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SO_2-ethanol-water fractionation and enzymatic hydrolysis of forest biomass

Minna Yamamoto

Aalto University

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SO_2-ethanol-water fractionation and enzymatic hydrolysis of forest biomass

Minna Yamamoto

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Abstract

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Abstract

Utilization of forest harvest residues in a biofuel production process was studied. The chemical composition of forest biomass and its effects on processability were evaluated. The biomass was fractionated using the SO_2-ethanol-water (SEW) pulping technology and optimal processing conditions were determined. Conversion of the released cellulosic fibers into sugar monomers through enzymatic hydrolysis was also studied.

The raw materials included hardwood (HW) and softwood (SW) biomass which consisted mainly of branches and tree tops. The chemical composition differed clearly from that of stem wood due to the presence of bark and special tissues, such as reaction wood, in biomass. SEW fractionation efficiently dissolved lignin and hemicelluloses from biomass while cellulose remained mostly intact. The degradation of dissolved hemicelluloses was minimal and thus, compounds inhibiting the fermentation were not produced in notable quantities at normal operating conditions. However, sugar degradation was observed at severe treatment conditions. Hemicellulose dissolution and HW delignification were comparable to those of stem wood but SW delignification was clearly inferior. Especially the presence of polyphenolic acids, typical for coniferous bark, was found to reduce the degree of delignification. The negative effect of SW bark was also demonstrated by purposely adding bark to the feedstock, resulting in increased amounts of undigested wood rejects. Similar effect was not observed with HW biomass since its rejects consisted mainly of undigested bark.

Both HW and SW SEW fibers were effectively hydrolyzed by commercial enzymes although SW biomass fibers required significantly higher enzyme dosages. Especially a high residual lignin content in the SEW treated fibers reduced the enzymatic hydrolysis and this explains in part the high recalcitrance of SW biomass. Besides lignin, polyphenolics present in SW bark also bind to the enzymes thereby reducing their hydrolytic activity. SW bark was found to notably impair hydrolysis while HW bark only had a negative effect on enzymatic hydrolysis at very high bark content. Based on this study it was estimated that the best method for overcoming the negative effects of bark was to apply surfactants during enzymatic treatment. It was also speculated that lignosulfonates produced in the SEW fractionation could be utilized as enzyme enhancers instead of commercial surfactants.

Keywords bark, biorefinery, enzymatic hydrolysis, forest biomass, SO_2-ethanol-water (SEW) fractionation

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Tiivistelmä

Työssä tutkittiin metsähakkuutähteiden käyttöä eräässä biopolttoaineen valmistusprosessissa. Erityisesti perehdyttiin raaka-aineen kemialliseen koostumukseen sekä sen vaikutuksiin prosessissa. Metsäbiomassaa fraktioitiin SO_2-etanoli-vesi-keitolla, ja prosessille määriteltiin suotuisat olosuhteet. Lisäksi tutkittiin fraktioinnista saatavan kiinteän jakeen eli kuitujen entsymaattista hydrolyysiä.

Raaka-aineena käytettiin sekä havu- että lehtipuubiomassaa, joka koostui pääosin puun oksista ja latvuksista. Biomassan kemiallinen koostumus poikkesi selvästi runkopuusta johtuen sekä hakkuutähteiden sisältämistä kuoresta että erilaisista solukoista, kuten reaktiopuusta. Tutkittu fraktiointimenetelmä liuotti biomassasta tehokkaasti sekä hemiselluloosaa että ligniiniä, mutta selluloosa ei hajonnut fraktioinnin aikana. Keittonesteeseen liuenneiden sokerien hajoaminen käsittelyn aikana oli erittäin vähäistä, ja näin ollen fermentaatiota estäviä kemikaaleja ei syntynyt merkittävästi normaaleissa käsittelyolosuhteissa. Voimakkaampi käsittely nosti kuitenkin hajoamistuotteiden pitoisuutta. Hemiselluloosan liukeneminen ja lehtipuubiomassan delignifiointi olivat verrattavissa runkopuun vastaaviin tuloksiin, mutta havupuubiomassan delignifiointi oli huomattavasti heikompaa johtuen etenkin havupuukuoren fenolihapoista. Havupuukuoren haitallinen vaikutus todistettiin myös tutkimalla kuoripitoisuuden kasvattamisen vaikutusta rejektien määrään ja laatuun: puurejektin määrä lisääntyi selvästi kuoripitoisuuden noustessa. Lehtipuulla vastaavaa vaikutusta ei havaittu, vaan rejekti koostui pääosin kuoresta.

Sekä havu- että lehtipuukuidut hydrolysoituivat tehokkaasti kaupallisen entsyymiliuoksen avulla, mutta havupuubiomassa vaati huomattavasti korkeampia entsyymiannoksia. Tutkitulla fraktiointimenetelmällä valmistettujen kuitujen ominaisuuksista erityisesti korkean ligniinipitoisuuden todettiin heikentävän entsymaattista hydrolyysiä, mikä osaltaan vaikeutti havupuukuitujen käsittelyä. Ligniinin lisäksi myös kuoren fenolihapot voivat sitoa entsyymejä heikentäen niiden tehokkuutta. Havupuukuori heikensi entsymaattista hydrolyysiä merkittävästi, kun taas lehtipuukuorella oli negatiivinen vaikutus vain huomattavan korkeissa kuoripitoisuuksissa. Lisäksi tutkimuksessa arvioitiin, että pinta-aktiivisten aineiden käyttö entsymaattisessa hydrolyysissä on paras tapa vähentää kuoren haittavaikutuksia. Näiden kaupallisten kemikaalien sijaan fraktioinnissa syntyviä lignosulfonaatteja voitaisiin mahdollisesti hyödyntää saantojen parantamisessa.

Avainsanat biojalostamo, entsymaattinen hydrolyysi, kuori, metsäbiomassa, SO_2-etanoli-vesi-fraktiointi

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Preface

This study was carried out between November 2008 and June 2014 in the Department of Forest Products Technology at Aalto University, School of Chemical Technology (former Helsinki University of Technology). The work was performed within the Bioforest and SEWIBE projects funded by Finnish Funding Agency for Innovation (TEKES) and industrial sponsors. The funding and contribution of industrial parties are gratefully acknowledged. Additionally, the Walter Ahlström foundation and Finnish Paper Engineer's association are acknowledged for their financial support during the research.

I am grateful to my supervisor, Professor Adriaan van Heiningen, for giving me the opportunity to join his research group with a very interesting research topic and guiding my work throughout the years. Despite his simultaneous professorship at the University of Maine, I always received rapid and detailed advice regarding my work. I would also like to thank him for giving me the great opportunity to visit his laboratory for a month research period in Maine. I am indebted also to Professor Tapani Vuorinen who recommended me to continue my studies after the Master's thesis and introduced me to Professor van Heiningen.

I would like to sincerely thank my colleague and instructor Mikhail Iakovlev for his constant and patient support in both laboratory work and writing the manuscripts. Mikhail, your contribution was outstanding – I will always feel thankful for all your help, kindness and encouraging attitude towards my research. It was such a pleasure to get to know you and co-operate with you.

I am thankful also to all my co-authors and the researchers of the Bioforest and SEWIBE projects, especially Evangelos, German, Kristian, Shrikant and Tom. I appreciate the opportunity to work with you in creating a new biorefinery process. Sandip is greatly appreciated for his efforts to carry out successful fermentation trials. I would also like to thank Tuomas for continuing the research during my maternity leave and conducting an excellent study on the effects of bark. Sefik is thanked for all his help and guidance during my stay at the University of Maine. I also wish to thank Hanna who was one of my closest colleagues from the beginning of this work.

I would like to express my gratitude to all present and former colleagues and personnel in our department. The working atmosphere was really pleasant and supportive. Thanks to the laboratory technicians for their excellent assistance with the laboratory work. I would also like to thank all members of the Biorefineries research group – it was nice to be part of such an innovative and

talented group, not to mention conference trips and all great activities outside office hours.

Thanks to all my dear friends for joyful moments, laughter and support in countless of matters throughout the years. Your kind encouragement and positive attitudes helped me a lot during my PhD studies. I am also truly thankful to my parents, siblings and relatives for their caring support and encouragement in my life and studies throughout the years.

Finally, my deepest gratitude to my husband and daughter for so much love, happiness and joy every day! Akio, your constant encouragement during my doctoral studies was invaluable.

Espoo, August 20th, 2014
Minna Yamamoto

List of Publications

This doctoral dissertation consists of a summary of the following publications which are referred to in the text by their Roman numerals. Please notice the change in the family name (current Yamamoto, née Rakkolainen).

Paper I Rakkolainen, M., Iakovlev, M., Teräsvuori, A-L., Sklavounos, E., Jurgens, G., Granström, T., van Heiningen, A. (2010) SO_2-ethanol-water fractionation of forest biomass and implications for biofuel production by ABE fermentation. *Cellulose Chemistry and Technology,* 44(4-6), 139-145.

Paper II Yamamoto, M., Iakovlev, M., van Heiningen, A. (2011) Total mass balances of SO_2-ethanol-water (SEW) fractionation of forest biomass. *Holzforschung,* 65(4), 559–565.

Paper III Yamamoto, M., Iakovlev, M., van Heiningen, A. (2014) Kinetics of SO_2-ethanol-water (SEW) fractionation of hardwood and softwood biomass. *Bioresource Technology*, 155, 307-313.

Paper IV Yamamoto, M., Iakovlev, M., van Heiningen, A. (2014) The effect of chemical and physical characteristics of spruce SEW pulps on enzymatic hydrolysis. *Cellulose,* DOI 10.1007/s10570-014-0396-y.

Paper V Yamamoto, M., Iakovlev, M., Bankar, S., Tunc, M.S., van Heiningen, A. (2014) Enzymatic hydrolysis of hardwood and softwood harvest residue fibers released by sulfur dioxide-ethanol-water fractionation. *Bioresource Technology*, 167, 530-538.

Paper VI Yamamoto, M., Niskanen, T., Iakovlev, M., Ojamo, H., van Heiningen, A. (2014) The effect of bark on sulfur dioxide-ethanol-water fractionation and enzymatic hydrolysis of forest biomass. *Bioresource Technology*, 167, 390-397.

Author's Contribution

Paper I Minna Yamamoto was responsible for the experimental design with the co-authors and performed the experimental work related to raw materials and fractionation. She analysed the results and wrote the manuscript with the co-authors (responsible author).

Papers II-IV Minna Yamamoto was responsible for the experimental design with the co-authors, performed most of the experimental work and analysed the results. She wrote the manuscript with the co-authors (responsible author).

Paper V Minna Yamamoto was responsible for the experimental design with the co-authors and performed the experimental work except for the ABE fermentation and feedstock pretreatment work. She analysed the results and wrote the manuscript with the co-authors (responsible author).

Paper VI Minna Yamamoto was responsible for the experimental design with the co-authors and supervised the experimental work which was mostly carried out by Tuomas Niskanen. Minna Yamamoto wrote the manuscript with the co-authors (responsible author).

Other publications that are not included in this thesis but to which the author has contributed:

Paper VII Sklavounos, E., Iakovlev, M., Yamamoto, M., Teräsvuori, L., Jurgens, G., Granström, T., van Heiningen, A. (2011) Conditioning of SO_2-ethanol-water spent liquor from spruce for the production of chemicals by ABE fermentation. *Holzforschung*, 65(4), 551-558.

List of Abbreviations and Symbols

ABE	Acetone, butanol, ethanol
AVAP®	American Value Added Pulping
CBH	cellobiohydrolase
CED	cupriethylenediamine
DIP	deinked pulp
DNS	dinitrosalisylic acid
DP	degree of polymerization
FPU	filter paper unit
GC-FID	gas chromatography with flame ionisation detector
HMF	hydroxymethylfurfural
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
HW	hardwood
IC	ion chromatography
IFBR	Integrated Forest Biorefinery
LCC	lignin-carbohydrate complex
L:W	liquor-to-wood
o.d.f.	oven-dried feedstock
PAD	pulse amperometric detector
SEW	SO_2-ethanol-water
SHF	separate hydrolysis and fermentation
SPORL	sulfite pretreatment to overcome recalcitrance of lignocelluloses
SSF	simultaneous saccharification and fermentation
SW	softwood
YSI	Yellow Springs Instrument

Contents

1. Introduction

Global concerns regarding climate change, energy security and depletion of easily available fossil fuel resources have attracted wide interest into the production of renewable fuels, chemicals and energy. Simultaneously, population growth and increasing standard of living are forecasted to substantially increase the energy and fuel demand worldwide (International Energy Agency, 2012). The need to improve energy efficiency, and develop renewable energy technologies suitable for replacing fossil based energy, liquid fuels and chemicals, is evident and urgent. Thus, a large amount of scientific research has been focused on these areas. Also local subsidies and initiatives have promoted the intensive research actions in the field, as well as strongly supported the first production facilities (Balan et al., 2013). Production of biochemicals, materials and fuels from forest biomass also opens new opportunities to the traditional pulp and paper industries in temperate climate areas (Ragauskas et al., 2006), which have been struggling economically due to fluctuating profitability in the areas of mature and declining markets such as newsprint and printing and writing papers.

Especially lignocellulosic biomass is promising for renewable fuels and chemicals since it is an abundant, sustainable and cost competitive feed stock (Pu et al., 2008). The first generation biofuels are mainly produced from edible sources, such as corn, sugarcane and vegetable oils. However for the future lignocellulosic resources are strongly favoured since they do not compete with food or feed production. Such materials include forestry residues, wood processing mill residues and agricultural residues, used for the so-called second generation biofuels. Nevertheless, conversion processes of lignocellulosics are more complicated and costly than those based on starch due to their recalcitrant nature since they consist of a dense and highly organized matrix of cellulose, hemicellulose and lignin which is very difficult to penetrate by degrading enzymes and microbes (Himmel et al., 2007). Bringing down the lignocellulosic biofuel production costs will be the key factor for their success and extensive and innovative research is required to reach techno-economic and environmentally sound solutions. However, due to the progress in technical development and clear human and global driving forces in the liquid transportation field, a gradual but sustained shift towards biobased economy is predicted.

Several processing options exist for the conversion of lignocellulosic biomass into liquid biofuels. The major pathways include thermochemical processes,

such as gasification and pyrolysis (Digman et al., 2009), and biochemical conversion (enzymatic hydrolysis and fermentation) after fractionation or pretreatment of biomass (Kumar et al., 2008). Whereas currently about half of the wood used in pulping industry is converted to energy, novel biorefineries aim at a wider variety of end products by utilizing all the biomass compounds more efficiently. Ideally, each biomass component should be converted to a product at a value significantly above its energy content, and the new processes be integrated with existing pulp or paper production facilities to improve the profitability and reduce investment costs (Van Heiningen, 2006).

In this work, the focus will be on SO_2-ethanol-water (SEW) fractionation technology and enzymatic hydrolysis of forest biomass which meets the criteria of a successful biorefinery concept. The research was carried out in a project where a whole biorefinery process from feedstock processing to final biofuel product was developed. The focus of this thesis was to study the charasteristics of softwood (SW) and hardwood (HW) harvest residues as raw materials, and their treatment by SEW fractionation (Papers I-III). Also the enzymatic digestibility of SEW treated biomass was studied: Paper IV concentrated on the effect of the characteristics of SEW pulps on hydrolysis, whereas in Paper V, mainly the differences between HW and SW biomass hydrolysis were discussed. Finally, the effect of bark on SEW fractionation and enzymatic hydrolysis was discussed in Paper VI. Cellulose and hemicellulose sugars released from biomass were also converted into a mixture of solvents through acetone-butanol-ethanol (ABE) fermentation by *Clostridia* bacteria (Paper V). These research topics were selected since sustainable production of biofuel from renewable sources, especially from low-grade lignocellulosic biomass, is timely and essential for the future. Techno-economically sound solutions are still under investigation despite the extensive research efforts already carried out in the field. The selected process concept was believed to offer a competitive and economical production of biofuel from a wide variety of lignocellulosics. The whole process was demonstrated in laboratory scale and its strengths and weaknesses were evaluated throughout to estimate its potential.

