I and II, unless there is a surplus of animal feed.

In the study presented in Paper II, only LWM, i.e. the stream containing glucose polymers, was added to SSCF of SPWS. A xylose-fermenting strain of *S. cerevisiae* (TMB3400) was used to study whether the sugar released from LWM during saccharification could facilitate xylose uptake.

The main reason for investigating the integration of the saccharified wheat meal with the enzymatically hydrolysed SPWS (Paper III) was to take advantage of the possibility of feeding SWM (wheat hydrolysate) into the fermentation step of SHCF,

to facilitate glucose and xylose co-fermentation. The benefits of controlled sugar release in the co-fermentation of glucose and xylose in the studies presented in Papers II and III are discussed in detail in Chapter 6.

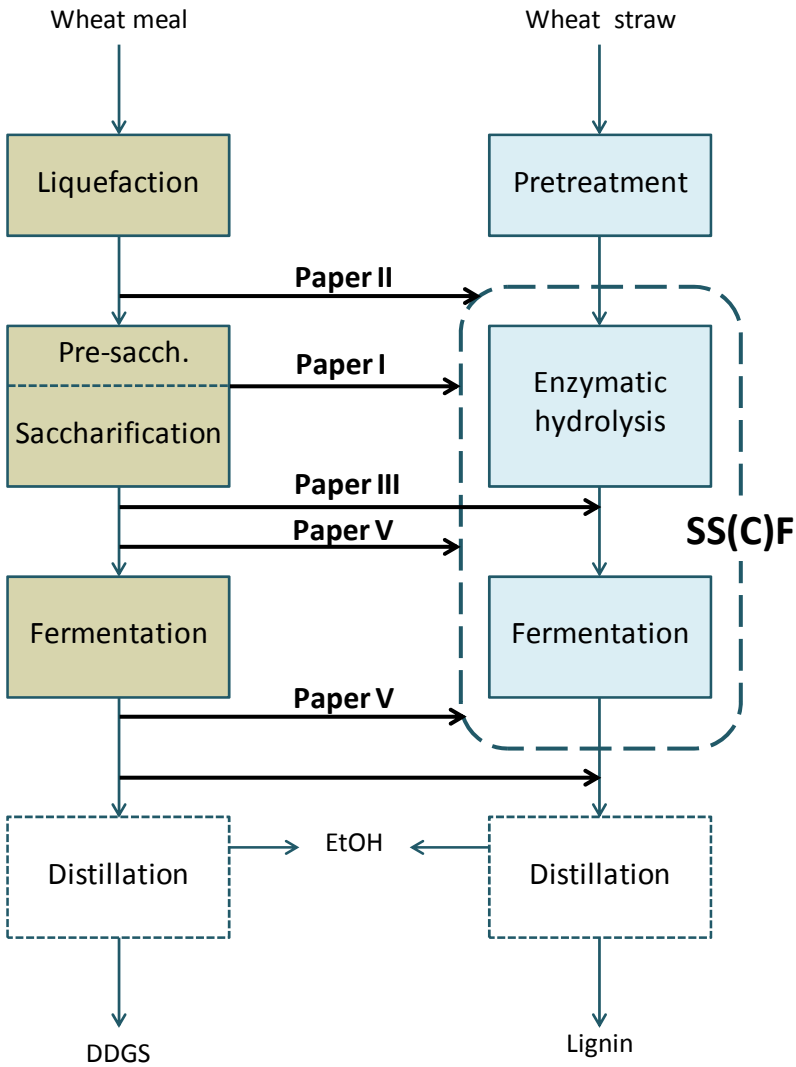

Figure 5.1 Alternatives investigated for the integration of the starch-based (1G) and lignocellulose-based (2G) ethanol production processes.

The study described in Paper V, in which SWM was subjected to SSF or SSCF, was performed to investigate the possible recovery of the protein-rich residue. In this case, a mutant of *S. cerevisiae* TMB3400 (KE6-12) xylose-fermenting microorganism was used to investigate whether the co-fermentation of glucose and xylose could be facilitated by adding SWM. In another part of this study, FWM was subjected to SSF to determine whether a stream containing an already high concentration of ethanol could be used for dilution of the lignocellulose stream, thereby increasing the ethanol concentration.

5.2 Influence of integration on SSF

Since SSF has become a low-cost, high-yield fermentation technique in most modern 1G plants (Destexhe et al. 2004), and the SSF of lignocellulose is also considered to be advantageous in many respects, the first integration scenario investigated was to mix the materials before SSF (Papers I and II). Pretreatment of the raw materials is required in both cases: liquefaction of the meal breaks down the starch, while pretreatment of the straw partially breaks down the lignocellulose to sugar polymers. In the SSF step these polymers are converted to sugars and simultaneously fermented to ethanol. In this way, sugar inhibition and osmotic stress could be avoided. If the pretreated straw residue and the liquefied meal are mixed, saccharification and fermentation of both materials can take place in one fermentor. The effects of integrating the two streams on ethanol productivity and concentration, but most importantly on the ethanol yield, were investigated, and the factors influencing the outcome were examined.

The main results obtained using SSF are discussed below, based on the results presented in Papers I, II and V. SSF was carried out using compressed *S. cerevisiae* (Paper I) (and dry *S. cerevisiae* (Paper V)). Although a genetically modified xylose-fermenting strain of *S. cerevisiae* was used in SSCF in the study presented in Paper II, the results of integration based on glucose utilization only are presented here.

5.2.1 Ethanol yield and concentration

Ethanol yield is an important measure of how well the biomass is utilized, i.e., how much of the sugar that is released from the cellulose in enzymatic hydrolysis is converted into ethanol during fermentation. In SSF, the yield represents the combination of these two processes. Ethanol yields are expressed here as the fraction of the maximum theoretical ethanol yield of 0.51 g/g glucose. In this section, the ethanol yields presented are based on all the glucose (glucan in the WIS and both

mono- and oligomers in the liquid fraction) from both cellulose and starch added to SSF (Papers I and V) or SSCF (Paper II).

As the cost of feedstock is one of the major costs of lignocellulosic ethanol (up to 30-40% of the total production cost) (Sassner et al. 2008a; Wingren et al. 2008), it is crucial to maximize biomass utilization. The aim is thus to achieve a high ethanol concentration at the end of the conversion process, by ensuring that the ethanol yield is as high as possible.

The concept of using the sugar-rich wheat starch-based hydrolysate to dilute the lignocellulosic substrate, i.e. reducing the concentration of inhibitors, while increasing the concentration of the sugars, was confirmed by the study presented in Paper I. In this study, SPWS was mixed with increasing proportions of PWM, at a total WIS of 5% (w/w) in the mixtures, in SSF. Since PWM contained the fibre residue of the kernels, the proportions mixed were based on the WIS content of each material. However, it should be kept in mind that PWM represents a greater amount of sugars added per unit WIS when added to SSF than SPWS, as the glucose concentration is higher in the material originating from starch than in that from wheat straw. The amount of sugars added to the SSF experiments with respect to their origin is described in detail in Paper I.

The ethanol yield from the various mixtures of substrates evaluated was higher than the yields from the separate substrates, see Figure 5.2. In this study, the yield observed for pure SPWS was about 70% of the theoretical, which is in the range reported in other studies on wheat straw (Linde et al. 2008), and slightly lower than for corn stover (Ohgren et al. 2006b). The yield observed for pure PWM was about 90%, which is also typical for SSF of starch-based materials (Destexhe et al. 2004). In mixtures of the two substrates the yield increased as the proportion of PWM was increased. This is probably due to the decreasing amount of inhibitors present in SSF. However, the most important observation is that the highest yield obtained for the mixture with equal amounts of PWM and SPWS (based on WIS), was higher than the yield obtained from pure PWM, when no inhibitors were present. Similar observations of improved ethanol yield with low inhibitor concentrations, compared with cases with no inhibitors, have been reported previously (Pampulha and Loureiro-Dias 1989; Taherzadeh et al. 1997). Both weak acids (Larsson et al. 1999) and furfural have been found to improve the ethanol yield at low concentrations (Palmqvist et al. 1999; Palmqvist et al. 1996b). Despite the higher ethanol yield, furfural has been shown to lower the ethanol productivity (Palmqvist et al. 1999). In the present study, the highest ethanol yield was obtained when the acetic acid and the furfural concentrations were about 1.0 g/L and 0.6 g/L, respectively.

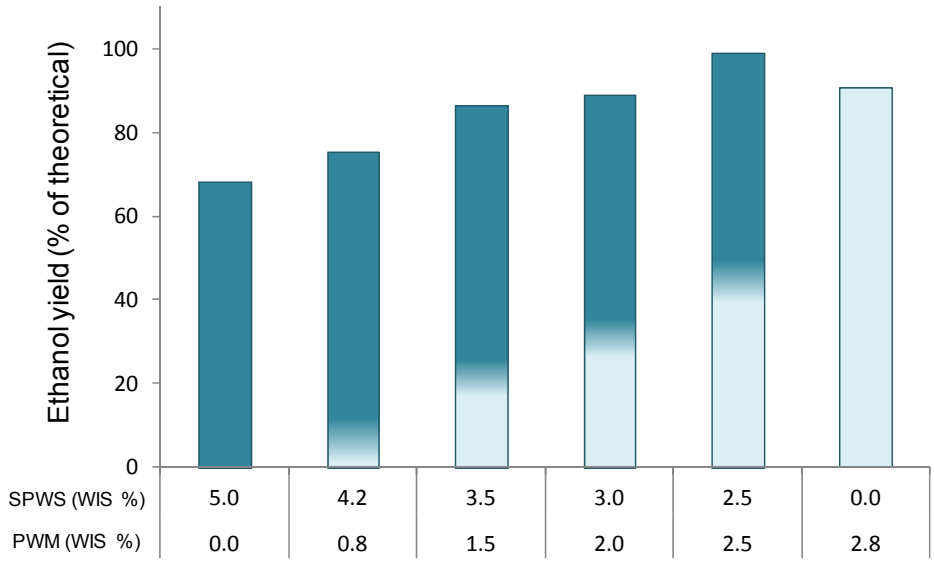

Figure 5.2 Ethanol yield obtained in SSF of pure steam-pretreated wheat straw (SPWS- dark shading) pre-saccharified wheat meal (PWM-light shading) and mixtures of the two (toned shading) at a total water insoluble solids (WIS) concentration of 5% (w/w). (Adapted from Paper I.)

To increase the proportion of sugars arising from the wheat straw in SSF, higher WIS loadings of 7.5-10% (w/w) SPWS were applied in the studies described in Papers II and V. Fairly high ethanol yields, over 90% of theoretical, were achieved in SSF of pure SPWS in both studies, however, the yields decreased slightly when the WIS loading was increased to 10% from 7.5% in the study presented in Paper II, see Table 5.1. Increasing the WIS loading from 7.5% to 10% has also been found to decrease the ethanol yield from barley straw (Linde et al. 2007), while it remained unchanged for corn stover (Ohgren et al. 2006b). The addition of LWM resulted in a slight decrease in ethanol yield, which was more pronounced at 10% WIS. Although inhibitor concentrations were higher with higher WIS loading, they were the same in the substrates used for SSF with and without the addition of the starch-based material (both LWM and SWM). Therefore, other factors must have caused the decrease in ethanol yield. At higher WIS loading, the combined effect of several stresses, from inhibitors and increased osmotic stress caused by increased glucose concentration,

might have led to the observed results. As the sugars from LWM were released almost immediately (see Paper II), osmotic stress may have occurred. Osmotic stress is also indicated by the higher glycerol production of the yeast in response to the osmotic pressure created by a higher glucose concentration. Intracellular glycerol concentration is increased to maintain the cell functionality of enzymes under such conditions (Albertyn et al. 1994). Although increased glycerol production was observed when SWM was added to SSF, the ethanol yield increased slightly (Paper V). This may be due to the greater proportion of readily available glucose. Another possible explanation is that LWM, as opposed to SWM, contains WIS that contain some glucan and other polymers, such as lignocellulose. However, this material had not been steam pretreated, which means that the glucan from this fibre material could only be hydrolysed to a minor extent by cellulases and thus decreases the yield.

Since the inhibitor concentrations, which probably have the greatest influence on the ethanol yield, were the same in SSF when the starch-based materials (both LWM and SWM) were added, only small differences were observed compared with the reference experiments. However, if the concept described in Paper I had been used, i.e. using the starch-based material for dilution, the inhibitor concentrations in the mixture would have been lower. According to this concept, ethanol yields obtained from SSF of SPWS with 7.5% WIS diluted by the addition of LWM with 2.5% WIS, should be compared with the yields obtained from SSF of SPWS with 10% WIS. In the same way yields obtained in SSF of SPWS with 8.8% WIS should be compared with the results from SSF of SPWS with 7.5% WIS with the addition of SWM, where the latter case, as expected shows slight improvement in ethanol yield. Based on the ethanol yields from SSF, the results obtained suggest that the addition of SWM may be a better option for integration purposes as it is a liquid and there is no WIS, as in LWM, which would increase the WIS content, but remaining unused.

