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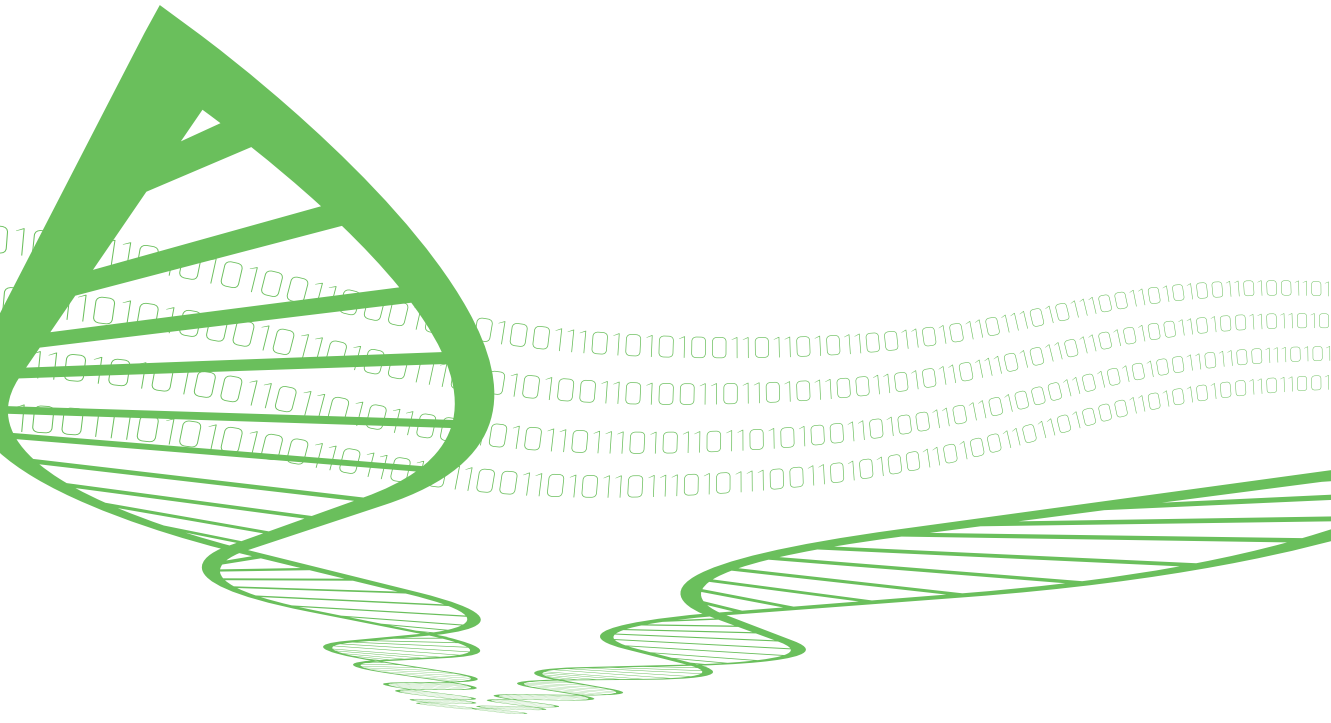
Dissertation
56

Development of pretreatment technology and enzymatic hydrolysis for biorefineries

Anne Kallioinen

VTT



VTT SCIENCE 56

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Anne Kallioinen

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Development of pretreatment technology and enzymatic hydrolysis for biorefineries

Esikäsittely- ja entsyymihydrolyysiteknologioiden kehittäminen biojalostamosovelluksiin.
Anne Kallioinen. Espoo 2014. VTT Science 56. 107 p. + app. 64 p.

Abstract

The growing demand for energy, materials and food, depletion of fossil raw material reservoirs and increasing environmental concerns have all increased interest in renewable resources. Lignocellulosic biomass is an alternative for replacing fossil raw materials in the production of fuels, materials and various chemicals. Lignocellulose present in plant cell walls consists mainly of polysaccharides, cellulose and hemicellulose, and aromatic lignin. These major components form a complex structure that is resistant to microbial and enzymatic activity. Due to the recalcitrant structure of plant cell walls, lignocellulosic raw materials must be pretreated before their enzymatic hydrolysis to monosaccharides. Various pretreatment methods; chemical, physical, biological or their combinations, have been developed. After pretreatment, polysaccharides can be hydrolysed enzymatically to monosaccharides, which in turn can be fermented to different products such as ethanol. Currently the first commercial scale lignocellulosic ethanol plants have started production. A secure supply of biomass is one of the key factors for a feasible biorefinery, and new alternative feedstocks are still required especially in northern climates in order to fulfil the raw material demands of biorefineries in a sustainable way. In addition, development of new pretreatment technologies and more efficient enzymatic hydrolysis are needed.

New lignocellulosic feedstocks and improved pretreatment methods were studied in the work described in this thesis. Reed canary grass and barley straw were found to be interesting carbohydrate-rich raw materials that could be pretreated by steam explosion and hydrolysed enzymatically with yields comparable to those obtained from wheat straw. Selection of the most favourable harvest time for reed canary grass, autumn or spring, was studied in relation to pretreatment and hydrolysis yields. Spring harvested reed canary grass was found to be the more suitable raw material as it had a higher cellulose content and the pretreated fibre was hydrolysed more efficiently compared to autumn harvested material.

A new pretreatment method using sodium carbonate and oxygen pressure was developed. The alkaline oxidation method fractionated biomass into a carbohydrate-rich fibre and a dissolved fraction containing most of the lignin. The produced carbohydrate-rich fibre could be efficiently hydrolysed by enzymes and the hydrolysis was also efficient at 12% dry matter content. Compared to the 52% total glucose yield obtained in enzyme hydrolysis of spruce after pretreatment by steam explosion, a significantly higher glucose yield of 84% was obtained in hydrolysis after alkaline oxidation. Different kinds of raw materials, such as spruce, birch and sugar cane bagasse, could be efficiently pretreated by alkaline oxidation.

The main effects of alkaline oxidation pretreatment were dissolution and partial degradation of lignin and hemicellulose. Some galactoglucomannan and xylan was solubilised and further oxidised to other products, and therefore relatively low yields of hemicellulose were obtained. Organic acids were formed as degradation products of lignin and carbohydrates. Process conditions were partially optimized using spruce as raw material in order to improve the efficiency of alkaline oxidation. The pretreatment could be accelerated by increasing the treatment temperature, by the use of copper-phenanthroline catalyst, and by decreasing the particle size of the raw material. Further optimization of *e.g.* alkali dosage and the solid to liquid ratio is still required to improve hemicellulose yield and economical feasibility.

Fibre fractions of alkaline oxidation could be hydrolysed by low enzyme dosages, 2–4 FPU/g dry matter. Significantly higher enzyme dosages were required in the hydrolysis of steam exploded materials, probably due to the inhibitory effect of the high residual lignin content after the pretreatment. The efficient hydrolysis of alkaline oxidised materials by low enzyme dosages can decrease enzyme costs or enable shorter hydrolysis time.

In order to further improve the hydrolysis efficiency and decrease the required enzyme dosage, enzyme mixtures were optimized regarding the major enzymes needed in biomass hydrolysis. Optimized mixtures of thermostable enzymes were found to have significantly different proportions of cellobiohydrolases, endoglucanases and xylanase than the optimized mixtures of *Trichoderma reesei* enzymes. Although different, the significant role of cellobiohydrolases was demonstrated in both types of mixtures. The results also indicated that high xylanase activity was required in the hydrolysis of pretreated materials having decreased enzyme accessibility to cellulose due to high xylan content or possibly due to drying of the substrate. The hydrolysis performance of optimized enzyme mixtures of five thermostable enzyme components was shown to be close to that of state-of-the-art commercial mixtures.

Keywords lignocellulose, pretreatment, enzymatic hydrolysis, optimal enzyme mixture

Esikäsittely- ja entsyymihydrolyysiteknologioiden kehittäminen biojalostamosovelluksiin

Development of pretreatment technology and enzymatic hydrolysis for biorefineries.
Anne Kallioinen. Espoo 2014. VTT Science 56. 107 s. + liitt.64 s.

Tiivistelmä

Kasvavat energian, materiaalien ja ruuan tarpeet, fossiilisten raaka-ainevarojen vähentyminen ja huoli ympäristöstä ovat lisänneet kiinnostusta uusiutuviin luonnonvaroihin. Lignoselluloosapohjainen biomassa on vaihtoehto fossiilisille raaka-aineille polttoaineiden, materiaalien ja monien kemikaalien tuotannossa. Kasvien soluseinän lignoselluloosa koostuu pääosin polysakkarideista, kuten selluloosasta ja hemiselluloosasta, sekä aromaattisesta ligniinistä. Nämä pääkomponentit muodostavat monimutkaisen rakenteen, joka on hyvin kestävä mikrobien ja entsyymien hajotukselle. Koska kasvien soluseinat ovat lujia, lignoselluloosapohjaiset raaka-aineet täytyy esikäsitellä ennen entsymaattista hydrolyysia monosakkarideiksi. Erilaisia kemiallisia, fysikaalisia ja biologisia esikäsittelymenetelmiä tai niiden yhdistelmiä on kehitetty. Esikäsittelyn jälkeen polysakkaridit voidaan hydrolysoida entsymaattisesti monosakkarideiksi, jotka voidaan edelleen fermentoida erilaisiksi tuotteiksi, kuten etanoliksi. Tällä hetkellä ensimmäiset kaupallisen mittakaavan lignoselluloosapohjaista bioetanolia valmistavat tehtaat ovat aloittaneet tuotannon. Koska kannattava biojalostamo vaatii turvatut raaka-ainelähteet, uusia ja vaihtoehtoisia biomassoja tarvitaan edelleen erityisesti pohjoisessa ilmastossa täyttämään biojalostamoiden raaka-ainetarve. Myös uusien esikäsittelytekniikoiden kehitystä ja nykyistä tehokkaampaa entsyymihydrolyysiä tarvitaan.

Tässä työssä tutkittiin uusia lignoselluloosaraaka-aineita ja kehitettiin esikäsittelymenetelmiä. Tutkimuksessa havaittiin, että ruokohelpi ja ohran olki olivat kiinnostavia korkean hiilihydraattipitoisuuden omaavia raaka-aineita, jotka voitiin esikäsitellä höyryräjäytyksellä ja hydrolysoida entsymaattisesti vehnän olkeen verrattavilla saannoilla. Ruokohelven korjuuajankohdan vaikutusta esikäsittelyyn ja hydrolyysisaantoon tutkittiin syksyllä ja keväällä korjatulla ruokohelvellä. Keväällä korjatun ruokohelven havaittiin olevan sopivampi raaka-aine, sillä sen selluloosapitoisuus oli suurempi ja siitä saatu esikäsitelty kuitu hydrolysoitui paremmin verrattuna syksyllä korjattuun materiaaliin.

Tutkimuksessa kehitettiin uusi esikäsittelymenetelmä, joka perustui alkaliseen käsittelyyn hapettavissa olosuhteissa käyttämällä kemikaaleina natriumkarbonaattia ja happea. Alkalihapetus fraktioi biomassan hiilihydraattipitoiseen kuituun ja ligniinipitoiseen liuenneeseen fraktioon. Tuotettu kuitu voitiin hydrolysoida tehokkaasti entsyymeillä, ja hydrolyysi oli tehokas myös 12 %:n kuiva-ainepitoisuudessa. Verrattuna höyryräjäytetyllä kuusella saatuun 52 %:n glukoosisaantoon esikäsittelyssä ja hydrolyysisssä, merkittävästi korkeampi 84 %:n glukoosisaanto saatiin käyttämällä alkalihapetusta esikäsittelynä. Menetelmä soveltui erilaisten raaka-aineiden, kuten kuusen, koivun ja sokeriruokobagassin, esikäsittelyyn.

Alkalihapetuksen päävaikutukset olivat ligniinin ja hemiselluloosan liukeneminen ja osittainen hajoaminen. Osa galaktoglukomannaanista ja ksylaanista liukeni ja hapettui edelleen muiksi tuotteiksi, ja siksi hemiselluloosasaanto oli suhteellisen alhainen. Alkalihapetuksessa muodostui ligniinin ja hiilihydraattien hajoamistuotteina orgaanisia happoja. Prosessiolosuhteita optimoitiin alkalihapetuskäsittelyn tehokkuuden parantamiseksi käyttämällä kuusta raaka-aineena. Esikäsittelyä voitiin tehostaa nostamalla käsittelylämpötilaa, käyttämällä kupari-fenantroliinikatalyyttiä ja pienentämällä raaka-aineen partikkelikokoa. Hemiselluloosasaannon ja taloudellisen tehokkuuden parantamiseksi tarvitaan esimerkiksi alkaliannostuksen ja kiintoaine-neste-suhteen lisäoptimointia.

Alkalihapetus-esikäsittelystä saadut kuitufraktiot hydrolysoituivat tehokkaasti jo alhaisilla entsyymiannoksilla, 2–4 FPU/g kuiva-ainetta. Merkittävästi korkeampia entsyymiannoksia tarvittiin höyryräjäytettyjen materiaalien hydrolyysissä, mikä johtui todennäköisesti korkean ligniinipitoisuuden aiheuttamasta inhiboivasta vaikutuksesta. Alkalihapetettujen materiaalien tehokas hydrolyysi alhaisilla entsyymiannostuksilla voi alentaa entsyymikustannuksia tai mahdollistaa lyhyen hydrolyysiajan.

Biomassan hydrolyysissä tarvittavien pääentsyymien seoksien koostumusta optimoitiin, jotta hydrolyysiä voitaisiin edelleen tehostaa ja alentaa entsyymiannoksia. Lämpöstabiilien entsyymien optimoiduissa seoksissa oli eri suhteissa sellobiohydrolaaseja, endoglukanaaseja ja ksylanaasia kuin *Trichoderma reesei* -homeen entsyymeistä koostetuissa optimoiduissa seoksissa. Sellobiohydrolaasit olivat kuitenkin merkittävin entsyymi molemmissa seoksissa. Saadut tulokset viittaavat siihen, että korkeaa ksylanaasiaktiivisuutta tarvitaan sellaisten esikäsiteltyjen materiaalien hydrolyysissä, joissa entsyymien pääsy selluloosaan on heikentynyt korkeasta ksylaanipitoisuudesta tai mahdollisesti raaka-aineen kuivaamisesta johtuen. Viiden termostabiilin entsyymin optimaaliset seokset vastasivat hydrolyysitehokkuudeltaan kaupallisia entsyymiseoksia.

Avainsanat lignocellulose, pretreatment, enzymatic hydrolysis, optimal enzyme mixture

Preface

The studies presented in this thesis were carried out at VTT Technical Research Centre of Finland during the years 2005–2012. Financial support of the Finnish Funding Agency for Technology and Innovation, ClimBus programme (AGROETA 40333/05), Biorefine programme (projects Pre-Cu 2366/31/07 and SugarTech 40282/08), EU 7th framework programme (EU-HYPE, 213139), and VTT is gratefully acknowledged.

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Espoo, March 2014

Anne Kallioinen

Academic dissertation

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List of publications

This thesis is based on the following original publications, which are referred to in the text as I–IV. The publications are reproduced with kind permission from the publishers. Some additional unpublished results are included.

I Kallioinen, A., Uusitalo, J., Pahkala, K., Kontturi, M., Viikari, L., von Weymarn, N., Siika-aho, M., 2012. Reed canary grass as a feedstock for 2nd generation bioethanol production. Bioresour. Technol.123, 669–672.

II Rovio, S., Kallioinen, A., Tamminen, T., Hakola, M., Leskelä, M., Siika-aho, M., 2012. Catalysed alkaline oxidation as a wood fractionation technique. Bioresources 7, 756–776.

III Kallioinen, A., Hakola, M., Riekkola, T., Repo, T., Leskelä, M., von Weymarn, N., Siika-aho, M., 2013. A novel alkaline oxidation pretreatment for spruce, birch and sugar cane bagasse. Bioresour. Technol. 140, 414–420.

IV Kallioinen, A., Puranen, T., Siika-aho, M. Mixtures of thermostable enzymes show high performance in biomass saccharification. Appl Biochem. Biotechnol. In press.

Author’s contributions

I Anne Kallioinen had the main responsibility for preparing and writing the article, and is the corresponding author. She planned the study together with the co-authors. She designed the experiments with respect to enzymatic hydrolysis and carbohydrate analysis and supervised the experimental work and was mainly responsible for the interpretation of the results.

II Anne Kallioinen wrote the article together with the other co-authors. She planned the study together with the co-authors. She had the main responsibility in the design of oxidation treatments and supervised the carbohydrate analysis. She interpreted the results together with the co-authors.

III Anne Kallioinen had the main responsibility for preparing and writing the article, and is the corresponding author. She planned the study together with the co-authors. She had the main responsibility in the design of experiments and supervised the experimental work. She was mainly responsible for the interpretation of the results.

IV Anne Kallioinen had the main responsibility for preparing and writing the article, and is the corresponding author. She planned the study together with the co-authors. She designed the experiments, supervised the experimental work and carried out statistical analysis of the data. She was mainly responsible for the interpretation of the results.

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List of abbreviations

AA	Auxiliary activities (family)
Ac	Acetyl
AlkOx	Alkaline oxidation
AFEX	Ammonia fibre explosion
Ara	Arabinose
ARP	Ammonia recycle percolation
At Cel7A	*Acremonium thermophilum* cellobiohydrolase I
βG	β-glucosidase
CDH	Cellobiose dehydrogenase
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CBP	Consolidated bioprocessing
Cel	Cellulase
CrI	Crystallinity index
DDGS	Dried distillers' grains with solubles
d.m.	dry matter
DNS	dinitrosalicylic acid
DP	Degree of polymerization
EG	Endoglucanase
EU	European Union
FPU	Filter paper unit
G	Guaiacyl unit

Gal	Galactose
GH	Glycoside hydrolase
Glc	Glucose
GlcA	Methyl glucuronic acid
H	4-Hydroxyphenyl unit
HPAEC	High performance anion exchange chromatography
LCC	Lignin-carbohydrate complex
LPMO	Lytic polysaccharide monooxygenase
Man	Mannose
MESP	Minimum ethanol selling price
M_w	Average molecular weight, mass average
Mt	Mega tons
nd	no data
PAD	Pulsed amperometric detection
RI	Refractive index
S	Syringyl unit
SAA	Soaking in aqueous ammonia
SE	Steam explosion
SEC	Size exclusion chromatography
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
Thermo mix	Mixture of thermostable enzymes
T. reesei	*Trichoderma reesei*
Tr Cel7B	*Trichoderma reesei* endoglucanase II
Tr Xyn10	*Trichoderma reesei* family 10 xylanase
TrMix	Mixture of *Trichoderma reesei* enzymes
UV	Ultra violet
Xyl	Xylose
Xylp	Xylopyranose
Xyn	Xylanase

1. Introduction

1.1 Biomass as a raw material for chemicals and fuels

Worldwide interest in sustainable production of energy, fuels and chemicals has increased for many reasons during recent decades. Energy, food and material demands are continuously increasing due to the growing global population, the reservoirs of fossil raw materials are known to be depleting, global climate change has caused worldwide environmental concern, and many countries have national aims to increase energy independence by their own energy production. To fulfil the growing need for energy resources, more efficient ways to use the remaining fossil resources by *e.g.* the recovery of oil sands and shale gases have attracted wide interest. Alternative ways and advanced technologies to produce chemicals, fuels and energy from renewable resources to decrease greenhouse gas emissions are however also needed. Against this background, various national, EU, and worldwide targets have been set to increase the proportion of renewable energy and transportation fuels and to reduce greenhouse gas emissions.

Biomass is a promising alternative to fossil raw materials for the production of liquid biofuels needed in transportation and for production of chemicals. A biorefinery is a facility which converts biomass to various products such as fuels, energy and chemicals. Bioethanol and biodiesel are examples of biorefinery products that are currently produced and blended with gasoline and diesel. In first generation biorefineries these products are mainly produced from biomass also suitable for food use, such as starch, sucrose from sugar cane or beet and vegetable oils. The sustainability of the utilization of agricultural land for production of fuels and chemicals in the first generation technologies has been questioned and the obtained greenhouse gas reductions are also under review.

Second generation biorefineries utilize more sustainable raw materials, such as various lignocellulosic side-streams, which are generated when sugar cane, sugar beet, corn, grains (e.g. wheat) and wood are processed. Dedicated energy crops as well as industrial and municipal solid wastes are also seen as sustainable raw materials. However, the division into 1st and 2nd generation technologies is not completely clear and lignocellulosic side-streams can also be considered as raw materials for production of animal feed and food (Villas-Bôas *et al.*, 2002). Lignocellulosic raw materials contain polysaccharides, cellulose and hemicellulose, which

can be hydrolysed to monosaccharides and converted to ethanol or various chemicals by a microbial conversion. Hydrolysis of carbohydrates in lignocellulosic materials can be carried out by acids or enzymes. Acid hydrolysis requires severe, corrosive process conditions. Yield losses can also occur, as well as formation of compounds inhibiting fermentation organisms. Enzymatic hydrolysis is currently considered to be more promising due to its mild process conditions and lower formation of inhibiting compounds. The first commercial scale lignocellulosic bioethanol plant is now operational in Italy and four facilities are under construction (International Energy Agency, 2013). However, the technology is still in a developmental phase and needs improvements. The major challenges are related to the recalcitrant, complex structure of plant biomass and the performance of enzymes. Pretreatment of lignocellulosic raw materials is required before the enzymatic hydrolysis to monosaccharides. Long hydrolysis time, high dosages and costs of enzymes are disadvantages of the enzymatic process. Low cost raw materials and efficient and optimized pretreatment and hydrolysis technologies are required for profitable production in sugar platform biorefineries.

1.2 Lignocellulosic raw materials

Cell walls of woody and gramineous plants represent a huge resource of fermentable carbohydrates that are potential sources for ethanol and other chemicals. It has been estimated that globally 10^{10} to 10^{11} tonnes of cellulose is synthesised and degraded annually (Hon, 1994). Botanically, lignocellulosic raw materials are divided into angiosperms and gymnosperms; angiosperms can then further be divided into monocotyledons and eudicotyledons. These three groups have different cell wall compositions. Gramineous plants (*i.e.* grasses, Poaceae) belong to monocotyledons, hardwoods to eudicotyledons and softwoods to gymnosperms. They all have cellulose, hemicellulose and lignin as the main constituents, although their proportions vary significantly (Table 1). Cellulose content can be 30–41% of dry matter in gramineous plants and 40–47% in soft- and hardwoods. Lignin content is especially high in softwoods and in some hardwoods such as eucalyptus and poplar. In addition to the contents of the main components, the composition of hemicellulose (Section 1.2.2) and lignin (Section 1.2.3) varies considerably between hardwoods, softwoods and grasses. Pectins, proteins, minerals, and various lipophilic compounds (or extractives) are also present as minor constituents in lignocellulosic biomass.