It was determined that SEW fractionation is well suited for the conversion of harvest residues into its principal components. Hemicellulose sugars and lignin were rapidly removed from the biomass at relatively mild reaction conditions, while the sugar degradation and formation of inhibitors was negligible. Released cellulosic fibers were efficiently converted to glucose by subsequent enzymatic hydrolysis, using cellulase dosages comparable to the competing technologies. The main difficulties observed in the biomass processing included inferior delignification of SW harvest residues during SEW treatment, which was due to its higher content of bark and the specific features of conifereous bark, including the presence of polyphenolic acids which are insoluble during SEW fractionation. High lignin content of the resulting fibers impaired subsequent enzymatic hydrolysis. In fact, it was shown that especially SW bark was inhibiting the enzyme activities whereas the SEW delignification and the digestibility was less affected by HW bark.

However, surfactants were found to increase SW hydrolysis yields and thus to improve the potential of SW harvest residues as a feedstock for biochemical conversion.

2. Background

This chapter will provide background information on the structure and composition of lignocellulosics, forest residues as a raw material, and on SEW fractionation and enzymatic hydrolysis.

2.1 Structural components of lignocellulosic biomass

Lignocellulosic biomass consists of three main polymers: cellulose, hemicellulose and lignin. They form a complex and tightly organized matrix that contributes to the high recalcitrance of biomass to degradation, and thereby hinders biomass disassembly in industrial processing (Himmel et al., 2007). In addition, small amounts of extractives, inorganic compounds and proteins are present in wood tissues. The composition of biomass is highly dependent on its origin, i.e. there are large variations in the composition between different species and growth regions.

2.1.1 Cellulose

Cellulose is the most abundant renewable polymer in the world. It is a strictly linear homopolysaccharide consisting of 1-4-linked β-D-glucopyranose units. Cellulose is the main component in wood where its content is about 40-47% (Koch, 2006). The molecular structure of cellulose is shown in Figure 1.

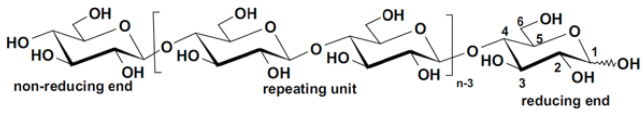

Figure 1. Molecular structure of cellulose chain (Koch, 2006).

In native wood, the degree of polymerization (DP) of cellulose is around 10000. Cellulose molecules have a strong tendency to both intra and intermolecular bonding, enabled by their linear structure, leading to the formation of aggregates called microfibrils. Microfibrils form both tightly organized crystalline areas, as well as amorphous areas. The high crystallinity of cellulose contributes to its high strenght and high resistance towards chemical treatments, such as pulping (Alén, 2000).

2.1.2 Hemicellulose

Hemicelluloses are group of heteropolysaccharides with either branched or linear structure. They constitute hexose (D-glucose, D-mannose and D-galactose) and pentose (D-xylose and D-/L-arabinose) units linked together at different ratios. Additionally, uronic acids are present in the side chains of xylans. Hemicelluloses are not crystalline and the DP of hemicelluloses is notably lower (100-200) compared to cellulose which results in lower chemical and thermal stability. Thus, hemicelluloses are easily dissolved in pulping/pretreatment processes. Specific hemicelluloses, such as arabinogalactan present especially in larch, even dissolve in water either fully or partially (Alén, 2000). Chemical structures of some hemicelluloses are shown in Figure 2.

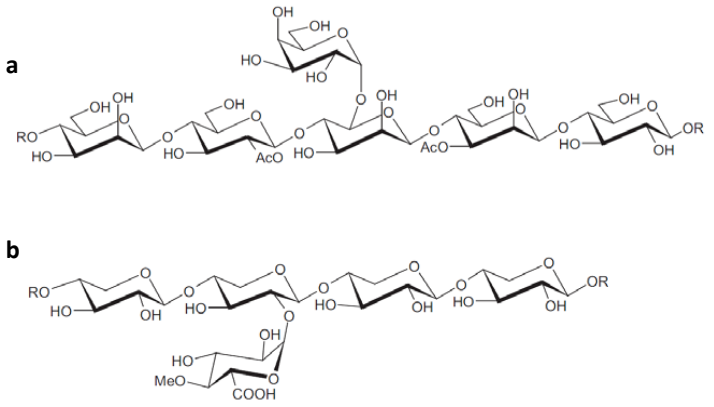

Figure 2. Examples of the chemical structures of hemicelluloses: a) softwood glucomannan and b) hardwood xylan (adapted from Koch, 2006).

The hemicellulose composition of HW and SW differ from each other as well as between different species. Typically SW hemicelluloses contain more mannose and galactose units compared to HW while HW has a higher content of xylan and acetylated hydroxyl groups (Koch, 2006). Xylan is also the dominant hemicellulose in agricultural residues. HWs have generally a somewhat higher content of hemicelluloses compared to SW (30-35 and 25-30% for HW and SW, respectively). The acetyl group content in SW galactoglucomannan is about 6%, while SW xylan has no acetyl groups. In HW glucuronoxylan, the acetyl group content varies from 8 to 17% whereas HW glucomannan is not acetylated. Other polysaccharides present in wood include starch, callose and pectic substances (galacturonans, galactans and arabinans). Different galactans are present especially in reaction wood (compression wood in SW and tension wood in HW) (Alén, 2000).

2.1.3 Lignin

Lignin is a complex amorphous heteropolymer which consist of three phenylpropanoid units, derived from *p*-coumaryl, coniferyl and sinapyl alcohol. These precursors are randomly linked either by ether linkages or

carbon-carbon bonds (Figure 3). The composition of lignin varies greatly between species. HW lignin consists typically of quaiacyl (coniferyl alcohol) and syringyl (sinapyl alcohol) units at about equal percentages, whereas guaiacyl is the main component in SW lignin. Grass lignins also contain significant amounts of structural units derived from *p*-coumaryl alcohol, besides sinapyl and coniferyl (Alén, 2011). The chemical reactivity of lignin is affected by the proportions of these structural units (Koch, 2006).

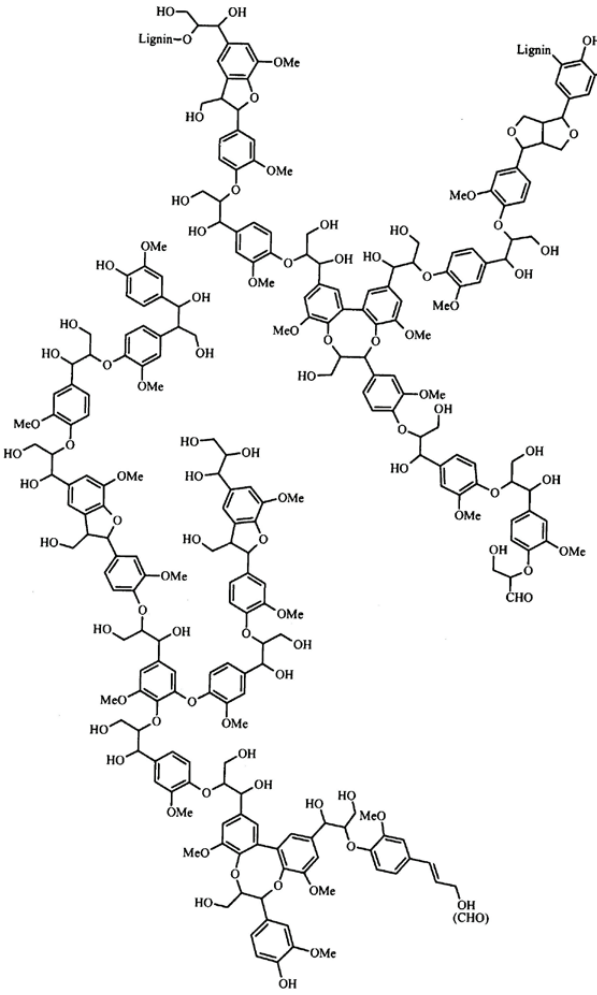

Figure 3. Molecular structure of quaiacyl lignin in softwood (Brunow et al., 1998).

The lignin content varies notably between wood species and is also affected by the location within a tree. In general, SWs have a lignin content of 26-32% and HWs 20-28% (Sjöström, 1981), while agricultural residues have a lignin content comparable to that of hardwood. There is also a substantial difference between the lignin structures of HW and SW. For instance, SW lignin is more branched and cross-linked, has a higher molecular weight and higher share of carbon-carbon bonds, i.e. it is more condensed (Sjöström, 1981; Achyuthan et

al., 2010). These differences are crucial in pretreament processes and likely responsible for the higher recalcitrance of SW biomass.

Lignin and carbohydrate molecules can bond with each other mainly through covalent bonds and a term lignin-carbohydrate complex (LCC) is used for such structures. LCCs are present in native wood but can also form during the delignification. Lignin is mainly bound to hemicelluloses through their side groups arabinose, galactose and 4-O-methylglucuronic acid (Fengel and Wegener, 1989). The chemical stability of LCC bonds depends on the type of the linkage and on the chemical structures of lignin and hemicellulose units associated with the linkage (Lawoko et al., 2005).

2.1.4 Extractives

Extractives in wood consist of several thousands of compounds, including resin acids, fats, terpenes, tannins and a variety of phenolic compounds. They mainly have a low molecular mass and are generally either water-soluble (hydrophilic substances) or soluble in neutral organic solvent (lipophilic substances). Extractives affect the odor, color and taste of wood. Their function in wood is to be energy sources and protect the wood against microbial attack and insects. The composition and content of extractives varies within the tree species and also within the different parts of the tree. In addition, the growth conditions and age of the tree affect the extractives content. For typical Nordic tree species such as pine (*Pinus sylvestris*), spruce (*Picea abies*) and birch (*Betula pendula*) the extractives content is about 2.5-4.5, 1.0-2.0 and 1.0-3.5%, respectively. (Alén, 2000)

Phenolic compounds, like pinosylvin in pine, has a tendency to form cross-links with lignin in acid sulfite pulping causing impaired delignification. One method to reduce the negative effects of extractives in sulfite pulping is subjecting the chips to long storage time which reduces the content and changes the composition of extractives (Sjöström, 1981). Phenolic substances are prevalent in bark and heartwood. Polyphenolic acids can account for 40-50% of the bark weight (Erman and Lyness, 1965; Goldstein, 1975). They are not soluble in any common organic solvents (Jensen et al., 1963; Hergert et al., 1965; Dietrichs et al., 1978) but are highly soluble in alkaline solutions (Fengel and Wegener, 1989).

Extractives cause problems in pulping and papermaking processes but they are also considered valuable compounds utilized for by-products, such as turpentine and tall-oil. Especially bark and bark-containing harvest residues are rich in extractives and those complicate the chemical processing and utilization of harvest residues. However, the potential of extractives in bark or knots for a variety of valuable niche applications (chemicals, materials, pharmaceuticals) has also been emphasized (Holmbom et al., 2003; Feng et al., 2013).

2.1.5 Inorganics

The inorganics content of wood harvested in temperate regions, measured as ash, accounts approximately for 0.1-1.0% on a dry basis. In tropical and subtropical woods the content can be much higher, up to 5%. The location within a tree and growth conditions (site fertility and climate) also influence the inorganics content. Potassium, calcium and magnesium are typically the most common inorganic elements in softwoods and hardwoods (Fengel and Wegener, 1989). The latter can add up to 80% of the total inorganics with the remainder being a wide variety of other elements (Alén, 2000).

Some inorganics are crucial for tree growth and thus critical for fertilisation and soil conservation. However, in energy production and pulping, inorganics are often harmful for instance by causing scaling or interfering in bleaching reactions. Also, bark and forest residues have a higher content of inorganics than wood. The ash content decreases in the order of bark to tiny roots, twigs, roots and branches and finally stem wood (Fengel and Wegener, 1989). Sometimes logging and harvest procedures increase the amount of inorganic contaminants, by entrainment of materials such as sand (Alén, 2000).

2.2 Forest residues as a raw material

Forest residues from the thinning and logging operations, as well as mill residues from wood processing offer an abundant and sustainable raw material source for the production of biochemicals and energy. Unfortunately, the fluctuations in the properties of forest residues are significantly larger than that of stem wood and thereby negatively affects their processability.

2.2.1 Differences compared to stem wood

Composition

Forest harvest residues consist of branches, twigs, tree tops and stump wood which cannot be utilized for conventional pulping and papermaking or timber production. Bark is present as separate particles or still attached to woody particles. The content of bark is highly dependent on tree species, as well as on growth conditions and age of the tree (Sjöström, 1981). Impurities, like humus and sand derived from the forest land, may reduce the quality of the residues.

The chemical composition of the feedstock affects the operation of many energy production processes. On the other hand, flexibility in raw material quality would be beneficial because it widens the use of possible feedstock resources. There are distinct differences in the composition of stem wood, bark and forest residues which are discussed in detail in the next sections. Table 1 presents the chemical composition of wood, bark and forest residues.

Table 1. The chemical compostion of wood, bark (inner and outer) and forest residue (% of the feedstock dry solids) (Alén, 2011).

Component	Wood	Bark [a]	Forest residue [a]
Cellulose	40-45	20-30	35-40
Hemicelluloses	25-35	10-15	25-30
Lignin	20-30	10-25	20-25
Extractives	3-4	5-20	~5
Other organics	~1	5-20 [b]	~3
Inorganics	<0.5	2-5	~1

[a] Depends greatly on the wood species.

[b] Containing mainly suberin (2-8%) and polyphenols (2-7%) as well as proteins and starch (1-5%)

Woody tissues

Compared to stem wood, branches have a higher share of special wood tissues, such as reaction wood and juvenile wood, leading to lower quality and yield of pulp. For SW species, juvenile wood present in branches and especially in tree tops have higher lignin content compared to stem wood, while HW tree tops benefit from higher glucan content (Hakkila, 1989). Compression wood present in SW branches has a higher density, higher lignin content and lower cellulose content compared to normal wood. Typically, cellulose is less crystalline and lignin more condensed in compression wood. The glucomannan content is about half of that in normal wood while galactan content is higher. On the other hand, tension wood in HW is characterized by lower lignin and xylan content but higher cellulose and galactan content than normal wood (Alén, 2011). Reaction wood tissue has also been observed in roots and bark (Höster and Liese, 1966). Thick cell walls and narrow lumina are typical for compression wood, leading to high density (Hakkila, 1989) and possibly reduced penetration of chemicals.

Compared to non-wood feedstocks (bagasse, straw etc.), which are possible alternative feedstocks for biorefineries, forest residues usually have a somewhat higher lignin content and lower hemicellulose content, although differences in these can be small and are highly species dependent. However, benefits of forest residues compared to non-wood feedstocks are their higher bulk density, lower inorganics and extractives content, as well as lower content of silica (SiO_2) which is known to cause problems in alkaline processes due to scaling. Unlike wood, non-wood feedstocks are also rich in proteins (Alén, 2011).

Bark

Bark protects the wood from mechanical damage, microbiological attack and variations in humidity and temperature (Sjöström, 1981). The bark content of stem wood is about 10-20%, depending on the species, age and growing conditions. The proportion of bark is higher in the branches, tree tops, stumps and roots (Fengel and Wegener, 1989). In general, the share of bark in the branches of large coniferous trees varies from 20-45% and most hardwood species are in the same range. The smaller the coniferous branch diameter is, the higher the bark content (Hakkila, 1989). There are also clear differences in

the outer and inner layers of bark: in general, the content of extractives and carbohydrates is reported to decrease and the content of lignin and polyphenolics to increase from the inner bark to outer bark (Fengel and Wegener, 1989).

The chemical composition of bark is complicated and varies a lot among different species. Bark is rich in extractives (20-40% of the dry weight) and inorganics (2-5%) while the carbohydrate content is notably lower than in wood (Sjöström, 1981). Usually HW bark has a higher content of inorganics compared to SW (Jensen et al., 1963). Calcium and potassium are the dominant species (Sjöström, 1981). The amount and type of extractives varies significantly between different wood species, and their determination requires several extraction sequences utilizing different solvents. Polyphenols consist of several compounds of which polyphenolic acids are probably the most harmful for processing due their chemical stability and low solubility: they are soluble only in 1% NaOH. They have a high content of carboxyl groups, contributing to their alkaline solubility, while the content of methoxyl groups is lower than in lignin. Suberin is an insoluble compound in outer bark, and its content is high especially in birch bark (20-40%) (Sjöström, 1981). The polysaccharide structures are similar in wood and bark but there are some differences in their ratios (Fengel and Wegener, 1989). In addition to chemical differences, SW and HW bark are also characterized by structural differences (Sjöström, 1981).

Determination of lignin content in bark is complicated due to the presence of polyphenolics which contribute to the lignin content, if not removed previously by alkaline extraction. Wood and bark lignin have similar structures, although some differences are observed in the ratios of the structural components (Fengel and Wegener, 1989). Isolated bark lignin is claimed to have a notably more heterogenous structure than that of wood lignin (Jensen et al., 1963).