Significant differences in the initial ethanol productivity were observed between SSF of pure SPWS and SSF of mixtures containing various proportions of the various starch-derived streams, i.e., PWM (Paper I), LWM (Paper II) or SWM (Paper V). The initial ethanol production rates were always higher when starch-based material was added to SPWS (Table 5.1). (The initial rates were calculated during the first two hours of SSF, when glucose was still detected in the pure SPWS medium in all cases, indicating that enzymatic hydrolysis was not the rate-limiting step.) The increase in ethanol productivity could be explained not only by the decrease in inhibitor concentration (Paper I), but also by the presence of higher amounts of readily available glucose, which was probably the reason for the increase in initial ethanol productivity in the studies described in Papers II and V (see Table 5.1). In these cases, the inhibitor concentrations were identical in the experiments with and without the addition of LWM or SWM.

Table 5.1 Ethanol and glycerol concentrations and ethanol yields after 120 hours of SSF of SPWS with the addition of LWM (Paper II) or SWM (Paper V). The values for experiments with mixed substrates are mean values of duplicate experiments.

SPWS	Material added	EtOH	Glycerol	EtOH prod.[a]	EtOH yield[b]
% (WIS)		g/L	g/L	g/L h	%
7.5	-	26.8	3.7	1.5	93
7.5	LWM	43.4	4.8	2.3	91
10.0	-	34.6	4.3	1.8	88
10.0	LWM	52.8	5.6	2.6	82
7.5	-	24.9	1.9	0.7	91
7.5	SWM	59.5	3.8	0.9	95
8.8	-	26.3	1.2	0.3	79
8.8	SWM	59.7	5.6	0.6	90

[a]Calculated from data for the first two hours of SSF.

[b]Based on stoichiometric conversion (0.51 g/g) of the total amount of glucose in SSF.

The ethanol concentration increased with increasing glucose content in the SSF experiments (Figure 5.3). The highest ethanol concentration obtained was 57 g/L, when SSF was performed with a mixture of 2.5% WIS SPWS and 2.5% WIS PWM (Paper I). The ethanol concentration was also high (53 g/L) when SPWS with 10% WIS was mixed with LWM (Paper II), due to the higher glucose potential of SPWS than LWM. Increasing the proportion of starch-based material in SSF with SPWS at a high WIS value had considerable potential to further increase the ethanol concentration, as demonstrated in the study presented in Paper V, where SWM was added to SSF of SPWS. In that study, the amount of starch- and cellulose-derived material in the mixture was based on equal amounts of raw materials, which resulted in a higher glucose potential of the starch-based materials. SSF was performed at 8.8% WIS with the addition of SWM. A final average ethanol concentration of about 60 g/L was achieved, corresponding to an ethanol yield of about 90%. Ethanol concentrations of about 6% (w/v) are in the range that would allow energy-efficient distillation, thus contributing to a more economically feasible biomass-to-ethanol process.

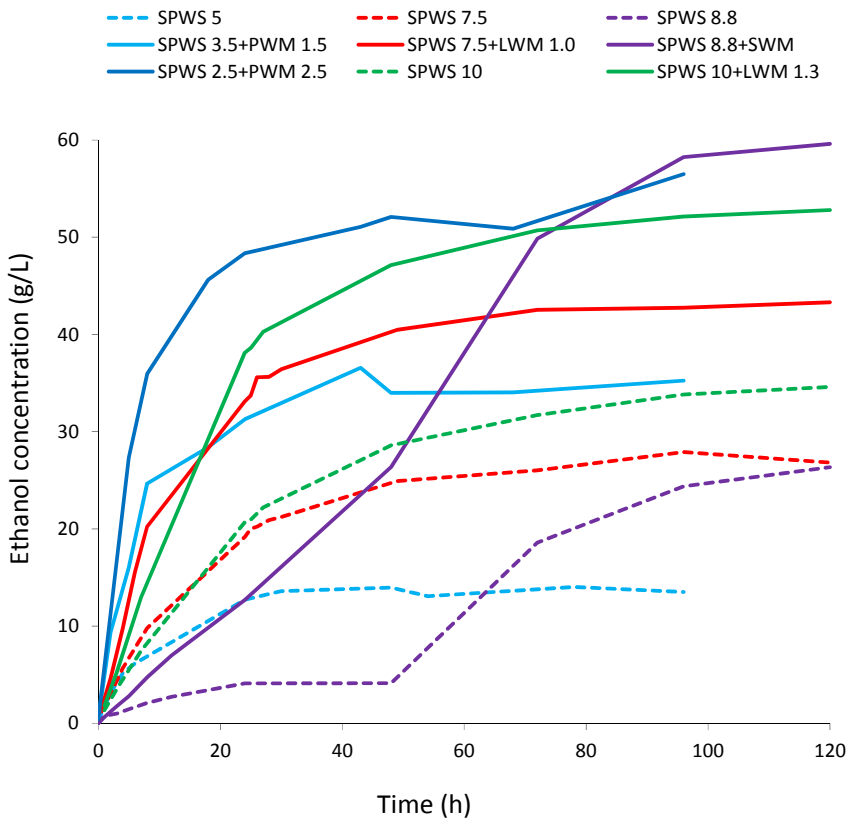

Figure 5.3 Ethanol concentration as a function of time during SSF of steam pretreated wheat straw (SPWS), mixtures of SPWS and pre-saccharified wheat meal (PWM) and mixtures of SPWS and liquefied wheat meal (LWM). Numbers are the % WIS. (Adapted from Papers I, II and V.)

5.2.2 FWM addition

One option for the integration of 1G and 2G ethanol production processes is to use fermented wheat meal (FWM), which already contains a high concentration of ethanol, for dilution of the lignocellulosic stream in SSF, in order to increase the ethanol concentration in the broth. FWM addition in batch and fed-batch modes was investigated in the study presented in Paper V.

SSF was first studied in batch mode. SPWS was diluted with FWM to a WIS content of 7.5%. The initial ethanol concentration, 33.6 g/L, increased to only about 40 g/L resulting in a very low ethanol yield from SSF, on average 27% of the theoretical, based on the glucose available in the SPWS (Figure 5.4). Glucose accumulation to 54% of that theoretically available indicates strong inhibition of the yeast. This was probably the result of the addition of ethanol and other fermentation products with FWM to batch SSF, since baker's yeast could ferment most of the glucose in the same medium diluted with water, see Figure 5.4. Ethanol inhibition must be greater when ethanol is added initially, since baker's yeast could perform fermentation in media with up to 60 g ethanol/L in other cases in this study. Ethanol concentrations reported to have metabolic effects (Hallsworth 1998; Piper 1995) or to cause complete inhibition of growth (Casey and Ingledew 1986) are considerably higher than the initial concentration in these experiments. This indicates that ethanol is probably not solely responsible for the inhibition. Although FWM contained only small amounts of lactic acid and acetic acid, apart from ethanol, other weak organic acids may be present in the broth, but these were not determined. Furthermore, as discussed previously, pretreated wheat straw contains several compounds that are inhibitory to the yeast. The combined synergistic effect of all the inhibitors present can explain the severe toxicity when FWM is added to SSF. The interaction between ethanol and acetic acid has been reported to cause stronger inhibition than each compounds separately due to the synergistic effects (Pampulha and Loureiro 1989). The sensitivity of the yeast to ethanol has been shown to differ in different phases of growth (Casey and Ingledew 1986; Day et al. 1975). Although cells are unlikely to grow in lignocellulose hydrolysate (Palmqvist and Hahn-Hägerdal 2000b); cells that are adapted to the environment may be less sensitive to higher ethanol concentrations.

To avoid the combined effects of toxicity during the critical first two hours, FWM addition at a later stage of SSF was considered, i.e., after 24 hours. Despite the higher WIS content at the beginning of SSF (11.7%), fermentation started with a high initial productivity, 1.6 g/L·h, and most of the glucose released during this time had been fermented to ethanol by the time feeding started. However, the accumulation of glucose released during the fed-batch phase indicates that the addition of FWM caused immediate inhibition, even in this late phase of SSF. The increase in ethanol concentration seen in Figure 5.4 is attributed solely to the ethanol addition and an ethanol concentration of over 50 g/L could be reached. The fermentation ceased after

24 hours; either due to the additional toxic effect of the added ethanol, or because cell death had already occurred before the addition of FWM. Since the addition of FWM was found not to be beneficial in the SSF process, it was concluded that this scenario was not feasible. If the liquid from fermented wheat meal is to be mixed with the fermented wheat straw, it should be done after fermentation, i.e. before the distillation step. However, this will require that the SSF of wheat straw is performed at a high WIS content in order to achieve a high final ethanol concentration in the mixed feed for distillation.

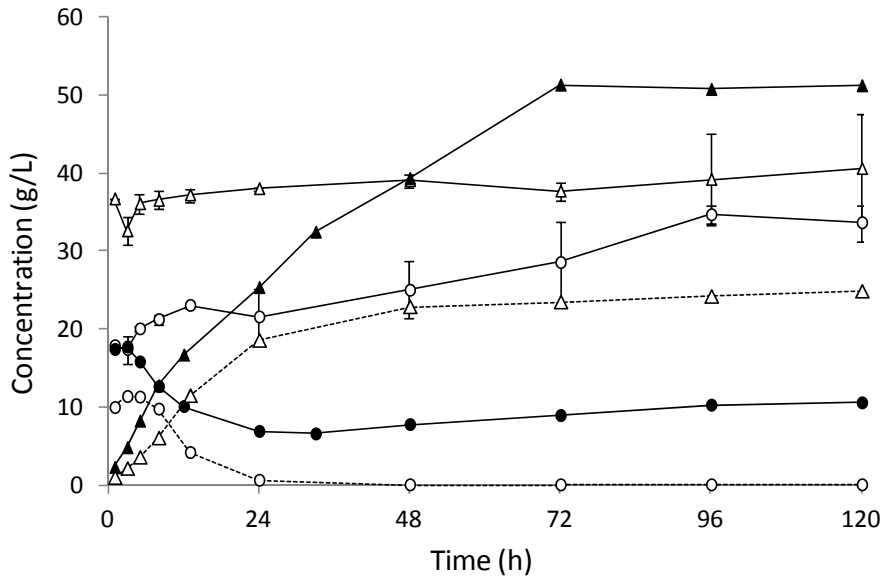

Figure 5.4 Ethanol (triangles) and glucose (circles) concentration during batch (empty) and fed-batch (filled) addition of FWM (solid lines). The reference experiment without the addition of LWM is indicated by the dashed line. Batch experiments were performed in duplicate. Error bars show standard deviation of the mean values. Feeding of FWM in the fed-batch experiment was performed between 24 and 96 hours.

6 Glucose and xylose co-fermentation

High ethanol yield was described in the previous chapter as one of the most important factors in achieving an economically viable ethanol production process. To obtain a high ethanol yield, most of the biomass must be converted to fermentable sugars. Agricultural residues contain a large amount of xylose in the hemicellulose fraction. The main sugar constituents of wheat straw are glucose and xylose, approximately 40% and 20%, respectively (see Table 3.1). Utilization of the xylose would, therefore, significantly increase the ethanol yield of the process. The cellulosic ethanol process could be substantially simplified if a single microorganism could efficiently convert both glucose and xylose to ethanol under industrial conditions. This has driven research on the genetic engineering of *S. cerevisiae*, the most robust glucose-fermenting strain, to provide a strain that can also utilize pentoses (Hahn-Hägerdal et al. 2007; Jeffries 2006; Kim et al. 2013). Several industrial strains of *S. cerevisiae* are available today that can ferment both glucose and xylose in lignocellulosic hydrolysates (Almeida et al. 2011).

6.1 Xylose fermentation by engineered S. cerevisiae

Two heterologous xylose-assimilating pathways are currently being used for the genetic engineering of *S. cerevisiae* (Figure 6.1): the xylose isomerase (XI) pathway (Karhumaa et al. 2007b; Kuyper et al. 2005) and the xylose reductase (XR) and xylitol dehydrogenase (XDH) pathway (Hahn-Hägerdal et al. 2007; Kötter et al. 1990). Xylulokinase (XK) must be overexpressed in both pathways to connect xylulose to the pentose phosphate pathway of *S. cerevisiae* (Eliasson et al. 2000; Jin et al. 2005; Wahlbom et al. 2003a) by xylulose-5-phosphate (Figure 6.1), and to ensure substantial xylose fermentation. When heterologous XI is introduced into yeast, xylose can be converted to xylulose in one step, although it has proven difficult to obtain a high activity of XI in *S. cerevisiae*. In xylose-utilizing strains, where *Pichia stipitis* genes encoding XR and XDH have been expressed in yeast, the initial step in xylose metabolism is the reduction of xylose to xylitol by XR, whereupon xylitol is

oxidized by XDH to xylulose. Xylitol is a by-product generally accumulated under anaerobic conditions when using *S. cerevisiae* strains harbouring the XR/XDH/XK pathways due to different co-factor preferences of XR and XDH. XR requires both NADH or NADPH as a co-factor, that prevents complete regeneration of NAD^+ that is needed for the XDH reaction (Kötter and Ciriacy 1993). NAD^+ has been shown to be regenerated by glycerol formation (Jeppsson et al. 2003).