Cell walls are built up of several layers: middle lamella, primary and secondary cell walls, and the warty layer (Sjöström, 1993). These layers differ from one another with respect to their ultrastructure as well as their chemical composition. The highly lignified middle lamella is located between cells and it binds them together. The thin primary cell walls consist of cellulose microfibrils (Section 1.2.1) that are embedded in a matrix consisting of hemicellulose, lignin and pectins (Harris and Stone, 2008). The primary cell wall is supported by the thicker secondary cell wall. Secondary cell wall consists of several sublayers: S_1, S_2, and S_3 that are com-

posed of cellulose, hemicellulose and lignin (Harris and Stone, 2008; Sjöström, 1993). The secondary cell wall layers differ in respect to orientation of the cellulose microfibril network. A schematic presentation of the lignocellulosic cell wall structure is presented in Figure 1.

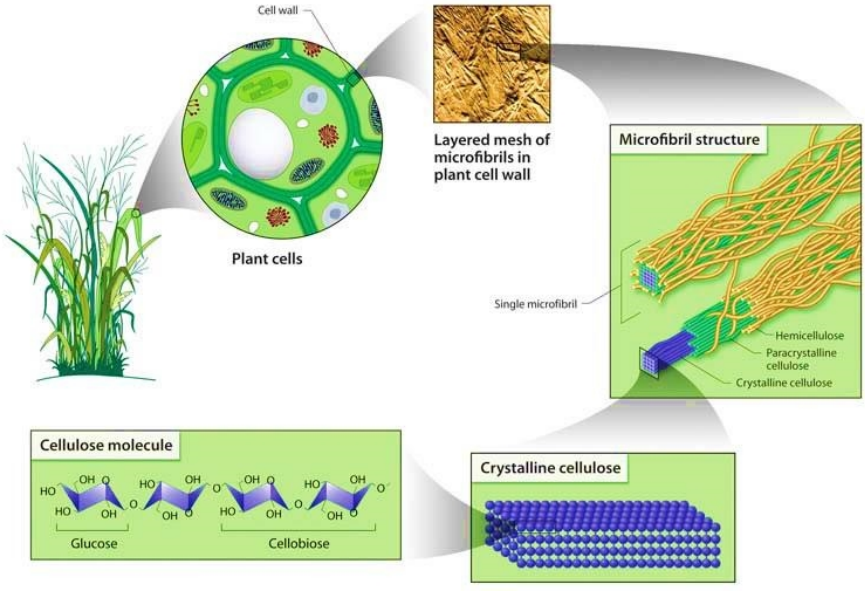

Figure 1. Schematic presentation of a lignocellulose structure. Cellulose microfibrils with crystalline and paracrystalline (amorphous) regions are embedded into a matrix consisting of lignin and hemicellulose in the cell wall (US DOE, 2005).

Table 1. Chemical composition of various lignocellulosic raw materials (percent of dry matter).

Raw material	Cellulose	Hemicellulose	Lignin	Extractives	Ash	References
Softwoods						
Norway spruce	45–47	24–26	27–28	0.4–0.9	nd	Bertaud and Holmbom, 2004
Pitch pine	43	24	29	nd	nd	Park and Kim, 2012
Hardwoods						
Eucalyptus	42	19	30	nd	nd	Park and Kim, 2012
Birch	40	27	25	nd	nd	Goshadrou *et al.*, 2013
Aspen	45	21	21	nd	0.5	Xu and Tschirner, 2012
Salix	43	21	26	nd	1.0	Sassner *et al.*, 2006
Poplar	44	20	29	3.6	1.1	Wyman *et al.*, 2009
Gramineous plants						
Sugar cane bagasse	41	21	26	0.6	1.6	Rabelo *et al.*, 2011
Wheat straw	35	22	16	nd	7	Østergaard Petersen *et al.*, 2009
Corn stover	38	26	18	nd	7	Kim and Lee, 2006
Switchgrass	30–35	23–26	19–22	nd	3–4	Kim *et al.*, 2011

nd = no data

1.2.1 Cellulose

Cellulose is one of the most abundant materials in the natural world and it is the structural material in plants. Cellulose is a linear polymer of glucose composed of glucopyranose units coupled to each other by β-(1→4)-glycosidic bonds. The number of glucose units in cellulose molecules varies and the degree of polymerization ranges from 300 to 15000 depending on the source and treatments carried out (Fengel and Wegener, 1989). Every glucose unit is 180° rotated with respect to its neighbours in the cellulose chain and thus the repeating unit is a cellobiose residue. In nature, the individual cellulose chains adhere to each other along their lengths by intra- and intermolecular hydrogen bonding and van der Waals interactions to form elementary fibrils (Nishiyama *et al.*, 2002; Parthasarathi *et al.*, 2011). The hydrogen bonding is not homogeneous, but differs from the centre chains to the outer chains. Although not fully confirmed, the elementary fibrils are considered to consist of 24–36 cellulose chains adhered to each other. These considerations are based on scattering data and information on cellulose synthase proteins (Endler and Persson, 2011; Fernandes *et al.*, 2011). The elementary fibrils are highly crystalline and they further aggregate to form microfibrils. However, the structure of cellulose is not uniform. There are crystalline and more disordered or amorphous regions in the structure and in addition there are several types of surface irregularities (Cowling, 1975; Fan *et al.*, 1980).

In native cellulose the crystal structure contains tens of glucan chains in a parallel orientation with their reducing chain ends at one terminus of the crystal and their non-reducing chain ends at the other. Seven different crystal structures have been identified for cellulose, which are designated as Iα, Iβ, II, III_I, III_{II}, IV_I and IV_{II} (O'Sullivan, 1997). In nature cellulose is present in two forms, Iα and Iβ, which are the most abundant crystal forms generally available. Cellulose Iα and Iβ differ mainly in the packing arrangement of their hydrogen bonded sheets (Nishiyama et al., 2003). The other structures are formed from native cellulose after chemical or thermal treatments, but how the changes in crystal structure occur is not yet fully understood.

Cellulose is almost always present in nature together with other biopolymers, primarily with lignin and hemicellulose. The cellulose produced by the bacterium *Acetobacter xylinum* is an example of pure cellulose (Marchessault and Sundararajan, 1993). The crystallinity and the matrix structure make the cellulosic structure highly recalcitrant.

1.2.2 Hemicellulose

In contrast to cellulose, hemicelluloses are heteropolymers consisting of the pentoses D-xylose and L-arabinose, the hexoses D-mannose, D-glucose and D-galactose, and uronic acids (Saka, 1991). Hemicelluloses can be extracted from plant cell walls by dilute alkali. The average degree of polymerization of hemicelluloses varies between 70 and 200 depending on the source (Fengel and Wegener,

1989). Hemicelluloses can be grouped into xylans, mannans, xyloglucans and mixed linkage glucans on the basis of the main sugar residue in the backbone. Xylans and mannans are the main groups of hemicellulose. The basic structures of xylan and mannan are presented in Figure 2.

Xylans, polymers of (1→4) -linked β-D-xylopyranosyl (Xylp) units, are abundantly found from many cell wall types in both hardwoods, softwoods and gramineous plants. The xylan backbone is usually substituted by side chains of L-arabinose or glucuronic acids (Ebringerová and Heinze, 2000; Timell, 1967). In hardwoods, xylans constitute 10–35% of the wood and are substituted by methyl glucuronic acid residues at every tenth Xylp residue (Whistler and Chen, 1991; Willför *et al.*, 2005b). Hardwood xylan is esterified with acetyl groups (~3.5–7 acetyl groups per ten Xylp residues). In softwoods, xylans consist of 7–15% of the wood. Softwood xylan contains methyl glucuronic acid substituents at every 5–6 Xylp residues and arabinose substituents at every 8 Xylp residues (Sjöström, 1993; Timell, 1967; Willför *et al.*, 2005a). Softwood xylans are not acetylated. In gramineous plants arabino(methylglucurono)xylans are prevalent and the content of xylan can vary from 20% in the primary cell wall up to 50% in the secondary cell wall (Vogel, 2008). The xylan backbone contains arabinose and glucuronic acid substituents and oligosaccharide side chains consisting of arabinose, xylose and galactose (Naran *et al.*, 2009; Wende and Fry, 1997; Wilkie, 1979). Ferulic acid and small amounts of p-coumaric acid and sometimes sinapinic acid are esterified to arabinose groups as single substituents or in oligosaccharide side chains (Harris and Trethewey, 2010; Iiyama *et al.*, 1994; Wende and Fry, 1997).

Mannans, galactoglucomannans and glucomannans, are found particularly in softwoods, where they are usually the predominant non-cellulosic polysaccharides (Sjöström, 1993; Timell, 1967). Galactoglucomannans comprise approximately 12–18% of softwood. Galactoglucomannans have a linear chain of (1→4) linked β-D-mannopyranosyl and β-D-glucopyranosyl residues, with α-D-galactose substituents (Lundqvist *et al.*, 2002). The ratio of glucose:mannose:galactose varies in the approximate range 1 : 3–4 : 0.1–1.0 (Sjöström, 1993). The higher galactose content is found in the water soluble fraction and the lower galactose content in the alkali soluble fraction of softwood galactoglucomannan. In addition, galactoglucomannans are acetylated, the degree of acetylation depending on the species. Hardwoods contain 2–5% of glucomannan, which is not substituted with galactose (Timell, 1967). The glucose-mannose ratio is usually 1:2 and acetyl groups can be attached to mannose (Teleman *et al.*, 2003). Only low amounts of glucomannan are present in gramineous plants (Vogel, 2008).

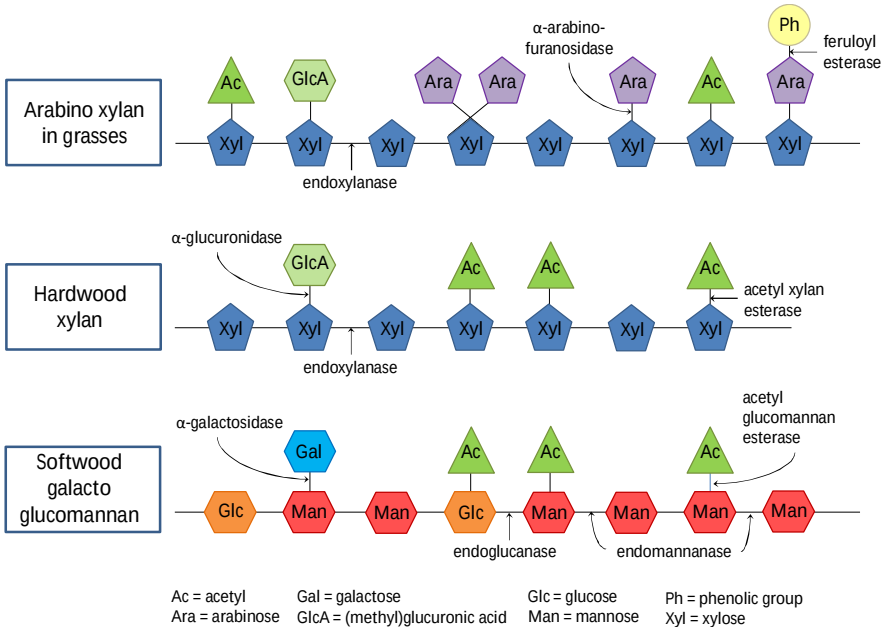

Figure 2. Schematic structures of polymeric xylan and galactoglucomannan and presentation of enzymes participating in their hydrolysis.

1.2.3 Lignin

Lignin is a complex aromatic polymer present abundantly in nature and is an integral component of plant cell walls. Softwoods contain approximately 27–29% of lignin, whereas hardwoods, grasses and cereals usually have lower amounts (Table 1). The exact structure of lignin in the native form is still unclear. The structure of isolated lignins is always modified to a certain extent. Lignin is a three dimensional heteropolymer of methoxylated phenyl propane units, for which the precursors, monolignols, are p-coumaryl, coniferyl and sinapyl alcohol. Monolignols differ in respect to methoxylation. The corresponding aromatic constituents of these alcohols in the polymer are called 4-hydroxyphenyl (H), guaiacyl (4-hydroxy-3-methoxyphenyl, G) and syringyl (4-hydroxy-3,5-dimethoxyphenyl, S) units. The composition of lignin varies widely with species. Softwood lignin is dominated by G units, whereas hardwood lignin is a mixture of G and S units (Fengel and Wegener, 1989; Sjöström, 1993). Lignin from grasses typically contains all three types of monolignols with different ratios (Buranov and Mazza, 2008). Different types of linkages connect the phenylpropane units. The most common is the β-O-4´ linkage making up more than half of the lignin linkages in soft- and hardwoods (Dorrestijn *et al.*, 2000; Sjöström, 1993). The amount, composition, and chemical bonds of lignin vary between plants, in different plant parts and cell types or even within a single cell wall (Campbell and Sederoff, 1996). In lignocellulosic biomass lignin is

cross-linked with carbohydrates by ether or ester linkages via *e.g.* arabinose-ferulic acid or glucuronic acid (Takahashi and Koshijima, 1988).

1.3 Enzymes degrading lignocellulose

Lignocellulosic biomass is degraded and utilized in nature by the action of micro-organisms as part of the carbon cycle (Lundell *et al.*, 2010; Lynd *et al.*, 2002). Fungi and bacteria produce and secrete enzymes that are needed to degrade lignocellulosic materials into a more widely utilisable form. Cellulose is degraded by an enzyme system consisting of hydrolytic cellulases. Recent studies also suggest that oxidative mechanisms participate in the degradation of cellulose (see Sections 1.3.1 and 1.3.2). Hydrolysis of hemicellulose requires various enzyme activities, *e.g.* xylanases and mannanases (Section 1.3.3). Several enzymes modifying and degrading lignin through oxidative mechanisms are also produced by microbes, *e.g.* laccases, lignin peroxidases and manganese peroxidases (Hatakka, 1994). One of the most characterized producers of biomass-degrading enzymes is the filamentous fungi *Trichoderma reesei*. It is also largely used in industrial production of cellulases and hemicellulases as well as being a heterologous host for protein production.

Cellulases and hemicellulases are often modular (Henrissat and Davies, 2000). They consist of at least one catalytic domain, which may be attached to a carbohydrate binding module (CBM) via a highly glycosylated peptide linker (Tomme *et al.*, 1988). CBMs have been shown to improve the hydrolysis of insoluble substrates (Tomme *et al.*, 1988) and they have also been proposed to contribute to amorphogenesis (Arantes and Saddler, 2010). Cellulases, other glycoside hydrolases and auxiliary enzymes have been classified into different families. Enzymes in the same family share similar protein folding and three dimensional structure as well as similar reaction mechanisms (Henrissat, 1991; Levasseur *et al.*, 2013). Today, 133 families of glycoside hydrolases (GH) and 11 auxiliary activity (AA) families including other enzymes participating in the degradation of lignocellulose have been identified and listed in the Carbohydrate Active Enzyme database (CAZy, 2013). Enzymes known to play a role in cellulose degradation are found at least in 20 glycoside hydrolase families, in families 1, 3, 5, 6, 7, 8, 9, 10, 12, 18, 19, 26, 30, 44, 45, 48, 51, 74, 116 and 124.

1.3.1 Cellulases

Cellulases are glycoside hydrolases that catalyse the cleavage of β-(1→4) glycosidic bonds in cellulose and produce glucose, cellobiose and cello-oligosaccharides as primary products. They are found in various organisms, predominantly in *Prokaryotae* and Fungi. Cellulases have traditionally been divided into cellobiohydrolases, endoglucanases and β-glucosidases.

Cellobiohydrolases (exo- 1,4-β-glucan cellobiohydrolases, CBHs) sequentially release cellobiose units from the cellulose chain either from the reducing end (EC

3.2.1.176) or the non-reducing end (EC 3.2.1.91). They can attack the crystalline parts of the substrate, primarily producing cellobiose and decreasing the substrate degree of polymerization (DP) only very slowly (Irwin *et al.*, 1993; Kleman-Leyer *et al.*, 1996). The CBHs attack cellulose crystals on the hydrophobic faces (Liu *et al.*, 2011). Most CBHs belong to GH families 6 and 7. Cellobiohydrolases have been shown to have a tunnel shaped active site composed of several loops, which limits their activity to chain ends (Divne *et al.*, 1994; Rouvinen *et al.*, 1990). On the other hand, CBHs can have some endo-type activity (Kurašin and Väljamäe, 2011; Ståhlberg *et al.*, 1993). CBHs are generally considered as processive enzymes, initiating their action from the ends of the cellulose chains and continuing along the cellulose chain until the end (Divne *et al.*, 1994; Teeri *et al.*, 1998). In strongly processive cellobiohydrolases (*e.g*. *Trichoderma reesei* CBHI) a substrate binding tunnel enhances the probability for the enzyme to remain bound after a catalytic cycle. *T. reesei* CBHII is suggested to be less processive (Igarashi *et al.*, 2009). The processive action may be restricted by obstacles formed by other molecules on the cellulosic substrates which prevent the CBHs from gliding along cellulose chains (Kurašin and Väljamäe, 2011). The blocked CBH molecule can also prevent action of other CBH molecules (Igarashi *et al.*, 2011).

Endoglucanases (endo-1,4-β-glucanase, EGs, EC 3.2.1.4) attack the cellulose chain at internal positions (Medie *et al.*, 2012), resulting in a rapid decrease in the DP of the substrate (Kleman-Leyer *et al.*, 1994; Kleman-Leyer *et al.*, 1996). Because the glucan chains can remain associated with the rest of the cellulose crystal after a single bond cleavage at the surface, it takes a relatively long time before soluble products are observed after an endo-type of attack. Endoglucanases are present in many GH families *e.g.* in 5, 6 and 7. Endoglucanases usually have an open active site, a substrate binding cleft, which can bind and act in the middle of the chain (Henriksson *et al.*, 1996; Kleywegt *et al.*, 1997). The cellulose chain segment interacts with multiple subsites (4–7) in the cleft. Processive endoglucanases have also been reported (Irwin *et al.*, 1993; Wilson and Kostylev, 2012).

β-Glucosidases (β-D-glucoside glucohydrolase, βGs, EC 3.2.1.21) have an important role in the hydrolysis of cellobiose and short oligosaccharides to glucose, thereby decreasing the product inhibition of cellobiohydrolases (Gusakov and Sinitsyn, 1992). The hydrolysis rates of βGs decrease markedly as the substrate DP increases (Zhang and Lynd, 2004). βGs can be found in GH families 1, 3, 9, 30 and 116, although most of the microbial βGs employed in cellulose hydrolysis belong to GH family 3. Structures of βGs have not been thoroughly studied. Family 1 βGs have been shown to have loops around active cavities (Hakulinen *et al.*, 2000). The loops are suggested to be involved in substrate binding. The GH1 βGs having broader cavities are suggested to have wider substrate specificity. The GH3 βGs have been reported to have clear differences in structure (Suzuki *et al.*, 2013). Crystal structures of *Aspergillus aculeatus* βG contained a long cleft suitable for longer oligosaccharides, whereas *Hordeum vulgare* βG had a binding pocket suitable for various disaccharides. Because cellobiose is a strong inhibitor of CBHs, the βG activity in cellulase mixtures must be optimized to overcome the product inhibition of CBHs (Gusakov and Sinitsyn, 1992). The inhibition of βGs by

glucose must also be considered because accumulation of glucose will lead to the accumulation of cellobiose and CBH inhibition (Seidle *et al.*, 2004; Teugjas and Väljamäe, 2013). Many βGs also have transglycosylation activity, which competes with hydrolysis (Bohlin *et al.*, 2013; Seidle *et al.*, 2004).

1.3.2 Auxiliary enzymes for degradation of cellulose

In 2007 Merino and Cherry found that a *Thielavia terrestris* GH61 protein enhanced the activity of *T. reesei* cellulases and in 2008 Moser *et al.* reported the same with a *Thermobifida fusca* CBM33 protein. Since then the structure, mechanisms and role of fungal GH61 and bacterial CBM33 proteins in cellulose degradation have been intensively studied. It is currently known that these new oxidoreductive enzymes, lytic polysaccharide mono-oxygenases (LPMOs, AA9 and AA10, formerly referred to as GH61 and CBM33, no EC classification) participate in the degradation of cellulose in addition to CBHs and EGs in the conventional models. They catalyse the oxidative cleavage of cellulose using low molecular weight reducing agents such as ascorbate, gallate, reduced glutathione, and even fragments from lignin (Quinlan *et al.*, 2011; Westereng *et al.*, 2011). LPMOs have been shown to cleave the cellulose chain in crystalline cellulose by an oxidative mechanism. Oxidation of hydroxyl groups at the C1, C4 or C6 position has been reported (Beeson *et al.*, 2012; Langston *et al.*, 2011; Quinlan *et al.*, 2011).

Cellobiose dehydrogenases (CDH, EC 1.1.99.18) are known to catalyse oxidation of the reducing end of cellobiose, cellodextrins, or certain other oligosaccharides to the corresponding lactone, which spontaneously converts to the aldonic acid (*e.g*. cellobionic acid) (Cameron and Aust, 2001; Henriksson *et al.*, 2000; Zamocky *et al.*, 2006). CDHs employ a wide spectrum of electron acceptors in oxidation reactions (Henriksson *et al.*, 2000). Recently it has been reported that CDH enhances the depolymerisation of crystalline cellulose in a synergistic mechanism together with LPMOs (Langston *et al.*, 2011; Sygmund *et al.*, 2012). However, not all enzyme systems contain CDHs and their exact role in the degradation of lignocellulose is not known. The proposed model for cellulose degradation is presented in Figure 3. CBHs hydrolyse the cellulose chains from reducing or non-reducing chain ends, endoglucanases form new chain ends in the amorphous regions and LPMOs in the crystalline parts of cellulose. βGs further hydrolyse the cellobiose formed into glucose.