In biomass processing, the specific chemical characteristics of bark complicate the processing compared to stem wood. Since fungicides are present in the bark and the function of several extractives in bark is to protect the wood against biological damage, it is likely that these compounds interfere with the actions of enzymes and microorganisms. Bark has also been shown to greatly increase the consumption of cooking chemicals in both sulfite and soda pulping. Phenolic acids consume a lot of alkali in soda pulping. Especially slash pine was detrimental in sulfite pulping since its bark consumed twice the amount of sulfite based on the same amount of wood (Jensen et al., 1963). While the specific chemical characteristics of bark affect both pretreatments and biochemical conversion, the high ash content also causes problems in thermochemical conversion processes.

2.2.2 Abundance

Cellulose present in different lignocellulosics is the most abundant biopolymer in the world and thus represents a widely available source for biochemical and fuel production. For the creation of the new bioeconomy, it is important to ensure the continuous availability of biomass at low enough cost and

environmental impact. Efficient forest management is needed to maintain the productivity of forest land and to secure sufficient annual growth.

Ecological, economical and technical constraints affect the amount of forest biomass available for processing. In 2012, Finland used 17.8 million m^3 solid wood (harvest residues, bark, saw dust, industrial wood waste) for heat and energy production, and the utilization rate has been increasing steadily throughout the past decade (Ylitalo, 2013). In the US, the availability of forest resources for bioenergy is estimated to be 33-119 million dry tons in 2012, strongly dependent on the price paid (higher availability at higher price) (U.S. Department of Energy, 2011). Only a modest increase in the supply of forest biomass over time is predicted, while agricultural resources could provide considerably higher quantities which are also expected to increase over time. It has been estimated that local biomass resources in the European Union are not able to meet their 2020 biofuel targets, and thus significant import of biomass is required (Balan et al., 2013).

The amount of harvested residual biomass is strongly affected by many factors, like tree size, species and forest management. In Scandinavian forests, unmerchantable stem wood in the harvest of mature stemwoods is 4-5% of industrial roundwood volume. The volume of residues from the tree crown (mainly branches) is about 5-20% (Hakkila, 2004). Additionally, annual surplus forest growth could be utilized in biorefineries, although stem wood likely remains primarily as the source for conventional lumber and value-added fiber products. Also insect-attacked wood is suitable for bioconversion processes.

Biorefineries should always rely on local raw material sources to maintain short and economic transport distances. In fact, location is one of the key factors affecting economical viability, especially as a result of logistics and biomass availability (Stephen et al., 2010). Since the available raw material supply and accessability vary notably within different regions, also biorefineries must be adapted to local conditions. Thus, different processing options optimized for local resources are needed since the raw material quality determines the most suitable process. Ideally, a biorefinery should be flexible with respect to raw material quality because it widens the feed stock supply, as well as reduces seasonal variations in the supply. Notable advantages of forest residues over agricultural residues are its year round harvest and lower variation in seasonal availability. Additionally, forest residues have a higher bulk density leading to lower transportation costs and improved logistics (Zhu and Zhuang, 2012).

2.2.3 Current use and other aspects

The utilization rate of harvest residues varies a lot within countries. Traditionally harvest residues are either left to decompose in the forest to maintain the nutrient balance of the forest land or they are combusted for energy production. Nordic countries have been at the forefront in energy production from wood residues, for example by utilizing logging residual chips for district heating. However, it has been claimed that the energy potential

from harvest residues has not been reached in any industrialized country (Richardson, 2002).

The positive aspects of utilizing forest residues more efficiently have been overshadowed by the fact that their harvest is expected to have permanent effects on the forest ecosystem. Especially foliage and needles, containing the greatest concentrations of nutrients (Werkelin et al., 2005), are believed to be crucial for the fertility of forest land and maintaining productivity of the growth site. Research on the effects of whole-tree harvesting requires long-term research efforts but it is clear that these aspects must be considered and negative effects on the forest ecosystem be minimized. Another drawback is the low density of lignocellulosic materials, which increase the costs for transportation and storage (Stephen et al., 2010).

Nevertheless, among the alternative raw material sources to replace fossil fuels, forestry residues represent a cost-competitive, sustainable and abundant resource for second generation biofuels. First generation biofuels based on edible sources are unsustainable and ethically not sound since they increase food prices and decrease food availability, and require significant fertilation and energy. Also the net greenhouse gas emissions of first generation biofuels are notably higher than that of biofuels derived from forestry residues. Thus, the exploitation of forestry residues to fulfill the energy and fuel demand in the future is highly desirable.

2.3 SO_2-ethanol-water (SEW) fractionation

2.3.1 Principles of SEW fractionation

SO_2-ethanol-water (SEW) pulping can be considered a hybrid between solvent and acid sulfite pulping processes. It fractionates the lignocellulosic feedstock into its principal components: cellulose, hemicellulose and lignin. The process was originally developed for pulping and introduced by Schorning (1957). Several studies about SEW pulping were published also by Ukrainian researchers (Eliashberg et al., 1960; Primakov et al., 1979) and some other research groups (Puumala, 1991; Pylkkänen, 1992). Recently, SEW process has been extensively studied at Aalto University by our group under the guidance of Professor Adriaan van Heiningen. The SEW technology is also part of a patented process termed American Value Added Pulping (AVAP®) by American Process Inc. (Retsina and Pylkkanen, 2011) – a company which is developing large scale biofuel and chemical production based on this method. As will be discussed below, the features of the SEW technology are highly suitably for lignocellulosics processing and thus, it is believed to have several advantages over other conversion technologies.

The cooking liquor of SEW process constitutes of ethanol, water and dissolved sulfur dioxide. Pulping is carried out under moderate temperatures from 130-160°C. The effect of temperature and SO_2 concentration on SEW fractionation kinetics has been extensively studied by Iakovlev et al. (Iakovlev et al., 2011; Iakovlev and van Heiningen, 2012b). Cellulose remains mostly

resistant to hydrolysis whereas hemicelluloses undergo acid hydrolysis and yield about equal amounts of monosugars and oligosaccharides. Lignin becomes water soluble through sulfonation reactions. The presence of ethanol facilitates rapid penetration of the cooking liquor into wood chips, thereby shortening the treatment time and preventing condensation reactions of lignin. The pH of the cooking solution decreases with increasing cooking time due to formation of strong lignosulfonic acids. However, ethanol reduces the effective acidity of the liquor and therefore minimizes carbohydrate degradation. Thus, sugar degradation and formation of inhibitory compounds are modest.

Chemical recovery is relatively simple due to absence of a base and relies on straightforward distillation of ethanol and unreacted SO_2. A significant benefit of SEW fractionation is also the omnivorous character, i.e. it has been shown that the process can be applied to various lignocellulosic feedstocks including annual plants (Iakovlev et al., 2011). An additional important advantage compared to many other processes is its flexibility in terms of potential final products: the process can be used either for the production of good quality paper pulp, dissolving pulp or biochemicals and biofuels, depending on the operation conditions. The properties of SEW pulps are similar to that of acid sulfite pulps. Compared to kraft pulps, they have excellent z-directional strength, higher brightness before bleaching and higher bleachability, but lower tear strength. SEW pulps have been demonstrated to be suitable for paper/tissue, dissolving pulp and nanocellulose production (Iakovlev et al., 2010, 2014b; Morales et al., 2014).

Drawbacks of the SEW method include ethanol flammability, corrosiveness of the spent liquor, possible SO_2 losses to atmosphere and the requirement of minimal ethanol losses to maintain profitability. Also the toxicity of SO_2 which poses safety and health risks is a distinct drawback of the SEW process, although SO_2 is used in many existing industrial processes. If the process is used for pulp production, the tear strength of pulp is lower and drainability worse compared to kraft pulp (Iakovlev et al., 2009, 2010). In addition, relatively complicated spent liquor conditioning is required prior to fermentation to butanol in order to make the liquor fermentable (Sklavounos et al., 2014). However for ethanol production by sugar fermentation, simple SO_2/ethanol evaporation and neutralisation would likely be adequate.

2.3.2 Comparison of SEW to competing technologies

A gradual transfer from fossil based fuels towards biofuels is presently ongoing supported by extensive research efforts directed especially towards the development of biomass pretreatment technologies. Pretreatment is still considered as one of the most expensive process stages in biomass conversion to fermentable sugars. It is required since without any pretreatment the enzymatic digestibility of most lignocellulosic biomass is very low, below 20%. Examples of pretreatment processes are steam explosion, acid hydrolysis, organosolv and Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL). These methods, however, are different from

fractionation processes such as SEW because the latter method separates the biomass in its principal components. Traditional acidic sulfite pulping, on the other hand, is comparable to the SEW process. Several other biomass pretretment methods (Mosier et al., 2005; Carvalheiro et al., 2008) have been developed, but they are not discussed here.

In short, steam explosion is a method where biomass is treated with high-pressure saturated steam followed by rapid depressurization which opens the biomass structure through an explosive decompression. It is generally carried out at high temperature (160-260°C) lasting from several seconds to a few minutes, and it can be either uncatalyzed or catalysed, e.g., by addition of SO_2 or H_2SO_4. Novel steam explosion technologies aim to utilize lower temperatures to improve feasibility (Jedvert et al., 2012). Acid pretreatment can be operated over a wide range of temperatures either at low or high acid concentration. It improves the enzymatic digestibility mainly through dissolution of hemicelluloses but the acid recovery and/or acid losses and corrosive nature of the acid makes it a relatively expensive pretreatment method. Organosolv processes rely on organic or aqueous organic solvent mixtures with or without acid catalyst (HCl or H_2SO_4) to carry out simultaneous delignification and prehydrolysis of hemicelluloses. Also alkaline organosolv pulping has been studied, although the requirement of chemical recovery system for alkali would complicate the process. Besides a variety of possible solvents and catalysts, the conditions in organosolv processes also vary widely depending on the feedstocks. However, in general the temperature is in the range of 180-195°C, time 30-90 min and ethanol concentration 35-70% (Kumar et al., 2009). The SPORL process treats wood chips with an aqueous sulfite solution followed by mechanical size reduction using disk refining. The temperature applied is 165-180°C, optimal chemical concentrations 2-4% H_2SO_4 and 8-10% Na_2SO_3 and duration 20-30 min (excluding the impregnation and heat-up time) (Zhu et al., 2009; Zhou et al., 2013b). Acid sulfite pulping is currently the only commercially operated wood fractionation process where dissolved hemicelluloses are converted to ethanol through fermentation (Jurgens et al., 2012). In sulfite pulping, an aqueous sulfur dioxide solution with base is used for the delignification of wood. The amount of base in the cooking liquor determines the pH during digestion. Cooking temperatures range from 125 to 150°C in the acidic process. The mechanisms of the main pulping reactions are similar to those in SEW process but the absence of ethanol requires slow impregnation (at 110-120°C) and thus very long overall treatment times (up to 12 hours) (Fengel and Wegener, 1989; Sixta et al., 2006). A qualitative comparison of these processes is presented in Table 2.

Table 2. Qualitative comparison of some pretreatment and fractionation technologies (Jurgens et al., 2012).

Pretreatment or fractionation	Full utilization of hemicelluloses	Low energy need	No sticky lignin issue	Omnivorous	Simple recovery
Steam explosion	No	No	Intermediate	No	Yes
Acid hydrolysis	Intermediate	Intermediate	No	No	No
Lignol (EtOH-H_2O)	Intermediate	No	Yes	No	Yes
SPORL	Intermediate	Intermediate	Yes	Intermediate	No
SEW (SO_2-EtOH-H_2O)	Yes	Yes	Yes	Yes	Intermediate
Sulfite pulping	Intermediate	Yes	Yes	Intermediate	Intermediate

Advantages of the SEW process over the aforementioned pretreatment technologies include full utilization of hemicelluloses due to negligible sugar degradation, lower energy needs due to lower operation temperatures, and capability of treating a wide range of biomass types, including SW. The latter is different for dilute acid and the uncatalyzed organosolv and steam explosion pretreatment technologies which are limited to the use of hardwoods and agriculture residues. In addition, the SEW process does not suffer from sticky lignin precipitates which impair steam pretreatment and dilute acid hydrolysis. Chemical recovery in the SEW process is simple compared to the dilute acid and SPORL treatments. Another benefit of SEW fractionation is its applicability to both green and air-dried feedstocks (Iakovlev et al., 2014a) whereas hornification caused by drying is considered to reduce the accessibility of feedstock to steam and chemicals during pretreatment. For example steam explosion is shown to work better at higher moisture content than with air-dry feedstock (Cullis et al., 2004). Nevertheless, several different technologies are being developed since none of them can be considered to be clearly superior. Furthermore, different raw materials and targeted final products require different processing severities for optimal pretreatment.

2.3.3 Example of a biorefinery process utilizing SEW fractionation

During the past 6 years, a biorefinery concept based on SEW fractionation, enzymatic hydrolysis and ABE fermentation has been developed at Aalto University. Figure 4 shows the flow diagram of the biorefinery process and also illustrates the different product options.

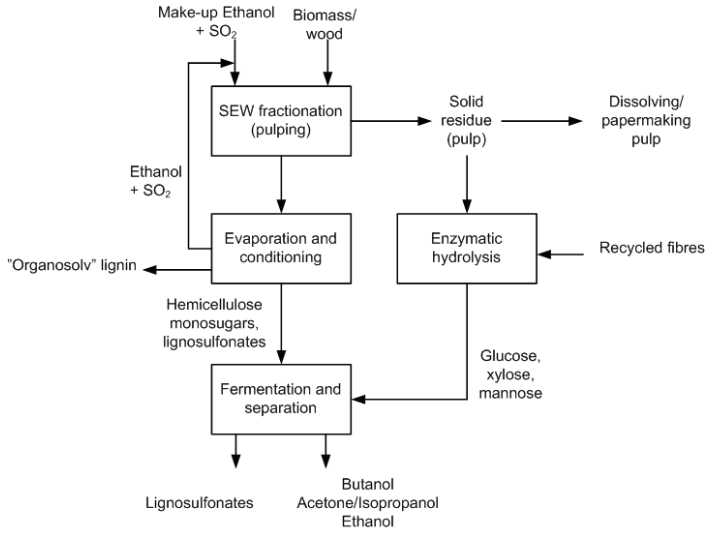

Figure 4. Process diagram of a biorefinery concept utilizing SEW fractionation.

Harvest residues were used as primary raw material and their suitability for this process were studied. The most relevant results with harvest residues are presented in this thesis. Additionally, a conditioning scheme was developed for the spent fractionation liquors (Sklavounos et al., 2011, 2013a, 2013b). Efficient chemical recovery is necessary for process economics, and the subsequent butanol fermentation stage sets strict requirements on the conditioned liquors, such as maximum allowable SO_2 and dissolved lignin concentrations. Improvements in the conditioning procedure in terms of lignin removal are still desirable however.

The fermentation process studied in the project was bacterial ABE fermentation originally developed by Weizmann (1915). Fermentability of the conditioned spent liquors by ABE fermentation utilizing *Clostridium acetobutylicum* bacteria was demonstrated in several publications, although dilution of the liquor was necessary to reach low enough dissolved lignin concentrations (Survase et al., 2011b; Sklavounos et al., 2013b). Also continuous plug flow fermentation with the *Clostridia* bacteria immobilized in a “rolled-up” bed of wood fibers was studied (Survase et al., 2011a, 2011c). Furthermore, the *Clostridia* bacterial strain was genetically modified in order to produce isopropanol instead of acetone which would not be suitable as a biofuel component (Jurgens et al., 2010). In summary, the overall results obtained in this project showed that the SEW biorefinery concept was a feasible pathway for the conversion of lignocellulosic biomass to value added products. The economical aspects are discussed shortly in section 4.5.

2.4 Enzymatic hydrolysis

2.4.1 Enzymes needed for lignocellulose conversion

Three main enzyme types are required for efficient enzymatic degradation of cellulose polymers into monomeric glucose units: endoglucanase, cellobiohydrolase (exoglucanase) and β-glucosidase (Enari, 1983). Cellulases act synergistically and each has a specific function in the disassembly of cellulose chains. Endoglucanases randomly shorten the cellulose chains by attacking amorphous regions and reducing the DP of cellulose. Cellobiohydrolases (CBH) act either on the reducing (CBHI) or non-reducing end (CBHII) of the cellulose chain and release cellobiose units consisting of two glucose monomers. Finally, β-d-glucosidase breaks down the soluble cellodextrins and cellobiose units into glucose monomers (Lynd et al., 2002). Figure 5 shows the action of different cellulases during cellulose hydrolysis.