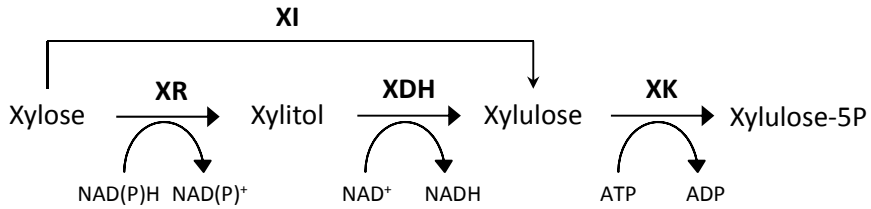

Figure 6.1 Two xylose-assimilating pathways: xylose isomerase (XI) and xylose reductase/xylitol dehydrogenase (XR/XDH). Xylulokinase (XK) produces xylulose-5P, which can enter the pentose phosphate pathway.

Transport proteins are needed to transport both xylose and glucose in yeast. It has been found that in *S. cerevisiae* xylose is transported by glucose transporters (Kilian and Uden 1988; Meinander and Hahn-Hägerdal 1997), which have approximately a 200-fold lower affinity for xylose than for glucose (Kötter and Ciriacy 1993). Since xylose transport into the cell is inhibited by glucose, the glucose concentration must be kept low for the successful fermentation of xylose (Meinander et al. 1999; Pitkänen et al. 2003). This can be attributed to the induction of transport systems (Bertilsson et al. 2008; Meinander et al. 1999; Pitkänen et al. 2003), the induction of glycolytic enzymes (Boles et al. 1996) and improved co-factor generation (Pitkänen et al. 2003).

TMB3400 (Wahlbom et al. 2003b), is an industrial *S. cerevisiae* strain containing genes that encode for XR/XDH/XK, and has been used in the work presented in this thesis (Papers II-IV). It has been shown to be able to co-ferment xylose and glucose in lignocellulosic hydrolysate (Hahn-Hägerdal et al. 2007; Sonderegger et al. 2004). As a low glucose concentration is required, a slow release of glucose is beneficial for efficient xylose fermentation using TMB3400. SSCF has therefore become an interesting process option since glucose is released from the WIS during hydrolysis. SSCF using TMB3400 has been thoroughly investigated on various steam-pretreated raw materials, for example, sugarcane bagasse (Rudolf et al. 2008), corn stover

(Ohgren et al. 2006a), spruce (Bertilsson et al. 2009) and wheat straw (Olofsson et al. 2008b). Although sugars are continuously released during SSCF, several ways of controlling the release of glucose from the solid fraction into the broth have recently been studied. Xylose utilization has been shown to be improved by pre-fermentation, during which much of the glucose is consumed (Bertilsson et al. 2009), or by controlled feeding of cellulases (Olofsson et al. 2010b), as well as by enzyme feeding strategies combined with fed-batch fermentation (Olofsson et al. 2010a) in SSCF. Another alternative, to ensure a sufficiently high level of glucose in the broth even after the cellulose is fully degraded, studied in the present work was the addition of liquefied or saccharified starch-based material to the lignocellulosic substrate to induce xylose uptake.

6.2 Integrated processes to facilitate xylose utilization

Various process configurations were investigated to study the effects of process integration on xylose utilization. The addition of LWM or SWM to SSCF of pretreated wheat straw is described in Papers II and V, respectively, while the co-fermentation of glucose and xylose in SHCF with the addition of wheat starch hydrolysate is presented in Paper III.

6.2.1 SSCF

The effect of adding LWM to SSCF of pretreated straw using *S. cerevisiae* TMB3400 was studied in order to investigate whether increasing the amount of glucose released from lignocellulose polymers could facilitate xylose uptake (Paper II). LWM, which contains mostly glucose oligomers and only a small amount of monomer glucose, was added to SSCF of SPWS (Figure 5.1). LWM was added at the beginning of batch mode SSCF together with cellulases (Celluclast 1.5L) supplemented with β-glucosidase (Novozym 188), and an amyloglucosidase (AMG) preparation (Spirizyme Fuel, Novozymes A/S, Denmark). In another experiment, the AMG preparation was added to SSCF after 24 hours to delay the release of glucose from the starch. However, in experiments performed on SPWS with either 7.5% or 10% WIS, rapid release of all the glucose from the starch was observed within minutes of starting SSCF, even when AMG was added after 24 hours, see Figure 6.2.

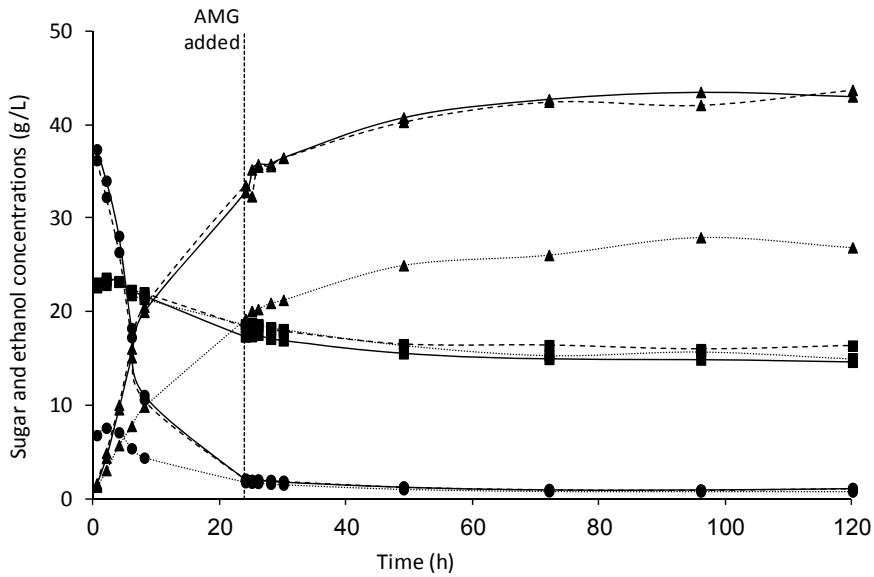

Figure 6.2 Ethanol (triangles), glucose (circles) and xylose (squares) concentrations during batch mode SSCF of SPWS (7.5% WIS) without LWM (reference; dotted lines) and with LWM (1% WIS). Amyloglucosidase (AMG) was added at the start of SSCF (dashed lines) or after 24 hours (solid lines). (Adapted from Paper II.)

To investigate the cause of the undesired release of glucose, separate hydrolysis of LWM was performed with each enzyme preparation, including new cellulases released by the manufacturer (Novozymes A/S). Figure 6.3 shows the glucose concentrations during the course of hydrolysis of LWM, showing clearly that the enzyme preparation Novozym 188 has a very high AMG activity, especially at the loading used for lignocellulosic material. Although Novozym 188 is mainly used for its β-glucosidase activity, this enzyme preparation contains large amounts of AMG and α-amylase proteins produced by *Aspergillus niger* (Banerjee et al. 2010). Oberoi et al. have also reported high AMG activity of Novozym 188 (Oberoi et al. 2010). This may be beneficial when only glucose fermentation occurs (as there is no need for an additional AMG preparation), but this enzyme preparation is unsuitable for this integration scenario and mode of operation. The use of controlled feeding of enzymes may be one possibility, as the method has been used successfully in other studies on SSCF of lignocellulosic material. However, it will probably be difficult to control the glucose release from both lignocellulose and starch-based material at the same time, as

the activity of the enzymes differ depending on the material, which may not be suitable on a larger scale. Therefore, a better solution is to use enzyme mixtures with low AMG activity for cellulase degradation.

Newly developed cellulase enzyme preparations were also investigated. Cellic Ctec and Htec showed no AMG activity, while Cellic Ctec2 released about 16 g glucose/L during a period of 24 hours (less than 1 g/L·h). These enzyme preparations were therefore deemed suitable for integrated configurations such as SSCF, when the slow release of glucose is required.

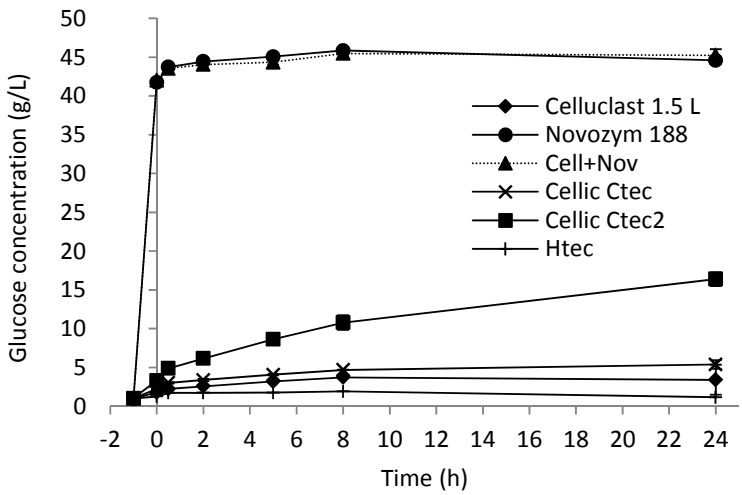

Figure 6.3 Glucose concentration during enzymatic hydrolysis of LWM using different enzyme preparations. (Adapted from Paper II.)

In order to obtain information on the glucose release profile from LWM by the action of the AMG enzyme preparation Spirizyme Fuel, separate enzymatic hydrolysis of LWM was performed in the same way as described in Paper II, using Spirizyme Fuel at different dilutions. Spirizyme Fuel was dosed as recommended by the producer, 0.5 mL/kg DM wheat meal. The enzyme preparation was then diluted by factors of 5, 10, 20 and 50, and the same volumes of these solutions were added to the hydrolysis step. The concentration of glucose during 72 hours of enzymatic hydrolysis and the glucose productivity, calculated from the data obtained, are shown in Figure 6.4. At the recommended dose of Spirizyme Fuel, hydrolysis was completed

in 48 hours, resulting in the release of 93% of the theoretically available glucose (data not shown). The initial glucose productivity was 8 g/L·h, which decreased to less than 2 g/L·h after the first hour.

To obtain efficient glucose and xylose co-fermentation with *S. cerevisiae* TMB3400, slow glucose release in the broth is preferable, as discussed above. Attempts have been made in several studies to determine the appropriate "low, but non-zero" level of glucose. Modelling of simultaneous glucose and xylose uptake has shown that a glucose feed rate between 5 and 10 g/L·h would be suitable to obtain the maximal xylose uptake rate with a yeast cell concentration of 5 g/L (Bertilsson et al. 2008), while Olofsson et al. reported an 80% xylose uptake in SSCF of lignocellulosic substrate using controlled feeding of cellulases giving a glucose release rate of 2 g/L at a yeast concentration of 4 g/L (Olofsson et al. 2010b). Therefore, it was decided to reduce the amount of AMG to half of the recommended dose, with the aim of obtaining slower glucose release from LWM, but at the same time ensuring that most of the glucose was released by the end of SSCF.

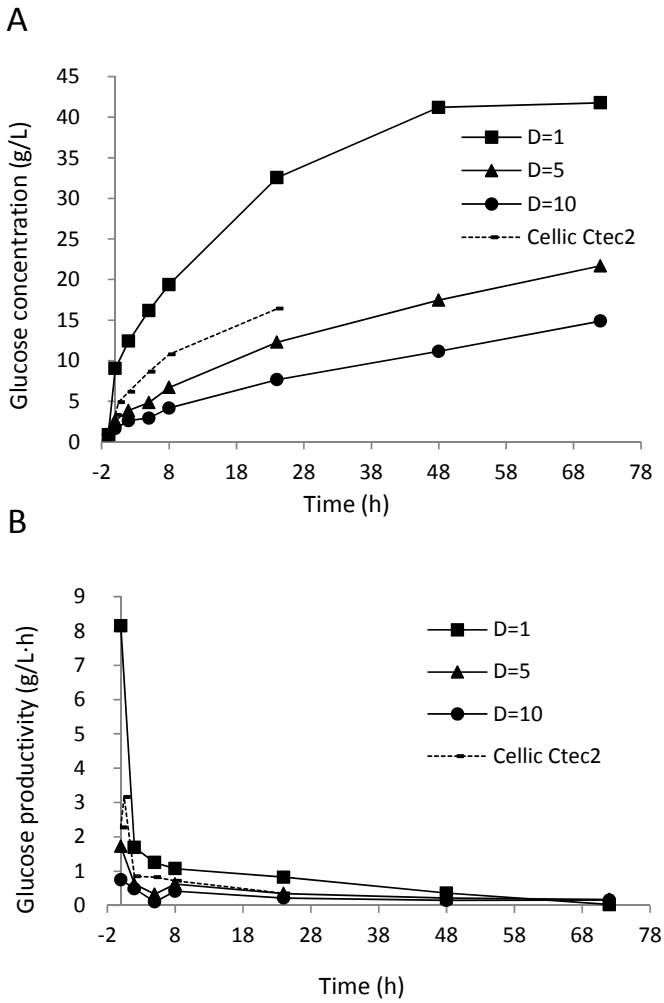

Figure 6.4 Glucose concentration (A) and productivity (B) during separate hydrolysis of LWM with different dilutions (D) of the AMG enzyme preparation, Spirizyme Fuel. Glucose productivity from LWM using Cellic Ctec2 is also shown for comparison.