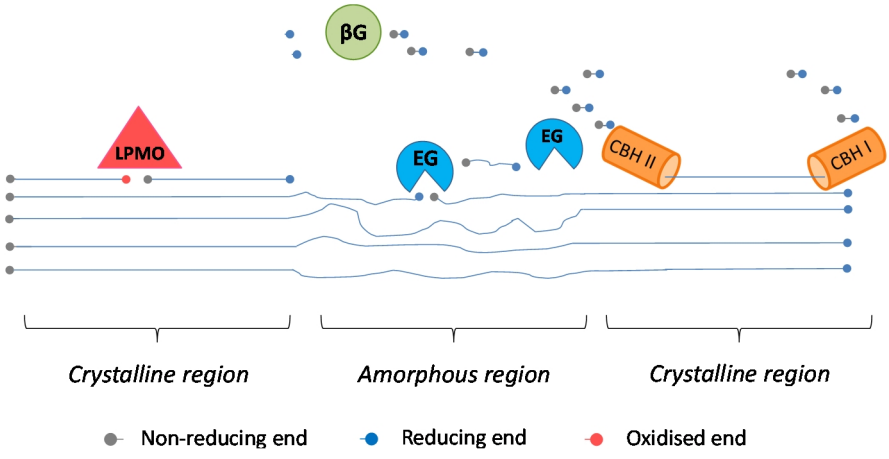

Figure 3. Synergistic model for enzymatic degradation of cellulose.

Swollenins and expansins have also been suggested to participate in cellulose degradation. Fungal swollenins have exhibited clear effects on insoluble cellulosic substrates, such as swelling and decrease in particle size and crystallinity, without the formation of reducing sugars (Chen *et al.*, 2010; Jager *et al.*, 2011; Saloheimo *et al.*, 2002). Disruption of the hemicellulosic fraction has also been reported (Gourlay *et al.*, 2013). Bacterial expansin exhibits similar cellulose binding and weakening activities (Kim *et al.*, 2009). No catalytic activity has been detected so far except for a weak endoglucanase activity (Chen *et al.*, 2010). Combination of swollenins and expansins with cellulases and xylanases has been reported to accelerate the hydrolysis of cellulose and xylan (Gourlay *et al.*, 2013; Kim *et al.*, 2009).

1.3.3 Hemicellulolytic enzymes

Hemicellulose is a group of carbohydrate polymers consisting of different monosaccharides, sugar acids and non-carbohydrate subunits, and having different branching and structures. A variety of enzymes is needed for the complete hydrolysis of hemicellulose.

Depolymerizing enzymes act on the backbone sugar chain *i.e.* xylan, glucomannan or glucan chain. Endo-1,4-β-xylanases (EC 3.2.1.8) solubilize xylan whereas an endo-1,4-β-mannanase (EC 3.2.1.78) cleaves the galactoglucomannan main chain to produce oligosaccharides (Sørensen *et al.*, 2007; Stålbrand *et al.*, 2004; Vrsanská *et al.*, 2007). The produced mixed xylo- and manno-oligosaccharides are hydrolysed by debranching enzymes and by the enzymes hydrolysing the oligomers. α-L-Arabinofuranosidases (EC 3.2.1.55) remove arabinose groups bound to the xylan backbone and can be specific for either mono- or disubstituted xylose residues and short or long oligomers (Kühnel *et al.*, 2011; Van Laere *et al.*, 1997). α-Glucuronidases (EC 3.2.1.139) are needed for the removal

of glucuronic acid groups from xylan (Tenkanen and Siika-aho, 2000) and α-galactosidase (EC 3.2.1.22) for the removal of galactose substituents in galactoglucomannans (Luonteri *et al.*, 1998). Acetyl groups ester-linked in xylan and glucomannan can be hydrolysed by acetyl xylan esterases (EC 3.1.1.72) or acetyl glucomannan esterases (EC 3.1.1.6), respectively (Biely, 2012; Stålbrand *et al.*, 2004). In addition, feruloyl esterases (EC 3.1.1.73) and p-coumaroyl esterases (EC 3.1.1.-) are needed to cleave the corresponding ester linkages in xylan (McCrae *et al.*, 1994). The depolymerizing and debranching enzymes acting on xylan and galactoglucomannan are presented in Figure 2. β-Glucosidases (EC 3.2.1.21), β-xylosidases (EC 3.2.1.37) and β-mannosidases (EC 3.2.1.25) are essential for the complete hydrolysis of hemicellulosic oligosaccharides to monosaccharides (Ademark *et al.*, 1999; Rahman *et al.*, 2003). Cellulases can also have hemicellulolytic activity towards xylan and other hemicelluloses (Tomme *et al.*, 1995; Vlasenko *et al.*, 2010). Several family 7 endoglucanases are active against xylan, arabinoxylan and xyloglucan, whereas family 5 endoglucanases have activity on mannan and galactomannan (Vlasenko *et al.*, 2010). Having a broad specificity may be beneficial for a biomass-hydrolysing enzymatic system, because of the heterogeneity of the substrate.

1.3.4 Synergism in the hydrolysis of cellulose

Cellulose degradation requires sets of secreted enzymes that work in synergy. Synergism occurs when the activity exhibited by mixtures of components is greater than the sum of the activity of these components evaluated separately (Henrissat *et al.*, 1985; Walker and Wilson, 1991).

It is known that endoglucanases and cellobiohydrolases work synergistically in the so called endo-exo synergy. Endoglucanases produce new chain ends by cleaving the cellulose chains, thus creating new starting points for cellobiohydrolases (Henrissat *et al.*, 1985; Nidetzky *et al.*, 1994; Nidetzky *et al.*, 1993). On the other hand, CBHs make the substrate more accessible to EGs (Irwin *et al.*, 1993; Väljamäe *et al.*, 1999). Two cellobiohydrolases (CBHI and CBHII) have also reported to have synergy due to their different specificity for reducing and non-reducing ends (Fägerstam and Pettersson, 1980; Igarashi *et al.*, 2011; Medve *et al.*, 1994; Nidetzky *et al.*, 1994). β-Glucosidase reduces inhibition by cellobiose and has a synergistic effect on the hydrolysis with cellobiohydrolases or endoglucanases (Gruno *et al.*, 2004; Lamed *et al.*, 1991).

Some oxidoreductases have recently been shown to synergistically enhance the hydrolysis by cellulases. Lytic polysaccharide monooxygenases (LPMOs) in family AA9 and some members in the family AA10 can act synergistically with cellulases (Forsberg *et al.*, 2011; Harris *et al.*, 2010). The synergistic effect appears to be related to the ability of LPMOs to act on highly crystalline areas, generating new ends for hydrolases and disrupting the crystallinity of cellulose (Langston *et al.*, 2011). Synergy of LPMOs with cellobiose dehydrogenases has

also been reported and the combination is able to significantly stimulate the degradation of cellulose by cellulases or even by β-glucosidase (Langston *et al.*, 2011).

Hydrolysis of hemicellulose by hemicellulolytic enzymes can synergistically improve the hydrolysis of cellulose. Addition of xylanase, feruloyl esterase and acetyl xylan esterases has increased the release of glucose in addition to xylose (Hu *et al.*, 2011; Kumar and Wyman, 2009; Murashima *et al.*, 2003; Selig, 2008; Zhang *et al.*, 2011). The boosting effect of xylanases and mannanases is also reported even with lignocellulosic substrates having only a low xylan content (Hu *et al.*, 2011; Varnai *et al.*, 2011a; Várnai *et al.*, 2010) and may be due to removal of residual hemicellulose on the cellulose fibres, or increased porosity and fibre disintegration (Arantes and Saddler, 2010; De Jong *et al.*, 1997; Suurnäkki *et al.*, 1997).

1.3.5 Optimal enzyme compositions for hydrolysis of lignocellulosic materials

The hydrolysis of lignocellulosic substrates requires numerous enzymes working in synergy. The variation in structure and composition between raw materials from different sources and after different types of pretreatment further increases the complexity. The optimal enzyme composition for a given raw material can vary due both to the different chemical bonds to be broken and to structural limitations such as crystallinity, pore size, fibrillation, and content and location of hemicellulose and lignin. In addition, the applied enzyme components, their specificity and synergistic interactions as well as their varied susceptibility to inhibition, inactivation or non-productive binding on lignin can affect the optimal enzyme composition.

The aim of enzyme mixture optimization is to hydrolyse lignocellulosic materials efficiently using lower enzyme dosages. Enzyme mixture optimization has been conducted for various substrates (Table 2). In addition to almost pure cellulosic substrates (Andersen *et al.*, 2008; Baker *et al.*, 1998; Boisset *et al.*, 2001; Gusakov *et al.*, 2007; Kim *et al.*, 1998), optimized minimal enzyme mixtures have been developed for lignocellulosic substrates such as pretreated barley straw (Rosgaard *et al.*, 2007), pretreated wheat straw (Billard *et al.*, 2012), pretreated corn stover (Banerjee *et al.*, 2010a; Banerjee *et al.*, 2010b; Banerjee *et al.*, 2010c; Gao *et al.*, 2010a; Gao *et al.*, 2010b; Zhou *et al.*, 2009), pretreated switchgrass, Miscanthus, distillers' grains, poplar wood (Banerjee *et al.*, 2010b) and pretreated douglas fir (Gusakov *et al.*, 2007).

Studies on the optimization of enzyme mixtures for the hydrolysis of lignocellulosic substrates indicated that the composition of enzyme mixtures can vary considerably. It has been claimed that the optimal ratio of cellulolytic activities in the hydrolysis of lignocellulose can differ remarkably from that of the cellulases secreted by *T. reesei* (Rosgaard *et al.*, 2007). Generally, a high proportion of Cel7A (CBHI) and Cel7B (EGI) has been reported in studies carried out using the main *Trichoderma* cellulases and endoxylanase (Banerjee *et al.*, 2010b; Banerjee *et al.*, 2010c; Billard *et al.*, 2012; Gao *et al.*, 2010a; Rosgaard *et al.*, 2007). On the other hand, the optimal proportions of xylanase, β-glucosidase and Cel6A (CBHII) appear to vary considerably depending on the study and raw material.

Banerjee *et al.* developed optimal enzyme mixtures for the hydrolysis of various pretreated substrates (Banerjee *et al.*, 2010b). They found that the optimal mixture of *T. reesei* enzymes for the hydrolysis of NaOH-pretreated corn stover and switchgrass contained clearly less endoxylanase than for the ammonia fibre explosion (AFEX) and alkaline peroxide pretreated materials, and that the increased proportion of xylanase was generally associated with lower proportions of Cel7A (CBHI) and Cel7B (EGI). In the case of barley straw, hydrolysis of material prepared by acid-catalysed steam explosion required less endoglucanase than material pretreated by non-catalysed steam explosion or hot water treatment (Rosgaard *et al.*, 2007).

The number of enzyme components in reported studies has varied from 3 to 16 and both purified and commercial enzyme mixtures have been used as mixture components (Table 2). The optimizations have been performed for the main cellulase activities (Zhou *et al.*, 2009), for cellulases and xylanases (Gao *et al.*, 2010b), for multi-component mixtures containing several accessory enzymes (Banerjee *et al.*, 2010c), or for mixtures of commercial enzymes (Berlin *et al.*, 2007; Garlock *et al.*, 2012). Banerjee *et al*. found that the optimal enzyme mixture varies substantially depending on how many and which enzyme components were included in the optimization in addition to a core set of enzymes (Banerjee *et al.*, 2010b). In fact, the number of enzyme components and the raw material appeared to have more significant effect on the enzyme proportions than the pretreatment carried out for the raw material (Banerjee *et al.*, 2010b; Banerjee *et al.*, 2010c; Billard *et al.*, 2012; Gao *et al.*, 2010a; Zhou *et al.*, 2009). Thus it is important to include all the most essential enzymes in experimental design, although this makes the design more complex.

The optimal mixtures have been shown to be clearly different for glucose and xylose release (Banerjee *et al.*, 2010a), and to be dependent on the total enzyme loading (Gao *et al.*, 2010a). Despite relatively high enzyme dosages (10–30 mg/g of dry substrate), many of the optimization studies resulted in very low hydrolysis yields (10–50%; Table 2). Recalcitrant raw materials and inefficient pretreatment appear to be the most probable reasons for low hydrolysis yields. In such a situation no significant benefit can be obtained by optimization. In addition to the degree of hydrolysis, the enzyme dosage can affect the optimal enzyme ratio. Gao *et al.* found that within *T. reesei* enzymes the role of Cel7B (EGI) was more important in the case of lower total protein loadings (Gao *et al.*, 2010b). The hydrolysis time also influenced the optimal enzyme ratio (Billard *et al.*, 2012). In the beginning of the hydrolysis of steam exploded wheat straw less xylanase and Cel5A (EGII) were needed than in the later stages of the hydrolysis.

Table 2. Studies on optimal enzyme compositions for hydrolysis of lignocellulosic raw materials.

Raw material	Pretreatment [a]	Source of enzyme components	Number of enzyme components	Statistical method (+/-)	Hydrolysis conditions (d.m. content / hydrolysis time / mixing speed)	Max. yield [b]	Reference
Avicel	-	*Chrysosporium lucknowense, T. reesei, Aspergillus japonicus*	7	-	5% / 24–72 h / n.d.	90% Glc	Gusakov *et al.*, 2007
Cotton	-	*Chrysosporium lucknowense, T. reesei, Aspergillus japonicus*	7	-	2.5% / 24–72 h / n.d.	84% Glc	Gusakov *et al.*, 2007
Filter paper	-	*Thermomonospora fusca, T. reesei*	7	+	0.85% / 24–72 h / n.d.	60%	Kim *et al.*, 1998
Bacterial cellulose	-	*Humicola insolens, A. niger*	4	-	0.1% / 1–14 h /0 rpm	56–90%	Boisset *et al.*, 2001
Barley straw	SE (acid impr.)	*T. reesei, A. niger* (Novozym188)	5	+	1% / 24 h / n.d.	56% Glc	Rosgaard *et al.*, 2007
	SE (water impr.)	*T. reesei, A. niger* (Novozym188)	5	+	1% / 24 h / n.d.	33% Glc	Rosgaard *et al.*, 2007
	Hot water extraction	*T. reesei, A. niger* (Novozym188)	5	+	1% / 24 h / n.d.	50% Glc	Rosgaard *et al.*, 2007
Wheat straw	SE	*T. reesei, A. niger*	6	+	1% / 0.5–48 h / 175 rpm	66%	Billard *et al.*, 2012

Raw material	Pretreatment [a]	Source of enzyme components	Number of enzyme components	Statistical method (+/-)	Hydrolysis conditions (d.m. content / hydrolysis time / mixing speed)	Max. yield [b]	Reference
Rice straw	NaOH	Celluclast, *A. aculeatus* (enzyme exract), *Bacillus subtilis* (expansin)	3	+	1% / 48 h / 200 rpm	78%	Suwannarangsee *et al.*, 2012
Corn stover	AFEX	*A. nidulans, A. niger* *T. reesei*,	6	+	0.2% glucan / 24 h / 200 rpm	80% Glc, 56% Xyl	Gao *et al.*, 2010a
	AFEX	*Dictyoglomus turgidum,* *C. thermocellum,* *Geobacillus spp.* *T. reesei*	6	-	0.2% glucan / 24 h / 200 rpm	94% Glc, 62% Xyl	Gao *et al.*, 2010b
	AFEX	*T. reesei, A. niger* *T. longibrachiatum,*	6–16	+	0.2% glucan/ 48 h / 10 rpm	44%–52% Glc	Banerjee *et al.*, 2010a; Banerjee *et al.*, 2010b; Banerjee *et al.*, 2010c
	NaOH	*T. reesei, A. niger* *T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	41% Glc	Banerjee *et al.*, 2010b
	Alkaline peroxide	*T. reesei, A. niger* *T. longibrachiatum*	6–16	+	0.2% glucan/ 48 h / 10 rpm	58%–69% Glc	Banerjee *et al.*, 2010b
	SE	*T. viride*	7	+	5% / 72 h /210 rpm	80% Glc	Zhou *et al.*, 2009
	Dilute acid	Celluclast1.5 Novozym188 Multifect xylanase Multifect pectinase	4	+	0.6% / 24 h / 600 rpm	99% Glc, 88% Xyl	Berlin *et al.*, 2007
	Ball milling	Accellerase1000 CellulaseZSL-1300 Xylanase	3	+	10% / 95 h/ 120 rpm	95%	Lin *et al.*, 2011

Raw material	Pretreatment [a]	Source of enzyme components	Number of enzyme components	Statistical method (+/-)	Hydrolysis conditions (d.m. content / hydrolysis time / mixing speed)	Max. yield [b]	Reference
Switchgrass	AFEX	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	24% Glc	Banerjee *et al.*, 2010b
	AFEX	Novozym188, Spezyme SP, Multifect xylanase Multifect pectinase	4	+	1% glucan /72 h / 200 rpm	80% Glc, 76% Xyl	Garlock *et al.*, 2012
	NaOH	*T. reesei, A. niger T. longibrachiatum*	6	+	0.2% glucan/ 48 h / 10 rpm	26% Glc	Banerjee *et al.*, 2010b
	Alkaline peroxide	*T. reesei, A. niger T. longibrachiatum*	6	+	0.2% glucan/ 48 h / 10 rpm	39% Glc	Banerjee *et al.*, 2010b
Miscanthus	AFEX	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	23% Glc	Banerjee *et al.*, 2010b
	NaOH	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	18% Glc	Banerjee *et al.*, 2010b
	Alkaline peroxide	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	32% Glc	Banerjee *et al.*, 2010b
DDGS[c]	AFEX	*T. reesei, A. niger T. longibrachiatum,*	6–16	+	0.2% glucan/ 48 h / 10 rpm	23%–52% Glc	Banerjee *et al.*, 2010b
	NaOH	*T. reesei, T. longibrachiatum, A. niger*	6	+	0.2% glucan / 48 h / 10 rpm	24% Glc	Banerjee *et al.*, 2010b
	Alkaline peroxide	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	30% Glc	Banerjee *et al.*, 2010b

Raw material	Pretreatment [a]	Source of enzyme components	Number of enzyme components	Statistical method (+/-)	Hydrolysis conditions (d.m. content / hydrolysis time / mixing speed)	Max. yield [b]	Reference
Poplar	AFEX	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan/ 48 h / 10 rpm	14% Glc	Banerjee *et al.*, 2010b
	NaOH	*T. reesei, A. niger T. longibrachiatum,*	6	+	0.2% glucan / 48 h / 10 rpm	10% Glc	Banerjee *et al.*, 2010b
	Alkaline peroxide	*T. reesei, A. niger T. longibrachiatum,*	6–16	+	0.2% glucan/ 48 h / 10 rpm	11% Glc	Banerjee *et al.*, 2010b
Douglas fir	Organosolv	*Chrysosporium lucknowense, T. reesei, A. japonicus*	7	-	5% /24–72 h / n.d.	96% Glc	Gusakov *et al.*, 2007

[a] SE = steam explosion, AFEX = ammonia fibre explosion
[b] Glc = glucose yield, Xyl = xylose yield
[c] DDGS = dried distillers grains with solubles
n.d. = no data

1.4 Pretreatment of lignocellulosic substrates

Lignocellulosic matrix is an important structural element in plants, and provides resistance against *e.g.* wind and cell rupture caused by osmotic pressure. Plants also have different kinds of defence mechanisms for protection against microbial and enzymatic attack, for example waxy layers on leaves and bark (Himmel *et al.*, 2007). The cell wall matrix as such is also resistant to microbial degradation. The structure of cellulose, with highly ordered and water-excluding crystallites, retards the action of cellulases. Cell wall microfibrils are surrounded by hemicellulose that is covalently linked to lignin. The recalcitrant matrix of polymers is the main reason why plant biomass has high resistance to chemical, mechanical and enzymatic treatment.

For an efficient and complete hydrolysis, biomass needs first to be pretreated. Various pretreatment methods have been developed to decrease the recalcitrance of lignocellulosic materials, and to make the cellulose more susceptible to enzymatic hydrolysis (Mosier *et al.*, 2005b; Sun and Cheng, 2002; Zhao *et al.*, 2012). Pretreatments can also be used to fractionate the lignocellulosic raw materials by separating lignin and hemicellulose from cellulose. The aim of the pretreatment is to open up the cell wall structure and make cellulose and hemicellulose more accessible to enzymes during hydrolysis. Cell wall structure can be opened up by reducing the particle size and by increasing the pore size and accessible surface area. The pretreatments should also modify lignocellulose matrix structure by reducing the cellulose crystallinity, and by modification and solubilisation of lignin and hemicellulose. From a process technology and economy point of view, the overall aim of the pretreatment of lignocellulose is to get high product yield in the whole process, including pretreatment, hydrolysis, fermentation and downstream processing. To achieve this, pretreatment should minimize the degradation or loss of carbohydrates, and avoid formation of by-products that are inhibitory to enzymes and fermentation microbes. In addition, pretreatment should have low capital and processing costs and be suitable for various different raw materials.

1.5 Pretreatment methods

Various different kinds of processes have been developed for the pretreatment of lignocellulosic biomass (Galbe and Zacchi, 2012; Haghighi Mood *et al.*, 2013; Mosier *et al.*, 2005b). The methods can be approximately grouped into physical, chemical, physico-chemical and biological pretreatment processes, although it is in many cases difficult to place a specific method into only one category. For example, thermal treatments can cause chemical changes in biomass and on the other hand it might also be difficult to avoid shear force effects during chemical treatments.

1.5.1 Physical and biological pretreatments

Physical pretreatments can be defined as methods using physical processing on biomass. Thus mechanical and thermal processes and methods using irradiation and ultrasound treatments can be defined as physical pretreatments. Mechanical treatments aim at particle size reduction by chipping, milling and grinding. Comminution techniques include *e.g.* wet disk milling, ball milling and hammer milling (da Silva *et al.*, 2010; Mani *et al.*, 2004). Milling is also often combined with other pretreatments (Gao *et al.*, 2012; Lin *et al.*, 2010; Teramoto *et al.*, 2008). In addition to particle size reduction, decrease in crystallinity of the lignocellulosic materials has been observed (Chang and Holtzapple, 2000; da Silva *et al.*, 2010). High energy requirements are typical for efficient mechanical treatments, thus decreasing the economic feasibility of these methods.