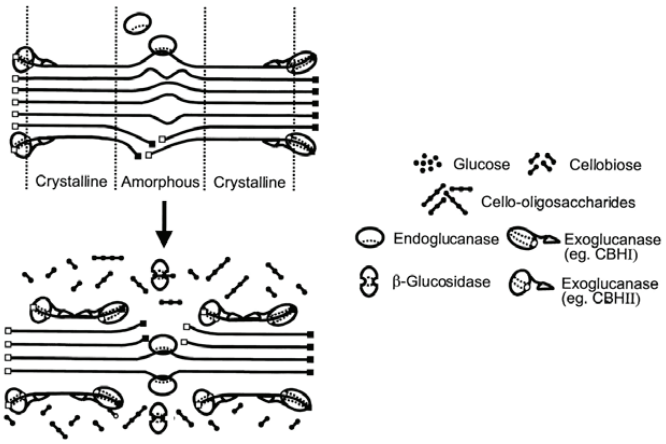

Figure 5. Schematic image of cellulose hydrolysis by the cellulases. The solid and open squares represent reducing ends and nonreducing ends, respectively. Cellulose, enzymes, and hydrolytic products are not shown to scale (adapted from Lynd et al., 2002).

Enzymes secreted by *Trichoderma reesei* are among the most studied and widely exploited cellulolytic enzymes, and their industrial strains are highly efficient enzyme producers. Cellulases produced by *T. reesei* are important especially considering the production of second generation biofuels (Schmoll and Schuster, 2010). Correct ratios of each cellulase type in the enzyme preparation are critical since cellobiohydrolases are inhibited by cellobiose (Jørgensen et al., 2007). In addition, β-glucosidases are inhibited by glucose. Cellulose-binding domains (or carbohydrate-binding modules) attach the cellulases on the cellulose surface, increasing the effective concentration of cellulases, as well as the time they remain on the cellulose surface. Cellulose-binding domains particularly improve the hydrolysis of crystalline cellulose, although they are also responsible for the unwanted non-productive binding of cellulases on lignin, thereby reducing the enzyme efficiency and recycling potential (Viikari and Alén, 2011).

Besides cellulases, efficient lignocellulose hydrolysis requires also hemicellulases which hydrolyze hemicelluloses that often cover cellulose fibrils. The required hemicellulases vary according to the substrate characteristics and they have synergistic interactions similar to cellulases (Várnai et al., 2011). Similarly to cellulases, endoenzymes are needed to randomly cleave the hemicellulose backbone, whereas exoenzymes cleave polymers created by endoenzymes. Xylanases act on xylan which is present in both HW and SW materials. SW hydrolysis also benefits from mannanases which break down glucomannan. In addition, several auxiliary enzymes are involved in the hydrolysis of hemicellulose side chains (Viikari and Alén, 2011), although those are quite often cleaved already during the pretreatment stage. Nevertheless, enzyme development for lignocellulose hydrolysis is an ongoing research area, and for example β-mannosidase crucial for mannan disaccharide hydrolysis is not commercially available yet.

2.4.2 Structural features affecting the lignocellulose digestibility

Several structural features of lignocellulosic biomass influence the ability to convert them into monomeric sugars by enzymatic hydrolysis. In general, pretreatment and fractionation methods carried out prior to enzymatic hydrolysis aim to improve the digestibility of lignocellulosics via chemical and/or structural changes.

Important features affecting the digestibility include surface area and cellulose accessibility to enzymes, cellulose crystallinity and lignin content and distribution. In addition, hemicellulose content, pore volume, lignin structure and functional groups, particle size, DP of cellulose and acetyl content have been reported to affect the digestibility (Mansfield et al., 1999; Leu and Zhu, 2013). Moreover, the type of raw material has a clear effect on digestibility: HW materials and agricultural resources are shown to be easier digestible than SW (Mansfield et al., 1999; Nakagame et al., 2010; Yu et al., 2011). Some of these features and their relative effects on digestibility are discussed more in the results section of this thesis.

2.4.3 Current status

Currently, both simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) are studied for biofuel production processes. Especially SSF processes are promising due to hydrolysis and fermentation combined in one reactor, likely leading to substantially lower processing costs. This approach also avoids end-product inhibition caused by glucose. Extensive research has been ongoing for the development of lignocellulosic hydrolysis by enzymes; however significant barriers are still to be overcome. Lignocellulosic biomass, especially SW, is highly recalcitrant and requires expensive pretreatments to be digestible, contrary to readily hydrolysable starch containing feedstocks. Despite the development of low cost cellulases, the enzyme cost for lignocellulosics is still considered too high especially in comparison with the enzymes hydrolysing starch. A clear

drawback of cellulases is also the slow reaction rates and thereby leading to large reactor volumes for commercial plants (Schubert, 2006).

Despite the technoeconomic difficulties in biomass conversion by enzymes, several positive aspects enhance their use in future biofuel production. Currently, bioethanol production through the biochemical conversion route is the main process strategy in both the EU and US (Balan et al., 2013), although it has been concluded that both the biochemical and thermochemical pathways seem economically equally feasible (Wright and Brown, 2007). Enzyme prices have been reduced thereby improving the economic potential of biochemical processing, although even higher cost reductions are still necessary (Schubert, 2006). Innovations in enzyme recycling would also lead to improved techno-economics. In addition, development of more efficient enzymes and enzymes having better stability at different process conditions, including higher temperature and wider pH range tolerance, is ongoing. Besides, genetic engineering of lignocellulosics to produce a less recalcitrant feed stock by modifying the plant cell wall, such as the concentration and type of lignin, is also discussed as a possible method to improve the potential of biobased fuels (Himmel et al., 2007).

An alternative method for enzymatic hydrolysis of cellulose is acid hydrolysis, although currently enzymatic hydrolysis is considered the more promising option. The advantages of enzymatic hydrolysis compared to acid hydrolysis are mild reaction conditions (low temperature and pH close to neutral), high yields without production of inhibitory compounds and lower maintenance costs due to the absence of corrosion-related problems. Enzymatic hydrolysis is also more environmentally friendly. In addition, cost reductions are expected due to technology development while acid hydrolysis is considered a mature technology with less potential for cost reductions. However, one of the main benefits of acid hydrolysis is the fast reaction rate - it takes only a few minutes to a few hours while enzymatic treatment requires several days (Hamelinck et al., 2005; Taherzadeh and Karimi, 2007a, 2007b).

3. Experimental

This chapter presents an overview of the raw materials and methods used in this dissertation. More detailed information on materials and methods can be found in Papers I-VI.

3.1 Materials

3.1.1 Forest biomass

The main raw materials included in this study were SW and HW harvest residue chips which consisted mainly of branches and tree tops. The materials are later refered to as SW and HW biomass. SW biomass consisted mostly of spruce (*Picea abies*) and some pine (*Pinus sylvestris*), while hardwood biomass consisted mainly of birch (*Betula pendula*). Biomass was harvested in midwinter from central Finland and stored in the freezer before use. Bark was present in biomass both attached to wood chips and as separate particles. In the preliminary experiments (Paper I-II), the HW biomass was used as such. SW biomass was screened (SCAN-CM 40:01, utilizing screens: Ø3, Ø7, Ø13, //8 and Ø 45 mm) to remove the substantial amount of humus and needles present in the obtained feedstock. Chips above Ø7 mm size were accepted but particles larger than 42 mm were manually rejected due to the size restrictions of the pulping bombs. Experiments were carried out on green biomass (dry matter content 48-55%). Deinked pulp (DIP) was studied briefly as alternative raw material in the preliminary studies (Paper I and II). However, the results are not discussed in this thesis.

Variations in the dry matter content of green chips were estimated to negatively affect the accuracy of the results. To improve the reproducibility of the experiments carried out with the very heterogenous biomass feedstocks, further experiments (Paper III-V) were done on air-dried biomass having narrower particle size distribution. Accepted chips size included the fractions collected from Ø7 and Ø13 mm screens (Figure 6). Iakovlev et al. (2014a) have shown that the dry matter content of the feedstock does not affect the SEW treatment efficiency. This finding was confirmed also on SW biomass: air-drying had no effect on pulp yield, reject content, viscosity or kappa number (unpublished results).

Figure 6. Raw materials before and after the screening: a) unscreened and b) screened HW biomass; c) unscreened and d) screened SW biomass. Accepted particles were collected from screens Ø7 and Ø13 mm.

For Paper VI, a new batch of SW biomass was obtained which was used as such after air-drying. Other raw materials included spruce (*Picea abies*) and birch (*Betula pendula*) stem wood chips, which were screened using the screens Ø45; //8; //6; //4; //2 mm. The fractions from the screens //2 and //4 mm were used in the experiments. Air-dried and ground bark from pine (*Pinus sylvestris*) and birch (*Betula pendula*) was also included in the study.

The bark content of HW and SW biomasses was determined by manually removing the bark according to SCAN-CM 42:95, with the exception of using only 100-200 g chips per analysis.

3.1.2 Enzymes

Commercial enzyme preparation Cellic CTec2 obtained from Novozymes was used in the enzymatic hydrolysis experiments. The preparation contained cellulase and xylanase activities. Two different batches were used, the first one in Papers IV and V and the second one in Paper VI. In addition, Cellic HTec2 obtained from Novozymes and endomannanase obtained from AB enzymes were used in some experiments included in Paper V. Surfactant Tween 20 was studied as yield enhancer in Paper VI. Filter paper unit (FPU) activity was determined for the Cellic CTec 2 to facilitate dosing comparable with other publications. Additionally, protein content analyses were carried out on the second batch of Cellic Ctec2.

3.2 Methods

3.2.1 SO_2-ethanol-water (SEW) fractionation

SEW fractionation of biomass chips was done in silicon oil bath using 220 mL bombs each filled with 25 g (o.d. basis) biomass chips or 15 g (o.d.) ground biomass. SEW liquor was prepared by injecting gaseous sulfur dioxide into an ethanol-water solution (deionized water and ethanol ETAX A, 96.1%v/v). The composition and charge of the cooking liquor was mostly kept constant in the experiments (SO_2:EtOH:H_2O = 12:43.5:44.5, by weight; liquor-to-wood (L:W) ratio 6.0 L kg^{-1}), whereas fractionation time (20-180 min) and temperature (135-160°C) were varied to determine the optimal conditions. Heat-up time was 8-9 min and it is included in the reported durations. Fractionation was stopped by cooling the bombs in cold water. Then, solid residue was separated from the spent liquor by squeezing it in a nylon washing bag. Pulps were washed twice with 40% v/v ethanol-water at 60°C (L:W 2 L kg^{-1}) and twice with deionized water at room temperature (L:W 20 L kg^{-1}).

3.2.2 Analysis of feedstock and pulp properties

Chemical charasteristics of the feedstocks, solid residues of fractionation and spent fractionation liquors were analysed to establish complete mass balances of fractionation. Air-dried solid materials were ground by Wiley mill (20 mesh) prior to analysis of chemical composition. Analyses included acetone extractives content, carbohydrate content and composition (high performance anion exchange chromatography with pulse amperometric detector (HPAEC-PAD) and gas chromatography with flame ionisation detector (GC-FID)), lignin content and ash content. Cellulose content of the feedstock and pulps was calculated by subtracting the glucose present in hemicelluloses from the total glucose. Glucose in hemicelluloses was calculated based on the mannose-to-glucose ratio of 1.6 and 4.15 reported for HW and SW glucomannan, respectively (Janson 1974). Pulps were also analysed for solid yield and reject content. Spent liquors were analysed for dry solids, dissolved carbohydrate and lignin contents, ash content and sugar degradation products: aldonic acids (HPAEC), furfural and hydroxymethylfurfural (high performance liquid chromatography (HPLC)). Sulfur consumption was determined by analysing the sulfur content of feedstocks, pulps and spent liquors dry solids (as sulfate anions by ion chromatography (IC), after oxidation by oxygen and hydrogen peroxide).

In addition, the pulps were analysed for kappa number, intrinsic viscosity in cupriethylenediamine (CED) solution and degree of polymerization of cellulose. Selected pulps were analysed also for fiber saturation point and crystallinity. More detailed description of the analytical procedures can be found in the Papers I-VI.

3.2.3 Enzymatic hydrolysis of solid residues

Enzymatic hydrolysis experiments were mainly carried out in 40 mL glass bottles with 10 mL sample volume. Conditions were 50°C, 250 rpm mixing, 1.5% substrate consistency and pH 4.8 (0.05 M sodium citrate buffer). Sodium azide was used as antibiotic. After specific reaction times, the samples were boiled for 10 min to denature the enzymes and stop the reactions. Samples were centrifuged to separate solids, and the dissolved sugars in the supernatant were analysed by either Yellow Springs Instrument (YSI) glucose analyser or high performance anion exchange chromatography with pulse amperometric detector (HPAEC-PAD).

For Paper VI, hydrolyses were carried out in Eppendorf tubes with sample volume of 1.2 mL and cellulose consistency of 1.0%. Rotating shaker was used instead of magnetic stirring. Sugar analysis was done by analysing the reducing sugars through dinitrosalisylic acid (DNS) method.

3.2.4 Fermentation

The ABE fermentation of sugars released from SEW fractionated and enzymatically hydrolysed biomass was briefly studied. Fermentation was carried out on SW biomass hydrolyzate which was produced by enzymatic hydrolysis at 10% consistency and at 20 FPU/g cellulose enzyme dosage for 72 h. ABE fermentation was done as batch experiment using *Clostridium acetobutylicum* and carried out for 120 h at 37°C. Activated carbon treatment was done to the production medium before fermentation in order to remove the inhibition caused by sodium citrate buffer. Butanol, acetone and ethanol produced were quantified by GC-FID.

4. Results and discussion

The most important findings of this work are summarized in this chapter. More detailed results can be found in the attached Papers I-VI.

4.1 Raw materials

4.1.1 Screening and bark content

The main purpose of screening SW biomass was to remove humus and needles (fractions smaller than Ø3 mm) which contained up to 37 % of these materials (Figure 7). In practice, it is very important to leave the humus, foliage and needles in the forest during harvesting since these are rich in nutrients important for the forest ecosystem and soil productivity. Thus, harvest operations should be optimized to minimize the share of these fractions in the collected biomass.

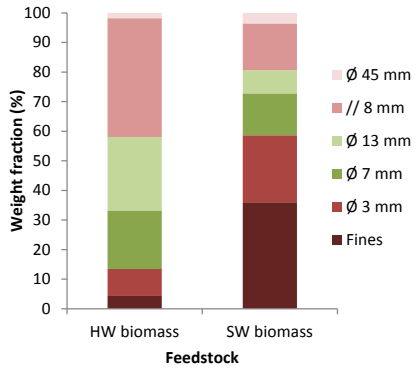

Figure 7. Size distribution of particles in original SW and HW biomass obtained for the experiments. Green areas describe the accept fractions used in the experiments for Papers III and V. For papers I and II, HW biomass was used as such while particles below 7 mm were rejected from SW biomass.

Biomass quality is also important in order to obtain a sufficiently high carbohydrate yield. For example SEW fractionation of unscreened SW biomass resulted in 33% lower sugar yield in the spent liquor and a considerably higher kappa number compared to screened SW biomass having particles larger than 7 mm (unpublished results). HW biomass, on the other hand, was readily usable and of higher quality due to its smaller fines content (Figure 7).

However, screening was also carried out on HW biomass to obtain a narrower particle size distribution in order to improve the reproducibility of the experiments by reducing the heterogenity of the feedstock. Also the small size of the batch pulping equipment (220 mL/batch, Ø42 mm) used in SEW fractionation required that larger particles must be removed.

There was a clear difference in the quality of the biomass types with HW having a higher content of woody chips while SW had a higher share of branch wood and bark. This was also evidenced by the fact that the analysed bark content was 28.0±2.3 and 7.2±1.1% for SW and HW, respectively (Paper III). Another SW biomass batch (Paper VI) had a bark content of 24.8±4.7%. The substantial difference in bark content naturally affects the processability of these feedstocks, in addition to leading to distinct differences in their chemical composition.