SSCF of SPWS at 7.5% WIS was performed (with and without the addition of LWM) using the cellulase enzyme preparation, Cellic Ctec2 to release glucose from the cellulose and Spirizyme Fuel to release glucose from LWM. Considering the

rather long hydrolysis time of the pretreated wheat straw, about 24 hours, LWM was added to SSCF after 24 hours. Because of the additional AMG activity of Cellic Ctec2 on LWM, AMG was added 4 hours after LWM addition to release the glucose from LWM and to ensure glucose was released from the LWM present in the broth after the glucose from the cellulose had been released and consumed (see Figure 6.5). A lower enzyme loading of cellulases was applied to decrease the hydrolysis rate of cellulose in the WIS of SPWS. The SSCF reaction time was extended to 144 hours.

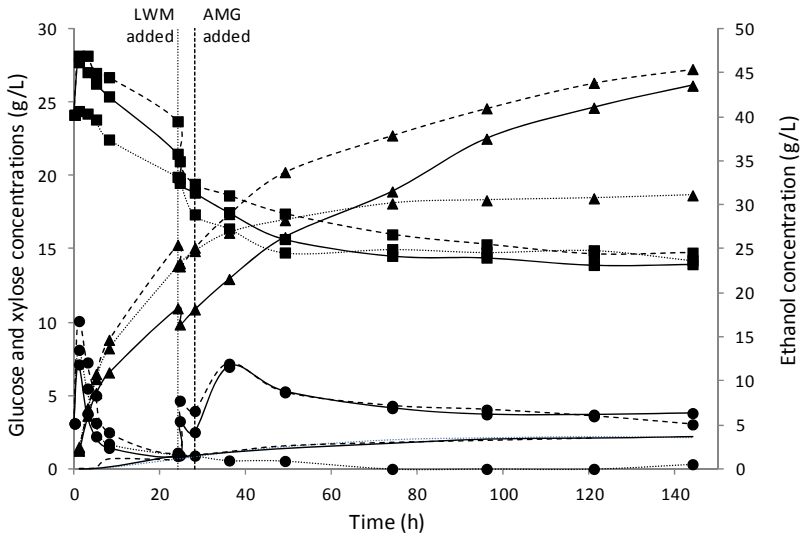

Figure 6.5 Glucose (circles), xylose (squares), ethanol (triangles) and xylitol (no symbol) concentrations during SSCF of SPWS with 7.5% WIS without LWM (reference; dotted lines) and with LWM (1% WIS) addition using cellulase at a loading of 20 (dashed lines) or 10 (solid lines) FPU/g cellulose. LWM and AMG were added 24 and 28 hours after the start of SSCF, respectively.

Although additional glucose was provided at a more constant concentration by the addition of LWM, no improvement in xylose utilization was observed, even when most of the glucose had been released from the cellulose. The imbalance between glucose and xylose concentration in the broth, which led to preferential utilization of glucose by TMB3400, probably explains the results. In the reference experiment without LWM addition, no glucose was available after 48 hours, which might have led to no more xylose being taken up after this time, which has also been reported previously by others (Ohgren et al. 2006a; Rudolf et al. 2008). When LWM was

added, the glucose concentration oscillated around 5 g/L, resulting in about 50% xylose being consumed, based on the total amount of xylose available. Although the experiment with the lower cellulase loading (10 FPU/g cellulose) showed lower ethanol productivity, the final concentration of ethanol was 43.6 g/L, which is almost as high as that achieved with a cellulase loading of 20 FPU/g cellulose, 45.4 g/L. Despite the higher ethanol concentration due to the addition of LWM, no increase in the ethanol yield was obtained; the maximal ethanol yield achieved was about 70%, based on all the available glucose and xylose.

The addition of SWM to SSCF of SPWS was investigated to establish whether this allowed well controlled glucose feeding. The glucose-rich SWM is easily fed by pumping (see Section 6.3). A mutated strain of TMB3400, *S. cerevisiae* KE6-12 (Albers et al. Manuscript in prep.), that harbours the same xylose utilization features as TMB3400, was used to investigate whether xylose uptake could be facilitated by supplying the broth with glucose at a low feeding rate in the manner described above.

Better xylose utilization was achieved with fed-batch addition of SWM (feeding from 48 to 96 hours), when the glucose had already been metabolized, than in the batch-wise experiments performed in the same study (Paper V). Very little xylose was taken up until the glucose had been depleted. After 48 hours, the low glucose concentration led to xylose uptake, and 31% of the total amount of xylose was utilized. Although this uptake is higher than that achieved with batch addition of SWM (see Figure 6.6), the xylose utilization was no better than in previous SSCF experiments with batch LWM addition (Paper II) or delayed LWM and AMG addition, where 54% of the xylose was utilized. The reason for this is probably related to the process design, which resulted in differences in inhibitor concentrations during the first 24-48 hours. The proportions of SPWS and SWM used in the study described in Paper V were based on equal amounts of straw and starch, while in the experiments involving the addition of LWM a smaller amount of the starch-based material was added. The WIS content of the pretreated material determines how much extra liquid can be added to the process. In the study with LWM addition, water was used for dilution to adjust the WIS content in SSCF to 7.5%, and was added at the beginning of SSCF. On the other hand, before adding SWM, SSCF had to be operated at a higher WIS loading of SPWS (around 11%), since the mixture of SWM and the liquid used to wash the solid residue of the starch material was used for feeding (Paper V). Due to no dilution of the SPWS at the beginning of SSCF, the yeast had to cope with higher inhibitor concentrations. This resulted in significant glucose accumulation, up to 20 g glucose/L over 24 hours, due to the longer lag phase, which was inhibitory for xylose uptake, at least during the first 48 hours.

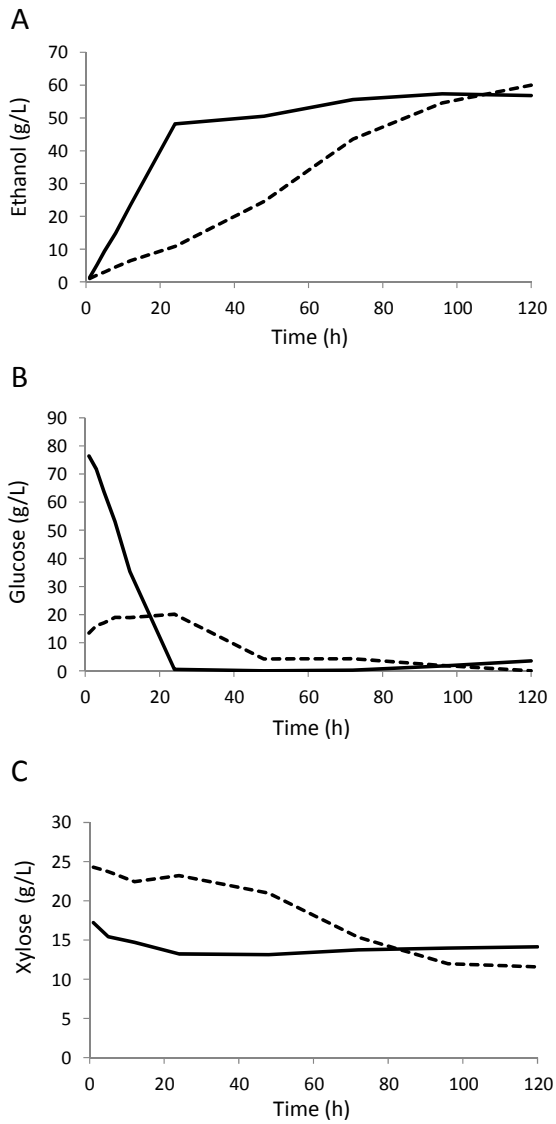

Figure 6.6 Measured concentrations of ethanol (A), glucose (B) and xylose (C) during SSCF of SPWS with batch (solid lines) or fed-batch (dashed lines) addition of SWM.

Alternatively, part of the mixture of the SWM and the washing liquid could have been used at an early stage to dilute SPWS. However, this would have led to a higher initial glucose concentration, but lower concentrations of inhibitors. The amount of SWM used in the feed can also be reduced, leaving more space for dilution with water, which would certainly result in a lower ethanol concentration. Furthermore, fed-batch addition of the lignocellulosic substrate could provide a suitable means of maintaining a low inhibitor concentration and of controlling the glucose concentration during the first 48 hours. Substrate feeding has been successfully applied in other studies (Bertilsson et al. 2009; Olofsson et al. 2010a). Better control of the xylose-to-glucose ratio may be an interesting option in combination with the addition of SWM as a source of glucose. Difficulties in glucose control in SSCF require other integration scenarios, such as SH(C)F as a potential method for glucose and xylose co-fermentation.

6.2.2 SHCF

The integrated process scenario using SHF in the lignocellulose-to-ethanol process, and its advantages and disadvantages have been discussed earlier in this thesis, but the effects of applications on glucose and xylose co-fermentation in SHCF will be discussed below. The high initial glucose concentration may be unfavourable when glucose and xylose are to be fermented together. However, a high initial glucose concentration is only a problem when SHCF is performed in batch fermentation, i.e., enzymatic hydrolysis of the whole pretreated slurry followed by fermentation of the liquid. A small glucose supply would be needed when the glucose is depleted to facilitate xylose uptake, as previously demonstrated (Olofsson et al. 2010b). SWM (called wheat hydrolysate in Paper III) is a glucose-rich solution derived from the starch-to-ethanol process that could serve as a potential feed in the lignocellulosic process. In this way, integration would not only facilitate xylose conversion, but would also increase the final ethanol concentration in the process.

Other SHCF process configurations are possible, such as separate hydrolysis of only the solid fraction after pretreatment, which can provide a glucose-rich solution that may then be fed back into the process. In this case, enzymatic hydrolysis must be performed at a high WIS content as dilution may result in a lower yield.

Two different process alternatives for SHCF, combined with SWM feed, were investigated (Paper III), representing the above mentioned scenarios, as a means of improving the co-fermentation of glucose and xylose. In the first configuration (Configuration 1) enzymatic hydrolysis of SPWS at a WIS content of 7.5% was performed, followed by separation of the solid and liquid fractions. The liquid fraction was then fermented in batch mode until the glucose had been consumed. SWM was then fed into the system for 75 hours, based on results obtained previously

with a glucose solution feed (Paper III). The second configuration (Configuration 2) involved pressing SPWS to a high DM content, which allowed enzymatic hydrolysis to be performed with an 18.5% WIS. The solid residue after hydrolysis was separated from the liquid, and the latter was mixed with the SWM (mixed hydrolysates) and used as feed for fermentation. Feeding of the mixed hydrolysates started after one hour of batch fermentation of the liquid fraction of SPWS, and lasted for 48 hours. The effect of higher yeast loading (20 g/L) was investigated and compared to a loading of 5 g/L since SHCF offers the possibility of yeast recycling. High cell density fermentation has been shown to increase the volumetric ethanol productivity (Thomas and Ingledew 1990) (g/L·h), which may reduce the investment costs. However, it also increases the cost of cell mass production, which is a function of the price of the added nutrients (Hahn-Hägerdal et al. 2007). The amount of nutrients that must be added can be reduced if wheat-derived hydrolysate is used as a feed for glucose supply, as discussed in Chapter 1.

The main subject of this work, presented in Paper III was to investigate how xylose uptake is affected by the feeding strategies in the two integrated scenarios of SHCF dealing with the different sugar profiles. The SPWS hydrolysate used in Configuration 1 contained both glucose and xylose sugars at high concentrations already at the beginning of fermentation. Feeding SWM provided a further supply of glucose during the fed-batch period. In Configuration 2, the liquid fraction of SPWS contained mainly xylose, and some glucose, while the feed of mixed hydrolysates contained a high amount of glucose and some xylose.

The high initial glucose concentration in Configuration 1 initially inhibited xylose uptake, but did not influence the final result. The xylose was taken up completely within 48 hours, resulting in an ethanol concentration of about 43 g/L, when using a yeast concentration of 5 g/L (Figure 6.7A). This corresponds to an average final ethanol yield of 95% of the theoretical, based on the available glucose and xylose. The low feeding rate of glucose, 0.8 g/L·h, was shown to be suitable for inducing xylose uptake, however, 20% of the xylose was used to produce xylitol.

In Configuration 2, xylose was initially taken up much faster, probably due to the very low initial glucose concentration, see Figure 6.7B. However, the rate of uptake decreased when feeding of the mixed hydrolysates started, as this also contained some xylose. The xylose uptake during feeding varied between only 58% and 76% with a yeast load of 5 g/L, which corresponds to ethanol yields of about 83% and 87%, probably due to the higher glucose feed rate, 4.4 g/L. Xylitol formation was also very low in relation to the xylose uptake (< 10%). The xylose uptake of TMB3400 has previously been investigated at different glucose feeding rates. Lower feed rates of glucose, 0.21 g/L·h and 0.45 g/L·h, on barley hydrolysate resulted in only 74% and 51% consumption of the total available xylose (Linde 2007). A glucose feeding rate of around 2 g/L·h has been shown to be most suitable for xylose-fermenting *S. cerevisiae*

(Krahulec et al. 2010; Olofsson et al. 2010b). Increasing the yeast concentration in Configuration 2 improved the xylose utilization, which increased to 90%, but did not improve the ethanol yield, which was around 85% of the theoretical (based on available glucose and xylose), probably due to the simultaneous increase in xylitol and glycerol (Paper III).