Biological pretreatment uses microorganisms, mainly brown, white or soft-rot fungi, to degrade lignin and hemicellulose. Biological pretreatments have been applied for gramineous plants such as wheat straw (Hatakka, 1983; Lopez-Abelairas *et al.*, 2012; Zeng *et al.*, 2011) and corn stover (Song *et al.*, 2013). The suggested advantages of biological methods are low energy consumption and the absence of chemical demands, mild process conditions and low capital costs (Alvira *et al.*, 2010; Talebnia *et al.*, 2010). On the other hand, pretreatment with microorganisms is slow and significant yield losses occur. Addition of metal ions and Tween has been suggested to improve the pretreatment efficiency by increasing the production of lignin-degrading enzymes (Song *et al.*, 2013; Zeng *et al.*, 2011). Combination of biological pretreatment with other pretreatments such as organosolv cooking is also one proposed option to improve its efficiency (Itoh *et al.*, 2003).

1.5.2 Thermo-chemical pretreatments

Chemical processes alone are not very often applied and chemicals are usually combined at least with thermal processing. Acid pretreatments are typically carried out with dilute mineral acids such as sulphuric acid at temperatures ranging from 120 to 200°C (Nguyen *et al.*, 2000; Saha *et al.*, 2005). The residence time can vary from minutes to an hour. Acid degrades hemicellulose, cellulose and lignin and toxic degradation products can be formed (Chundawat *et al.*, 2010; Larsson *et al.*, 1999). Alkaline treatments (Section 1.5.3.2) are usually carried out at lower temperatures and using longer residence times than acid pretreatments. Ozone treatments are purely oxidative treatments that solubilize lignin and small amounts of hemicellulose at room temperature and normal pressure, but their efficiency appears to be limited to grasses or agricultural residues such as sugar cane bagasse, wheat straw or rye straw (García-Cubero *et al.*, 2012; Lee *et al.*, 2010; Travaini *et al.*, 2013). Due to the low temperatures in ozone treatment, formation of toxic compounds such as furfural or hydroxymethylfurfural is low (Travaini *et al.*, 2013). The amount of ozone required in the treatment is important for the process

economy. Oxidative chemicals can be applied as such, or more usually combined with alkaline treatments (Section 1.5.3.3).

One attractive chemical pretreatment method is organosolv pretreatment, which includes systems containing combinations of solvents *e.g*. ethanol, acetic acid, acetone, butanol or formic acid (Arato *et al.*, 2005; Huijgen *et al.*, 2010; Rio *et al.*, 2010; Teramoto *et al.*, 2009; Vanderghem *et al.*, 2011) and acidic, neutral or alkaline catalysts (Mesa *et al.*, 2010; Park *et al.*, 2010). Treatment temperature can vary from 80°C to 210°C and time from 20 min to a few hours. Organosolv pretreatments remove extensively lignin and almost completely hemicelluloses (Perez-Cantu *et al.*, 2013; Rio *et al.*, 2010; Teramoto *et al.*, 2009). They are also effective for biomass with a high lignin content. Treatments with organic acids cause esterification of cellulose (Pan *et al.*, 2006; Zhao *et al.*, 2009). At the same time, degradation products such as furfural and hydroxymethylfurfural, acetic acid and formic acid are formed (Huijgen *et al.*, 2010; Rio *et al.*, 2010; Teramoto *et al.*, 2009). Disadvantages of organosolv treatments are the high cost of reactors and solvents that need to be recycled.

Use of ionic liquids (ILs) as solvents for pretreatment of cellulosic biomass has recently received much attention. ILs are salts, typically composed of large organic cations and small inorganic anions, which exist as liquids at temperatures below 100°C (Dadi *et al.*, 2007; Stark, 2011). Their solvent properties can be varied by adjusting the anion and the cation. In the pretreatment with ionic liquids, biomass is dissolved in IL and then precipitated by addition of antisolvent *e.g.* water (Dadi *et al.*, 2006; Dadi *et al.*, 2007). After washing, the precipitate can be hydrolysed by cellulases. Carbohydrates and lignin can be dissolved in ILs simultaneously. The first studies were carried out with pure crystalline cellulose (Dadi *et al.*, 2006; Dadi *et al.*, 2007; Kuo and Lee, 2009), but pretreatments with lignocellulosic biomass, even wood, have also been reported (Kilpeläinen *et al.*, 2007; Liu and Chen, 2006; Singh *et al.*, 2009). The pretreatment can be carried out at moderate temperatures (100–120°C) and the treatment time ranges from 30 min to 3 h. The disadvantages of ILs in general are related to the price, purity, toxicity and recyclability of the ILs (Stark, 2010). The ILs also have limited compatibility with cellulases, which hinders their use as a pretreatment for enzymatic hydrolysis (Datta *et al.*, 2010).

1.5.3 Physico-chemical pretreatments

Combinations of physical and chemical methods are currently regarded as the most promising approaches. These technologies include *e.g.* steam pretreatments (Section 1.5.3.1), ammonia fibre explosion (AFEX), and wet oxidation (Section 1.5.3.3).

The AFEX process includes ammonia treatment and steam explosion at temperatures from 90 to 180°C and high pressure (Garlock *et al.*, 2012; Holtzapple *et al.*, 1991). AFEX is a dry process and does not produce any separate liquid stream. In contrast, other pretreatment methods yield separate solid and liquid fractions. The main effects of AFEX are physical disruption, swelling and decrys-

tallization of cellulose and repositioning of cell wall decomposition products (Chundawat *et al.*, 2011a). Low formation of inhibitors has also been reported (Chundawat *et al.*, 2010). Recovery of ammonia is required for an economical process. Ammonia recycle percolation (ARP) and soaking in aqueous ammonia (SAA) are different variants of ammonia treatment occurring in aqueous solution (Kim *et al.*, 2003; Yoo *et al.*, 2013).

The following sections present a deeper description of steam treatments, alkaline, and alkaline oxidative pretreatments that were also studied in this work.

1.5.3.1 Steam pretreatments

Steam pretreatment is a general term used in this thesis to describe physico-chemical technologies exploiting high pressure steam. These methods include steam explosion and hydrothermal and liquid hot water treatments. The main difference between steam explosion and hydrothermal or hot water treatments is that steam explosion generally involves a higher dry matter content, whereas hydrothermal and liquid hot water treatments have more liquid water present during the pretreatment (Allen *et al.*, 2001; Bondesson *et al.*, 2013; Kristensen *et al.*, 2008; Kumar *et al.*, 2010). Steam pretreatments involve treatment of the biomass at high temperature (160–260°C, typically 180–215°C) and pressure. High-pressure and high-temperature steam is introduced into a reactor containing lignocellulosic material. The treatment time can vary from seconds to several minutes (typically 5 to 15 minutes). In steam explosion the pressure is rapidly released, causing the steam to expand in the lignocellulosic matrix, whereas in hydrothermal and liquid hot water treatments a similar explosion is not described in the literature, although the pressure will drop after the reaction. Steam treatments can be intensified with chemical catalysts such as SO_2 or sulphuric, acetic, phosphoric or lactic acid (Linde *et al.*, 2006; Pitarelo *et al.*, 2010; Tengborg *et al.*, 1998; Xu *et al.*, 2009b). Acid increases the solubilisation of hemicellulose sugars and it improves the enzymatic hydrolysis of the solid fraction (Hahn-Hägerdal *et al.*, 2006). In steam pretreatments acids are also formed from acetyl groups present in biomass, causing autohydrolysis (Fengel and Wegener, 1989). Steam treatment can be applied for a wide range of raw materials including agricultural residues, softwoods and hardwoods (Bondesson *et al.*, 2013; Kumar *et al.*, 2010; Schütt *et al.*, 2011). Softwoods usually require more drastic steaming conditions than hardwoods and straws.

During the steam pretreatment hemicellulose is solubilised, and after the treatment it is located in the liquid phase as oligomers and monomeric sugars (Cui *et al.*, 2012; Østergaard Petersen *et al.*, 2009). Increase of accessibility of lignocellulosic biomass by steam treatments appears to be mainly due to the removal of hemicelluloses (Mosier *et al.*, 2005b). On the other hand, steam treatments have been shown to have a major effect on the distribution and the chemical structure of lignin (Marchessault, 1991; Ramos, 2003). Degradation of β-ether linkages of lignin and of the chemical bonds in lignin-carbohydrate complex (LCC) have been reported, as well as chemical modifications of lignin. Depolymerisation of lignin in lower severity steam treatments can occur, whereas in more severe conditions

repolymerization has been reported (Li *et al.*, 2007; Wang *et al.*, 2012). Most of the lignin remains insoluble but the solubility of syringyl lignin increases more than that of guaiacyl lignin (Shimizu *et al.*, 1998) and the S/G ratio has been shown to decrease in steam explosion (Rahikainen *et al.*, 2013). Steam explosion has also been reported to increase the oxygen/carbon–ratio and thus to remove surface lignin and reduce the hydrophobicity of biomass (Kumar *et al.*, 2009; Negro *et al.*, 2003).

Structurally, steam treatments reduce biomass particle size and increase pore volume after explosive decompression (Mosier *et al.*, 2005b). Increased crystallinity index (CrI) of the substrate has been reported due to removal of hemicellulose (Kumar *et al.*, 2009). Significant decrease in the DP of cellulose in addition to shortening of cellulose fibrils has also been observed.

Steam treatments cause partial hemicellulose degradation and the generation of inhibitors that may affect the subsequent hydrolysis and fermentation (Allen *et al.*, 2001; Kim *et al.*, 2013; Palmqvist *et al.*, 1996). The major inhibitors generated during steam pretreatments are furan derivatives (furfural and hydroxymethylfurfural), weak acids and fragments from lignin. Treatment temperature and the amount of acid should be optimized to minimize the formation of inhibitors. Varying steam treatment temperature profiles (Monavari *et al.*, 2010) and two step steam explosion have been developed to decrease degradation of pentoses and formation of inhibitors (Söderström *et al.*, 2003; Zhang *et al.*, 2012). Mosier *et al.* reported that maintaining the pH value in the hydrothermal method between 4 and 7 during the process decreased the amount of inhibitors formed (Mosier *et al.*, 2005a).

1.5.3.2 Alkaline pretreatments

Alkaline treatments are widely used in the fractionation of wood in pulp and paper manufacturing. The dominating alkaline pulping method is sulphate cooking, the Kraft process, that is carried out using sodium hydroxide and sodium sulphide as cooking chemicals (Fengel and Wegener, 1989). The temperature is typically 155–175°C and the pressure 7–11 bar. The cooking time varies between 2 and 5 hours. For lignocellulose pretreatments various alkaline treatments have also been developed using varying temperatures and times. Alkaline pretreatment can be performed at room temperature and pressure conditions, when the time ranges from hours to days (Kim and Holtzapple, 2006; Park and Kim, 2012; Sharma *et al.*, 2013). The treatment time can be shortened to 0.5–10 hours by applying higher temperatures (85–160 °C) (Chang *et al.*, 1998; Chen *et al.*, 2013; Sharma *et al.*, 2013). Alkaline treatments can be combined with oxidative conditions (see Section 1.5.3.3) to improve the efficiency of high lignin content materials. Sodium, potassium, calcium and ammonium hydroxides as well as sodium carbonate are possible chemicals for alkaline pretreatments (Chen *et al.*, 2013; Jin *et al.*, 2013; Park and Kim, 2012; Sharma *et al.*, 2013). Typically, chemical dosages are high, with a range as high as 5–100% of biomass (w/w). High chemical consumption in alkaline pretreatments decreases the profitability of the method (Chen et al., 2013) and thus recycling of alkali is needed.

Alkaline treatments cause significant changes in the chemical composition of lignocellulose. They have been reported to dissolve lignin (Kim and Holtzapple, 2006; Kumar *et al.*, 2009; Sun *et al.*, 1995) and to cause the depolymerization of lignin molecules by cleavage of inter-molecular α- and β-aryl ether linkages, which essentially contributes to lignin degradation (Gierer, 1982; Gierer and Noren, 1980). In addition to lignin, dissolution of hemicellulose has also been observed (Jin *et al.*, 2013; Sun *et al.*, 1995). Alkaline pretreatments have also been shown to remove acetyl-substituents from hemicellulose (Kim and Holtzapple, 2006; Selig *et al.*, 2009), which has been reported to improve xylan and cellulose accessibility and hydrolysis by xylanases and cellulases (Grohmann *et al.*, 1989; Kumar and Wyman, 2009; Selig *et al.*, 2009). On the other hand, deacetylation and removal of arabinosyl residues might increase the adsorption of xylan to cellulose (Kabel *et al.*, 2007). Alkaline conditions also cause modification of uronic acid groups in xylan (Teleman *et al.*, 1995).

Due to its crystallinity and linearity, cellulose is more resistant to chemical changes than hemicellulose. However, degradation of cellulose also occurs in alkaline conditions by peeling from the reducing ends and alkaline hydrolysis of glycosidic bonds (Theander, 1980). The degree of polymerization of cellulose has been reported to decrease after NaOH and lime treatments (Chundawat *et al.*, 2011b; Kumar *et al.*, 2009; Mittal *et al.*, 2011).

Structural changes of lignocellulose are also remarkable after alkaline treatments. The crystallinity of lignocellulosic materials has been reported to increase due to removal of less crystalline lignin (Kim and Holtzapple, 2006; Kumar *et al.*, 2009). The ratio of amorphous and crystalline cellulose has been shown to increase in lime treatment of corn stover and poplar (Kumar *et al.*, 2009). Swelling and crystal change from cellulose I to cellulose II occurs during NaOH treatments, whereas ammonia treatments cause change from cellulose I to cellulose III_I or III_{II} (Mittal *et al.*, 2011; O'Sullivan, 1997).

1.5.3.3 Alkaline oxidative pretreatments

Alkaline oxidative conditions are applied in pulp bleaching processes, although they could also be applied for pretreatment or fractionation of biomass. Different kinds of oxidative chemicals such as ozone, peroxides and peracetic acid, or oxygen can be used to modify lignocellulose in alkaline conditions. Hydrogen peroxide and peracetic acid are strong oxidative chemicals that have been applied in alkaline conditions at temperatures ranging from room temperature to 150°C (Ayeni *et al.*, 2013; Saha and Cotta, 2007; Zhao *et al.*, 2007). The efficiency of alkaline treatments can be improved in high-lignin materials when pressurized oxygen and catalysts are added (Chang *et al.*, 2001; Hakola *et al.*, 2010). In wet oxidation the lignocellulosic material is treated with water and high pressure oxygen or air (10–12 bars) at elevated temperatures (120–200°C). The wet oxidation can also be carried out either in acidic or partly alkaline conditions in the presence of oxygen (Bjerre *et al.*, 1996).

Similarly to alkaline treatment, alkaline oxidative conditions deacetylate hemicellulose and cause delignification, but the effect has been reported to be more intense in oxidative conditions (Kim and Holtzapple, 2006). Lignin is at least partly solubilized and degraded by oxidation reactions (Ayeni *et al.*, 2013; Schmidt and Thomsen, 1998; Zhao *et al.*, 2007). The heating value of lignin is decreased due to the oxidation. Hemicellulose is also partly solubilized and removed, which increases cellulose accessibility. Usually the oxidant is not selective and losses of hemicellulose and cellulose can occur. Inhibitors can be formed as soluble aromatic compounds are produced by degradation of lignin. However, no furfural or hydroxymethylfurfural has been reported to be formed by peroxide or wet oxidation in alkaline conditions (Bjerre *et al.*, 1996; Saha and Cotta, 2007).

1.6 Techno-economic considerations for sugar platform biorefineries

Polysaccharides in the lignocellulosic raw materials can be hydrolysed to sugars and fermented to fuels and biochemicals in so-called sugar platform biorefineries. These biorefineries require raw materials that are processed by pretreatment, hydrolysis and fermentation before the product recovery. The requirements and the challenges of these processes are diverse. Raw materials should be utilized efficiently in the process, the costs of processing should be low, and products should be produced in high yields. To reach these targets, various economic and technical aspects need to be considered in the development of feasible processes.

1.6.1 Raw materials

The availability of a cheap raw material in sufficient amounts is a very important issue for feasible biorefineries. Various raw materials such as wood residues, straws, corn stover, sugar cane bagasse, wood waste and switchgrass are regarded as potential substrates and are used by the current commercial and demonstration plants for lignocellulosic biofuel production in Europe and the USA (Balan *et al.*, 2013). Table 3 presents the annual production of selected agricultural and forest residues that are available for biorefineries.

Costs related to raw materials are significant with respect to the process economy of a sugar platform biorefinery. In ethanol production feedstock costs can be up to 40% of the total production costs (Gnansounou and Dauriat, 2010; Hamelinck *et al.*, 2005; Table 4). The prices of feedstocks vary according to the type of feedstock, storage requirements, location, season, local supply-demand conditions and transportation needed. For forest residues, the transport costs are considered to dominate the costs (de Wit and Faaij, 2010). The harvest of forest residues within a 100–200 km radius of the end use appears to be economical and costs of 30–86 €/t dry matter including the transportation costs have been reported in Europe (Asikainen *et al.*, 2008). For agricultural residues approximately 100 km radius has been considered economical (Kudakasseril Kurian *et al.*, 2013; Lindh *et*

al., 2009). In Finland the price of straw was estimated to be 52 €/t including the costs of 50 km transportation (von Weymarn, 2007).

Table 3. Annual production of lignocellulosic residues.

Raw material	Area	Production (dry Mt/a)	References
Agricultural residues (corn stover, straw)	Europe	217	Kim and Dale, 2004
	USA, Canada	103–214	Kim and Dale, 2004; Mabee *et al.*, 2011
	World	1370	Kim and Dale, 2004
Sugar cane bagasse	Brazil	84	Corrêa do Lago *et al.*, 2012
	World	180–210	Gudoshnikov, 2009; Kim and Dale, 2004
Wheat straw	Europe	133	Kim and Dale, 2004
	World	354–430	Kim and Dale, 2004; Talebnia *et al.*, 2010
Forest residues	USA, Canada	72	Mabee *et al.*, 2011
	Europe	90	Asikainen *et al.*, 2008

1.6.2 Pretreatment

Pretreatment of lignocellulosic biomass is a crucial step in a sugar platform biorefinery to modify the raw material into a hydrolysable form. Pretreatment also has a significant impact on the process economy. The capital costs of pretreatment consist of reactors and equipment required in a pretreatment process and in recycling of chemicals. The properties of treatment chemicals and the applied conditions affect the materials of construction needed. The proportion of pretreatment in the total capital costs varies between 2 and 27% depending on the pretreatment method (Table 4). Although the differences are significant, the total capital costs of lignocellulosic ethanol processes show only relatively small differences between pretreatments within each study (Eggeman and Elander, 2005; Kazi *et al.*, 2010; Tao *et al.*, 2011). The differences between different studies are more significant. The assumed raw material prices, enzyme costs and other process assumptions are proposed to be the main reason for differences (Kazi *et al.*, 2010).

Table 4. Techno-economical evaluation of the most promising pretreatment methods.

Pretreatment method	Development stage [a]	Applicability to different feedstocks	Raw material used in economic evaluation	Raw material cost (% of MESP)	Pretreatment (% of total capital costs)	MESP (EUR/l EtOH)[b]	Reference
Dilute acid	Commercial	Yes	Corn stover	22	12	0.35	Eggeman and Elander, 2005
			Corn stover	32	10	0.76	Kazi *et al.*, 2010
			Switchgrass	39	23	0.62	Tao *et al.*, 2011
			Corn stover	34	7	0.48	Humbird *et al.*, 2011
Steam explosion	Commercial	Yes	Switchgrass	34	19	0.66	Tao *et al.*, 2011
			Sugar cane bagasse	nd	nd	0.63–1.25	Macrelli *et al.*, 2012
			Spruce	34–37	nd	0.47–0.51	Wingren *et al.*, 2008
Hot water	Demonstration	No	Corn stover	24	2	0.44	Eggeman and Elander, 2005
			Corn stover	33	2	1.02	Kazi *et al.*, 2010
			Switchgrass	39	11	0.74	Tao *et al.*, 2011
Alkaline (Lime)		Yes/No	Corn stover	40	14	0.43	Eggeman and Elander, 2005
			Switchgrass	38	27	0.71	Tao *et al.*, 2011

Pretreatment method	Development stage [a]	Applicability to different feedstocks	Raw material used in economic evaluation	Raw material cost (% of MESP)	Pretreatment (% of total capital costs)	MESP (EUR/l EtOH)[b]	Reference
Organosolv	Demonstration	Yes	Softwood	nd	nd	1.58	Hagman *et al.*, 2012
AFEX	Laboratory	No	Corn stover	24	12	0.38	Eggeman and Elander, 2005
			Switchgrass	39	16	0.62	Tao *et al.*, 2011
			Corn stover	34	8	0.82	Kazi *et al.*, 2010
Aqueous ammonia	Commercial	No	Corn stover	24	23	0.44	Eggeman and Elander, 2005
			Switchgrass	38	23	0.92	Tao *et al.*, 2011

[a] Advanced Ethanol Council, 2013; Balan *et al.*, 2013

[b] MESP = Minimum ethanol selling price; prices were adjusted to 2013 EUR value using consumer price index data (US Bureau of Labor Statistics, 2013) and 1 EUR=1.33 US$ and 1 EUR = 8.7 SEK.

AFEX = ammonia fibre explosion

nd = no data

Operating costs of pretreatment typically include costs of chemicals that are used in pretreatments and neutralization, and steam and other utilities. Besides the direct costs, pretreatment also impacts on the costs of the other process steps such as hydrolysis and fermentation, waste treatment and product separation. For example, the dry matter content in pretreatment affects the product concentration and separation. Similarly to total capital costs, economic evaluations showed that the obtained minimum ethanol selling prices (MESP) by the pretreatment methods had only relatively small differences within each study, whereas the differences between different economic evaluations are significant (Table 4). It appears that the low costs in pretreatment are counterbalanced by low product yields or increased costs in other steps such as hydrolysis and product recovery. Organosolv treatment, which is considered to have relatively high investment costs of reactors and solvent recycling, has not been intensively studied with respect to the economy of ethanol production. However, it has been estimated that the total capital cost for ethanol organosolv treatment is 1.4-fold and the MESP 2.6-fold compared to steam explosion, and high additional value from organosolv-lignin and other by-products was required to achieve a similar MESP as for steam explosion (Hagman *et al.*, 2012).