4.1.2 Chemical composition

Knowledge of the chemical composition of raw materials is important for process development and for understanding their behaviour and suitability as a feedstock. The chemical composition of SW and HW biomass is different similar to that of the composition of the corresponding stem wood. SW and HW biomass differ in their hemicellulose composition (galactoglucomannan being dominant in SW and arabino-4-O-Me-glucuronoxylan in HW) and amount (HW has higher content) and in lignin content (lower in HW). The content of acetyl groups is higher in HW biomass, as is typical for stem HW species. SW has a notably higher content of bark (28.0±2.3 vs. 7.2±1.1% for HW) which leads to a higher content of lignin, inorganics/ash and extractives. The chemical composition determined for SW and HW biomass and Spruce and Birch stem wood after screening (retained on screens Ø7 and Ø13 mm, Papers III and V) is shown in Table 3. All results are based on weight % of oven-dried feedstock (% o.d.f.). The SW and HW biomass results differ from that of the batches used and presented in Papers I and II but are considered to be more representative of the present study due to the same particle size distribution.

Table 3. Chemical composition of SW and HW biomass (Paper III) and corresponding stem wood materials. Chemical composition of spruce and birch stem wood is based on previous publications (Testova et al., 2011; Iakovlev and van Heiningen, 2012a). Carbohydrates are shown as anhydrosugars.

Components	SW biomass	Spruce stem wood	HW biomass	Birch stem wood
	% o.d.f.			
Carbohydrates	57.3	67.8	71.1	75.0
Cellulose	31.5	39.9	36.2	37.2
Hemicelluloses	25.8	27.9	34.9	37.8
Non-cellulosic glucan	1.4	3.1	1.2	1.1
Xylan	8.5	5.3	21.7	26.1
Mannan	5.9	12.8	1.9	1.8
Galactan	3.2	2.2	0.8	0.7
Arabinan	1.7	0.9	0.7	0.3
Rhamnan	0.4	0.2	0.5	n.d.
Galacturonic acid	2.6	1.4	2.8	n.d.
4-O-Me-glucuronic acid	0.8	1.0	1.5	3.1
Extractives	4.1	1.8	2.8	2.0
Lignin	33.9	27.7	25.8	25.9
Acid insoluble	32.7	27.4	21.9	21.4
Acid soluble	1.2	0.3	3.9	4.4
Ash	2.6	0.4	0.5	0.30
Acetyl groups	1.3	1.1	3.8	4.8
Total analyzed	97.9	97.8	100.3	103.2

The carbohydrate content of SW biomass is significantly lower than that of stem wood. Also, the cellulose to hemicellulose ratio in biomass is clearly lower than that of stem wood. Compared to spruce stem wood, SW biomass has a somewhat higher content of lignin, extractives and ash, mainly due to the presence of bark. The main compounds in ash are calcium, silica and potassium (Paper II). Also, the mannan content in SW biomass is only about half of that of spruce stem wood, likely due to presence of compression wood in branches since the glucomannan content of compression wood is reported to be about half of that in normal wood (Alén, 2011). Also the higher content of galactan is explained by the presence of compression wood.

HW biomass has a cellulose content comparable to stem wood which is explained by the relatively low content of bark. A high content of cellulose in tension wood in HW branches might also contribute to the high cellulose content observed (Alén, 2011). In addition, a lower xylan content is typical for tension wood, as is observed here. The comparable lignin content in HW biomass and birch stem wood is explained by the fact that the lignin content of birch bark (27.9%, Miranda et al. (2013)) is only slightly higher than that of stem wood. The main compounds in HW biomass ash (Paper II) are calcium and potassium, in accordance with literature data (Sjöström, 1981).

The composition for SW and HW biomass in Table 3 is the composite sum of bark and woody components However, the composition of bark obtained from pine and birch are reported in Paper VI. These results show significant differences with bark analyses reported by other researchers (Miranda et al., 2012, 2013). This suggests that the present analytical methods applicable for wood are not optimal for the analysis of bark. Specifically, the removal of extractives from bark requires comprehensive extraction stages, not only to improve the accuracy of the extractives content analysis but also to enhance the specificity of subsequent analyses, such as lignin analysis. Based on these

statements, it is also advisable to carefully reconsider the suitability of wood chemical analysis methods for harvest residues having high bark content.

4.2 SEW fractionation

4.2.1 Mass balances

Determination of detailed mass balances is an important aspect of optimizing the fractionation and determining the most favourable operating conditions for a given feedstock. Overall mass balances of SEW fractionation were measured by determining the oven-dry weights of the solid residue and spent liquor straight after fractionation (Paper II-III, V). SW mass balances were mostly close to 100%, indicating no significant losses in the course of fractionation experiments. However, HW mass balances were consistently in the range of 92-95%, mostly due to the analytical procedure of oven drying which resulted in the evaporation of volatile components, such as acetic acid originating from acetyl groups present in high amount in HW. Pulp yields obtained on biomass were systematically lower compared to those observed for stem wood due to the fact that the cellulose-to-hemicelluloses ratio and carbohydrate content of biomass are lower than those of stem wood. Figure 8 presents the mass balances of SW and HW, showing also the dissolution pattern of individual biomass components.

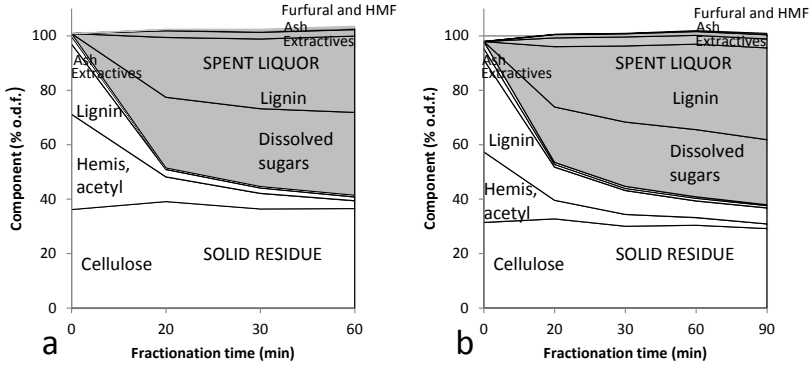

Figure 8. Mass balances of a) SW biomass and b) HW biomass (Paper III). SEW fractionation was carried out at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C.

The reject contents of biomass pulps varied from 0.5-1.2% for HW to 0.4-4.7% for softwood (Papers II-III, V). As expected, the reject content decreased with increasing intensity of fractionation. HW rejects mainly consisted of film-like reddish brown bark particles whereas SW rejects included also undigested dense branches or twigs, besides small bark pieces. More discussion related to the rejects is included in section 4.2.6.

4.2.2 Carbohydrate dissolution

Dissolution of hemicelluloses

One of the main factors affecting the viability of pretreatment and fractionation methods is sugar recovery from the raw material. Ideally, hemicellulose sugars are dissolved in high yield as monosaccharides and the degradation of liberated sugars is minimal. Dissolution of hemicelluloses is also of high importance in processes where the pretreatment is followed by enzymatic hydrolysis: hemicelluloses physically covering the cellulose chains are known to hinder the hydrolysis (Mansfield et al., 1999; Várnai et al., 2010). In most of the common pretreatment methods, cellulose remains intact due to the use of acidic pH or (for alkaline processes) of relatively low temperatures.

Dissolution of carbohydrates during SEW fractionation was discussed in Papers I-III. Paper III described the optimal fractionation conditions for biomass. Cellulose and hemicellulose dissolution from SW and HW biomass in the course of SEW fractionation is shown in Table 4.

Table 4. Cellulose and hemicellulose dissolution in SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C (Paper III). All carbohydrates are given as anhydrosugars.

Feedstock	**SW biomass**					**HW biomass**			
Fractionation time	**0**	**20**	**30**	**60**	**90**	**0**	**20**	**30**	**60**
	Carbohydrates in biomass or pulp, % o.d.f.								
Cellulose	31.5	32.8	30.1	30.4	29.2	36.2	39.2	36.5	36.6
Non-cellulosic glucan	1.4	0.4	0.3	0.2	0.1	1.2	0.6	0.4	0.3
Xylan	8.5	3.4	2.1	1.5	0.8	21.7	6.1	4.0	2.0
Mannan	5.9	1.8	1.3	0.9	0.6	1.9	0.9	0.7	0.4
Galactan	3.2	0.1	0.1	0.0	0.0	0.8	0.0	0.0	0.0
Arabinan	1.7	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0
Rhamnan	0.4	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
Galacturonic acid	2.6	n.m.	0.2	0.0	0.0	2.8	n.m.	0.0	0.0
4-O-Me-glucuronic acid	0.8	n.m.	0.2	0.1	0.0	1.5	n.m.	0.1	0.1
Acetyl groups	1.3	0.3	0.1	0.1	0.0	3.8	0.6	0.4	0.1
	Carbohydrates in spent liquor, % o.d.f.								
Glucose		2.0	2.5	2.9	3.4		0.8	1.2	2.0
Xylose		6.5	6.8	7.8	6.2		15.6	16.7	17.6
Mannose		3.4	4.2	4.6	5.5		0.9	1.1	1.5
Galactose		2.8	3.4	2.5	3.4		0.8	0.9	0.9
Arabinose		1.8	1.7	1.7	1.4		0.6	0.8	0.6
Rhamnose		0.4	0.4	0.4	0.3		0.5	0.5	0.5
Galacturonic acid		n.m.	2.4	2.3	1.4		n.m.	2.2	1.7
4-O-Me-glucuronic acid		n.m.	1.0	1.1	0.9		n.m.	1.7	1.9

Cellulose was mostly preserved in the solid residue for all the studied feedstocks, including stem wood studied previously. Hemicellulose sugars were rapidly dissolved: within 20 to 30 min fractionation, 74 and 84% of HW and SW biomass hemicelluloses were released into spent liquor, respectively. Dissolution rates of xylan and mannan are comparable for both SW and HW biomass, as well as for stem wood (Figure 9).

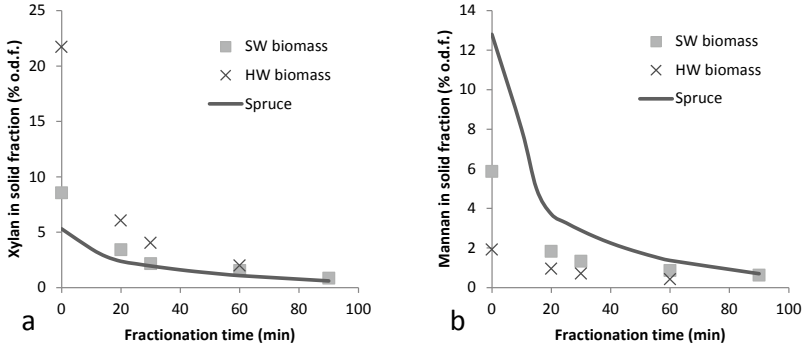

Figure 9. Residual xylan (a) and mannan (b) in the pulps produced from different feedstocks by SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C (Paper III). The curves for spruce are calculated according to the earlier published fractionation kinetics (Iakovlev et al., 2014a).

Arabinose and galactose, which are polysaccharide units located in the hemicellulose side chains, are fully dissolved within 20 min SEW treatment. However, 4-O-Me-glucuronic acid in the side chains of xylan is not fully hydrolysed due to the relative stability of the glucuronide bond towards acid hydrolysis (Sjöström, 1981). Pectin components (galacturonic acid and rhamnose) are almost fully dissolved within 30 min treatment.

Depending on the feedstock and processing conditions, approximately 50% of the dissolved hemicellulose sugars are present as monomers in the spent fractionation liquor which is a distinct advantage of SEW process. Their share increases further during the conditioning stages due to the removal of ethanol which favors acid hydrolysis reactions in the remaining aqueous solution (Sklavounos et al., 2011, 2013b).

Sugar degradation

Sugar degradation in the course of SEW fractionation was discussed in Papers II and III. Increased fractionation temperature and prolonged time boost the formation of sugar degradations products, such as furfural and hydroxymethylfurfural (HMF) formed from the liberated hemicellulose monosaccharides (Figure 10a). Due to the higher inorganics content of biomass, the increased formation of bisulfite could also lead to significant oxidation of the dissolved sugars to aldonic acids (Sixta et al., 2006). Formation of degradation products should be avoided to maintain sugar yields and to prevent formation of inhibitory compounds for fermentation. Furfural would be partially removed during conditioning stages by co-evaporation with steam prior to fermentation but non-volative HMF would be enriched during spent liquor concentration (Sklavounos et al., 2011, 2013b). However, an important finding of the present study is that sugar dehydration during SEW treatment of SW and HW biomass is minimal (see Figure 10a) and concentrations are generally well below *Clostridia* tolerance levels: 1.0 and 1.5 g/L for furfural and HMF, respectively (Teräsvuori, 2010).

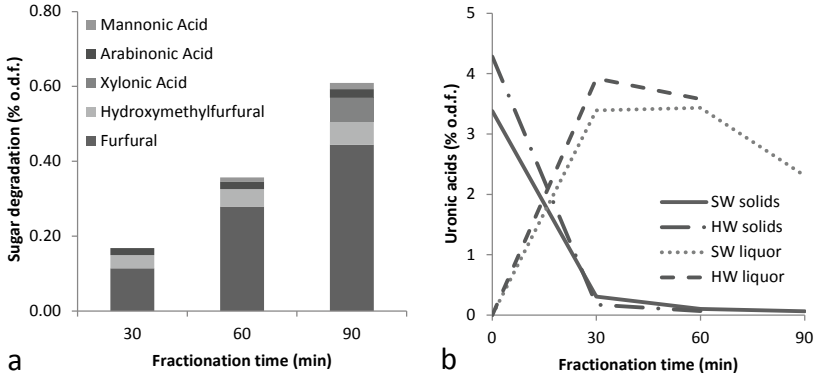

Figure 10. a) Sugar degradation products formed from dissolved hemicelluloses; b) Dissolution and degradation of uronic acids in the course of SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C.

Another negative effect resulting from unfavourable fractionation conditions is the degradation of uronic acids. As shown in Figure 10b, uronic acids are rapidly dissolved from wood in the course of SEW fractionation. However, after the dissolution galacturonic acid is possibly converted into CO_2 through decarboxylation causing pressure increase in the process. Also furfural and insoluble humins can be formed from uronic acids (Feather and Harris, 1966). Uronic acids could be utilized in fermentation and thus, it is beneficial to avoid their degradation by using short fractionation times.

4.2.3 Pulp viscosity and DP

In the course of fractionation, pulp viscosity and DP of cellulose are decreased due to hydrolysis of glycosidic bonds in the cellulose chains. Contrary to hemicelluloses, the crystallinity of cellulose protects it from extensive degradation despite the acidic conditions. Hydrolysis of cellulose is, however, pronounced at high temperature and longer fractionation times.

Cellulose DP is determined based on the intrinsic viscosity of cupriethylenediamine solutions of pulps. The effect of hemicelluloses on DP can be accounted for by the formula by da Silva Perez and van Heiningen (2002). Figure 11 shows the DP of cellulose as a function of fractionation time for the diffferent feedstocks studied (Paper III). The differences in DP between the different feedstocks are small towards the end of fractionation but in the beginning the DPs for biomass feedstock pulps are only approximately half of those in stem wood. It may be that the initial DP of biomass is lower than that of stem wood, although no literature values are available for DP in branches. However, bark is reported to have lower cellulose DP than that of wood (Fengel and Wegener, 1989), and also the crystallinity of cellulose is lower in compression wood than in normal wood (Alén, 2011).

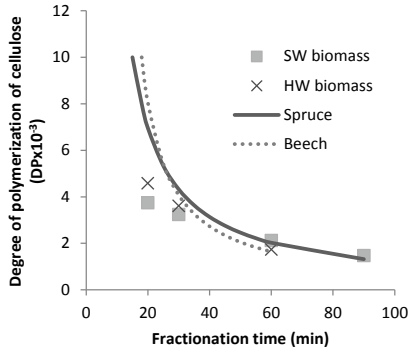

Figure 11. Viscosity-average cellulose DP of the pulps produced from different feedstocks by SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C (Paper III). The curves for spruce and beech are calculated according to the earlier published fractionation kinetics (Iakovlev et al., 2011; Iakovlev et al., 2014a).

Another observation based on Figure 11 is the slightly faster decrease in the DP of HW biomass. A similar observation was made in a stem wood study and it was speculated that this could be caused by easier accessibility of the amorphous regions of HW cellulose (Iakovlev et al., 2011). There is also some evidence that higher residual hemicellulose content, as observed on HW pulps, would lead to lower cellulose crystallinity in pretreated lignocellulose (Xu et al., 2012). Easier accessibility of HW cellulose in general is also evidenced by its easier enzymatic digestibility. However, the faster delignification and lower final lignin content of HW cellulose fibers are mostly responsible for its improved digestibility (see section 4.3.1).