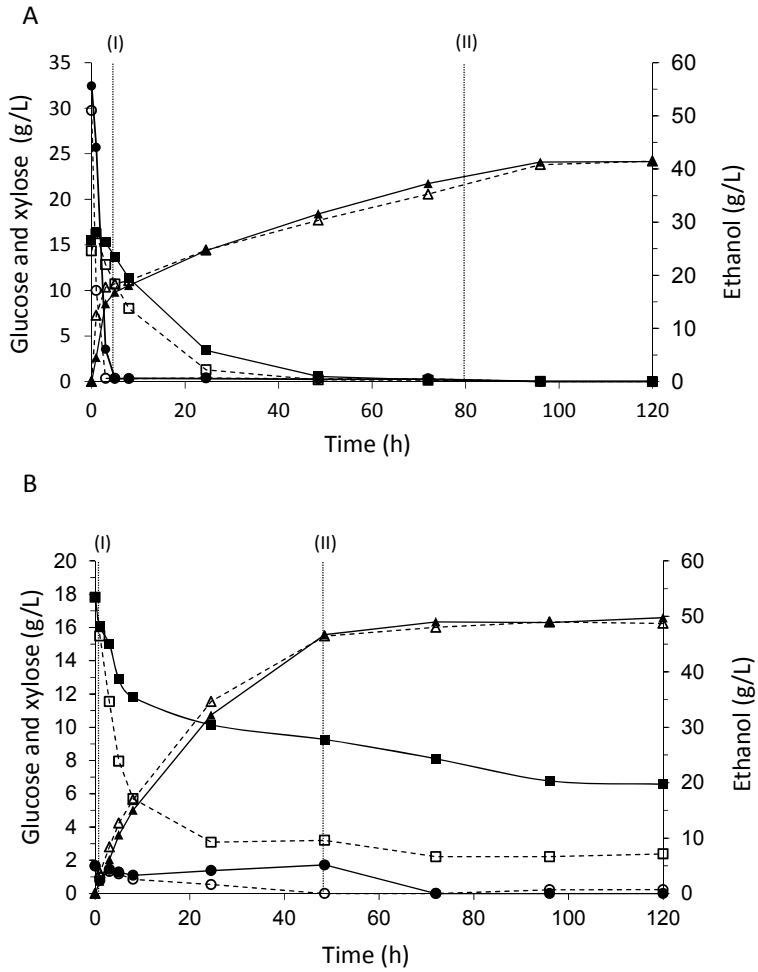

Figure 6.7 Concentrations of glucose (circles), xylose (squares) and ethanol (triangles) measured during SHCF with Configuration 1 (A) and Configuration 2 (B), using a yeast load of 5 (solid lines) and 20 g/L (dashed lines) (for details see text). SWM or the hydrolysate mixtures were fed between (I) and (II) in the two configurations. (Adapted from Paper III.)

The incomplete xylose uptake in Configuration 2 may also be due to higher amounts of inhibitors, mostly during the first part of fermentation, as the medium used for dilution was fed into the fermentor at a later time. The effect of inhibitors on the fermentation of xylose-fermenting *S. cerevisiae* is discussed in detail in Section 6.3. The fact that it was possible to obtain efficient xylose utilization in SHF, but not in SSCF (Papers II and V), at the same WIS content may suggest that removing the solid particles may have a positive effect on fermentation.

SHCF as a possible alternative for integrated process configuration resulted in efficient xylose utilization and a high ethanol yield. Configuration 2 showed a higher initial xylose uptake, but traditional hydrolysis and fermentation (Configuration 1) was more successful, resulting in more efficient xylose uptake and a higher ethanol yield, based on all the sugars added, despite the high initial glucose concentration. This process configuration is also easier to implement due to the simpler process scheme, and this may influence the investment cost.

6.3 Fermentation using mutants of S. cerevisiae TMB3400

S. cerevisiae TMB3400 was used in SSCF (Paper II) and in SHCF (Paper III) due to its proven ability to ferment glucose and xylose (Bertilsson et al. 2009; Ohgren et al. 2006a; Olofsson et al. 2008b; Rudolf et al. 2008). However, further improvement of this strain was necessary to increase its xylose utilization and inhibitor tolerance, and to decrease by-product formation. Two mutated strains, KE6-12 and KE6-13i, derived from TMB3400, exhibiting xylose utilization and inhibitor tolerance, were developed elsewhere (Albers et al. Manuscript in prep.) and their performance was studied in fermentation. Two different process configurations were used to perform batch and fed-batch fermentation at three different levels of inhibitor concentration. Batch fermentation was performed using the liquid pressed from SPWS, while fed-batch fermentation was fed with the hydrolysate obtained from enzymatic hydrolysis of the solid fraction of SPWS.

Inhibitor intolerance is a serious problem in the fermentation of sugars derived from pre-treated lignocellulosic material by microorganisms, as the slurry contains toxic compounds that affect yeast metabolism. The undiluted SPWS liquid contained furfural, HMF and acetic acid at concentrations of 2.8 g/L, 0.3 g/L and 3.6 g/L, respectively (Table 4.1). The tolerance of the strains TMB3400, KE6-12 and KE6-13i was assessed in batch fermentation at different levels of inhibitors, in non-diluted, 1.5 and 2 times diluted SPWS liquid fraction (mainly comprising hemicellulose sugars), while fed-batch fermentation was performed using only 1.5 and 2 times

diluted SPWS liquid. The final inhibitor concentrations in the batch and fed-batch fermentation experiments were the same at each dilution. Figure 6.8 shows how the strains performed in different modes of fermentation at different inhibitor levels.

All strains were affected by the inhibitors in the hydrolysates. In batch fermentation, both the xylose fermentation rate and ethanol yield clearly improved as a result of diluting the hydrolysates (see Table 6.1). Monomeric glucose was rapidly consumed in all cases. TMB3400 could ferment about 53% of the xylose in undiluted SPWS liquid, which increased to 72% and 93% when the inhibitors were diluted 1.5 and 2 times, respectively, which also resulted in increasing ethanol yields. The same strain has also been found to be able to ferment about 70% of the total xylose in the liquid fraction of pretreated sugarcane bagasse, which contained about 1.8 g/L acetic acid and 0.2 g/L furfural (Carrasco et al. 2010), and all the xylose within 30 hours when the medium contained only 10% of the liquid fraction of pretreated spruce (Sonderegger et al. 2004).

KE6-12 performed worse than the original strain, regarding ethanol yield and xylose consumption, while KE6-13i performed better in undiluted hydrolysates. Both the mutated strains were less sensitive at lower inhibitor concentrations, being able to convert over 90% of the xylose. However, since more xylose was converted to xylitol and the glycerol production also increased, poor ethanol yields were achieved with KE6-12, the highest being about 0.25 g/g total sugar. The ethanol yield using KE6-13i was about 0.3 g/g in 2 times diluted liquid. Other evolved strains of TMB3400 have also been investigated using spruce hydrolysate containing different amounts of inhibitors (Koppram et al. 2013). In that study, the specific consumption rate of sugars and the specific ethanol productivity improved, but xylose consumption was not enhanced. In another study, improved strains of TMB3400 obtained by genetic engineering exhibited an improved affinity for xylose, but the ethanol yield remained unchanged (Fonseca et al. 2011).

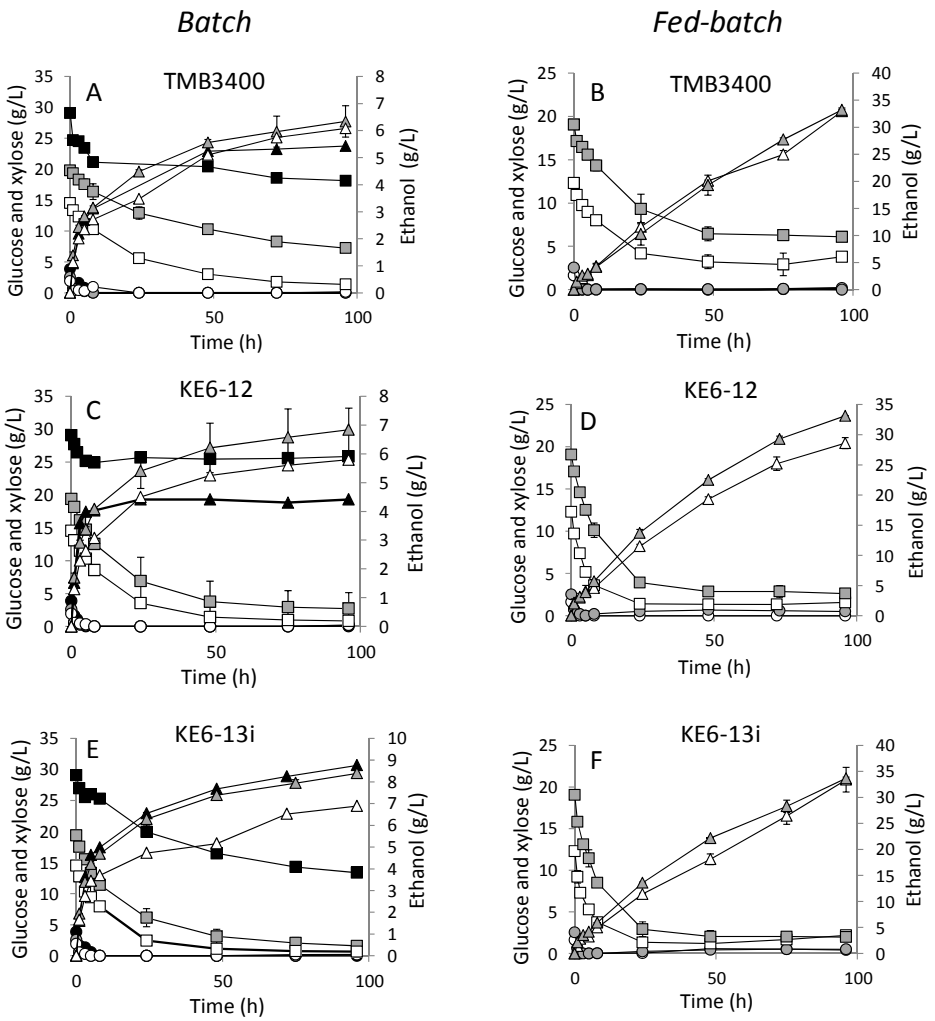

Figure 6.8 Concentrations of glucose (circles), xylose (squares) and ethanol (triangles) during batch (A, C, E) and fed-batch (B, D, F) fermentation of S. cerevisiae TMB3400 KE6-12 and KE6-13i at different dilutions: undiluted (filled), 1.5 times (shaded) and 2 times (empty) diluted SPWS liquids.

As shown in Paper IV, fed-batch fermentation generally resulted in higher ethanol yields, together with higher glycerol and lower xylitol production. Xylitol yields, based on consumed xylose, were reduced to half, probably due to the significant increase in glycerol production, the concentration of which increased by a factor of 2. Similar behaviour has been observed in XR/XDH modified strains in previous studies, where xylitol production was found to be lower (Karhumaa et al. 2007a; Ohgren et al. 2006a). This was attributed to glycerol production which regenerates NAD^+ for the XDH reaction, which in turn balances the redox reactions for the xylose pathway (Jeppsson et al. 2003).

Although more xylose was not consumed, higher xylose uptake rates were observed in fed-batch fermentation (Figure 6.8) at the same inhibitor levels. This is related to the relatively higher amount of yeast loading at the beginning of fermentation, due to a lower starting volume, which is a great advantage of fed-batch operation. Fermentation experiments were performed using a similar process design (Paper III), showing comparable behaviour patterns, and ethanol yields obtained using TMB3400 were in the same range at similar dilutions.

The significantly higher ethanol productivity and ethanol yield exhibited by the mutant strain KE6-13i clearly demonstrates its greater inhibitor tolerance. Xylose consumption was most enhanced in undiluted liquids. KE6-12 could not ferment xylose very well, but in more diluted liquids it showed a somewhat better performance than the original strain.

Table 6.1 Results obtained after 120 hours of fermentation. Mean values of duplicate experiments are given for both dilutions in fed-batch mode and 1.5x dilution in batch mode.

Strain	Mode.	D	Xyl. cons.	Xyl. cons.	Xylitol[b]	Glycerol	EtOH yield
			g/L	%[a]	% (g/L)	g/L	g/g[c]
TMB3400	Batch	Undil.	20.0	53	15 (3.1)	2.0	0.12
		1.5x	18.2	72	22 (3.9)	3.3	0.21
		2x	17.7	93	21 (3.7)	2.4	0.26
	Fed-batch	1.5x	21.6	78	13 (2.9)	8.1	0.44
		2x	18.6	83	16 (3.0)	6.3	0.48
KE6-12	Batch	Undil.	12.3	32	14 (1.8)	1.4	0.10
		1.5x	22.7	90	28 (6.4)	3.5	0.23
		2x	18.2	96	24 (4.4)	2.6	0.25
	Fed-batch	1.5x	25.1	91	13 (1.5)	9.4	0.44
		2x	20.8	93	12 (2.5)	8.4	0.41
KE6-13i	Batch	Undil.	24.7	65	24 (6.0)	1.7	0.19
		1.5x	23.8	94	29 (6.9)	3.3	0.28
		2x	18.4	96	28 (5.2)	2.8	0.30
	Fed-batch	1.5x	25.7	93	14 (3.4)	9.3	0.45
		2x	20.2	90	13 (2.7)	7.0	0.49

D: dilution, Undil: undiluted, Xyl: xylose, EtOH: ethanol

[a]Relative to all xylose added, including monomers and oligomers.