The costly pretreatment step is one factor that hinders the commercialization of lignocellulosic ethanol. Integration with other production facilities such as sugarcane biorefineries, pulp and paper processes or repurposing of closing Kraft mills are potential alternatives to improve energy efficiency and to decrease the high investment costs related to cellulosic ethanol (Corrêa do Lago *et al.*, 2012; Fornell *et al.*, 2012; Phillips *et al.*, 2013). The risk for investment might also decrease as a result of experience obtained with the commercial scale plants that will soon start production.

1.6.3 Process configurations for hydrolysis and fermentation

Hydrolysis and fermentation are the key processing steps in a sugar platform biorefinery. There are several process configurations for enzyme production, hydrolysis and fermentation: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP). In SHF, hydrolysis and fermentation can be carried out in different conditions and reactors and there is no need to compromise between different optimal hydrolysis and fermentation conditions. Separate reactors have also been suggested to improve process control (Hamelinck *et al.*, 2005). However, the accumulation of end products can reduce the efficiency of hydrolysis in SHF. Improved SHF can potentially be obtained by the use of higher temperatures in the hydrolysis and by selecting β-glucosidases with decreased product inhibition (Teugjas and Väljamäe, 2013).

In simultaneous saccharification and fermentation the hydrolysis and fermentation occur at the same time in the same reactor, thus decreasing the product inhibition of enzymes and reducing investment costs (Drissen *et al.*, 2009; Wingren *et*

al., 2003). The limitation in SSF is that the optimal temperatures of enzymes in the hydrolysis are different from that of fermentation with conventional yeasts. Thermostable microorganisms have been suggested to enable operation in higher or varied temperatures, thus improving the hydrolysis and ethanol production in SSF (Kang *et al.*, 2012). A prehydrolysis before SSF can provide a possibility to operate in elevated and optimal conditions in hydrolysis and to decrease viscosity before fermentation (Szijarto *et al.*, 2011; Viikari *et al.*, 2007). Gradual or stepwise addition of substrate *i.e.* fed-batch processing is also one option to decrease the viscosity, content of inhibitors, and enzyme inhibition (Liu *et al.*, 2010; Zhang *et al.*, 2010).

In SHF and SSF, enzymes for the hydrolysis are produced separately, whereas in CBP at least some of the enzymes are produced by the fermenting organism. CBP combines the steps of enzyme production, polysaccharide hydrolysis, and fermentation of sugars into one unit operation performed by one or several organisms. The technology is still under development for production of cellulosic ethanol, but CBP is foreseen to provide significant savings in production costs (Lynd, 2005).

Hydrolysis and fermentation at high dry matter is a requirement to achieve high product concentrations without further concentration steps and to decrease reactor volumes. High dry matter hydrolysis produces high concentrations of glucose and cellobiose, which inhibit cellulases (Gruno *et al.*, 2004; Takagi, 1984). On the other hand, high dry matter and the high viscosity can cause problems in mixing and in mass and heat transfer (Pimenova and Hanley, 2003). Hydrolysis at high dry matter content can be increased by efficient mixing (Palmqvist *et al.*, 2011). Different kinds of reactors providing efficient mixing at high dry matter content have been developed (Jorgensen *et al.*, 2007; Zhang *et al.*, 2010).

Enzyme costs are still important in the production of lignocellulosic ethanol, although the costs have been significantly decreased in the past few years. It has been estimated that enzyme costs can be 16% of ethanol production costs in a process using corn stover raw material and dilute acid pretreatment (Humbird *et al.*, 2011). Decreased enzyme costs can be obtained by development of improved production strains producing enzymes more efficiently or with improved properties and composition of enzymes (Gusakov, 2011; Zhang *et al.*, 2006). Cheaper raw materials in enzyme production or on-site production of enzymes are also possible alternatives to decrease enzyme costs (Humbird *et al.*, 2011). Enzyme consumption can be decreased by efficient pretreatments, by improved enzymes and enzyme mixtures working synergistically (see Section 1.3.4), by adding enzyme components such as LPMOs (see Section 1.3.2), by consolidated bioprocessing, or by enzyme recycling.

Enzyme recycling has been studied for many years. The main approaches are recycling of enzymes attached to insoluble solids (Ramos *et al.*, 1993; Weiss *et al.*, 2013) or recycling of desorbed enzymes in the liquid phase (Tu *et al.*, 2007; Wu *et al.*, 2010). The main difficulties with respect to recycling are the inactivation and binding of cellulases to lignin (Rahikainen *et al.*, 2011; Tu *et al.*, 2009). Enzyme desorption can be improved by efficient delignifying pretreatments (Lee *et al.*, 1995; Varnai *et al.*, 2011b) and by the use of surfactants (Tu *et al.*, 2007). In addition, it appears that the cellulases without CBM can hydrolyse as efficiently as

intact enzymes in high dry matter content, but with less binding to the substrate, thus making recycling possibly easier (Varnai *et al.*, 2013).

1.6.4 Multi-product biorefineries

Future biorefineries can be efficient production plants similar to oil refineries that utilize all the raw material components and produce various kinds of products with minimal or no waste. Typically in lignocellulosic biorefineries, sugars are used to produce cellulosic ethanol or other products and the residues are burned to generate steam and electricity. For example in ethanol production the process costs are high (see MESP in Table 4), whereas the ethanol selling price is relatively low. Therefore the process should also produce other products in addition to ethanol. The combined production of biofuels and high-value products in a biorefinery would enhance the economy of biomass processing (Zhang, 2008). To be economically feasible, multi-product biorefineries should have well developed fractionation technology to separate biomass into valuable components or their intermediates. As integral parts of a biorefinery, efficient recovery and recycling of chemicals, water recirculation as well as waste water treatment are also needed.

In addition to ethanol, various other products such as other alcohols, polymers, lactic acid, and glutamic acid can be derived from cellulose in either biochemical or chemical conversion processes (Menon and Rao, 2012). Hemicellulose can be fermented to ethanol, other alcohols or used for the production of ferulic acid or furfural. Hemicellulose can also be utilized in a polymeric form in different applications. Although lignin is in many cases burned in biorefinery concepts, there are various other options for lignin. For example, lignin-derived compounds can be used in resins, as a substitute for polymeric materials, as a glue in composites, as dispergents, and for the production of syngas, hydrocarbons, formaldehyde, phenols, oxidised products or carbon fibre (Menon and Rao, 2012; Zhang, 2008). In addition to biomaterials, chemicals and fuels, biorefineries can produce energy and electricity to the grid.

In the near future, the experience obtained with the commercial scale plants will provide essential knowledge about the bottlenecks in the processes and properties of the process streams. As a result of this learning and experience, improved equipment will be developed. Together with cheap and adequate renewable lignocellulosic raw materials, improved fractionation technologies, energy and process integration, decreased enzyme costs, and more valuable products, future biorefineries can become efficient, profitable and sustainable production facilities.

2. Aims of the present study

The overall aim of the work was to develop technologies for future biorefineries by improving the enzymatic hydrolysis of various lignocellulosic materials, by developing pretreatment methods, and by optimizing enzyme mixtures for the hydrolysis of pretreated raw materials. More specifically, the aims were:

1. To analyse the suitability of reed canary grass and barley straw – both potential lignocellulosic feedstocks in northern climates – for enzymatic hydrolysis and ethanol production in a lignocellulosic biorefinery.

2. To develop an improved alkaline oxidative pretreatment method that fractionates lignocellulose efficiently, is flexible with respect to different raw materials and produces material that can be hydrolysed efficiently by enzymes.

3. To optimize enzyme mixture composition for the hydrolysis of pretreated materials in order to decrease enzyme dosage in hydrolysis.

3. Materials and methods

A summary of the materials and methods used in the thesis is presented in this Section. Detailed descriptions can be found in the original publications I–IV.

3.1 Lignocellulosic substrates and pretreatments

3.1.1 Preparation of materials

Various lignocellulosic raw materials were pretreated by hydrothermal treatment, steam explosion, alkaline oxidation or catalytic oxidation. A summary of the pretreatment conditions applied for different raw materials is presented in Table 5. More detailed descriptions can be found in publications I–IV.

Table 5. Studied raw materials and pretreatments.

Raw material	Pretreatment	Pretreatment conditions (Catalyst, temperature, reaction time, other parameters)	Publication
Reed canary grass (spring or autumn harvest)	Steam explosion	2% SO_2, 190°C, 5 min	I
Barley straw	Steam explosion	2% SO_2, 190–200°C, 5 min	I
Wheat straw	Hydrothermal	3 g/l acetic acid, 190°C, 12 min	IV
Sugar cane bagasse	Steam explosion	0.5% H_2SO_4, 200°C, 5 min	III, IV
	Alkaline oxidation	5.4 mol Na_2CO_3/kg substrate (d.m.), 10 bar O_2, 120°C, 20 h, reactor fill 10% of total volume	III, IV
Norway spruce	Steam explosion	2% H_2SO_4, 205°C, 15 min or 2% SO_2, 215°C, 5 min	III
	Alkaline oxidation	5.4 mol alkali /kg substrate (d.m.), 10 bar O_2, 120–140°C, 4–20 h, alkali Na_2CO_3, KOH, NaOH or $Ca(OH)_2$, reactor fill 10%–75%)	II, III
	Catalytic oxidation	5.4 mol Na_2CO_3 /kg substrate (d.m.), 120–140°C, 1–20 h, reactor fill 10%–75% of total volume	II, III
Birch	Steam explosion	0.5% H_2SO_4, 200°C, 5 min	III
	Alkaline oxidation	5.4 mol Na_2CO_3/kg substrate (d.m.), 10 bar O_2, 120°C, 20 h, reactor fill 10% of total volume	III

3.1.2 Analytical procedures for biomasses

Several methods were applied to characterize the chemical composition of raw materials and pretreated materials (Table 6).

Table 6. Analysis methods to characterize the raw materials, pretreated solids and dissolved material.

Analysis	Description of the method	Publication
Characterization of raw materials and pretreated solids		
Carbohydrate content and composition of monosaccharides	Two step sulphuric acid hydrolysis (first step 70%, second step 4% H_2SO_4) of polysaccharides to monosaccharides followed by high performance anion exchange chromatography (HPAEC) of monosaccharides with pulse amperometric detection (PAD)	I–IV
Lignin content	Gravimetric analysis of the residue remaining after the two step sulphuric acid hydrolysis (Klason lignin) combined to a UV-spectroscopic analysis of acid soluble lignin	III
Ash content	Gravimetric analysis of the residue after burning at 550°C	III
Extractives	Gravimetric analysis of the extract obtained by Soxhlet extraction with heptane and evaporation of the solvent	III
Characterization of material dissolved in pretreatments		
Carbohydrate content and composition of monosaccharides	Dilute sulphuric acid (4%) or enzymatic hydrolysis of oligosaccharides to monosaccharides followed by HPAEC-PAD analysis	I–III
Carboxylic acid content and composition	Capillary electrophoretic separation of samples and photodiode array UV detection	II
Lignin content	UV spectroscopic detection at 280 nm using an absorptivity of 20 l/g/cm and gravimetric analysis by precipitation of lignin by acidification	II
Molecular weight distribution	Size exclusion chromatography with UV and refractive index (RI) detection	II

3.2 Enzymatic hydrolysis

3.2.1 Enzymes

The studied commercial, pre-commercial and purified enzymes are presented in Table 7 and in Table 9. The protein content of the enzyme samples was measured using a Bio-Rad protein assay kit based on the Lowry method (Lowry *et al.*, 1951). Bovine serum albumin was used as a standard. The enzyme activity assays that were used to characterize the enzymes are summarized in Table 8.

Table 7. Commercial enzymes used in the hydrolysis of lignocellulosic raw materials.

Commercial product name	Manufacturer	Main activities	Protein content (mg/ml)	Studied in publication
Celluclast 1.5L	Novozymes	cellulases, 69 FPU/ml	125	I, III
Novozym 188	Novozymes	β-glucosidase, 6360 nkat/ml	181	I, III
Cellic Ctec2	Novozymes	cellulases, 124 FPU/ml	183	III, IV
Cellic Htec	Novozymes	xylanase, 98300 nkat/ml	36	III, IV
Spezyme CP	Genencor	cellulases, 64 FPU/ml	111	I
Econase CE	Roal	cellulases, 69 FPU/ml	110	I

Table 8. Enzyme activity assays to characterize enzymes.

Activity method	Substrate	Reference
Total cellulase activity	Filter paper	Ghose, 1987
β-Glucosidase activity	*p*-nitrophenyl-β-glucopyranoside	Bailey and Linko, 1990
Endoxylanase activity	Birch wood xylan	Bailey *et al.*, 1992

Table 9. Pre-commercial and purified enzymes used in the hydrolysis of lignocellulosic raw materials.

Enzyme	GH family	Origin	Abbreviation	Purification	Publication
Cellobiohydrolase I (EC 3.2.1.176)					
	Cel7A	*Trichoderma reesei*	TrCel7A (CBHI)	Suurnäkki *et al.*, 2000	I, IV
	Cel7A	*Thermoascus aurantiacus + CBM/TrCel7A*	TaCel7A (CBHI)	Heat treated at 60°C, 2 h/ purified according to IV	IV
	Cel7A	*Acremonium thermophilum*	AtCel7A (CBHI)	Heat treated at 60°C, 2 h	IV
Cellobiohydrolase II (EC 3.2.1.91)					
	Cel6A	*Trichoderma reesei*	TrCel6A (CBHII)	Suurnäkki *et al.*, 2000	I, IV
	Cel6A	*Chaetomium thermophilum*	CtCel6A (CBHII)	Heat treated at 60°C, 2 h / purified according to IV	IV
Endoglucanases (EC 3.2.1.4)					
	Cel7B	*Trichoderma reesei*	TrCel7B(EGI)	Suurnäkki *et al.*, 2000	I, IV
	Cel5A	*Trichoderma reesei*	TrCel5A (EGII)	Suurnäkki *et al.*, 2000	I, IV
	Cel5A	*Thermoascus aurantiacus + CBM/CtCel7A*	TaCel5A (EGII)	Heat treated at 60°C, 2 h	IV
β-glucosidase (EC (3.2.1.21)					
	Cel3A	*Aspergillus niger*	AnCel3A(βG)	Sipos *et al.*, 2010	IV
	Cel3A	*Thermoascus aurantiacus*	TaCel3A(βG)	Heat treated at 60°C, 2 h	IV
Xylanase (EC 3.2.1.8)					
	Xyn10A	*Thermoascus aurantiacus*	TaXyn10	Heat treated at 60°C, 2 h	IV
	Xyn11A	*Trichoderma reesei*	TrXyn11(XYLII)	Tenkanen *et al.*, 1992	I, IV
	Xyn11A	*Nonomurea flexuosa*	NfXyn11A	Heat treated at 60°C, 2 h	IV

3.2.2 Hydrolysis experiments

The hydrolysis experiments were typically carried out at 1% dry matter (d.m.) content, at 45°C, pH 5.0 in a 50 mM sodium acetate buffer for various time periods. For thermostable enzymes higher temperatures, 52–55°C were applied. In addition, hydrolysis at higher d.m. content (8–12%) was studied (Publications I, IV). The cellulase dosage was 2–20 FPU/g d.m (Publications I, III) or 4–15 mg protein/g d.m (Publications III, IV). β-Glucosidase dosage was 100–200 nkat/g d.m. Hydrolysis reactions were stopped by boiling samples for 5 min (Publications I, III, IV) or by adding 10 M NaOH (Publication IV). The hydrolysates were diluted to 1% d.m. content (when necessary) and supernatants were collected by centrifugation.

The release of soluble sugars in the enzymatic hydrolysis was monitored by analysing the reducing sugars by the DNS-method (Bernfeld, 1955) with glucose as a standard (Publications I, III, IV) or by high performance anion exchange chromatography (HPAEC) to detect the content of monosaccharides and oligosaccharides in the hydrolysates (Publications I, III).

The optimal enzyme mixtures were determined by statistically designed experiments using Modde software (Umetrics, Sweden) for hydrothermally pretreated wheat straw, alkaline oxidised bagasse and for steam exploded bagasse. The design of experiments is presented in Appendix 5 (Tables 1–4). After hydrolysis experiments the hydrolysis results were analysed and modelled by Modde as described in publication IV.

3.3 Ethanol fermentation

The fermentation of pretreated lignocellulosic substrates was carried out as simultaneous saccharification and fermentation at 10–12% d.m. content after 6–24 h prehydrolysis in oil lock shake flasks as described in publications I and III. Prehydrolysis was carried out with a commercial enzyme mixture Celluclast + Novozym 188 (10 FPU/g+100 nkat/g) or Cellic Ctec2+Htec (13.5 mg/g+1.5 mg/g). Prehydrolysed substrates were inoculated using 3.5 g/l of yeast strains VTT B-03339 or Red Star (LeSaffre). Fermentation was followed by measuring the mass loss due to formation of CO_2 and analysing ethanol from the broth by HPLC as described in publications I and III.

4. Results and discussion

4.1 Potential Nordic raw materials for a sugar platform biorefinery

The availability and the costs of raw materials are important factors in the feasibility of sugar platform biorefineries. Switchgrass and residues from the production of corn (*e.g.* corn stover) belong to the most studied gramineous raw materials for sugar platform biorefineries. In northern climates the growth conditions significantly limit the selection of potential raw materials. Cultivation of many grasses is not feasible, or even possible due to climate conditions. On the other hand, a commercial scale production plant requires a constant supply of raw materials irrespectively of the harvesting season. It is probable that several different raw materials are required to meet the needs in different production seasons. Thus, new feedstocks especially suitable for northern climates are required.

Reed canary grass (*Phalaris arundinacea L.*) is a perennial grass species that can be cultivated in different kinds of low value areas such as bogs after peat production, and in fields which are not needed for food production. The annual production can be 7–8 tons dry matter per hectare (Saijonkari-Pahkala, 2001). Reed canary grass has attracted considerable interest as an energy crop for the production of heat and electricity by combustion and as a cellulosic raw material for paper manufacturing (Hadders and Olsson, 1997; Saijonkari-Pahkala, 2001). Reed canary grass has also been proposed as a raw material for the production of ethanol (Belkacemi *et al.*, 1997; Dien *et al.*, 2006; Digman *et al.*, 2010) and biogas (Lakaniemi *et al.*, 2011). The cultivation of reed canary grass has been relatively limited and the cultivated area was approximately 15 000 ha in Finland in 2011 (TIKE, 2012), corresponding to an annual production of 0.1 Mt. Wheat straw and barley straw are important side streams from grain production in Europe and their production, 133 and 44 Mt/a, respectively, is significantly higher than the production of reed canary grass.

Reed canary grass and barley straw were found to have a high carbohydrate content, which is essential for a raw material in a sugar platform biorefinery (Publication I). The carbohydrate contents of the studied reed canary grasses were 56–64%. Similar values have also been reported in the literature (Table 10; Finell *et al.*,

2011), and the value is comparable to the contents of wheat straw (57–59%) and barley straw (56–68%). The glucose and xylose yields of reed canary grass after steam explosion and enzymatic hydrolysis were 82–89% and 87–94%, respectively, whereas for barley straw the yields were slightly higher, 90% and 97%. The yields reported in the literature for wheat and barley straws are at a similar level. The fermentation yields from glucose were 82% and 74% for pretreated reed canary grass and barley straw, respectively. These results were similar to those reported in the literature for reed canary grass, barley and wheat straws. Due to the relatively high pentose content, high potential improvement in ethanol concentration could be obtained by using a yeast that could ferment pentoses efficiently.

In Finland, reed canary grass is typically harvested in the spring for combustion purposes. When harvested in the spring, the water content of the biomass has decreased to a level enabling storage without additional drying. This so called delayed harvesting also supports the density and survival of the grass plantation (Saijonkari-Pahkala, 2001). On the other hand, dry matter losses as well as sugar losses occur during the winter time (Hadders and Olsson, 1997; Landström *et al.*, 1996). The ash content of the biomass decreases, whereas the proportion of stem and fibre content increases during the winter (Pahkala and Pihala, 2000). Consistently with this, the cellulose content of reed canary grass harvested in the spring was significantly higher than that of the autumn-harvested crop (Publication I). On the other hand, the autumn-harvested crop contained fructose, which may have originated from water soluble fructan polysaccharides or from sucrose (Dien *et al.*, 2006). The degree of hydrolysis of reed canary grass harvested in the spring was higher than that of the autumn-harvested crop, probably due to more efficient xylan removal in the pretreatment. Ageing in the field during winter might also have brought about chemical and physical changes improving the efficiency of the steam explosion.

Results indicated that reed canary grass and barley straw are potential lignocellulosic feedstocks for a sugar platform biorefinery. The reed canary grass harvested in the spring had a higher degree of hydrolysis than the autumn-harvested crop. Thus, in addition to the other advantages of the spring harvest, reed canary grass harvested in the spring is a favourable raw material for a sugar platform biorefinery. Other grasses and reeds, such as common reed or surplus forage, would be interesting options for future studies.

Table 10. Reed canary grass, wheat straw and barley straw as raw materials for sugar platform biorefineries.

Raw material		Carbohydrate content (% of d.m.)	Yield (%) [a]		Ethanol yield (%) [b]	Reference
			Glucose	Xylose		
Reed canary grass	**Autumn**	56	82	94	nd	Publication I
	Spring	64	89	87	82	Publication I
		52–60	nd	nd	83	Dien *et al.*, 2006; Digman *et al.*, 2010
Wheat straw		57–59	75	69	93[c]	Østergaard Petersen *et al.*, 2009; Thomsen *et al.*, 2008
Barley straw		68	90	97	74	Publication I
		56–60	92	67	82	Linde *et al.*, 2007; Linde *et al.*, 2006

[a] Yield in pretreatment and hydrolysis from raw material glucose and xylose
[b] Ethanol yield calculated from glucose
nd= no data available
[c] high enzyme dosage of 30 FPU/g was applied compared to 10 FPU/g in publication I

4.2 Development of alkaline oxidation pretreatment

Various pretreatment methods have been developed to improve enzymatic hydrolysis of lignocellulosic raw materials. Economic evaluation of the most promising methods has shown that none of them are clearly superior (Table 4). Thus new methods still need to be developed. In this work, a new alkaline oxidation pretreatment was developed to efficiently fractionate lignocellulosic materials and to produce a carbohydrate fraction with high enzymatic hydrolysability (Publications II, III).