4.2.4 Delignification

Delignification of biomass and stem wood

The objective of most lignocellulosics pretreatments or fractionations is to achieve efficient delignification in order to notably reduce the biomass recalcitrance and to improve enzymatic digestibility. SEW delignification of biomass was discussed in each of the papers (Papers I-VI). In general, the initial delignification during SEW fractionation is rapid for both SW and HW biomass. However, clear differences are observed between these feedstocks during the residual delignification phase and thus in final lignin content. Whereas HW biomass allows efficient and almost complete delignification comparable to stem wood feedstocks, SW biomass suffers from high residual lignin contents (Figure 12). SW biomass delignification was also inferior to spruce stem wood delignification, indicating that bark compounds might be responsible.

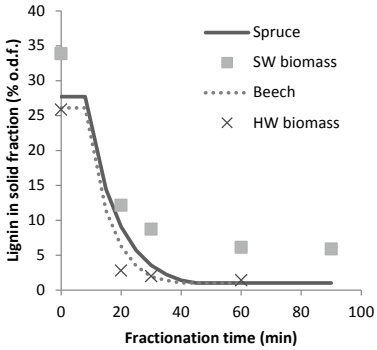

Figure 12. Delignification during SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1} and 150°C (Paper III). The curves for spruce and beech are calculated according to the earlier published fractionation kinetics (Iakovlev et al., 2009; Iakovlev et al., 2011; Iakovlev et al., 2014a).

There are several differences in lignin structure and delignification chemistry between SW and HW (Achyuthan et al., 2010) which contribute to the higher recalcitrance of SW in both pretreatments and enzymatic hydrolysis (Mansfield et al., 1999). In case of bark containing feedstocks, high lignin and polyphenolics content in bark further increases the biomass recalcitrance, and condensation reactions between lignin and extractives are typical. Inorganic compounds may also impair hydrolysis by neutralizing the lignosulfonic acids or by converting SO_2 into thiosulfate anions that can condense with lignin. Also, compression wood in branches has more highly condensed lignin that may impair the delignification (Alén, 2011). Another possible cause of inferior delignification could be the presence of pine wood in SW biomass. There is no data available on pine SEW fractionation but it is well known that acid sulfite pulping can not process pine, partially due to condensation reactions involving stilbenoid pinosylvin (Sixta et al., 2006). Also, the fact that initial delignification of SW is comparable to stem wood indicates condensation reactions – dissolved lignin or lignin-like compounds are first dissolved but later condense and precipitate on the fibers leading to higher "lignin" content. In addition, the presence of covalent lignin-carbohydrate bonds may have affected the inferior delignification of SW. Lawoko et al. (2005) have stated that whereas the xylan-linked LCC is degraded to a large extent, glucomannan-linked LCC lignin is more susceptible to condensation.

Condensation reactions are not pronounced in SEW pulping if there is sufficient SO_2 in the liquor. The presence of SO_2 promotes lignin sulfonation at the alpha carbon site which is subject to condensation. For the experiments carried out at for 90 min at 160°C and with only 6% SO_2 content it was noticed that the solid residue was very dark (kappa number ~150) indicating condensation due to lack of SO_2 and harsh conditions (unpublished results). The intrinsic viscosity also decreased to 200 mL/g, indicating the severe acidic conditions which are also favourable for lignin condensation (high temperature combined with long duration and low SO_2 charge). Thus the results obtained at lower SO_2 concentrations in Paper IV are indicative of

lignin condensation since the residual lignin contents remained elevated despite the high fractionation intensity.

The effect of ash, inorganics and polyphenolic acids on delignification

Since HW biomass delignification was comparable to beech and spruce stem wood delignification, it was suspected that SW bark is the main component impairing the delignification of SW. Especially polyphenolic acids, extractives and inorganics in bark were considered possible causes of impaired delignification. Thus, a more detailed study was carried out to find out the reasons behind inferior delignification of SW in Paper V. Results are displayed in Table 5.

Table 5. The effect of 0.1M HCl extraction, acetone extraction and 1% NaOH extraction on the yields and chemical characteristics of SW biomass and SW biomass pulps (Paper V). All extractions were performed on ground wood prior to SEW fractionation, unless otherwise stated. Yields are based on weight % on oven dry feedstock (original SW biomass).

Material	Extraction method	Total yield	Kappa number	Total lignin	Carbohydrates	Ash
SW biomass	-	100		33.8[a]	50.1[a]	2.5
	HCl	96.6		33.8[b]	50.1[c]	1.3
	Acetone	93.8		33.8	50.1[c]	
	Acetone, NaOH 98°C	63.7		19.9	41.7	
	Acetone, NaOH 25°C	84.4		27.1		
SW biomass pulps	-	36.6	64.9	7.3	29.4	
	HCl	35.5	74.6	7.5	26.2	
	Acetone	36.3	63.9	6.1	30.3	
	Acetone, NaOH 98°C	30.0	37.3	2.7	24.2	
	Acetone, NaOH 25°C	38.4	46	4.3	32.7	
	Acetone, NaOH 98°C postextraction	22.7	53.2	4.6	19.5	

[a] Measured after acetone extraction.

[b] Assumed the same as for acetone-extracted SW biomass.

[c] Assumed the same as for the original SW biomass.

It can be seen that removal of ash from 2.5 to 1.3% by acidic (HCl) leaching did not improve the delignification of SW biomass (lignin in pulp of 7.5 vs 7.3% based on original biomass) but resulted in notable carbohydrate losses (26.2 vs 29.4% or 3.2% loss based on original biomass). Also, removal of acetone extractives caused only slight improvement in the delignification (6.1% vs 7.3% residual lignin). However, the combination of acetone extraction and removal of polyphenolic acids prior to fractionation by 1% NaOH treatment at 98°C led to substantial improvement in SEW delignification (residual lignin content of pulp 2.7 vs. 7.3% on original SW biomass), indicating that the persistent phenolic acids were responsible for the inferior delignification observed on SW. Thus, it was concluded that a significant fraction of the acid-insoluble lignin in SW samples in fact constituted of polyphenolics or other lignin-like material soluble only in alkaline treatment. It is likely that the structure of polyphenolics without the presence of alpha-carbon hydroxyls on the phenolic structures does not allow degradation or sulfonation reactions during SEW treatment, thereby remaining in the treated residual fibers.

NaOH treatment was also carried out at room temperature in order to improve the selectivity and to reduce the substantial carbohydrate losses observed at 98°C (residual carbohydrates of 24.2 vs 29.4% based on original biomass). It can be seen in Table 5 that the lower temperature treatment reduced the carbohydrate dissolution (residual carbohydrate content of 32.7%) and thus, enhanced the pulp yield. However, delignification was not as efficient as after high temperature NaOH treatment with the residual lignin content increasing to 4.3% vs 2.7% at 98°C. NaOH post-extraction was also investigated but it led to higher residual lignin contents compared to pre-extraction. This finding supports the earlier statements about condensation during SEW treatment of SW – condensed lignin would be more difficult to extract after the fractionation.

Therefore it can be concluded that the presence of polyphenolics in SW are the main reason for its inferior delignification. However, the removal of polyphenolics at industrial scale would likely not be viable with the current alkaline extraction approach and a better pretreatment method should be found. In particular if the bark content of the feedstock could be reduced, the negative effects would be diminished and bark could even be used for higher value products. Unfortunately the removal of bark from harvest residues is technically difficult.

4.2.5 Sulfonation of lignin

Stable lignosulfonic acids are formed in the reactions between SO_2 and lignin during SEW fractionation, and those are present in both the liquid and solid phases. The degree of sulfonation in SEW fractionation of harvest residues was studied in order to estimate the amount of recoverable SO_2 and determine the residual lignin properties (Paper III). A similar study was previously carried out on stem wood (Iakovlev and van Heiningen, 2012a) and thus the present results are compared to those of the previous study.

The sulfur content of lignin (S/C_9) in the solid residue and in solution after SEW treatment of SW biomass was somewhat higher than that of the corresponding stem wood (Table 6). The higher sulfonation degree was thought to be due to the higher content of inorganics in the harvest residue which results in a higher content of hydrosulfite ions that promote sulfonation during fractionation. However, also the higher temperature used in biomass fractionation might have affected the sulfonation degree. Nevertheless, it can be concluded that despite the high initial charge of SO_2, the sulfonation degree of lignin dissolved during SEW treatment is notably lower than in sulfite pulping (S/C9 0.36 and 0.6, respectively) (Rydholm, 1965). The considerable remaining amount of the sulfur dioxide applied in the process is recoverable by distillation (Iakovlev and van Heiningen, 2012a).

Table 6. Sulfur content of feedstocks, residual lignin in pulps and dissolved lignin in spent liquors (Paper III). Fractionation conditions were SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L/W ratio 6 L kg^{-1}, 150°C, 30 min (biomass) and 135°C, 80 min (spruce).

Feedstock	Sulfur content						
	in biomass	in residual lignin		in dissolved lignin		total	
	% o.d.f.	% o.d.f.	S/C9	% o.d.f.	S/C9	% o.d.f.	S/C9
SW biomass	0.03	0.16	0.11	1.53	0.36	1.69	0.30
HW biomass	0.01	0.04	0.11	1.46	0.36	1.49	0.34
Mixed biomass		0.09	0.12	1.32	0.31	1.41	0.28
Spruce[a]	0.004	0.05	0.08	1.01	0.25	1.06	0.23
Spruce, sulfite[b]		0.2	0.35	2-3	0.5-0.7	2-3	

Assumed lignin molar mass is 190 g mol^{-1} (Rydholm, 1965)

[a] (Iakovlev and van Heiningen, 2012a)

[b] (Rydholm, 1965)

4.2.6 The effect of bark

The chemical composition of bark present in forest residues differs notably from that of wood and thus may markedly influence the biomass processability. The study carried out on mixed wood chips and ground bark of both SW and HW origin (Paper VI) revealed that for HW, SEW fractionation of wood is not impaired by an increased percentage of bark. Contrary to HW, SW fractionation with increasing percentage of bark clearly impaired the fractionation of wood by increasing the amount of woody rejects (Figure 13). It was speculated that lignin and extractives in SW bark lead to condensation reactions resulting in inferior fractionation.

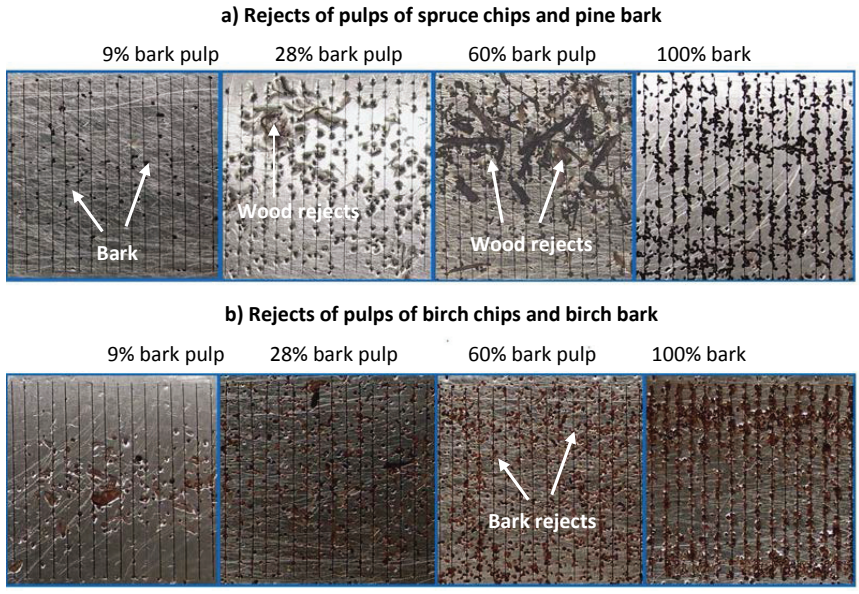

Figure 13. The rejects of the pulps prepared from a) spruce chips and pine bark; b) birch chips and birch bark. Wood rejects are visible for spruce pulps while birch rejects are mainly bark pieces (Paper VI).

In both cases, the increased share of bark linearly raised the reject content (Figure 14b). For SW, a maximum in reject content was observed at 60% bark content due to a significant fraction of undigested wood, while with HW bark the reject content increased linearly with bark content from 0 to 100%. The high yield and reject content observed with HW (Figure 14a and 14b) corresponds to the the low solubility and relative inertness of HW bark in SEW fractionation.

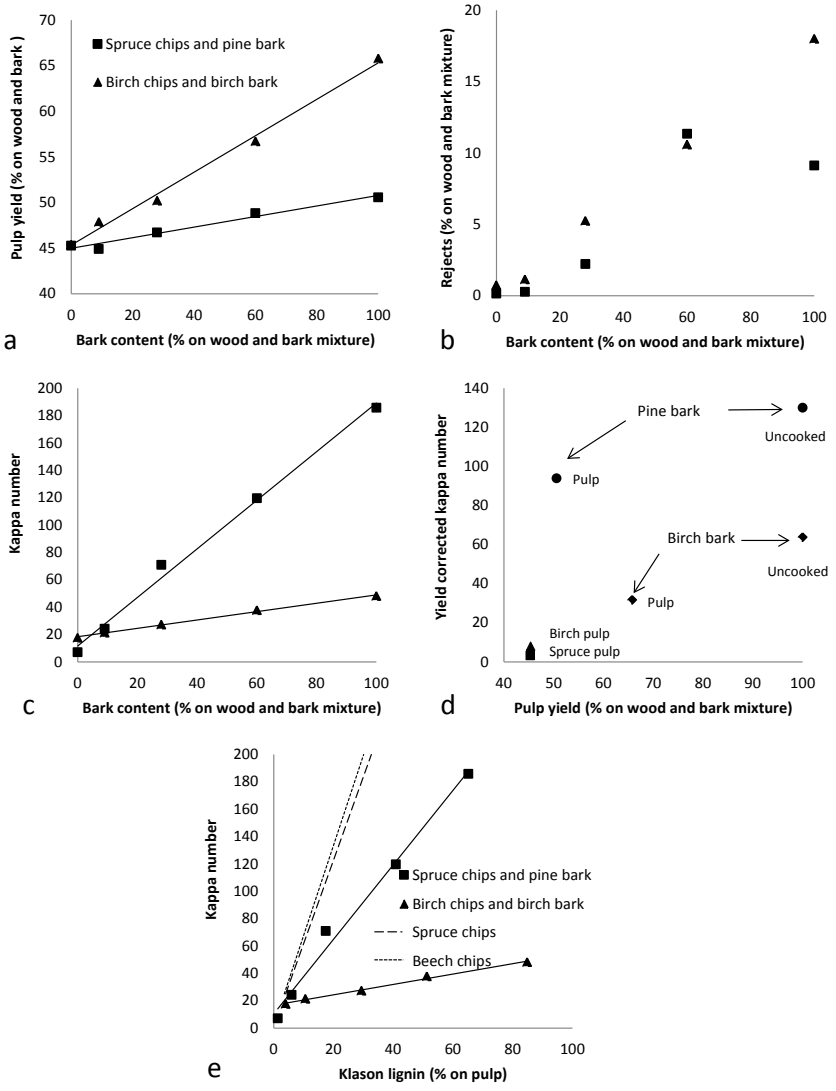

Figure 14. SEW fractionation of mixtures of bark and wood chips: a) Pulp yield versus bark content; b) Pulp reject content versus bark content; c) Kappa number versus bark content; d) Yield-corrected kappa number versus pulp yield; e) Kappa number versus Klason lignin (Paper VI). SEW fractionation was performed at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L:W ratio 6 L kg^{-1}, 150°C and 30 min (HW) / 60 min (SW). Spruce and beech chips results in Figure 1e are calculated based on linear relationship between Klason lignin and kappa numbers (Iakovlev and van Heiningen, 2011).

Kappa numbers were especially high for SW (Figure 14c and 14d), as observed in earlier studies as well (section 4.2.4). The high lignin content of bark contributed to the high kappa values. Also, especially SW bark is rich is oxidisable compounds as determined by kappa number analysis, such as polyphenolic acids. Nevertheless, it is important to note that the kappa number of biomass residues does not quantify the lignin content in same manner as for stem wood pulps (Figure 14e). The correlation between kappa number and lignin content is quite similar for both spruce and beech stem wood but it varies notably for biomass. Also, the correlation for SW and HW biomass is significantly different.