[b]Relative to the amount of xylose consumed.

[c]Based on glucose and xylose added.

7 Conclusions and future outlook

The purpose of this work was to develop integrated processes for wheat straw- and wheat starch-based ethanol production, and to improve the co-fermentation of glucose and xylose from steam-pretreated wheat straw using integrated process configurations.

Several alternatives for integration in upstream processes have been demonstrated, and a better understanding of the effects of mixing has been obtained. Furthermore, valuable information has been obtained on the performance of novel mutants of *S. cerevisiae* TMB3400, regarding their xylose utilization ability and inhibitor tolerance.

The major findings of this work are summarised below.

Integration of the cellulose- and starch-based processes

- Mixed streams of wheat straw and pre-saccharified wheat meal were beneficial for both processes, as SSF of the mixtures showed better utilization of the biomass than with any of the substrates alone. Thus, mixed substrates are favourable in terms of final ethanol yield, probably due to the stress on *S. cerevisiae* caused by weak acids present in SPWS. At the same time, it is also easier to achieve high ethanol concentrations using mixtures than when using only wheat straw as a raw material.
- The addition of saccharified starch-based material to wheat straw improved the ethanol productivity and ethanol yield, making this integration scenario a promising alternative, especially when considering the possibility of separating the solid residue from the wheat meal and the utilization of that fraction as animal feed.
- Neither batch nor fed-batch SSF benefited from the addition of fermented wheat meal due to severe inhibition by ethanol and other inhibitory compounds. It was thus concluded that this scenario is not suitable for integration in SSF.

Glucose and xylose co-fermentation

- Xylose uptake could not be improved by the addition of liquefied wheat meal to SSCF, since the β-glucosidase enzyme preparation used for the cellulose process had considerable side activities that prevented slow glucose release from the starch, which could have facilitated xylose utilization. Although glucose was provided at a more constant level by other enzyme preparations, no improvement in xylose utilization was achieved.
- It has been demonstrated that separate hydrolysis and co-fermentation facilitates xylose utilization when applying feed from the starch stream. Higher ethanol yields were achieved in co-fermentation of glucose and xylose in SPWS hydrolysate when only wheat starch hydrolysate was used as feed, than in co-fermentation of the liquid fraction of SPWS fed with a hydrolysate mixture from wheat meal and wheat straw.
- Differences in the performance of two mutated strains of *S. cerevisiae* TMB3400 have been revealed. It has been shown that KE6-13i was more tolerant to inhibitors, while KE6-12 performed better in less inhibitory environments.

Integration of first-generation and second-generation ethanol production processes has been demonstrated to increase the ethanol concentration. This will result in a reduction in the cost of distillation, thus improving the process economics, while maintaining a high yield.

Large-scale production of second-generation bioethanol has recently started, and an intensive learning period is expected in the near future. A great deal of work clearly remains to be done on both research and development level. To reduce production costs, the integrated scenarios must be optimized by minimizing the amount of enzyme and yeast, while the use of chemicals can be reduced, by utilizing wheat hydrolysate as a nutrient supplement in fermentation. Techno-economic evaluation, based on the data produced in this work, must be performed and the most promising integrated scenarios suggested must be tested on process development unit and pilot scale to verify their applicability in commercial production.

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References

Agroetanol. Lantmännen, Agroetanol. http://agroetanol.se, February 18, **2013**.

Albers E., Halpin R., and Olsson L. **Manuscript in prep.** Evolutionary engineering for development of improved xylose utilization capacity and inhibitor tolerance in an industrial Saccharomyces cerevisiae strain.

Albertyn J., Hohmann S., Thevelein J.M., and Prior B.A. **1994**. GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. *Mol. Cell. Biol.* 14, 4135-4144.

Almeida J.R.M. **2009**. Improving the response of Saccharomyces cerevisiae to lignocellulosic hydrolysate inhibitors in ethanolic fermentation / João Ricardo M. de Almeida. Lund: Division of Applied Microbiology, Lund University, 2009.

Almeida J.R.M., Runquist D., Nogue V.S.I., Liden G., and Gorwa-Grauslund M.F. **2011**. Stress-related challenges in pentose fermentation to ethanol by the yeast Saccharomyces cerevisiae. *Biotechnol. J.* 6, 286-299.

Bacovsky D., Dallos M., and Wörgetter M. **2010**. Status of 2nd generation biofuels demonstration facilities in June 2010. IEA Bioenergy Task 39: Report T39-P1b.

Baffes J., and Haniotis T. **2010**. Placing the 2006/08 Commodity Price Boom into Perspective. http://econ.worldbank.org.

Bai F.W., Anderson W.A., and Moo-Young M. **2008**. Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnol. Adv.* 26, 89-105.

Balat M. **2011**. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. *Energy Conversion and Management* 52, 858-875.

Balat M., Balat H., and Oz C. **2008**. Progress in bioethanol processing. *Progress in Energy and Combustion Science* 34, 551-573.

Ballesteros I., Negro M.J., Oliva J.M., Cabanas A., Manzanares P., and Ballesteros M. **2006**. Ethanol production from steam-explosion pretreated wheat straw. *Appl. Biochem. Biotechnol.* 129-132, 496-508.

Banerjee G., Car S., Scott-Craig J., Borrusch M., and Walton J. **2010**. Rapid optimization of enzyme mixtures for deconstruction of diverse pretreatment/biomass feedstock combinations. *Biotechnol. Biofuels* 3, 22.

Béguin P., and Aubert J.-P. **1994**. The biological degradation of cellulose. *FEMS Microbiology Reviews* 13, 25-58.

Bertilsson M., Andersson J., and Lidén G. **2008**. Modeling simultaneous glucose and xylose uptake in *Saccharomyces cerevisiae* from kinetics and gene expression of sugar transporters. *Bioprocess Biosyst. Eng.* 31, 369-377.

Bertilsson M., Olofsson K., and Lidén G. **2009**. Prefermentation improves xylose utilization in simultaneous saccharification and co-fermentation of pretreated spruce. *Biotechnol. Biofuels* 2, 1-10.

BlueFire. BlueFire Ethanol. http://bfreinc.com/, February 18, **2013**.

Boles E., Muller S., and Zimmermann F. **1996**. A multi-layered sensory system controls yeast glycolytic gene expression. *Mol. Microbiol.* 19, 641 - 642.

Borregaard. http://borregaard.com, February 18, **2013**.

Bothast R.J., and Schlicher M.A. **2005**. Biotechnological processes for conversion of corn into ethanol. *Appl. Microbiol. Biotechnol.* 67, 19-25.

Bowyer J.L., and Stockmann V.E. **2001**. Agricultural Residues. *Forest Products Journal* 51, 10.

Brandberg T., Karimi K., Taherzadeh M.J., Franzen C.J., and Gustafsson L. **2007**. Continuous fermentation of wheat-supplemented lignocellulose hydrolysate with different types of cell retention. *Biotechnol. Bioeng.* 98, 80-90.

Börjesson P. **2009**. Good or bad bioethanol from a greenhouse gas perspective – What determines this? *Applied Energy* 86, 589-594.

Börjesson P., and Tufvesson L.M. **2011**. Agricultural crop-based biofuels – resource efficiency and environmental performance including direct land use changes. *Journal of Cleaner Production* 19, 108-120.

Cardona C.A., and Sánchez Ó.J. **2007**. Fuel ethanol production: Process design trends and integration opportunities. *Bioresour. Technol.* 98, 2415-2457.

Carrasco C., Baudel H.M., Sendelius J., Modig T., Roslander C., Galbe M., Hahn-Hägerdal B., Zacchi G., and Lidén G. **2010**. SO2-catalyzed steam pretreatment and fermentation of enzymatically hydrolyzed sugarcane bagasse. *Enzyme. Microb. Technol.* 46, 64-73.

Carvalheiro F., Duarte L.C., and Girio F.M. **2008**. Hemicellulose biorefineries: a review on biomass pretreatments. *J. Sci. Ind. Res.* 67, 849-864.

Casey G.P., and Ingledew W.M.M. **1986**. Ethanol Tolerance in Yeasts. *Crc Critical Reviews in Microbiology* 13, 219-280.

Chakrabortty A. **2008**. Internal World Bank study- biofuel caused food prices. http://www.guardian.co.uk/environment/2008/jul/03/biofuels.renewableenergy. In: *The Guardian*.

Chang V.S., Burr B., and Holtzapple M.T. **1997**. Lime pretreatment of switchgrass. *Appl. Biochem. Biotechnol.* 63-5, 3-19.

Chiaramonti D., and Recchia L. **2010**. Is life cycle assessment (LCA) a suitable method for quantitative CO2 saving estimations? the impact of field input on the LCA results for a pure vegetable oil chain. *Biomass Bioenerg.* 34, 787-797.

Dahlbacka B. **2009**. Sweden Biofuels Annual Report http://gain.fas.usda.gov/.

Day A., Anderson E., and Martin P.A. Ethanol tolerance of brewing yeast; 1975; Oxford. IRL press.

de Vries S.C., van de Ven G.W.J., van Ittersum M.K., and Giller K.E. **2010**. Resource use efficiency and environmental performance of nine major biofuel crops, processed by first-generation conversion techniques. *Biomass Bioenerg.* 34, 588-601.

Destexhe A., Peckous L., and Picart L. **2004**. Using enzymes in ethanol production. Novozymes A/S.

Domsjö. http://www.domsjo.adityabirla.com/, February 18, **2013**.

Dunlop A. **1948**. Furfural formation and behavior. *Industrial & Engineering Chemistry* 40, 204-209.

Elander R.T., and Putsche V.L. **1996**. Ethanol from corn: technology and economics. In: *Handbook on Bioethanol: Production and Utilization*. Washington: Taylor & Francis. 329-347 p.

Eliasson A., Christensson C., Wahlbom C.F., and Hahn-Hägerdal B. **2000**. Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1 , XYL2 , and XKS1 in mineral medium chemostat cultures. *Appl. Environ. Microbiol.* 66, 3381-3386.

Energimyndigheten. Swedish Energy Agency, 2011: Energy in Sweden.

Entrix C. **2010**. Current state of the U.S. ethanol industry. National Renewable Energy Laboratory.

Esteghlalian A., Hashimoto A.G., Fenske J.J., and Penner M.H. **1997**. Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresour. Technol.* 59, 129-136.

Fazio S., and Monti A. **2011**. Life cycle assessment of different bioenergy production systems including perennial and annual crops. *Biomass Bioenerg.* 35, 4868-4878.

Fengel D., and Wegener G. **1989**. Wood: chemistry, ultrastructure, reactions. Berlin: Walter de Gruyter.

Fonseca C., Olofsson K., Ferreira C., Runquist D., Fonseca L.L., Hahn-Hägerdal B., and Lidén G. **2011**. The glucose/xylose facilitator Gxf1 from Candida intermedia expressed in a xylose-fermenting industrial strain of *Saccharomyces cerevisiae* increases xylose uptake in SSCF of wheat straw. *Enzyme. Microb. Technol.* 48, 518-525.

Galbe M., Sassner P., Wingren A., and Zacchi G. **2007**. Process engineering economics of bioethanol production. In: *Biofuels*. Leipzig: Springer Berlin Heidelberg. 303-327 p.

Galbe M., Wallberg O., and Zacchi G. **2011**. 6.47 - Techno-Economic Aspects of Ethanol Production from Lignocellulosic Agricultural Crops and Residues. In: *Comprehensive Biotechnology (Second Edition)*. Burlington: Academic Press. 615-628 p.

Galbe M., and Zacchi G. **2012**. Pretreatment: The key to efficient utilization of lignocellulosic materials. *Biomass Bioenerg.* 46, 70-78.

Galbe M., and Zacchi G. **2002**. A review of the production of ethanol from softwood. *Appl. Microbiol. Biotechnol.* 59, 618-628.

García-Cubero M.T., González-Benito G., Indacoechea I., Coca M., and Bolado S. **2009**. Effect of ozonolysis pretreatment on enzymatic digestibility of wheat and rye straw. *Bioresour. Technol.* 100, 1608-1613.

Gauss W., Suzuki S., and Takagi M.; Bio Research Center Company Limited, assignee. 1976. Manufacture of alcohol from cellulosic materials using plural ferments. USA patent 3990944.

Geohive. World: production of crops in metric tonnes. http://www.geohive.com/charts/ag_crops.aspx, December 14, **2012**.

Gírio F.M., Fonseca C., Carvalheiro F., Duarte L.C., Marques S., and Bogel-Łukasik R. **2010**. Hemicelluloses for fuel ethanol: A review. *Bioresour. Technol.* 101, 4775-4800.