4.2.1 Optimizing the treatment conditions for alkaline oxidation

Alkaline oxidation of spruce was studied in various treatment conditions with temperature, amount of oxygen, treatment time, presence of catalyst and alkali source as variables (Publications II, III). The effects of the different treatment conditions on the fractionation of lignocellulose and on enzymatic hydrolysability are summarized in Table 11.

In alkaline oxidation, a clearly lower temperature, 120–140°C was required as compared to steam explosion which was conducted at 200–215°C. On the other hand, the residence time in alkaline oxidation was relatively long, typically 4–20 h, compared to about 5–15 min in steam explosion. The oxidative conditions were provided by using 10 bar oxygen pressure and by a varying degree of reactor fill (10–75%). Alkaline conditions were achieved by addition of sodium carbonate to a concentration of 0.25 mol/l. The most significant difference compared to wet oxidation treatment was that the conditions were alkaline throughout the reaction, whereas wet oxidation is carried out with lower alkali dosages and the reactions have been reported to occur at least partly in acidic or neutral conditions (Klinke *et al.*, 2002). However, the alkali dosage was not optimized in this study.

Alkaline oxidation (AlkOx) was efficient in the fractionation of spruce, birch and sugar cane bagasse into a cellulose-rich solid fraction and a solubilized fraction (Publication III). In contrast to steam explosion, a state-of-the-art technology that was studied for comparison, alkaline oxidation dissolved lignin and part of the hemicellulose, whereas steam explosion dissolved mainly hemicellulose. Cellulose remained mainly in the solid fraction after treatment by both methods. The chemical reactions in alkaline oxidation are described in more detail in Section 4.2.4.

Table 11. Effect of different parameters in the alkaline oxidation treatment of spruce on dissolution of lignin and hemicellulose and on enzymatic hydrolysability. (+) increasing, (-) decreasing, and (+/-) no significant effect. nd = no data.

Change in conditions	Dissolution of lignin and hemicellulose	Formation of organic acids	Enzymatic hydrolysability	Reference
Increased temperature (from 120°C to 140°C)	+	nd	+	Publication III, unpublished data
Increased degree of reactor fill (from 10% to 50%)	-	-	+/-	Publication II, unpublished data
Increased temperature with decreased reactor fill	+	+	+	Publication II, unpublished data
No oxygen (Argon pressure)	-	nd	-	Publication III
Decreased particle size	+	nd	+	Unpublished data
Decreased time from 20 h to 4–5 h with chips	-	nd	-	Publication II, unpublished data
Copper-phenanthroline catalyst	+	+	+	Publications II, III
Alkali source (Na_2CO_3, NaOH, KOH, $Ca(OH)_2$)	+/-	+/-	+/-	Publications II, III

The cellulose in the alkaline oxidised materials was very easily hydrolysed to glucose: in pretreatment and hydrolysis by a commercial enzyme mixture (Celluclast 1.5L + Novozym188), 84%, 91% and 97% yield was obtained within 72 hours from spruce, birch and sugar cane bagasse, respectively, using an enzyme dosage of 10 FPU/g d.m.+100 nkat/g. After steam explosion (SE) the corresponding values were clearly lower for spruce (52%) and bagasse (78%), whereas with birch the yields were similar (93%). The applied alkaline oxidation conditions were similar for spruce, bagasse and birch. Thus, alkaline oxidation showed flexibility with respect to raw material. However, the conditions for sugar cane bagasse and birch were not optimized and the optimum might be milder due to the lower lignin content compared to spruce.

4.2.2 Hydrolysis and fermentation of alkaline oxidised materials

The alkaline oxidised fibre fraction could be efficiently fermented to ethanol after a short prehydrolysis with commercial enzymes (Figure 4; Publication III). Alkaline oxidised sugar cane bagasse and spruce were fermented efficiently in 12% d.m. content using a commercial yeast (RedStar, LeSaffre) able to utilize only C6 sugars. The ethanol yield from hexoses in the pretreated fibre fraction was 80% of the theoretical yield in 1–3 days fermentation. On the other hand, ethanol production from steam exploded and prehydrolysed bagasse and spruce in the corresponding conditions was slow and 39% and 32% ethanol yields from hexoses were obtained respectively after 6 days SSF. The highest ethanol concentration, 49 g/l, was obtained with alkaline oxidised spruce at 12% d.m. content of fibre. The ethanol concentration obtained from alkaline oxidised bagasse was clearly lower, 38 g/l, due to the lower content of hexoses in the substrate. The enzymatic hydrolysis limited significantly the fermentation of steam exploded materials. The most probable reason for this was non-productive adsorption and decreased accessibility of enzymes caused by residual lignin. Steam explosion has been shown to increase enzyme adsorption on lignin (Rahikainen *et al.*, 2013). By contrast, hydrolysis of alkaline oxidised materials evidently did not limit the fermentation to the same extent as the hydrolysis of steam exploded material, and ethanol production from hexoses was efficient.

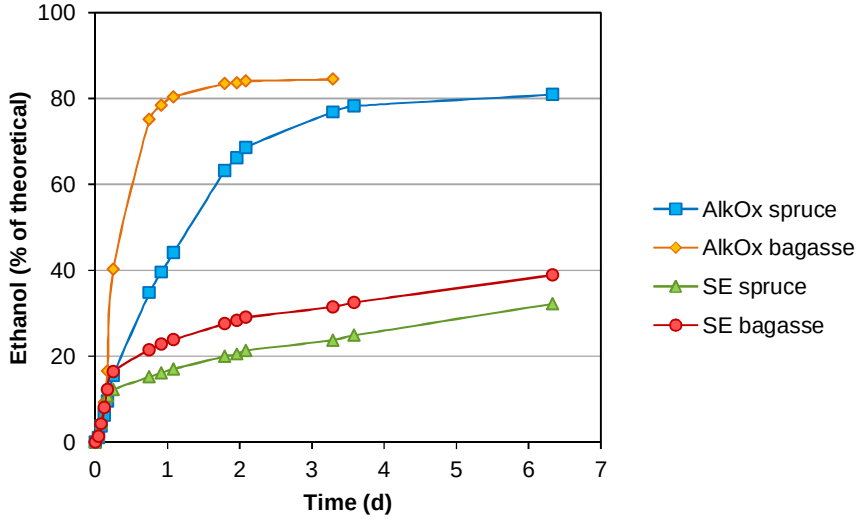

Figure 4. SSF of alkaline oxidised (AlkOx) spruce and sugar cane bagasse and steam exploded (SE) spruce and bagasse at 12% d.m. content at 35°C. 6 hours prehydrolysis was carried out with Cellic Ctec2+Htec enzyme mixture (enzyme ratio was 90:10; total dosage 15mg/g) at 50°C, pH 5 before inoculation with a commercial yeast, Red Star. Ethanol yields were calculated from the measured mass loss during fermentation.

4.2.3 Effect of particle size in alkaline oxidation pretreatment

Mechanical pretreatments such as chipping, cutting and grinding are often applied before chemical pretreatments to improve the mass transfer and pretreatment efficiency. Due to the high energy consumption of mechanical pretreatments, excessive particle size reduction should be avoided. When the efficiency of 4 hours alkaline oxidation was studied in the fractionation of spruce chips either with or without a catalyst, the treatment yielded a non-homogeneous material consisting of dark brown spruce chips in a matrix of yellow pulp (Publication II). Fractionation and dissolution of lignin was less efficient than with sawdust. Therefore, it appeared that the mass transfer of alkali and oxygen in these larger particles was limited to the surfaces and the smallest particles.

Particle size reduction was studied as a method to improve the efficiency of short alkaline oxidations (Figure 5, unpublished results). Spruce chips were ground coarsely in a hammer mill or powdered in a hammer mill followed by circulation (5 x) in a sieve plate press. After mechanical pretreatments, materials were alkaline oxidised at 120°C as described in publications II and III but 5 hours oxidation time was applied instead of 20 h oxidation. In addition, alkaline oxidation of sawdust was carried out for comparison.

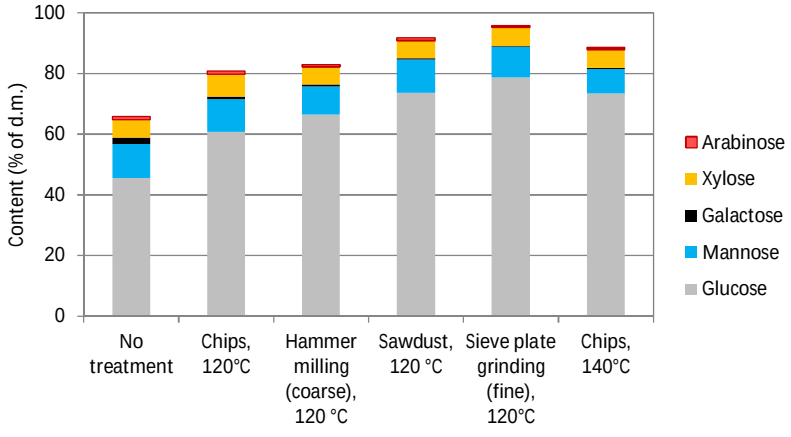

Figure 5. Effect of mechanical pretreatments and treatment temperature on alkaline oxidation of spruce. Carbohydrate composition of spruce raw material without alkaline oxidation (no treatment) and washed fibre fractions after alkaline oxidation at 120°C (or 140°C), for 5 hours. Unpublished results.

Particle size reduction had a clear effect on delignification in alkaline oxidation, and on carbohydrate content and composition of the produced fibre fraction (Figure 5). Compared to the reference treatment, spruce chips processed at 120°C, all the treatments including reduced particle size produced material having higher cellulose content and evidently lower lignin content. Part of the hemicellulose, both galactoglucomannan and arabinoglucuronoxylan, was solubilised and

the proportion of hemicellulose to total carbohydrate content in the treated solids decreased with decreasing particle size. Interestingly, the content of hemicellulose in the fibre fraction remained almost constant regardless of particle size. Powdering of wood by sieve plate grinding increased delignification and solubilisation of hemicellulose in alkaline oxidation, whereas with the other particle size reduction methods the impact was less pronounced.

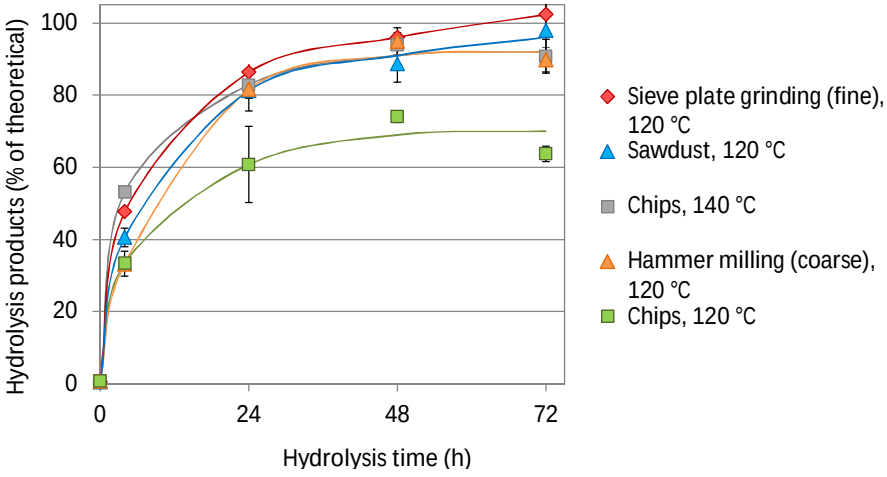

Figure 6. Effect of mechanical pretreatments and oxidation temperature on enzymatic hydrolysis of washed fibre fraction after alkaline oxidation at 120°C (or 140°C), for 5 hours. Hydrolysis in test tubes at 45°C, 1% d.m. content with enzymes Celluclast 1.5L (10 FPU/g d.m.) and Novozym 188 (100 nkat/g d.m.). Hydrolysis products were analysed as reducing sugars. Unpublished results.

Particle size reduction before alkaline oxidation also improved the enzymatic hydrolysis of the obtained solid fraction (Figure 6). The degree of hydrolysis was increased by over 25% compared to the degree of hydrolysis obtained with spruce chips when using a treatment temperature of 120°C. The highest hydrolysis yield was obtained with the smallest particle size, *i.e.* after sieve plate grinding. A slightly lower degree of hydrolysis was obtained with hammer milled spruce and sawdust with a particle size of approximately 2–5 mm. Consistently with this, grinding to a particle size below 1 mm was reported to give no additional improvement in lime treatment of corn stover (Chang *et al.*, 1997). Although hammer milled spruce and sawdust had about the same particle size, delignification was slightly more efficient with sawdust, probably due to different shapes and surface areas of the particles obtained by the two methods. However, no significant difference was found in enzymatic hydrolysability. Improved accessibility of oxygen and alkali to the target compounds in alkaline oxidation treatment, and more homogeneous processability are the most probable reasons for increased pretreatment efficiency with reduced particle sizes.

4.2.4 Chemical reactions and modification of biomass in alkaline oxidation

Alkaline oxidation combines the effects of alkaline and oxidative treatments, and several reactions take place simultaneously during the treatment. Hemicellulose and lignin are dissolved, oxidised and partially degraded, and carboxylic acids are formed. The effect of alkaline oxidation on the main chemical components was followed with spruce (Publication II).

Polysaccharides can degrade in alkaline oxidative conditions through peeling reactions from chain ends and by random chain cleavages (Theander, 1980). In alkaline oxidation, either with or without a catalyst, cellulose remained mostly in the solid fraction throughout the 20-hour oxidation. Although cellulose is more resistant to degradation than hemicellulose, glucan losses did occur, ranging from 0 to 16% of raw material glucose (Publication II, Table 2). Taking into account the glucose present in galactoglucomannan, up to 11% cellulose losses occurred. Swelling, crystal structure change from cellulose I to cellulose II, decrease in the crystallinity of cellulose and improved hydrolysis have been observed as a result of alkaline pretreatments (Mittal *et al.*, 2011). These changes can also contribute to high enzymatic hydrolysability of alkaline oxidised materials. On the other hand, extensive oxidation of reducing ends in cellulose has been reported to decrease significantly the susceptibility of the substrate to CBH activity (Xu *et al.*, 2009a). Steam explosion resulted in high cellulose yield in pretreatment, but significantly lower enzymatic hydrolysability (Publication III, Tengborg *et al.*, 1998).

Hemicellulose was partly solubilized during alkaline oxidation of spruce. Significant dissolution of hemicellulose, both galactoglucomannan and arabinoxylan, was observed already after one hour of treatment (Publication II, Figure 5). Approximately half of the hemicellulose was dissolved in the first four hours. Size exclusion chromatography (SEC) with RI detection showed that polysaccharides were mainly dissolved as small fragments of oligosaccharides with M_w ranging between 600 and 1600 (Publication II, Figure 3). In addition to solubilisation of hemicellulose, removal of acetyl substitutions has been reported with alkaline pretreatments; both of these effects can significantly enhance the enzymatic hydrolysis (Selig *et al.*, 2009). The carbohydrate yields after fractionation in various conditions showed that significant hemicellulose losses occurred during alkaline oxidation (Publication II, Table 2). Especially the yield of galactoglucomannan was low, 40–72% and 23–79% for mannose and galactose, respectively. The xylose yield varied between 66% and 92%. A larger proportion of galactoglucomannan than that of xylan was solubilized in alkaline oxidation of spruce, which probably made mannans more susceptible to oxidation. Oligosaccharides and monosaccharides can be oxidised to organic acids or even CO_2 and water in alkaline oxidation, whereas no furfural is produced in alkaline conditions (Klinke *et al.*, 2002). Steam explosion was also efficient in the dissolution of hemicellulose (Publication III). However, in acidic conditions degradation of pentoses and formation of furfurals can occur, especially with increasing severity (Stenberg *et al.*, 1998).

During alkaline oxidation of spruce, oxidation, degradation and dissolution of lignin was observed. The lignin content of the dissolved fraction ranged from 6–20% of raw material d.m. after oxidations. In catalysed alkaline oxidation, one third of lignin in the raw material was dissolved already in one hour reaction time, and two thirds after four hours, but after that only minor changes occurred in the lignin content (Publication II, Table 4). Oxidative conditions have been shown to increase dissolution of lignin in lime treatments (Chang *et al.*, 2001). The catalyst in alkaline oxidation, copper-phenanthroline complex, increased the degradation of lignin: precipitation yields of the dissolved lignin were higher for alkaline oxidation without catalyst than in the presence of catalyst, indicating increased degradation of lignin by the catalyst. Size exclusion chromatography with UV detection confirmed that the solubilized lignin had a lower molecular weight distribution and was thus more degraded after 20 h alkaline oxidation in the presence of catalyst than without catalyst (Publication II, Table 3). Removal of lignin has been suggested to increase the access of enzymes to the remaining polysaccharides and to decrease the non-productive binding of cellulases (Kumar *et al.*, 2012).

The degradation products, organic acids, were analysed from the dissolved fraction. After alkaline oxidation, the total content of organic acids varied from 10% to 22% of raw material d.m. Formic and acetic acids were the dominating organic acids. A similar composition of degradation products has been observed in wet oxidation of wheat straw (Bjerre *et al.*, 1996). Organic acids can be formed in alkaline oxidative conditions by hydrolysis of acetyl groups from hemicellulose, and through oxidation of carbohydrates and lignin (Rovio *et al.*, 2011; Sjöström, 1993). Almost 50% of the maximum content of acetic acid and formic acid was generated during the first hour of catalytic oxidation and the rest was formed during the following 3–7 hours. The formation of the other main carboxylic acids, including glycolic, oxalic, and 2,5-dihydroxy pentanoic acid, also occurred mainly during the first 4 hours.

4.2.5 Process conditions in alkaline oxidation

The lignocellulosic raw materials were fractionated by alkaline oxidation. The compositions of different fractions obtained by alkaline oxidation of spruce at 120–140°C for 4–20 h are presented in Table 12.

High water and chemical consumption increases the costs of the pretreatment. Alkaline oxidations were carried out at relatively low d.m. contents of 5% using a liquid/wood ratio of 19–20. A significant amount of alkali, 0.5 g/g (Na_2CO_3/wood d.m.) was applied in alkaline oxidation. In addition to sodium carbonate, several other alkalis, $Ca(OH)_2$, NaOH or KOH, were also shown to be possible alternatives (Publications II, III). The process should be optimized to minimize the amount of alkali required in the treatment. Efficient recovery and recycling of alkali are also needed to minimize the costs of chemicals in commercial processes.

Table 12. Raw materials, chemicals and the composition of product streams in alkaline oxidation of spruce (Publications II, III).

Component	Content (% of raw material d.m.)
Raw materials	
Spruce	100
Water	1900–2000
Oxygen	8–12
Na_2CO_3	53
$CuSO_4$ (in catalytic oxidation)	0.45
Phenanthroline (in catalytic oxidation)	0.59
Solid fraction	
Hexoses	40.5–56.8
Pentoses	2.0–5.9
Lignin (insoluble)	10.4
Ash	4.5
Extractives (heptane extraction)	0.5
Dissolved fraction	
Hexoses	0.4–3.2
Pentoses	0.5–3.1
Lignin (dissolved)	10.0–19.5
Organic acids	9.9–21.7

The function of a catalyst is to increase the rate of the reaction. Consistently with this, catalytically assisted alkaline oxidation by a copper-phenanthroline complex dissolved more carbohydrates than oxidation without a catalyst, especially those originating from galactoglucomannan and arabinoxylan (Publication II). Increased degradation of lignin was also observed. Thus, catalysed alkaline oxidation might enable shorter reaction time than the alkaline oxidation without the catalyst. On the other hand, the use of a catalyst increases chemical costs, and the used catalyst components, phenanthroline and $CuSO_4$, are toxic, which emphasizes the need for recycling. The recycling would need additional equipment and process development.

The role of oxygen is essential in alkaline oxidation. Oxygen pressure of 10 bars was applied and oxygen consumption, based on the measured pressure drop in alkaline oxidation, was 80–120 g O_2/kg wood. The alkaline treatment using argon gas pressure instead of oxygen revealed that oxidative conditions enhanced radically both solubilisation in the pretreatment and enzymatic hydrolysability of the produced solid fraction (Publication III). Only 9% glucose yield (% of glucose in the fibre) in the enzymatic hydrolysis of the fibre fraction was obtained from pretreatment of spruce in argon pressure compared to over 90% yield with material pretreated in oxygen pressure. In lime pretreatment, oxygen addition has been shown to increase the pretreatment efficiency with materials having high lignin content, such as poplar and softwood (Chang *et al.*, 2001). Oxidation of lignin is the most probable reason for improved dissolution and delignification.

The effect of the oxygen concentration on pretreatment efficiency was studied by filling the reactor to different levels of substrate-liquid suspension. The reactor fill *i.e.* liquid/gas ratio had a clear impact on the solubilisation and oxidation during the alkaline oxidation in the presence or absence of the catalyst. More carbohy-

drates and lignin were dissolved in 4 and 20 hour oxidations with a lower reactor fill (Publication II, Figure 1). These effects were also reflected as improved enzymatic hydrolysis. The formation of acids was also significantly higher when a lower reactor fill (10% and 25% of total volume) was used compared to using higher reactor fill (50% and 75%) (Publication II, Figure 4). In addition to higher oxygen concentration, the lower reactor fill might have improved mixing of the suspension, decreased concentration gradients, and increased the solubilisation of oxygen throughout the reaction. From a process economy point of view, the lower reactor fill would mean larger reactors and subsequently increased capital costs. To scale-up the alkaline oxidation method, development of special reactors enabling efficient mixing and oxygen transfer would be required.

Residence time of the process should be as short as possible in order to minimize process and investment costs. Alkaline oxidation could be shortened from 20 h to 5 h by particle size reduction. Alternatively, the higher temperature (140°C) in alkaline oxidation enhanced the efficiency of the pretreatment (Figure 5, Figure 6). However, both grinding and higher reaction temperature would increase the energy consumption.

Preliminary techno-economic evaluation of process concept using alkaline oxidation pretreatment has been carried out outside this study (Biorefine programme report, 2012). The feasibility study indicated that the key cost elements for the concept were raw material costs and the capital costs related to alkaline oxidation pretreatment and energy production. Significant savings in capital costs of alkaline oxidation could be obtained by repurposing kraft mills and utilizing existing infrastructure.