It should be noted that in these experiments, SW and HW were delignified at different fractionation times because shorter times were considered optimal for HW biomass. Nevertheless, the study revealed the main effects caused by the presence of bark in these feedstocks.

4.2.7 Simultaneous fractionation of SW and HW biomass

Simultaneous treatment of SW and HW biomass was briefly studied since flexibility in raw material quality and origin is a distinct advantage for a biorefinery process. Also the previously reported delignification rates on spruce, beech and straw were comparable at any particular temperature, indicating the omnivorous character of the SEW process (Iakovlev et al., 2011). In Paper III, it was demonstrated that simultaneous treatment of SW and HW resulted in pulp properties which were in agreement with the average of the properties obtained by separate treatment of two feedstocks. A similar conclusion was also made for DIP which was included as a third feedstock (Paper II). Thus, based on these brief studies simultaneous processing of different types of lignocellulosics in SEW fractionation seems a promising option.

4.3 Enzymatic hydrolysis

4.3.1 Hardwood versus softwood hydrolysis

Enzymatic hydrolysis of SW and HW biomass pulps was performed in order to determine the digestibility of SEW pulps (Paper V). Almost complete conversion of cellulosic residues to monomeric sugars should be accomplished at low enzyme dosage in order to obtain an economically viable process. The low dosage is required because the enzyme cost is high, and this is especially important for SW feedstocks which require higher enzyme dosages than HW (Klein-Marcuschamer et al., 2012).

The results obtained on SW and HW pulps fractionated at the same conditions revealed clear differences in their digestibility (Figure 15). Whereas SW required 19 FPU/g cellulose enzyme dosage to reach sufficient 80% glucose yield, HW pulp was efficiently hydrolysed at a much lower enzyme dose (5 FPU/g cellulose). HW cellulose was also more accessible since complete conversion was reached by excessive enzyme addition, unlike for SW.

The notably high lignin content of SW (18.4% vs. 4.1% for HW) contributed to its low digestibility. The reasons behind inferior digestibility of SW compared to HW are partially related to distinct differences in lignin structure and chemistry (Achyuthan et al., 2010) since even at equal lignin contents, hydrolysis of SW is worse (Yu et al., 2011). Apparently, hemicelluloses and lignin occupy smaller spaces in SW resulting in a smaller increase in pore volume upon their removal or redistribution in the cell wall which leads to impaired digestibility (Mansfield et al., 1999).

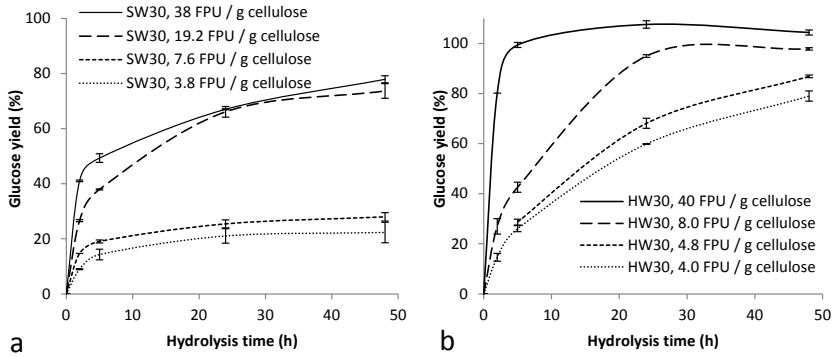

Figure 15. Enzyme dosage curves for a) SW and b) HW biomass pulps produced by SEW fractionation at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L:W ratio 6 L kg^{-1}, 30 min and 150°C (Paper V). Glucose yield is based on total glucose in pulp.

In the present study the enzymatic hydrolysis yields are reported as glucose monomer yields analysed by YSI glucose analyser or HPAEC. Xylose yields were comparable with the glucose yields in all the samples which were also analysed for hemicelluloses content. Also mannose yields were well comparable with those of cellulose to glucose, but over 50% of the dissolved mannose was always present as oligomers due to lack of β-mannosidase in the enzyme preparation. Glucose and xylose were mostly present as monomers.

The effect of SEW fractionation time (20-60 min) on the enzymatic digestibility was also studied for HW and SW biomass (Figure 16). However, the changes in chemical composition caused by longer fractionation were moderate, leading to negligible differences in digestibilities as well. A similar observation was made for steam pretreatment of spruce forest residues by Janzon et al. (2014) who stated that pretreatment time had only a very small influence on carbohydrate yields, while SO_2 concentration and temperature were the main factors affecting the yields. Therefore the present finding also supports the use of a short fractionation time which earlier was shown to be more beneficial in terms of limited carbohydrate degradation than longer fractionation times.

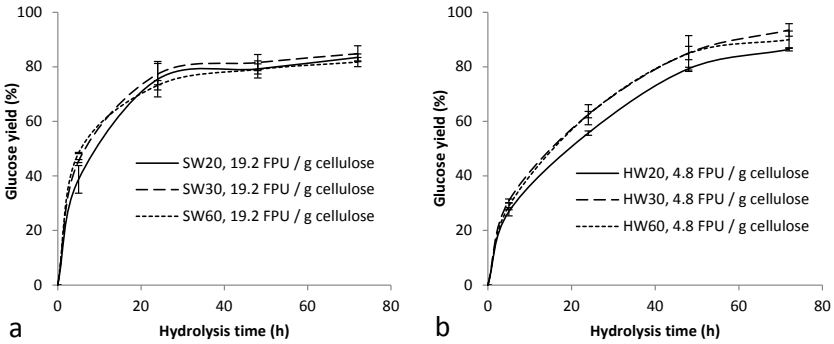

Figure 16. The effect of fractionation time (20-60 min) on enzymatic hydrolysis of a) SW and b) HW biomass SEW pulps produced at conditions: SO_2:EtOH:H_2O = 12:43.5:44.5 (by weight), L:W ratio 6 L kg^{-1} and 150°C (Paper V).

4.3.2 The effect of lignin content and quality on digestibility

Lignin content

Lignin is one of the main inhibitors of enzymatic hydrolysis since enzymes can non-productively bind on the lignin surface (Palonen et al., 2004; Kumar et al., 2012). Lignin also covers carbohydrate surfaces limiting their accessibility to enzymes and restricts swelling of the substrate (Mooney et al., 1998). It has also been revealed that at the same lignin content, SW is more resistant to enzymatic hydrolysis than HW (Yu et al., 2011) and cellulases associated with SW have less potential for enzyme recycling (Gregg and Saddler, 1996). These facts emphasize the importance of lignin origin. Also, agricultural residues are shown to undergo more efficient hydrolysis compared to similarly treated woody feedstocks (Arantes and Saddler, 2011). In addition, there are also notable differences in digestibility related to the quality of lignin as determined by the delignification or pretreatment method (Pan et al., 2005). Thus, it is important to separately evaluate the digestibility of biomasses pretreated by different processes.

SEW pulps with varying chemical compositions were prepared from SW stem wood chips in order to study the effect of lignin content and other properties on enzymatic digestibility (Paper IV). Analysis of the results is somewhat complicated due to the fact that several parameters of lignocellulosics such as lignin content, hemicellulose content and cellulose DP are simultaneously modified making the assessment of the relative importance of individual factors difficult. However, some important conclusions can be made based on the observations in this study. First of all, it is clear that the lignin content of SEW pulps strongly affects the digestibility (Figure 17). A strong correlation between lignin content and digestibility can be seen especially at the lower range of residual lignin content (1-5%) while the digestibility was less affected by changes at higher lignin contents.

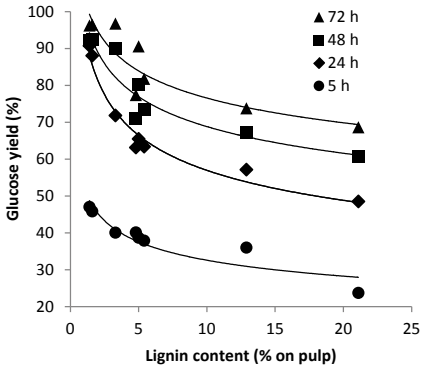

Figure 17. The effect of lignin content on glucose yields of spruce pulps in enzymatic hydrolysis (Paper IV). Enzyme dosage was 10 FPU/g cellulose.

Other properties, such as hemicellulose content and DP of cellulose, showed a weaker correlation with glucose yield. Thus, it was concluded that lignin content is the main factor affecting digestibility of SEW pulps although in general, the effect of hemicellulose and DP on enzymatic digestibility is also well recognized and discussed in many studies (Mansfield et al., 1999; Várnai et al., 2010; Hallac and Ragauskas, 2011). Additionally, the strong covalent bonds between residual lignin and carbohydrates might have retarded the digestibility. The requirement of a low residual lignin content to achieve efficient hydrolysis can be considered a weakness of the SEW process since biomass pretreated by SPORL can achieve over 90% yield within 24 h at a charge of 15 FPU on spruce fibers containing 33% residual lignin (Shuai et al., 2010).

The negative effect of SEW pulp lignin content on enzymatic hydrolysis has also been confirmed for SEW lignocellulose nanofibrils (Morales et al., 2014). It was also found that nanofibrillated fibers had approximately 10% higher digestibility than normal SEW pulp fibers at similar lignin content. This finding suggests that nanofibrils might be more accessible to enzymes due to the desconstruction of the cell wall structure and increased surface area. This statement is in good agreement with the positive effects of enhanced cellulose accessibility (Arantes and Saddler, 2011).

Lignin quality

Besides lignin content and origin, the properties and quality of lignin as determined by the delignification method play a significant role in digestibility. Lignin sulfonation, which introduces hydrophilic sulfonic acid groups into lignin, has been shown to improve enzymatic hydrolysis since the improved hydrophilicity reduces the non-productive binding of enzymes on lignin (Zhu et al., 2009; Lou et al. 2013). Sulfonation also results in a more swollen cell wall with improved accessibility (Scallan, 1977). It has been concluded that the SEW pulps produced at higher SO_2 charge in the fractionation liquor have somewhat higher sulfonation degrees (Iakovlev and

van Heiningen, 2012a). Thus, comparison of the digestibilities of SEW pulps produced at different SO_2 contents can predict the effect of sulfonation, especially at constant lignin content (Paper IV). Based on Figure 18, it can be concluded that sulfonation improves the hydrolysis yield, in agreement with other studies. It's important to note that the pulp with highest digestibility had also the highest hemicellulose content and DP – thus, the effect of those can be disregarded.

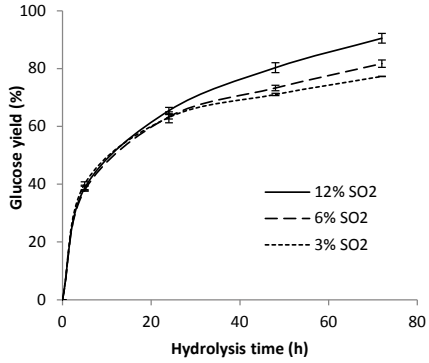

Figure 18. The effect of SO_2 charge in SEW fractionation on glucose yields of spruce pulps (Paper IV). The pulps produced at different SO_2 charge had approximately equal lignin contents (4.8-5.4%). Enzyme dosage was 10 FPU/g cellulose.

Contrary to sulfonation, lignin condensation increases the hydrophobicity of lignin due to elimination of hydrophilic hydroxyl groups in the α-position of lignin unit and affects negatively affects enzymatic hydrolysis (Zhu et al., 2009; Pielhop et al., 2012). Condensation prevents delignification since it forms covalent carbon-carbon bonds between the lignin units. Besides lignin, also some phenolic extractives may participate in condensation reactions. In SEW fractionation, lignin condensation occurs especially at lower SO_2 concentrations that also require longer fractionation time. Thus, the poor digestibility that is observed at lower SO_2 concentrations (Figure 18) may be partially related to lignin condensation. Shuai et al. (2010) have demonstrated low digestibility of dilute-acid pretreated biomass having condensed lignin compared to sulfonated SPORL lignin. It was also suggested that, instead of costly extensive delignification, modification of lignin properties might be a more feasible way to achieve efficient hydrolysis. For the SEW process, it is clear that high SO_2 concentration is preferable due to advantages both in fractionation and in enzymatic hydrolysis.

4.3.3 The effect of bark content

The effect of SW and HW bark content on SEW fractionation and enzymatic hydrolysis was studied in Paper VI. The results of SW and HW are not fully comparable due to the different enzyme dosages selected based on earlier optimization studies. However, SW bark was found to significantly impair the enzymatic hydrolysis whereas HW bark only had a negative effect when its

share exceeded 28% (Figure 19). Based on high kappa numbers, SW bark had notably more oxidisable structures, such as lignin and polyphenolic acids, and those are crucial considering the tendency of enzymes to non-productively adsorb on them, thereby decreasing their enzymatic activities. Polyphenolic acids are reported capable of forming complexes with proteins, similarly to humic acids (Jensen et al., 1963). In addition, the bark used in the experiments was mainly outer bark which is especially rich in lignin and polyphenolics (Fengel and Wegener, 1989). Another possible explanation for the difficulties caused by bark is the presence secondary metabolites which are known to have several different functions in plant physiology, including defense against pathogenic microbes, fungi or insects. For example terpenes, phenolics including lignin, tannins and flavonoids, widely present in bark and extractives, are all classified as secondary metabolites. In intact plants some of these compounds have defensive roles and strong antimicrobial activity (Taiz and Zeiger, 2002). Therefore, such compounds may also cause reduced enzymatic activities or inhibition of yeast and bacteria in further processing. For example, tannins can bind with proteins or form chelates with metals inhibiting the growth of microorganisms (Scalbert, 1992).

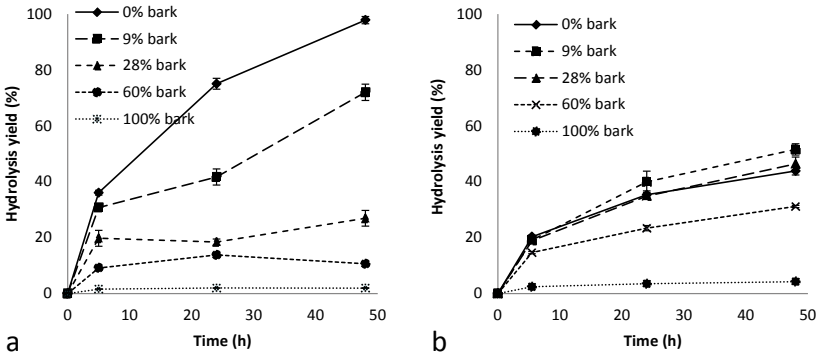

Figure 19. The effect of bark content on enzymatic hydrolysis of a) spruce chips and pine bark and b) birch chips and birch bark (Paper VI). Enzyme dosage was 10 FPU/g cellulose on spruce pulps and 5 FPU/g cellulose on birch pulps. Hydrolysis yield is expressed as reducing sugars as glucose per total sugars in the pulp.

A previous study on SPORL pretreatment of Douglas-fir (*Pseudotsuga menziesii*) revealed a similar effect of bark: the presence of 14.3% bark reduced substrate digestibility by 16% compared to a bark-free sample (Zhang et al., 2012). However, notable differences were observed in the digestibility of bark since in the present study it was close to 0% while in the SPORL process it was 41%. The twofold enzyme dosage partly may explain the higher digestibility of SPORL bark but differences in the origin and treatment method of the bark (SEW vs. SPORL) may play a significant role. Nevertheless, it is clear that bark impairs the digestibility and to improve the potential of forest residues as a feedstock, these negative effects should be overcome.

To reduce the inhibition of enzymatic hydrolysis by bark, it would be beneficial to reduce the bark content. However, in case of forest harvest

residues with bark still attached to the wood particles, this might be impractical, technically difficult and uneconomical. It was demonstrated in the laboratory that free bark particles tend to sediment by time in water but in practice, this would not offer notable benefits due to simultaneous carbohydrate yield loss (Paper VI). Another method that has been suggested for the reduction of both the bark and ash content is physical fractionation since amorphous and friable bark makes smaller particles that can be separated through sieving (Zhang et al., 2012). If carried out at the harvest site, the bark rich fraction could be left in the forest to maintain soil fertility which makes this method very desirable. However, perhaps the most promising method for enhancing hydrolysis of bark-rich biomass is the use of surfactants to reduce irreversible adsorption of enzymes on bark, as will be discussed in the next paragraph.