Govumoni S.P., Koti S., Kothagouni S.Y., Venkateshwar S., and Linga V.R. **2013**. Evaluation of pretreatment methods for enzymatic saccharification of wheat straw for bioethanol production. *Carbohydr. Polym.* 91, 646-650.

Hahn-Hägerdal B., Karhumaa K., Fonseca C., Spencer-Martins I., and Gorwa-Grauslund M.F. **2007**. Towards industrial pentose-fermenting yeast strains. *Appl. Microbiol. Biotechnol.* 74, 937-953.

Hallsworth J.E. **1998**. Ethanol-induced water stress in yeast. *Journal of Fermentation and Bioengineering* 85, 125-137.

Hansen M.A.T., Kristensen J.B., Felby C., and Jørgensen H. **2011**. Pretreatment and enzymatic hydrolysis of wheat straw (Triticum aestivum L.) – The impact of lignin relocation and plant tissues on enzymatic accessibility. *Bioresour. Technol.* 102, 2804-2811.

Holtzapple M.T., Jun J.H., Ashok G., Patibandla S.L., and Dale B.E. **1991**. The ammonia freeze explosion (AFEX) process- a practical lignocellulose pretreatment. *Appl. Biochem. Biotechnol.* 28-9, 59-74.

Hoyer K., Galbe M., and Zacchi G. **2009**. Production of fuel ethanol from softwood by simultaneous saccharification and fermentation at high dry matter content. *J. Chem. Technol. Biotechnol.* 84, 570-577.

IEA. **2012**. 2012: Key world energy statistics. http://www.iea.org/publications/freepublications/publication/name,31287,en.html.

IEA. IEA Bioenergy Task 39, Status of 2nd Generation Biofuels Demonstration Facilities. http://demoplants.bioenergy2020.eu/, February 14, **2013**.

IEA. **2010**. World Energy Outlook. http://www.iea.org/publications/freepublications/publication/name,27324,en.html.

IEA. **2011**. World Energy Outlook, Executive Summary. http://www.iea.org/Textbase/npsum/weo2011sum.pdf.

Ingledew W.M. **1999**. Alcohol production by Saccharomyces cerevisiae: a yeast primer. In: *The alcohol textbook*. Nottingham, United Kingdom: Nottingham University Press. 49-87 p.

IPCC. **2007**. Climate change 2007: Synthesis report. http://www.ipcc.ch/pdf/assessment-report/ar4/syr/ar4_syr.pdf.

Jeffries T. **2006**. Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.* 17, 320 - 326.

Jeppsson M., Träff K., Johansson B., Hahn-Hägerdal B., and Gorwa-Grauslund M.F. **2003**. Effect of enhanced xylose reductase activity on xylose consumption and product

distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 3, 167-175.

Jin Y.S., Alper H., Yang Y.T., and Stephanopoulos G. **2005**. Improvement of xylose uptake and ethanol production in recombinant Saccharomyces cerevisiae through an inverse metabolic engineering approach. *Appl. Environ. Microbiol.* 71, 8249-8256.

Jones A.M., and Ingledew W.M. **1994a**. Fermentation of very high gravity wheat mash prepared using fresh yeast autolysate. *Bioresour. Technol.* 50, 97-101.

Jones A.M., and Ingledew W.M. **1994b**. Fuel alcohol production: Assessment of selected commercial proteases for very high gravity wheat mash fermentation. *Enzyme. Microb. Technol.* 16, 683-687.

Jonsson L., Alriksson B., and Nilvebrant N.-O. **2013**. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels* 6, 16.

Jorgensen H., Vibe-Pedersen J., Larsen J., and Felby C. **2007**. Liquefaction of lignocellulose at high-solids concentrations. *Biotechnol. Bioeng.* 96, 862-870.

Jørgensen H. **2009**. Effect of Nutrients on Fermentation of Pretreated Wheat Straw at very High Dry Matter Content by Saccharomyces cerevisiae. *Appl. Biochem. Biotechnol.* 153, 44-57.

Karhumaa K., Fromanger R., Hahn-Hägerdal B., and Gorwa-Grauslund M. **2007a**. High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant Saccharomyces cerevisiae. *Appl. Microbiol. Biotechnol.* 73, 1039 - 1046.

Karhumaa K., Sanchez R., Hahn-Hägerdal B., and Gorwa-Grauslund M.F. **2007b**. Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microbial Cell Factories* 6, 1-10.

Kelsall D.R., and Lyons T.P. **2003**. Grain dry milling and cooking procedures: extracting sugars in preparation for fermentation. In: *The alcohol textbook*. Nottingham, United Kingdom: Nottingham University Press. 9-21 p.

Kendall A., and Chang B. **2009**. Estimating life cycle greenhouse gas emissions from corn–ethanol: a critical review of current U.S. practices. *Journal of Cleaner Production* 17, 1175-1182.

Kilian S.G., and Uden N. **1988**. Transport of xylose and glucose in the xylose-fermenting yeast *Pichia stipitis*. *Appl. Microbiol. Biotechnol.* 27, 545-548.

Kim S.R., Park Y.-C., Jin Y.-S., and Seo J.-H. **2013**. Strain engineering of Saccharomyces cerevisiae for enhanced xylose metabolism. *Biotechnol. Adv.* http://dx.doi.org/10.1016/j.biotechadv.2013.03.004.

Klinke H., Olsson L., Thomsen A., and Ahring B. **2003**. Potential inhibitors from wet oxidation of wheat straw and their effect on ethanol production of Saccharomyces cerevisiae: wet oxidation and fermentation by yeast. *Biotech Bioeng* 81, 738 - 747.

Koppram R., Nielsen F., Albers E., Lambert A., Wannstrom S., Welin L., Zacchi G., and Olsson L. **2013**. Simultaneous saccharification and co-fermentation for bioethanol production using corncobs at lab, PDU and demo scales. *Biotechnol. Biofuels* 6, 2.

Krahulec S., Petschacher B., Wallner M., Longus K., Klimacek M., and Nidetzky B. **2010**. Fermentation of mixed glucose-xylose substrates by engineered strains of Saccharomyces cerevisiae: role of the coenzyme specificity of xylose reductase, and effect of glucose on xylose utilization. *Microbial Cell Factories* 9, 16.

Kuyper M., Hartog M., Toirkens M., Almering M., Winkler A., van Dijken J., and Pronk J. **2005**. Metabolic engineering of a xylose-isomerase-expressing Saccharomyces cerevisiae strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res.* 5, 399 - 409.

Kötter P., Amore R., Hollenberg C.P., and Ciriacy M. **1990**. Isolation and characterization of the *Pichia stipitis* xylitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Current Genetics* 18, 493-500.

Kötter P., and Ciriacy M. **1993**. Xylose fermentation by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 38, 776-783.

Larsson S., Palmqvist E., Hahn-Hägerdal B., Tengborg C., Stenberg K., Zacchi G., and Nilvebrant N.O. **1999**. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme. Microb. Technol.* 24, 151-159.

Larsson S., Quintana-Sainz A., Reimann A., Nilvebrant N.-O., and Jonsson L. **2000**. Influence of lignocellulose-derived aromatic compounds on oxygen-limited growth and ethanolic fermentation by Saccharomyces cerevisiae. *Appl. Biochem. Biotechnol.* 84, 617 - 632.

Linde M. **2007**. Process development of bioethanol production from wheat and barley residues: Lund University.

Linde M., Galbe M., and Zacchi G. **2007**. Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration. *Enzyme. Microb. Technol.* 40, 1100-1107.

Linde M., Galbe M., and Zacchi G. **2006**. Steam pretreatment of acid-sprayed and acid-soaked barley straw for production of ethanol. *Appl. Biochem. Biotechnol.* 130, 546-562.

Linde M., Jakobsson E.L., Galbe M., and Zacchi G. **2008**. Steam pretreatment of dilute H_2SO_4 -impregnated wheat straw and SSF with low yeast and enzyme loadings for bioethanol production. *Biomass Bioenerg.* 32, 326-332.

Liu C.-F., and Sun R.-C. **2010**. Chapter 5 - Cellulose. In: *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels*. Amsterdam: Elsevier. 131-167 p.

Lynd L.R., Weimer P.J., van Zyl W.H., and Pretorius I.S. **2002**. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506-577.

Lynd L.R., Zyl W.H.v., McBride J.E., and Laser M. **2005**. Consolidated bioprocessing of cellulosic biomass: an update. *Curr. Opin. Biotechnol.* 16, 577-583.

Mabee W.E., and Saddler J.N. **2010**. Bioethanol from lignocellulosics: Status and perspectives in Canada. *Bioresour. Technol.* 101, 4806-4813.

Martin C., Galbe M., Wahlbom C.F., Hahn-Hägerdal B., and Jönsson L.J. **2002**. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilising Saccharomyces cerevisiae. *Enzyme. Microb. Technol.* 31, 274-282.

Meinander N., Boels I., and Hahn-Hägerdal B. **1999**. Fermentation of xylose/glucose mixtures by metabolically engineered Saccharomyces cerevisiae strains expressing XYL1 and XYL2 from Pichia stipitis with and without overexpression of TAL1. *Bioresour. Technol.* 68, 79-87.

Meinander N.Q., and Hahn-Hägerdal B. **1997**. Influence of cosubstrate concentration on xylose conversion by recombinant, XYL1-expressing *Saccharomyces cerevisiae*: a comparison of different sugars and ethanol as cosubstrates. *Appl. Environ. Microbiol.* 63, 1959-1964.

Merali Z., Ho J.D., Collins S.R.A., Gall G.L., Elliston A., Käsper A., and Waldron K.W. **2013**. Characterization of cell wall components of wheat straw following hydrothermal pretreatment and fractionation. *Bioresour. Technol.* 131, 226-234.

Milbrandt A. **2005**. A geographic perspective on the current biomass resource availability in the United States. National Renewable Energy Laboratory, Golden, CO.

Montesinos T., and Navarro J.M. **2000**. Production of alcohol from raw wheat flour by amyloglucosidase and *Saccharomyces cerevisiae Enzyme. Microb. Technol.* 27, 362-370.

Mosier N., Wyman C., Dale B., Elander R., Lee Y.Y., Holtzapple M., and Ladisch M. **2005**. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* 96, 673-686.

Nguyen Q.A., Tucker M.P., Keller F.A., Beaty D.A., Connors K.M., and Eddy F.P. **1999**. Dilute acid hydrolysis of softwoods. *Appl. Biochem. Biotechnol.* 77-9, 133-142.

Nguyen Q.A., Tucker M.P., Keller F.A., and Eddy F.P. **2000**. Two-stage dilute-acid pretreatment of softwoods. *Appl. Biochem. Biotechnol.* 84-86, 561-576.

Nidetzky B., Steiner W., Hayn M., and Esterbauer H. **1993**. Enzymatic hydrolysis of wheat straw after steam pretreatment: Experimental data and kinetic modelling. *Bioresour. Technol.* 44, 25-32.

O'Sullivan A.C. **1997**. Cellulose: the structure slowly unravels. *Cellulose* 4, 173-207.

Oberoi H.S., Vadlani P.V., Brijwani K., Bhargav V.K., and Patil R.T. **2010**. Enhanced ethanol production via fermentation of rice straw with hydrolysate-adapted *Candida tropicalis* ATCC 13803. *Process Biochem.* 45, 1299-1306.

Ohgren K., Bengtsson O., Gorwa-Grauslund M.F., Galbe M., Hahn-Hägerdal B., and Zacchi G. **2006a**. Simultaneous saccharification and co-fermentation of glucose and xylose in steam-pretreated corn stover at high fiber content with *Saccharomyces cerevisiae* TMB3400. *J. Biotechnol.* 126, 488-498.

Ohgren K., Bura R., Lesnicki G., Saddler J., and Zacchi G. **2007**. A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochem.* 42, 834-839.

Ohgren K., Galbe M., and Zacchi G. **2005**. Optimization of steam pretreatment of SO2-impregnated corn stover for fuel ethanol production. *Appl. Biochem. Biotechnol.* 121, 1055-1067.

Ohgren K., Rudolf A., Galbe M., and Zacchi G. **2006b**. Fuel ethanol production from steam-pretreated corn stover using SSF at higher dry matter content. *Biomass Bioenerg.* 30, 863-869.

Olofsson K., Bertilsson M., and Liden G. **2008a**. A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. *Biotechnol. Biofuels* 1, 7.

Olofsson K., Palmqvist B., and Lidén G. **2010a**. Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. *Biotechnol. Biofuels* 3, 17.

Olofsson K., Rudolf A., and Lidén G. **2008b**. Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of Saccharomyces cerevisiae. *J. Biotechnol.* 134, 112-120.

Olofsson K., Wiman M., and Lidén G. **2010b**. Controlled feeding of cellulases improves conversion of xylose in simultaneous saccharification and co-fermentation for bioethanol production. *J. Biotechnol.* 145, 168-175.

Olson D.G., McBride J.E., Shaw A.J., and Lynd L.R. **2012**. Recent progress in consolidated bioprocessing. *Curr. Opin. Biotechnol.* 23, 396-405.