4.3 Improved enzymatic hydrolysis by efficient pretreatment and optimization of enzyme mixtures

Despite intensive development of cellulolytic and hemicellulolytic enzymes, enzyme cost is still an important factor in processes hydrolysing lignocellulose to monosaccharides. The expenses can be decreased by lowering the enzyme price or by reducing their consumption. The use of enzymes can be reduced by several ways as described in Section 1.6.3. In this study, two ways to decrease enzyme dosages, namely efficient pretreatments and optimized enzyme compositions, were studied.

4.3.1 Decrease in enzyme dosages by alkaline oxidation pretreatment

Lignocellulosic raw materials are modified structurally and chemically by pretretment. An efficient pretreatment produces material with good enzymatic hydrolysability and thus also enables decreased enzyme dosages or shorter hydrolysis time and affects process costs and feasibility.

Enzymatic hydrolysis with lower enzyme dosages was studied using alkaline oxidised and steam exploded materials (Publication III). Cellulase dosage was varied, whereas the β-glucosidase dosage was kept constant at 100 nkat/g d.m. Enzymatic hydrolysability of sugar cane bagasse and birch was good with both pretreatments using a dosage of 10 FPU/g d.m., which is a typical cellulase dos-

age used for hydrolysis of various materials. The hydrolysis yield was already 100% in 24 h hydrolysis of pretreated sugar cane bagasse, whereas 80–100% yield was reached with pretreated birch. The hydrolysis rate and the degree of hydrolysis of alkaline oxidised bagasse decreased with decreased enzyme dosages. Hydrolysis yields of 90% and 70% were obtained in 72 h with enzyme dosages of 4 and 2 FPU/g, respectively (Figure 7). On the other hand, the hydrolysis of steam exploded bagasse using the lower enzyme dosages stopped almost completely after 4 hours and even lower hydrolysis yields of 70% and 40% were obtained with enzyme dosages of 4 FPU/g and 2 FPU/g, respectively. Lignin has been shown to decrease the accessibility of enzymes to polysaccharides and to cause unproductive binding of cellulases (Kumar *et al.*, 2012; Palonen *et al.*, 2004; Rahikainen *et al.*, 2013; Várnai *et al.*, 2010). Easily hydrolysable material, oligosaccharides or small particles with high surface area probably increased the hydrolysis rate of steam exploded bagasse in the first 4 hours of hydrolysis. High surface area has been shown to increase overall protein adsorption and to give a higher initial rate of hydrolysis (Piccolo *et al.*, 2010). With steam exploded and alkaline oxidised birch, the hydrolysis levels were similar with similar dosage of enzymes for 24 hours but after that the hydrolysis rate of steam exploded birch decreased more steeply.

With steam exploded spruce, enzymatic hydrolysability was low and was further decreased with lower enzyme dosages. By contrast, alkaline oxidised spruce had high enzymatic hydrolysability and the degree of hydrolysis was clearly decreased only when the enzyme dosage was lowered to 2 FPU/g. Although the pretreatment conditions used (205°C, 15 min) were not optimal for spruce, steam exploded spruce has often been found to be a challenging material to hydrolyse. It has been reported that the enzymatic hydrolysis of steam pretreated softwood can be limited even with enzyme dosages of 10–15 FPU/g without delignifying post-treatments (Kumar *et al.*, 2010; Kumar *et al.*, 2011; Tengborg *et al.*, 1998; Várnai *et al.*, 2010). The decreased hydrolysis of steam exploded spruce can be due to decreased accessibility of enzymes to their substrates due to the high lignin content, or to unproductive binding of cellulases. The lignin in steam exploded spruce has been observed to be especially inhibitory to enzymes (Rahikainen *et al.*, 2013). Denaturation of enzymes in prolonged contact with lignin at hydrolysis temperature has also been observed (Rahikainen *et al.*, 2011). Obviously, with steam exploded spruce having a high lignin content, these effects were more profound than with alkaline oxidised spruce. The results clearly showed that alkaline oxidation pretreatment enabled the use of lower enzyme dosages or shorter hydrolysis time compared to steam exploded materials.

Further decrease in enzyme costs can be obtained by enzyme recycling. Cellulases have been shown to adsorb to substrate in the beginning of hydrolysis (Boussaid and Saddler, 1999; Varnai *et al.*, 2011b). With materials having high lignin content, cellulases remain bound, which limits the recycling of enzymes. On the other hand, after delignifying pretreatments desorption of enzymes has been observed (Boussaid and Saddler, 1999; Varnai *et al.*, 2011b). Increased desorption of enzymes in alkaline oxidised materials could make recycling of enzymes possible.

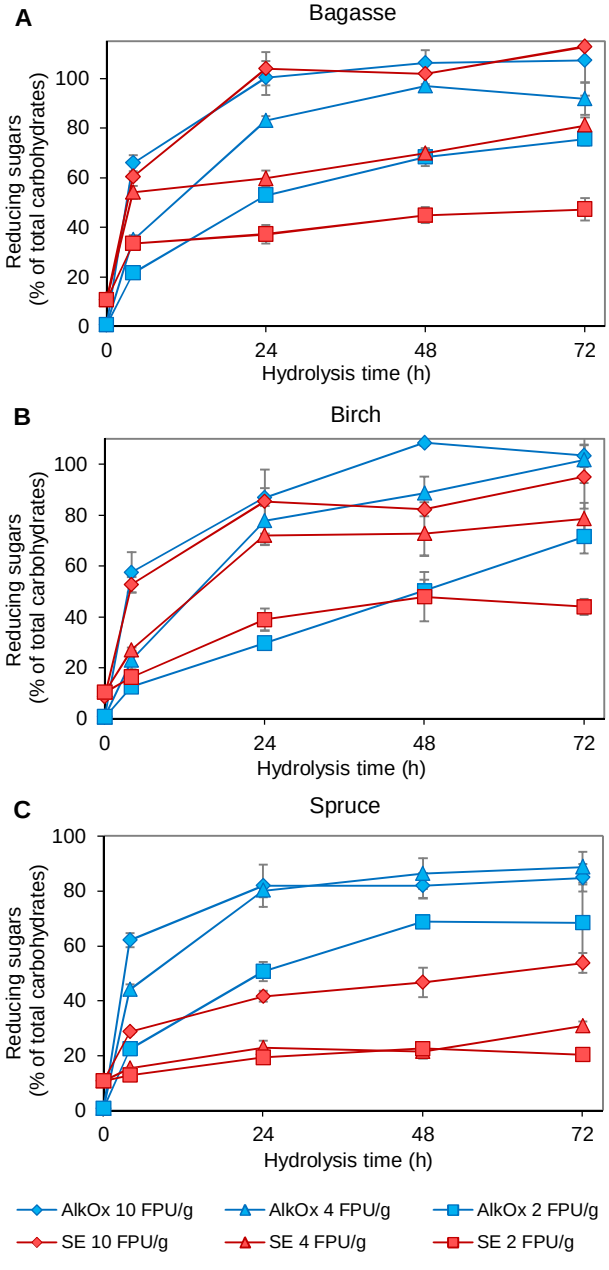

Figure 7. Effect of enzyme dosage and pretreatment type on enzymatic hydrolysability of steam exploded (SE) and alkaline oxidised (AlkOx) bagasse (A), birch (B), and spruce (C). Enzymatic hydrolysis at 1% d.m. consistency, 45°C, pH5, with Celluclast1.5L(2–10 FPU/g) and Novozym 188 (100 nkat/g). (Publication III)

4.3.2 Enzyme mixture optimization

Optimal enzyme composition can provide maximal synergistic effect and thus an improved degree and rate of hydrolysis can be obtained with a minimal enzyme loading. Therefore the use of optimal enzyme compositions for a particular pretreated raw material can minimise enzyme dosages and the costs of enzyme treatment. There are several options in designing optimal enzyme compositions for the hydrolysis of lignocellulosic raw material. In this thesis, supplementation of a commercial enzyme mixture with the rate limiting enzyme activities (Publication I), and development of optimized enzyme mixtures from monocomponent enzymes (Publication IV) were studied in the hydrolysis of pretreated materials.

4.3.2.1 Supplementation of commercial enzyme mixtures

β-Glucosidase activity is essential in the total hydrolysis of lignocellulosic substrates, although conventional *Trichoderma reesei* mixtures are known to contain insufficient β-glucosidase activity. Supplementation of commercial *T. reesei* enzyme mixtures with a commercial β-glucosidase (Novozym 188) was studied in the hydrolysis of pretreated reed canary grass and barley straw. Hydrolysis by all three different conventional commercial cellulases showed clear improvement by the addition of commercial β-glucosidase (Publication I, Table 3). In addition to improved production of glucose, the yield of xylose was also slightly enhanced by additional Novozym 188 due to some minor hemicellulase activities present in the commercial enzyme. It has been observed that the increased hydrolysis of cellulose synergistically enhances the hydrolysis of xylan in the complex lignocellulose material (Varnai *et al.*, 2011a). The hydrolysis of side groups of xylan and mixed xylo-oligomers might also have been improved by the side activities in the β-glucosidase preparation. Arabinose analysis of the hydrolysates supported this hypothesis, as detectable concentrations of arabinose were obtained only when β-glucosidase was applied. Synergy between xylanases and cellulases in the hydrolysis of pretreated raw materials has frequently been observed (Hu *et al.*, 2011; Kumar and Wyman, 2009).

The possibility to further enhance the hydrolytic performance of a commercial *T. reesei* cellulase mixture (Econase CE+Novozym 188) by the addition of potential rate-limiting enzymes was studied in the hydrolysis of steam exploded reed canary grass by overdosing the mixture with the major cellulases and xylanase (Publication I). It has been reported that the optimal enzyme composition for total hydrolysis can be significantly different from that produced by *T. reesei* (Rosgaard *et al.*, 2007; Zhou *et al.*, 2009). The addition of Cel6A (CBHII) at a level of 5 mg/g cellulose improved the hydrolysis of the washed solid fraction of steam exploded reed canary grass most significantly, by 13% after 48 hours hydrolysis (Figure 8). This increase was observed in all time points, although standard deviations were relatively high in some points. The hydrolysis yield was also enhanced by the addition of Cel7B (EGI). In addition to endoglucanase activity, *Tr* Cel7B is also reported to have strong hemicellulolytic side activities (Bailey *et al.*, 1993; Vlasen-

ko *et al.*, 2010). Consistently with this, a high proportion of Cel6A and Cel7B has been observed in optimized mixtures for the hydrolysis of steam pretreated barley straw and corn stover (Rosgaard *et al.*, 2007; Zhou *et al.*, 2009). Supplementation with Cel7A (CBHI) and xylanase Xyn11A had a lower effect in 48 h hydrolysis, and supplementation with Cel5A (EGII) had practically no effect in the hydrolysis. Thus the amount of Cel5A was not considered to limit the rate or degree of hydrolysis of steam exploded reed canary grass.

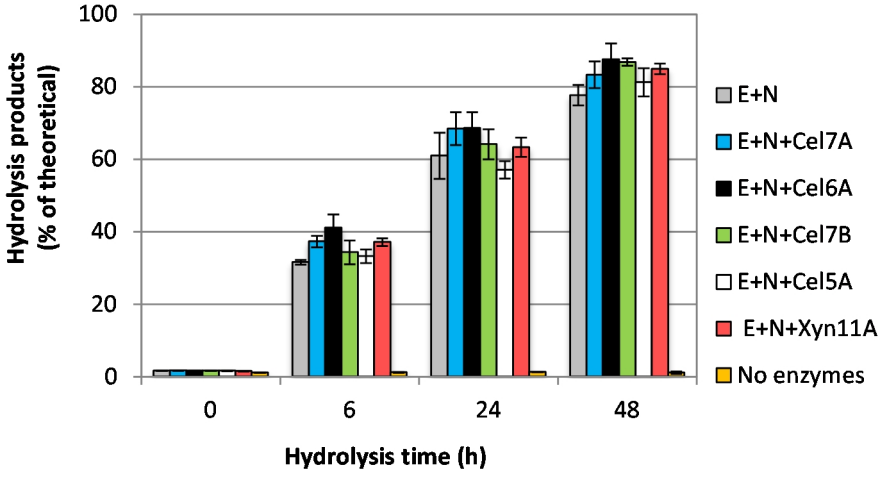

Figure 8. Effect of supplementation of a commercial enzyme mixture (Econase CE +Novozym 188, E+N) with purified *T. reesei* enzymes Cel7A, Cel6A, Cel7B, Cel5A, or Xyn11A in the hydrolysis of washed solid fraction of steam exploded reed canary grass (spring harvest). Enzyme dosages were for Econase CE 10 FPU/g cellulose, for Novozym 188 100 nkat/g cellulose, and for the purified enzymes 5 mg/g cellulose. Hydrolysis conditions were 1% cellulose content, 45°C, pH 5. Hydrolysis products were analysed as reducing sugars (Publication I).

4.3.2.2 Optimization of enzyme mixtures from monocomponent enzymes for the hydrolysis of pretreated raw materials

Optimal enzyme mixtures were developed for the hydrolysis of alkaline oxidised and steam exploded sugar cane bagasse and hydrothermally pretreated wheat straw by statistically designed experiments (Publication IV). Most of the optimizations of enzyme mixtures studied in the literature have been carried out using cellulases from *Trichoderma sp.* In this work, optimal enzyme mixtures were determined by using thermostable enzymes Cel7A from *Acremonium thermophilum*, Cel6A from *Chaetomium thermophilum*, Cel5A, Cel3A and Xyn10A from *Thermoascus aurantiacus* and the optimal mixture was compared to the best mixture obtained using *Trichoderma reesei* enzymes. The enzyme mixture compositions and the hydrolysis yields are presented in Table 13. In addition, the results of selected optimization studies in the literature are presented for comparison.

For the different pretreated raw materials and hydrolysis times the optimal enzyme mixtures of thermostable enzymes contained 49–73% Cel7A (CBHI) of total protein, 10–30% Cel6A (CBHII), 6–16% Cel5A (EGII), 1–12% xylanase (Xyn10A), and 2–5% Cel3A (βG). The optimal mixture of *T. reesei* enzymes for the hydrolysis of pretreated wheat straw contained 39–42% Cel7A (CBHI), 32–34% Cel6A (CBHII), 20–26% Cel7B (EGI), 1–2% Cel5A (EGII), 1% xylanase (Xyn11A), and 1–2% Cel3A (βG). The results of the statistically designed experiments were mathematically modelled by the Modde program to describe the effect of each component on the hydrolysis efficiency. Contour plots of the models showed broad optimal areas (Publication IV, Figures 3 and 4) for the enzyme mixtures. Thus, relatively high variations in the proportions of enzyme mixture components were possible without significant impact on the degree of hydrolysis.

The optimal enzyme mixtures were dominated by cellobiohydrolases, the proportion being 70–88% of total enzyme dosage in all the tested enzyme mixtures. This is significantly higher than the proportion analysed from the commercial enzyme mixture Celluclast and higher than that naturally produced by *T. reesei* (Shoemaker *et al.*, 1983; Sipos *et al.*, 2010). High proportions of cellobiohydrolases have also been recommended in various other optimization studies carried out on steam exploded raw materials (Billard *et al.*, 2012; Rosgaard *et al.*, 2007; Zhou *et al.*, 2009). The main component in all the enzyme mixtures was cellobiohydrolase Cel7A. With thermostable enzymes the proportion of Cel7A was clearly higher than that of Cel6A, whereas in *T. reesei* mixtures the role of Cel6A seemed to be more significant. It is possible that the ratio of Cel7A and Cel6A is not as important as the total content of cellobiohydrolases (Cel7A+Cel6A), as the optimal ranges for cellobiohydrolases were relatively broad (Publication IV).

The proportion of endoglucanases and xylanase showed more variation in the optimized mixtures. It appeared that hydrolysis of hydrothermally pretreated wheat straw and alkaline oxidised bagasse required a significant amount of xylanolytic activity, which was provided by either xylanase or by endoglucanase Cel7B. In *T. reesei* enzyme mixture Cel7B appeared to be able to replace both Cel5A and xylanase almost completely, whereas significantly higher proportions, 13–16% and 8–12% of Cel5A and xylanase, respectively, were present in the optimized thermostable enzyme mixture.

Table 13. Optimal enzyme mixtures of various pretreated lignocellulosic substrates (Publication IV).

Raw material	Pretreatment	Content (% of total protein dosage)							Hydrolysis yield (%)	Ref.
		Cel7A (CBHI)	Cel6A (CBHII)	Cel7B (EGI)	Cel5A (EGII)	Xyn10A or Xyn11A	Cel3A (βG)	Other		
Sugar cane bagasse	SE	57–73	10–24	-	9–11	1–4	3–5	-	84–98	[a]
Sugar cane bagasse	AlkOx	49–58	14–30	-	5–13	12	3	-	67–88	[a]
Wheat straw	hydrothermal	52–56	17–19	-	13–16	8–12	2–3	-	51–74	[a]
Wheat straw	hydrothermal	39–42	32–34	20–26	1–2	1	1–2	-	37–56	[b]
Wheat straw	SE	40	27	15	6	12	*	0	68	[c]
Barley straw	SE	27	47	27	0	-	*	*	56	[d]
Barley straw	Hot water	20	43	37	0	-	*	*	56	[d]
Corn stover	SE	27	35	21	-	-	6	11	36	[e]
Corn stover	AFEX	29	19	35	-	14	*	3	51	[f]
Corn stover	AFEX	35	4	26	-	19	12	4	44	[g]
Corn stover	Alkaline	49	4	34	-	4	5	4	41	[g]
Corn stover	Alk. peroxide	43	4	30	-	11	8	4	58	[g]

AlkOx = alkaline oxidation, SE = steam explosion, AFEX = ammonia fibre explosion
* were present in the hydrolysis but were not in variables of optimization
[a] 24–72 h hydrolysis with thermostable enzymes (this study).
[b] 24–72 h hydrolysis with *Trichoderma reesei* and *Aspergillus niger* enzymes (this study).
[c] 48 h hydrolysis with *T. reesei* and *A. niger* enzymes (Billard *et al.*, 2012).
[d] 24 h hydrolysis with *T. reesei* cellulases and Novozym 188 (Rosgaard *et al.*, 2007).
[e] 72 h hydrolysis with *T. viride* cellulases (Zhou *et al.*, 2009)
[f] 24 h hydrolysis with *T. reesei, A. nidulans* and *A. niger* enzymes (Gao *et al.*, 2010a).
[g] 48 h hydrolysis with *T. reesei, T. longibrachiatum* and *A. niger* enzymes (Banerjee *et al.*, 2010b).

On the basis of the studies carried out using thermostable enzymes some conclusions could be drawn about the effect of pretreatment and raw material on enzyme mixture composition. Firstly, more *Acremonium thermophilum* Cel7A (CBHI) was needed in the optimal mixture for steam exploded bagasse than for alkaline oxidised bagasse. Secondly, the hydrolysis of alkaline oxidised bagasse required at least three times more xylanase than the hydrolysis of steam exploded bagasse. The difference can be explained by the chemical composition of the materials. Bagasse pretreated by alkaline oxidation had a very high xylan content, 24%, whereas the xylan content of steam exploded bagasse was only 3%. Banerjee *et al.* (2010b) observed that more xylanase (*Tr* Xyn10) was needed after AFEX than after NaOH or alkaline peroxide pretreatments. Although no composition of pretreated raw materials was presented in the study of Banerjee *et al.* , it is probable that AFEX treated material had a higher lignin content than NaOH and alkaline peroxide treated materials and also potentially a higher hemicellulose content. Therefore it appears that substrates with either high hemicellulose or high lignin content require more xylanase to increase the accessibility of substrates to cellulases. Thirdly, comparison of two raw materials, wheat straw and bagasse, pretreated with relatively similar methods showed that more *At* Cel7A and a lower proportion of xylanase were needed with steam exploded bagasse than with hydrothermally pretreated wheat straw, although the carbohydrate compositions were similar. The composition of the optimal enzyme mixture might be affected by the higher enzyme dosage and degree of hydrolysis with steam exploded bagasse than with wheat straw. Wheat straw substrate was used as dried sheet discs, whereas the more homogeneous steam exploded bagasse was pipetted as a never-dried slurry. Drying has been shown to decrease enzyme accessibility by closing of larger pores, the effect being more profound with substrates containing lignin (Esteghlalian *et al.*, 2001; Luo and Zhu, 2011). It appears that drying-induced decrease in accessibility has increased the role of xylanases in improving the accessibility of cellulose to cellulase enzymes. Cell wall anatomy and microstructure can have significant impacts on optimal enzyme proportions in addition to the content of lignin, cellulose and hemicellulose.

The performance of the optimal enzyme mixtures of thermostable enzymes was evaluated by comparing the hydrolysis with that obtained using a mixture of *Trichoderma reesei* enzymes and using commercial enzymes (Figure 9; Publication IV). The optimal mixture of thermostable enzymes produced 64% yield in 48 hours using an enzyme dosage of 6 mg/g, whereas the mixture of *Trichoderma reesei* enzymes produced only 51% hydrolysis yield. The hydrolysis yield obtained by the commercial enzyme mixture, Celluclast-Novozym 188 (10 mg/g+1000 nkat/g), was 68%. Novozym 188 preparation is reported to contain *e.g.* various cell wall degrading glucanases, amyloglucosidases, and some xylanolytic activity (Banerjee *et al.*, 2010b). The higher dosage and the accessory enzymes that were included in the commercial mixture improved the hydrolysis result. Thus the optimal enzyme mixture of thermostable enzymes performed much better than the commercial enzyme mixture in the hydrolysis of hydrothermally pretreated wheat straw.

The optimization experiments of wheat straw substrate were carried out using dried wheat straw discs, as this made it possible to carry out a large number of small scale experiments with the same substrate. The never-dried wheat straw substrate was hydrolysed more easily than the dried substrate and the hydrolysis yield was 86% in 48 hours (Figure 9). Drying has been shown to decrease enzyme accessibility as discussed in the previous paragraph (Esteghlalian *et al.*, 2001). However, the degree of hydrolysis was clearly decreased when the dry matter content was increased to 12%. It is probable that in the higher dry matter content the hydrolysis requires a different optimal enzyme mixture.