4.3.4 Methods to enhance enzymatic hydrolysis

Auxiliary enzymes or chemicals are often found to efficiently improve the yields of enzymatic hydrolysis. The lignocellulose matrix consists of several compounds tightly assembled together. The layered structure of hemicellulose and cellulose restricts the hydrolysis unless their simultaneous removal is enabled (Várnai et al., 2011). Thus, a mixture of several enzymes is generally needed for total hydrolysis of lignocellulosics. In this work, a commercial enzyme preparation containing xylanase was used and the overall hydrolysis rates were high. However, addition of endomannanase boosted the hydrolysis of cellulose and allowed a reduction in the dosage of the enzyme preparation (Paper V). Thus, development of enzyme preparations suitable for different applications and feedstocks is crucial in order to optimize the yields and minimize the enzyme consumption. For example, preparations optimized especially for SW feedstocks are required due to its clear differences as compared to HWs and herbaceous feedstocks. A benefit of the SEW process in this respect is therefore the efficient dissolution of hemicelluloses (discussed in section 4.2.2) which facilitates rapid hydrolysis without strict requirements for auxiliary enzymes.

The problem with lignocellulosics having high lignin content is the non-productive binding of enzymes on the lignin surface. This causes reduction in the enzyme activity and consequently a lower hydrolysis rate and yield. Proteins, like bovine serum albumin (BSA), and surface active chemicals, such as Tween or polyethylene glycol (PEG), dosed before the addition of enzymes have been found to effectively improve hydrolysis and reduce irreversible adsorption of the enzymes on lignin (Alkasrawi et al., 2003; Yang and Wyman, 2006; Börjesson et al., 2007; Kumar et al., 2012). In Paper VI, the non-ionic surfactant Tween 20 was shown to give a substantial increase in the hydrolysis yield of bark containing SW biomass (Figure 20). A 2% (w/w) addition was adequate and more than doubled the hydrolysis yield. Doubling the dosage of the enzyme preparation to achieve a similar effect would be uneconomical.

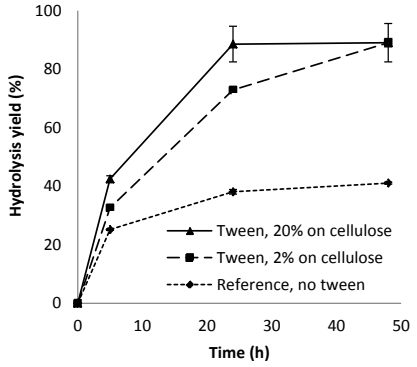

Figure 20. Enzymatic hydrolysis of SW harvest residue (bark content 24.8±4.7%) pulp and addition of surfactant Tween 20 (Paper VI). Enzyme dosage was 10 FPU/g cellulose. Results on 20% Tween dosage were obtained at 47°C, others at 50°C.

Also lignosulfonates have been found to improve the enzymatic digestibility (Wang et al., 2013; Zhou et al., 2013a), likely through blocking the binding to lignin by a similar mechanism as the additives described above. Contrary to lignin itself, lignosulfonates are largely hydrophilic, and therefore are expected to have less affinity to cellulases. Since lignosulfonates are produced as by-product in SEW treatment, possibilities to utilize these to improve enzymatic hydrolysis were briefly evaluated. Separated lignosulfonates could be added directly to hydrolysis but a more efficient way would to reduce the degree of washing after fractionation, and thereby carry-over the lignosulfonates to the enzymatic hydrolysis stage. Less washing would offer significant economic benefits as well. A simple study was carried out where the washing efficiency was notably reduced compared to the standard washing procedure adopted in this work (Paper VI). The results are shown in Figure 21.

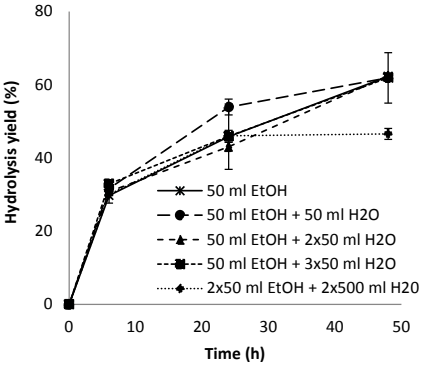

Figure 21. The effect of reduction of washing efficiency after SEW fractionation on enzymatic digestibility (Paper VI). Study was carried out on SW harvest residue and enzyme dosage was 10 FPU/g cellulose.

Digestibility was improved when the washing efficiency was reduced indicating that SEW lignosulfonates in the carry-over liquor might act as

enhancers. However, the study was carried out at low substrate consistency leading to significant dilution of the carry-over liquor. Thus, to confirm the industrial viability, this approach should also be demonstrated at commercially feasible high consistencies since the enrichment of wood-derived inhibitors might affect both the hydrolysis and fermentation processes.

4.3.5 Optimization of hydrolysis conditions

In the present study, the enzymatic hydrolysis experiments were carried out following a standardized procedure (Selig et al., 2008). However, careful optimization of the hydrolysis conditions would likely improve the obtained hydrolysis yields. For feedstocks with high lignin content, a lower temperature (45°C vs. 50°C) has been found beneficial due to a decrease in non-productive cellulase binding to lignin (Rahikainen et al., 2013; Zheng et al., 2013). In Paper IV, this effect was confirmed on SEW pulps showing an increase of about 3% in glucose yield at the lower temperature. In addition, elevated pH (5.2-6.2) reportedly improves the hydrolysis yields, also by reducing the non-productive binding (Lan et al., 2013; Lou et al., 2013). The yield enhancement due to elevated pH was found significant especially on pulps containing highly sulfonated lignin.

On the other hand, the present experiments were carried out at low consistency (1.5%) which is known to lead to higher yields than that at industrially viable higher consistencies (Kristensen et al., 2009). Thus, the use of high consistencies would likely lead to somewhat lower yields. Nonetheless, a moderate increase in consistency (from 1.5% to 5%) was shown to have negligible effect on yield (Paper IV) and 90% yield on SW biomass pulp was obtained even at 10% consistency (20 FPU/g cellulose, Paper V). One reason for the good yields even at high consistencies could be the constant and efficient mixing practice employed: pulp was gradually added to the enzyme-buffer solution within the first two hours after sufficient liquefaction of the fiber suspension. A similar observation on the effect of an appropriate mixing scheme to maintain high yields at 20% consistency hydrolysis has been reported by Xue et al. (2012).

Another significant matter is the selection of substrate-specific auxiliary enzymes for each application since the total hydrolysis of lignocellulosics requires a variety of different enzyme activities which are somewhat feedstock specific. Currently, there are no commercial preparations available which were optimized for SW feedstocks but it is expected that extensive research in the field will lead to their development.

4.4 Fermentation of enzymatic hydrolyzate

4.4.1 ABE fermentation of SW hydrolyzate

Fermentation of the dissolved sugars and solvent recovery are the final stages in biotechnological conversion of lignocellulosics to ABE. ABE fermentation was successfully carried out on the enzymatic hydrolyzate produced from SW

harvest residue pulp (Paper V). The hydrolyzate was diluted two times to decrease the sugar concentration to the optimal level for fermentation, 56 g/L (52 g/L glucose, 2.2 g/L xylose and 2.2 g/L mannose). Also, activated carbon treatment was required to remove the inhibition caused by sodium citrate used in the enzymatic hydrolysis stage. In a glucose control fermentation, sodium citrate was found highly inhibitive at 0.05M concentration. Possibly other fermentation inhibitors derived from wood were removed simultaneously. The lignin content of the hydrolyzate prior to dilution was only 0.28 g/L which is well below the *Clostridia* tolerance level of about 1 g/L. It has been reported that ABE fermentation is inhibited when the soluble lignin compounds exceed 1.77 g/L, while concentrations below 0.89 g/L were found beneficial to improve fermentation (Wang and Chen, 2011). Results of the ABE fermentation are shown in Table 7.

Table 7. Solvent yields in ABE fermentation trial of SW biomass enzymatic hydrolyzate (Paper V). Initial sugar concentration was 60g/L for the glucose control and 56 g/L for SW hydrolyzate.

Sample	Time (h)	Acetone (g/l)	Ethanol (g/l)	Butanol (g/l)	Total solvents (g/l)
Glucose control	48	1.35	0.41	4.25	6.01
	120	2.86	0.96	8.86	12.68
SW hydrolyzate	48	1.63	0.59	4.98	7.20
	120	3.07	1.05	8.21	12.33

The 120 h fermentation yield of SW hydrolyzate was 12.3 g/L of ABE solvents in total (equal to 0.22 g/g sugars), which compares well with the yield obtained in the glucose control experiment. Similar yields have been reported earlier on conditioned spruce and SW biomass SEW spent liquors (Sklavounos et al., 2011, 2013b) while the maximum theoretical yield would be close to 0.4 g/g sugars. Low solvent concentration compared to, for example, ethanol production is one commonly recognized weakness of butanol production (Jurgens et al., 2012). Nevertheless, this result further justifies the potential of the present biorefinery approach for biofuel production.

4.4.2 Ethanol fermentation as an alternative

The main difficulties in fermentative butanol production are solvent toxicity and low solvent concentrations and yields (Jones and Woods, 1986). Additional difficulties related to the current approach of butanol production from SEW treated biomass are the low tolerance of *Clostridia* bacteria to lignin degradation products and other phenolic compounds. Thus, several conditioning stages are required to produce a fermentable solution having a low enough concentration of inhibitors. On the other hand the advantages of butanol production are the utilization of pentose sugars and superior fuel properties of butanol compared to ethanol (Bankar et al., 2013). Nevertheless, it is important to consider production of ethanol from SEW spent liquor and the enzymatic hydrolyzate since the conditioning is certainly less complicated in particular due to the better lignin tolerance of the yeasts. Thus, competing pretreatment or fractionation technologies can only be objectively compared on the basis of the same fermentation approach.

For example, sulfite pretreatment to overcome recalcitrance of lignocellulosics (SPORL) has been demonstrated as an efficient pretreatment for the production of ethanol from wood without detoxification (Zhou et al., 2013a) and also harvest residues are suitable feedstocks (Zhang et al., 2012; Leu et al., 2013). Also, ethanol fermentation of bark-containing Douglas fir hydrolyzate pretreated by SO_2-catalysed steam explosion has been carried out successfully by Robinson et al. (2002). They showed that bark content up to 30% had negligible effect on the fermentation although sugar recovery in pretreatment was lower and the prehydrolysate had a higher content of lipophilic extractives. Thus the same advantages may be applicable to SEW fractionation when followed by yeast fermentation to produce ethanol. Considering this, as well as other advantages of SEW treatment, such as almost full recovery of hemicelluloses, absence of base, relatively simple recovery of the chemicals and feedstock/product flexibility, the potential of this fractionation method are very promising.

4.5 Economical aspects of the developed biorefinery process

For the production of an ABE solvent mixture from forest biomass, the process utilizing SEW fractionation and enzymatic hydrolysis followed by spent liquor conditioning and ABE fermentation has been shown to be economically viable through a detailed process simulation (Melin and van Heiningen, 2013). For a process utilizing 700 000 tonnes (o.d.) of biomass annually and producing 74 000 tonnes butanol, an annual profit was calculated of approximately 18 Million Euro corresponding to a payback time of about 5 years. According to the sensitivity analysis conducted, the changes in product yield, butanol and biomass price and capital costs are the most crucial factors affecting the profitability. The energy in the recovered lignin, biogas and hydrogen gas produced as by-products, was estimated to be sufficient to satisfy the heat and electricity requirement of the entire process.

The water use in fractionation should be minimized through low L:W ratio and efficient washing stages since it strongly affects the energy consumption. Also, efficient recirculation of water in the process is important to reduce the amount of waste water streams which require purification. Process simulation was done based on a L:W ratio of 3:1 L kg^{-1} in fractionation which is half of that used in the present laboratory study. Nevertheless, this has been proven to be adequate by Sklavounos et al. (2013b). Almost full recovery of the cooking chemicals, ethanol and SO_2, is crucial for both economical and environmental aspects and thus a simple recovery technology is beneficial. SO_2 consumed by lignin represents only approximately 1 w/w % as sulfur/o.d. wood.

A high yield of fermentable sugars from fractionation and enzymatic hydrolysis is one of the key criteria for successful biofuel production. SEW fractionation benefits from the full utilization of hemicelluloses, unlike several other pretreatment processes (see Table 2). There are no sugar losses in fractionation, but hemicellulose losses occur during liquor conditioning and those were considered as being 10% for the economical evaluation. For

enzymatic hydrolysis, high consistency treatment is required to reduce the use of water and to obtain fermentable sugars at high concentration. 10% consistency was demonstrated in the laboratory scale but ideally 20% consistency used in the economical analysis should be employed in the industrial scale.

The process discussed is able to utilize all biomass components for either valuable products or for the generation of heat and electricity. Also the ability of the SEW process to simultaneously treat different lignocellulosics is a clear benefit and improves its viability. In addition, the process tolerates biomass of a wide range of moisture contents. However, biomass processing is done in much smaller scale than fossil-based transportation fuels which leads to higher capital costs per final product. Spent liquor conditioning including evaporation, steam stripping and membrane filtration needs to be carefully optimized for improved economic performance. Besides, solvent toxicity in ABE fermentation results in low product concentrations and high product recovery costs.

The several possible co-products can improve the feasibility of the whole process. Other effective means of improving the profitability include the use of cheaper raw materials, as well as reduction in enzyme use and capital costs. In addition, targeting the products to high value markets such as cosmetics and specialty solvents would be beneficial. Process integration with existing facilities should also be considered. The concept of Integrated Forest Biorefinery (IFBR) presented by van Heiningen (2006) is believed to notably improve the technoeconomic potential of biomass based fuels. This concept aims to combine new processes and products with the traditional ones in already existing pulp and paper industry facilities. For example, existing logistics, storage and effluent treatment plants could be utilized to reduce the capital costs.

5. Concluding remarks

SO_2-ethanol-water (SEW) fractionation was shown to be an efficient method to treat forest harvest residues prior to enzymatic hydrolysis and ABE fermentation. Both HW and SW residues were rapidly defiberized through efficient dissolution of hemicelluloses and lignin. The hemicellulose dissolution rate was comparable for both HW and SW, as well as for stem wood. However, it was clear that short fractionation time and relatively low temperature are beneficial in order to avoid degradation of dissolved hemicelluloses. Formation of furfural and HMF and degradation of uronic acids were promoted at extended fractionation time, leading to sugar losses.

HW biomass delignification was comparable to stem wood and resulted in a residual lignin content of below 2% on wood. However, SW delignification was impaired due to the high content of bark and specific chemical characteristics of coniferous bark. Delignification was efficient at the initial stage of cooking but ceased after 20 min resulting in pulp with a high residual lignin content. Especially polyphenolic acids present in coniferous bark were found to impair the delignification, whereas the effect of acetone extractives and inorganics was insignificant. In addition, the presence of bark was shown to increase woody rejects in SW, unlike for HW. This indicates that condensation reactions are occurring during SW fractionation.

Enzymatic hydrolysis using commercial enzymes was successful on both HW and SW fibers, although similar to previous findings in the literature, HW was less recalcitrant and lower enzyme dosages were sufficient for HW. Besides the higher lignin content in SW and differences in lignin quality, also the higher bark content of SW feedstock impaired SW hydrolysis.

Lignin content was shown to be a critical parameter for the digestibility of SEW fibers especially in the lower range (1-5%) of residual lignin content. Besides the lignin origin and content, the quality of lignin as determined by the delignification conditions, also contributed to digestibility. Sulfonation was shown to improve hydrolysis, likely due to improved hydrophilicity and decreased enzyme binding on lignin. On the contratry, lignin condensation and increased hydrophobicity of lignin was speculated to impair the hydrolysis yield.

Especially SW bark was detrimental for enzymatic hydrolysis whereas HW bark inhibited hydrolysis only when its share in the feedstock exceeded 28%. High enzyme dosage or reduction of the bark content improved the hydrolysis yields on SW but the most efficient solution was shown to be the addition of surfactants prior to enzymatic hydrolysis. Adsorption of surfactant on the

lignin surface prior to enzyme addition notably reduces the loss of enzyme activity and allows the attainment of high hydrolysis yields at relatively low enzyme dosage.

The combination of SEW fractionation, enzymatic hydrolysis and ABE fermentation can be considered a viable option of biofuel production. Both conditioned SEW spent liquors and enzymatic hydrolyzate have been successfully fermented with *Clostridia* to produce mixtures of butanol, acetone and ethanol. Benefits of the approach include the possibility to process wide a range of raw materials, almost full utilization of hemicelluloses, product flexibility and relatively simple chemical recovery, as well as distinct benefits of butanol over ethanol as a fuel.

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