Olsson L., and Hahn-Hägerdal B. **1993**. Fermentative performance of bacteria and yeasts in lignocellulose hydrolysates. *Process Biochem.* 28, 249-257.

Osman Y.A., and Ingram L.O. **1985**. Mechanism of ethanol inhibition of fermentation in *Zymomonas mobilis* CP4. *J. Bacteriol.* 164, 173-180.

Pacini H., and Silveira S. **2011**. Consumer choice between ethanol and gasoline: Lessons from Brazil and Sweden. *Energy Policy* 39, 6936-6942.

Palmqvist E., Grage H., Meinander N., and Hahn-Hägerdal B. **1999**. Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. *Biotech Bioeng* 63, 46-55.

Palmqvist E., and Hahn-Hägerdal B. **2000a**. Fermentation of lignocellulosic hydrolysates, II: inhibitors and mechanisms of inhibition. *Bioresour. Technol.* 74, 25-33.

Palmqvist E., and Hahn-Hägerdal B. **2000b**. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. *Bioresour. Technol.* 74, 17-24.

Palmqvist E., Hahn-Hägerdal B., Galbe M., Larsson M., Stenberg K., Szengyel Z., Tengborg C., and Zacchi G. **1996a**. Design and operation of a bench-scale process development unit for the production of ethanol from lignocellulosics. *Bioresour. Technol.* 58, 171-179.

Palmqvist E., Hahn-Hägerdal B., Galbe M., and Zacchi G. **1996b**. The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme. Microb. Technol.* 19, 470-476.

Pampulha M.E., and Loureiro-Dias M.C. **1989**. Combined effect of acetic-acid, ph and ethanol on intracellular ph of fermenting yeast. *Appl. Microbiol. Biotechnol.* 31, 547-550.

Pampulha M.E., and Loureiro V. **1989**. Interaction of the effects of acetic-acid and ethanol on inhibition of fermentation in *Saccharomyces cerevisiae*. *Biotechnol. Lett.* 11, 269-274.

Park N., Kim H.-Y., Koo B.-W., Yeo H., and Choi I.-G. **2010**. Organosolv pretreatment with various catalysts for enhancing enzymatic hydrolysis of pitch pine (Pinus rigida). *Bioresour. Technol.* 101, 7046-7053.

Percival Zhang Y.H., Himmel M.E., and Mielenz J.R. **2006**. Outlook for cellulase improvement: Screening and selection strategies. *Biotechnol. Adv.* 24, 452-481.

Pérez J., Munoz-Dorado J., De la Rubia T., and Martinez J. **2002**. Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview. *Int. Microbiol.* 5, 53-63.

Piper P.W. **1995**. The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. *FEMS Microbiology Letters* 134, 121-127.

Pitkänen J.P., Aristidou A., Salusjärvi L., Ruohonen L., and Penttilä M. **2003**. Metabolic flux analysis of xylose metabolism in recombinant *Saccharomyces cerevisiae* using continuous culture. *Metabolic Engineering* 5, 16-31.

Power R.F. **2003**. Enzymatic conversion of starch to fermentable sugars. In: *The alcohol textbook*. Nottingham, United Kingdom: Nottingham University Press. 23-32 p.

Ralph J., Lundquist K., Brunow G., Lu F., Kim H., Schatz P.F., Marita J.M., Hatfield R.D., Ralph S.A., Christensen J.H. and others. **2004**. Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenyl- propanoids. *Phytochem. Rev.* 3, 29-60.

Ramirez E.C., Johnston D.B., McAloon A.J., Yee W., and Singh V. **2008**. Engineering process and cost model for a conventional corn wet milling facility. *Industrial Crops and Products* 27, 91-97.

RFA. Renewable Fuel Association, World Fuel Ethanol Production. http://ethanolrfa.org/pages/World-Fuel-Ethanol-Production, December 30, **2012**.

Ross Morrow W., Gallagher K.S., Collantes G., and Lee H. **2010**. Analysis of policies to reduce oil consumption and greenhouse-gas emissions from the US transportation sector. *Energy Policy* 38, 1305-1320.

Rudolf A., Baudel H., Zacchi G., Hahn-Hägerdal B., and Lidén G. **2008**. Simultaneous saccharification and fermentation of steam-pretreated bagasse using *Saccharomyces cerevisiae* TMB3400 and *Pichia stipitis* CBS6054. *Biotechnol. Bioeng.* 99, 783-790.

Saha B.C., Iten L.B., Cotta M.A., and Wu Y.V. **2005**. Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. *Process Biochem.* 40, 3693-3700.

Sanchez B., and Bautista J. **1988**. Effects of furfural and 5-hydroxymethylfurfural on the fermentation of *Saccharomyces cerevisiae* and biomass production from *Candida guilliermondii Enzyme. Microb. Technol.* 10, 315-318.

Sassner P., Galbe M., and Zacchi G. **2006**. Bioethanol production based on simultaneous saccharification and fermentation of steam-pretreated Salix at high dry-matter content. *Enzyme. Microb. Technol.* 39, 756-762.

Sassner P., Galbe M., and Zacchi G. **2008a**. Techno-economic evaluation of bioethanol production from three different lignocellulosic materials. *Biomass Bioenerg.* 32, 422-430.

Sassner P., Mårtensson C.-G., Galbe M., and Zacchi G. **2008b**. Steam pretreatment of H2SO4-impregnated Salix for the production of bioethanol. *Bioresour. Technol.* 99, 137-145.

SEKAB. http://www.sekab.com/birefinery/demo-plant, February 18, **2013**.

Sims R.E.H., Mabee W., Saddler J.N., and Taylor M. **2010**. An overview of second generation biofuel technologies. *Bioresour. Technol.* 101, 1570-1580.

Singh D., Zeng J., Laskar D.D., Deobald L., Hiscox W.C., and Chen S. **2011**. Investigation of wheat straw biodegradation by Phanerochaete chrysosporium. *Biomass Bioenerg.* 35, 1030-1040.

Sipos B., Reczey J., Somorai Z., Kadar Z., Dienes D., and Reczey K. **2009**. Sweet sorghum as feedstock for ethanol production: enzymatic hydrolysis of steam-pretreated bagasse. *Appl. Biochem. Biotechnol.* 153, 151-162.

Sjöström E. **1993**. Wood chemistry; fundamentals and applications. San Diego, CA: Gulf Professional Publishing.

Sonderegger M., Jeppsson M., Larsson C.r., Gorwa-Grauslund M.F., Boles E., Olsson L., Spencer-Martins I., Hahn-Hägerdal B.l., and Sauer U. **2004**. Fermentation performance of engineered and evolved xylose-fermenting *Saccharomyces cerevisiae* strains. *Biotechnol. Bioeng.* 87, 90-98.

Sun R., Lawther J.M., and Banks W.B. **1997**. A tentative chemical structure of wheat straw lignin. *Industrial Crops and Products* 6, 1-8.

Sun Y., and Cheng J. **2002**. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* 83, 1-11.

Söderström J., Galbe M., and Zacchi G. **2005**. Separate versus Simultaneous Saccharification and Fermentation of Two-Step Steam Pretreated Softwood for Ethanol Production. *Journal of Wood Chemistry and Technology* 25, 187-202.

Söderström J., Pilcher L., Galbe M., and Zacchi G. **2003**. Two-step steam pretreatment of softwood by dilute H2SO4 impregnation for ethanol production. *Biomass Bioenerg.* 24, 475-486.

Taherzadeh M.J., Gustafsson L., Niklasson C., and Liden G. **2000**. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 53, 701-708.

Taherzadeh M.J., and Karimi K. **2007**. Acid-based hydrolysis processes for ethanol from lignocellulosic materials: a review. *Bioresources* 2, 472-499.

Taherzadeh M.J., Niklasson C., and Liden G. **1997**. Acetic acid - friend or foe in anaerobic batch conversion of glucose to ethanol by *Saccharomyces cerevisiae*? *Chemical Engineering Science* 52, 2653-2659.

Tang Y., Zhao D., Cristhian C., and Jiang J. **2011**. Simultaneous saccharification and cofermentation of lignocellulosic residues from commercial furfural production and corn kernels using different nutrient media. *Biotechnol. Biofuels* 4, 22.

Tengborg C., Galbe M., and Zacchi G. **2001**. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood. *Enzyme. Microb. Technol.* 28, 835-844.

Thomas K.C., and Ingledew W.M. **1990**. Fuel alcohol production- effects of free amino nitrogen on fermentation of very high gravity wheat mashes. *Appl. Environ. Microbiol.* 56, 2046-2050.

Tomás-Pejó E., Alvira P., Ballesteros M., and Negro M.J. **2011**. Chapter 7 - Pretreatment Technologies for Lignocellulose-to-Bioethanol Conversion. In: *Biofuels*. Amsterdam: Academic Press. 149-176 p.

Ulbricht R.J., Northup S.J., and Thomas J.A. **1984**. A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fundamental and Applied Toxicology* 4, 843-853.

UNFCCC. United Nations Framework Convention on Climate Change. https://unfccc.int/, February 10, **2013**.

Wahlbom C.F., Otero R.R.C., van Zyl W.H., Hahn-Hagerdal B., and Jonsson L.J. **2003a**. Molecular analysis of a Saccharomyces cerevisiae mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. *Appl. Environ. Microbiol.* 69, 740-746.

Wahlbom C.F., van Zyl W.H., Jonsson L.J., Hahn-Hägerdal B., and Otero R.R.C. **2003b**. Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. *FEMS Yeast Res.* 3, 319-326.

Walker G.M. **1998**. Yeast physiology and biotechnology. Chichester, England: John Wiley &Sons.

Wang K., and Sun R.-C. **2010**. Chapter 7.5 - Biorefinery Straw for Bioethanol. In: *Cereal Straw as a Resource for Sustainable Biomaterials and Biofuels*. Amsterdam: Elsevier. 267-287 p.

Verduyn C., Postma E., Scheffers W., and Van Dijken J. **1992**. Effect of benzoic acid on metabolic fluxes in yeast: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 8, 501-517.

Verduyn C., Postma E., Scheffers W.A., and Vandijken J.P. **1990**. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. *Journal of General Microbiology* 136, 395-403.

Viegas C., and Sa-Correia I. **1991**. Activation of plasma membrane ATPase of Saccharomyces cerevisiae by octanoic acid. *J Gen Microbiol* 137, 645-651.

Viikari L., Vehmaanperä J., and Koivula A. **2012**. Lignocellulosic ethanol: From science to industry. *Biomass Bioenerg.* 46, 13-24.

Wingren A., Galbe M., and Zacchi G. **2008**. Energy considerations for a SSF-based softwood ethanol plant. *Bioresour. Technol.* 99, 2121-2131.

Wingren A., Galbe M., and Zacchi G. **2003**. Techno-economic evaluation of producing ethanol from softwood: Comparison of SSF and SHF and identification of bottlenecks. *Biotechnol. Prog.* 19, 1109-1117.

WRI. World Resources Institute, GHG mitigation in the EU: An overview of the current policy landscape. http://www.wri.org, February 10, **2013**.

Wyman C.E. **1994**. Ethanol from lignocellulosic biomass: Technology, economics, and opportunities. *Bioresour. Technol.* 50, 3-15.

Wyman C.E., Dale B.E., Elander R.T., Holtzapple M., Ladisch M.R., and Lee Y.Y. **2005**. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresour. Technol.* 96, 2026-2032.

Wyman C.E., Decker S.R., Himmel M.E., Brady J.W., Skopec C.E., and Viikari L. **2004**. Hydrolysis of Cellulose and Hemicellulose. In: *Polysaccharides: Structural Diversity and Functional Versatility*. New York: Dekker.

Yang B., Dai Z.Y., Ding S.Y., and Wyman C.E. **2011**. Enzymatic hydrolysis of cellulosic biomass. *Biofuels* 2, 421-450.

Zacchi G., and Axelsson A. **1989**. Economic-evaluation of preconcentration in production of ethanol from dilute sugar solutions. *Biotechnol. Bioeng.* 34, 223-233.

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Development of integrated cellulose- and starch-based ethanol production and process design for improved xylose conversion

Transportation fuels from renewable resources such as ethanol are one of the alternatives to ensure energy security and decrease the net emission of carbon dioxide. First-generation ethanol production from sugar- and starch-based raw materials (1G) is today well established in many countries, and the focus of research has thus shifted to the development and demonstration of the production of second-generation bioethanol from lignocellulose (2G).

The purpose of this work was to develop integrated processes for wheat straw- and wheat starch-based ethanol production, and to improve the co-fermentation of glucose and xylose from steam-pretreated wheat straw using integrated process configurations. Mixing steam-pretreated wheat straw (SPWS) and pre-saccharified or saccharified wheat in simultaneous saccharification and fermentation, using baker's yeast, Saccharomyces cerevisiae was shown to be beneficial for both 1G and 2G ethanol production. The most promising configuration for co-fermentation of glucose and xylose was separate hydrolysis and co-fermentation of SPWS, using the genetically modified strain, S. cerevisiae TMB3400, as this allowed the glucose concentration to be controlled by the wheat-starch hydrolysate feed.

In the last part of the work, differences in the performance of two mutated strains of S. cerevisiae TMB3400 were revealed, regarding their xylose utilization ability and inhibitor tolerance.

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