Alkaline oxidation pretreated bagasse was hydrolysed very easily in high dry matter content of 8% and the degree of hydrolysis was 90–100% with both the optimized enzyme mixture and the commercial enzyme mixture (Figure 9). However, slightly faster hydrolysis was obtained using the commercial Cellic enzyme mixture. With steam exploded bagasse the degree of hydrolysis was significantly lower than in the hydrolysis of alkaline oxidation pretreated bagasse at 8% dry matter content despite the higher enzyme dosage. The inhibitory effect of lignin is the most probable reason for this. The optimal enzyme mixture for the hydrolysis of steam exploded bagasse was slightly more efficient in the beginning of the hydrolysis but after 48 hours a similar degree of hydrolysis, 81–82%, was obtained with the commercial enzyme mixture (Publication IV). The thermostable enzyme mixture was composed of five enzyme component preparations. The accessory enzymes present in Cellic but not in the thermostable enzyme might have improved the rate and degree of hydrolysis. The accessory enzymes have been found to be more important in the later stages of hydrolysis (Banerjee *et al.*, 2010c). Thus optimal enzyme mixtures could further be improved by including other enzymes, such as arabinofuranosidases or LPMOs, needed for efficient and complete hydrolysis of cell wall carbohydrates.

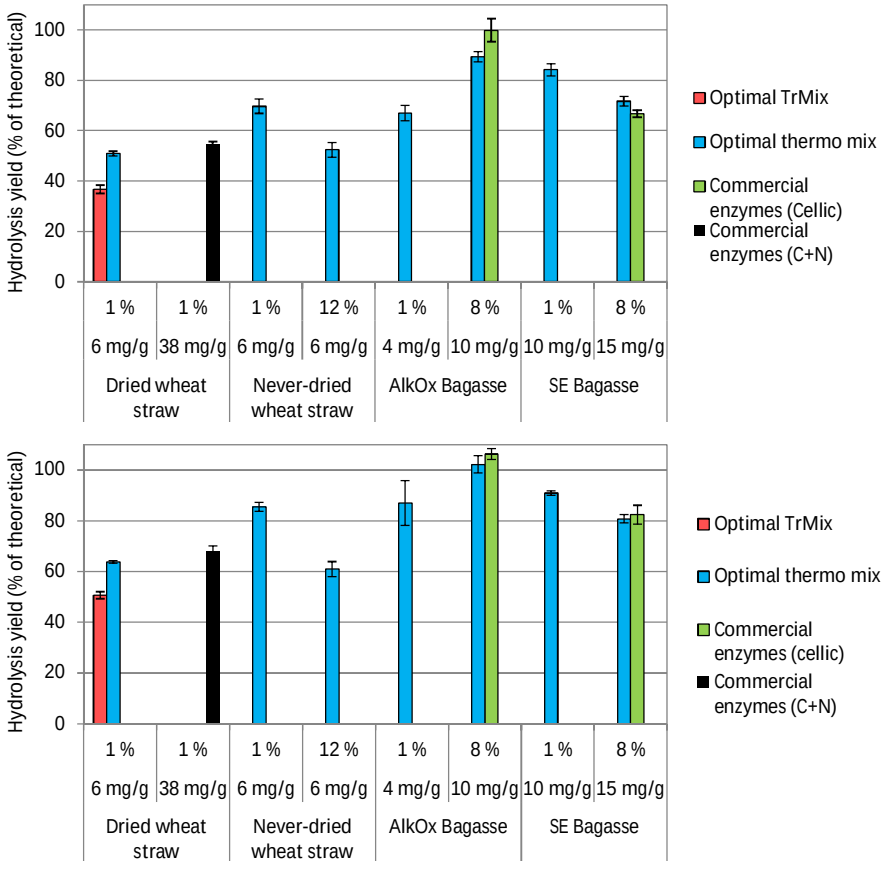

Figure 9. Hydrolysis of hydrothermally pretreated wheat straw, alkaline oxidised (AlkOx) sugar cane bagasse and steam exploded (SE) bagasse by an optimal enzyme mixture of *T. reesei* enzymes (TrMix), thermostable enzymes (thermo mix) and commercial enzymes for 24 h (A) and 48 h (B). The dry matter content was 1–12% and enzyme dosage 4–38 mg/g d.m. depending on the experiment. In the hydrolysis of AlkOx and SE bagasse the commercial enzyme mixture was Cellic Ctec2+Htec, 85%+15% of total protein and in hydrolysis of wheat straw a mixture of Celluclast +Novozym 188 (C+N,10 mg/g +1000 nkat/g d.m). Hydrolysis temperature was 45°C for TrMix and for C+N, and 52°C in the hydrolysis of wheat straw with thermostable enzymes. The hydrolysis temperature with pretreated bagasses was 52°C and 50°C, at 1% and 8% d.m. contents, respectively. Hydrolysis yield was measured as reducing sugars.

5. Conclusions and recommendations for future studies

Alternative raw materials for biorefineries, a novel alkaline oxidation pretreatment method and optimization of enzyme mixture compositions were studied in this work. Reed canary grass and barley straw were shown to have high carbohydrate content similarly to wheat straw, and they could thus be interesting alternatives or supplementary raw materials for the production of sugars in a biorefinery, especially in a northern climate. Reed canary grass harvested in the spring had higher cellulose content, more xylan was removed from it by steam explosion and the pretreated fibre was hydrolysed more efficiently compared to the autumn harvested material. The modification of the raw material in the field during winter might work as a partial pretreatment and bring out other advantages in raw material properties, such as decreased moisture and ash contents. Thus, spring was found to be a more suitable harvest time for the production of sugars from this material in a biorefinery. Although the cultivation of reed canary grass is currently low, it could be increased significantly *e.g.* in marginal land, without competing with food production. The other potential raw materials such as common reed and surplus forages would be interesting options for future studies.

Alkaline oxidation was found to be an efficient pretreatment method, fractionating biomass into a carbohydrate-rich fibre fraction and a dissolved fraction containing most of the lignin. Alkaline oxidised fibre showed high enzymatic hydrolysability and the hydrolysis was also efficient at relatively high 12% dry matter content. Compared to the 52% glucose yield obtained by steam explosion pretreated spruce in pretreatment and hydrolysis, a significantly higher glucose yield of 84% was obtained after alkaline oxidation pretreatment. The efficiency of alkaline oxidation was also shown with different types of raw materials, such as spruce, birch and sugar cane bagasse.

After alkaline oxidation, cellulose remained mainly in the solid fraction. Cellulose losses were 0–11%, depending on the treatment conditions. The losses were probably due to oxidation of cellulose. The structural changes of cellulose in alkaline oxidation, the oxidation of cellulose and changes in cellulose crystallinity require further studies. Part of the hemicellulose, both galactoglucomannan and xylan, was solubilised and further oxidised to other products and therefore hemicellulose yields were relatively low. Up to 60% of mannan and 34% of xylan was

lost in the pretreatment. The hemicellulose losses due to oxidation should be minimized by further optimization of process conditions.

In addition to carbohydrates, alkaline oxidation treatment dissolved and oxidised lignin. Alkaline oxidation produced a new type of lignin, which is oxidised and sulphur-free, unlike the lignin separated from black liquor of the Kraft pulping process. Sulphur-free oxidised lignin is a very interesting material and its properties and suitability for different applications should be investigated. However, the heating value of lignin generated by alkaline oxidation is decreased due to the oxidative nature of the pretreatment. Organic acids were formed in alkaline oxidation as degradation products of both lignin and carbohydrates. Their formation should be minimized by optimizing process conditions. The recovery and economical exploitation of organic acids should also be considered.

Various process conditions were studied using spruce as raw material to improve the alkaline oxidation pretreatment. The pretreatment efficiency could be improved by reducing the particle size of the raw material, by increasing the treatment temperature from 120°C to 140°C, and by catalysing the reaction with a copper-phenanthroline catalyst. The alkaline treatment of spruce without oxygen pressure revealed that oxidative conditions enhanced radically both solubilisation in the pretreatment and the enzymatic hydrolysability of the produced solid fraction. In order to improve the feasibility of alkaline oxidation, optimized mechanical pretreatments and oxygen supply should further be developed. In addition, alkali dosages, solid to liquid ratios and treatment time and temperature should be optimized for different raw materials in order to obtain high enzymatic hydrolysability and yield. Advanced reactor systems providing efficient oxygen and mass transfer throughout the reaction would enable higher solid to liquid ratios and possibly also lower alkali dosages. Feasibility of the optimized alkaline oxidation process should be evaluated in future studies.

Enzyme consumption and costs can be decreased by enhanced pretreatments. Especially with spruce, alkaline oxidation pretreatment enabled the use of decreased enzyme dosages in the hydrolysis and thus significant reduction in enzyme costs or hydrolysis time could be obtained. With steam exploded materials, high enzyme dosages were required to obtain high hydrolysis yield, most probably due to the inhibitory effect of high lignin content.

The significant role of cellobiohydrolases in the hydrolysis of pretreated gramineous raw materials was demonstrated in experiments studying the supplementation of commercial mixtures with purified enzymes as well as in optimization of mixtures of individual enzyme components. The results indicated that cellobiohydrolase activity can be the limiting activity in the hydrolysis by commercial mixtures. With thermostable enzymes the proportion of Cel7A in the optimal mixture was at least 50%, whereas with *Trichoderma reesei* enzymes almost equal amounts of Cel6A and Cel7A were required. The results also indicated that materials having decreased accessibility to cellulose due to their high xylan content required higher xylanolytic activity in optimal mixtures. The structural collapse caused by drying of substrate might also have increased the need for high xylanolytic activity in the case of pretreated wheat straw. It would be interesting in the

future to study the effects of the addition of accessory enzymes such as debranching enzymes, different activities such as LPMOs and swollenins, or enzymes with improved properties, in enzyme mixtures. The effect of higher dry matter content as well as other hydrolysis conditions on enzyme mixture composition should be evaluated in future studies.

The performance of optimal enzyme mixtures in hydrolysis was compared with that of the commercial enzymes Celluclast and Cellic. The results showed that significant improvement in the hydrolysis efficiency can be obtained by optimizing the enzyme mixtures. Optimized thermostable enzyme mixtures of five components hydrolysed pretreated materials as efficiently as commercial enzymes containing a whole spectrum of supplementary activities. The results indicated significant flexibility in the proportions of enzyme components leading to high hydrolysis yields. Thus, instead of using monocomponent enzyme mixtures, optimized ratios of commercial enzyme preparations are also one option to achieve efficient hydrolysis of lignocellulosic materials with minimum enzyme consumption.

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Title	**Development of pretreatment technology and enzymatic hydrolysis for biorefineries**
Author(s)	Anne Kallioinen
Abstract	The growing demand for energy, materials and food, depletion of fossil raw material reservoirs and increasing environmental concerns have all increased interest in renewable resources. Lignocellulosic biomass is an alternative for replacing fossil raw materials in the production of fuels, materials and various chemicals. Lignocellulose present in plant cell walls consists mainly of polysaccharides, cellulose and hemicellulose, and aromatic lignin. These major components form a complex structure that is resistant to microbial and enzymatic activity. Due to the recalcitrant structure of plant cell walls, lignocellulosic raw materials must be pretreated before their enzymatic hydrolysis to monosaccharides. Various pretreatment methods; chemical, physical, biological or their combinations, have been developed. After pretreatment, polysaccharides can be hydrolysed enzymatically to monosaccharides, which in turn can be fermented to different products such as ethanol. Currently the first commercial scale lignocellulosic ethanol plants have started production. A secure supply of biomass is one of the key factors for a feasible biorefinery, and new alternative feedstocks are still required especially in northern climates in order to fulfil the raw material demands of biorefineries in a sustainable way. In addition, development of new pretreatment technologies and more efficient enzymatic hydrolysis are needed. New lignocellulosic feedstocks and improved pretreatment methods were studied in the work described in this thesis. Reed canary grass and barley straw were found to be interesting carbohydrate-rich raw materials that could be pretreated by steam explosion and hydrolysed enzymatically with yields comparable to those obtained from wheat straw. Selection of the most favourable harvest time for reed canary grass, autumn or spring, was studied in relation to pretreatment and hydrolysis yields. Spring harvested reed canary grass was found to be the more suitable raw material as it had a higher cellulose content and the pretreated fibre was hydrolysed more efficiently compared to autumn harvested material. A new pretreatment method using sodium carbonate and oxygen pressure was developed. The alkaline oxidation method fractionated biomass into a carbohydrate-rich fibre and a dissolved fraction containing most of the lignin. The produced carbohydrate-rich fibre could be efficiently hydrolysed by enzymes and the hydrolysis was also efficient at 12% dry matter content. Compared to the 52% total glucose yield obtained in enzyme hydrolysis of spruce after pretreatment by steam explosion, a significantly higher glucose yield of 84% was obtained in hydrolysis after alkaline oxidation. Different kinds of raw materials, such as spruce, birch and sugar cane bagasse, could be efficiently pretreated by alkaline oxidation. The main effects of alkaline oxidation pretreatment were dissolution and partial degradation of lignin and hemicellulose. Some galactoglucomannan and xylan was solubilised and further oxidised to other products, and therefore relatively low yields of hemicellulose were obtained. Organic acids were formed as degradation products of lignin and carbohydrates. Process conditions were partially optimized using spruce as raw material in order to improve the efficiency of alkaline oxidation. The pretreatment could be accelerated by increasing the treatment temperature, by the use of copper-phenanthroline catalyst, and by decreasing the particle size of the raw material. Further optimization of *e.g.* alkali dosage and the solid to liquid ratio is still required to improve hemicellulose yield and economical feasibility. Fibre fractions of alkaline oxidation could be hydrolysed by low enzyme dosages, 2–4 FPU/g dry matter. Significantly higher enzyme dosages were required in the hydrolysis of steam exploded materials, probably due to the inhibitory effect of the high residual lignin content after the pretreatment. The efficient hydrolysis of alkaline oxidised materials by low enzyme dosages can decrease enzyme costs or enable shorter hydrolysis time. In order to further improve the hydrolysis efficiency and decrease the required enzyme dosage, enzyme mixtures were optimized regarding the major enzymes needed in biomass hydrolysis. Optimized mixtures of thermostable enzymes were found to have significantly different proportions of cellobiohydrolases, endoglucanases and xylanase than the optimized mixtures of Trichoderma reesei enzymes. Although different, the significant role of cellobiohydrolases was demonstrated in both types of mixtures. The results also indicated that high xylanase activity was required in the hydrolysis of pretreated materials having decreased enzyme accessibility to cellulose due to high xylan content or possibly due to drying of the substrate. The hydrolysis performance of optimized enzyme mixtures of five thermostable enzyme components was shown to be close to that of state-of-the-art commercial mixtures.
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Nimeke	**Esikäsittely- ja entsyymihydrolyysiteknologioiden kehittäminen biojalostamosovelluksiin**
Tekijä(t)	Anne Kallioinen
Tiivistelmä	Kasvavat energian, materiaalien ja ruuan tarpeet, fossiilisten raaka-ainevarojen vähentyminen ja huoli ympäristöstä ovat lisänneet kiinnostusta uusiutuviin luonnonvaroihin. Lignoselluloosapohjainen biomassa on vaihtoehto fossiilisille raaka-aineille polttoaineiden, materiaalien ja monien kemikaalien tuotannossa. Kasvien soluseinän lignoselluloosa koostuu pääosin polysakkarideista, kuten selluloosasta ja hemiselluloosasta, sekä aromaattisesta ligniinistä. Nämä pääkomponentit muodostavat monimutkaisen rakenteen, joka on hyvin kestävä mikrobien ja entsyymien hajotukselle. Koska kasvien soluseinat ovat lujia, lignoselluloosapohjaiset raaka-aineet täytyy esikäsitellä ennen entsymaattista hydrolyysia monosakkarideiksi. Erilaisia kemiallisia, fysikaalisia ja biologisia esikäsittelymenetelmiä tai niiden yhdistelmiä on kehitetty. Esikäsittelyn jälkeen polysakkaridit voidaan hydrolysoida entsymaattisesti monosakkarideiksi, jotka voidaan edelleen fermentoida erilaisiksi tuotteiksi, kuten etanoliksi. Tällä hetkellä ensimmäiset kaupallisen mittakaavan lignoselluloosapohjaista bioetanolia valmistavat tehtaat ovat aloittaneet tuotannon. Koska kannattava biojalostamo vaatii turvatut raaka-ainelähteet, uusia ja vaihtoehtoisia biomassoja tarvitaan edelleen erityisesti pohjoisessa ilmastossa täyttämään biojalostamoiden raaka-ainetarve. Myös uusien esikäsittelytekniikoiden kehitystä ja nykyistä tehokkaampaa entsyymihydrolyysiä tarvitaan. Tässä työssä tutkittiin uusia lignoselluloosaraaka-aineita ja kehitettiin esikäsittelymenetelmiä. Tutkimuksessa havaittiin, että ruokohelpi ja ohran olki olivat kiinnostavia korkean hiilihydraattipitoisuuden omaavia raaka-aineita, jotka voitiin esikäsitellä höyryräjäytyksellä ja hydrolysoida entsymaattisesti vehnän olkeen verrattavilla saannoilla. Ruokohelven korjuuajankohdan vaikutusta esikäsittelyyn ja hydrolyysisaantoon tutkittiin syksyllä ja keväällä korjatulla ruokohelvellä. Keväällä korjatun ruokohelven havaittiin olevan sopivampi raaka-aine, sillä sen selluloosapitoisuus oli suurempi ja siitä saatu esikäsitelty kuitu hydrolysoitui paremmin verrattuna syksyllä korjattuun materiaaliin. Tutkimuksessa kehitettiin uusi esikäsittelymenetelmä, joka perustui alkaliseen käsittelyyn hapettavissa olosuhteissa käyttämällä kemikaaleina natriumkarbonaattia ja happea. Alkalihapetus fraktioi biomassan hiilihydraattipitoiseen kuituun ja ligniinipitoiseen liuenneeseen fraktioon. Tuotettu kuitu voitiin hydrolysoida tehokkaasti entsyymeillä, ja hydrolyysi oli tehokas myös 12 %:n kuiva-ainepitoisuudessa. Verrattuna höyryräjäytetyllä kuusella saatuun 52 %:n glukoosisaantoon esikäsittelyssä ja hydrolyysisssä, merkittävästi korkeampi 84 %:n glukoosisaanto saatiin käyttämällä alkalihapetusta esikäsittelynä. Menetelmä soveltui erilaisten raaka-aineiden, kuten kuusen, koivun ja sokeriruokobagassin, esikäsittelyyn. Alkalihapetuksen päävaikutukset olivat ligniinin ja hemiselluloosan liukeneminen ja osittainen hajoaminen. Osa galaktoglukomannaanista ja ksylaanista liukeni ja hapettui edelleen muiksi tuotteiksi, ja siksi hemiselluloosasaanto oli suhteellisen alhainen. Alkalihapetuksessa muodostui ligniinin ja hiilihydraattien hajoamistuotteina orgaanisia happoja. Prosessiolosuhteita optimoitiin alkalihapetuskäsittelyn tehokkuuden parantamiseksi käyttämällä kuusta raaka-aineena. Esikäsittelyä voitiin tehostaa nostamalla käsittelylämpötilaa, käyttämällä kupari-fenantroliini-katalyyttiä ja pienentämällä raaka-aineen partikkelikokoa. Hemiselluloosasaannon ja taloudellisen tehokkuuden parantamiseksi tarvitaan esimerkiksi alkaliannostuksen ja kiintoaine-neste-suhteen lisäoptimointia. Alkalihapetus-esikäsittelystä saadut kuitufraktiot hydrolysoituivat tehokkaasti jo alhaisilla entsyymiannoksilla, 2–4 FPU/g kuiva-ainetta. Merkittävästi korkeampia entsyymiannoksia tarvittiin höyryräjäytettyjen materiaalien hydrolyysissä, mikä johtui todennäköisesti korkean ligniinipitoisuuden aiheuttamasta inhiboivasta vaikutuksesta. Alkalihapetettujen materiaalien tehokas hydrolyysi alhaisilla entsyymiannostuksilla voi alentaa entsyymikustannuksia tai mahdollistaa lyhyen hydrolyysiajan. Biomassan hydrolyysissa tarvittavien pääentsyymien seoksien koostumusta optimoitiin, jotta hydrolyysiä voitaisiin edelleen tehostaa ja alentaa entsyymiannoksia. Lämpöstabiilien entsyymien optimoiduissa seoksissa oli eri suhteissa sellobiohydrolaaseja, endoglukanaaseja ja ksylanaasia kuin *Trichoderma reesei* -homeen entsyymeistä koostetuissa optimoiduissa seoksissa. Sellobiohydrolaasit olivat kuitenkin merkittävin entsyymi molemmissa seoksissa. Saadut tulokset viittaavat siihen, että korkeaa ksylanaasiaktiivisuutta tarvitaan sellaisten esikäsiteltyjen materiaalien hydrolyysissä, joissa entsyymien pääsy selluloosaan on heikentynyt korkeasta ksylaanipitoisuudesta tai mahdollisesti raaka-aineen kuivaamisesta johtuen. Viiden termostabiilin entsyymin optimaaliset seokset vastasivat hydrolyysitehokkuudeltaan kaupallisia entsyymiseoksia.
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Development of pretreatment technology and enzymatic hydrolysis for biorefineries

The growing demand for energy, materials and food, finite fossil raw material reservoirs and increasing environmental concerns have all increased interest in renewable resources. Lignocellulosic biomass is a widely available alternative for replacing fossil raw materials in the production of fuels, materials and various chemicals. Lignocellulose present in plant cell walls has a recalcitrant structure and therefore needs to be pretreated before enzymatic hydrolysis. Various pretreatment methods; chemical, physical, biological or their combinations, have been developed. After pretreatment, polysaccharides can be hydrolysed enzymatically to monosaccharides, which in turn can be fermented to different products such as ethanol.

A secure supply of biomass is one of the key factors for a feasible biorefinery, and thus new feedstocks are required. In addition, development of new pretreatment technologies and more efficient enzymatic hydrolysis are needed. In this work new alternative lignocellulosic raw materials – reed canary grass and barley straw – were investigated as feedstocks for a biorefinery in northern climates. In addition, an alkaline oxidative pretreatment method was developed which can fractionate the main components of lignocellulose and provide a well-hydrolysable cellulose fraction. Furthermore, optimal enzyme mixtures were developed for the hydrolysis of pretreated materials.

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